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A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects

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The scientific basis for many traditionally accepted causes of recurrent miscarriage (RM) is weak. A significant proportion of RM cases with relatively few miscarriages can presumably be attributed to the random occurrence of consecutive chromosomally abnormal conceptions. New insights in the immunological interactions taking place at the feto–maternal interface provide us with the opportunity to propose detailed pathophysiological models for immunologically mediated RM. Scientific support for the theory that RM is a consequence of graft rejection-like alloimmune reactions against paternal human leukocyte antigens on the fetus is sparse. Conversely, there is considerable evidence that decidual natural killer cells play a role in the implantation and early invasion of the trophoblast and in the pathogenesis of RM. T helper (T_h) cells from women with RM react against trophoblast antigens *in vitro* with the secretion of mainly interleukin-2 and interferon-γ (a so-called T_h1 response), which are known to inhibit trophoblast growth. The predisposition to a T_h1 response against a given antigen may be determined by an individual's class II histocompatibility genes. In accordance with this, case-control, prospective and family studies indicate that maternal histocompatibility haplotypes comprising DR1 and DR3 alleles confer susceptibility to RM. The frequent occurrence of autoantibodies in women with RM is compatible with the theory of a T_h1 response against trophoblast as a cause of the syndrome, but the autoantibodies themselves probably do not cause RM.

Key words: anticardiolipin antibodies/autoimmunity/HLA/pregnancy/recurrent spontaneous abortion

Introduction

> 'There are few subjects of such clinical importance as recurrent miscarriage so bedevilled by inconsistency, imprecision and unwarranted assumption. This applies particularly to the fundamental question of etiology in which physicians all too frequently confuse "association" with "cause".'
>
> G.M.Stirrat, 1992

When a woman has experienced a series of at least three involuntary losses of intrauterine pregnancies before the completion of the 28th gestational week she qualifies for the diagnosis recurrent miscarriage (RM; Crosignani and Rubin, 1991). Many readers of recent literature will get the impression that the diagnostic possibilities with regard to RM have improved during the last decades because of the more widespread use of hysteroscopy, microbiological investigations and measurements of autoantibodies and hormones, and the appearance of new diagnostic tools such as high-resolution ultrasound. This larger array of diagnostic possibilities is expected to result in better and more specific treatments. It was thus disappointing when Plouffe *et al.* (1992) compared the total livebirth rate in the next pregnancy in a cohort of RM patients who received specific treatment (surgical, anticoagulation, hormones) between 1968 and 1977 with a similar group treated between 1987 and 1991. Despite the fact that fewer of the cases in the more recent group were thought to remain unexplained after completion of the investigation programme, no im-

provement (but rather a decline) in the total livebirth rate was detected. The possible reasons for this could include (i) classification of the cases into different aetiological categories is erroneous, or (ii) the treatments are not effective.

With regard to the first reason, many researchers seem to exhibit a rather lax attitude to the concept 'cause', and this is responsible for the introduction of several undocumented causes of RM. Regarding the second reason, the majority of interventions in RM patients have not been tested in prospective, placebo-controlled trials; despite this lack of scientific evidence of efficacy, they have obtained acceptance for general use.

A critical discussion of the criteria for assigning causes and accepting treatments of RM will be the main topics of this review. In this regard, both non-immunological and immunological abnormalities presumed to cause RM will be discussed. However, non-immunological abnormalities will be reviewed rather superficially and only to the extent necessary to illustrate that the documentation for these factors is, in general, not better than for many of the immunological factors.

Emphasis will be laid on the discussion of possible immunological causes of RM because the bulk of new knowledge on feto–maternal relations in early pregnancy published recently relates to immunology. An attempt will be made to integrate results from relevant epidemiological, immunohistochemical and immunogenetic studies into a general theory of the aetiology of immunologically mediated RM.

Epidemiological considerations

Only a few epidemiological studies have been carried out with regard to RM, and the results have been integrated, only to a very limited degree, with the theories of the aetiologies of RM that have developed from, for example, immunological studies.

Systematic and non-recurrent reasons for RM

The RM syndrome is defined by the repeated occurrence of an event, miscarriage, which in its sporadic form occurs frequently in the normal population. Clinical intrauterine pregnancy loss occurs in 12–14% of all pregnancies (Fertility and Employment, 1979; Wilcox *et al.*, 1988; Regan *et al.*, 1989) and can, in most instances, probably be attributed to random factors (often fetal de-novo chromosomal abnormalities). Accepting a 14% pregnancy loss rate, it can be deduced that many women ($\sim 0.14^3 = 0.3\%$) will be diagnosed as suffering RM for non-recurrent reasons — the women have simply been unlucky three times consecutively (Huisjes, 1984; Redman, 1990). It is estimated that the prevalence of clinical RM in the population is 0.5–0.8% (Fertility and Employment, 1979; Alberman, 1988). The number of women with RM for non-recurrent reasons will decline exponentially with the number of previous miscarriages, because their risk of a new miscarriage in their fourth or fifth pregnancy is still only 14% compared with the 35–50% risk in the whole group of RM patients with a similar number of previous miscarriages (Workshop Reports, 1991; Cauchi *et al.*, 1995). The consequence will be as shown in Figure 1. Patients with a systematic problem will comprise an increasingly larger fraction of all RM patients with an increased number of previous pregnancy losses.

Table I. Prevalence of recurrent miscarriage (RM) in relatives of RM patients and controls

Authors	Types of relatives studied	RM rate (%)		
		Relatives	Controls	
Johnson *et al.* (1988)	Blood relatives	12.2	7.3	
Alexander *et al.* (1988)	Mothers and sisters	7.0[a]	0.0[a]	
Ho *et al.* (1991a)	First-degree relatives of the couple	1.4[b]	0.2[b]	
Christiansen *et al.* (1990a)			Control 1	Control 2
	Mothers	8.0[c,d]	1.4[c]	1.8[d]
	Sisters	10.6[e,f]	1.4[e]	1.8[f]
	Brothers' wives	6.3	1.4	1.8

Control 1 = control group from Stray-Pedersen and Lorentzen-Styr (1979); $n = 5901$. Control 2 = control group from Fertility and Employment (1979); $n = 493$.
[a]$P < 0.02$; [b]$P < 0.0001$; [c]$P < 0.0002$; [d]$P < 0.001$; [e]$P < 0.00005$; [f]$P < 0.0005$.

