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Abnormal T-cell activation in chronic hepatitis B viral infection: a consequence of monocyte dysfunction?

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SUMMARY

The process of T-cell activation in chronic hepatitis B virus (HBV) carriers has been investigated by measurement of membrane expression of lymphocyte-activation markers in response to a variety of mitogenic stimuli in order to delineate further the abnormality of T-cell-mediated immunity present in such patients. A substantial proportion of unstimulated T cells from the peripheral blood of patients but not controls expressed HLA-DR; in contrast the IL-2 and transferrin receptors were rarely expressed spontaneously in either group and there was no difference in spontaneous lymphocyte transformation. After stimulation with monocyte-dependent T-cell mitogens, phytohaemagglutinin (PHA) or anti-T3, patients had significantly reduced expression of the IL-2 and transferrin receptors and of HLA-DR in association with impaired lymphocyte transformations compared to controls. In contrast, lymphocyte activation was normal in response to the monocyte-independent T-cell mitogen phorbol-myristate-acetate (PMA). These data confirm that the process of T-cell activation is abnormal in chronic HBV carriers but suggest that the T cell is intrinsically normal. In allogeneic co-cultures, monocytes from patients inhibited the transformation of normal and patients' lymphocytes in response to PHA, suggesting that defects of T-cell-mediated immunity in chronic HBV carriers may be a consequence of monocyte dysfunction.

INTRODUCTION

Exposure to the hepatitis B virus (HBV) results in a chronic carrier state in approximately 10% of previously healthy adults (Barker & Murray, 1971). Chronic HBV infection develops more frequently in neonates or infants (Beasley *et al.*, 1983; Gerety *et al.*, 1974), those with Down's syndrome (Stoller & Collmann, 1965; Blumberg, Sutnick & London, 1970) and patients on immunosuppressive therapy (Sagnelli *et al.*, 1980) than would otherwise be expected by chance, and is thought to be a consequence of a relative impairment of cellular immunity in these groups. Further evidence for the view that abnormal cellular immunity may be related to the carrier state is that T-cell-mediated immunity (Hanson *et al.*, 1984) has been shown consistently to be abnormal in chronic HBV carriers in whom there is no evidence of, or reason to suspect, a pre-existing immune deficit and which, in these instances, is presumed to be secondary to chronic HBV infection or chronic liver disease.

The mechanisms underlying defective T-cell-mediated immunity in otherwise healthy adults with chronic HBV infection are unclear, and some of the documented abnormalities are in apparent contradiction. Suppressor cell control of T-cell proliferation is impaired (Chisari *et al.*, 1981), lymphocyte transformation in response to mitogens is low (Giustino, Dudley & Sherlock, 1972) or normal (Viola *et al.*, 1982), and IL-2 activity in culture supernatants of peripheral blood mononuclear cells (PBMC) is reduced (Saxena *et al.*, 1985). In contrast, the proportion of T cells isolated from peripheral blood that express HLA-DR prior to exposure to mitogens is increased (Fukiu *et al.*, 1984), a finding that has been interpreted as indicative of *in vivo* activation.

In order to determine whether the observed reduction in IL-2 activity is a consequence of monocyte function, a direct consequence of T-cell dysfunction or due to impaired monocyte–T-cell interaction, we have investigated the process of T-cell activation in response to phytohaemagglutin (PHA) and phorbol-myristate-acetate (PMA), mitogens that have fundamentally different modes of action.

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MATERIALS AND METHODS

Patients

Forty-three patients sero-positive for HBsAg for at least 12 months but negative for antibody to the human immunodeficiency virus (HIV) have been investigated. The liver biopsy appearances in 25 cases showed chronic active hepatitis, and in

15 of these cirrhosis was already present. Three were seropositive for anti-HBe, while the remainder had evidence of active viral replication with HBeAg or DNA-polymerase in serum. Thirteen patients had chronic persistent hepatitis (inflammation confined to the portal tracts) and 12 were male. Nine were homosexual. All of these had HBeAg or DNA-polymerase in serum. Five patients (1 female) had inactive liver disease, one with cirrhosis, and all of these were anti-HBe positive.

Normal controls were recruited from laboratory staff.

