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T Cell Hyperreactivity to IL-6 in Chronic Nonviremic HBV Carriers Despite Normal IL-6 Receptor or gp130 Expression

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ABSTRACT

We previously showed that T cells from chronic nonviremic HBsAg carriers activated with immobilized OKT3 MAb are hyperreactive to monocyte accessory signals, mainly to interleukin-6 (IL-6). We have further characterized this T cell hyperreactivity using phytohemagglutinin (PHA) as the primary activating signal. PHA-stimulated T cells from nonviremic patients had a significantly higher response to addition of monocytes, monocyte supernatants, and IL-6 alone or combined with IL-1β when compared to controls. We examined if these effects could be mediated by a differential expression of IL-6 receptor (p80) or gp130 on resting or PHA-stimulated T cells. We found that PHA, IL-6, IL-1β, or IL-2 induced only small changes of the dull p80 expression on T cells. In contrast, we found a significant increase of gp130 expression on PHA-activated T cells compared to unstimulated T cells, which was down-regulated by the presence of IL-6. However, no significant differences in p80 or gp130 expression were detected between patients and controls within all the culture conditions tested. Our results confirm that IL-6 is involved in the *in vitro* T cell hyperreactivity of nonviremic HBV carriers and indicate that this effect is not mediated by disturbances of IL-6 receptor expression.

INTRODUCTION

Two types of chronic hepatitis B virus (HBV) carriers have been described, the so called "viremic HBsAg carrier," who is highly infectious presenting positive serum HBeAg and HBV-DNA, and the "nonviremic HBV carrier," who shows negative HBeAg and HBV-DNA serological markers associated to normal ALT activity (1). Failure to clear HBsAg from the blood continues to be the hallmark of both types of chronic HBV infection (5,9). In this regard, we have previously shown the existence of anti-CD3-activated T cells in peripheral blood from chronic viremic and nonviremic HBV carriers. Moreover, highly purified

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T cells from chronic nonviremic HBsAg carriers were hyperreactive to signals provided by monocytes and/or monocyte-soluble factors (2). We also found that IL-6, which acts as a helper signal in T cell activation (3,4,13,18,19), was the main soluble factor associated with this sustained T cell hyperreactivity probably related to ongoing viral clearence (2). The receptor mediating the biological activities of IL-6 has been identified as two different membrane glycoproteins, an 80-kDa protein, referred to as the ligand-binding protein (IL-6R), and a 130-kDa protein, referred to as the signal-transducing protein (16,21). It has been demonstrated that despite its lack of IL-6 binding property, gp130 is involved in the formation of high-affinity IL-6 binding sites and in IL-6 signal transduction (6,17).

Since the effect of IL-6 as a helper factor on T cell activation is more sensitive in a mitogen-stimulated system (4,11,13,18), we have further investigated the IL-6-dependent functional T cell hyperreactivity in the nonviremic HBV patients using PHA as the primary activating signal. Moreover, we also examined IL-6R and gp130 expression on pure T cells activated with PHA and the cytokines IL-6, IL-1β, and IL-2.

MATERIALS AND METHODS

Patients. Thirteen asymptomatic chronic nonviremic HBV carriers, 9 males and 4 females (mean age, 40 years; range, 25–55 years) were studied following a previously standardized protocol (22). All patients were anti-HBc positive, anti-HBe positive, anti-HBs negative, IgM anti-HBc negative, and HBeAg negative. ALT and AST were within normal ranges and serum HBV-DNA was undetectable by polymerase chain reaction (PCR). All the patients were negative for antibodies to hepatitis C (anti-HCV) and to HIV. Twenty healthy individuals, seronegative for HBV, HCV, and HIV markers, were selected as the control group. Viral markers for hepatitis B were determined by commercially available kits (Hepanostika, Organon-Teknika, Turnhout, Belgium). Anti-HCV and anti-HIV were detected by enzyme-immune assays (Ortho HCV ELISA Test Systems, 2nd Generation, Germany; Abbott Laboratories, North Chicago, IL, respectively). Serum HBV-DNA was detected by PCR assay as described elsewhere (2,10).

Cell separation. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradients. After three washings in RPMI-1640 (GIBCO BRL, Gaithersburg, MA), the cells were resuspended in complete culture medium containing RPMI-1640 supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS). Adherent cells as a source of monocytes, monocyte supernatants, and highly purified T cells were obtained as previously described (2). The T cell preparations contained more than 98% $CD3^+$, less than 1–2% $CD16^+$, and less than 0.5% $CD14^+$ cells as determined by flow cytometry analysis in an EPICS Profile II (Coulter Corp. Hialeah, FL).

