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A randomized, double-blind, placebo-controlled trial of intravenous immunoglobulin in the prevention of recurrent miscarriage: evidence for a therapeutic effect in women with secondary recurrent miscarriage

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BACKGROUND: Previous trials of intravenous immunoglobulin (IvIg) treatment of women with recurrent miscarriage (RM) have provided diverging results. This may be due to different inclusion criteria and suboptimal treatment protocols in some trials. METHODS: According to a computer-generated list, 58 women with at least four unexplained miscarriages were randomly assigned to receive infusions of high doses of IvIg or placebo starting as soon as the pregnancy test was positive. RESULTS: In the intention-to-treat analysis, a 45% live birth rate was found in both allocation groups. In patients with secondary RM, 50% in the treatment group and 23% in the placebo group had successful pregnancies (*P* = not significant). When data from the present and a previous placebo-controlled trial of the same treatment were combined, 15/26 (58%) of the patients with secondary RM in the treatment group versus 6/26 (24%) in the placebo group had successful outcomes (*P* < 0.02). Only 7% of the karyotyped abortuses were abnormal. CONCLUSIONS: IvIg may improve pregnancy outcome in patients with secondary RM. A new placebo-controlled trial focusing on this subgroup should be conducted to confirm the results.

Keywords: abortion/HLA-DR/HY antigen/intravenous immunoglobulin/recurrent spontaneous abortion

Introduction

Approximately 10% of all clinical pregnancies result in miscarriage (Regan, 1988), and 43% of these losses seem to be caused by aneuploidy of the fetus (Creasy, 1988). Recurrent miscarriage (RM), which is defined as three or more consecutive miscarriages, occurs in 0.5–1.0% of women who desire a child (Alberman, 1988), but in this group fetal aneuploidy seems to be an infrequent cause of miscarriage (Ho *et al*., 1991). There are indications that the frequency of fetal aneuploidy decreases with the number of previous miscarriages (Ogasawara *et al*., 2000), suggesting that other causes of RM become more dominant with an increased number of previous losses. Several studies have reported that the prevalence of immunological disturbances (Christiansen *et al*., 1998) and of particular HLA alleles (Christiansen *et al*., 1994; Pfeiffer *et al*., 2001) increase with the number of previous pregnancy losses. So far, no clinical or laboratory diagnostic criteria (except for fetal and parental chromosomal abnormality) have been able to discriminate adequately between the various causes of RM, and most cases of RM are probably the result of the action of several immunological and non-immunological factors.

Since immunological disturbances seem to be a risk factor in many cases of RM, various immunotherapeutical interventions have been tested in RM. The efficacy of immunization with allogeneic leukocytes (most often of paternal origin) is disputed, since published trials have been in favour (Recurrent Miscarriage Immunotherapy Trialists Group, 1994) as well as not in favour of the treatment (Ober *et al*., 1999).

Intravenous immunoglobulin (IvIg) exhibits a documented effect in many disorders caused by immunological abnormalities (Ronda *et al*., 1993) which may be mediated partially through induction of apoptosis in activated peripheral blood lymphocytes (Prasad *et al*., 1998). It has also been tested in the treatment of women with RM (The German RSA/IVIG Group, 1994; Christiansen *et al*., 1995; Coulam *et al*., 1995; Marzusch *et al*., 1996; Perino *et al*., 1997; Stephenson *et al*., 1998; Jablonowska *et al*., 1999). So far, six placebo-controlled trials of IvIg treatment have been published with very diverging results. Two trials (Christiansen *et al*., 1995; Coulam *et al*., 1995) showed a 24–29% therapeutic gain from IvIg compared with placebo, whereas the other trials (The German RSA/IVIG Group, 1994; Perino *et al*., 1997; Stephenson *et al*., 1998; Jablonowska *et al*., 1999) could not find any benefit.

The contradictory results of the trials of IvIg treatment

of RM might be explained by different selection criteria of the patients, especially with regard to the number of previous fetal losses, different starting time of the infusions and different IvIg doses. RM patients with only three previous miscarriages generally exhibit a good prognosis, whereas the prognosis worsens and the evidence for an immunological cause increases in women with a higher number of miscarriages (Christiansen *et al*., 1994, 1998; Pfeiffer *et al*., 2001). The latter patients are thus potentially the best candidates for an expensive immunological treatment. In several previous trials, the starting time of infusions was at the first ultrasonic detection of fetal heart action in gestational week 6–7 (The German RSA/IVIG Group, 1994; Jablonowska *et al*., 1999). Initiation of treatment at that time may be too late to prevent early miscarriages because it may take weeks for the effects of IvIg to be complete, whereas the spontaneous chance of live birth increases after the detection of fetal heart action. The IvIg doses generally given in published trials have been significantly lower than those given in most autoimmune diseases (Ronda *et al*., 1993).

