Administration of high-dose intact immunoglobulin has an anti-resorption effect in a mouse model of reproductive failure

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Administration of high-dose intact human immunoglobulin (IH-Ig) has been applied to treat a variety of inflammatory and autoimmune diseases, and is expected to have beneficial effects on human fecundity. In the present study, we investigated the mechanism of the effect by examining the mRNA expression of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-10, IL-4 and TGF-β1 in spleens and placenta from the resorption-prone model treated with IH-Ig, by reverse transcription (RT)–polymerase chain reaction (PCR). Administration of high-dose IH-Ig significantly reduced the fetal resorption rate from 55% to 10%. This anti-resorption effect, however, was not detected in mice administered with Fab fragments of human Ig. We then performed adoptive transfer experiments to examine whether cellular components could transfer the effect. A remarkable anti-resorption effect was seen in poly (I:C)-injected pregnant recipients transferred with spleen cells from IH-Ig-treated donor mice. The RT–PCR study showed that IH-Ig reduced the expression of IFN-γ and TNF-α mRNA in placenta of poly (I:C)-injected pregnant mice. The present findings demonstrate that intact Ig, particularly its Fc portion, possesses anti-resorption activity. The effect might be attributed to the suppressed production of pro-inflammatory cytokines at the maternofetal interface.

Keywords: immunoglobulin; fetal resorption; spleen; mouse model

Introduction

It is widely acknowledged that intravenous use of a high dose of immunoglobulin (Ig) G (HIVIg) is practically effective, and this therapy has long been applied to a variety of immune-mediated diseases such as idiopathic thrombocytopenic purpura, Guillain–Barré syndrome, Kawasaki’s disease and myasthenia gravis (Imbach et al., 1981; Fehr et al., 1982; Dwyer, 1992; Kazatchkine and Kaveri, 2001). Several mutually non-exclusive mechanisms of action, which include suppression of inflammation and modification of Fc receptor, T cell, B cell or macrophage functions, are proposed to account for the immunoregulatory effects of the HIVIg therapy (Kazatchkine and Kaveri, 2001).

Recurrent miscarriage (RM) is defined as the experience of three or more consecutive spontaneous abortions, caused by various pathologic conditions such as uterine abnormality, anti-phospholipid antibody, chromosomal translocation or endocrine or metabolic disorders. However, the majority of RM is of unknown etiology, and therefore designated as unexplained RM. The pathologic basis of some of the unexplained RM may be explained by immunologic abnormalities including over activation of NK cells (Yamada et al., 2001, 2003a, 2004), dysregulation of helper T cells and/or immunody-strophism (Shimada et al., 2003a, 2004).

To assess the efficacy of this therapeutic option in women with unexplained RM, randomized, double-blind, and placebo-controlled trials of medium dose of intravenous Ig therapy, in which 20–40 g of Ig/person is infused weekly or every 2–4 weeks (wk) during early pregnancy, have been performed in many countries (The German RSA/IVIG Group, 1994; Christiansen et al., 1995; Coulam et al., 1995; Perino et al., 1997; Stephenson et al., 1998; Jablonowska et al., 1999). However, conclusions drawn from these Ig trials have been controversial or rather negative. On the other hand, our group tried HIVIg therapy in which a high dose (totally 100 g/person) of Ig was infused intravenously over the course of 5 days during early pregnancy. In non-randomized trials, we observed a high live birth rate among women who had a history of severe unexplained RM (Yamada et al., 1998; Morikawa et al., 2001; Yamada and Morikawa, 2005). Recent publications on the timing of treatment and selection of patients with immunological abnormalities (Clark et al., 2006), and from a meta-analysis of secondary RM (Hutton et al., 2007), seem to be concordant with our data. Yet, the pharmacodynamic
mechanism of the Ig therapy in RM has not been fully investigated in any experimental animal models of reproductive failure.

