Anti-CD3-Activated T Cells from Chronic Nonviremic HBV Carriers Are Hyperreactive to Monocytic Accessory Signals[1]

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We analyzed T cell responses through the CD3 activation pathway in a group of chronic HBV carriers. PBMC stimulated with the mAb OKT3 showed higher proliferative response in HBV-DNA(−) carriers compared to HBV-DNA(+) carriers and to controls. In contrast, no differences in proliferative responses were observed between HBV-DNA(−) carriers and controls in cell cultures stimulated with immobilized 64.1 mAb (SPB-64.1) which induces proliferation in the absence of monocytes. We further examined T cell responses in the presence of monocytes and their soluble factors to immobilized OKT3 mAb (SPB-OKT3). Purified T cells did not proliferate to SPB-OKT3. When autologous monocytes were added, higher proliferative response, IL-2 production, and IL-2 receptor expression were observed in HBV-DNA(−) carriers than in controls. An enhanced cell proliferation was also obtained when monocyte supernatants were added to T cells cultured with SPB-OKT3. Moreover, when IL-6 alone or combined with IL-1 was added to SPB-OKT3-stimulated T cell cultures, a significantly higher increase in T cell proliferation was detected in HBV-DNA(−) carriers. Our results thus show a T cell hyperreactivity to accessory signals from monocytes (mainly IL-6) in HBV-DNA(−) carriers, that is probably related to an ongoing viral clearance. © 1993 Academic Press, Inc.

INTRODUCTION

The replicative or viremic phase of hepatitis B virus (HBV) infection is identified by the presence in serum of hepatitis e antigen (HBeAg) and HBV-deoxyribonucleic acid (HBV-DNA) which are markers of active viral replication (1). There are also chronic HBV carriers who are in nonreplicative or nonviremic phase as identified by the presence of HBeAg antibody (anti-HBe) and the disappearance from serum of HBV-DNA, usually associated with inactive liver disease (1–3). This latter group have hepatitis B surface antigen (HBsAg) which may be produced by the hepatocyte without secretion of complete viral particles (2, 4). During the disease outcome those nonviremic HBV-DNA-negative carriers may spontaneously present signs of liver disease with reappearance of serum markers of HBV replication (1). It is believed that defective cellular immune competence could be the major cause of the tolerance to HBV antigens (1, 2, 5). Hyporeactivity to mitogenic stimuli (6), hyporesponsiveness to HBV surface proteins (7, 8), decreased cytokine synthesis (6, 9), and monocyte dysfunction (10) have all been implicated in the immunopathogenesis of the chronic HBV carrier state. Thus far, in vitro experiments utilizing different sources of HBV-encoded antigens seem to reveal that T cell immune responses in chronic HBV-infected individuals vary depending on the stage of infection (7, 8). Conclusive evidence regarding the functional status of the peripheral blood T cells stimulated through the T cell antigen receptor complex in those infected individuals has not yet been clearly established (10, 11). The T cell antigen receptor (TCR) is closely associated with the CD3 polypeptide complex (12). Under appropriate conditions, monoclonal antibodies (mAbs) to CD3 are mitogenic for T cells and they mimic the effects of the binding of antigen to TCR in inducing signal transduction events and T cell activation (12–14). The requirement of monocyte accessory signals for T cell activation is well known (15). Two monocyte-derived cytokines, IL-1 and IL-6, have previously been identified as accessory signals which can act synergistically in the process of T cell activation (16–19). The functional outcome and efficacy of T cell activation thus depends not only on appropriate TCR triggering, but also on accessory signals provided by antigen-presenting cells. The present investigation was undertaken to assess T lymphocyte responses and the effects of monocytes and accessory cell-secreted soluble factors when T cells from chronic HBV carriers are activated through the CD3 pathway.

MATERIALS AND METHODS

Patients. Twenty chronic HBsAg-positive carriers (15 males, 5 females; mean age, 42 years; range, 28–55 years) were studied following a previously standarized

protocol (20). All patients were antiHBc positive, anti-HBs negative, and IgM-antiHBc negative. Seven were HBeAg positive and HBV-DNA positive (HBV-DNA(+)) either through slot blotting or by the polymerase chain reaction (PCR) (>50 to >100 pg/ml) with a mean ALT 3.9-fold over the normal range (147 vs 37 IU/liter) and a mean AST 2.2-fold over superior limit (90 vs 40 IU/liter). Among this group five have chronic active hepatitis and two have active cirrhosis as shown by liver biopsy. Thirteen individuals showed anti-HBe positive, ALT and AST within normal ranges, and serum HBV-DNA undetectable by slot blotting and by PCR assay (HBV-DNA(−)). No patient was receiving antiviral drugs prior entering to the study. All the patients were negative for antibodies to C hepatitis (anti-HCV) and to HIV. Twenty-two healthy individuals, seronegative for HBV, HCV, and HIV markers, were selected as control group.

Viral markers. 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 (Abbott Laboratories, North Chicago, IL), the former through the second generation kit.

