Reduction in serum levels of antimitochondrial (M2) antibodies following immunoglobulin therapy in severe combined immunodeficient (SCID) mice reconstituted with lymphocytes from patients with primary biliary cirrhosis (PBC)

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SUMMARY

The effect of gammaglobulin treatment on autoantibody production was investigated in SCID mice reconstituted with human peripheral blood mononuclear cells (PBMC) obtained from patients with PBC. All reconstituted mice displayed the presence of human antimitochondrial antibodies (αM2Ab) of both IgG and IgM types before treatment with human immunoglobulin. Two weeks after i.p. injection of 20×10^6 PBMC into SCID mice, i.p. treatment with various preparations of human immunoglobulin was initiated. In control animals treated with saline, serum levels of human αM2Ab of the IgG type increased with time, peaking around 4 weeks after reconstitution. In contrast, human IgG autoantibodies rapidly decreased in all animals treated with human IgG. Treatment with a human IgM preparation had no effect on serum levels of αM2Ab of the IgG type. The results may suggest that the pronounced reduction of specific IgG autoantibodies was due to an increased catabolism of human IgG, including the autoantibodies, in the gammaglobulin-treated mice. Although the production of human αM2Ab in reconstituted mice could be easily shown, PBC-specific liver lesions or bile duct destruction were not observed, irrespective of treatment protocol.

Keywords primary biliary cirrhosis   SCID mice   immunoglobulin therapy   autoimmune disease   antimitochondrial antibodies

INTRODUCTION

PBC is a chronic, progressive cholestatic liver disease often diagnosed in middle-aged women and characterized by ongoing inflammatory destruction of interlobular and septal bile ducts [1–3]. The disease is generally progressive and usually leads to chronic cholestasis, fibrosis/cirrhosis, portal hypertension, and often premature death secondary to liver failure unless liver transplantation is performed [4–6]. While the etiology of PBC remains unknown, the disease is thought to be related to abnormalities in immune regulation [7]. PBC is serologically characterized by the presence of autoantibodies to mitochondria which can be detected in more than 95% of patients [2]. A family of antimitochondrial antibodies has been identified. The M2 antibody, which reacts with a trypsin-sensitive antigen on the inner mitochondrial membrane, is found most closely in association with PBC [8,9].

The homozygous C.B-17 scid/scid (SCID) mutant mouse strain

exhibits a severe immunodeficiency involving both T and B cells [10,11]. Several approaches have been developed to establish *in vivo* models for human haematopoiesis by transplanting human haematopoietic cells into SCID mice. SCID mice can be successfully engrafted with human lymphoid tissues [12], human bone marrow [13] and human peripheral blood mononuclear cells (PBMC) [14]. Such chimaeric mice present certain characteristics of the human immune system and can be used as *in vivo* models of human haematopoiesis, infectious diseases, autoimmunity, tumourigenesis, and primary immunodeficiencies [12,15–19].

In one of the initial reports on repopulation of SCID mice, Krams *et al.* found significant levels of human anti-M2 antibodies (αM2Ab) of the IgM and all IgG subclasses in SCID mice receiving PBMC from patients with PBC [20]. However, although the histological lesions in the liver resembled those seen in graft-*versus*-host disease (GVHD) during hepatic allograft rejection, it was suggested as a possible animal model of PBC.

During the past decade, treatment with intravenous immunoglobulin (IVIG) has become an established form of therapy in a

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number of autoimmune diseases such as idiopathic thrombocytopenic purpura [21,22]. The mechanism underlying the therapeutic effect still remains elusive, but IVIG may exhibit a number of biological properties in addition to its well recognized antimicrobial effect [23,24].

Theoretically, IVIG may have beneficial effects in a variety of potentially autoimmune disorders. However, the therapeutic effect of immunoglobulins has been tested only in a limited number of these conditions. In the present study, we used the SCID mouse model of PBC to examine the effect of immunoglobulin therapy on circulating αM2Ab as well as on histological liver lesions.

MATERIALS AND METHODS

Preparation of human PBMC

PBMC from buffy coats donated by four patients with well established diagnosis of PBC, were isolated by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation. All patients, one in stage 2 (patient 1), two in stage 3 (patients 2 and 3), and one in stage 4 (patient 4) of the disease, had detectable circulating αM2Ab of both IgG and IgM types (Table 1). SCID mice were injected intraperitoneally with 20×10^6 PBMC in 0·5 ml volume of PBS.