Reagents

Mouse monoclonal antibodies. Anti-Tac was a generous gift of Dr T. Waldmann (National Health Institute, Bethesda, MD). Phycoerythrin (PE)-conjugated anti-IL-2 receptor, biotinylated anti-Leu 3a, biotinylated anti-Leu 2a, rhodamine-conjugated anti-HLA-DR antibodies (this monoclonal antibody does not cross-react with either HLA-DP or HLA-DQ; Beckton-Dickinson) fluorescein isothiocyanate (FITC)-conjugated anti-transferrin (TRF)-receptor and avidin fluorescein-conjugate were obtained from Beckton-Dickinson (Laboratory Impex Ltd, London, Middlesex). OKT3, OKT4, OKT8 and OKT9 monoclonal antibodies were obtained from Ortho Diagnostics Systems Inc. (Mountain View, CA).

Other reagents. FITC-conjugated polyclonal goat anti-mouse Ig was obtained from Dako (Dakopatts, Mercia Brocades Ltd, Weybridge, Surrey). 4β-phorbol-12β-myristate-13α-acetate (PMA) (Sigma, London) was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1 mg/ml and stored in small aliquots at $-20°$. The final dilution of PMA was made in RPMI-1640. PHA was obtained from Sigma.

Cell preparation and culture

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood by standard Ficoll–Triosil (Nyegaard & Co., As, Oslo, Norway) density gradient centrifugation (Boyum, 1968). After washing three times with Hank's balanced salt solution (HBSS) (Wellcome, Beckenham, Kent), PBMC were adjusted at a concentration of 1×10^6 cells/ml in 10% fetal calf serum (FCS) (Gibco, Paisley, Renfrewshire) in RPMI-1640 (Gibco) containing 2 mM glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml amphotericin B. The viability, according to trypan blue exclusion, was greater than 97%. PBMC, 3×10^6, were cultured in Falcon (Scientific Supplies, London) test tubes (T2001) in the presence of either 2 μg/ml PHA, 50 ng/ml PMA, a combination of PHA and PMA, 25 ng/ml anti-T3 or no additive for 2 days at 37° in 5% CO_2 and 100% humidified air. The concentrations of PMA and anti-T3 used were those found to maximally stimulate normal PBMC; in addition the concentration of PHA was identical to that used in studies of IL-2 activity (Saxena *et al.*, 1985). To assess DNA synthesis, 2×10^5 PBMC/well were cultured in flat-bottomed 96-well microtitre plates (Sterilin, Middlesex) in the presence or absence of the above mitogens for 3 days in the conditions described above. Total T lymphocytes were separated by E-rosetting of PBMC as required.

Allogeneic co-culture experiments

Monocytes were separated from whole blood or PBMC using a monocyte gradient ('Nycodenz Monocytes', Nyegaard & Co., Oslo, Norway) and co-cultured with monocyte-depleted PBMC at a ratio of 1 : 20 in the presence or absence of PHA. The purity of monocytes was 85–92% as assessed by staining with the monoclonal antibody Leu M3 (CD14).

Staining

T-cell phenotypic markers and T-cell activation markers (CD4, CD8, IL-2 and TRF receptors and HLA-DR antigen) were examined using a variety of techniques. Fresh or mitogen-stimulated PBMC were first incubated with biotinylated monoclonal antibodies to Leu 3a or Leu 2a (40 min at 4°), subsequently with avidin fluorescein-conjugate (40 min at 4°) and thereafter with PE-conjugated anti-IL-2 receptor under similar conditions. The PBMC were washed three times in HBSS after each incubation period, and finally stained cells were counted using a Leitz fluorescence microscope. Alternatively, total PBMC or T cells separated were first incubated with monoclonal antibodies (40 min at 4°), washed three times in HBSS and then incubated with FITC-conjugated anti-mouse Ig. As controls, PBMC were stained with FITC-conjugated anti-mouse Ig alone (binding $< 1\%$).

Blast transformation

Cell cultures were pulsed with 1 μCi [^{3}H]thymidine (6-[^{3}H]TdR; 20 Ci/mM; Amersham Int. PLC, Amersham, Bucks) for 4 hr before harvest, after a 3-day culture. Incorporation of [^{3}H]TdR was measured by a standard liquid scintillation counting technique after harvesting filters. The mean count per minute (c.p.m.) was determined for triplicate cultures.

Statistical analysis

The data were non-parametric and thus all values have been expressed as the median (range) or geometric mean $\pm$ SEM; subsequently, the Rank sum test and the Spearman rank correlation were used as appropriate.