HBV-DNA assay. HBV-DNA was detected by slot blotting and the PCR assay as described elsewhere (20, 21). We used one set of oligonucleotides specific for the region encoding the pre-core:core sequence on the HBV genome (21). Semiquantitation of viral DNA was carried out using HBV-DNA (3.2 kb in length) derived from plasmid pAM (kindly donated by Dr. Robert Purcell, NIH, USA). Samples of 5, 10, 50, and 100 pg of total viral DNA were amplified by PCR, analyzed by agarose gel electrophoresis and stained with ethidium bromide. The amount of HBV-DNA was estimated in the range of 5 to 100 pg by comparing the DNA band intensity in the positive serum samples with the band intensity of the controls.

Monoclonal antibodies, cytokines, and other reagents. The mAb OKT3 and 64.1, directed to the CD3 antigen (22), were purchased from Ortho Diagnostic System (Raritan, NJ) and Oncogen (Seattle, WA), respectively. Mouse ascites fluid containing anti-Tac mAb (directed to the p55 chain of the IL-2 receptor, CD25) was a generous gift from Dr T. Waldmann (NIH, Bethesda, MD) (23). The mAb clonab IL-2R FITC (CD25) was obtained from Biotest Diagnostics (Dreieich, Germany) and used for the detection of membrane IL-2R in fluorescence studies. MO2 (CD14), B4 (CD 19), T3 (CD3) in FITC-conjugated form, were purchased from Coulter Corp. (Hialeah, FL). Leu11b-FITC (CD16) was purchased from Becton Dickinson (San Jose, CA). Affinity-purified goat anti-mouse IgG was obtained from Tago (Burlingame, CA). Recombinant IL-1 and IL-6 were purchased from Genzyme (Boston, MA), indomethacin

was from Sigma (St. Louis, MO), and [³H]thymidine (specific activity, 2 mCi/mmol) was purchased from New England Nuclear (Boston, MA).

Cell separation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood 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). As a source of monocytes, adherent cells were obtained by incubating PBMC (30 × 10⁶ in 10 ml of culture medium plus 20% FBS) in culture grade plastic petri dishes (Falcon, Becton Dickinson, NJ) at 37°C for 1 h. Nonadherent cells were decanted and monocytes were collected with a rubber stab. Enriched T cells were obtained by passage of the plastic nonadherent cells through a nylon wool column (24). Highly purified T cells were obtained by further treatment of the enriched T cell fraction with lympho-Kwik-T (One Lambda Inc., Los Angeles, CA), supplemented with the complement-fixing mAb anti-CD14 (MO2, Coulter Corp., Hialeah, FL) and anti-CD16 (Leu11b, Becton Dickinson, San José, CA) as previously described (25, 26). Briefly, nylon wool non-adherent cells (10–15 × 10⁶) were resuspended in 200 μl of complete medium and were incubated for 30 min at 4°C, with the reagent mixture and washed once. The T-lympho-Kwik reagent (0.8 ml) was then added. This reagent contains anti-monocyte and anti-B cell mAbs and complement. The mixture was incubated for 1 hr at 37°C. Cells were centrifuged at 1000g for 5 min, washed twice, and resuspended in complete culture medium. The resulting cells contained more than 98% CD3(+), less than 1–2% CD16(+), and less than 0.5% CD14(+) cells.

Production of monocyte culture supernatants. Cell-free monocyte culture supernatants were obtained by incubating adherent cells (30 × 10⁶ PBMC) in petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) at 37°C overnight with 6 ml of RPMI 1640 plus 20% FBS and indomethacin (1 μg/ml) in order to avoid prostaglandin production (16). The supernatants were recovered, centrifuged, and passed through 0.45-μm pore size filters (Sigma). Supernatants were obtained from monocytes cultures from all normal donors and patients. The concentrations of IL-1 and IL-6 were measured by ELISA (Quantikine, R & D Systems, MN). The supernatants did not contain measurable amounts of IL-2 (<0.05 U/ml).

Coating of culture plates with anti-mouse IgG. Culture plates were uncoated or coated with affinity-purified goat anti-mouse IgG. To this end, 100 or 500 μl of antibody (10 μg/ml) diluted in bicarbonate buffer (pH

9.6) were put in flat-bottomed culture wells of a 96-well microculture plate and in wells of a 24-well culture plate (Falcon, Becton Dickinson), respectively, and incubated overnight at 4°C. They were washed two times with RPMI medium. This solid-phase anti-mouse IgG replaces monocyte Fc receptor and provides a frame for binding of the Fc part of anti-CD3 mAbs (27). When cells were cultured with OKT3 or Ab 64.1 on these coated plates, we used the term "solid-phase-bound OKT3" (SPB-OKT3) or "solid-phase-bound 64.1" (SPB-64.1).

Proliferation studies. PBMC and T cells were cultured in complete medium in 200-μl volumes at a concentration of 5×10^5 cells/ml or 2.5×10^5 cells/ml respectively in flat-bottomed microtiter plates (Falcon 3872, Becton Dickinson) that were either uncoated or coated with anti-mouse IgG. Predetermined optimal doses of mAbs OKT3 (10 ng/ml) and 64.1 (100 ng/ml) were added. All cultures were performed in triplicates and incubated for 4 days at 37°C in a 5% CO_2 atmosphere. Cultures were pulsed with 1 μCi of [^{3}H]thymidine per well, 18 hr (PBMC) or 8 hr (T cells) before the end of incubation. Cells were harvested on glass fiber filter and thymidine incorporation was measured in a liquid scintillation counter (1205 Betaplate, LBK Wallac, Turku, Finland).