SCID mice

Homozygous C.B-17 SCID mice were obtained from Stanford University (Stanford, CA) by courtesy of Drs M. Lieberman and J. M. McCune, and were bred and maintained under specific pathogen-free conditions at Huddinge University Hospital. The animals received antibiotics as previously described [12]. Eighty-two SCID mice were used for reconstitution experiments at 6–10 weeks of age. Mice of same sex and age were used for reconstitution with cells from a single patient.

Detection of human total and specific immunoglobulins in mouse sera

Following PBMC inoculation, serum samples were obtained biweekly before the treatments with immunoglobulins were given, i.e. 1 week after previous immunoglobulin substitution.

The detection of total human IgG and IgM as well as αM2Ab in the mouse serum was performed using ELISA. For measurement of total human IgG and IgM, rabbit anti-human μ and γ immunoglobulin heavy chains (Dakopatts, Glostrup, Denmark) were used as the capture agents and AP-conjugated affinity-purified rabbit anti-human μ and γ (Dakopatts) were used for detection, respectively.

For detection of αM2Ab, ELISA plates were coated with 100 μl (12 μg/ml) of pyruvate dehydrogenase (PDH; Sigma Chemical Co., St Louis, MO) in carbonate coating buffer and incubated at 4°C overnight. The plates were then washed three times with PBS containing 0·05% Tween 20. Different dilutions of sera from reconstituted mice and from patients, as well as standard serum and normal human and mouse control sera were dispensed into individual wells in a volume of 100 μl in PBS containing 0·05% Tween 20 and incubated for 1 h at room temperature. The standard serum was a serum pool from patients with PBC which is routinely used in our laboratory as a standard for quantitative measurement of IgG and IgM αM2Ab. The arbitrary standard contained 6000 U of either IgG or IgM αM2Ab in undiluted form. After washing, the plates were incubated for 1 h with 100 μl of AP-conjugated affinity-purified rabbit anti-human γ and μ (Dakopatts) for detection of IgG or IgM αM2Ab, respectively. Due to the differences in serum levels of αM2Ab in the patients and in order to simplify the comparisons in different groups, the levels of αM2Ab in reconstituted mice are displayed as percentages of the levels measured before immunoglobulin therapy 2 weeks after cell transfer.

Human immunoglobulin therapy in reconstituted mice

All immunoglobulin injections were given intraperitoneally in 0·5–1 ml volume of PBS. Control mice received i.p. injections of 0·5 ml PBS at each injection occasion. The first treatment was given 2 weeks after injection of human PBMC and was repeated once a week until the last treatment, which was given 9 weeks after transfer of cells. Mice reconstituted with PBMC from the first three patients were divided into two groups: (i) a control group receiving only PBS; and (ii) a gammaglobulin-treated

Table 1. Serum levels of total and specific antimitochondrial IgG and IgM in four patients with PBC and in SCID mice 2 weeks after reconstitution with cells from patients (prior to treatment with human immunoglobulin preparations)

	Serum immunoglobulin levels in patients with PBC				Serum immunoglobulin levels in reconstituted SCID mice (mean ± s.e.m.)			
	Patient 1 (stage 2)	Patient 2 (stage 3)	Patient 3 (stage 3)	Patient 4 (stage 4)	Recipients of patient 1 ($n = 17$)	Recipients of patient 2 ($n = 10$)	Recipients of patient 3 ($n = 32$)	Recipients of patient 4 ($n = 23$)
Total IgG (μg/ml)	19 000	13 000	8000	23 000	2120 ± 256	1571 ± 234	741 ± 78	2630 ± 364
Total IgM (μg/ml)	14 800	3900	8700	5600	346 ± 39	165 ± 38	145 ± 16	164 ± 27
Anti-M2 IgG (arbitrary unit)*	906	1869	4212	6065	34 ± 5·6	67 ± 8·5	175 ± 14	237 ± 22
Anti-M2 IgM (arbitrary unit)*	6346	4919	9216	1061	159 ± 25	95 ± 21	445 ± 48	13 ± 0·6

* The arbitrary unit for anti-M2 IgG is different from the arbitrary unit for anti-M2 IgM.

group. Gammaglobulin-treated animals received 50 mg/week purified human polyclonal IgG (Gammagard; N. V. Baxter S. A., Hyland Division, Lessines, Belgium). Mice reconstituted with cells from patient 3 were divided into four groups treated weekly with either PBS, 50 mg Gammagard, 50 mg Pentaglobin (Biotest, Pharma GmbH, Dreieich, Germany; containing 38 mg IgG, 6 mg IgM, and 6 mg IgA), or 50 mg IgM-enriched immunoglobulin preparation (prepared from Pentaglobin by column fractionation; consisting of 5 mg IgG, 33 mg IgM, and 12 mg IgA).

