Immunology 1998 **94** 410–416

Human/BALB radiation chimera engrafted with splenocytes from patients with idiopathic thrombocytopenic purpura produce human platelet antibodies

B. DEKEL,* H. MARCUS,* B. SHENKMAN,‡ A. SHIMONI,‡ Y. SHECHTER,‡ A. CANAAN,* A. BERREBI,† D. VARON‡ & Y. REISNER* **Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel, †Institute of Haematology, Kaplan Hospital, Rehovot, Israel, and ‡Institute of Haematology, Sheba Medical Centre, Tel Hashomer, Israel*

SUMMARY

We have previously shown that lethally irradiated normal strains of mice, radioprotected with severe combined immunodeficient (SCID) bone marrow, can be engrafted with human peripheral blood mononuclear cells (PBMC). The human/mouse radiation chimera can mount marked humoral and cellular responses to recall antigens, as well as primary responses. In the present study, we adoptively transferred splenocytes from patients with chronic immune thrombocytopenic purpura (ITP) into lethally irradiated BALB/c mice, radioprotected with SCID bone marrow. High titres of total human immunoglobulin appeared as early as 2 weeks post-transplant and declined after 6 weeks, while human anti-human platelet antibodies were detected 2–8 weeks after the transfer of splenocytes. The immunoglobulin G (IgG) fraction contained antibodies against glycoprotein (GP) IIb/IIIa (CD41) or GPIb/IX (CD42). The human platelet antibodies showed a low level of cross-reactivity with mouse platelets, and thrombocytopenia in the animals was not observed. Splenocytes from individual ITP patients differed in their capacity to produce either human platelet antibodies or total human immunoglobulin. Furthermore, antibodies produced in the murine system were not always identical to the original antibodies present in the serum of the patients. The study of the serological aspects of autoantibodies against human platelets in an animal model might be useful for the investigation of potential therapeutics in ITP.

INTRODUCTION

Immune thrombocytopenic purpura (ITP) is principally a disorder of increased platelet destruction mediated by autoantibodies directed against platelet-membrane antigens.[1] The hypothesis that autoantibodies have a pathogenic role and are of paramount importance is supported by early observations that infusions of plasma from patients with ITP caused acute thrombocytopenia in normal subjects.[2] Autoantibodies against the major membrane glycoproteins (GP) can be identified in about 80% of patients with ITP.[3,4] The majority of these antibodies target epitopes on either GPIIb/IIIa (CD41) or the GPIb/IX/V (CD42) complex.[5] First-line treatment options consist mainly of glucocorticoid therapy and high doses of intravenous gamma globulin, and splenectomy is a typical second-line treatment.[6] Recently, antibody concentrations on patients' platelets have been shown to diminish in response to effective treatment or to increase upon relapse.[3,6] In the past few years, human/mouse chimera based on Mosier's peripheral blood lymphocyte–severe combined immunodeficient (PBL–SCID) model[7] have proved to be useful for studies of autoimmune disease. Lymphocytes from humans with several autoimmune diseases, such as primary biliary cirhosis, rheumatoid arthritis, autoimmune thyroid disease and systemic lupus erythematosus, were injected into SCID mice, thus 'transferring' these human diseases to the mice, and allowing the study of autoantibody production and other pathogenic mechanisms.[8–11]

Recently, Lubin *et al.*[12,13] described a new approach enabling engraftment of human peripheral blood mononuclear cells (PBMC) in normal strains of mice and rats following split-dose lethal irradiation. Irradiated animals are initially converted into SCID-like animals by means of bone marrow transplantation from SCID donors and subsequently are infused with $50–100 \times 10^6$ human PBMC. These chimeric animals allow an effective and rapid engraftment of human cells, enabling their functional study early after transplantation. Unlike the hu-PBL-SCID mouse model described by Mosier,[7] dissemination of engrafted lymphocytes is very rapid and both T and B lymphocytes are found in significant numbers in the lymphoid tissues as early as 24 hr following transplantation,[12,14] enabling their functional study early after transplantation. High levels of human immunoglobulin were

Received 15 January 1998; revised 10 March 1998; accepted 10 March 1998.

Abbreviations: ITP, idiopathic thrombocytopenic purpura; PBMC, peripheral blood mononuclear cells; SCID, severe combined immune deficiency.

Correspondence: Dr Y. Reisner, Department of Immunology, The Weizmann Institute of Science, Rehovot 76 100, Israel.

found as early as 10 days post-transplant of human PBMC in conditioned mice and the human/BALB chimera were capable of mounting a significant memory as well as primary response against different antigens.[15] The apparent activity of the human B cells in the former chimeric mice was explained, in part, by their ability to migrate readily from the peritoneum into the spleen and lymph nodes (LN), where the establishment of structures similar to germinal centres were found to be formed.[14] Moreover, in contrast to human SCID mice, human/BALB chimera do not develop Epstein–Barr virus lymphono.[16]

A vigorous anti-allogeneic human cytotoxic T-lymphocyte (CTL) response also could be generated in these mice, by immunizing them with foreign antigens or with allogeneic cells, respectively.[17] In addition, models of human disease, including those for human B-chronic lymphocytic leukaemia (CLL)[18] and human renal allograft rejection[19,20] have been established in these chimeras. In the present study we attempted to generate a human/BALB radiation chimera with splenocytes from ITP patients. We further investigated, in such radiation chimera, the extent of human anti-platelet antibody production, including specific immunoglobulin G (IgG) autoantibodies, and the relative autoantibody production potential of splenocytes from ITP patients at different stages of disease activity.