IL-2 production. PBMC (1×10^6/ml) or T cells (0.5×10^6/ml) stimulated with optimal dose of OKT3 or 64.1 mAb were cultured in complete medium in flat-bottomed wells of a 24-well plate (Falcon 3047, Becton Dickinson), uncoated (PBMC) or coated (T cells) with anti-mouse IgG. The anti-Tac mAb (1/10.000 final dilution of ascites fluid) was added to block the IL-2 receptor and to prevent IL-2 consumption (28). The supernatants were collected after 48 hr. IL-2 activity was tested on an IL-2-dependent mouse cell line CTLL (29). The culture supernatants were added in serial dilutions to 1×10^4 CTLL cells in 200 μl culture medium containing 5×10^{-5} M mercaptoethanol in flat-bottomed microculture plates. After 24 hr of culture, [^{3}H]thymidine (1 μCi/well) was added for 6 hr. The CTLL cells were harvested and radioactivity on the filter paper was counted. Results are expressed in milliunits per milliliter by comparison with a standard curve, obtained with serial dilutions of an IL-2 reference preparation provided by the Biological Response Modifiers Program (NCI, Bethesda, MD).

IL-2 receptor expression. PBMC (1×10^6/ml) or T cells (0.5×10^6/ml) were cultured under the same conditions described above for IL-2 production but in the absence of anti-Tac. After 48 hr culture at 37°C, the cells were washed twice and resuspended in 100 μl of PBS containing 1% bovine serum albumin. The cell suspension was incubated for 40 min at 4°C with 10 μl of FITC-conjugated anti-IL-2R (p55 chain) mAb. Controls include cells incubated with FITC-conjugated mouse IgG2a. The cells were washed twice with PBS and fixed in 2% paraformaldehyde. Specific immunofluorescence was analyzed by flow cytometry (EPICS 753, Coulter Corp.).

Statistical analysis. The statistical significance between groups was assessed by Student t test for unpaired samples.

RESULTS

Proliferative responses, IL-2 production, and IL-2 receptor expression of PBMC to anti-CD3 mAbs. We first examined the effect of optimal concentrations of soluble mAbs OKT3 or 64.1 on cultures of PBMC from chronic HBV carriers and controls. PBMC from the HBV-DNA(−) group showed a significantly higher proliferative response than that from controls ($P < 0.001$) and HBV-DNA(+) patients when stimulated with soluble OKT3 (Fig. 1). The response of PBMC from HBV-DNA(+) patients to OKT3 was not different from that of controls. When PBMC were stimulated with the 64.1 mAb, which does not require a second signal to induce T cell proliferation, no difference was observed between HBV-DNA(−) patients and controls while the HBV-DNA(+) group demonstrated significantly lower responses than controls (Fig. 1). PBMC were also cultured on goat anti-mouse-coated plates in order to provide a solid phase for binding and crosslinking of the Fc part of the anti-CD3 mAb. This solid-phase anti-mouse IgG replaces monocyte Fc receptors minimizing differences due to an unequal expression of these Fc receptors among subjects. As shown in Fig. 1 the results observed in the three groups when PBMC were stimulated with SPB-OKT3 or SPB-64.1 mAbs were higher than those with soluble mAb, but the differences between the three study groups were comparable to those obtained with the soluble anti-CD3 mAbs. The differences in T cells responses thus are not related to differences in Fc receptor expression and/or function.

Regarding IL-2 production, PBMC from HBV-DNA(−) carriers consistently and significantly demonstrated increased IL-2 production to both soluble anti-CD3 mAbs compared to controls, while the HBV-DNA(+) patients showed elevated levels of IL-2 only when exposed to soluble OKT3 (Table 1). An increased IL-2 receptor expression (p55 chain) was also found in the HBV-DNA(−) patients compared to controls when cultured with soluble OKT3 mAb (Table 1). The phenotypic distribution of PBMC subpopulations was similar in both groups of HBV carriers and controls. The percentage of CD3 + T cells (HBV-DNA(−), 66 ± 2; HBV-DNA(+), 69 ± 4; controls, 60 ± 2) and monocytes (HBV-DNA(−), 19 ± 2; HBV-DNA(+), 16 ± 2; controls, 15 ± 1; means ± SEM) were comparable in either study group. T cells from HBV-DNA(−) carriers then
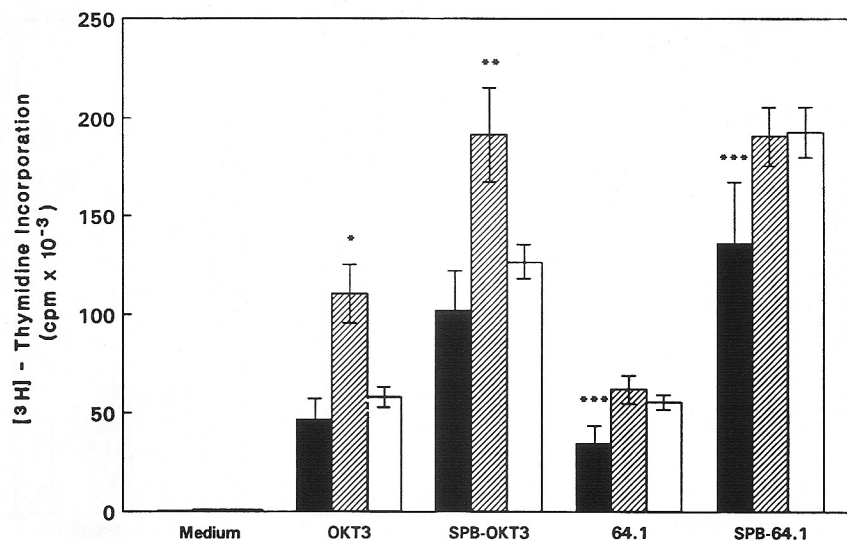
FIG. 1. Proliferative responses induced by anti-CD3 mAbs in peripheral blood mononuclear cells from HBV-DNA(+) carriers, ■ (n = 7), HBV-DNA(−) carriers, ▨ (n = 10), and healthy controls, □ (n = 14). PBMC (0.5×10^6/ml) were cultured in complete medium with optimal doses of mAb OKT3 (10 ng/ml) or with mAb 64.1 (100 ng/ml). mAb OKT3 and 64.1 were used either in soluble or in immobilized form with SPB anti-mouse IgG. After 72 hr at 37°C, cell proliferation was determined by [³H]thymidine incorporation and results were expressed in mean cpm $\times 10^{-3} \pm$ SEM. Statistical significance: *$P < 0.001$ versus controls; **$P < 0.01$ versus controls; ***$P < 0.05$ versus controls and HBV-DNA(−) carriers.