Histopathology and immunohistochemistry
All mice were killed 10 weeks after injection of cells. Pieces of liver tissues were fixed in 4% formaldehyde. Paraffin sections were stained with haematoxylin and eosin. Aliquots of liver tissues were also snap frozen in liquid nitrogen and stored in −70°C until cryosectioning. Sections (6 μm) were fixed in acetone and stored at −20°C. Before immunostaining, endogenous peroxidase was blocked using 3% H_2O_2 in TBS for 10 min. MoAbs against human CD45 (LCA) and CD20 (L26) were obtained from Dako-patts. A three-step indirect peroxidase method was used, with both secondary and tertiary reagents enzyme-conjugated (Dakopatts). The peroxidase reaction was developed using the DAB-method (Sigma).

Statistical analysis
Levels of total human and specific immunoglobulins in serum are expressed as mean ± s.e.m. The non-parametric Wilcoxon test was used for statistical comparison of immunoglobulin levels at each time point. Probability values ⩽ 0·05 were regarded as significant.

RESULTS

Total and specific IgG and IgM levels in the patients
As shown in Table 1, all patients had high serum levels of specific antibodies to mitochondrial M2 antigen. Anti-M2 antibodies of both IgG and IgM types could be detected, although a variation was found in the proportion of specific IgG and IgM in different patients. The levels of both total and specific IgG and IgM in serum samples from reconstituted SCID mice at 2 weeks correlated to the levels measured in patient sera (Table 1).

Presence of human αM2Ab in reconstituted mice
All reconstituted SCID mice were serologically analysed for the presence of human αM2Ab of both IgG and IgM types by ELISA. In initial pretreatment samples obtained from the mice 2 weeks after reconstitution, there was a variability in αM2Ab levels between groups reconstituted with cells from different patients. The serum αM2Ab levels in animals correlated to the levels of αM2Ab in patient sera (Table 1). In addition to this interpatient variability, mice reconstituted with cells from a single patient also showed a slight variation in levels of αM2Ab. The latter variability tended to increase over time.

In control mice reconstituted with cells from patients 1, 2 and 4, levels of αM2Ab of IgG type initially increased, with a peak 4 weeks after reconstitution (Fig 1a,c,e). The levels then declined and returned to the initial values around 8–10 weeks after reconstitution. Levels of αM2Ab of IgM type usually tended to decrease in control animals, with the exception of mice reconstituted with cells from patient 4, where a slight increase in levels

of IgM autoantibodies was noted 10 weeks post-transplant compared with levels obtained 2 weeks after reconstitution (Fig. 1b,d,f).

The levels of αM2Ab in non-repopulated mice as well as in transplanted mice with 20 × 10^6 PBMC from two normal blood donors were below detection range (<10 U) at any time after reconstitution.

Effect of immunoglobulin therapy on serum levels of αM2Ab
The serum level of αM2Ab of both IgG and IgM type was compared in two groups of reconstituted SCID mice. Mice reconstituted with PBMC from patients 1, 2 and 4 were either treated with human polyclonal IgG (Gammagard) or with PBS. Levels of αM2Ab of IgG type were significantly lower in mice treated with human polyclonal IgG than in control mice (Fig. 1a,c,e).

The presence of αM2Ab of IgG type was further analysed in mice injected with cells from patient 3. These animals were treated with various human immunoglobulin preparations. As shown in Fig. 2, the reduction of specific IgG antibodies was more pronounced in mice treated with purified polyclonal IgG (Gammagard) than in those treated with immunoglobulin preparation containing less IgG but with additional IgM and IgA (Pentaglobin). Furthermore, treatment with purified human IgM did not have any significant effect on levels of αM2Ab of IgG type compared with levels found in control mice (Fig. 2a).

Levels of αM2Ab of IgM type did not seem to be affected by treatment with human polyclonal IgG (Fig. 1b,d,f). In addition, treatment with the other human immunoglobulin preparations also containing IgM did not influence the levels of αM2Ab of IgM type (Fig. 2b).