In the present study, a placebo-controlled trial of IvIg in the treatment of RM was carried out where only patients with at least four previous miscarriages were included, and a very intensive treatment protocol was followed in order to optimize the chance of detecting a possible treatment effect.

Materials and methods

Protocol

Each patient had to meet the following criteria to be eligible for participation in the trial: (i) a history of four or more confirmed miscarriages before the end of the 26th gestational week, of which the last three had been consecutive; (ii) no uterine or parental chromosomal abnormality; (iii) regular menstruations with cycle length between 21 and 35 days; (iv) written informed consent; and (v) a positive pregnancy test carried out at the hospital. The exclusion criteria were as follows: (i) total IgA deficiency; (ii) autoimmune rheumatic disease; (iii) insulin-dependent diabetes mellitus; (iv) pregnancy obtained by IVF or controlled ovarian stimulation; and (v) application to participate in the trial later than 7 days after the expected menstruation. The patients could only participate in the trial once.

All miscarriages stated by the patients were confirmed by searching hospital records or by contacting the practitioners. All women had normal findings by hysterosalpingography or hysteroscopy, and all couples had normal chromosomes by ordinary G-band technique. Since all women had regular menstruations and could conceive spontaneously within a few months the presence of significant endocrinological disturbances with a potential impact on fecundity were unlikely. However, as part of our routine work-out, all patients were screened for serum thyroxine in one cycle and serum mid-luteal serum progesterone (s-P) in two cycles. All measurements were within the normal range.

The first infusion of study drug was given immediately after randomization which was done according to a computer-generated list. At each intravenous infusion until the 20th gestational week, a total of 0.8 g study drug per kilogram bodyweight (measured at the time of the first infusion) was administered. From gestational weeks 20 to 26, 1.0 g of study drug per kilogram bodyweight was given. From inclusion in week 5 until week 10, weekly infusions were given, and infusions were subsequently carried out every second week. After the 26th gestational week no further infusions were undertaken, and this resulted in a total of 14 infusions in successful pregnancies. All infusions were given on an outpatient basis at the Department of Obstetrics and Gynaecology, Aalborg Hospital.

The active drug was Nordimmun® (HemaSure A/S, Copenhagen, Denmark); this is a human IgG preparation manufactured from plasma screened for HIV, hepatitis B and hepatitis C virus and virus-inactivated by pasteurization at 60°C and caprylic acid precipitation. Nordimmun contained 4.6% human IgG, 1.5% human albumin, 4.6% sucrose and 0.15 mol/l sodium. The placebo drug contained 1.5% human albumin, 4.6% sucrose and 0.15 mol/l sodium.

All parts of the study were conducted according to the regulations of Good Clinical Practice (GCP) for trials on medical products in the European Community (Brussels 1990, Document II/3976/8-8EN). The trial was approved by the local ethics committee and conducted in accordance with Helsinki Declaration II

Assignment

Before inclusion, all patients had a case report made at our clinic comprising their reproductive history and results of previous investigations. If the criteria for participation in the trial were met, patients were informed of the procedures and possible side effects before giving their written informed consent. Patients were instructed to contact the clinic immediately if the menstruation was 3 days overdue, in order to have a pregnancy test conducted. If the test proved positive, the patients were included in the trial.

When a serum or urine β-HCG test was positive, the patient was allocated a consecutive number of randomization; this identified the patient and her treatment with either IvIg or placebo. Allocation to the treatment arms was made according to a computer-generated randomization list which was retained by HemaSure A/S, Copenhagen, during conduct of the trial.

Masking

The randomization code was blinded to the patients and hospital staff (including the authors) until after the last included patient had given birth and all data had been entered into a computer database in April 2000 by an independent clinical research organization (Ecron Wiedey GmbH, Konstanz, Germany). The placebo drug could not be distinguished visually from the active drug. Bottles containing either Nordimmun or placebo were marked with their allocation numbers, but otherwise were identical. Measurements of immunological parameters in the patients' blood samples (including immunoglobulins) were not made until the trial had been concluded.

