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Increased IgM B cell differentiation lymphokine production by T lymphocytes from patients with primary biliary cirrhosis

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Hypergammaglobulinemia, mainly due to increased serum immunoglobulin M concentrations, is a common and distinctive feature of primary biliary cirrhosis. T-B cell cooperation plays a pivotal role in the regulation of immunoglobulin secretion. In this paper, the production of regulatory B lymphokines by T cells, as well as the functional response of B lymphocytes to these molecules, was investigated in patients with primary biliary cirrhosis. T cells from patients with primary biliary cirrhosis have an enhanced ability to produce lymphokines that regulate the proliferation of B cells and their differentiation to immunoglobulins G- and M-secreting cells. In contrast, the cellular production of lymphokines involved in the induction of immunoglobulin A-secreting cells was normal. Simultaneously, the proliferation and differentiation of purified B cells in response to stimulation with surface immunoglobulin ligands and lymphokines were normal. These results suggest that the elevated serum levels of immunoglobulins M and G found in patients with primary biliary cirrhosis could be ascribed to an enhanced lymphokine-mediated T-B cooperation. © Journal of Hepatology.

Key words: B-cell differentiation; Hypergammaglobulinemia; Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is a chronic progressive disease of unknown etiology, characterized by the inflammatory destruction of intrahepatic bile ducts. Several abnormalities of the immune system have been described in patients with this disorder (1–6). Characteristically, hypergammaglobulinemia, mainly due to an enhancement of immunoglobulin (Ig) M, is a hallmark of PBC (1–6). Nonetheless, the lymphocyte abnormalities in the physiopathology of this Ig alteration remain unknown.

The synthesis of Igs is a complex process with several phases (7–11). First, T and B cells are activated due to the interaction of the antigen and the T cell receptor or the B cell membrane Igs, respectively. Second, activated T cells synthesize several lymphokines that modulate the mitogenesis of the B lymphocytes and their differentiation into plasma cells, after binding to the specific receptors expressed by activated B cells. Finally, the plasma cells secrete Igs. The effect of the interaction of the antigen and the T and B lymphocytes can be avoided by stimulation with polyclonal activators such as plant lectins – phytohemagglutinin (PHA) (11) – and immunoglobulin ligands – *Staphylococcus aureus* Cowan I (SAC) (12) –, respectively.

A markedly deficient proliferative interleukin 2 (IL2) pathway has been shown in T lymphocytes from PBC patients (13). In this study, we investigated T-B cooperation in Ig secretion in PBC patients. The ability of the T lymphocytes in these patients to regulate the proliferation of B lymphocytes and their differentiation into Ig-secreting cells is studied here. At the same time, the responsiveness of B cells to T lymphocyte factors that modulate their blastogenesis and differentiation has been assessed in PBC patients.

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Material and Methods

Population

Twenty patients with PBC and 20 age- and sex-matched controls were studied (Table 1). All gave informed consent to the experimental protocol. The diagnosis of PBC was reached on the basis of the following criteria (1): 1) the presence of a histopathologically compatible lesion was the major criterion; 2) the four minor criteria included: a) elevation of serum IgM levels; b) increase in serum alkaline phosphatase activity to more than twice the upper limit of normal; c) the presence of antimitochondrial antibodies (AMA) at titers equal to or greater than 1/40; and d) the permeability of the biliary tract demonstrated by endoscopic retrograde cholangiopancreatography and/or echography. The diagnosis of PBC was reached if the major and at least three minor criteria were present. The stage, determined by the histological study of the biopsies, was: I in 20% of cases; II in 30%; III in 30%; and IV in 20% (14).

Exclusion criteria for this study were: 1) prior treatment with immunosuppressors (steroids, cyclosporin A, D-penicillamine, etc.); 2) the existence of clinical, epidemiological, serological or pathological signs of liver disease other than PBC; 3) the presence of diseases or drugs that could modify the results of the tests performed.