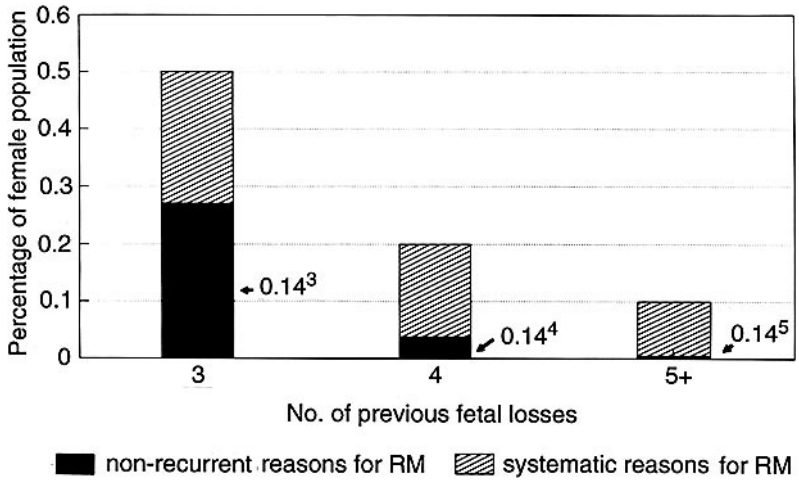

Figure 1. Theoretical distribution of recurrent miscarriage (RM) patients with three, four and five or more consecutive previous fetal losses in the female population. The summarized prevalence of the three subgroups is 0.8%, and it is suggested that 40% of patients with three fetal losses and 50% of patients with four fetal losses will experience fetal loss in their next pregnancy. The shaded areas of the columns indicate the calculated proportion of patients in each subgroup who, by sheer bad luck, had three, four or five fetal losses for non-recurrent reasons.

Partner specificity

The theory that RM is a consequence of increased human leukocyte antigen (HLA) sharing between spouses and its treatment with partner lymphocytes was founded on the belief that RM is a partner-specific disorder. Only one study has been published on the topic of partner specificity (Christiansen *et al.*, 1990a), which found that RM women who had achieved pregnancies with two different spouses and displayed an almost 100% rate of miscarriage with their second spouse had also had a highly increased 72% rate of miscarriage [95% confidence interval (CI) 50–88%] with their first spouse. These results argue against the concept of RM as a partner-specific condition.

Familial predisposition

A few studies have focused on the occurrence of RM among relatives of women with RM. All found an increased prevalence of RM among first-degree blood relatives (Table I). Odds ratios (OR) of RM among first-degree relatives of RM patients were in the interval 2–7 compared with the background population, suggestive of the existence of a familial predisposition to RM.

Non-immunological causes

Association and cause

RM patients and their spouses are traditionally subjected to a screening programme comprising search for anatomical, chromosomal, endocrine, microbiological and, for the last decade, immunological abnormalities. After conclusion of the screening programme, causes of RM have often been assigned according to the principle that if a result fell outside the normal range it was considered to be cause. By these means, distributions of the relative frequencies of the aetiologies of RM have been proposed, but there is little agreement as to how large these frequencies are. Anatomical causes have been claimed to account for 1 (Coulam, 1991) to 28% (Stray-Pedersen and Stray-Pedersen, 1984) of RM cases, luteal phase deficiency for 3 (Stray-Pedersen and Stray-Pedersen, 1984), 20 (Fritz, 1988) and 40% (Daya *et al.*, 1988), and infectious agents for 1 (Plouffe *et al.*, 1992), 15 (Stray-Pedersen and Stray-Pedersen, 1984) and 48% (Harger *et al.*, 1983) of RM cases. Disagreement on the classification of RM into various categories according to presumed causality is partially because some physicians consider a factor found statistically associated with RM in a few case-control studies to be a cause, whereas others have more rigorous criteria for accepting causality. Among such criteria might be a demand for the demonstration of plausible pathophysiological mechanisms linking the suspected risk factor and pregnancy loss, and a demand for a demonstration, in prospective studies, that patients with the suspected factor, compared with those without, display a significantly higher risk of pregnancy loss after adjustment for confounding variables. Because very few proven causes of RM are known, the term ‘risk factor’ will be used instead of ‘causes’ for the remainder of this paper.

Anatomical risk factors

Anatomical investigations are normally performed by hysterosalpingography, hysteroscopy or ultrasound scans. Müllerian duct fusion anomalies in the uterus have tradition-

ally been considered responsible for cases of RM and premature deliveries. However, the finding of a uterine anomaly in a woman with RM is not a direct indication that it is the cause of the problem because untreated Müllerian anomalies are, in most cases, compatible with normal pregnancy outcome (Rock and Jones, 1977). Most estimates of the prevalence of congenital uterine anomalies in the normal fertile population are in the interval 3–10% (Hay, 1958; Greiss and Mauzy, 1961; Stampe Sørensen, 1988). Hence, many women with RM who have other causes for their pregnancy losses will, by sheer coincidence, also be diagnosed as having a Müllerian fusion anomaly. Various surgical procedures (Strassmann, 1907; Jones and Jones, 1953) have been claimed as increasing the rate of successful pregnancy outcome, but no prospective randomized controlled trial has assessed their efficacy. Indeed, a study comparing patients with Müllerian anomalies who underwent a metroplasty procedure with matched non-surgically treated patients found similar success rates in the two groups (Kirk *et al.*, 1993). Because a 30% infertility rate after abdominal metroplasty has been reported (Bennett, 1987), reticence to carry out these procedures is recommended.

Women with second trimester miscarriages or preterm deliveries preceded by symptomless dilatation of the cervix have traditionally been diagnosed as having insufficiency of the uterine cervix. There exist no generally accepted objective criteria for the diagnosis. Women with a diagnosis of cervical insufficiency are often 'treated' by a cervical cerclage (McDonald, 1987). Three randomized controlled trials of the efficacy of this intervention have been carried out: none could demonstrate any significant effect with respect to decreasing perinatal mortality, in patients with at least three previous second trimester miscarriages or stillbirths (Rush *et al.*, 1984; Winisdoerffer *et al.*, 1989; MRC/RCOG Working Party on Cervical Cerclage, 1993). Stirrat (1992) concluded that 'empirical cervical cerclage can still, therefore, not be recommended'.

Chromosomal risk factors

Chromosomal investigations are usually carried out by Q or G banding techniques on the metaphase chromosomes of peripheral blood cells from the couple or fetal tissue. It has been reported that as many as 50% of culturable tissue samples from miscarriages occurring sporadically have chromosomal abnormalities (Creasy, 1988). These non-inheritable de-novo abnormalities arise from random errors in meiosis, the first mitoses or from fertilization of the ovum by two spermatozoa. Significant chromosomal abnormalities (often balanced translocations) are found in 2.6–4.0% of couples with RM (Stray-Pedersen and Stray-Pedersen, 1984; Tharapel *et al.*, 1985; Warburton and Strobino, 1987). It is expected that these couples will display an increased risk of chromosomally abnormal conceptions. However, when parental chromosomes are normal in RM couples it seems that the risk of chromosome abnormalities in the next pregnancy is relatively low. Warburton and Strobino (1987) found a 17% rate of abnormal karyotypes among aborti of women with RM, whereas the corresponding rate was 33% in women with sporadic miscarriages. In prospective studies, Ho *et al.* (1991b) and Christiansen *et al.* (1993) found no karyotypically abnormal conceptions among a total of 25 karyotyped aborti of women with unexplained RM.