A murine model of spontaneous abortion was developed with resorption-prone mating between CBA/J (H-2k) female and DBA/2J (H-2d) male mice. In this particular combination, 20–25% of fetuses are spontaneously resorbed (Clark et al., 1980), and the immunologic mechanisms of resorption have been extensively studied with this model. It has been found in these murine models that Th1 cytokines are harmful to pregnancy and cause resorption, whereas Th2 cytokines produced at the maternofetal interface are beneficial to the maintenance of pregnancy by suppressing cellular cytotoxicity (Wegmann et al., 1993). Indeed, Th1 cytokines such as IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α are dominant in the placentas (Tangri et al., 1994), whereas production of Th2 cytokines, including IL-4 and IL-10, is quantitatively deficient in the resorption-prone CBA/J × DBA/2J mating model as compared with the non-resorption prone CBA/J × BALB/c mating combination (Chao et al., 1995). In addition, the fetal resorption rate increased up to 50–60% per litter after administration of polyinosinic-polycytidylic acid [poly (I:C)]; a synthetic double-stranded RNA molecule that activates macrophages and NK cells via toll-like receptor (TLR) 3 (de Fougerolles and Baines, 1987; Cavanaugh et al., 1996; Shimada et al., 2003b), suggesting involvement of natural immunity in the RM.

In the present study, we employed the CBA/J × DBA/2J mating model, where the resorption rate was augmented by poly (I:C) administration, and examined the preventive effect of the intact human Ig (IH-Ig) administration on immune reproductive failure. We demonstrate herein that the IH-Ig administration significantly reduces the resorption rate. Furthermore, adoptive transfer of spleen cells from IH-Ig-treated non-pregnant female CBA/J mice to the CBA/J × DBA/2J mating model prevents resorption, suggesting that a certain cellular mechanism is involved in the suppression.

Materials and Methods

Mice and mating

CBA/J females aged 4–6 wk and DBA/2J males aged 6 wk were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6 females aged 7 wk were purchased from the Shizuoka Laboratory Animal Corporation (Hamamatsu, Japan). These mice were maintained in a specific pathogen-free condition, with a 12 h light/12 h dark cycle, and used at the age of 7 wk or above. Female CBA/J mice were mated to male DBA/2J mice (one CBA/J female was housed with a single DBA/2J male for 12 h from 9 o’clock in the evening to 9 o’clock in the morning), and the presence of a vaginal plug was checked. The day of confirmation of plug was defined as gestational day 0.5 (day 0.5). All animal care and experimental procedures conformed to the regulation of the Committee of Hokkaido University on Animal Experimentation.

Poly (I:C) injection and evaluation of fetal resorption

Poly (I:C) (Sigma Chemical Co., St Luis, MO, USA) which induces an abortifacient effect was administered to pregnant CBA/J mice with a single intraperitoneal (i.p.) injection of 200 µg dissolved in 500 µl of sterile phosphate-buffered saline (PBS) on day 7.5 as previously reported (Shimada et al., 2003b). Evaluation of fetal resorption was performed on day 13.5 with macroscopic examination by their size and accompanying necrotic and/or hemorrhagic appearance as previously described (de Fougerolles and Baines 1987). The fetal resorption rate was calculated as follows: resorption rate (%) = (number of resorbed fetuses/number of resorbed fetuses + viable fetuses) × 100.

Ig administration

IH-Ig (Globben-In) and Fab fragment of human Ig (Fab-Ig) (Gamma-Vein in P) were provided by Nihon Pharmaceutical Co. (Tokyo, Japan) and Aventis Pharma Ltd (Tokyo, Japan), respectively. Each Ig was dissolved in PBS, and i.p. administered to pregnant CBA/J mice that had received 200 µg of poly (I:C), with several kinds of administration schedules. Pregnant CBA/J mice without treatment following i.p. injection of poly (I:C) (200 µg) served as controls. All the pregnant CBA/J mice were sacrificed on day 13.5 to assess the fetal resorption rate (Fig. 1A).

Adoptive transfer of spleen cells from IH-Ig-treated non-pregnant CBA/J mice to pregnant CBA/J mice

Spleen cells for adoptive transfer were prepared from non-pregnant female CBA/J mice (donor mice). The donor mice received i.p. injections of 0.8 g/kg/day of IH-Ig for 3 days along with a period of day 1.5 to day 3.5 of the pregnant CBA/J mice, mating combination (recipient mice). Four days after the last IH-Ig injection, spleens were harvested from donors, and a single cell suspension was prepared. After lysis of erythrocytes with Tris–NHCl solution, the cells were washed three times with complete medium (RPMI 1640 containing 10% fetal calf serum (FCS), 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin), and resuspended in PBS at 5 × 10^7/0.3 ml before injection. Spleen cells derived from non-pregnant female CBA/J mice without treatment were also prepared as controls. On day 7.5, 1 h after i.p. injection of poly (I:C) (200 µg), the spleen cells or control cells were transferred to the recipients, pregnant CBA/J mice via tail vein. All the recipient mice were sacrificed on day 13.5 for the assessment of the fetal resorption rate. Pregnant CBA/J mice without treatment following i.p. injection of poly (I:C) (200 µg) were also sacrificed to examine the resorption rate (Fig. 2A).

