Clin. exp. Immunol. (1985) **61**, 297–304.

In vitro studies on the mechanism of increased serum IgM levels in primary biliary cirrhosis

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(Accepted for publication 5 March 1985)

SUMMARY

To evaluate the mechanisms underlying the increase in serum IgM in primary biliary cirrhosis (PBC) studies were designed to examine IgM production *in vitro* and to assess the relative contribution of intrinsic B cell activity and immunoregulatory T cell balance to IgM synthesis. The number of peripheral blood lymphocytes (PBL) producing IgM (spontaneous and pokeweed mitogen (PWM) stimulated) at the end of a seven day culture period was similar in PBC patients and control subjects while the amount of IgM synthesized (spontaneous and PWM stimulated) during this period was significantly greater in the patient group, implying that the amount of IgM produced per B cell was increased in PBC. Co-culture of autologous and allogeneic T and B lymphocytes and irradiation of T lymphocytes from patients and normal subjects clearly implicated abnormal suppressor T cell function, rather than autonomous B cell hyperactivity, as the cause of the increased IgM synthesis. Direct studies of T cell function indicated that although concanavalin A (Con A) activated suppressor cells inhibited proliferation of IgM producing B cells in the majority of PBC patients, they were unable to inhibit IgM synthesis. The demonstration of a disparity between IgM synthesis and the proliferation of IgM-producing B cells, together with the observation that the abnormality of T cell function is largely confined to the control of IgM secretion, is consistent with the presence of at least two different suppressor subpopulations regulating IgM production. In PBC the main suppressor cell abnormality seems to affect regulation of IgM secretion rather than B cell proliferation.

Keywords immunoglobulin M Con A induced suppression primary biliary cirrhosis

INTRODUCTION

Primary biliary cirrhosis (PBC) is a disease of unknown aetiology characterized by a progressive destruction of bile ducts in which aberrant cellular and humoral immune responses are thought to play a major pathogenetic role (James *et al.*, 1981). Abnormalities of the cellular immune response include enhanced *in vitro* cytotoxicity for a variety of target cells including hepatocytes (Vierling *et al.*, 1977), sensitization to bile and liver specific antigens (McFarlane *et al.*, 1979; Tsantoulas *et al.*, 1980), bile-duct disruption by mononuclear cells (Schaffner *et al.*, 1982) and defective suppressor T-cell function (Zetterman *et al.*, 1980). Abnormalities of the humoral immune response are reflected by increased serum levels of IgM and by the presence of serum autoantibodies (Doniach *et al.*, 1966), notably mitochondrial antibody, circulating immune complexes (Wands *et al.*, 1978) and accelerated complement turnover (Wands *et al.*, 1978). Although these data are consistent with

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excessive uncontrolled activity of immunoglobulin (Ig) producing B cells the mechanisms involved in the production of hyperglobulinaemia in patients with PBC are unknown. In particular, it is unclear why IgM is the major contributor to the increase in globulin levels in the majority of patients with PBC, when in most other chronic liver and autoimmune diseases the associated hyperglobulinaemia is due largely to an increase in serum IgG.

It is now appreciated that immune regulation is dependent upon an elaborate network of interacting immunocompetent cells (Allison *et al.*, 1971) and that B-cell Ig production is the result of a delicate balance between T-suppressor and T-helper lymphocytes (Reinherz *et al.*, 1980). The present study examines IgM production by lymphocytes from patients with PBC and assesses the relative contribution of immunoregulatory T-cell balance and intrinsic B-cell function to IgM synthesis.

MATERIALS AND METHODS

Patients. The clinical, immunoserological and histological features of the 36 patients selected for the study, who met the international criteria for the diagnosis of PBC (Sherlock *et al.*, 1973) are shown in Table 1. Ig production and suppressor T-cell function were studied in 23 patients of whom six were receiving D-penicillamine (1·2 gm/day), four azathioprine (1 mg/kg/day) and 13 no specific therapy. While there were no significant differences between treated and untreated patients, those receiving D-penicillamine tended to have more advanced disease with higher serum bilirubin levels and more advanced histology (Table 1). In the remaining 13 patients (11 untreated, two azathioprine treated), autologous and allogeneic lymphocyte co-culture experiments were performed (see below) to assess the relative contributions of T-cell and B-cell activity to IgM synthesis.