Endocrine risk factors

Luteal phase insufficiency has for decades been considered a cause of RM. When repeated serum progesterone concentration measurements in the luteal phase are below a defined value (e.g. 10 ng/ml) or when a luteal phase endometrial biopsy is >2 days out of phase, a diagnosis of luteal insufficiency is often made. There is very little agreement on the diagnosis of luteal insufficiency (Karamardian and Grimes, 1992), and no trial has assessed the effect of progestational agents on the maintenance of pregnancy in patients selected by this diagnosis. A meta-analysis of the effect of progestational agents when administered to women with previous adverse pregnancy outcomes did not disclose any effect on the rate of fetal survival (Goldstein *et al.*, 1989); the same result was obtained in a subset of patients with repeated miscarriages.

With respect to the effect of human chorionic gonadotrophin (HCG), administration in early pregnancy to women with RM in a multicentre placebo-controlled trial failed to confirm previous suggestions of a therapeutic effect (Harrison, 1992).

The presence of polycystic ovary syndrome and a luteinizing hormone (LH) concentration >10 IU/l on day 8 of the proliferative phase has in prospective studies been shown to be associated with a higher rate of miscarriage, and thus been suggested as a risk factor for RM (Regan *et al.*, 1990). Based on theoretical considerations, a suppression of endogenous LH secretion using gonadotrophin-releasing hormone (GnRH) agonists may be beneficial (Rai *et al.*, 1996), but no results from controlled trials of this treatment have been published so far.

Microbiological risk factors

A number of micro-organisms, e.g. toxoplasmosis, listeria, cytomegalovirus and parvovirus, have been suggested to cause sporadic miscarriages and stillbirths. For a micro-organism to cause RM it must be presumed to persist in the genital tract for a long time. This is not very likely and there

is no proof that such organisms play any role in RM. A series of bacteria, e.g. group B streptococci, will often grow in cultures from aborted tissue, but this is generally believed to reflect a secondary intrauterine colonization after death of the fetus (Warburton and Strobino, 1987; Stirrat, 1990). At present there is no evidence from controlled trials of any effect of antibiotics in the prevention of RM.

The immune system: a short description

Considerable knowledge concerning the immunological architecture at the feto–maternal interface has accumulated during the last decade. It has been derived primarily from histological studies of tissue from normal pregnancies (induced abortions). Some basic features of the immune system in general and the immunological architecture of the feto–maternal interface in particular will be briefly described below. This description will provide a clearer understanding of a later discussion of the probable importance of immunological interactions for a successful pregnancy.

The elements of the immune system

The main elements of the human immune system are the T and B lymphocytes, granulocytes, monocytes, macrophages, natural killer (NK) cells and a variety of non-cellular factors secreted by the cells [immunoglobulins, interleukins (IL), complement factors]. The main task of the immune system is to protect the individual against pathogenic micro-organisms. Immune responses depend on the ability of the immune system to recognize foreign molecules — antigens — on potential pathogens, and then to mount an appropriate reaction to eliminate the source of the antigen. This is undertaken by two different functional divisions of the immune system: the innate and the adaptive immune system.

The innate immune system comprises granulocytes, macrophages and NK cells, and acts as a first line of defence. It can react against foreign antigens without prior sensitization. NK cells primarily attack cells expressing no or low levels of the classic HLA antigens HLA-A, -B, -DR, -DQ and -DP (Ljunggren and Karre, 1990). However, the expression of HLA-G and/or HLA-C molecules might protect against NK cell attack (Loke and King, 1996).

The cellular branch of the adaptive immune system comprises the T and B lymphocytes. T lymphocytes recognize foreign HLA and non-HLA in conjunction with the individuals own HLA. The adaptive immune system needs some time to be activated after the first encounter with an antigen. This will activate a specific clone of T helper (T_h) or T cytotoxic (T_c) cells, and be followed by an effector phase mediated by a variety of cell types in concert with immunoglobulins, complement factors and IL. Finally, a very specific (and usually effective) immune reaction will eradicate the source of the antigen.

Histocompatibility antigens

The HLA genes are the main genetic determinants of the repertoire of possible immune responses of an individual. They comprise a series of closely linked genes located on chromosome 6 encoding for polypeptide molecules expressed on the majority of human nucleated cells. The so-called classic HLA loci are divided into class I loci coding for HLA-A, -B and -C antigens, and class II loci coding for HLA-DR, -DQ and -DP antigens. The genes in these HLA loci are characterized by an extensive polymorphism, with the result that two unrelated individuals will very rarely be HLA identical within more than a few loci. The immunological role of classic HLA molecules is to bind foreign antigens on the surfaces of cells and present the antigen–HLA complex to the host's T lymphocytes. In the case of endogenous antigens (viruses, etc.), the antigens are presented on the surface of the infected cell in context with its class I HLA molecules. T_c lymphocytes will recognize this complex and attack the cell. In the case of an extracellular antigen, this is endocytosed by macrophages and subsequently expressed on the surface of this antigen-presenting cell (APC) in context with the host's class II HLA antigens. This complex will be presented to T_h lymphocytes, which will result in an immune response. The existence of a series of non-classic HLA genes (HLA-E, -F and -G) has been recognized recently. These genes display very limited polymorphism and their function, if any, is unknown. In particular, it is not clearly established whether they can complex with foreign antigens on the surface of target cells and elicit a T_c lymphocyte-mediated response.

Immunology of the feto–maternal interface

The only site of direct contact between fetally derived cells and maternal immune-competent cells is at the feto–maternal interface in the uterus. The intact trophoblast has for decades been considered to constitute a barrier for the transport of maternal and fetal cells (Medawar, 1953). The cellular elements of this interface consist, on the fetal side, of various subsets of trophoblast cells and, on the maternal side, of immune-competent cells in peripheral blood and decidual tissue. It is expected that the majority of potential immune reactions of importance for fetal survival will take place between these cells. Consequently, the antigens and other immunological markers expressed by them will be of considerable interest.

Table II. Hypothetical models for the pathogenesis of recurrent miscarriage (RM)

	Model A	Model B	Model C
For the theory	Miscarriage caused by graft rejection-like alloimmunity	Miscarriage caused by the innate immune system	Miscarriage caused by organ-specific autoimmunity
		Abundant presence of $CD56^+$ cells in the decidua	Increased prevalence of autoantibodies in RM patients Predominant T_h1-type immunological response to trophoblast in women with RM
		In-vitro evidence that lymphokine-stimulated $CD56^+$ cells can kill trophoblast	Association between RM and particular maternal class II HLA alleles
		Increased NK cell number and activity in RM patients indicate a poor prognosis	
Against the theory	The HLA antigens responsible for strong graft-rejections are not expressed on trophoblast		
	The degree of HLA antigen sharing between spouses has no impact on the probability of fetal survival		
	No correlation between in-vitro reactions of maternal T cells or antibodies against paternal HLA and pregnancy outcome		

HLA = human leukocyte antigen; NK = natural killer; T_h = T helper.