Detection of human IgG on spleen cells by flow cytometry

On day 7.5, spleen cells were prepared from the non-pregnant female C57BL/6J mice which had received the i.p. injection of IH-Ig (0.8 g/kg, daily from day 1 to day 3.5).
1.5 to 3.5) as described earlier. After hemolysis treatment, the cells were washed and resuspended with PBS/NaNO3/bovine serum albumin (BSA) buffer for flow cytometry. Prepared cells were incubated with 2.4G2 monoclonal antibody (mAb) to block non-specific binding of mAb, and labeled with following reagents: (i) phycoerythrin (PE)-conjugated anti-mouse CD45R/B220 and fluorescein isothiocyanate (FITC)-conjugated anti-human IgG; (ii) PE anti-mouse CD11b/Mac-1, and FITC anti-human IgG; (iii) PE anti-mouse CD11c and FITC anti-human IgG. Analysis was carried out using the FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) and the CellQuest Software.

Detection of the transferred cells in spleens and placentas of recipient mice transferred with carboxyfluorescein diacetate succinimidyl ester-labeled spleen cells

A single cell suspension was prepared from the spleens of non-pregnant female CBA/J donors without treatment. After hemolysis treatment and a wash with PBS, the cells were resuspended into PBS (1 x 10^7/ml), and incubated with 0.5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min. Labeling was stopped by an addition of FCS and the stained cells were washed with complete medium, and then transferred to the pregnant CBA/J recipients on day 7.5 via tail vein as a 5 x 10^7/0.3 ml PBS solution. All the recipient mice were sacrificed 3–5 days after the transfer for obtaining the spleens and placentas (Fig. 3A).

Spleen cells were prepared as described earlier, and placental mononuclear cells (PMC) were isolated by density-gradient centrifugation. In brief, the pooled placentas were carefully homogenized and filtered through a stainless

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Figure 3: Results of flow cytometric analyses for the detection of the transferred cells in spleens and placentas of recipient mice that had been transferred with CFSE-labeled donor spleen cells (CFSE-labeled SPC) (A) Resume of the experimental system of analyses for the detection of CFSE-positive cells in the recipient tissues. (B) Representative dot plots of flow cytometric analyses for the detection of CFSE-positive cells in spleen cells and PMC from recipients. Pregnant CBA/J mice transferred with CFSE-free, non-labeled spleen cells (non-labeled SPC) served as controls. The numbers represent the percentage of CFSE-positive cells in total spleen cells or total PMC from recipients. Data are mean ± SE of three independent experiments. (C) Representative dot plots of flow cytometric analyses for the detection of CD11b-positive cells in the CFSE-positive population in total spleen cells or total PMC from recipients. The numbers represent the percentage of CD11b-positive or negative cells in the CFSE-positive population in total spleen cells or total PMC from recipients.
mesh to obtain a cell suspension. PMC were purified by density-gradient centrifugation on Lympholyte-M™ (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada). The prepared spleen cells or PMC were resuspended in PBS/Na3/BSA buffer for flow cytometry. After an incubation with 2.4G2 mAb, the cells were labeled with following reagents: (i) PE anti-mouse CD4, (ii) PE anti-mouse CD45R/B220, (iii) PE anti-mouse CD11b/Mac-1, (iv) PE anti-mouse CD11c and (v) PE anti-mouse DX5. Pregnant CBA/J mice transferred with non-labeled, CFSE-negative spleen cells served as controls. Analyses were performed with the FACScalibur flow cytometer and the CellQuest Software (BD Bioscience).