Total human IgG and IgM levels in reconstituted mice
SCID mouse serem levels of total human IgG and IgM were measured at 2, 4, 6, 8 and 10 weeks after i.p. transfer of cells. The first sample at 2 weeks was obtained before any treatment. The level of human IgG seen in sera from reconstituted SCID mice was patient-dependent, although some differences were seen between levels in individual control mice repopulated with cells from a single patient. As shown in Fig. 2c, the highest levels of human IgG in control mice reconstituted with cells from patient 3 were obtained 6 weeks after injection of cells. Serum IgG levels in control mice reached 1·6 mg/ml, while mice treated weekly with 50 mg human polyclonal IgG (Gammagard) displayed up to 10 mg/ml human IgG at the same time point. In general, mice treated with human IgG-containing preparations had three to five-fold higher levels of human IgG compared with control animals when examined 6–8 weeks after reconstitution (Fig. 2c).

Serum total human IgM was also measured in reconstituted mice at the same time points as human IgG. In tumour-free mice, the mean serum level of human IgM was not significantly different in mice treated with human polyclonal IgG (Gammagard) compared with control mice at any time point, indicating that the treatment did not influence the level of engraftment. However, there was a more significant individual variation in serum levels of human IgM, particularly at later time points, compared with IgG levels. Serum total human IgM was significantly higher in mice treated with Pentaglobin or with the immunoglobulin preparation enriched for IgM compared with levels seen in control mice or in those treated with polyclonal human IgG (Fig. 2d). The highest levels of human IgM were
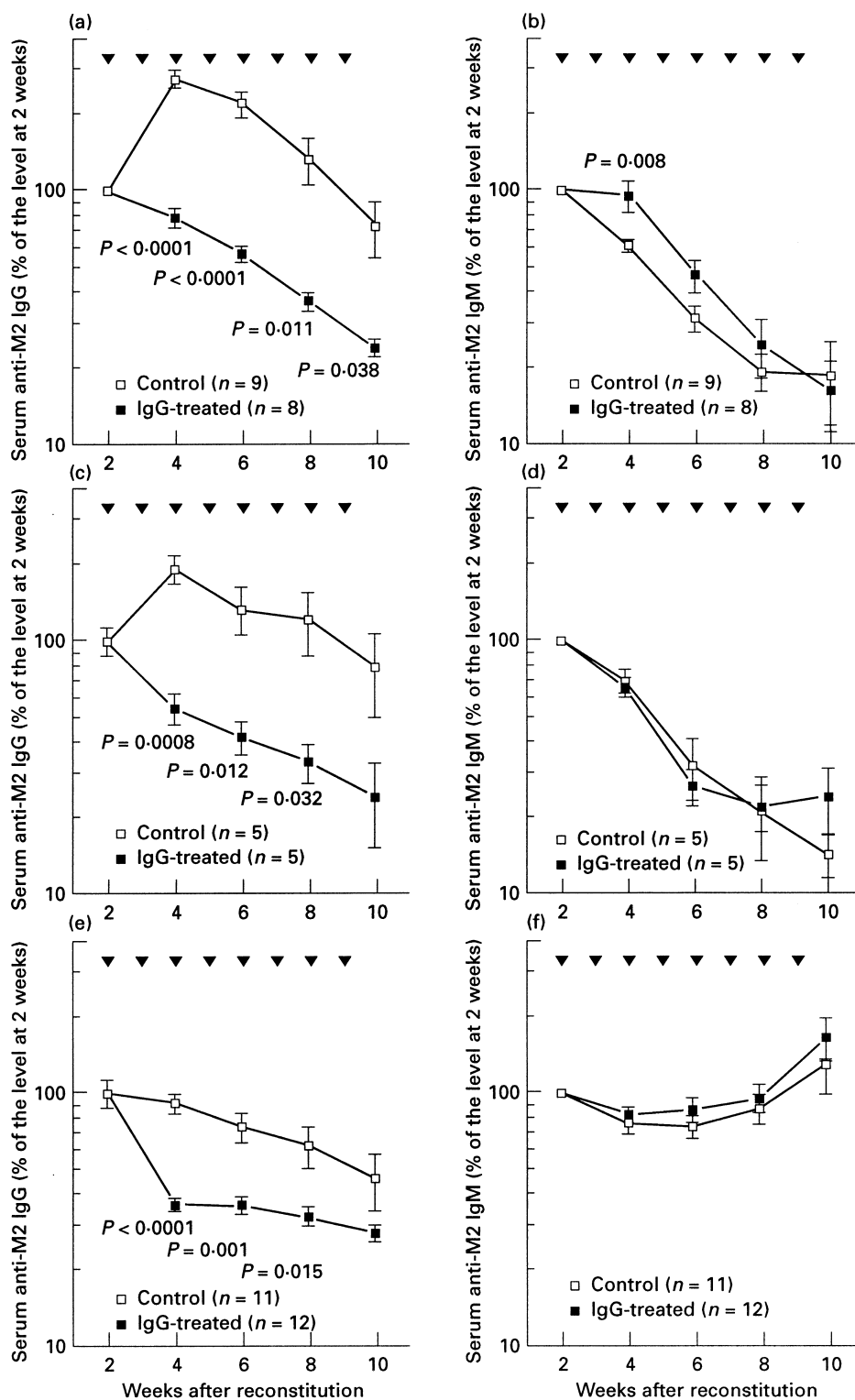
Fig. 1. Levels of specific human αM2Ab of IgG (a, c, and e) and IgM types (b, d, and f) in serum samples from SCID mice reconstituted intraperitoneally with 20×10^6 peripheral blood mononuclear cells (PBMC) obtained from patients 1 (a,b), 2 (c,d), and 4 (e,f) with PBC. Mice reconstituted with cells from each patient were divided into two groups receiving either PBS (□) or 50 mg Gammagard (■) on each treatment (▼). Serum samples were taken before the treatments were given. Levels shown are mean ± s.e.m.