Proliferation studies. T cells (0.25×10^6/ml) were cultured in complete medium in flat-bottomed microtiter plates (Falcon 3872, Becton Dickinson, Lincoln Park, NJ) alone or in the presence of 0.5 μg/ml of PHA (Wellcome Diagnostics, Dartford, England). This concentration was optimal for stimulation of PBMC cultures. Predetermined optimal doses of recombinant IL-1β and IL-6 (Genzyme, Boston, MA) and IL-2 (Biotest Diagnostics, Dreieich, Germany) were added as indicated in results. All cultures were performed in triplicate and incubated for 4 days at 37°C in a 5% CO_2 atmosphere. Cultures were pulsed with 1 μCi of [^{3}H]thymidine (NEN Products, Boston, MA) per well, 8 h before the end of incubation. Cells were harvested on glass fiber filter and thymidine incorporation was measured in a liquid scintillation counter (1205 Betaplate, LKB Wallac, Turku, Finland).

IL-6 receptor expression studies. T cells were cultured as described above and after 48 h at 37°C, the cells were washed twice and resuspended in 100 μl of PBS containing 1% bovine serum albumin. The IgG_{2b} monoclonal antibody (MAb) MT18, directed against the IL-6R (p80) and the IgG_1 mouse antihuman gp130 MAb AM64 have been characterized previously (6,7). These MAbs were kindly provided by Dr. T. Taga (Osaka University, Osaka, Japan). MT18 MAb was used at 5 μg/ml, adding 25 μl to 0.25×10^6 T cells and AM64 MAb was used as 50 μg/ml, adding 10 μl to 0.25×10^6 T cells. The cell suspensions were incubated for 1 h at 4°C with MT18 or AM64 MAbs and controls included cells incubated with mouse IgG_{2b} or IgG_1, respectively. Biotin-conjugate goat antimouse IgG_{2b} or biotin-conjugate goat antimouse IgG_1

(Amersham Life Science, Buckinghamshire, UK) were added to the cells followed by streptavidin-fluorescein (Amersham Life Science, Buckinghamshire, UK). Specific immunofluorescence was analyzed by flow cytometry (EPICS Profile II, Coulter Corp, Hialeah, FL).

Statistical analysis. The statistical significance between groups was assessed by Student's *t* test for paired or unpaired samples.

RESULTS AND DISCUSSION

Proliferative T cell response to PHA and the effects of accessory cells and of cytokines. As shown in Figure 1, purified T cells from nonviremic HBV carriers and controls proliferated poorly when stimulated with an optimal dose of PHA. The addition of increasing monocyte concentrations (1, 10, and 20%) enhanced T cell proliferation and a significantly higher proliferative response was achieved in the patient group compared to controls. These results and our previous data (2) suggested that the higher response of T cells from HBV nonviremic carriers involved monocyte-produced soluble factors. Thus, we also did experiments to analyze T cell response to monocyte supernatants. We observed that PHA-stimulated T cells from nonviremic carriers had a significantly higher response to autologous (9106 ± 2573, mean cpm ± SEM, $n = 10$; $p < 0.005$) or allogeneic (8497 ± 2324, mean cpm ± SEM, $n = 12$; $p < 0.05$) monocyte supernatants compared to T cell responses from controls to either autologous (3292 ± 484, mean cpm ± SEM, $n = 16$) or allogeneic (3494 ± 2003, mean cpm ± SEM, $n = 13$) monocyte supernatants.

Both cytokines, IL-6 and IL-1β, have been shown to represent helper accessory signals for T cell activation. However, the requirement of IL-6 and/or IL-1β to induce T cell proliferation seems to be variable and will depend upon the *in vitro* T cell activation system. For instance, a clearer helper effect of IL-1β on T cell activation is seen when cross-linking CD3 with anti-CD3 MAb is used to stimulate T cell growth (20), while IL-6 has been identified as an essential helper factor for human T cell stimulation with PHA (4) and anti-CD2 (12). In contrast, IL-1β is unable to support PHA or anti-CD2-induced T cell growth (4,12). Therefore, we examined the direct effect of IL-6 alone or plus IL-1β on PHA-stimulated T cells. As shown in Figure 2, the addition of an optimal dose of IL-6 enhanced PHA-induced T cell proliferation, while IL-1β and IL-6 combined have a synergistic effect on PHA-induced T cell proliferation. A signifi-