Examination of cytokine mRNA expression by reverse transcription–polymerase chain reaction

After 1 h or more from the last IH-Ig administration, spleens and placentas were removed from pregnant CBA/J mice that had received i.p. injection of poly (I:C) (200 μg) on day 7.5 with or without Ig administration (0.8 g/kg of IH-Ig daily from day 7.5 to 9.5) on day 9.5. Control spleens and placentas were obtained from pregnant CBA/J mice without treatment (Fig. 4A). Spleens and placentas, carefully detached from uterine wall, were snap-frozen with liquid nitrogen and kept at −80°C. Then, ~100 mg of tissue (spleen or placentas) were soaked in 2 ml of TRIzol™ (Invitrogen Co., Carlsbad, CA,

![Figure 4](image)

**Figure 4:** mRNA expressions of IFN-γ, TNF-α, IL-10, IL-4 and TGF-β in spleens and placentas of pregnant CBA/J mice

(A) Resume of the experimental system for the analyses of each mRNA expression. mRNA expression of IFN-γ (B), TNF-α (C), IL-10 (D), IL-4 (E) and TGF-β (F) in spleens and placentas from CBA/J mice. Results of respective groups; mice without treatment (control, n = 5), mice with poly (I:C) injection (n = 5) and mice with IH-Ig administration following poly (I:C) injection (n = 5) are shown. Respective columns represent the standardized ratios of the band intensity of each PCR product divided by that of HPRT. Data are mean ± SE from five mice in each group. Statistical significance was calculated by non-repeated measures ANOVA and Student–Newman–Keuls test (*P < 0.05 and **P < 0.01).
USA), and homogenized (Polytron, Kinematics AG, Littau, Switzerland). Total RNA was purified with TRIzol/chloroform method, and precipitated with 99.5% ethanol. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA using random hexamers and reverse transcriptase, Superscript II™ (Invitrogen Co.), in the presence of dNTPs and RNase inhibitor, RNasin™ (Promega Co., Madison, WI, USA) according to manufacturer’s protocol.

Amplification for IFN-γ, TNF-α, IL-10, IL-4, TGF-β1, and HPRT massages was performed with Taq polymerase, Go Taq™ (Promega) with thermal cycling programs as follows: IFN-γ and HPRT: 5 min at 94°C as initial denaturation step, 30 sec at 94°C, 30 sec at 50°C and 1 min at 72°C for 30 cycles; TNF-α: 5 min at 94°C as initial denaturation step, 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C for 30 cycles; IL-10: 5 min at 94°C as initial denaturation step, 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C for 32 cycles; IL-4: 5 min at 94°C as initial denaturation step, 30 sec at 94°C, 30 sec at 65°C and 1 min at 72°C for 35 cycles; TGF-β1: 5 min at 94°C as initial denaturation step, 30 sec at 94°C, 30 sec at 51°C and 1 min at 72°C for 30 cycles. Each program was followed by an additional extension course of 72°C for 10 min.

The following primer sequences were used: IFN-γ (sense) 5'-ACT GGC AAA AGG ATG TTG AC-3'; (antisense) 5'-TGA GCT CAT TGA ATG CT-3'; TNF-α (sense) 5'-CCA GAC CTT CCT CAC ACT CAG AT-3'; (antisense) 5'-AAC ACC CAT TCT CTC CAC AG-3'; IL-10 (sense) 5'-TAC CTG GTA GAA GTG ACC CC-3'; (antisense) 5'-CAT GAT GTA TGC TAT GC-3'; IL-4 (sense) 5'-AGC GAG ATG ATG GTG CCA AAC GTC-3'; (antisense) 5'-CGA GTA ATC TTG CAT GAT GC-3'; TGF-β1 (sense) 5'-ACC ATG CCA CCT TCT GTC TG-3'; (antisense) 5'-CGG GTT GTG TTG GTA GA-3'; HPRT (sense) 5'-GGT GGA TAC AGG CCA GAC TTT GGT G-3'; (antisense) 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'.

PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide, and the bands were visualized under ultraviolet light. The size of the amplified DNA fragments was determined using a 100 bp DNA ladder (Promega). The PCR products were excised from the agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen, USA), and sequenced at the University of Virginia DNA Core Facility.

**Results**

**Ig administration and suppression of fetal resorption**

In the present study, we employed 200 μg of poly (I:C) for the enhancement of fetal resorption based on the report by de Fougerolles and our own work (Shimada et al., 2003b). Among the various doses (60–400 μg) tested, the 200 μg dose showed the maximal effect with the CBA/J × DBA/2J mating.