Results of the conventional clinical and biochemical studies performed in these patients are presented in Table 1. The serum levels of IgM, IgG and IgA were also determined in PBC patients and in healthy controls. IgM concentrations in the serum of the PBC group (531±252 mg/dl) were found to be significantly higher than in the group of healthy controls (129±41 mg/dl; $p<0.001$). Serum IgG levels were also significantly increased in PBC patients (1652±479 mg/dl) compared to those of healthy controls (1020±219 mg/dl; $p<0.001$). However, serum IgA levels were only slightly elevated in PBC patients compared to the control group (275±98 vs 178±63; $p<0.05$).

TABLE 1

Characteristics of the PBC population dealt with in the study

Age (years)	56	(34–77)*
Duration of the disease (months)	62	(56–80)
Clinical manifestations:		
Asymptomatic	5	(20%)**
Jaundice	6	(30%)
Hepatomegaly	8	(40%)
Portal hypertension	6	(30%)
Ascites	2	(10%)
Laboratory data:		
Bilirubin (mg/dl)	4.3	(5.6)***
Alkaline phosphatase (U/dl)	433	(245)
ALT (U/dl)	100	(46)
AST (U/dl)	114	(62)
GGT (U/dl)	191	(145)
Prothrombin activity (%)	93	(3.8)
AMA>1/40	20	(100%)**
ANA>1/40	8	(40%)

*Mean (range); **Number of patients (percentage of the whole); ***Mean (standard deviation).

Reagents

The following monoclonal antibodies (MoAb), that recognize specific molecules on the surface of certain cellular subpopulations, were used in the studies: OKT11 (CD2), OKT3 (CD3), OKT4 (CD4), OKT8 (CD8) (Ortho-mune, Orthodiagnostic System, NJ, USA), anti-B1 (CD20), anti-Mo2 (CD14), anti-TAC (CD25) (Coulter Clone, Coulter Immunology, FL, USA), BAC 1 (donated by M.D. Cooper, Alabama University, Birmingham, AL, USA) (15), 4F2 (donated by A.S. Fauci, NIH, MD, USA), and Leu 11b (CD16) (Becton Dickinson, CA, USA). Recombinant IL2 (rIL2) was the gift of Hoffman-La Roche, NJ, USA).

Biological material

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque gradient. T cells were purified by double-rosetting as previously described (16), and the purity was greater than 95% in every case. B cells were purified from the non-rosetting fraction by excluding plastic-adherent cells and by treatment with the MoAb OKT3, Mo2, Leu11b and rabbit complement (Behring Institute, Marburg, Germany), as previously described. The purity of $B1^+$ cells was greater than 90%, as determined by flow cytometry. Tonsillar T and B cells were prepared in a similar manner after disaggregation of human tonsils obtained from therapeutic tonsillectomies in 6- to 21-year-old individuals (17).

Cells were washed and resuspended in RPMI 1640 (Flow Lab, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY, USA), 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM) and 1% penicillin-gentamicin (complete medium), and checked for viability by trypan blue exclusion. Experiments with less than 90% viability were discarded.

Proliferation studies

Purified B cells (2×10^6/ml) were cultured in complete medium in the presence of SAC (1/25000 vol/vol) from Calbiochem (Behring Corp, CA, USA) in 24-macrowell plates (Costar, Cambridge, MA, USA) for 48 h. The SAC-stimulated B cells were washed twice in complete medium and placed in 96-microwell plates (5×10^4 cells/well) with or without either the B cell growth factor (BCGF) preparation (see below) (20% vol/vol) or rIL2

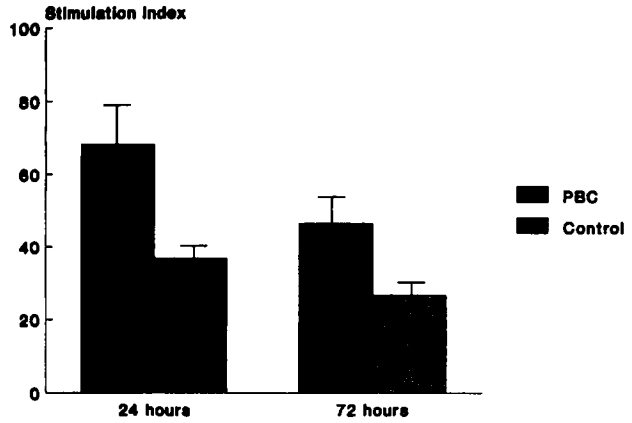

Fig. 1. Synthesis of B cell growth factor (BCGF) activity by T cells of patients with PBC and controls after activation with PHA for 24 and 72 h. Results are expressed as the stimulation index (SI) of the tonsillar *Staphylococcus aureus* Cowan I-activated B lymphocytes after supplementation of T cell supernatants. Significant differences ($p<0.05$) were detected between the BCGF activity synthesized by the T cells from PBC patients and that of controls at both 24 and 72 h of culture. SI: (cpm after stimulation−basal cpm)/basal cpm.