Cell preparation. Peripheral blood was mixed with dextran 6% (w/v) in 0·15% sodium chloride and preservative-free heparin (10 U/ml of blood) and the red cells allowed to sediment for 45 min at 37°C. Mononuclear leucocytes were isolated from the leucocyte-rich plasma by centrifugation on Ficoll-Triosil (Böyum, 1968) (400*g* for 20 min). These cells were harvested and incubated at 37°C for 45 min in plastic Petri dishes to deplete adherent cells; the resulting preparation contained 3–5% monocytes as assessed by peroxidase staining. The viability of lymphocytes was greater than 99%, as determined by trypan blue exclusion. The lymphocytes were washed three times in Hank's Balanced Salt Solution (HBSS) (Wellcome Laboratories, Beckenham, Kent, England) and were resuspended at a concentration of 1×10^6 cells/ml in 20% heat inactivated fetal calf serum (FCS) (GIBCO Europe, Paisley, Scotland) in RPMI 1640 (GIBCO) containing 2 mM glutamine, 200 U/ml of penicillin, 100 μg/ml of streptomycin and 2 μg/ml of amphotericin B.

Preparation of T and B cell enriched fractions. Peripheral blood lymphocytes (PBL) were

Table 1. Serological and histological features of 36 patients with primary biliary cirrhosis

Patient group	Number	Serum bilirubin (μmol/l)	IgG g/l (normal < 15·0)	IgM g/l (normal < 2·0)	Antimitochondrial antibody titre	Histological stage I	II	III	IV
Untreated	24	13 (5–191)	17·1 (4·8–28·9)	4·1 (0·9–12·2)	640 (10–2560)	4	2	6	12
Azathioprine	6	24 (10–38)	18·8 (8·6–24·3)	4·7 (1·7–7·3)	100 (20–160)	0	0	0	6
D-Penicillamine	6	43 (14–293)	19·3 (11·4–35·9)	5·0 (2·3–9·7)	160 (40–640)	0	0	1	5

Values are expressed as median and (range)

prepared as described above and resuspended at a concentration of 4×10^6 cells/ml in 20% FCS in RPMI 1640. 4 ml (16×10^6 cells) were incubated with an equal volume of 1% 2-amino ethylisothiouronium bromide hydrobromide (AET) (Gelfand *et al.*, 1979) (Sigma Ltd, Poole, Dorset, England) treated sheep red blood cells for 5 min at 37°C. Following centrifugation (250*g* for 5 min) the cells were incubated at 4°C for 2 h and T- and B-enriched lymphocyte fractions isolated by centrifugation on Ficoll-Triosil (400*g* for 20 min). B-enriched lymphocytes from the interface layer were washed three times in HBSS and resuspended at a concentration of 1×10^6 cells/ml, in 20% FCS in RPMI 1640. The pellet containing the T-enriched lymphocyte population was suspended in 10 ml of 0·83% (w/v) ammonium chloride for 5 min at 37°C, washed three times in HBSS and resuspended at a concentration of 1×10^6 cells/ml in 20% FCS in RPMI 1640. Re-rosetting with AET was used to assess the composition of the lymphocyte populations isolated from the interface layer and pellet. The T- and B-enriched fractions contained >95% and <5% AET rosette forming cells respectively.

Cell culture. PBL were cultured at a concentration of 2×10^5 cells per well in a flat-bottomed microplate (Sterilin, England) with and without 20 μg/ml of pokeweed mitogen (PWM) (Sigma Ltd) to stimulate B-cell proliferation and were incubated for 7 days at 37°C in humidified air containing 5% CO_2, 12% O_2 and 83% N_2 (Nouri-Aria *et al.*, 1982).