Data analysis

The primary effect parameter was the proportion of all randomized patients in the two allocation groups discharged from the birth clinic with at least one living child. This was the so-called live birth rate (LBR) calculated by an intention-to-treat (ITT) analysis without any exclusion of patients once allocated to the trial.

Three secondary effect parameters were also planned to be analysed and compared between the patients in each allocation group: (i) the LBR after exclusion of patients who had a s-P level <35 nmol/l and a β-HCG level <100 U/l at the time of inclusion before the first infusion; (ii) the LBR after exclusion of patients who had ectopic pregnancies, miscarriages with a chromosome abnormal fetus or fetal losses happening immediately after invasive prenatal diagnostics; and (iii) the LBR after exclusions in both (i) and (ii). A fourth secondary effect parameter, the LBR among patients with secondary RM, was added after publication of a meta-analysis of IvIg treatment in RM (Daya *et al*., 1999).

The following tertiary effect parameters were analysed: gestational

age at the time of delivery, birthweight, Apgar scores, the LBR in relation the maternal HLA-DR types, and the level of ACAs early in pregnancy.

Maternal safety parameters evaluated were clinical symptoms, in addition to laboratory screening tests for transmission of hepatitis and HIV and for signs of hepatic and renal affection during and 3 months after end of treatment. In addition to neonatal parameters, fetal safety parameters were the health and development of the child evaluated by questionnaires sent to the parents at 3 and 12 months after birth.

Sample size calculation

The spontaneous prognosis for a completed pregnancy among the women suitable for inclusion in the trial was estimated to be 30% according to the results of previous studies. After treatment with Nordimmun, an increase in the LBR from 30 to 70% would be clinically relevant. It was calculated that the inclusion of 56 patients (28 in each group) would be necessary to detect this difference with a probability of 90%, accepting a type II error of 10% and a type I error of 5%.

It was planned that for each patient excluded from the analysis of the secondary effect parameters, one additional patient should be included in the trial in order to ensure that the statistical power of this analysis remained intact.

Statistical analysis

Categorical variables were analysed using either the χ^2 test or Fisher's exact test. Unpaired continuous variables were analysed by the Mann–Whitney test, since a normal distribution of the variables was not evident. Relative risks (RR) and their 95% confidence limits (95% CI) were given when appropriate. Comparisons of data stratified according to the number of previous miscarriages were performed using the Mantel–Haenzel test. Paired continuous variables were analysed by Wilcoxon's test for paired variables. All calculations were made using a MEDSTAT statistical package (Astra, Denmark). Testing of deviations of sex distributions from 1:1 of abortuses and infants was carried out using the binomial distribution. A *P*-value (two-sided) < 0.05 was considered significant.

Laboratory analyses

Blood samples were withdrawn before each infusion of study drug, centrifuged, and the plasma was stored immediately at –80°C until analysed. Analysis of immunological parameters was carried out after completion of the trial, using the samples taken at three time points: gestational week 5 (sample 1); gestational week 8 or the last preceding infusion if the patients miscarried before this time (sample 2); and gestational week 12 or at the last preceding infusion if the patient miscarried between weeks 8 and 12 (sample 3). Testing for hepatitis B antigens and antibodies against hepatitis C and HIV 1 and 2 was done on fresh samples at the time of inclusion, in week 24, and at approximately 3 months after birth or the last infusion in case of miscarriage. The activated partial thromboplastin time (APTT) was measured in fresh blood samples from weeks 5, 8 and 12, and alanine aminotransferase (ALAT) in samples from weeks 5, 8, 12 and 24 and at 3 months after birth or the last infusion. Serum creatinine was measured in weeks 5 and 24, and at 3 months after birth or last infusion. If any of the above-mentioned parameters were abnormal they were investigated at each subsequent infusion until they had normalized. If the APTT was >40 s an investigation for lupus anticoagulant was undertaken using commercially available kits (Viper Quick LA-test and Viper Quick LA-check; Organon Technics, Durham, NC, USA). ALAT was monitored using reaction kinetic photometry, with a cut-off value of 35 U/l. Antibodies against hepatitis C and HIV 1 and 2 and HbsAg virus were monitored using an enzyme immunoassay (EIA).