RESULTS

T-cell activation markers on unstimulated PBMC

The median proportions of unstimulated PBMC from patients with chronic hepatitis B virus infection expressing the IL-2 or the TRF receptors were 0·7% and 1·3%, respectively. Neither the IL-2 nor the TRF receptors were detectable on unstimulated PBMC from normal subjects ($P < 0{\cdot}01$). Using double staining, the median proportion of Leu 3a$^+$ (inducer/helper) cells from patients with chronic hepatitis B virus infection expressing the HLA-DR antigen was 12·0%, and that for Leu 2a$^+$ cells (suppressor/cytotoxic) was 17·3%. The median proportion of total T cells from normal subjects expressing the HLA-DR antigen was 1·2% (Table 1).

Although both Leu 3a and HLA-DR are present in monocytes, the intensity of Leu 3a staining on monocytes is substantially less and could be easily differentiated from CD4$^+$ cells expressing HLA-DR. Spontaneous lymphocyte transformation was similar in both patients and controls [median c.p.m. (range) 399 (98–3701) and 410 (193–3108), respectively, P = NS].

T-cell activation markers and lymphocyte transformation following stimulation with:

PHA. The median proportion of PBMC expressing the IL-2 receptor was significantly lower in patients compared to normal

Table 1. Median percentage unstimulated mononuclear cells expressing early and late T-cell activation markers

	IL-2 receptor (PBMC)	Transferrin receptor (PBMC)	HLA-DR (T cells) Leu 3a$^+$	Total T cells	HLA-DR (T cells) Leu 2a$^+$
Chronic HBV infection	0·7* (0–6) $n=13$	1·3* (0–4·3) $n=8$	12·0 (0–27) $n=6$	ND	17·3 (8–24) $n=6$
Normal Subjects	0 $n=4$	0 $n=4$	ND	1·2% (0–2) $n=4$	ND

The IL-2 and transferrin receptors were sought on PBMC and HLA-DR antigen on T cells separated by E-rosetting from patients with chronic HBV infection and normal subjects.

* $P<0·01$.

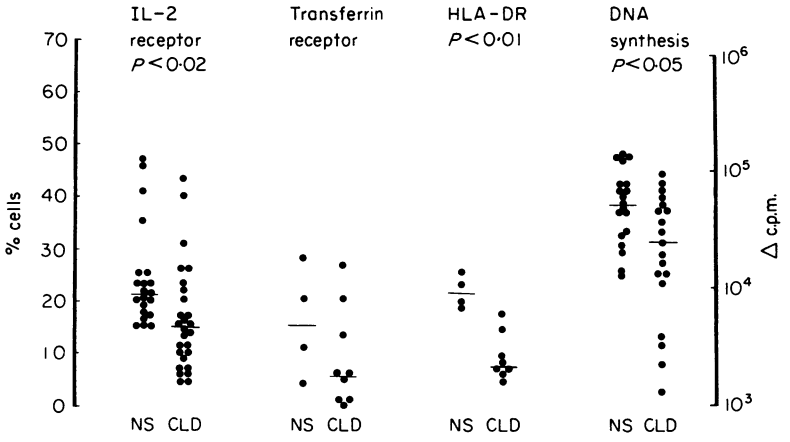

Figure 1. Percentage T cells expressing activation markers (after 2 days of culture) and lymphocyte proliferation (after 3 days of culture) measured as DNA synthesis (on a log scale) following stimulation of PBMC with PHA. Bars represent median values; NS, normal subjects; CLD, chronic HBV carriers.

Table 2. Median percentage IL-2 receptor expression on T-cell subsets following stimulation of PBMC WITH PHA

	Leu 3a$^+$	Tac/Leu 3a$^+$	Leu 2a$^+$	Tac/Leu 2a$^+$
Chronic liver disease ($n=7$)	30 (14–44)	9* (6–32)	27 (20–35)	25† (0–53)
Normal subjects ($n=4$)	36·5 (23–46)	50·5 (50–51)	35 (15–55)	49·5 (41–61)

PBMC from patients with chronic HBV infection and normal subjects were stimulated with PHA (see the Materials and Methods) and IL-2 receptor was sought on Leu 3a$^+$ and Leu 2a$^+$ T-cell subsets.

* $P<0·01$; † $P<0·05$.

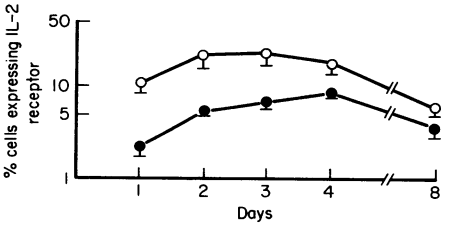

Figure 2. Kinetics of IL-2 receptor expression (1–8 days) on PBMC following stimulation with PHA in five HBV carriers and six normal subjects. Closed and open circles represent percentage IL-2 receptor-positive PBMC in patients and controls, respectively. Values shown are geometric mean − SD on a log scale.