(100 U/ml) for 3 days. The cultures were pulsed with 1 μCi of ^{3}H-thymidine (Radiochemical Centre, Amersham, UK) for the last 18 hours of culture, and uptake was measured by standard liquid scintillation counting techniques after harvesting by a Skatron device. Other aliquots of B cells (50 000 cells/well) were maintained for 5 days with the medium, but with no stimulus. Cultures were performed at 37°C in a humidified atmosphere containing 5% CO_2. Results were expressed as mean counts per minute (cpm)±standard deviation of triplicate cultures.

Immunoglobulin production

The experimental system was similar to that described earlier. After incubation of B lymphocytes (2×10^6/ml) with SAC 1/25 000) for 48 h, SAC-activated B cells (50 000/ml) were cultured with or without either the B cell differentiation factor (BCDF) preparation (20% vol/vol) (see below) or rIL2 (100 U/ml) for 5 days. Other aliquots of B cells (50 000 cells/well) were maintained for 5 days

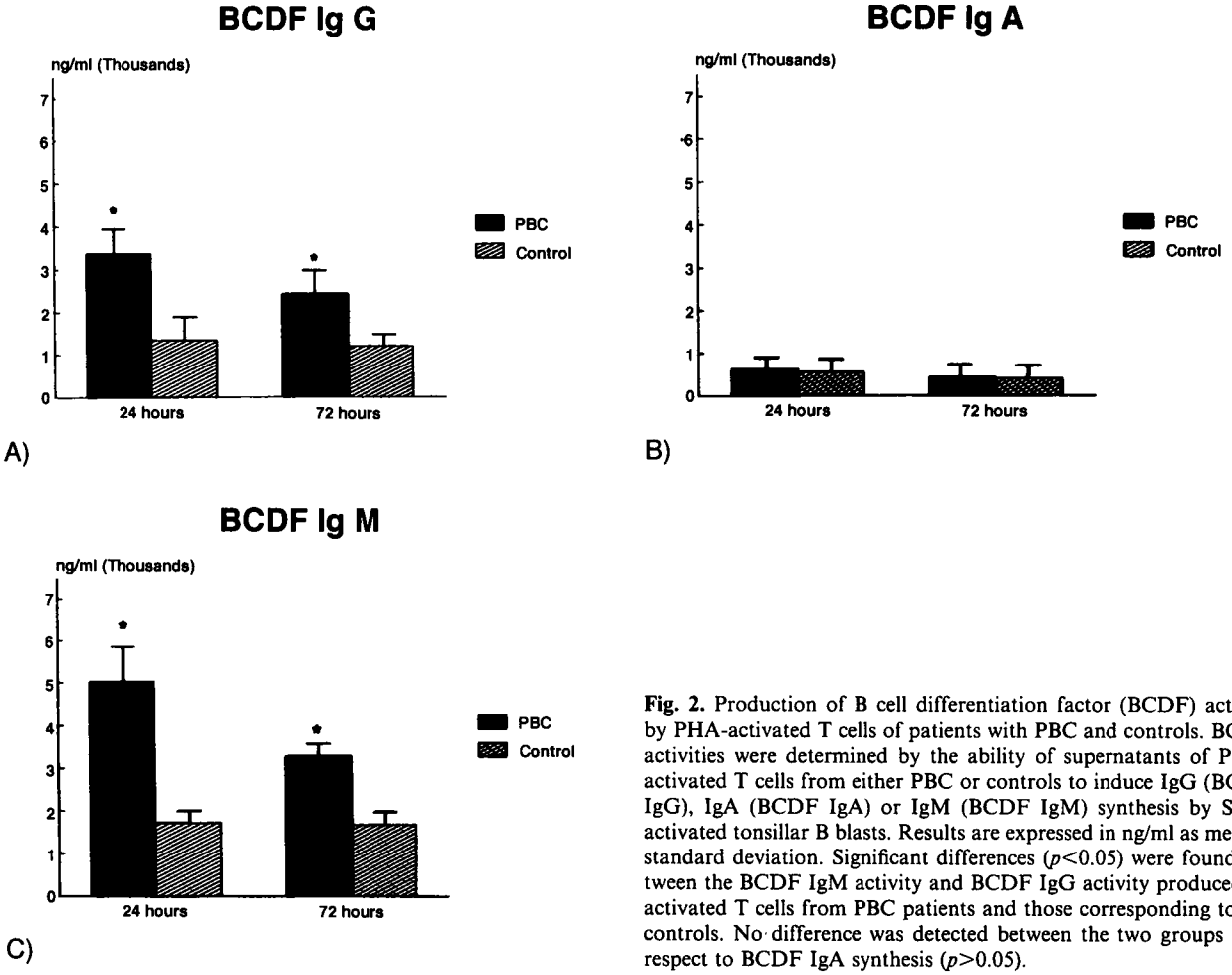

Fig. 2. Production of B cell differentiation factor (BCDF) activity by PHA-activated T cells of patients with PBC and controls. BCDF activities were determined by the ability of supernatants of PHA-activated T cells from either PBC or controls to induce IgG (BCDF IgG), IgA (BCDF IgA) or IgM (BCDF IgM) synthesis by SAC-activated tonsillar B blasts. Results are expressed in ng/ml as mean+standard deviation. Significant differences ($p<0.05$) were found between the BCDF IgM activity and BCDF IgG activity produced by activated T cells from PBC patients and those corresponding to the controls. No difference was detected between the two groups with respect to BCDF IgA synthesis ($p>0.05$).