detected 10 weeks after reconstitution in mice treated with human immunoglobulin preparation enriched for human IgM. Tumour development was accompanied by increased levels of both human IgG and IgM, although the increase in IgM levels was more prominent. However, in no single case did we notice a rise in αM2Ab of either the IgG or the IgM class coinciding with tumour development, strongly suggesting that transformed clones did not contribute to the levels of αM2Ab formed.
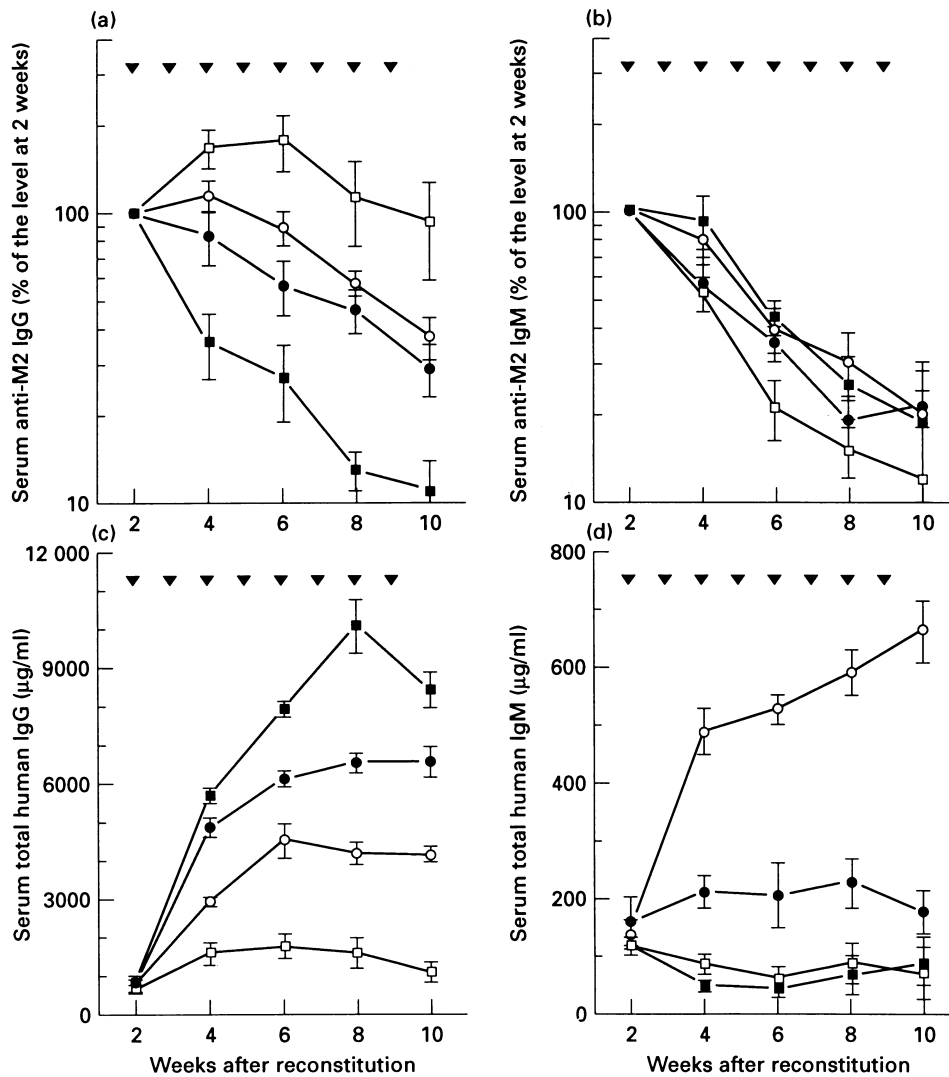
Fig. 2. Levels of specific human αM2Ab of IgG and IgM types (a,b, respectively) and total human IgG and IgM (c,d, respectively) in serum samples from SCID mice reconstituted intraperitoneally with 20×10^6 peripheral blood mononuclear cells (PBMC) obtained from patient 3 with PBC. Mice were divided into four groups receiving either PBS (□, $n = 7$), 50 mg Gammagard (■, $n = 8$), 50 mg Pentaglobin (●, $n = 9$), or 50 mg IgM-enriched immunoglobulin preparation (○, $n = 8$) at each treatment occasion (▼). Serum samples were taken before the treatments were given. Levels shown are mean ± s.e.m. The levels of specific human αM2Ab of IgG type were significantly lower at 4, 6, 8, and 10 weeks after reconstitution in the Gammagard-treated as well as in the Pentaglobin-treated groups compared with the control group ($P < 0.005$ and $P \leqslant 0.05$, respectively).