Anticardiolipin antibodies (ACA) were measured using an enzyme-linked immunosorbent assay (ELISA) (VRA-ELISA cardiolipin IgG, IgM; Pharmacia-Upjohn). Reference values established were <7 M-phospholipid (MPL)-U/ml and <22 G-phospholipid (GPL)-U/ml for IgM and IgG ACAs respectively. Screening for antinuclear antibodies (ANA) was carried out using immunofluorescence on murine fibroblasts and confirmation of positive findings on HEP2 cells. Positive samples were semi-quantified as weakly, moderate, or strongly positive. Anti-double-stranded DNA antibodies were investigated using an ELISA method with a reference value ⩽100 kU/l. Investigation of HLA-DR alleles was undertaken in patients of Caucasian origin using a PCR single-strand polymorphism (PCR-SSP) technique with 24 HLA-DR allele-specific primers (Dynal Biotech, Oslo, Norway).

Quantification of serum β-HCG and s-P was undertaken using an automated ELISA method (Bayer Immuno I, Terrytown, USA).

Ultrasound and chromosomal investigations

Between the 6th and 12th gestational weeks, vaginal ultrasound examinations were carried out every second week at the authors' clinic by skilled ultrasonographists to verify fetal heart action (FHA) and confirm the duration of gestation. Quantitative β-HCG measurements were undertaken weekly until week 10. If the β-HCG increase was insufficient, or if the patient experienced vaginal haemorrhage, then weekly ultrasound examinations were undertaken. Abdominal ultrasound examinations including screening for fetal malformations were performed in gestational weeks 18 and 26. After the 26th gestational week, surveillance of pregnancy was undertaken at the patients' local hospitals. Pregnancy losses were considered to be pre-embryonic if they occurred before the documentation of FHA, embryonic if they occurred after documentation of FHA but before the end of the 10th gestational week, and fetal if they occurred after documentation of FHA and after the 10th gestational week.

Every possible effort was undertaken to karyotype abortuses. The investigations were carried out at university-affiliated genetic institutes by conventional G-banding techniques.

Results

Participant flow and follow-up

A total of 68 patients with RM were considered eligible for participation if they achieved pregnancy during the study period, and were informed about the trial. Fifty-eight of these patients achieved pregnancy before the conclusion of the trial and were randomized. Fifty-six patients were included in the trial in accordance with the primary sample size calculation, but as two of these pregnancies (one ectopic, one with a chromosomally abnormal fetus) could not be evaluated for the secondary effect parameters, according to the protocol two further patients were included in order to maintain the statistical power of the trial. As the ectopic and chromosomally abnormal pregnancies occurred during the first half of the study, the decision to extend the trial with two patients was taken 4 years before its conclusion and unblinding of the randomization code.

The couples originated from all parts of Denmark and Norway. Fifty-six patients (97%) and 55 (95%) of their husbands were of Caucasian ethnic origin. The first patient was enrolled in June 1994, and the last in June 1999 (giving birth in January 2000). All patients complied completely with

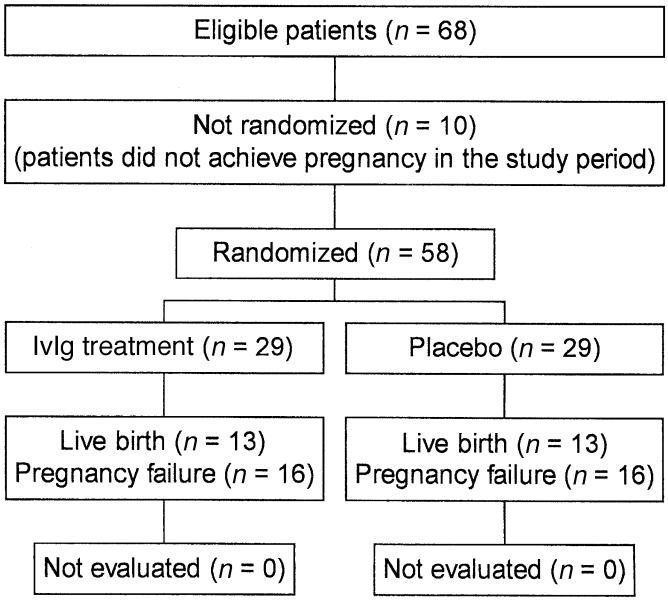

Figure 1. Diagram of participant flow and follow-up.