Trophoblast-related antigens

Trophoblast cells exhibit some unique immunological features because almost all classic HLA class I and II antigens expressed on the majority of other human cell lineages are not expressed on any subset of trophoblast cells (Sunderland *et al.*, 1981). The only HLA expressed in the compartments of the trophoblast seem to be HLA-G, -E and -C. HLA-E displays very limited polymorphism (two functional alleles) but is expressed on most human cell lineages (Ulbrecht *et al.*, 1992), including amnion epithelial cells (Houlihan *et al.*, 1995). Most attention has been attracted by HLA-G, because its expression seems to be almost restricted to extravillous trophoblast (Ellis *et al.*, 1990; Kovats *et al.*, 1990). In Caucasians, HLA-G exhibits limited polymorphism (Morales *et al.*, 1993), whereas it seems to exhibit extensive polymorphism in African-Americans (van der Ven and Ober, 1994). A recent finding is that the classic HLA-C is also expressed on trophoblast cells (King *et al.*, 1996).

Other trophoblast-expressed antigens may have a function in preventing complement-mediated immunological destruction of the trophoblast [membrane co-factor protein = CD46; decay-accelerating factor = CD55; Vince and Johnson (1995)]. However, published studies have been contradictory with regard to the role of these proteins in RM (Cunningham and Tichenor, 1995; Hill *et al.*, 1995a).

Decidual factors

The maternal part of the feto–maternal interface exhibits some unique characteristics. The most prominent leukocyte type in secretory phase endometrium and in the decidua of the first trimester is the large granulated lymphocyte (LGL). Using monoclonal antibodies, these cells stain very intensely for the NK cell-specific marker CD56, whereas they seem to be negative for other more common NK cell markers (CD16) and T cell markers. CD56-positive decidual LGL comprise >70% of decidual leukocytes compared with only 1–2% of peripheral blood lymphocytes (King and Loke, 1990a). Unstimulated decidual LGL exhibit in-vitro cytotoxic activity against classic NK target cells, but not against trophoblast cells (King *et al.*, 1989). When co-cultured with IL-2, the decidual LGL can be transformed into lymphokine-activated killer cells that can kill trophoblast cells (King and Loke, 1990b). This is the only cell type known to be cytotoxic to trophoblast cells.

The immune system and RM

Possible models of immunologically mediated RM

Having the immunological architecture of the feto–maternal interface in mind, the immunological reactions against fetal antigens that might theoretically result in RM can be simplified into three main models (Table II): model A, graft rejection-like alloimmune reactions against paternal HLA on the trophoblast; model B, reactions of the innate immune system against antigens on the trophoblast; and model C, autoimmunity-like reactions against trophoblast antigens. The relevance for RM of these hypothetical models will be evaluated below in the context of results from studies of peripheral blood immune reactions and immunogenetic and epidemiological studies carried out among patients with RM and their relatives.

Immunological findings in peripheral blood among RM patients

Alloimmune reactions against paternal HLA (model A)

In transplantation immunology, a low mixed lymphocyte reaction (MLR) between the T lymphocytes of an organ recipient and a potential organ donor suggests a high degree of HLA compatibility between recipient and donor and assures a low graft rejection rate. However, MLR can also decline as a consequence of prior immunization against HLA (Unander *et al.*, 1985). Lauritsen *et al.* (1976) and Beer *et al.* (1981) reported that women with unexplained RM exhibited lower one-way MLR responses against paternal lymphocytes than women with normal pregnancies or explained miscarriages. Beer *et al.* (1981) proposed that RM was caused by a defect in the elicitation of a maternal cellular immune response to paternal HLA.

However, Sargent *et al.* (1988) discovered no association between the level of maternal–paternal one-way MLR and pregnancy outcome in normal and RM women. Neither could they find evidence of either a rapid (secondary) response in one-way MLR timecourse studies or a cytotoxic T lymphocyte response against paternal antigens during normal pregnancies and pregnancies in women with RM. This emphasizes that maternal T cell-mediated sensitization to paternal antigens is not a regular event during normal pregnancy nor is it associated with RM.

On the other hand, a maternal antibody response against paternal HLA is a frequent event. Intact fetal blood cells may enter the maternal circulation in the case of feto–maternal haemorrhage, and soluble HLA molecules might diffuse into the maternal blood. It was proposed previously that because cytotoxic antibodies directed against paternal HLA are often found in multiparae and rarely in women with RM, they might be prerequisites for normal pregnancy outcome and their absence could be a marker for an inappropriate maternal immune recognition of the fetus (Taylor and Faulk, 1981; Mowbray *et al.*, 1985). However, numerous studies have found that the development of anti-paternal cytotoxic antibodies in normal women or RM patients was not associated with the outcome of the next pregnancy (Sargent *et al.*, 1988; Smith and Cowchock, 1988; Regan *et al.*, 1991; Coulam, 1992; Kilpatrick and Liston, 1993; Christiansen *et al.*, 1994a).

A plethora of so-called blocking antibodies has been claimed to be of relevance to pregnancy outcome in patients with RM (Takakuwa *et al.*, 1986; Unander and Lindholm, 1986; Beer *et al.*, 1985). Maternal sera with a positive blocking effect, when added to cellular in-vitro assays, were defined as exhibiting some degree (e.g. 20%) of inhibition of the reactions. The assays used for detection comprise one-way MLR (Unander and Lindholm, 1986; Takakuwa *et al.*, 1986), the rosette inhibition test (Power *et al.*, 1983) and assays for the production of migration inhibitory factor (Rocklin *et al.*, 1976; Stimson *et al.*, 1979). However, the majority of studies have found that blocking antibodies have no predictive value with respect to pregnancy outcome (Sargent *et al.*, 1988; Coulam, 1992; Cowchock and Smith, 1992). Neppert *et al.* (1989) showed that the detection of blocking antibodies was poorly reproducible between laboratories: all the detected blocking antibodies were directed against HLA specificities and almost all proved to be conventional cytotoxic antibodies when tested in sufficiently sensitive assays.

The prevalent view today is that neither cytotoxic nor blocking antibodies (which might be the same) have any impact on pregnancy outcome in normal women or RM patients. Anti-HLA antibodies in pregnant women are considered to be the consequences of successful pregnancies. Their infrequent presence in women with RM compared with women who reproduce normally is because of the failure of the former group to carry the gestation sufficiently long to produce a response.

HLA compatibility between the mother and the father (model A)

HLA are the primary targets for the strongest alloimmune reactions. Thus HLA-G, -E and -C, which are the only HLA expressed on trophoblast, are of particular interest for reproductive immunologists. HLA-G was believed, until recently, to be non-polymorphic at the level of the expressed protein (Kovats *et al.*, 1990), meaning that all fetuses would express an HLA-G antigen on their trophoblast cells that would be identical to that of their mothers. Consequently, a graft rejection-like alloimmune reaction mediated by maternal T_c cells would not be possible. The recent finding that HLA-G exhibits polymorphism among African-Americans complicates the situation. However, there is no evidence that African-Americans exhibit higher miscarriage rates than other ethnic groups, which suggests that feto–maternal incompatibility for HLA-G does not render the trophoblast susceptible to maternal alloimmune reactions. Studies on the localization of the polymorphic amino acid residues (van der Ven and Ober, 1994) in the three-dimensional model of the HLA-G molecule suggest that HLA-G during the course of evolution has developed to be a non-functional molecule which has probably not retained the ability to present antigens to T_c cells (Parham, 1995). That the polymorphic HLA-C and -E antigens seem to be expressed on trophoblast will probably not render the trophoblast susceptible to maternal T_c lymphocytes, because experience from transplantation immunology is that these antigens elicit no or only weak graft rejection reactions.