were hyperreactive in terms of proliferation, IL-2 production, and IL-2 receptor expression in response to an accessory cell-dependent polyclonal stimulus (OKT3). In contrast, T cells from HBV-DNA(+) carriers had a decrease T cell response to an accessory signal-independent stimulus (mAb 64.1).

T cell response to immobilized anti-CD3 mAbs and the role of accessory signals. The results above suggested that the higher response of T cells from HBV-DNA(−) carriers was due to accessory signals. To further investigate the T cell hyperreactivity to OKT3 found in the HBV-DNA(−) patients, we examined the effect of monocytes and their soluble factors on the proliferative response of highly purified T cells using SPB-OKT3 as the primary activating signal. As shown in Fig. 2, purified T cells from HBV-DNA(−) carriers and controls were poorly responsive to SPB-OKT3 alone.

This is in accordance with our previous results (30). When increasing concentrations of autologous monocytes (1, 10, and 20%) were added, a significantly higher proliferative response was achieved in the HBV-DNA(−) group than that in the controls. On the other hand, using SPB-64.1 anti-CD3 mAb which induces proliferation of purified T cells in the absence of monocytes, no significant difference in the proliferative response was found between the group of HBV-DNA(−) patients and controls (Fig. 2). The latter demonstrates that the intrinsic reactivity of T cells to anti-CD3 is not altered. We also set up experiments to analyze T cell reactivity to autologous (AuMoS) or allogeneic (AlMoS) monocyte supernatants. As depicted in Fig. 3, SPB-OKT3 stimulated T cells from HBV-DNA(−) carriers displayed a striking and significantly higher response in the presence of either AuMoS (patients) or AlMoS (controls) compared to con-

TABLE 1

IL-2 Production and IL-2 Receptor Expression of PBMC Stimulated with Anti-CD3 mAbs in HBV Chronic Carriers

	IL-2 production (mU/ml)			% IL-2 receptor (+) cells		
	HBV-DNA(+)	HBV-DNA(−)	Controls	HBV-DNA(+)	HBV-DNA(−)	Controls
Medium	72 ± 32	57 ± 8	58 ± 10	16.4 ± 2.3	18.0 ± 2.6	13.9 ± 0.9
OKT3	1760 ± .640*	2330 ± 520**	825 ± 100	38.0 ± 5.0	49.0 ± 4.5***	39.0 ± 2.6
64.1	737 ± 270	1610 ± 350**	488 ± 80	36.4 ± 4.2	49.0 ± 4.2	42.0 ± 2.8

Note. PBMC (1×10^6/ml) were stimulated with optimal doses of soluble OKT3 (10 ng/ml) or mAb 64.1 (100 ng/ml) for 48 hr. IL-2 was measured in the supernatants using a bioassay on CTLL. Results are expressed in milliunits per milliliter. IL-2 receptor expression was measured by immunofluorescence with anti-IL-2 receptor (p55 chain) mAb and analyzed by flow cytometry. Mean ± SEM of 7 patients HBV-DNA(+), 13 patients HBV-DNA(−), and 22 controls are represented.