Autologous and allogeneic co-culture studies. Co-culture experiments, using autologous and allogeneic T and B lymphocyte enriched fractions from patients and normal subjects were performed to determine whether the increased IgM production was a result of autonomous B-cell hyperactivity, abnormal T-cell function or a combination of these effects. T- and B-enriched populations of lymphocytes were prepared from patients and normal subjects as described above and designated TpBp and TnBn respectively. T and B lymphocytes were mixed in a ratio of 4:1 respectively to a final concentration of 1×10^6 cells/ml and cultured at a concentration of 2×10^5 cells per well in a flat-bottomed microplate with 20 μg/ml of PWM and incubated for 7 days at 37°C as described above. Lymphocytes were co-cultured in the following combinations: TnBn, TpBn, TnBp and TpBp.

Evaluation of IgM production.

Plaque assay. After 7 days of culture, the cells were washed three times in RPMI 1640 and resuspended at a concentration of 1×10^5 cells/ml. A 50 μl aliquot of cells was mixed at 46°C with 50 μl of sheep red cells coated with staphylococcal protein A (Pharmacia, Fine Chemical Company, Sweden), 50 μl of an appropriately diluted fraction of rabbit anti-human IgM (Miles Laboratories, Slough, England) and 700 μl of Agarose A37 (Indubiose) (Pharmindustrie, Villeneuve La Garenne, France); poured into plastic Petri dishes, diameter 5 cm, and incubated at 37°C for 4 h. To each dish was added 1 ml of guinea pig complement (Flow Laboratories, Scotland) diluted to 1:25 in RPMI 1640 and the mixture incubated for an additional 2 h at 37°C. Discrete concentric areas of haemolysis were counted visually in indirect light and the results expressed as the number of IgM producing cells/10^6 viable lymphocytes (Hammerstrom *et al.*, 1979).

Fluorometric assay. Supernatant from the microplate wells was removed at the end of the 7-day culture period and stored at −20°C until required for analysis. Following thawing and centrifugation at 2400*g* for 10 min to remove cell debris the amount of IgM was determined using an immunofluorescent assay (Immuno-Fluor, BioRad Laboratories, Richmond, California). After the supernatant had been incubated for 90 min with immunobeads coated with anti-IgM, fluorescein-labelled anti-IgM was added. Following incubation for a further 60 min, the beads were washed three times in buffer and resuspended in 2·5 ml buffer. Reference IgM standards were included in each assay and the fluorescence of each sample determined using a fluorescence spectrophotometer (Perkin-Elmer, High Wycombe, Buckinghamshire, England) at excitation and emission wavelengths of 485 and 525 nm, respectively. The results were expressed, as the amount of IgM synthesized per 10^6 lymphocytes (Schwartz, 1980).

Evaluation of suppressor cell function. To stimulate T cells (Shou *et al.*, 1976), 5×10^6 lymphocytes were incubated with 20 μg/ml of concanavalin A (Con A; Sigma Ltd) at 37°C for 24 h under the conditions described above. Following incubation the Con A-treated lymphocytes were washed three times in HBSS containing 0·3 M α-methyl-D-mannoside (Sigma Ltd) to remove Con A and then washed twice in HBSS and reconstituted to 1×10^6 cells/ml in 20% FCS in RPMI 1640.

Equal numbers of Con A-stimulated and -unstimulated lymphocytes were co-cultured in the presence of 20 μg/ml of PWM and incubated at 37°C for 6 days as described above. The results of the suppressor-cell assay were expressed as percentage suppression, using the following formula:

$$\% \text{ suppression} = \left[1 - \frac{\text{No. of plaque forming cells or amount of IgM produced by PWM stimulated cells + Con A}}{\text{No. of plaque forming cells or amount of IgM produced by PWM stimulated cells}} \right] \times 100$$

Statistics. Wilcoxon's rank sum test was used to test the statistical significance of differences in the number of IgM-producing cells, IgM synthesis and suppressor-cell function in patients and control subjects. Linear regression analysis was used to assess the relationship between *in vitro* observations and serological parameters.