Table I. Baseline characteristics of patients according to allocation group

	IvIg (n = 29)	Placebo (n = 29)	P
Clinical characteristics			
Primary RM	17/29 (59)	16/29 (55)	NS
Secondary RM	12/29 (41)	13/29 (45)	NS
Age (years)			
Median	31	31	NS
Range	24–38	24–41	
No. of previous losses			
Median	5.0	4.0	NS
Range	4–9	4–0	
No. with 4 previous losses	14 (48)	19 (66)	NS
No. with 5 previous losses	8 (28)	5 (17)	NS
No. with ⩾6 previous losses.	7 (24)	5 (17)	NS
Immunological characteristics			
Autoantibody-positive[a]	14/29 (48)	11/29 (38)	NS
Anticardiolipin-positive	11/29 (38%)	11/29 (38)	NS

Values in parentheses are percentages.
[a]IgM and IgG anticardiolipin, lupus anticoagulant, antinuclear antibody, anti-ds-DNA.
NS = not significant.

the infusion schedule until the pregnancy was concluded by a birth, miscarriage or an ectopic pregnancy. Thus, all patients who were randomized received the allocated intervention, none discontinued the intervention, none were lost to follow-up, and all randomized patients were included in the primary ITT analysis (Figure 1). No patient concomitantly received therapies used in the management of RM such as progesterone or HCG supplementation, aspirin, heparin, prednisone and allogeneic lymphocyte immunization.

Although there was a tendency for more previous miscarriages among patients allocated to Nordimmun, no significant differences in the baseline characteristics of patients according to the allocation group could be detected (Table I). All patients had had at least four consecutive miscarriages immediately before inclusion, except for three patients who had a miscarriage preceding a live birth which was then followed by three consecutive miscarriages. The levels of β-HCG and s-P before the first infusion, the median body weight and the median amount of study drug given were not different between the two allocation groups (Table II). The plasma concentration of IgG increased (as expected) significantly in the Nordimmun group after the infusions had been undertaken, whereas no change could be detected in the placebo group (Table II). There were no differences in plasma IgG concentrations between the patients who subsequently gave birth and those who miscarried (data not shown).

Table II. Pregnancy-related hormone levels at inclusion, median body weights, amount of study drug given at each infusion and subsequent serum IgG concentrations according to allocation group

	IvIg (n = 29)	Placebo (n =29)	P
Hormones at randomization			
Sereum β-HCG (U/l)	548	721	NS
Serum progesterone (nmol/l)	71	64	NS
Median body weight (kg)	67.0	64.8	NS
Treatment			
Study drug given at each infusion (g)[a]	55	50	NS
Serum IgG in sample 1 (g/l)	11.4	11.4	NS
Serum IgG in sample 2 (g/l)	23.3[b]	10.7	–
Serum IgG in sample 3 (g/l)	21.7[b]	10.4	–

[a]Until 20th week gestation.
[b]$P < 0.00001$ versus sample 1.
NS = not significant.

The LBRs were completely similar in the Nordimmun and placebo groups after ITT analysis, and only differed by 3–5% after predefined exclusions (Table III). Among patients with secondary RM (RM after a pregnancy which progressed to at least the 26th gestational week), a 27% higher success rate could be detected in the Nordimmun group than in the placebo group, but this was not statistically significant. Statistical analysis stratifying for the number of previous miscarriages gave similar adjusted P-values (P_{adj}-values).

Two miscarriages, both in the placebo group, occurred in the second trimester (weeks 20 and 21), whereas all remaining pregnancy losses were in the first trimester.

The LBR was 45%, irrespective of the patients being positive or negative for IgG and/or IgM ACA at inclusion into the trial (Table IV). More than 95% of those shown as ACA-positive in blood sample 1 were also ACA-positive in blood sample 2 taken 1–3 weeks later. Two patients were positive for lupus anticoagulant, but they were also positive for high titres of IgG ACA. Caucasian patients positive for one of the HLA-DR alleles (HLA-DR1, -DR3 or -DR10) exhibited a significantly lower LBR than those negative for all three alleles ($P = 0.03$).