with the medium only and with no stimulus. After incubation, Ig synthesis was detected by ELISA, with purified goat $F(ab)_2'$ anti-heavy chain of human IgA, IgG and IgM, as previously described (17).

Each reagent was tested in dose/response titrations before use.

BCGF and BCDF production and detection assays

Purified T cells (5×10^6/ml) from patients and controls were cultured for 24 or 72 h with or without PHA (10 μg/ml) in 1 ml of complete medium, in round-bottomed culture tubes (Falcon, Oxnard, CA, USA). The supernatants were collected and stored at −20°C until tested.

BCGF activity was assessed as previously described (18). Briefly, tonsillar B cells were cultured for 48 h with SAC, as described in the proliferation assays. SAC-activated blasts (5×10^4 in 100 μl of complete medium per well) were incubated with serial dilutions of the sample supernatants and cultured for 72 h. Cells were pulsed with 1 μCi/ml of ^{3}H-thymidine over the last 18 h and ^{3}H-thymidine uptake was measured.

BCDF activity was measured using a similar experimental system. SAC-activated tonsillar B cells (5×10^4 cells/well) were recultured for 5 days with serial dilutions of the sample supernatant. Total IgA, IgG and IgM in culture medium were measured by ELISA, as described in the Ig production assays, and will be referred to as BCDF IgA, BCDF IgG and BCDF IgM.

A standard BCGF-containing preparation was obtained after double-stimulus with mixed lymphocyte culture and PHA supplementation of tonsillar T cells from a pool of 10 control individuals. Similarly, a standard BCDF-containing preparation was obtained. The supernatants were collected and stored at −20°C until used. BCGF and BCDF supernatants were tested in dose/response titrations and a concentration of 20% (v/v) was considered optimum.