RESULTS

IgM synthesis

The median *number* of lymphocytes spontaneously producing IgM at the end of the 7-day culture period was similar in patients and control subjects (110 and 147/10^6 cells respectively; $P > 0{\cdot}05$ (Fig. 1)). Following stimulation with PWM there was a similar increase in the median number of IgM producing cells in patients and control subjects (1812 and 1798/10^6 cells respectively; $P > 0{\cdot}05$). In contrast the median *amount* of IgM produced by lymphocytes from PBC patients during the culture period was significantly greater than that produced by lymphocytes from normal subjects both spontaneously (914 and 298 ng/10^6 cells respectively; $P < 0{\cdot}05$) and after PWM stimulation (1887 and 796 ng/10^6 cells respectively; $P < 0{\cdot}05$ (Fig. 1)). The calculated median amount of IgM spontaneously produced per B lymphocyte in patients and control subjects was 8·4 and 2·5 ng per cell respectively ($P < 0{\cdot}02$).

IgM synthesis during autologous and allogeneic co-culture of T and B lymphocytes

PWM-stimulated co-culture experiments using T and B lymphocytes from patients (TpBp) and normal subjects (TnBn) clearly showed that abnormal T-cell function and not autonomous B-cell hyperreactivity was responsible for the increased IgM synthesis (Fig. 2). Thus when Bn were co-cultured with Tp, IgM synthesis approached levels seen when autologous Bp and Tp were co-cultured (median values of 827 ng and 850 ng/10^6 cells respectively). Conversely, when Bp were co-cultured with Tn, IgM synthesis fell to levels seen when autologous Bn and Tn were co-cultured (557 ng and 538 ng/10^6 cells respectively). These changes in IgM synthesis during the culture period

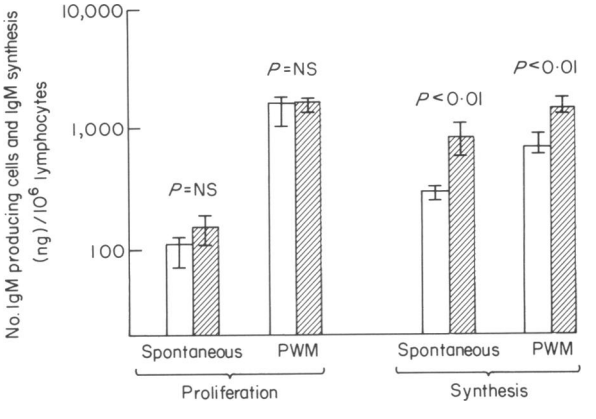

Fig. 1. Spontaneous and pokeweed mitogen stimulated IgM producing B-cell proliferation and IgM synthesis by lymphocytes from normal subjects (□) and PBC patients (▨). (Values shown are median and range.) NS = not significant.

were not associated with any significant change in the number of IgM-producing cells on the seventh day. To determine if increased IgM synthesis was related to defective suppressor T cells or to enhanced helper T-cell function, the co-culture experiments described above were repeated using irradiated and unirradiated T cells from six normal subjects and six patients. When irradiated T lymphocytes from normal subjects were co-cultured with allogeneic B lymphocytes from either normals or patients, IgM synthesis increased (Fig. 3). In contrast irradiation of T lymphocytes from patients prior to co-culture with allogeneic B cells was not associated with any further increase in IgM synthesis. Since irradiation inactivates suppressor cells, these data are consistent with a suppressor, rather than helper T-cell defect in PBC.