  * $P < 0.02$ versus controls.
 ** $P < 0.001$ versus controls.
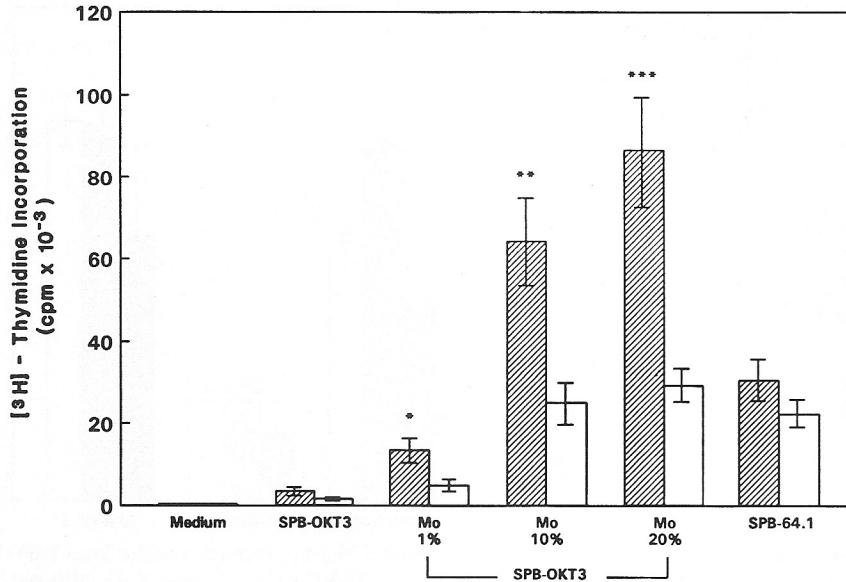*** $P < 0.005$ versus controls.

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FIG. 2. Effect of monocytes on the proliferation of T cells stimulated with SPB-OKT3 from HBV-DNA(−) carriers, ▨ (n = 13), and controls, □ (n = 16). Peripheral blood T cells (0.25 × 10^6/ml) were cultured on anti-mouse IgG-coated plates with mAb OKT3 (10 ng/ml) without or with autologous monocytes (1, 10, or 20%). T cells were also cultured with mAb 64.1 (100 ng/ml) in the absence of monocytes. After 4 days at 37°C [³H]thymidine incorporation was measured for 8 hr. Results are expressed in mean cpm × 10⁻³ ± SEM. Statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001, for differences between patients and controls.

trol T cells. The concentrations of IL-1 and IL-6 found in the cell-free monocyte culture supernatants from 13 patients and 15 controls were similar (IL-1, 249 ± 30 vs 208 ± 28 pg/ml; IL-6, 864 ± 104 vs 711 ± 106 pg/ml, respectively; mean ± SEM, P > 0.05). Altogether, these results suggest that T cells from HBV-DNA(−) carriers are more sensitive to accessory signaling by monocyte-synthesized soluble factors.

IL-2 production and IL-2 receptor (IL-2R) expression of T cells stimulated with immobilized anti-CD3 mAbs. IL-2 production in unstimulated T cell cultures was always low and similar in the HBV-DNA(−) carriers and controls (Table 2). Stimulation with SPB-OKT3 alone induced a small increase of IL-2 production in these two groups. However, we found a strongly increased IL-2 secretion when monocytes were added to
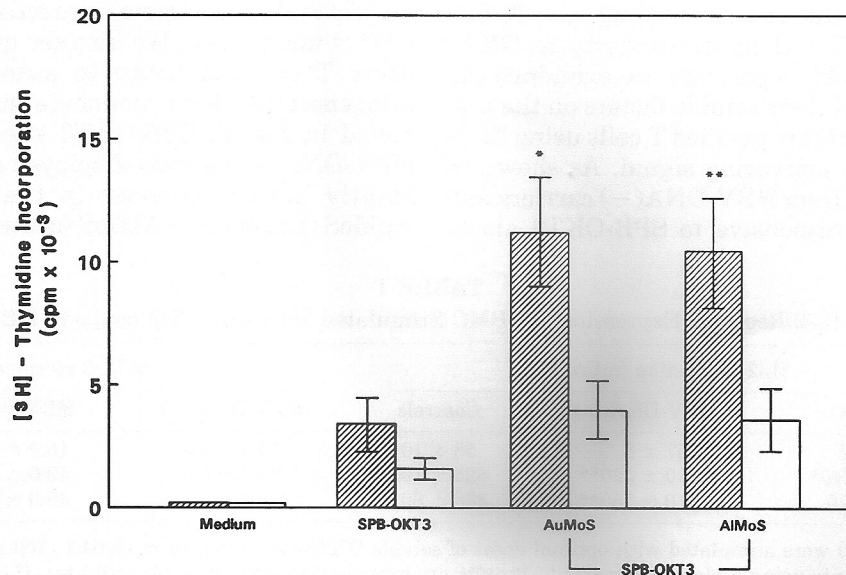

FIG. 3. Effect of monocyte cell-free supernatants on T cell proliferation induced by SPB-OKT3 in HBV-DNA(−) carriers, ▨ (n = 13), and controls, □ (n = 16). Peripheral blood T cells (0.25 × 10^6/ml) were cultured on anti-mouse IgG-coated plates with mAb OKT3 (10 ng/ml) without or with autologous (AuMoS) or allogeneic (AlMoS) monocyte supernatants (10% v/v). After 96 hr at 37°C, cell proliferation was determined by [³H]thymidine incorporation and results were expressed in mean cpm × 10⁻³ ± SEM. Statistical significance: *P < 0.01; **P < 0.02.