TABLE 2

Phenotypic study of the peripheral blood mononuclear and purified T cells from PBC patients and controls

Phenotype	PBC	Control
Peripheral blood mononuclear cells		
T lymphocytes ($CD3^+$)	65.2 (8.0)	66.8 (6.6)
B lymphocytes ($CD20^+$)	13.1 (3.7)	13.9 (6.7)
Monocytes ($CD14^+$)	21.9 (7.2)	20.8 (8.4)
NK cells ($CD16^+$)	17.1 (4.4)	13.7 (6.6)
Purified T lymphocytes		
$CD2^+$	95.6 (2.7)	96.0 (3.2)
$CD3^+$	87.4 (4.2)	88.2 (6.3)
$CD4^+$	56.3 (8.2)	54.7 (6.6)
$CD8^+$	29.5 (6.5)	30.5 (5.7)

Results are expressed as the mean (±standard deviation).

Immunofluorescence studies

Immunofluorescence staining of cell surface antigens was performed using a standard method as previously described (16). The fluorescence of 10000 viable lymphoid cells stained for each different MoAb was quantified using an EPICS C flow cytometer (Coulter).

Statistical analysis

The data from the groups were compared using the Mann-Whitney U test. For correlation studies, linear regression analysis with the least squares method was employed. A p value of less than 0.05 was considered to indicate a significant difference between groups.

Results

1. Production by T cells from PBC patients of lymphokines that regulate B cell proliferation and B cell differentiation to IgM and IgG-secreting plasma cells is enhanced

When activated by antigens or mitogens, T cells produce several lymphokines involved in the regulation of B cell proliferation and differentiation. The net effect of these lymphokines on B cell proliferation is described as B cell growth factor (BCGF), while their net effect on B cell differentiation to Ig-secreting cells is described as B cell differentiation factor (BCDF).

The BCGF activity present in the supernatants of cultures of PHA-activated T cells from PBC patients was significantly increased when compared with that of the healthy controls ($p<0.05$) (Fig. 1).

There were obvious differences between the BCDF activities present in supernatants of the cultures of PHA-

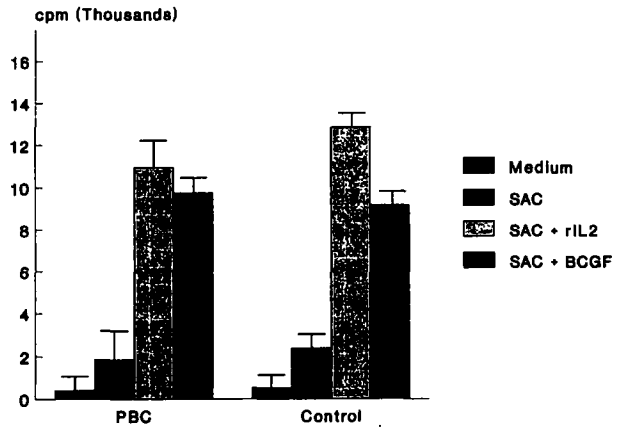

Fig. 3. Proliferative response of B cells from PBC patients and healthy controls, cultured in the absence of stimulation (medium alone) and after stimulation with SAC in the presence or absence of either rIL2 or a BCGF preparation (BCGF). Results are expressed in cpm as mean+standard deviation. No differences were detected between the two groups under the conditions analyzed ($p>0.05$).