Suppression of IgM synthesis

Inhibition of proliferation of autologous IgM-producing B cells by Con A-activated suppressor cells was similar in patients and control subjects (mean % suppression = 73·8 ± s.d. 35·7 and 91·4 ± s.d. 9·8 respectively; $P > 0{\cdot}05$) although two patients had a profound defect in suppressor cell function (Fig. 4). In contrast inhibition of the amount of IgM synthesized was significantly less in

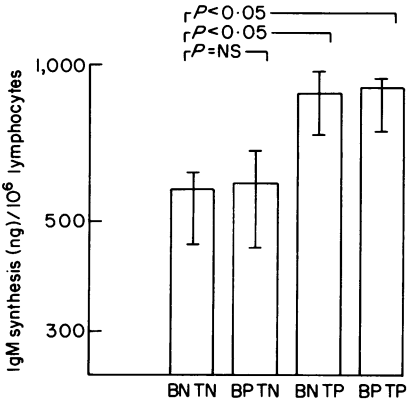

Fig. 2. Amount of IgM synthesized (ng) during co-culture of T and B lymphocytes from patients (BpTp) and normal control subjects (BnTn). (Values shown are median and range.) NS = not significant.

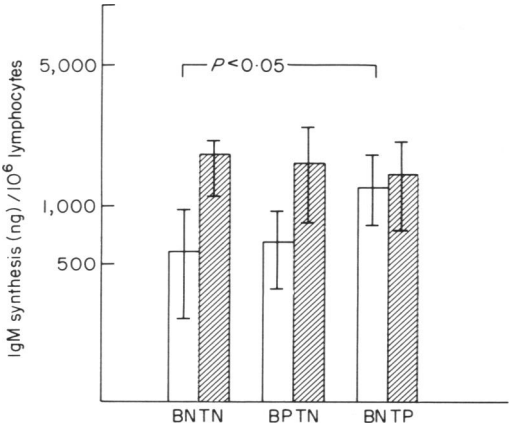

Fig. 3. Amount of IgM synthesized (ng) during co-culture of T and B lymphocytes from patients (BpTp) and normal control (BnTn). ■ Irradiated T lymphocytes. □ Unirradiated T lymphocytes. (Values shown are median and range.) NS = not significant.

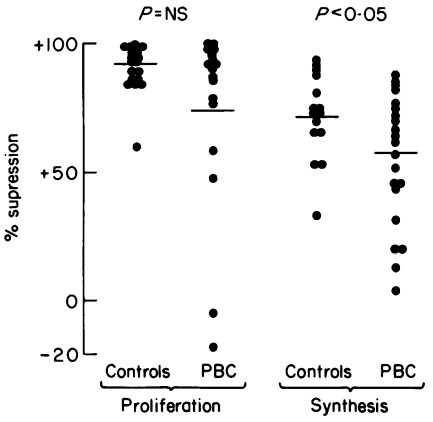

Fig. 4. Concanavalin A activated suppressor cell inhibition of proliferation of IgM producing B cells and amount of IgM synthesized by lymphocytes isolated from control subjects and patients with PBC. Horizontal bars represent mean values. NS = not significant.

patients than in control subjects (mean % suppression = 52·1 ± s.d. 25·5 and 69·2 ± s.d. 16·7 respectively; $P < 0{\cdot}01$).

Correlations

There was a weak, but significant correlation between the number of B cells producing IgM following PWM stimulation (as assessed by the haemolytic plaque assay and serum IgM levels in patients with PBC ($r = 0{\cdot}50$; $P < 0{\cdot}05$). No association was found between the activity of Con A-stimulated suppressor cells regulating the proliferation of B cells secreting IgM, using either the plaque or immunofluorometric assays and the titre of anti-mitochondrial antibody, serum bilirubin, alkaline phosphatase, aspartate transaminase levels and serum IgM or IgG levels in patients with PBC.