No significant difference in neonatal data could be detected between Nordimmun- and placebo-treated patients (Table V). However, due to the small number of births it cannot be excluded that the neonatal complication rate was higher in Nordimmun-treated pregnancies. In the Nordimmun group, four neonatal complications were observed: one infant had a slight deviation of the nose, one had a cleft-palate (the mother

Table III. Outcome of pregnancies according to allocation group

	Live birth rate		RR (95% CI)	*P*	P_{adj}
	IvIg (n = 29)	Placebo (n = 29)			
ITT analysis	13/29 (45)	13/29 (45)	1.0 (0.6–1.8)	NS	NS
After exclusions[a]	13/27 (48)	13/29 (45)	1.1 (0.6–1.9)	NS	NS
Appropriate hormones[b]	13/23 (57)	13/24 (54)	1.0 (0.6–1.7)	NS	NS
Both [a] and [b]	13/22 (59)	13/24 (54)	1.1 (0.7–1.8)	NS	NS
Secondary RM (ITT)	6/12 (50)	3/13 (23)	2.2 (0.7–6.8)	NS	NS

Values in parentheses are percentages.
[a]Predefined exclusions: one clinical ectopic pregnancy and one karyotypical abnormal abortion.
[b]Serum β-HCG >100 U/l and serum progesterone >35 nmol/l at inclusion.
ITT = intention-to-treat; NS = not significant; RM = recurrent miscarriage.

Table IV. Pregnancy outcome according to anticardiolipin status or HLA-DR type of the woman

	Live birth rate	RR	(95% CI)	*P*
Anticardiolipin-positive[a]	13/29 (45)	1.00	(0.59–1.77)	NS
Anticardiolipin-negative	13/29 (45)			
HLA-DR1 or -3 or -10 positive[b]	8 /28 (29)	0.50	(0.26–0.97)	0.03
HLA-DR1 and -3 and -10 negative[b]	16/28 (57)			

[a]Positive for IgM and/or IgG anticardiolipin antibodies.
[b]Only women of Caucasian ethnic origin.
NS = not significant.

Table V. Birth weight, gestational age at delivery, infants' sex and neonatal disorders according to treatment group

	IvIg (n = 13)	Placebo (n = 13)	*P*
Birth weight (g)			
Median	3150	3300	NS
Range	1490–4620	1940–3850	
Gestational age (weeks)			
Median	39	39	NS
Range	34–42	31–42	
Apgar score ⩽7 at 5 min	0	0	NS
Caesarean section	3	2	NS
Neonatal disorders	4	1	NS

Table VI. Data of karyotypes and gender of abortuses and infants lost/borne by the patients participating in the trial. Data expressed as sex of abortuses/infants (male/female/unknown)

	IvIg (n = 29)	Placebo (n = 29)	Total
Pregnancies in trial			
Sex (male/female/unknown)[a]			
Liveborn infants	4/9/0	5/8/0	9/17/0
Fetal pregnancy losses	0/3/0	1/0/1	1/3/1
Embryonic pregnancy losses	0/2/3	1/3/3	1/5/6
Pre-embryonic pregnancy losses	1/1/4	0/2/5	1/3/9
Total sex ratio	5/15/7	7/13/9	12/28/16[b]
Aneuploidic	1 (92xxyy)	0	
Ectopic	1	0	

[a]All abortuses except one were sex-determined by karyotyping.
[b]$P < 0.01$.

had previously borne a child with the same malformation), one had maldescended testes, and one child was born with alloimmune thrombocytopenia. The thrombocyte count quickly normalized post-partum after administering standard treatment (intravenous immunoglobulin) to the infant. One infant in the placebo group had a hygroma in the tongue. All children were healthy and thriving at 1 year after birth.

In the Nordimmun group, adverse events probably related to the infusions were reported 41 times, and in the placebo group four times. All adverse events but four were considered to be mild or moderate: local skin rash, headache, joint pains and slight fever. Three Nordimmun-treated patients and one placebo-treated patient were hospitalized for very short periods due to infusion-related symptoms. Tests for antibodies against hepatitis C and HIV remained negative in all patients during and after participation in the trial. Sixteen Nordimmun-treated and 10 placebo-infused patients had ALAT levels exceeding the upper normal value (35 U/l) in at least one measurement during pregnancy. In the majority of these cases the upper normal limit was only slightly exceeded, the maximum ALAT measurement in Nordimmun- and placebo-treated patients being 164 U/l and 105 U/l respectively. All patients had normal ALAT values 3 months after the infusions had ended. Other laboratory values (platelet count and serum creatinine values) remained normal during and after treatment.