MATERIALS AND METHODS

Mice

Animals used were 6–10 weeks old. BALB/c mice were obtained from Olac Farms (Bicester, UK), and SCID mice from the Weizmann Institute Animal Breeding Centre (Rehovot, Israel). All mice were kept in small cages (five animals in each cage) and fed sterile food and acid water containing ciprofloxacin (20 μg/ml).

Conditioning regimen

BALB/c mice were exposed to split-dose (4 Gy followed 3 days later by 10 Gy) total body irradiation (TBI), from a gamma beam 150-A^{60}Co source (produced by the Atomic Energy of Canada, Kanata, Ontario) with focal skin distance (FSD) of 75 cm and a dose rate of 0·7 Gy/min.

Preparation and transplantation of T cell-depleted bone marrow cells

Bone marrow cells were collected into phosphate-buffered saline (PBS) by flushing the shafts of the femur and tibia obtained from non-obese diabetic (NOD)/SCID mice (4–6 weeks old), and were fractionated by differential agglutination with soybean agglutinin (to remove T lymphocytes that may be present in occasional 'leaky' SCID mice), according to Reisner *et al.*[21] with minor modifications.[22] Recipient mice were injected (intravenously in 0·2 ml PBS) with $2–3 \times 10^6$ T-cell-depleted SCID bone marrow cells or non-depleted SCID bone marrow in some of the experiments 1 day after irradiation.

ITP patients

The patient group included five patients treated in the Sheba Medical Centre Haematology Institute, Tel Hashomer, Israel, and the Kaplan Hospital Haematology Institute, Rehovot, Israel. Diagnosis of ITP was based on low platelet count, bone marrow with hyperactive megakaryopoiesis, antibodies to platelets and the absence of other causes of thrombocytopenia. Splenectomy was required in all of the patients for either a relapse of their disease, after an initial response to glucocorticoids, or unresponsiveness to steroid therapy and inability to attain remission. Patient's charts were reviewed for age, disease duration, platelet count, levels and types of anti-platelet antibodies and treatment before the splenectomy.

Preparation and transplantation of human splenocytes and PBMC

Spleens were cut into pieces and pressed through stainless steel sieves to make a cell suspension in PBS. Cell suspensions and buffy coats from normal volunteers were layered onto Lymphoprep solution (Nycomed, Oslo, Norway) and spun at 400 ***g*** for 20 min. The interlayer was collected, washed twice, counted and resuspended in PBS (pH 7·4) to the desired cell concentration. Human splenocytes or PBMC ($70–100 \times 10^6$) were injected intraperitoneally (i.p.) into recipient BALB/c mice conditioned as described above. Control mice did not receive human PBMC.

Cells and plasma collection from human/mouse chimera

Animals were bled from the retro-orbital vein using heparin-coated glass capillaries. Plasma was kept for human immunoglobulin and human anti-platelet antibody determination. Peritoneal cells were obtained by lavage with 5–10 ml of PBS and cells were then isolated using Lymphoprep, washed with PBS containing 1% bovine serum albumin (BSA), and stained for fluorescence-activated cell sorter (FACS) analysis.

FACS analysis of human cell engraftment

Single-cell suspensions were incubated for 30 min on ice with a mixture of appropriate fluorescence-labelled monoclonal antibodies (mAb). After washing, two- or three-colour fluorescence analysis of human antigens was performed using a FACScan analyser (Becton Dickinson, Mountain View, CA). The following murine mAb were used to identify leucocyte subsets: CD45-fluorescein isothiocyanate (FITC) (pan human leucocyte antigen); CD3-peridinin chlorophyll protein (pan T cell); CD20-phycoerythrin (PE) (pan B cell), were all from Becton Dickinson (Mountain View, CA).

Human immunoglobulin determination

Sera were tested for antigen-specific human antibody and for total human immunoglobulin as well as human immunoglobulin subclass (IgG, IgM and IgA). Total human immunoglobulin and human subclass was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) using goat F(ab')$_2$-purified anti-human IgG + IgM + IgA (Zymed Laboratories, San Francisco, CA) as the capture agent and peroxidase-conjugated purified goat anti-human immunoglobulin (G + M + A) or each of the anti-immunoglobulin subclass alone (Zymed Laboratories) as the detection reagent. Human serum of known immunoglobulin concentration was used as the standard. Microplates (Nunc, Roskilde, Denmark) pre-coated with the capture reagent (5 μg/ml, 50 μl/well) and blocked with 1% BSA were incubated overnight at 4° with dilutions of plasma from 1 : 500–1 : 16 000, or the standard from 0·05 to 1 μg/ml, then washed three times with PBS–

Tween solution. The detection reagent was added and the plates were incubated for 2 hr at 37°, then washed again three times. Fresh substrate solution was added and, after peroxidase-catalysed colour development, the absorbance at 405 nm was quantified on an ELISA reader (Dynatech, Port Guernsey, Channel Islands, UK).