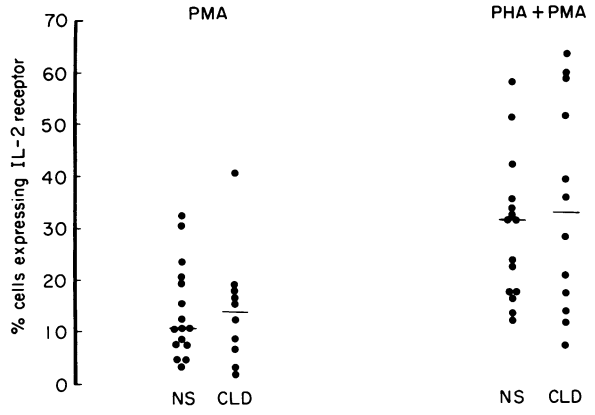

Figure 3. Percentage PBMC expressing IL-2 receptor, following 2 days stimulation of PBMC with PMA or PHA plus PMA. Bars represent median values. NS, normal subjects; CLD, chronic HBV carriers.

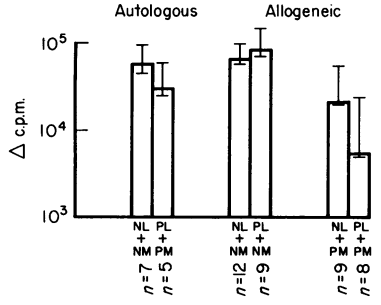

Figure 4. Lymphocyte transformation in HBV carriers and normal subjects in autologous and allogeneic co-cultures of monocytes and monocyte-depleted PBMC stimulated with PHA for 3 days. Values shown are geometric mean ± SEM on a log scale. NL, normal lymphocytes; NM, normal monocytes; PL, patients' lymphocytes; PM, patients' monocytes.

subjects (15·0% and 21·5%, respectively, $P<0·02$; Fig. 1). A similar pattern was seen with respect to the proportion of T cells expressing the TRF receptor (5·5% and 15·5%, respectively) or the HLA-DR antigen (7·0% and 21·5%, respectively, $P<0·01$). Using double staining, the proportions of Leu 3a$^+$ and Leu 2a$^+$ cells expressing the IL-2 receptor were significantly lower in patients (9·0% and 25·0%, respectively) than in normal subjects (50·5% and 49·5%, respectively, $P<0·01$ and $P<0·05$, respectively; Table 2). This effect was sustained throughout 8 days in patients in whom there was delay in the time taken to reach maximum stimulation (4 days and 2 days for patients and controls, respectively; Fig. 2).

DNA synthesis was also significantly lower in patients (median Δc.p.m. 19,829) than normal subjects (48,644, $P<0·05$). Similar results were obtained when anti-T3 monoclonal antibody, another macrophage-dependent T-cell mitogen, was added to cultures containing PBMC from three patients and three controls. Thus, the proliferative response was lower in patients [median Δ c.p.m. (range), 11,555 (11,193–31,666), than normal subjects, 104,201 (38,630–121,979)].

PMA. Using PMA, a mitogen that can substitute for macrophage function, the median proportion of PBMC expressing the IL-2 receptor was comparable in patients and normal subjects (13·5% and 10·5%, respectively). Lymphocyte proliferation in response to PMA was similar in patients and controls (median Δc.p.m. 34,060 and 21,035, respectively, $P=$NS; Fig. 3).

PMA and PHA. Stimulation of PBMC with both PMA and PHA resulted in an increase in the proportion of cells expressing the IL-2 receptor compared to that following stimulation with PHA alone in both patients (with PHA 12·5% and with PHA + PMA 33·5%) and in controls (with PHA 20·0% and with PHA + PMA 32·0%) (Fig. 3). The values obtained in patients and controls were not significantly different. Lymphocyte transformation in response to a combination of PHA + PMA was similar in patients (median Δc.p.m. 49,660, range 2866–122,028) and controls (39,768, range 6498–101,188, $P=$NS).

There was a highly significant association between the proportion of PBMC expressing the IL-2 receptor and the proliferative response to PHA or PHA + PMA in patients with chronic HBV infection ($R=0·517$, $P<0·01$).