DISCUSSION

Since immunoglobulin catabolism has been found to be normal in patients with chronic liver disease (Cohen, 1963), the increase in serum immunoglobulin levels in primary biliary cirrhosis (PBC) is likely to represent increased synthesis and indeed this has been directly demonstrated *in vitro* in the present studies. Two possible explanations for this observation deserve consideration. Increased IgM synthesis could have resulted if either B lymphocytes were unresponsive to normal T-cell control or there was a defect in T-cell control of B-lymphocyte function. The clear drop in IgM production when T lymphocytes from normal individuals were co-cultured with B lymphocytes from patients and the rise of IgM production when T lymphocytes from patients were co-cultured with B cells from normal subjects indicate that the primary abnormality in PBC is due to T-cell dysfunction and that patients B cells can respond to normal regulatory T-cell influences. This conclusion is in agreement with previous observations demonstrating normal B-cell function in PBC (James *et al.*, 1980).

Results of studies of immunoregulatory T-cell function *in vitro* in PBC have recently been reported which suggest that the primary abnormality of Ig production is due to defective suppressor T-cell function. Thus lymphocytes from PBC patients continued to synthesize increased quantities of Ig when co-cultured at high ratios of T cells to B cells (where suppressor-cell function normally predominates) and this effect could be reproduced in normal subjects if suppressor T cells were first inactivated by irradiation prior to culture (James *et al.*, 1980). This latter finding also implies that helper T-cell function is normal in PBC. Other studies have evaluated suppressor T-cell control of

B-cell Ig production with conflicting results. In one such study (James *et al.*, 1980), increased B-cell Ig synthesis was attributed to defective suppressor-cell function while a second (Dienstag *et al.*, 1981) had shown that suppressor cells from PBC patients and normal subjects inhibited B cell Ig production to the same extent. However, these studies are not directly comparable since the former assessed unstimulated suppressor-cell activity in an autologous cell-culture system while the latter examined the function of Con A-activated suppressor cells in allogeneic culture system. In the present study we have demonstrated that suppressor-cell regulation of IgM production is abnormal in PBC and that at least two subpopulations of suppressor T cells may be involved in the control of IgM synthesis. In PBC the increased IgM production may result from abnormalities of T lymphocyte subpopulations regulating IgM secretion.

Although defective suppression of IgM production in PBC might explain high serum IgM levels, observations of the *in vivo* response to immunization with bacteriophage ØX174 have demonstrated a failure of the normal transition from IgM to IgG synthesis (Thomas *et al.*, 1976) and have suggested that this switching defect could result in persistently increased IgM production. However, available data have clearly demonstrated that circulating B cells (which lack SIgD and do not form rosette with mouse erythrocytes (Kuritani & Cooper, 1982)) appeared to be precommitted to a particular isotype which they produce following PWM stimulation (Mayumi *et al.*, 1983). Thus, there is no evidence to indicate that switching from IgM to IgG synthesis occurs following PWM stimulation and the present results cannot be explained on the basis of such a switching defect.

REFERENCES

ALLISON, A.C., DENMAN, A.M. & BARNES, R.D. (1971) Cooperating and controlling function of thymus derived lymphocytes in relation to autoimmunity. *Lancet*, **ii**, 135.

BOYUM, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* (Suppl. 21) **97**, 77.

COHEN, S. (1963) γ-globulin metabolism. *Br. med. Bull.* **19**, 202.

DIENSTAG, J.L., WEAKE, J.R. & WANDS, J.R. (1981) Abnormalities of mononuclear cell regulation in vitro in primary biliary cirrhosis. *Liver*, **1**, 230.

DONIACH, D., ROITT, I.M., WALKER, J.G. & SHERLOCK, S. (1966) Tissue antibodies in primary biliary cirrhosis, active chronic (lupoid) hepatitis, cryptogenic cirrhosis and other liver disease and their clinical implication. *Clin. exp. Immunol.* **1**, 237.

GELFAND, E.W., SHORE, A., GREEN, B., LIN, M.T. & DOSCH, H.-M. (1979) The E rosette assay: A cautionary note. *Clin. Immunol. Immunopathol.* **12**, 119.

HAMMERSTROM, L., BIRD, A.G., BRITTON, S., SMITH, C.I.E. (1979) Pokeweed mitogen differentiation of human B cells: Evaluation by a protein A haemolytic plaque assay. *Immunology*, **38**, 181.