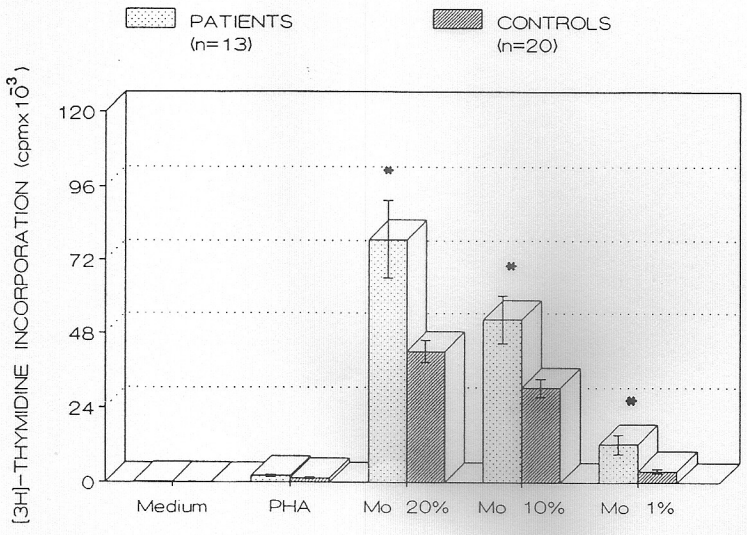

FIG. 1. Effect of monocytes on the proliferation of T cells stimulated with PHA. Purified T cells (0.25×10^6/ml) were cultured with PHA (0.5 μg/ml) alone or with autologous monocytes (1, 10, or 20%). After 4 days at 37°C [^{3}H]thymidine incorporation was measured for 8 h. Results are expressed in mean cpm ± SEM. Statistical significance *$p < 0.01$, for differences between patients and controls.

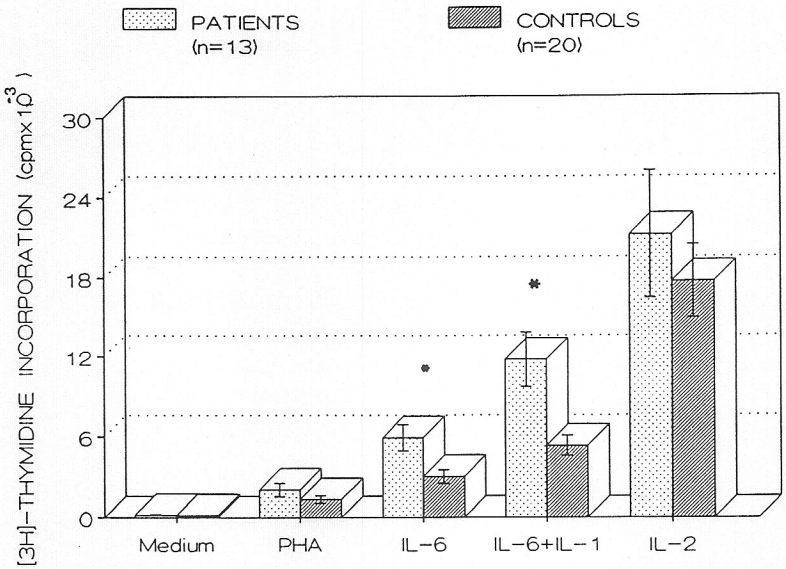

FIG. 2. Effect of cytokines on the proliferation of T cells stimulated with PHA. Purified T cells (0.25×10^6/ml) were cultured for 4 days at 37°C with PHA (0.5 μg/ml) alone or with IL-6 (100 U/ml), IL-1β (50 U/ml) plus IL-6 or IL-2 (10 U/ml). T cell proliferation was measured by [^{3}H]thymidine incorporation and the results are expressed in mean cpm $\times 10^{-3} \pm$ SEM. Statistical significance: $*p < 0.01$, for differences between patients and controls.