Determination of human anti-human and anti-mouse platelet antibodies and subclass analysis

The assay was performed according to Visentin *et al.*[23] Human platelets, obtained from the blood bank, or mouse platelets, collected from BALB/c mice, were washed three times in 50 µl of Ringers-citrate-dextrose buffer containing 50 ng/ml prostaglandin E_1 (RCD–PGE_1) and were then resuspended in the same buffer at a concentration of $2{\cdot}5 \times 10^8$ per ml. For the assay, 5×10^7 platelets were aliquoted into polypropylene tubes treated with 1% BSA. Sera collected from mice that were preconditioned as described above and injected with splenocytes from ITP patients or with normal PBMC and sera collected from preconditioned mice that were not injected with human lymphocytes, were added to a final volume of 400 µl and the mixture was incubated for 30 min at room temperature. Human or mouse platelets were then washed with RCD-PGE_1 buffer and incubated with FITC-labelled anti-human immunoglobulin, anti-human IgG and anti-human IgM, separately, at a dilution of 1:10, for 30 min at room temperature in the dark. After three additional washes in RCD–PGE_1 buffer, platelets were suspended in 400 µl of buffer. Platelet-bound immunoglobulin was measured by flow cytometry (Beckton Dickinson, Mountain View, CA) using an immunoglobulin-specific conjugate and the isotype was determined with specific reagents.

MAIPA studies

The MAIPA (monoclonal antibody-specific immobilization of platelet antigens) test was performed according to Kiefel *et al.*[24] with minor changes. Platelets from normal donors were pooled and stored in saline containing 0·5% azide. To $50–100 \times 10^6$ platelets, 20 µl of the serum to be tested was added and incubated at 37° for 30 min. After two washes of the samples with saline, 10 µl of the murine monoclonal antibodies against GPIIb/IIIa or GPIb/IX (Immunotech, Marseilles, France) was added, and the test tubes were incubated for 30 min at 37°. The samples were washed three times with saline and then solubilized in solubilization buffer for 30 min at 4°. The samples were centrifuged at 400 *g* for 30 min at 4°, diluted in PBS and 100 µl was transferred, in duplicate, into wells of microtitre plates coated with rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark), and incubated for 90 min at 4°. The well contents were then discarded, the wells washed six times with PBS and then 100 µl of alkaline phosphatase-labelled goat anti-human IgG, diluted in PBS, was added. Following incubation for 120 min at 4°, the wells were washed six times with PBS and then 100 µl of substrate solution (5 mg *p*-nitrophenyl phosphate in diethanolamine buffer) was added. After incubation at 37° in the dark the reaction was read at 405 nm.

Statistical analysis

Statistical analyses were performed using the Stat View II program (Abacus Concepts, Inc, Berkeley, CA) on a Macintosh IICi. Statistical significance was determined by the Student's *t*-test.

RESULTS

Engraftment of spleen cells from ITP patients in the human/mouse radiation chimera

Splenocytes from five patients with chronic ITP were transplanted into the peritoneal cavity of lethally irradiated BALB/c mice radioprotected with SCID bone marrow (each group comprising 10–15 animals). Engraftment of the human cells was evaluated by FACS analysis of peritoneal washes and by ELISA of total human immunoglobulin in the serum of transplanted mice at 14 days post-transplant. Mice were scored positive for engraftment when $CD3^+CD45^+$ and $CD20^+CD45^+$ human cells were detected in the peritoneal fluid 14 days following cell transfer, and the level of total human immunoglobulin in their serum was higher than 600 µg/ml (Figs 1a, 1b). Thus, as can be seen in Table 1, a high rate of engraftment was attained (42 out of 51 surviving mice) in four out of five experiments, in which spleen cells from five different patients were infused.

Total human platelet antibody production by splenocytes from ITP patients and by PBMC from normal donors

The level of human anti-human platelet antibodies was measured only in engrafted mice (see above). Background reactivity produced by serum samples from control mice, which did not receive lymphocytes, was subtracted from all measurements.

As can be seen in Figs 2(a), 2(b) and 2(c), the level of human anti-human platelet antibodies, in a typical mouse, 4 weeks after transfer of splenocytes from an ITP patient, was higher than that of control mouse engrafted with PBMC from a normal donor.