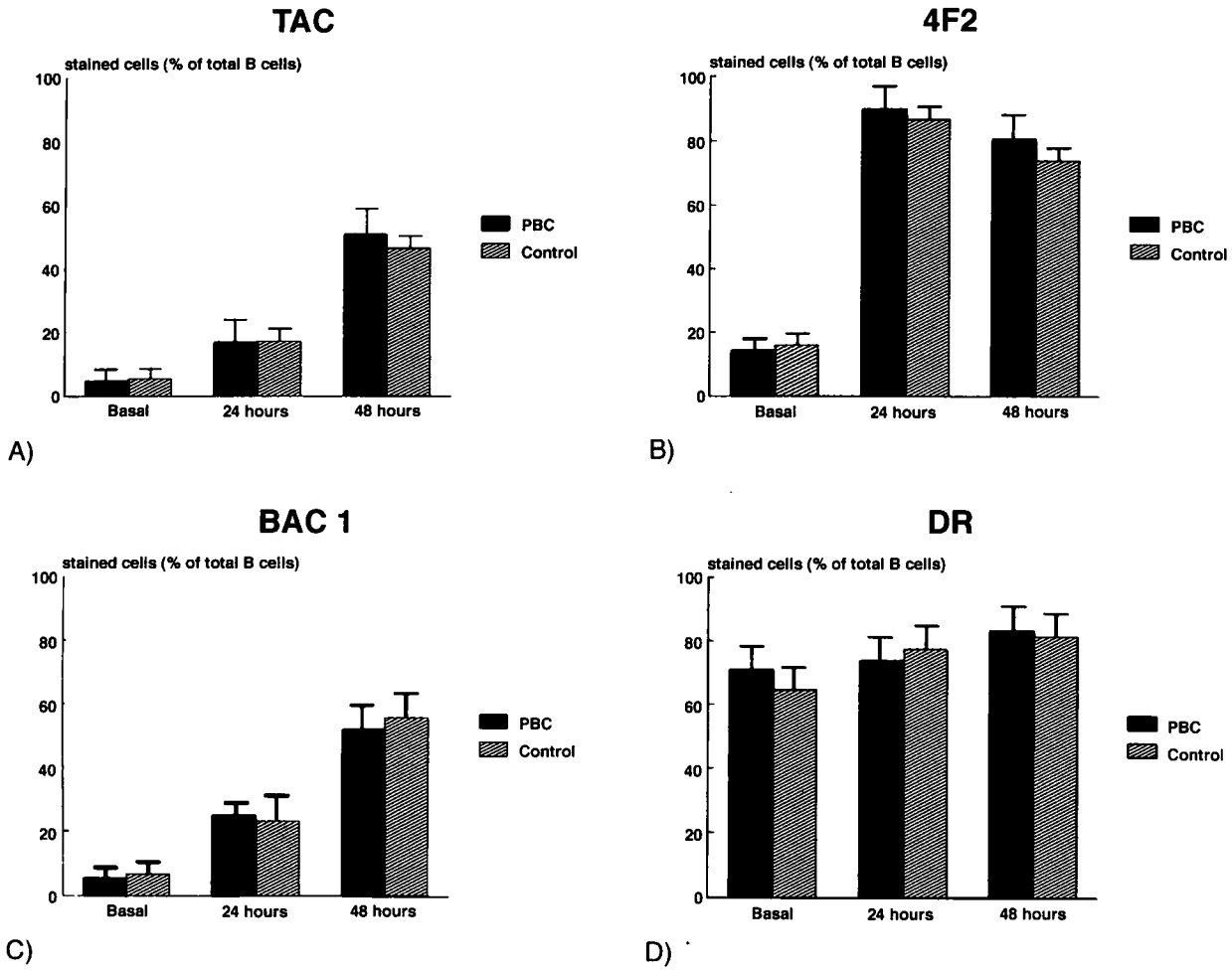

Fig. 4. Expression, on the cytoplasmic membrane of B cells from PBC patients and controls, of the IL2 receptor (TAC), the glycoprotein 4F2, the BAC-1 antigen and MHC-class II molecules (DR), spontaneously and after stimulation with SAC for 24 or 48 h. No differences were detected between the two groups under the conditions analyzed ($p>0.05$).

activated T cells from PBC patients and healthy controls. Significantly enhanced secretions of BCDF IgM and of BCDF IgG were observed in T cell cultures from PBC patients compared to healthy controls ($p<0.05$). Nevertheless, no statistically significant differences were found when BCDF IgA activity was analyzed in PHA-stimulated T cell cultures from the two groups of subjects ($p>0.05$) (Fig. 2). No relevant levels of BCGF and BCDF activities were observed in supernatants from cultures of nonstimulated T cells from patients or controls.

The T cell functional alterations cannot be ascribed to modifications in the percentages of $CD4^+$ and $CD8^+$ subsets of the T cell population present in PBC patients (Table 2).

2. B lymphocytes from PBC patients show normal lymphokine-conditioned proliferation and differentiation to IgM, IgG and IgA-secreting cells

When activated by antigens or immunoglobulin ligands, B lymphocytes express a set of receptor molecules to lymphokines secreted by T cells on their cytoplasmic membrane. After the lymphokine-receptor interaction, B cells progress in their cellular cycle (7–10,18).