TABLE 2

IL-2 Production and IL-2 Receptor Expression of T Cells Stimulated with SPB-OKT3 or SPB-64.1 in HBV Chronic Carriers

Culture addition	IL-2 production (mU/ml)		% IL-2 receptor (+) cells	
	HBV-DNA (−)	Controls	HBV-DNA (−)	Controls
Medium	77 ± 16	71 ± 13	16 ± 3*	9 ± 2
SPB-OKT3	191 ± 49	156 ± 20	34 ± 5**	17 ± 2
SPB-OKT3 + Mo 20%	981 ± 271*	477 ± 56	58 ± 6**	32 ± 4
SPB-OKT3 + AuMos 10%	257 ± 43	250 ± 28	40 ± 8	26 ± 3
SPB-64.1	355 ± 60	324 ± 30	52 ± 8	44 ± 5

Note. Isolated T cells (0.5 × 10^6/ml) were cultured on anti-mouse IgG-coated plates. Cells were stimulated with OKT3 (10 ng/ml) or mAb 64.1 (100 ng/ml) for 48 hr. IL-2 activity was measured in the supernatants using bioassay on CTLL. Results are expressed in milliunits per milliliter. IL-2 receptor expression was measured by immunofluorescence with anti-IL-2 receptor mAb and analyzed by flow cytometry. Means ± SEM of 6 patients HBV-DNA(−) and 13 controls are represented.

* $P < 0.05$ versus controls.
** $P < 0.001$ versus controls.

SPB-OKT3-stimulated T cells which was significantly higher in the group of HBV-DNA(−) patients compared to controls. In the presence of autologous monocyte supernatants, a slight increase of IL-2 production

was observed which was similar in both groups. As shown also in Table 2, IL-2 production in stimulated cultures with SPB-64.1 was comparable in HBV-DNA(−) and control groups. An increased spontaneous IL-2R expression (p55 chain) was detected in patient's nonstimulated T cells cultures compared to controls (Table 2). When T cells were cultured with SPB-OKT3 alone or in the presence of 20% autologous monocytes, a significantly higher IL-2R expression was also found in the group of HBV-DNA(−) patients. The percentage of T cells expressing IL-2R in T cell cultures stimulated with SPB-OKT3 plus autologous monocyte supernatants or SPB-64.1 alone was similar in both HBV-DNA(−) and control groups. IL-2R expression was studied by one-color flow cytometry analysis. The representative fluorescence histograms from one patient and one control are depicted in Fig. 4 to illustrate the latter findings.

Effect of IL-1 and IL-6 on SPB-OKT3-stimulated T cells. In order to identify the possible monocyte soluble factor(s) involved in the hyperreactive pattern observed in HBV-DNA(−) patients, we examined the effect of recombinant IL-1 and IL-6 alone or combined on SPB-OKT3-stimulated T cells. As shown in Fig. 5, addition of two different doses of IL-1 enhanced proliferation induced by SPB-OKT3 in patients as well as in controls. In aggreement with previous findings (30), we did not observe any effect of IL-6 alone on T cell pro-
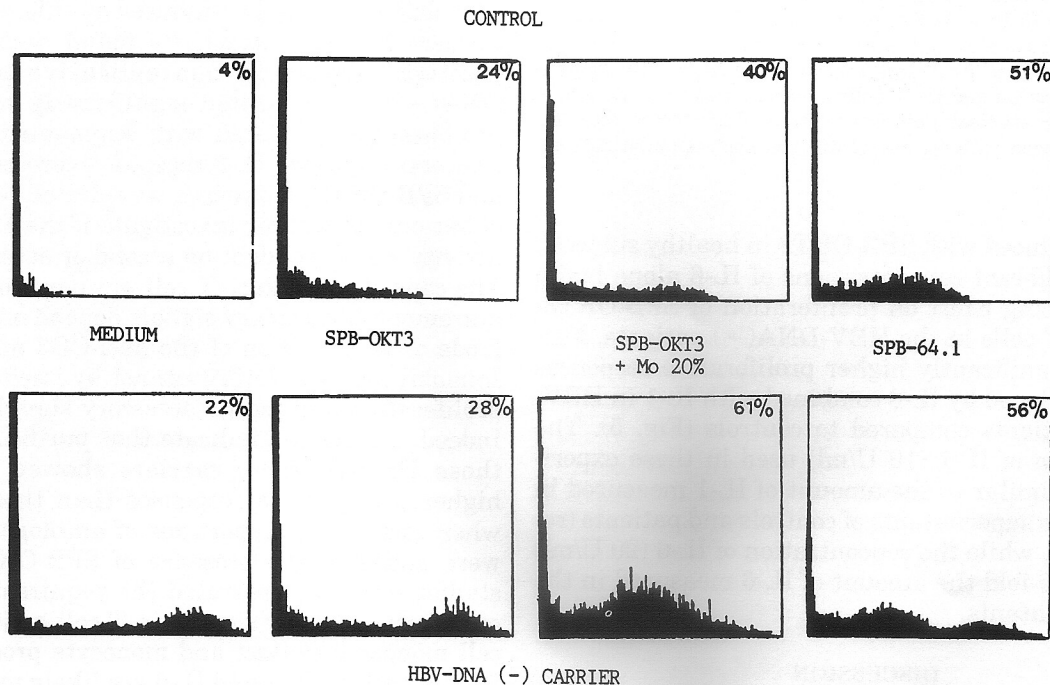