The intact trophoblast might thus, with the current knowledge, still be considered a barrier resistant to potential maternal alloimmune reactions against paternal classic and non-classic HLA. Despite this fact, theories of an inappropriate maternal immune response to paternal HLA or antigens encoded by HLA-linked genes (so-called trophoblast–lymphocyte cross-reacting antigens) as a cause of RM gained some acceptance in the 1980s (McIntyre and Faulk, 1982). The theories were founded on the suggestions that the spouses in RM couples shared more common HLA than fertile couples, thereby having a greater probability of conceiving a fetus which is HLA compatible with the mother. Such a fetus was suggested to be unable to elicit the formation of maternal blocking antibodies and it would subsequently be miscarried (Taylor and Faulk, 1981). The assumed pathophysiological mechanism is the opposite of that known to be responsible for graft rejection, and scientific evidence for it is sparse, as stated previously. A number of studies seemed to confirm the hypothesis of increased HLA sharing (Komlos *et al.*, 1977; Gerencer and Kastelan, 1983; Beer *et al.*, 1985; Coulam *et al.*, 1987; Ho *et al.*, 1990), but the majority were not able to reproduce this (Rocklin *et al.*, 1982; Oksenberg *et al.*, 1984; Cauchi *et al.*, 1988; Smith and Cowchock, 1988; Christiansen *et al.*, 1989a; Eroglu *et al.*, 1992; Laitinen *et al.*, 1993). Furthermore, there is agreement that the degree of HLA sharing in RM couples has no impact on the prognosis for the next pregnancy [Smith and Cowchock, 1988; Christiansen *et al.*, 1994a; Recurrent Miscarriage Immunotherapy Trialists Group (RMITG), 1994]. Almost all the HLA-sharing studies have carried out HLA typing by conventional serological and obsolete techniques. These techniques are characterized by an inability to distinguish alleles with different peptide-presenting capabilities, a poor ability to resolve broad antigens into important subtypes and a detection rate for some HLA-DR antigens <70% of the detection rate by DNA-based techniques (Doherty and Donaldson, 1991), resulting in the finding of a high number of false blank alleles in this locus. Interestingly, the only three published case-control studies of HLA sharing using DNA technology (Christiansen *et al.*, 1989a; Takakuwa *et al.*, 1992; Laitinen *et al.*, 1993) could not find any trend of increased HLA-DR and/or -DQ sharing in RM couples.

In conclusion, there are overwhelming arguments against the hypothesis of miscarriage being a consequence of graft rejection-like reactions (model A in Table II).

Investigations of the innate immune system in RM women (model B)

The accumulation of $CD56^+$ cells in the uterus during pregestation and early gestation suggests a role for these cells in implantation and early trophoblastic development. A certain level of NK cell-mediated cytotoxicity against trophoblast might be the factor restraining the trophoblast from a too deep invasion of the myometrium (Loke and King, 1996). The finding that IL-2-stimulated $CD56^+$ cells seem to be the only cell type capable of killing trophoblast cells *in vitro* (King and Loke, 1990b) renders this cell type even more interesting in the context of RM. In theory, miscarriage could occur if a pregnant woman has an excess of IL-2-stimulated $CD56^+$ cells in the decidua and/or if the trophoblast expresses a too low level of non-classic HLA molecules (in particular HLA-C and -G are interesting in this respect), which may protect it against NK cell attack (Loke and King, 1996). The quantitative expression of HLA-G on trophoblast is under genetic control and might vary between individuals (Schmidt *et al.*, 1993). At present there is no way of measuring the decidual NK cell activity in pregnant RM women, and measurements in tissue from miscarriages are unreliable because changes may be consequences of fetal death and necrosis. However, some studies of peripheral blood during pregnancy in patients with RM lend support to the hypothesis that there is an association between NK cell numbers and activity and the risk of miscarriage. A significantly increased risk of miscarriage has been reported in RM patients with a high pre-conceptional NK cell activity in peripheral blood (Aoki *et al.*, 1995a), a high frequency of $CD56^+$ cells in the blood (Coulam *et al.*, 1995a) and a high number of $CD16^+$ NK cells in the blood (Christiansen *et al.*, 1994a). RM women may also have a higher number of $CD56^+$ cells in the blood than fertile controls (Kwak *et al.*, 1995).

The theoretical and clinical evidence that NK cell activity plays a role in RM is summarized in Table II (model B). The extent of the role of the innate immune system in the aetiology of RM awaits further and larger studies.

Autoimmunity and RM (model C)

Autoimmunity is the immunological reaction against an individual's own tissue. When autoreactive T lymphocytes for several reasons escape clonal deletion in the thymus during the fetal or neonatal period, or when a previously sequestered antigen (towards which tolerance was never acquired) becomes accessible for T lymphocytes (Møller *et al.*, 1990), autoreactivity may develop. Autoreactivity can be mediated by autoantibodies and autoreactive T lymphocytes. When it results in tissue damage, non-organ-specific or organ-specific autoimmune disease (AID) may result. The classic example of a non-organ-specific AID is systemic lupus erythematosus (SLE), in which multiple organs are affected. Examples of organ-specific AID are Hashimoto's thyroiditis, Addison's disease, insulin-dependent diabetes

mellitus (IDDM), psoriasis and multiple sclerosis. In organ-specific AID it is often possible to find organ-specific autoantibodies in the blood (e.g. islet cell cytoplasmic antibodies in IDDM), but the prevalent view today is that these are in most circumstances without pathogenic importance (Møller *et al.*, 1990). Conversely, there is considerable evidence that the majority of organ-specific AID is caused by autoreactive T lymphocytes infiltrating the target organ (Bach, 1995). During recent years data have accumulated suggesting that an imbalance between T_h type 1 (T_h1) cells and T_h type 2 (T_h2) cells could predispose an individual to organ-specific AID (Bach, 1995). T_h1 cells secrete predominantly IL-2, interferon-γ and tumour necrosis factor-β, whereas T_h2 cells predominantly secrete IL-4, IL-6, IL-10 and IL-13. If an immunological response to an autoantigen is predominantly mediated by T_h1 cells, an AID corresponding to the autoantigen will develop, whereas a predominantly T_h2 response will hinder the autoimmune reaction. It is characteristic for organ-specific AID that they are, with very few exceptions, always associated with one or more HLA-DR or -DQ alleles or genomic sequences. These are thought to play an important role in the selection of T-cell repertoires in the thymus. It has been suggested that, through their role in thymic selection processes, HLA-DR and -DQ alleles determine whether a later immunological response to a given antigen will be predominantly T_h1 or T_h2 mediated (Zanelli *et al.*, 1995).