cant higher proliferative response was observed by adding IL-6 alone or combined with IL-1β in the nonviremic patients as compared to controls. The addition of IL-1β alone to T cell cultures stimulated with PHA had no or minimal influence on T cell proliferation (data not shown). These results further confirmed that IL-6 alone or in combination with IL-1β is involved in the T cell hyperreactivity demonstrated in the chronic nonviremic HBsAg carriers. As also observed in Figure 2, PHA-stimulated T cells from both groups similarly respond to IL-2 in the complete absence of monocytes or monocyte-produced soluble factors. Previous reports indicate that IL-6, in addition to its direct T cell growth promoting effect, also renders the T cells more responsive to IL-2 (11). Indeed, we also found that IL-6 further increases the response of PHA-stimulated T cells to IL-2 both in patients (49975 ± 6535 vs 21278 ± 4771, mean cpm ± SEM, $n = 5$) and controls (37821 ± 4027 vs 17769 ± 2772, mean cpm ± SEM, $n = 6$). Although a higher response was detected in the patients compared to controls, no significant difference was found between both groups. There is evidence that IL-6 induces an IL-2 independent pathway of T cell growth. Although IL-6 enhances T cell responsiveness to IL-2, no effect on IL-2 receptor expression or IL-2 production could be observed (11). Similar to these previous findings we did not detect enhanced IL-2 production and/or increased IL-2 receptor expression when adding IL-6 to PHA stimulated T cells from patients or controls.

IL-6R and gp130 expression on T cells activated with PHA and cytokines. Since IL-6R have been demonstrated in resting as well as in activated T-lymphocytes (7,23), we decided to examine whether differences in IL-6R expression could be involved in the T cell hyperreactivity phenomenon. Moreover, regarding IL-6R expression and its signal transducer gp130, no information has been reported on *in vitro* activated T cells with different cytokines. We used the MAbs MT18 and AM64 to detect IL-6R (p80) and gp130 expression, respectively, and incubation with biotynilated antimouse IgG followed by streptavidin fluorescein. We set up cultures of unstimulated and PHA-stimulated purified T cells in the presence or absence of the cytokines IL-6, IL-1β, and IL-2 alone or combined. Our first experiments were done to determine the optimal time to measure IL-6R (p80) expression. We found that the percentage of cells expressing these receptors was similar on unstimulated or PHA-stimulated T cells after 24 or 48 h of incubation, but decreased after 72 and 96 h of culture. For this reason, we performed all the T cell cultures for measuring IL-6R and gp130 expression for 48 h at 37°C. As shown in Figure 3A, the percentage of IL-6R positive cells on unstimulated T cells from patients (22 ± 2%) and/or controls (23 ± 2%) was similar. The