The proliferative response to lymphokines of SAC-activated B cells was studied in PBC patients. B cells from PBC and healthy controls were activated with SAC and cultures were subsequently supplemented with either a BCGF preparation or rIL2. The B cell proliferation from PBC patients was not significantly different from that of healthy controls in any of the different experimental conditions analyzed (Fig. 3).

The spontaneous and activation-dependent B cell expression of receptors to growth factors (IL2 receptor (10) and BAC1 (the putative BCGF receptor) (15)) as well as the activation markers 4F2 (12) and major histocompatibility (MHC) class II molecules (12) was also investigated. As shown in Fig. 4, B cells from PBC patients normally express and acquire these membrane antigens in basal conditions and after SAC stimulation for 1 and 2 days.

Next, the production of Igs by B cells from PBC patients was investigated. After antigenic challenge and surface Ig crossing, activated B cells express receptors to differentiation lymphokines on their cytoplasmic membrane. The lymphokine-receptor binding promotes the secretion of Igs (7,10,12,).

B cells from PBC patients and healthy controls were activated with SAC, and cultures were subsequently supplemented with either a BCDF preparation or rIL2. The secretion of IgM, IgG and IgA by SAC-stimulated B cells from PBC patients was similar to healthy controls in all the different experimental conditions analyzed ($p > 0.05$) (Fig. 5).

The results of this study could not be ascribed to contamination of the purified B cell preparations by T lymphocytes. In each experiment, this possibility was assessed by phenotypical and functional studies, to determine the presence of $CD3^+$ cells (<0.5%) and the absence of proliferative response to PHA stimulation, respectively.

No correlation was found between the *in vitro* tests performed and the severity of liver disease, as evaluated by Child-Pugh criteria and serum levels of aminotransferases, alkaline phosphatase, bilirubin and ceruloplasmin. Nor was any correlation found between the immunological tests performed and the serum levels of antimitochondrial antibodies or the histological stage of biliary cirrhosis.

Discussion

The involvement of a sequential process in the activation and differentiation of B cells is generally accepted (7–9,18). B lymphocytes are activated via the union and crossing of the surface Igs with either an antigen or a mitogen. After this interaction, B cells express several receptors on their cytoplasmic membrane. T cell lymphokines act on these receptors, inducing the progression of the B lymphocytes in the cellular cycle and enabling them to differentiate to plasma cells. On the basis of this model, T-B lymphocyte cooperation was investigated in patients with PBC.

The results of this study demonstrate that the increased serum IgM and IgG concentrations observed in PBC patients can be explained by alterations in the regulatory effects of T cells on the proliferation and differentiation