Histological analysis of liver tissues

Periportal leucocyte infiltration was seen in scattered portal triads in animals from all treatment groups. Neither the size, cellular composition, or relation to bile ducts were different in treatment groups, and human cells could not be demonstrated immunohistochemically in all portal infiltrates. In one animal from each treatment group receiving cells from patient 3, more extensive lymphoproliferative infiltrates developed in the peritoneal cavity. Also, in animals receiving cells from patient 4 (two in the Gammagard-treated and one in the control group), development of abdominal tumours was noticed. Infiltration of human CD45$^+$ lymphoid cells was also evident in the mesenchymal tissues of the hilar region of the liver and subcapsularly. In these animals, a higher frequency of human lymphoid cells was also seen around septal and a few interlobular bile ducts.

However, no distinct duct lesions or intraepithelial infiltrations were associated with these human lymphoid infiltrates. The B cell origin of cells in these infiltrates was indicated by the expression of CD20.

DISCUSSION

In the past few years, several investigators have utilized SCID mice for the study of human autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroiditis, and myasthenia gravis [25–28]. Although the presence of human autoantibodies could be shown in the sera of the reconstituted animals in most of these experiments, inconsistent results were obtained with respect to target tissue pathology [29].

In our experiments, conventional histological examination revealed no significant alteration in the liver of SCID mice reconstituted with cells from patients with PBC. Minute scattered periportal leucocyte infiltrates were seen in some, but not all, reconstituted animals. However, human leucocyte antigen-positive cells could not be consistently detected in these lesions, and some of the infiltrating cells were neutrophils, probably of mouse origin. From previous experience, a slight variability in the cellular infiltration is normally seen also in non-reconstituted SCID mice, probably related to environmental factors. In SCID mice receiving human PBMC intraperitoneally from Epstein–Barr virus (EBV)-positive donors, lymphoproliferative infiltrates regularly occur in the abdominal cavity [14,30]. Often these infiltrates are located in the hilar region of the liver, under the liver capsule and in septal areas, suggesting that the lymphatic flow in the mouse abdomen promotes this localization of injected human cells. In evaluating the potential for bile duct destruction following injection of PBMC from patients with PBC, several of these and other factors should be considered. Periportal infiltrates could be part of the normal mouse recirculation of leucocytes in the tissues with a slight variation over time. Some human cells might follow this lymphatic flow and be deposited along with mouse leucocytes. EBV-related lymphoproliferations may also be located in these areas without any relation to specific tissue homing or antigen recognition. Finally, injected human PBMC could develop a form of xenotransplantation reaction of GVHD type, as has been previously suggested [20].