Is there evidence that RM may be an AID? A prevalent opinion today is that a fraction of RM cases — namely those associated with the presence of antiphospholipid antibodies (APL) — is caused by autoantibodies. During the last decade, numerous case-control studies have reported an increased prevalence of APL in women with RM compared with fertile controls. The most frequently investigated APL are anticardiolipin antibody (ACL) and the lupus anticoagulant (LAC). The former antibody is detected by immunological tests such as the enzyme-linked immunosorbent assay (ELISA; Loizou *et al.*, 1985). The latter is detected by coagulation assays because LAC prolongs phospholipid-dependent coagulation tests, e.g. the activated partial prothrombin time (APTT). Table III shows an overview of the results of studies of the prevalence of ACL, LAC, anti-double-stranded DNA (a-ds-DNA) and antinuclear antibody (ANA) in women with RM and controls. It is evident that ACL, LAC and ANA are found with higher prevalence in RM patients than in controls. A series of organ-specific autoantibodies (e.g. antithyroid antibodies) has also been reported to be found with increased prevalence in RM patients (Stagnaro-Green *et al.*, 1990; Singh *et al.*, 1995).

Many authors have considered APL as a cause of RM because of the findings presented in Table III. However, a proof for a causative role for APL in RM requires the demonstration of (i) a plausible pathophysiological mechanism and (ii) a significantly higher rate of subsequent fetal losses in patients positive for APL compared with those negative (see Association and cause above).

With regard to the first point, there is little agreement as to the pathophysiological mechanisms suggested to cause RM. APL have often been associated with the risk of thrombosis. Experiments on rat tissue have shown that APL inhibited prostacyclin production, resulting in a high thromboxane:prostacyclin ratio in the placental vascular endothelium, which was suggested to predispose the individual to placental thrombosis and miscarriage (Carreras and Vermylen, 1982; De Castellarnau *et al.*, 1983). However, this hypothesis did not hold true for human endothelium (Dudley *et al.*, 1990). Whereas SLE patients with APL have a generally increased risk of venous and arterial thrombosis (Harris *et al.*, 1986), there is doubt that non-SLE patients with RM and APL without LAC activity contract an increased risk of thrombosis (Love and Santoro, 1990; Pattison *et al.*, 1993). The present author has investigated >30 RM women positive for ACL but negative for LAC; none had a history of venous or arterial thrombosis, whereas four LAC-positive RM patients had all experienced venous thromboses.

New hypotheses of a pathophysiological link between RM and APL have been proposed. There is some in-vitro evidence that antibodies against phosphatidylserine, which is a phospholipid expressed in the syncytiotrophoblast cell membrane, are capable of inhibiting fusion of cytotrophoblast to syncytiotrophoblast (Rote *et al.*, 1992). Conversely, cardiolipin and most other phospholipids are not expressed in the trophoblast cells, viable endothelial cells or non-activated thrombocytes, and thus they are not accessible to their respective antibodies. Being aware of this, it is hard to imagine how ACL and most other APL can play any pathophysiological role in RM.

With regard to the second requirement for accepting that APL play a causal role, a negative impact on future pregnancies among women positive for APL would be expected if causality was a reality. Few studies have investigated the impact of ACL on the prognosis for pregnancy in untreated women. Four studies found no or little difference between the rates of pregnancy loss between ACL-positive and -negative RM patients (Taylor *et al.*, 1990; Out *et al.*, 1992; Quenby and Farquharson, 1993; Melk *et al.*, 1995), whereas two (Christiansen *et al.*, 1992a; Tulppala *et al.*, 1993) found a 17–40% poorer (but not significantly decreased) outcome in ACL-positive RM patients compared with ACL-negative

patients. Two studies of women without a history of RM found higher rates of pregnancy loss in ACL-positive women compared with ACL-negative women (Lockwood *et al.*, 1989; Pattison *et al.*, 1993); however, two other studies could detect no impact of ACL on pregnancy outcome in women from the general population (Haddow *et al.*, 1991; Infante-Rivard *et al.*, 1991). It must be kept in mind that comparisons of pregnancy outcomes in ACL-positive patients from different geographical regions should be made with caution because the interlaboratory variations in the detection of ACL are significant (Peaceman *et al.*, 1992). The overall conclusion that can be drawn from the prospective studies is that the presence of ACL may display a negative impact of limited size on pregnancy outcome; however, live birth is also the most probable outcome in untreated ACL-positive patients with RM.

An evaluation of the true impact of APL on pregnancy outcome in RM patients would require control for the influence of potential confounding factors by a logistic regression analysis. The number of previous fetal losses (Kwak *et al.*, 1992b) and the presence of other autoantibodies (Kwak *et al.*, 1992a; Bagger *et al.*, 1993) are associated with the presence of APL. Control for the influence of these factors might eradicate the impact of APL on pregnancy outcome. Recent studies have focused on the detection of β_2-glycoprotein I-dependent ACL in RM patients. β_2-glycoprotein I is a phospholipid co-factor, and antibodies to the phospholipid–co-factor complex have been proposed to be associated more closely with RM than ACL, which are not directed to the complex (Aoki *et al.*, 1995b). However, published studies have so far not provided any strong support for the concept that β_2-glycoprotein I-dependent ACL are of greater significance for RM than ordinary ACL antibodies (Rai *et al.*, 1995).

Table III. Case-control studies of the prevalence (%) of various autoantibodies in non-systemic lupus erythematosus women with recurrent miscarriage (RM) and controls (C)

Reference	ACL		LAC		a-ds-DNA		ANA	
	RM	C	RM	C	RM	C	RM	C
Petri *et al.* (1987)[a]	11	3	9	0	2	0	16	20
Cowchock *et al.* (1986)[b]	13	0	–	–	–	–	30	14
Edelman *et al.* (1986)[b]	–	–	10	0	1	0	5	2
Barbui *et al.* (1988)[b]	8	0	14	0	–	–	–	–
Unander *et al.* (1987)[a]	23	8	–	–	–	–	–	–
Maier and Parke (1989)[a]	50	8	10	0	–	–	20	0
Christiansen *et al.* (1989b, 1992a)[a]	24	12	4	1	7	3	4	1
Kwak *et al.* (1992a)[a]	15	2	–	–	2	4	19	14
Bahar *et al.* (1993)[a]	12	0	–	–	4	10	8	10
Aoki *et al.* (1995b)[b,c]	8	3	–	–	–	–	–	–
Parazzini *et al.* (1991)[b]	19	3	7	0	–	–	–	–
Taylor *et al.* (1990)[a]	15	0	–	–	–	–	–	–
Xu *et al.* (1990)[b]	–	–	–	–	–	–	40	6
Howard *et al.* (1987)[a]	–	–	48	0	–	–	–	–
Costa *et al.* (1993)[a]	20	0	–	–	–	–	–	–
Tulppala *et al.* (1993)[a]	10	7	2	0	2	0	15	13
Mueller-Eckhardt *et al.* (1994)[a]	35	16	–	–	–	–	–	–
Harger *et al.* (1989)[b]	–	–	–	–	–	–	7	0
Parke *et al.* (1991)[a]	12	2	5	5	–	–	–	–
Out *et al.* (1991)[d]	21	10	–	–	–	–	9	1
Carolis *et al.* (1994)[b]	19	6	–	–	–	–	–	–
Konidaris *et al.* (1994)[a]	23	0	0	0	0	0	9	3

ACL = anticardiolipin antibody; LAC = lupus anticoagulant; a-ds-DNA = anti-double-stranded DNA; ANA = antinuclear antibody; Ig = immunoglobulin.
[a]Patients with at least three pregnancy losses.
[b]Patients with at least two pregnancy losses.
[c]Only immunoglobulin G class.
[d]Patients with at least one late or three early pregnancy losses.