The average levels of reactivity that were obtained with serum samples from mice which received ITP splenocytes ($n=42$) and from control mice (buffy coat-derived lymphocytes, $n=22$), at different time-points, is presented in Fig. 3. Low but significantly higher anti-human platelet antibody levels were already found within 2 weeks after transfer of splenocytes ($P<0{\cdot}042$), compared with anti-human platelet antibody levels detected in mice that were engrafted with human PBMC. Levels of platelet-reactive antibodies rose rapidly 3 weeks after

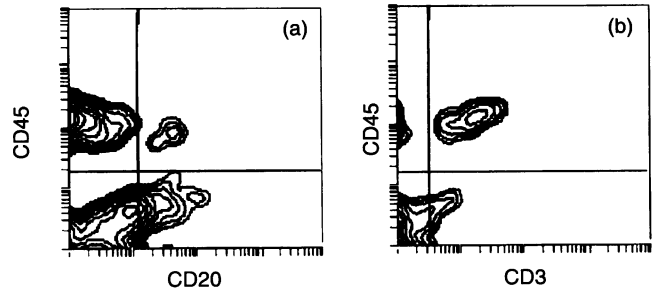

Figure 1. Engraftment of human cells, 14 days after transfer of ITP splenocytes into the SCID/BALB radiation chimera. Peritoneal cells were double stained by either CD3-peridinin chlorophyll protein or CD20-PE, and CD45-FITC. CD45 delineates their human origin.

Table 1. Human anti-platelet antibody and total human immunoglobulin production in groups of mice engrafted with splenocytes from different idiopathic thrombocytopenic purpura donors

			2 weeks	4 weeks	6 weeks
	Autoantibody production rate		Anti-platelet antibodies (%FPC)		
Spleen 1	12/12		3·77 ± 0·73	30·43 ± 2·45*	10·35 ± 1·90
Spleen 2	6/9		0·62 ± 0·20	19·10 ± 3·10	6·93 ± 0·90
Spleen 3	9/10		3·75 ± 1·90	30·90 ± 2·62*	14·22 ± 2·50
Spleen 4	9/11		3·19 ± 1·60	23·50 ± 5·80	5·93 ± 0·90
	Survival rate	Engraftment rate	Total human immunoglobulin (mg/ml)		
Spleen 1	12/18	12/14	4·70 ± 0·72	3·77 ± 0·68	1·69 ± 0·42
Spleen 2	9/18	9/12	2·90 ± 0·14	2·53 ± 0·29	4·35 ± 0·76
Spleen 3	10/18	10/13	6·31 ± 0·39	3·40 ± 0·49	3·61 ± 0·45
Spleen 4	11/18	11/12	4·68 ± 0·30	5·39 ± 0·94	2·70 ± 0·48

Transplantation of spleen cells was carried out in five experiments with five different donors out of which engraftment of human lymphocytes was achieved in four experiments.

%FPC, percentage of fluorescence-positive cells.

*$P < 0.05$ versus spleen 2 at 4 weeks, †$P < 0.05$ versus spleen 2 and spleen 4 at 6 weeks; data are expressed as mean ± SD.

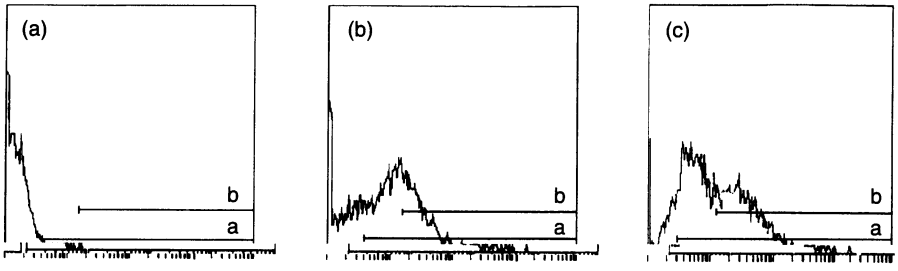

Figure 2. Detection of human anti-human platelet antibodies by FACS analysis in serum samples from SCID/BALB radiation chimera reconstituted with human PBMC (a) or ITP splenocytes (b). Serum analysed in (c) was taken from an ITP patient and served as a positive control. The percentage of fluorescence-positive human platelets (FPC%) was determined in region b.

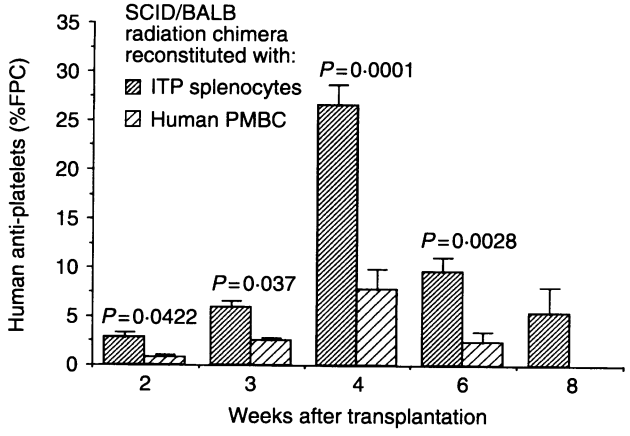

Figure 3. Levels of human platelet antibodies, at different time-points post-transplant, in SCID/BALB radiation chimera reconstituted with ITP splenocytes or with human PBMC. Statistical significance was determined using the Student's *t*-test. Error bars represent mean ± SE. Levels of human anti-platelet antibodies in recipient mice reconstituted with human PBMC were undetectable at 8 weeks.