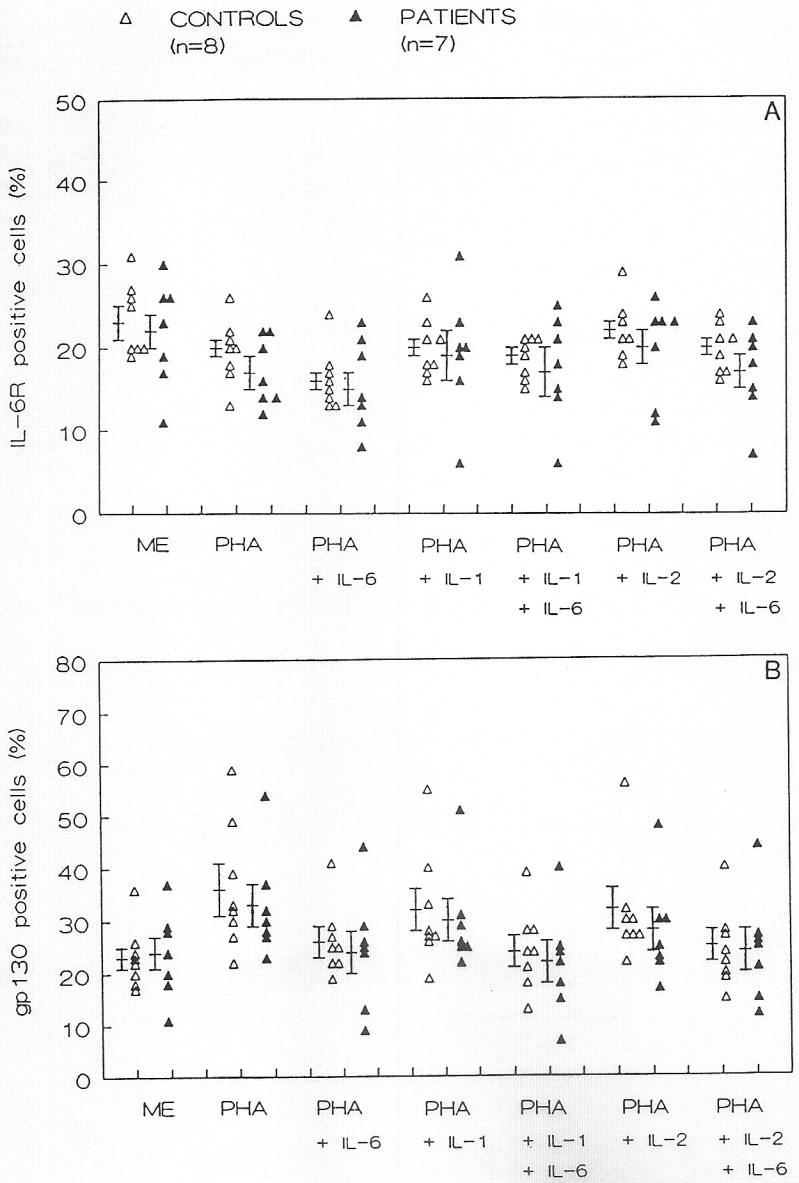

FIG. 3. IL-6 receptor expression (A) or gp130 expression (B) on unstimulated T cells and PHA-activated T cells from patients and controls. Purified T cells (0.25×10^6/ml) were cultured for 48 h with PHA (0.5 μg/ml) alone or in the presence of the cytokines IL-6 (100 U/ml), IL-1β (50 U/ml), and/or IL-2 (10 U/ml). Specific immunofluorescence was analyzed by flow cytometry as indicated in Materials and Methods. Percentages of IL-6R or gp130-positive cells are individually represented and $\overline{\underline{\mathrm{I}}}$ indicates the mean ± SEM.

level of IL-6R slightly decreased after stimulation with PHA alone or PHA + IL-6. The addition of IL-1β alone or IL-1β + IL-6 to PHA-activated T cells did not affect IL-6R expression. However, IL-2 induced a small increase of IL-6R compared to PHA-stimulated T cells. Further addition of IL-6 to activated T cells with PHA + IL-2 did not change IL-6R expression (Fig. 3A). When 10% of autologous monocytes were added to PHA-stimulated T cell cultures for 48 h, a slight increase of IL-6R was found in the control group (24 ± 2 vs 20 ± 1%) and in patients (22 ± 5 vs 17 ± 2%). No significant differences related to IL-6R expression between patients and controls were observed within all the stimulating conditions tested. These results are in agreement with a previous report where the levels of IL-6R detected with MAb MT18 on pe-

ripheral T lymphocytes did not significantly change after PHA stimulation (7). Therefore, the p80 chain does not seem to be a useful marker of T-lymphocyte activation under disease conditions.

As shown in Figure 3B, cell-surface gp130 expression on unstimulated T cells from patients (24 ± 3%) or controls (23 ± 2%) correlates with a similar distribution of IL-6R under the same conditions. However, in contrast to IL-6R expression, the gp130 IL-6 signal transducer seems to be modulated on PHA-activated T cells. First, a significant increase was observed on PHA-stimulated T cells from patients (33 ± 4%, $p < 0.05$) and controls (36 ± 5%, $p < 0.005$) compared to unstimulated T cells. When IL-6 alone or combined with IL-1β or IL-2 was added to PHA-stimulated T cells, the percentage of gp130-positive cells diminished to values comparable to those of unstimulated T cells. The same effect was observed when 10% of autologous monocytes were added to PHA-activated T cells. We did not detect interference of the MAb AM64 with IL-6 binding on PHA-activated T cells cultured with or without IL-6. The addition of IL-1β or IL-2 to PHA-stimulated T cells induced a slight decrease of gp130 expression compared to PHA alone (Fig. 3B). Similar to the findings on IL-6R expression, no significant differences in gp130 expression within the different culture conditions were found between patients and controls. We also analyzed the mean fluores-