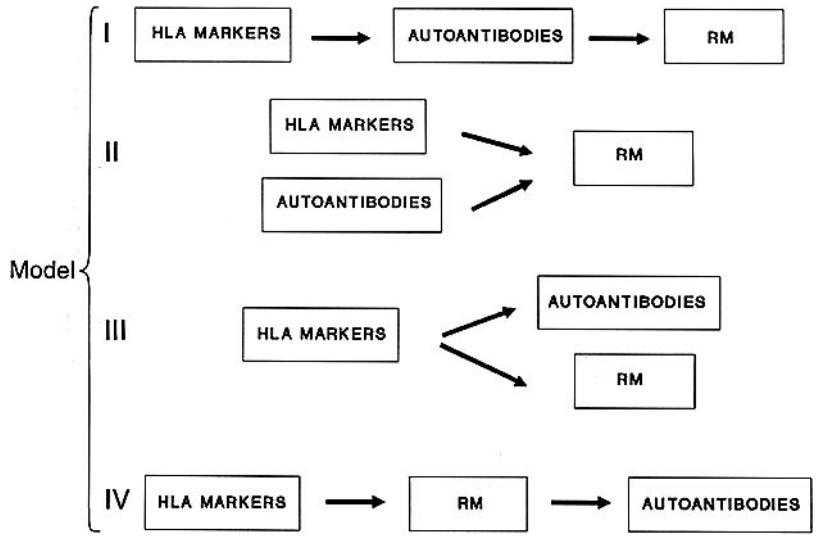

Figure 2. Four possible models that may explain the statistical associations between certain human leukocyte antigen (HLA) class II antigens (DR1 and/or DR3) and a series of autoantibodies (e.g. antiphospholipid antibodies) on one hand and recurrent miscarriage (RM) on the other. See text for further explanations.

The widespread acceptance of APL as causing RM is, in the author's opinion, too hasty and is another example of confusing association with cause. Figure 2 depicts four different models, all of which may explain the statistical association between autoantibodies (e.g. APL) and particular HLA class II markers (see below) on the one hand and RM on the other.

Only in models I and II are autoantibodies suggested to cause RM. In model III, the HLA alleles of women with RM are suggested to be genetic determinants for immunological conditions (other than autoantibody formation) that cause miscarriage. Such conditions might comprise the thymic selection of clones of T_h1 lymphocytes directed against a trophoblast-expressed protein. The same HLA alleles are also suggested to be associated with the concurrent formation of non-pathogenic autoantibodies (e.g. APL), which must be considered to be epiphenomena in this model.

In model IV, the formation of autoantibodies is suggested to be a consequence of the intrauterine retention of necrotic fetal tissue subsequent to RM (tissue necrosis has previously been reported to give rise to the formation of ACL antibodies in other categories of patient; Mattila *et al.*, 1989). Degradation of trophoblastic tissue may expose previously inaccessible autoantigens to the mother's immune system. The IL liberated during the initial T-cell response against one autoantigen is suggested to lead to the up-regulation of several other autoantigens. The result will be a secondary wave of humoral and cellular responses against a series of autoantigens (Lehmann *et al.*, 1993). This might explain the plethora of autoantibodies often seen in RM patients.

From the author's point of view, model III and to some extent model IV (Figure 2) are the most probable if the pathophysiological mechanisms underlying RM are analogous to those underlying the majority of organ-specific AID.

A theory that RM is caused by T lymphocyte clones directed against trophoblast antigens is supported by experimental data. In animal experiments, Wegmann *et al.* (1993) found that T_h1 lymphokines compromised pregnancies whereas T_h2 lymphokines improved fetal survival. Lymphocytes from 66% of women with RM when cultured with trophoblast antigen extracts were reported to secrete factors toxic to mouse embryos, and cells from 51% of the patients secreted large amounts of embryotoxic T_h1 lymphokines (in particular interferon-γ) (Hill *et al.*, 1992, 1995b). Lymphocytes from fertile women and men secreted predominantly non-embryotoxic T_h2 lymphokines in response to the same antigens. The conclusion was that women with RM react with a T_h1 response to trophoblast antigens, whereas normal pregnancy seems to be a T_h2-dominated phenomenon. Embryotoxic factors have also been described in the sera of women with RM and unexplained infertility (Dokras *et al.*, 1993)

HLA class II genes in women with RM (model C)

Almost all AID are associated with particular class II HLA alleles in both family and population studies (Theofilopoulos, 1995). The molecular background for this association has proved to be amino acid sequences at the peptide binding sites of the HLA molecules (Brown *et al.*, 1988; Todd *et al.*, 1988) which determine the HLA molecule's antigen

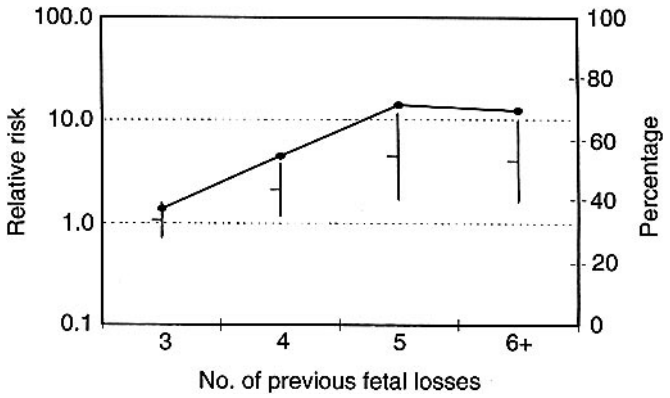

Figure 3. Graph indicating the percentage of women with recurrent miscarriage (RM) positive for the DR1 and/or DR3 antigens according to the number of previous fetal losses. The line relates to the right *y*-axis. $P < 0.002$ for the increased percentage of women with at least one of the two antigens with increased number of fetal losses (χ^2 test for trend, degrees of freedom = 3). Vertical bars relate to the left *y*-axis and indicate the relative risk (and its 95% confidence limits) of women with the same antigens of experiencing three, four, five and six or more fetal losses (logarithmic scale).

binding properties and thus the repertoire of antigens it can present to T_h lymphocytes. The alleles encoding molecules displaying a high affinity for an AID-associated autoantigen will be found with increased prevalence in diseased individuals.