Analysis of fetal karyotype and gender

There was an excess of females among the 14 abortuses which were found to have normal karyotype (n = 13) or sex-determined through examination by pathologists (n = 1) (Table VI). Furthermore, there was an excess of female live-born

Table VII. Combined results of two placebo-controlled trials of Nordimmun (IvIg) versus placebo in the prevention of recurrent miscarriage

	Successful pregnancy[a]		RR (95% CI)	*P*	P_{adj}
	IvIg	Placebo			
All patients (n = 92)					
ITT analysis	22/46 (48)	18/46 (39)	1.2 (0.8–2.0)	NS	NS
After exclusions	22/43 (51)	18/45 (40)	1.3 (0.8–2.0)	NS	NS
Secondary RM (n = 52)					
ITT analysis	15/26 (58)	6/26 (23)	2.5 (1.2–5.4)	<0.02	<0.02
After exclusions[b]	15/24 (63)	6/25 (24)	2.6 (1.2–5.6)	<0.01	<0.01

Values in parentheses are percentages.
[a]Progressing to at least the 26th gestational week.
[b]Predefined exclusions: two clinical ectopic pregnancies and one miscarriage after a severe car accident.
ITT = intention-to-treat; NS = not significant; RM = recurrent miscarriage.

infants compared with the expected 1:1 gender ratio. Overall, the male/female gender ratio of the sex-determined conceptions occurring in the trial was 12/28 = 0.43, which was significantly different from the expected 1:1 gender ratio ($P < 0.01$).

Combined analysis of two trials

The results of a previous placebo-controlled trial (Christiansen *et al.*, 1995) and the present trial are summarized in Table VII. In all patients, this gives non-significantly increased RRs for successful pregnancy in IvIg versus placebo-treated patients both after ITT analysis and after relevant exclusions (two clinical ectopic pregnancies, one chromosome abnormal loss and one miscarriage after a severe car accident). Stratified analysis did not change the *P*-values. Among 52 patients with secondary RM, the RR for a successful pregnancy in IvIg versus placebo-treated patients was 2.5 ($P < 0.02$) after ITT analysis and 2.6 ($P < 0.01$) after three relevant exclusions.

Discussion

For the present trial, a protocol was designed which took into account the possible weaknesses of previous trials of IvIg therapy in RM: all patients had had at least four previous pregnancy losses, infusions were started as soon as the sensitive pregnancy test was positive, and doses approaching those established in the treatment of autoimmune disease were given.

In the general population of RM patients, the results were disappointing. In the ITT analysis the success rate was identical whether IvIg treatment was given or not (Table III). Many pregnancies are non-viable from the time of conception because they are either genetically abnormal or ectopically located. Most of these pregnancies can be identified by a single s-P or β-HCG measurement (Stovall *et al.*, 1992). In a previous prospective trial it was found that a combined criterion of both s-P <35 nmol/l and β-HCG <100 IU/l would always indicate a non-viable pregnancy, and this was verified in the present trial. However, a secondary analysis of outcome in the present trial excluding all pregnancies with no potential ever to succeed only resulted in a non-significant 5% therapeutic gain from IvIg treatment.

In a meta-analysis (Daya *et al.*, 1999), only a small and non-significant effect of IvIg compared with placebo could be demonstrated among RM patients; however, in a subset with secondary RM the effect was greater. Although not defined as an effect parameter in the present protocol, these results prompted a search for outcome data among the present patients with secondary RM (defined as RM after a pregnancy which progressed to at least the 26th week). This analysis showed a large (27%) but not significant treatment effect. When the patients with secondary RM from the two placebo-controlled trials were combined (Table VII), the therapeutic efficacy of IvIg became statistically significant (RR for birth = 2.5, $P < 0.02$). It must be borne in mind that 45 out of the 52 (87%) secondary RM patients had had at least four previous miscarriages, so the therapeutic benefit of IvIg may apply only to secondary RM patients with at least this number of miscarriages. The first placebo-controlled trial (Christiansen *et al.*, 1995) on IvIg in preventing RM was also conducted and monitored according to GCP rules; here, Nordimmun was also the drug under test and was administered in doses that were ~60% those given in the present study, albeit at similar intervals. The study population comprised mainly patients with secondary RM, the allocation and concealment procedures were identical to those of the present trial, and in the final ITT analysis all randomized patients were evaluated without any exclusions or drop-outs.

Only one (7%) of the 14 miscarriages which were successfully karyotyped was chromosomally abnormal (Table VI). This is in accordance with other studies of fetal chromosome anomaly in RM, and emphasizes the fact that fetal aneuploidy is an infrequent cause of miscarriages in patients with multiple (e.g. four or more) miscarriages (Ho *et al.*, 1991; Ogasawara *et al.*, 2000).