FIG. 4. Immunofluorescence analysis of IL-2 receptor expression (p55 chain) on unstimulated T cells and anti-CD3 mAbs-activated T cells. Purified T cells (0.5 × 10^6/ml) were cultured for 48 hr with SPB-OKT3 (10 ng/ml) alone or in the presence of autologous monocytes (20%), or with SPB-64.1 (100 ng/ml) alone. The cells were stained with FITC-conjugated anti-IL-2R mAb. One-color fluorescence was analyzed on flow cytometry. Histograms of a representative HBV-DNA(−) carrier and one control are shown, with cell number on the Y-axis and fluorescence intensity on the X-axis. The percentage IL-2 receptor (+) cells is given in the upper-right corner of each histogram.
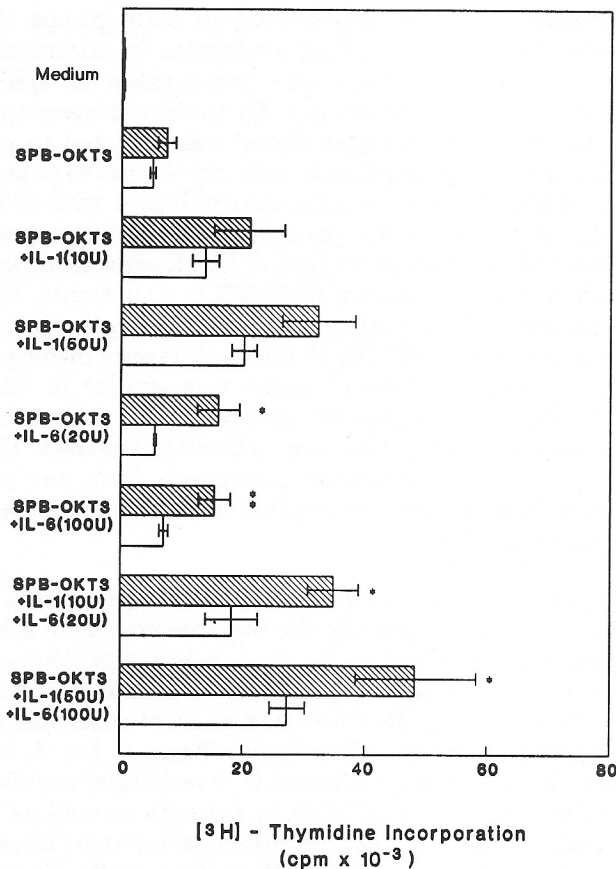
[³H] - Thymidine Incorporation
(cpm x 10⁻³)

FIG. 5. Effect of IL-1 and/or IL-6 on SPB-OKT3-stimulated T cells from HBV-DNA(−) carriers, ▨ (n = 6), and controls, ☐ (n = 7). Purified T cells (0.25 × 10⁶/ml) were cultured for 4 days on anti-mouse IgG-coated plates with mAb OKT3 (10 ng/ml) and different doses of IL-1 and IL-6. T cell proliferation was measured by [³H]thy-midine incorporation and the results are expressed in mean cpm × 10⁻³ ± SEM. Statistical significance: *P < 0.05; **P < 0.01, for differences between patients and controls at each stimulating combinations.

liferation induced with SPB-OKT3 in healthy subjects. However, different concentrations of IL-6 alone had a clear enhancing effect on proliferation of SPB-OKT3-stimulated T cells in the HBV-DNA(−) patients. Furthermore, significantly higher proliferative responses were also induced by IL-6 combined with IL-1 in HBV-DNA(−) patients compared to controls (Fig. 5). The concentration of IL-1 (10 U/ml) used in these experiments was similar to the amount of IL-1 measured in the monocyte supernatants of controls and patients (results, Fig. 3), while the concentration of IL-6 (20 U/ml) was about 10-fold the amount of IL-6 measured in the same supernatants.

DISCUSSION

In a group of chronic HBV carriers, we have investigated potential abnormalities of the peripheral T lymphocytes stimulated through the CD3 pathway. We found that the PBMC of either HBV-DNA(+) or HBV-