IH-Ig administration was performed on this protocol as depicted in Fig. 1A. When IH-Ig was injected from day 7.5 to 9.5 at 0.8 kg/day (Group B, n = 12) to pregnant CBA/J mice that had received 200 μg of poly (I:C), the fetal resorption rate was markedly reduced (10 ± 3%) on day 13.5 in comparison with that of mice without IH-Ig administration (control: 55 ± 5%, n = 7) (Fig. 1B). In mice that received a single injection of 0.8 kg of IH-Ig (Group A, n = 6) or a lesser dose per injection but more times (Group C, n = 4), the resorption rates were not significantly reduced in comparison with control group (39 ± 9% and 41 ± 8%, respectively, versus 55 ± 5%). To examine whether the antigen-binding portion of Ig was involved in the preventive effect, Fab-Ig (0.8 kg/day) was administered for 3 days (Group D, n = 4). With this regimen, the inhibitory effect was not demonstrated, suggesting that Fab-Ig lacked the important component required for the anti-resorption effect.

**Adoptive transfer of spleen cells and suppression of fetal resorption**

To examine whether the marked suppression of fetal resorption seen in IH-Ig-treated pregnant mice was attributed to the cellular components, adoptive transfer experiments were performed according to a regimen depicted in Fig. 2A. First, we performed flow cytometric analyses to examine whether the transferred cells carried administered human Ig. With the regimen we used, the human Ig was not detected on the cell surfaces on day 7.5 when spleen cells were prepared (data not shown).

In a group of pregnant CBA/J mice that had been transferred with spleen cells from IH-Ig-treated non-pregnant female CBA/J mice 1 h after i.p. injection of poly (I:C) (200 μg), the fetal resorption rate was dramatically reduced to 6 ± 3% (n = 5) on day 13.5 (Fig. 2B). On the other hand, when spleen cells from non-pregnant female CBA/J mice without treatment were transferred to pregnant CBA/J mice, the fetal resorption rate on day 13.5 was 37 ± 7% (n = 4). There was no significant reduction in comparison with the group of mice without treatment following poly (I:C) injection (47 ± 5%, n = 7) (Fig. 2B). These findings demonstrated that the certain cellular component in spleen cells from IH-Ig-treated CBA/J mice contributed to the suppression of resorption.

Furthermore, flow cytometric analyses demonstrated that CFSE-labeled transferred cells (more than 99% of transferred cells were labeled with CFSE: data not shown) were detected in PMC as well as in spleen cells from the recipients (Fig. 3B). Indeed, substantial proportions of CFSE-positive cells were clearly detected in the recipient tissues (Fig. 3B). It was also shown in Fig. 3B that 0.33–0.81% of total PMC and 0.75–2.45% of total spleen cells from the recipients were CFSE-positive. It should be noted that the proportion of CD11b-positive cells in the CFSE-positive population was higher in PMC than in spleen cells (8.5–45.0% versus 0–1.3%, Fig. 3C). These results suggest that CD11b-positive cells, perhaps cells of monocyte/macrophage lineage, are preferentially recruited to placentas compared with other cell populations. These CFSE and CD11b-positive cells were detected for at least 3 days in placentas.

**Suppression of IFN-γ and TNF-α mRNA expression in placentas from pregnant mice received IH-Ig administration**

To elucidate the mechanism of the anti-resorption effect with IH-Ig administration, we examined the expression of several kinds of cytokines in spleens and placentas from pregnant CBA/J mice treated with following regimen: (i) poly (I:C) (200 μg) i.p. on day 7.5 and (ii) poly (I:C) (200 μg) i.p. on day 7.5, followed by 0.8 g/kg of IH-Ig daily from day 7.5 to 9.5. Mice without treatment served as controls (Fig. 4A).

Administration of 200 μg of poly (I:C) to pregnant CBA/J mice significantly augmented mRNA expression of IFN-γ (Fig. 4B) and TNF-α (Fig. 4C) mRNA in placentas. Notably, the enhancement of these pro-inflammatory cytokines in placentas was significantly reduced by the administration of IH-Ig. A similar but not so impressive suppression of IFN-γ and TNF-α mRNA was demonstrated in spleens (Fig. 4B and C). These results suggested that the suppression of inflammatory responses with IH-Ig administration was evident at this period especially around the maternofetal interface. In spleens, but not in placentas, mRNA expression of IL-10 was significantly augmented after IH-Ig administration (Fig. 4D). On the other hand, neither IL-4 (Fig. 4E) nor TGF-β1 (Fig. 4F) mRNA expression was influenced by the IH-Ig administration.