injection, reaching maximal values 4 weeks after transfer of cells, and the significance of the difference in the level of platelet reactive antibodies between the two groups of mice was enhanced ($P < 0.037$ and $P < 0.0001$, respectively). Thereafter, levels declined but were still significantly higher at 6 ($P < 0.0028$) and at 8 weeks post-transplant. Few mice reconstituted with splenocytes showed a long presence of autoantibodies up to 9 weeks post-transplant.

The human anti-human platelet antibodies were primarily of the IgG subtype. The dominance of the IgG subtype was observed throughout the follow-up period. Anti-human platelet antibodies of the IgM subtype were not detectable in the chimeric mice (data not shown).

No evidence was obtained for a reduced survival of murine platelets, as determined by measuring platelet counts at regular intervals (data not shown). However, cannot be excluded that there might have been a state of compensated thrombocytolysis.

Engrafted splenocytes of individual ITP patients differ in their capacity to produce human platelet antibodies

Table 1 summarizes the characteristics of human anti-human platelet antibody production in four groups of mice engrafted with splenocytes from different ITP donors. In all four groups we detected significant levels of anti-human platelet antibodies in a high percentage of animals. However, each group of mice exhibited different human autoantibody production capacity.

Early autoantibody production was observed in groups 1, 3, and 4 in which, 2 weeks after transfer of splenocytes, a

small fraction of the chimeric mice had already sustained very high levels of autoantibodies (two of 12, two of 10 and one of 11, respectively). In contrast, none of the mice from group 2 showed high autoantibody levels at this time-point. Rapid elevation to maximal autoantibody values within 4 weeks of transplantation of splenocytes and a gradual decline afterwards was observed in all groups of mice.

We did not find a correlation between the ability to produce autoantibodies in chimeric mice and the severity of disease in respective ITP donors. Highest autoantibody levels were seen throughout the experiment in groups 1 and 3, while lowest levels were seen in groups 2 and 4. However, for example, ITP donor 1 had low disease activity (he sustained the highest platelet count (45 000/μl) compared with the other donors) while ITP donor 2 had a severe exacerbation of his disease (he sustained a minimal platelet count (<15 000/μl), which was the lowest among the ITP donors).

Total human immunoglobulin production by splenocytes from ITP patients and by PBMC from normal donors

Total human immunoglobulin was measured periodically in all of the BALB/c mice engrafted with splenocytes from ITP patients and in control mice (all conditioned as described above) receiving human PBMC from normal donors. Figure 4 summarizes the kinetics of total human immunoglobulin production in both of these groups of mice. As previously described by Lubin *et al.*,[12,13] we found, at day 14, a high serum level of human immunoglobulin in mice transplanted with splenocytes (4·7±0·28 mg/ml), and 5·37±0·9 mg/ml in mice transplanted with normal PBMC. Human immunoglobulin continued at this level until week 6 after transfer of cells, and declined to an average of ≈2 mg/ml at 2 months, in both groups of mice. Thus, the total human immunoglobulin production in mice receiving spleen cells from ITP patients was not significantly different from that found in mice receiving PBMC from normal donors. Human IgG was found to be the dominant subclass in both groups of chimeric mice (>90%), but IgM and IgA were also detected (data not shown).

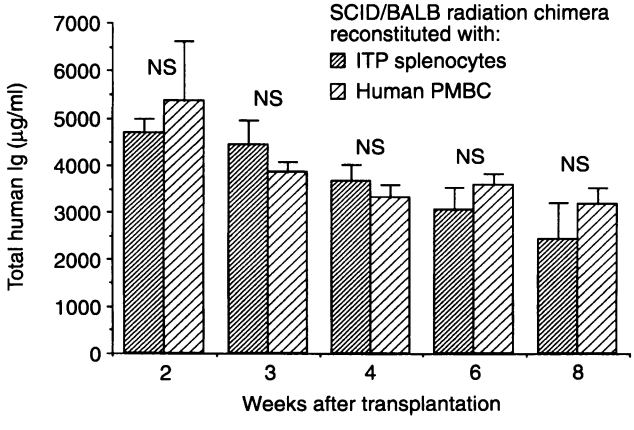

Figure 4. Levels of total human immunoglobulin, at different time-points post-transplant, in SCID/BALB radiation chimera reconstituted with ITP splenocytes or with human. Statistical significance was determined using the Student's *t*-test. Error bars represent mean±SE. NS, not significant.