DNA(−) patients were able to sustain proliferative responses, IL-2 production, and IL-2 receptor expression when stimulated with soluble or immobilized (SPB) OKT3 or 64.1 mAbs. Patients in high replicative phase (HBsAg⁺, HBeAg⁺, HBV-DNA(+)) and with active liver necroinflammation showed lower proliferative responses to both anti-CD3 mAbs compared to HBV-DNA(−) carriers. Early reports have found that HBsAg-positive carriers with serum HBeAg are hyporesponders to mitogens (6). The decreased reactivity obtained in those patients could be related to the immunomodulation induced by viral infection. For instance, the circulating excess of HBeAg as well as the presence of extrachromosomal or replicative forms of HBV-DNA in the mononuclear cells of the HBeAg-positive carriers have been implicated in the functional abnormalities found in those patients (10, 31). Thus, it is probable that an inhibitory effect of the antigenic excess might be mainly exerted on T lymphocyte proliferation. Certainly, in the experiments using mAb 64.1 which induced T cell growth partly independent of monocytes, the hyporesponsivenesses of the HBV-DNA(+) group was more relevant, suggesting a possible defective T cell response rather than a monocytic dysfunction. In this sense, PBMC hyporesponsiveness to 64.1 mAb was not associated with deficient IL-2 production probably reflecting a minor usage of this cytokine during T cell proliferative expansion. These results are in agreement with a previous report where the addition of exogenous IL-2 did not reconstitute defective mitogen-induced proliferation in HBV carriers (6). In contrast, we found that the HBsAg-positive carriers in nonreplicative phase (HBV-DNA(−)) demonstrated significantly higher PBMC proliferation correlated with augmented IL-2 production and increased IL-2 receptor expression to soluble and SPB-OKT3. Therefore, we selected this latter type of patients to further investigate if the T cell hyperreactivity was dependent on second or accessory signals. The capacity to induce T cell proliferation and the requirement of accessory signals depend on the type and mode of presentation of the anti-CD3 mAb used (30). Immobilized mAb OKT3 cannot by itself induce T cell proliferation and second accessory signals are needed. Indeed, our results indicate that purified T cells from those HBV-DNA(−) carriers showed significantly higher proliferative responses than those of controls when increasing proportions of autologous monocytes were added in the presence of SPB-OKT3. Previous studies have demonstrated the requirement of monocyte-derived helper signals in T cell activation (15). T cell monocyte contact and monocyte produced soluble factors such as IL-1 and IL-6 are likely to be important (16–19). Within this context, we searched for the effect of either autologous (patients) or allogeneic (controls) monocyte supernatants on lymphocyte proliferation in cultures stimulated with SPB-OKT3. A clear increased

T cell sensitivity to monocyte-soluble factors was obtained in the HBV-DNA(−) carriers. The possibility that an augmented monocyte function could be involved in the observed T cell hyperreactivity cannot be completely ruled out. However, the content of IL-1 and IL-6 measured in the cell-free supernatants was similar between HBV-DNA(−) patients and controls. Moreover, we showed that the addition of exogenous IL-6 alone or in combination with IL-1 was also able to induce higher proliferative responses in T cells of HBV-DNA(−) carriers through SPB-OKT3 activation. Since it has been shown that receptors for IL-6 are present on resting T cells (32), it will be of interest to extend such studies in those patients. Other secreted monocyte factors different from IL-1 or IL-6 could be involved in the T cell-hyperreactive pattern depending on the activation stimulus. For instance, tumor necrosis factor-α (TNF-α) might be another candidate to be tested on immobilized anti-CD3-induced T cell proliferation (33). Then the hyperreactivity seems to be dependent on an increased sensitivity to intact monocytes and to monocyte soluble factors such as IL-6. The results obtained using SPB-64.1 mAb support this hypothesis. This mAb is able to induce T cell growth in the complete absence of monocytes; thus, apparently 64.1 mAb acts as an accessory signal-independent stimulus for T cell activation (30). In this regard, no difference was found in T cell proliferative responses to SPB-64.1 between patients and controls.

We also found a significantly elevated IL-2 production when T cells from the HBV-DNA(−) carriers were cultured with SPB-OKT3 plus monocytes. There are conflicting results regarding IL-2 secretion in chronic HBV carriers (6, 9, 34). However, it should be pointed out that we measured IL-2 production by adding the mAb anti-Tac to the cultures in order to block IL-2 consumption, thus providing a more exact quantification of the secreted IL-2. This modification avoids differences in the capacity of the lymphocytes to consume IL-2 which might interfere with interpretation of the results under disease conditions (28). Thus, our findings confirm previous studies which have suggested a preserved IL-2 production of PBMC in patients seropositive for HBsAg (6, 34). Other T cell-secreted cytokines could be involved in the T cell hyperreactivity seen in the HBV-DNA(−) carriers. For instance, previous studies indicate that interferon-γ (IFN-γ) production is directly related to the proliferative effects of OKT3 (35), and, more recently, it has been showed that human IFN-γ up-regulates IL-2 receptor expression on mitogen-activated T cells (36). The analysis of the IL-2 receptor expression was also compatible with the higher T cell proliferative responses and with the augmented IL-2 production found in the HBV-DNA(−)-infected group. Moreover, a T cell population expressing increased density of IL-2 receptors was identified on unstimulated T cells suggesting a preactivated T cell status.

Prior studies of hepatitis B virus-infected subjects have documented that even in the absence of serum HBV-DNA, integrated forms of HBV genomes could be identified in hepatocytes and in peripheral blood lymphocytes (1, 2, 5, 31). Hepatitis B surface antigenemia due to translation of the integrated HBV-DNA persisted in these patients (5). There is also evidence that these carriers exhibit CD4$^+$ peripheral T lymphocytes able to proliferate to HBcAg and HBeAg (7, 8). Thus, the monocyte-mediated T cell hyperreactivity described here might be related to an ongoing viral clearance associated with persistent serum levels of anti-HBe. Therefore, peripheral T cells polyclonally activated through the CD3 pathway might contain T lymphocyte subpopulations that could be potentially expanded when stimulated with specific HBV peptides which are considered to play main roles in T cell tolerance and the resolution of HBV infection.

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