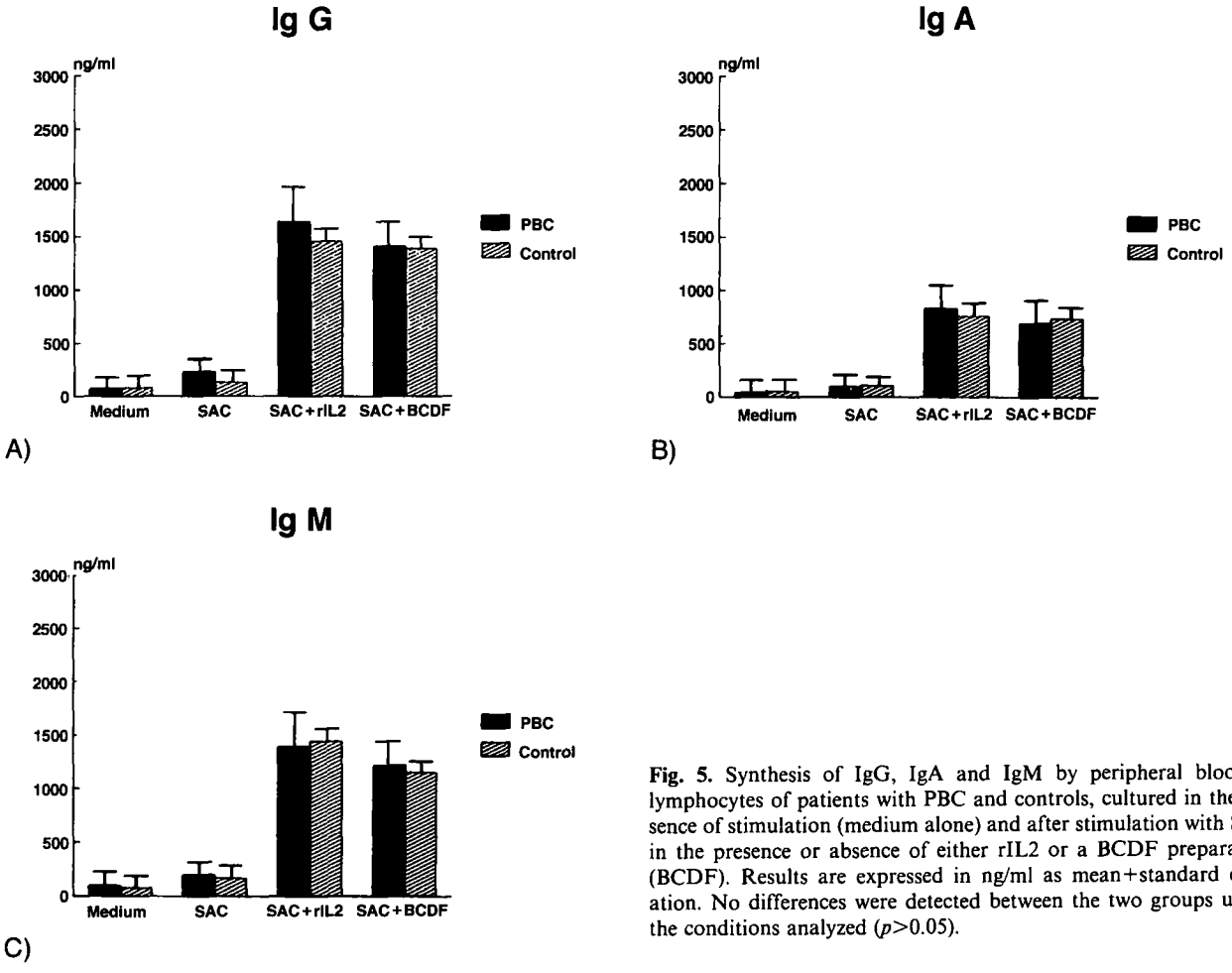

Fig. 5. Synthesis of IgG, IgA and IgM by peripheral blood B lymphocytes of patients with PBC and controls, cultured in the absence of stimulation (medium alone) and after stimulation with SAC in the presence or absence of either rIL2 or a BCDF preparation (BCDF). Results are expressed in ng/ml as mean+standard deviation. No differences were detected between the two groups under the conditions analyzed ($p > 0.05$).

of B lymphocytes. T cells from PBC patients show both enhanced production of lymphokines with BCGF activity and increased production of BCDF IgM and BCDF IgG activities, while no alteration in the IgA regulatory process was detected. In contrast to the T lymphocyte alterations, normal B cell function was observed in PBC patients.

The origin of these functional abnormalities in the T cell compartment may involve several mechanisms. First, PBC patients have been shown to have an unequivocal defect of the C3b receptor-mediated clearance function of Kupffer cells (19) Thus, an unspecific hyperstimulation of T cells by an increased antigenic charge may be involved in the induction of the T cell functional stage (19–21). It could also be claimed that these T cell alterations might be due to the metabolic disorder associated with the liver disease. However, the differences in the pattern of hyperimmunoglobulinemia (21–25) as well as the fact that the alterations of T and B cell functions in PBC patients and those of alcoholic cirrhosis patients do not overlap (26) argue against these hypotheses.

There is increasing evidence that PBC is an autoimmune disease. As reported with other autoimmune diseases, such as systemic lupus erythematosus (27), the results of this study clearly show that the deficient regulation of T cell function is associated with an anomalous T-B cooperation, with an enhancement of the lymphokine secretion. Defective lymphocyte functions have also been described (28–31). These abnormal immune regulations and/or immunodeficiencies may provoke an alteration in the dynamic equilibrium and internal connectivity of the immune system, and possibly contribute to abnormal autoreactivity and liver damage.

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