Human platelet antibody and total human immunoglobulin production do not correlate

Table 1 summarizes the total human immunoglobulin production in the different groups of mice engrafted with splenocytes from respective ITP donors. In engrafted mice, no correlation was found between total human immunoglobulin levels and platelet antibody levels. Mice in groups 2 and 4, which attained lower levels of platelet antibodies throughout the experiment, exhibited high levels of total human immunoglobulin, similar to the mice of groups 1 and 3, which displayed high levels of platelet antibodies. For instance, 4 weeks after transfer of splenocytes, mice of group 2 were found to have a significantly lower level of platelet antibodies ($P<0·05$), compared with mice in groups 1 and 3, but total human immunoglobulin level was not significantly reduced. Furthermore, at 6 weeks post-transplant, mice in groups 2 and 4 had significantly lower levels of anti-human platelet antibodies compared with mice in group 3 ($P<0·05$); nonetheless, they sustained similar levels of total human immunoglobulin compared with other groups of mice (Table 1).

Human platelet antibodies cross-react with mouse platelets

The possible presence of human anti-mouse platelet reactivity was evaluated by combining sera collected from 10 human/BALB chimera, which attained high autoantibody levels at 4 weeks post-transplant, with mouse platelets.

As shown, the level of human anti-mouse platelets antibodies in a typical mouse engrafted with ITP splenocytes (Fig. 5c), was higher than that found in control mouse engrafted with PBMC from a normal donor or in control preconditioned mouse which did not receive lymphocytes (Figs 5a, 5b).

A large fraction of the human/BALB chimera, generated by ITP splenocytes, exhibited a high response to mouse platelets (eight out 10 mice, mean level of 17±2·0% fluorescence-positive cells (%FPC)), while only two out of seven human/BALB chimera, generated by normal PBMC transplantation, were found to have high reactivity to mouse platelets (mean level of 6±1·3% FPC, $P<0·05$ compared with the latter). Human anti-mouse platelet reactivity was not found in preconditioned mice that did not receive lymphocytes.

Antigen specificity of platelet antibodies generated by ITP splenocytes

Selective detection of human platelet-reactive antibodies against membrane glycoprotein complexes IIb/IIIa and Ib/IX, was performed in mice reconstituted with either ITP splenocytes or human PBMC. Serum to be investigated was collected from group 3 and group 4 mice, 4 weeks after transfer of cells, in which levels of platelet autoantibodies were shown to be the highest. The serum of respective ITP donors contained either antibodies against GPIIb/IIIa and GPIb/IX (donor 3) or only against GPIIb/IIIa (donor 4). The serum of the majority of mice reconstituted with ITP splenocytes (11 of one, seven of eight and four of six mice injected with splenocytes from donors 3 and 4, respectively) contained human IgG antibodies against GPIIb/IIIa and/or GPIb/IX, but no reactivity was obtained by MAIPA with the serum samples obtained from control mice (Table 2). Transfer of splenocytes from ITP

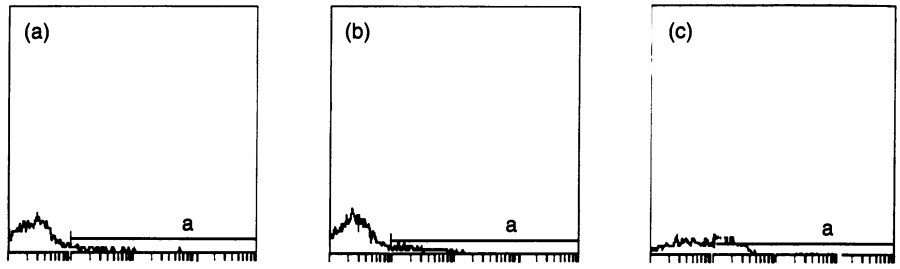

Figure 5. Detection of human anti-mouse platelet antibodies by FACS analysis in serum samples from SCID/BALB radiation chimera, which did not receive human cells (a), and in serum samples from SCID/BALB radiation chimera reconstituted with human PBMC (b) or ITP splenocytes (c). The percentage of fluorescence-positive mouse platelets (FPC%) was determined in region a.

Table 2. Antigen specificity of human anti-human platelet antibodies generated in human idiopathic thrombocytopenic purpura (ITP)/mouse radiation chimera (monoclonal antibody-specific immobilization of platelet antigens (MAIPA (assay)

Experiment	No.	IIbIIIa	Ib
Hu/ITP radiation chimera	4	+ +	+ +
	4	– –	+ +
	3	+ +	– –
	4	– –	– –
Hu/PBMC radiation chimera	5	– –	– –
ITP-positive donor	+ +	+ +	
ITP-negative donor	– –	– –	
PLA*	– –	+ +	
Anti-mouse IgG	– –	– –	

donor 3 into recipient animals resulted in simultaneous production of platelet antibodies against GPIIb/IIIa and GPIb/IX in four of eight mice, while in three of eight mice we detected antibodies against GPIb/IX. Interestingly, transfer of splenocytes from ITP donor 4 resulted in the production of platelet antibodies targeting not only GPIIb/IIIa (two of four) but also GPIb/IX (two of four).