JAMES, S.P., ELSON, C.O., JONES, E.A. & STROBER, W. (1980) Abnormal regulation of immunoglobulin synthesis *in vitro* in primary biliary cirrhosis. *Gastroenterology*, **79**, 242.

JAMES, S.P., VIERLING, J.M. & STROBER, W. (1981) The role of the immune response in the pathogenesis of Primary Biliary Cirrhosis. In: *Seminars in Liver Disease*, Vol. 1, No. 4, p. 322. Thieme-Stratton Inc., New York, NR 10016.

KURITANI, T. & COOPER, M.D. (1982) Human B cell differentiation. II. PWM response of B cells belong to a surface IgD negative subpopulation. *J. exp. Med.* **155**, 1561.

MAYUMI, M., KURITANI, T., KUBAGAURA, H. & COOPER, M.D. (1983) IgG subclass expression by human B lymphocytes and plasma cells. B lymphocytes precommitted to IgG subclass can be preferentially induced by polyclonal mitogens with T cell help. *J. Immunol.* **130**, 671.

MCFARLANE, I.G., WOJCICKA, B.M., TSANTOULAS, D.C., PORTMANN, B., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1979) Leukocyte migration inhibition in response to biliary antigens in primary biliary cirrhosis, sclerosing cholangitis and other chronic liver disease. *Gastroenterology*, **76**, 1333.

NOURI-ARIA, K.T., HEGARTY, J.E., ALEXANDER, G.J.M., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1982) Effect of corticosteroids on suppressor cell activity in autoimmune and viral chronic active hepatitis. *New Engl. J. Med.* **307**, 1301.

REINHERZ, E.L. & SCHLOSSMAN, S.F. (1980) Regulation of the immune response. Inducer and suppressor T lymphocyte subsets in human beings. *New Engl. J. Med.* **303**, (7), 370.

SCHAFFNER, F. & POPPER, H. (1982) Clinical pathological relations in primary biliary cirrhosis. In: *Progress in Liver Disease*. Vol. VII. (ed. by H. Popper & F. Schaffner) p. 529. Grune & Stratton, New York.

SCHWARTZ, S.A. (1980) Heavy chain specific suppression of immunoglobulin synthesis and secretion by lymphocytes from patients with selective IgA deficiency. *J. Immunol.* **124**, 2034.

SHERLOCK, S. & SCHEUER, P.J. (1973) The presentation and diagnosis of 100 patients with primary biliary cirrhosis. *New Engl. J. Med.* **289**, 674.

SHOU, L., SCHMARTZ, S.A. & GOOD, R.A. (1976) Suppressor cell activity after concanavalin A treatment of lymphocytes from normal donors. *J. exp. Med.* **143**, 1100.

THOMAS, H.C., HOLDEN, R., VERRIER JONES, J. &

PEACOCK, D.B. (1976) Immune response to ØX174 in man: Primary and secondary antibody production in primary biliary cirrhosis. *Gut*, **17**, 844.

TSANTOULAS, D., PERPERAS, A., PORTMANN, B., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1980) Antibodies to a human liver membrane lipoprotein (LSP) in primary biliary cirrhosis. *Gut*, **21**, 557.

VEIRLING, J.M., NELSON, D.L., STROBER, W., BUNDY, B.M. & JONES, E.A. (1977) *In vitro* cell-mediated cytotoxicity in primary biliary cirrhosis and chronic hepatitis; dysfunction of spontaneous cell-mediated cytotoxicity in primary biliary cirrhosis. *J. clin. Invest.* **60**, 1116.

WANDS, J.R., DIENSTAG, J.L., BAHN, A.K., FELLER, E.R. & ISSELBACHER, K.J. (1978) Circulating immune complexes and complement activation in primary biliary cirrhosis. *New Engl. J. Med.* **298**, 233.

ZETTERMAN, R.K. & WOLTJEN, J.A. (1980) Suppressor cell activity in primary biliary cirrhosis. *Dig. Dis. Sci.* **25**, 104.