**Discussion**

In the present study, we used a CBA/J × DBA/2J mating model, where approximately half of the litter was stably resorbed after administration of 200 μg of poly (I:C). It was previously reported that poly (I:C), a synthetic double-stranded RNA, activates macrophages and NK cells via TLR 3 (Cavanaugh et al., 1996; Alexopoulou et al., 2001; Applequist et al., 2002). Poly (I:C) also increases the number of CD11b (Mac-1)-positive cells at the maternofetal interface (Duclos et al., 1994), and augments expression of NO synthase,
IFN-γ, TNF-α and allograft inflammatory factor-1 mRNA in embryos and placentas of pregnant CBA/J (Haddad et al., 1997; Shimada et al., 2003b; Zhang et al., 2007). Furthermore, abortifacient effects in naive recipients can be induced by adoptive transfer of spleen cells from poly (I:C)-injected donor mice (Kinsky et al., 1990). The abortifacient effects of poly (I:C) in CBA/J × DBA/2J mating are markedly reduced by the administration of anti-asialo-GM1 antibody (de Fougérolles and Baines, 1987). Moreover, it has been suggested that decidual NK cell abnormalities are causally associated with RM as well as sporadic spontaneous abortion (Yamada et al., 2005). Anti-asialo-GM1 antibody may also delete NKT cells that can be involved in the fetal resorption of CBA/J × DBA/2J by enhancement of the ratio of Th1/Th2, 5 cytokines around the maternofetal interface (Clark and Croitoru, 2001). Decidual NK cells stimulated with their agonist, α-galactosylceramide on day 9.5 could cause fetal resorption of CBA/J × CBA/J or C57BL/6 × C57BL/6 syngeneic mating combinations, with an increase in serum TNF-α, IL-2 and IL-4 production (Boyson et al., 2006). Thus, it seems that poly (I:C) stimulates macrophages and then activates NK cells and/or NKT cells at the materno-fetal interface, causing fetal resorption in our mouse model of immune reproductive failure, with systemic elevation of pro-inflammatory cytokines. Thus, this mouse mating combination with poly (I:C) administration may be a useful model for the investigation of immune-mediated reproductive failure.

Using this model, we for the first time demonstrated that IH-Ig could exert a considerable anti-resorption effect in mice (Fig. 1B). The most effective regimen (0.8 g/kg/day for 3 days) seemed compatible with a high-dose therapy employed in human patients. On the other hand, no anti-resorption effect was detected in pregnant CBA/J mice treated with Fab-Ig (Fig. 1B). These findings suggest that a high dose of IH-Ig is required for the anti-resorption effect in the mouse model, and the Fc portion of Ig plays a critical role in this effect. Although this postulate needs to be confirmed in further investigations, the active role of the Fc portion implies that cellular interactions via Fc and Fc receptor may be involved in the efficacy of the IH-Ig. In a rat model of experimental autoimmune encephalitis (EAE), Fab-Ig reduced the clinical score of EAE, but not as much as IH-Ig did (Pashov et al., 1998). This result seems to support our postulate.

To examine whether cellular components were really involved in the anti-resorption effect, we performed adoptive transfer experiments. Spleen cells obtained from IH-Ig-treated donor mice could successfully transfer the anti-resorption effect to the pregnant recipients (Fig. 2B). The effect was not merely executed by IH-Ig borne on spleen cells, because no human Ig on the cell surface was detected with flow cytometric analyses (data not shown). To examine whether donor spleen cells were really present in the recipient tissues, we carried out the transfer experiments where CFSE-labeled spleen cells were transferred. The CFSE-labeled transferred cells were indeed detected in spleens and placentas of the recipients (Fig. 3B). Notably, a high proportion of CD11b-positive cells was detected in placentas (Fig. 3C). These results suggest that the transferred cells might have direct effects on the inhibition of fetal resorption around the maternofetal interface as well as an interaction with the immune cells in spleen and peripheral blood. Furthermore, since these cells were obtained from non-pregnant female CBA/J mice, it seems that generation of the regulatory/effector cells carrying the anti-resorption effect requires IH-Ig administration but not antigenic stimulation. The study to determine the actual effector subset of the donor spleen cells that confers anti-resorption effects is underway in our group.