DISCUSSION

Autoantibodies are of crucial importance in the pathogenesis of ITP.[1] In the present study, we demonstrated that the engraftment of human splenocytes, obtained from ITP patients, in preconditioned BALB/c mice, leads to the production of human platelet antibodies in these chimeric mice.

These results are in accordance with other investigations that showed the production of various human autoantibodies (anti-nuclear antibodies, anti- mitochondrial antibodies, anti-thyroglobulin antibodies, rheumatoid factor and anti-acetylcholine receptor) in SCID mice upon transfer of lymphocytes from patients with respective autoimmune disease.[8–11] In our model, levels of platelet antibodies did not follow the early high levels of total human immunoglobulin, peaking at a later time-point than that of the total human immunoglobulin. This is in contrast to the work of Macht *et al.*[10] who showed that thyroid autoantibodies peaked earlier than total immunoglobulin, but is in accordance with the work of Abedi *et al.*[25] who reported delayed appearance of anti-mitochondrial autoantibodies.

Peak levels of platelet antibodies 4 weeks after injection of cells, and the persistence of these autoantibodies 2 months after transfer of splenocytes, could be related to human antibodies against human and murine platelets. It has been recently suggested that in the hu–PBL–SCID mouse model, anti-mouse reactive human T cells are stimulated by the xenoevironment and this reactivity is a major factor involved in the selection of T-cell clones that populate chimeras at later stages.[26] Likewise, human T cells against murine platelets could influence further production of human anti-mouse platelet antibodies, after autoreactive pathological B lymphocytes have already disappeared from chimeric mice (such antibodies were indeed found in the serum of recipient animals). Human T cells directed against murine platelets might also exist in the human radiation chimera generated by transfer of normal PBMC. Minor reactivity is obtained with human platelets when 'naive' PBMCs are transferred into mice. Nonetheless, a substantial amount of platelet antibodies could not be found in the latter, suggesting the important role of the pathogenic B-cell clones, which are responsible for autoantibody production in the patients, in the transfer of the disease. Furthermore, polyclonal activation of human B cells during adoptive transfer into the radiation chimera[15] might drive the autoreactive clones for production of human platelet antibodies in the murine system, which are not present in the patients' serum. Therefore, is not surprising that in the different groups of mice, each engrafted with splenocytes from a respective ITP donor, correlation was not found between levels of human platelet antibodies and total human immunoglobulin. At the onset and at successive time intervals after transfer of human cells into recipient mice, different numbers of normal and autoreactive clones, among the donor's lymphocytes, survive and proliferate. It should also be noted that the number of γ-positive B cells in the spleen might be higher when compared with peripheral blood. The absence of platelet antibodies in the control mice might thus partially be explained by the limited number of γ-positive B cells that were transferred. However, similar levels of total human immunoglobulin, accompanied by a marked difference in the levels of platelet antibodies between assay and control mice, observed in several experiments, seem to validate our results.

Our observation that the level of autoantibody production detected in mice does not correlate with the clinical severity of the disease (the donor which had the lowest platelet count did not transfer highly autoantibody-producing splenocytes) further proves that the severity of thrombocytopenia in an ITP patient is, besides the quantity of antibody, determined

by the IgG subclass and the ability of the mononuclear phagocytic system to process immune complexes. Despite direct evidence of interaction between human autoantibodies and mouse platelets, and the characterization of the autoantibodies as being against platelet IIb/IIIa or Ib/IX glycoproteins, thrombocytopenia was absent in our chimeric system. Thus, thrombocytopenia was not observed, probably because the reduction of platelet survival was limited by an ineffective Fc receptor-mediated clearance of platelets sensitized with human antibodies and a mild reduction might have been compensated by increased thrombocytopoiesis. The hypothesis by Vladutiu *et al.*[27] that the amount of human autoantibodies in models of autoimmune disease in SCID mice was too small to cause target organ damage, might be less relevant in regard to the biological inactivity of the human platelet antibodies.

In summary, we have demonstrated that transfer of splenocytes from ITP patients to lethally irradiated BALB/c mice protected with SCID bone marrow leads to generation of human IgG antibodies against platelets. These data offer a new *in vivo* model for studying immune cells from patients with ITP and for the investigation of future potential therapeutics that might ameliorate autoantibody production in ITP.

ACKNOWLEDGMENTS

This study was partially supported by grants from XTL Biopharmaceuticals Ltd, Rehovot, Israel.