Co-culture studies

Mixture of normal monocytes and normal lymphocytes in an allogeneic system was, as expected, associated with a slight stimulatory effect in response to PHA in comparision to autologous mixture of monocytes and lymphocytes (DNA synthesis geometric mean c.p.m., 58,487 and 66,174 for autologous and allogeneic co-cultures, respectively). In striking contrast, patients' monocytes inhibited DNA synthesis by normal lymphocytes (21,386 c.p.m.) (Fig. 4), while normal monocytes increased the transformation of patients' lymphocytes when compared to autologous patients' lymphocytes and monocytes (c.p.m. = 83,311 and 31,237 for allogeneic and autologous, respectively). The effect of patients' monocytes on allogeneic patients lymphocytes was also inhibitory c.p.m. (5252 and 31,237 for allogeneic and autologous, respectively).

DISCUSSION

The present study demonstrates that in chronic HBV carriers the process of T-cell activation in response to PHA and anti-T3 differs markedly from that in normal controls. Expression of the IL-2 receptor, the TRF receptor and HLA-DR on T cells, as well as lymphocyte transformation, were all reduced. Both PHA and anti-T3 require the presence of accessory cells (AC) for antigen presentation (Hara, Fu & Hansen, 1985) in order for lymphocytes to undergo activation, and down-regulation of the IL-2 receptor expression and lymphocyte proliferation could be attributed broadly to abnormal AC function, directly to T-cell dysfunction or to a combination of these defects. Since the proportion of monocytes was similar in patients and controls, one possibility is that the AC fails to produce IL-1 or other monokine(s), or secretes in excess an inhibitory factor, e.g. PEG_2, which results in inhibition of the T-cell activation process and finally lymphocyte transformation. Although the addition of exogenous IL-1 or indomethacin to cultures of PBMC from patients did not affect DNA synthesis (Anastassakos *et al.*, 1987, 1988), co-culture experiments clearly indicated that monocytes from chronic HBV carriers suppress lymphocyte activation. Similar results were observed by Daniels *et al.* (1987), where addition of monocytes from HBV carriers to PBMC from

controls inhibited IL-2 production. Inhibition of T-cell activation requires a signal conveyed by *intact* monocytes (Davis & Lipsky, 1985), thus the defect in AC function in these patients may be a consequence of ingestion, or infection with, HBV antigens. HBV genome is indeed present in PBMC of at least two-thirds of HBV carriers, even when HBV-DNA is absent from serum, indicating that HBV genome can persist in such cells (Davison *et al.*, 1987). The functional relevance of the HBV-genome in such cells, however, is uncertain at present.

Addition of normal monocytes to patients' lymphocyte appeared to correct PHA responsiveness and DNA synthesis and therefore it is unlikely that the down-regulation of lymphocyte transformation in chronic HBV carriers is a consequence of the insufficient lymphokine production, e.g. IL-2 (Saxena *et al.*, 1985), α or γ interferons (Kato *et al.*, 1982). This hypothesis is supported by the studies of Anastassakos *et al.* (1987), where the addition of exogenous IL-2 or supernatants from mixed lymphocyte reactions obtained from normal controls to cultures containing PBMC from patients prior to stimulation with PHA did not affect lymphocyte transformation.

In order to assess intrinsic T-cell function in chronic HBV carriers, IL-2 receptor expression and lymphocyte proliferation were measured following stimulation with PMA, a mitogen that has been shown to substitute for ACs and which acts on T cells through a mechanism that differs from that of PHA (Hara & Fu, 1985). PMA bypasses all of the AC-dependent steps of T-cell activation by directly stimulating phospholipid-dependent protein kinase *C* activity (Deeper *et al.*, 1984), a signal that enhances cell cycle entry and progression of the stimulated cells. Both IL-2 receptor expression and lymphocyte transformation of PBMC from chronic HBV carriers were normal in response to PMA or a combination of PMA and PHA. These findings suggest that T cells from such patients are intrinsically normal and will respond normally if stimulated appropriately.

The observation that a substantial proportion of unstimulated circulating T cells bore HLA-DR (Fukiu *et al.*, 1984) and that similar cells have been identified in liver tissue (Collucci *et al.*, 1983; Pape *et al.*, 1983), particularly in the lobule where concentration of viral antigens is maximal, suggests that T cells from this group of patients are fully and normally activated. However, the absence of cells expressing the IL-2 receptor or TRF receptor from peripheral blood reported here as well as from liver tissue (Naoumov *et al.*, 1987) appears anomalous and two explanations may account for this paradox. Firstly, activation may have occurred through an alternative pathway, bypassing the early activation events, and secondly the HLA-DR may have been expressed as a consequence of local interferon-gamma secretion following viral infection.

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