To elucidate the mechanism for the anti-resorption effect of IH-Ig, we compared the expression of several cytokines in spleens and placentas of pregnant CBA/J mice received poly (I:C) injection with/without consecutive IH-Ig administration. We found that IH-Ig administration reduced the poly (I:C)-induced enhancement of both IFN-γ and TNF-α mRNA expression in placentas to the levels of control mice (Fig. 4B and C). Thus, the elevated expression of pro-inflammatory cytokines in placentas, which were involved in fetal resorption (Shimada et al., 2003b; Whiteside et al., 2003; Zhang et al., 2007), could be suppressed by IH-Ig administration. IH-Ig might directly suppress the expression of pro-inflammatory cytokines from immune cells in placentas, and/or suppress the expression through the interaction between the regulatory/effector cells generated in spleen and peripheral blood and the immune cells in placentas. In particular, IH-Ig-stimulated macrophages may attenuate the cytotoxic abilities of effector macrophages present around the maternofetal interface, and exert the anti-resorption effects; this theory has been suggested in an arthritis model (Bruhns et al., 2003). Alternatively, IH-Ig-stimulated spleen cells may deactivate NK cells at the materno-fetal interface in our mouse model, since HIV-Ig therapy induces NK cell suppression and Th1/2 modification in the circulation of women with unexplained RM (Morikawa et al., 2001; Yamada et al., 2003b).

Depletion of cells that express γδT cell receptor (γδ cells) by i.p. injection of anti-γδ1.1 antibody on day 5.5 inhibited fetal resorption of a CBA/J × DBA/2J mating model with the reduction of TNF-α-positive γδT cells. On the other hand, injection of the antibody on day 8.5 boosted fetal resorption, reducing TGF-β-positive γδ cells (Arck et al., 1999). This might be attributed to the shift of the decidual γδT cell subset from Th1 to Th2, along the time course of the pregnancy. It seems to us that the time course of the cells and cytokines present around the maternofetal interface may determine inhibition or enhancement of the fetal resorption.

In the present study, however, IH-Ig administration enhanced mRNA expression of IL-10 in spleens of poly (I:C)-injected mice, but not in placentas (Fig. 4D). Moreover, neither poly (I:C) injection nor IH-Ig administration following poly (I:C) injection influenced mRNA expression of IL-4 or TGF-β1 in either of spleens or placentas (Fig. 4E and F). This finding implied that these cytokines might not have key roles in the inhibition of fetal resorption by IH-Ig. Further studies are needed to formerly exclude the involvement of these cytokines.

It is known that suppression of T cell-driven local inflammatory responses to fetal alloantigens by indoleamine 2,3-dioxygenase (IDO) contributes to maintenance of pregnancy (Munn et al., 1998; Mellor et al., 2001). However, fetal resorption was not enhanced in the mating of IDO-deficient mice, suggesting that other immunosuppressive mechanisms might compensate for lack of IDO activity (Baban et al., 2004). The tolerance-signaling molecule CD200, which reduced resorption rates in the CBA/J × DBA/2J mating (Clark et al., 2001) and rendered CD200 receptor-positive macrophages immunosuppressive via an IDO-dependent mechanism (Gorczynski et al., 2002), might be necessary for IH-Ig-dependent suppression of human NK cells (Clark and Chaouat, 2005).

Additionally, it was recently discovered that Ig-treated CD11c-positive dendritic cells were responsible for the amelioration of mouse idiopathic thrombocytopenic purpura, and the effect was attributed to the interaction between Ig and FcγR on dendritic cells (Siragam et al., 2006).

However, the abovementioned explanations for the anti-resorption effect of IH-Ig are still hypothetical. Further studies are needed to determine the precise mechanisms underlying the IH-Ig effect in reproductive failure using mouse models, and to serve the rationale for utilizing Ig as a therapeutic option for RM patients.

Funding

This study was supported in part by a Grant-in-Aid for Scientific Research (S) (#14404010 to K.O.) from Japan Society for the
Promotion of Science (JSPS), a Grant-in-Aid for Scientific Research on Priority Areas (#18105003 to K.O.) by the Ministry of Education, Culture, Sport, Science and Technology (MEXT) Japan, and a Grant-in-Aid for the 21st Century COE program on ‘Topological Science and Technology’ (to H.Y.) from the MEXT, Japan. This study was also supported by the Tomakomai East Hospital Foundation (to K.O.).

Acknowledgements
We thank for the staffs of Nihon-Seiyaku Company and Aventis Pharma Co. Ltd. for providing intact human immunoglobulin and Fab immunoglobulin, respectively.

References

Anti-resorption effect of immunoglobulin

813


Submitted on July 3, 2007; resubmitted on August 14, 2007; accepted on August 21, 2007.