REFERENCES

1. George J., El-Haake M. & Raskob G. (1994) Chronic idiopathic thrombocytopenic purpura. *N Engl J Med* **331,** 1207.
2. Harrington W., Minnich V., Hollingsworth J. & Moore C. (1951) Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med* **38,** 1.
3. Berchtold P. & Wegner M. (1993) Autoantibodies against platelet glycoproteins in autoimmune thrombocytopenic purpura: their clinical significance and response to treatment. *Blood* **81,** 1246.
4. He R., Reid D., Jones C. & Shulman N. (1994) Spectrum of Ig classes, specificities, and titres of serum antiglycoproteins in chronic idiopathic thrombocytopenic purpura. *Blood* **83,** 1024.
5. Varon D. & Karpatkin S. (1983) A monoclonal anti-platelet antibody with decreased reactivity for autoimmune thrombocytopenic platelets. *Proc Natl Acad Sci USA* **80,** 6992.
6. Fujisawa K., Tani P., Piro L. & McMillan R. (1993) The effect of therapy on platelet associated autoantibody in chronic immune thrombocytopenic purpura. *Blood* **81,** 2872.
7. Mosier D.E., Gulizia R.J., Baird S.M. & Wilson D.B. (1988) Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* **335,** 256.
8. Krams S.M., Dorshkind K. & Gershwin M.E. (1989) Generation of biliary lesions after transfer of human lymphocytes into severe combined immunodeficient (SCID) mice. *J Exp Med* **170,** 1919.
9. Tighe H., Silverman G., Kozin F. *et al.* (1990) Autoantibody production by severe combined immunodeficient mice reconstituted with synovial cells from rheumatoid arthritis patients. *Eur J Immunol* **20,** 1843.
10. Macht L., Fukuma N., Leader K. *et al.* (1991) Severe combined immunodeficient (SCID) mice: a model for investigating human thyroid autoantibody synthesis. *Clin Exp Immunol* **84,** 34.
11. Duchosal M.A., McConahey P.J., Robinson C.A. & Dixon F.J. (1990) Transfer of human systemic lupus erythematosus in severe combined immunodeficient (SCID) mice. *J Exp Med* **172,** 985.
12. Lubin I., Segall H., Marcus H. *et al.* (1994) Engraftment of human peripheral blood lymphocytes in normal strains of mice. *Blood* **83,** 2368.
13. Lubin I., Segall H., Erlich P. *et al.* (1995) Conversion of normal rats into SCID-like animals by means of bone marrow transplantation from SCID murine donor allows engraftment of human peripheral blood lymphocytes. *Transplantation* **60,** 740.
14. Burakova T., Marcus H., David M. *et al.* (1997) Engrafted human T and B lymphocytes from mixed follicles in lymphoid organs of human/mouse and human/rat radiation chimera. *Transplantation* **63,** 1166.
15. Marcus H., David M., Cnaan A. *et al.* (1995) Human/mouse radiation chimera are capable of mounting a human primary humoral response. *Blood* **86,** 398.
16. Marcus H., David M., Boracova T., Shtsen E., Cnaan A. & Reisner Y. (1996) Human/mouse radiation chimera do not develop Epstein–Barr virus lymphoma. *Immunol Lett* **49,** 155.
17. Segall H., Lubin I., Marcus H., Canaan A. & Reisner Y. (1996) Generation of primary antigen specific human CTLs in human/mouse radiation chimera. *Blood* **88,** 721.
18. Shimoni A., Marcus H., Canaan A. *et al.* (1997) A model for human B-chronic lymphocytic leukemia in human/mouse radiation chimera: evidence for tumor-mediated suppression of antibody production in low stage disease. *Blood* **89,** 2210.
19. Dekel B., Burakova T., Marcus H. *et al.* (1997) Engraftment of human kidney tissue in rat radiation chimera: I. A new model of human kidney allograft rejection. *Transplantation* **64,** 1541.
20. Dekel B., Burakova T., Ben-Hur H. *et al.* (1997) Engraftment of human kidney tissue in rat radiation chimera: II. Human fetal kidneys display reduced immunogenicity to adoptively transferred human peripheral blood mononuclear cells and exhibit rapid growth and development. *Transplantation* **64,** 1550.
21. Reisner Y., Itzicovitch L., Meshorer A. & Sharon N. (1978) Hematopoietic stem cell transplantation using mouse bone marrow and spleen cells fractionated by lectins. *Proc Natl Acad Sci USA* **75,** 2933.
22. Schwartz E., Lapidot T., Gozes D., Singer T.S. & Reisner Y. (1987) Abrogation of bone marrow allograft resistance in mice by increased total body irradiation correlates with eradication of host clonable T-cells and alloreactive cytotoxic precursors. *J Immunol* **138,** 460.
23. Visentin G.P., Wolfmeyer K., Newman P.J. & Aster R.H. (1990) Detection of drug-dependent, platelet-reactive antibodies by antigen-capture ELISA and flow cytometry. *Transfusion* **30,** 694.
24. Kiefel V., Santoso S., Weisheit M. & Mueller E.C. (1987) Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood* **70,** 1722.
25. Abedi M.R., Hammarstrom L., Broome U., Angelin B., Smith C.I. & Christensson B. (1996) 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). *Clin Exp Immunol* **105,** 266.
26. Tary-Lehmann M., Saxon A. & Lehmann P. (1995) The human immune system in hu-PBL-SCID mice. *Immunol Today* **16,** 529.
27. Vladutiu A.O. (1993) The severe combined immunodeficient (SCID) mouse as a model for the study of autoimmune diseases. *Clin Exp Immunol* **93,** 1.