An unexpected finding was a very skewed sex-ratio of the children born in the trial, and among the abortuses that it was possible to sex-determine primarily by karyotyping. There was a highly significant ($P < 0.01$) excess of both female infants and female abortuses compared with the expected 1:1 ratio. The karyotypings were carried out in four different university-affiliated genetic institutes which were confident that fetal cells rather than maternal cells were karyotyped. A skewed sex-ratio of abortuses and children in pregnancies of RM patients

has not been previously reported. The gender distribution of infants and abortuses born/lost after RM has been reported in very few studies. In previous trials (Christiansen *et al*., 1992, 1995) of IvIg and RM, the boy/girl ratio was 11/14 (0.79) after RM. In another trial (Ober *et al*., 1999) comprising 131 pregnant women, the ratio of liveborn male/female infants after treatment was 0.82 in the lymphocyte immunization group and 0.86 in the placebo group. These data support to some extent the findings in the present study.

Primarily supported by data from our own studies, we propose the hypothesis that many women with RM have developed a harmful immunological reaction against male-specific antigens (HY-antigens) on the trophoblast, and that this results in a subsequent increased miscarriage rate of male conceptions (de Bueger *et al*., 1992; Goulmy, 1997; Warren *et al*., 2000). At a first glance, this appears not to be in concordance with the increased frequency of abortuses which could be karyotyped as female (Table VI). However, 16 of the abortuses were not karyotyped, mainly because they were pre-embryonic losses (including biochemical pregnancies) and uterine aspiration was thus avoided. If the majority of these losses indeed are male embryos, it is possible to make the reasonable assumption that at the time of conception there is a 1:1 sex-ratio. Currently, this hypothesis is being explored further by means of ongoing epidemiological and immunological studies, the results of which will be reported shortly.

The significant impact on pregnancy outcome of the HLA-DR1, -DR3 and -DR10 alleles detected in the present (Table IV) and several previous studies (Christiansen *et al*., 1994, 1999; Christiansen, 1996) confirm that these HLA alleles are genetic markers for increased susceptibility to RM. HLA-DR1, -DR3 and -DR10 expressed on antigen-presenting cells (e.g. macrophages) may be the best HLA molecules to present trophoblast-expressed HY-antigens to T lymphocytes which can subsequently develop cytotoxic anti-trophoblast responses (van Els *et al*., 1990). IvIg treatment may exert its possible effect by inducing apoptosis (Prasad *et al*., 1998) of T lymphocytes with reactivity against antigens on the trophoblast.

In some previous studies RM patients have been selected for IvIg treatment because of positivity for autoantibodies, and in particular antiphospholipid antibodies (Marzusch *et al*., 1996). We do not believe that it is relevant in trials of IvIg to divide the patients into autoantibody-positive and -negative categories. There is good evidence that RM may be caused by a T-helper type 1 cytokine excess at the feto-maternal interface, increased natural killer cell cytotoxicity against trophoblast or, as suggested herein, cytotoxicity against male- specific antigens on the trophoblast. Autoantibodies are found with increased prevalence in RM patients, but there is still no convincing evidence that these antibodies *per se* exert a pathogenic effect on pregnancy (Christiansen, 1996; Gleicher, 1998) and they are therefore probably not useful for selecting patients for IvIg therapy. The frequent finding of autoantibodies including antiphospholipid antibodies in RM patients with no or limited impact on pregnancy outcome (Table IV) might be a consequence of the well-recognized immunological phenomenon determinant spreading (Lehmann *et al*., 1993). When an autoimmune reaction is initiated by T lymphocytes, previously hidden antigens (e.g. phospholipids) may be exposed to immune-competent cells as a consequence of inflammation, and antibodies can be formed against these antigens as a secondary event.

In conclusion, there are two main findings in the present study. First, IvIg treatment might be efficacious in a subset of patients with secondary RM. We believe that the results support the need for a trial focusing on this subgroup for a final evaluation of the effect, since evaluation of the effect on secondary RM patients was not pre-specified in the present study protocol and a significant treatment effect became apparent only after the combination of two different yet similar trials. By comparing the results of the two trials, it appears that smaller IvIg doses may be as efficacious as those used in the present trial, but with fewer side effects and lower costs. Second, the gender distribution of children and abortuses of RM patients appears to very skewed, which may be caused by maternal immunization against male-specific antigens. Future studies should aim at confirming or rejecting these epidemiological findings and investigating whether T-lymphocyte clones directed against male-specific antigens are more prevalent in RM patients than in women with normal fecundity.

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