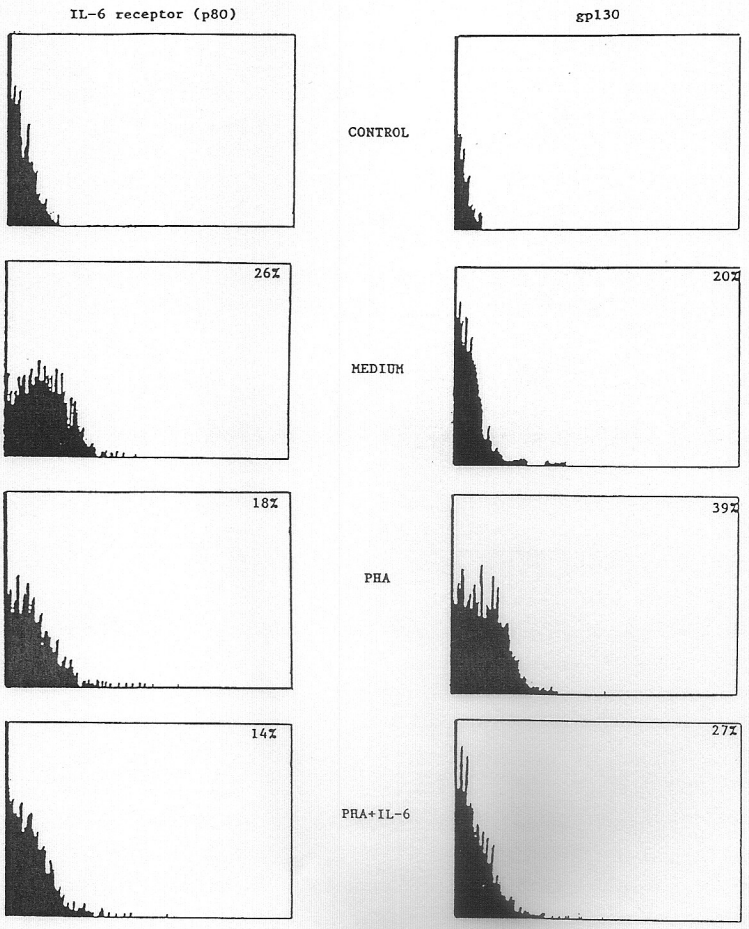

FIG. 4. Immunofluorescence analysis of IL-6 receptor and gp130 expression on PHA-activated T cells in the presence of IL-6. Purified T cells (0.25×10^6/ml) were cultured for 48 h with PHA (0.5 μg/ml) alone or in the presence of IL-6 (100 U/ml). The cells were stained with MAbs MT18 (IL-6 receptor) or AM64 (gp130) as indicated in Materials and Methods. Control cells were incubated with mouse nonspecific IgG_{2b} or IgG_1 isotype. One-color fluorescence was analyzed on flow cytometry. Histograms of a representative healthy subject are shown, with cell number on the *y*-axis and fluorescence intensity (log scale) on the x-axis. The percentage IL-6 receptor(+) or gp130(+) cells is given in the upper-right corner of each histogram.

cence intensity for IL-6R or gp130 expression in all the T cell cultures and no significant differences were found between the two groups. The representative fluorescence histograms of IL-6 receptor and gp130 expression from one healthy donor are depicted in Figure 4. They illustrate our previous findings on unstimulated and PHA-stimulated T cells alone or in the presence of IL-6.

Finally, we tested the ability of IL-6 to modulate IL-6R or gp130 expression on unstimulated T cells. When IL-6 was added to T cells for 48 h at 37°C, the percentage of IL-6R positive cells decreased 1.5- to 2-fold, while gp130 expression diminished 2- to 10-fold. No significant differences were observed between patients and controls. We did not find any effect of IL-1β or IL-2 on MT18 or AM64 staining under non-stimulating conditions. In conclusion, our results indicate that the enhanced IL-6 helper effect on PHA-induced T cell proliferation in chronic nonviremic HBV carriers is probably not mediated by disturbances of the IL-6R and/or its associated signal transducer gp130 chain. These results, however, do not exclude the possibility that higher amounts of soluble IL-6R and/or differences in the signal transduction events triggered by IL-6, could be involved in the increased T cell response to IL-6 seen in these patients. Previous studies have demonstrated the presence of natural soluble IL-6R, which are capable of binding IL-6 and mediating signals via membrane-anchored gp130 (8,15). Moreover, soluble human IL-6R enhances the effect of IL-6 on acute-phase protein production by hepatocytes presumably by formation of IL-6–gp80 complexes (14). It will be of interest to investigate whether nonviremic HBV carrier T cells and/or monocytes shed more amounts of gp80 than normal subjects and, therefore, the hyperreactivity to IL-6 could be due to IL-6–gp80 complex formation. Future experiments on this area will help to clarify the T cell hyperreactivity to IL-6 in chronic nonviremic HBV carriers.

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