Despite the lack of any direct evidence, the possibility of an antigen-specific disease-related immune attack of human lymphoid cells on mouse bile ducts should be considered. Such a disease mechanism, however, would require a cross-species interaction between adressins on human lymphoid cells and homing molecules in the mouse on endothelial and possibly epithelial cells. Furthermore, human T cells specific for a disease-related antigen in the autologous environment should be able to recognize and become activated by the corresponding mouse target tissue antigens presented by mouse MHC molecules, and possibly also be dependent on mouse-derived cytokines.

The present study was aimed at trying to evaluate the use of immunoglobulin therapy in the SCID mouse model of human PBC. IVIG was initially introduced as replacement therapy in antibody deficiency diseases [31]. In addition to its use as substitution therapy for primary and secondary immunodeficiencies, IVIG is increasingly being used as immunomodulating therapy in the treatment of patients with a variety of autoimmune and systemic inflammatory disorders [32,33]. However, the mechanism of action of IVIG in the treatment of these disorders remains poorly understood. Two main hypotheses have gained popularity: (i) Fc receptor modulation; and (ii) anti-idiotypic suppression of autoantibodies [32]. The presence of anti-idiotypic activity against disease-associated autoantibodies may contribute to the therapeutic effects of IVIG by directly blocking autoantibody activity *in vivo*, thereby causing a reduction of autoantibody titres [22,34]. Moreover, several reports have shown that regulatory T cell subsets are altered during high-dose IVIG therapy [35,36].

As previously described by Krams *et al.* [20], antimitochondrial antibodies were readily detected in the sera of SCID mice reconstituted with cells from patients with PBC. The levels of αM2Ab of IgG type were significantly higher than those of IgM type. This could be explained by the very short half-life of human IgM in SCID mice [37]. Interestingly, a continuous decline in serum levels αM2Ab of IgG type was observed in SCID mice reconstituted with cells from patients with PBC after treatment with human polyclonal IgG. However, injection of purified human IgM had no effect on the titres of specific IgG autoantibodies. If anti-idiotypic antibodies alone caused the reduction of αM2Ab in reconstituted animals, one might expect decreased levels of autoantibodies after treatment with both IgG and IgM. However, as anti-idiotypic regulation might be mediated via cellular mechanisms [38], a differential effect of anti-idiotypic IgG *versus* IgM antibodies on αM2Ab could be envisaged. The effect of immunoglobulin treatment on αM2Ab of IgM type in reconstituted animals should be interpreted with caution, as the half-life of human IgM in SCID mice is very short and minor effects thus may have been overlooked.

A major physiological factor that affects immunoglobulin catabolism is the plasma immunoglobulin concentration. The different classes of immunoglobulins differ significantly as regards the relationship between fractional catabolic rate and plasma concentration. The fractional rate of catabolism varies in direct proportion to the concentration of IgG in the plasma, while the half-life of IgM is independent of its plasma concentration [39]. The analysis of SCID mice receiving the IgM-enriched immunoglobulin preparation may suggest that the reduction of αM2Ab in reconstituted SCID mice treated with high-dose human IgG is due to an increased catabolism of IgG as such, including the autoantibodies. However, it would take appropriate labelling studies or assays of autoantibody-secreting cells in the recipient mice to establish whether this mechanism is indeed operative.

It is possible or even likely that a reduction of serum autoantibodies may result in improvement of the clinical course in selected autoimmune disorders. Several studies have demonstrated the usefulness of plasmapheresis in treatment of patients with autoimmune manifestations [40]. This effect has in many cases been ascribed to the lowering of autoantibody titres. In some autoimmune diseases such as myasthenia gravis, the effective use of IVIG has been favourably compared with plasmapheresis in treating the chronic phase of the disease [41,42]. In patients with PBC, antimitochondrial antibodies persist after liver transplantation, and recurrence of histological PBC in the allografted liver, although a rare occurrence [43], has been reported [44]. It is therefore possible that treatment of PBC patients with high-dose IVIG may be therapeutically beneficial and may result in remission of clinical symptoms or improvement of the pathological changes of the liver, and a controlled clinical trial could be useful for establishing the efficacy of IVIG treatment in PBC.

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