If an autoimmune aetiology is suspected in RM, studies of the prevalence of class II HLA alleles in these patients are highly relevant. However, only a few studies have investigated the distribution of HLA class II alleles in RM women. These studies have generally been small (<100 patients) and the statistical power to detect associations has consequently been limited. Furthermore, serological methods, which are now considered obsolete, have been used for the detection of class II alleles in the majority of studies. Christiansen *et al.* (1994b) carried out a study of 234 Danish women with unexplained RM and 360 controls using DNA techniques for HLA detection. The nomenclature for the HLA alleles assigned by these methods has in this paper been translated into serological antigen nomenclature to enable comparisons with other studies. Each of the HLA-DR1 and -DR3 antigens was significantly overrepresented among 97 RM patients with four or more previous fetal losses: they were found in 29 and 37% of RM patients compared with 16 and 21% of controls respectively ($P < 0.05$ for both antigens after correction for the number of antigens tested = 19). In the total group of RM patients the frequencies of the two antigens were not significantly increased compared with controls. Figure 3 shows that the frequency of patients with at least one of these two antigens increased significantly ($P < 0.002$) with the number of previous fetal losses. These findings are in line with the epidemiological considerations illustrated in Figure 1. Because non-recurrent causes for miscarriage probably explain the majority of RM cases among women with three miscarriages, the indicators of an autoimmune aetiology (e.g. particular HLA class II antigens) are expected to appear with increased prevalence with increased number of miscarriages. A meta-analysis of 14 published case-control studies (comprising a total of 1231 patients) of the distribution of HLA-DR antigens among Caucasian women with RM confirmed that HLA-DR1 and -DR3 (in conjunction with HLA-DR4 and -DR10) were significantly increased in women with RM compared with controls (O.B.Christiansen *et al.*, manuscript submitted).

If the putative RM susceptibility HLA alleles DR1 and DR3 are risk markers for RM, then their presence would be expected to result in a poorer pregnancy prognosis than their absence. This hypothesis was confirmed by results from Christiansen *et al.* (1993). The relative risk (RR) of miscarrying the next pregnancy was 2.2 (95% CI = 1.4–3.5; $P < 0.002$) for RM patients with the DR1 and/or the DR3 antigens compared with patients without. After adjustment for the number of previous pregnancy losses had been undertaken (other variables of prognostic significance were equally distributed), the RR for miscarriage became 1.8 (95% CI = 1.2–2.7, $P < 0.05$).

HLA studies in families of RM patients (model C)

A characteristic feature of AID is an increased prevalence of AID among first-degree family members of patients, with those carrying the disease-associated HLA haplotypes being at a particularly high risk of developing AID. With regard to IDDM, the risk of the disease in the background population is 0.5%, among all siblings of IDDM patients 5–10% and among HLA-identical siblings 17% (Rotter *et al.*, 1992). In analogy, if RM is an AID, it will be expected that (i) siblings are, in general, at a higher than normal risk of RM and

sporadic miscarriage, and (ii) the risk is even higher for siblings who prove HLA identical with the proband. The results in Table I are supportive of the former criterion. It can be calculated (Emery, 1986) that a family risk in this range is compatible with a polygenic mode of inheritance. Christiansen *et al.* (1995a) found that the rates of confirmed fetal losses among both sisters and brothers' wives were increased compared with the background population: 21 and 20% respectively. With respect to the latter criterion, Christiansen *et al.* (1990b) reported data which indicated that the susceptibility to miscarriage among siblings of RM patients segregates with HLA haplotypes. Sisters who shared both parental HLA haplotypes with the probands had themselves experienced a miscarriage rate of 44% in their pregnancies. This rate was significantly higher than the 24% rate in sisters who shared only one parental HLA haplotype and the 4% rate in sisters who shared no HLA haplotypes with the proband. A later study (Table IV) showed that sisters who were HLA-DR1 and/or -DR3-positive had an OR of 4.5 (95% CI = 2.2–8.9) for miscarrying their pregnancies compared with sisters without these HLA-DR alleles. The risk of miscarriage among the brothers' wives had no relationship to the brothers' HLA types or the degree of HLA identity with the proband. However, surprisingly, the risk of miscarriage among the brothers' wives was dependent on their own HLA-DR types. Brothers' wives who carried the HLA alleles DR1 and/or DR3 contracted an OR of 2.9 (95% CI = 1.1–7.9) of miscarriage compared with those who were negative for these alleles.

These results suggest an oligogenic or polygenic mode of inheritance of miscarriage among first-degree relatives of patients with unexplained RM. A possible model describing the pattern of inheritance of miscarriage among siblings of RM patients, and thus probably also in the probands themselves, is depicted in Table V. The model has similarities to models proposed for the pathogenesis of pre-eclampsia (Cooper *et al.*, 1988; Kilpatrick *et al.*, 1989; Arngrimsson *et al.*, 1990). A factor (entitled fetal factor) inheritable through both sisters and brothers seems to be an important determinant of miscarriage (Table V). It is suggested that the fetal factor is common in women with RM, rare in the background population and dominantly expressed. The penetrance of the fetal factor seems to be determined by the presence or absence of HLA-DR1 and/or -DR3 (or closely linked genes) in the genome of the pregnant woman. In the absence of these HLA markers, the risk of miscarriage seems to be similar to that of the background population (Table IV). The nature of the fetal factor is unknown — it might be one or several trophoblast-expressed antigens that function as targets for T_h1 cells or $CD56^+$ cells whose qualitative or quantitative expression is determined genetically. The evidence that HLA haplotypes comprising the HLA-DR1 and -DR3 alleles confer susceptibility to RM is thus extensive: the hypothesis was generated in a large case-control study and subsequently confirmed in a prospective study, a family study and a meta-analysis of all published studies of relevance. Thus the present author finds it justifiable to nominate these alleles (or closely linked DQ genes) as causes of RM. However, because of the suggested polygenic aetiology of RM, the RM-associated HLA alleles might cause RM only in conjunction with other maternal and fetal genes encoding factors of relevance for the immunological interactions at the feto–maternal interface. Candidate genes might be genes coding for Fc-γ-II receptor allotypes (Lorenz *et al.*, 1995) and genes determining mannan binding protein concentrations in serum (Kilpatrick *et al.*, 1995).

Table IV. Distribution of pregnancy outcomes among relatives of recurrent miscarriage (RM) patients according to the human leucocyte antigen (HLA)-DR type of a parent

Relative	+Risk HLA[a]	–Risk HLA	Odds ratio (95% confidence interval)	*P* value
Sisters				
Fetal losses	41 (35%)	13 (11%)	4.5 (2.2–8.9)	$<10^{-5}$
Live births	75	106		
Brothers[b]				
Fetal losses	18 (27%)	14 (17%)	1.8 (0.8–4.0)	NS
Live births	48	68		
Brothers' wives[b]				
Fetal losses	21 (28%)	6 (12%)	2.9 (1.1–7.9)	0.03
Live births	54	45		

NS = not significant.
[a]HLA-DR1 and -DR3.
[b]Numbers of pregnancies in brothers and brothers' wives are not identical because in some couples only the brother or the brother's wife was HLA typed.

Table V. Proposed model for two-locus-determined inheritance of the predisposition to pregnancy loss among relatives of patients with recurrent miscarriage

Type of couple	Fetal factor	Risk HLA marker[a]	Fetal loss risk
Sister	+	+	High
Brother-in-law[b]	–	+/–	
Sister	+	–	Low
Brother-in-law[b]	–	+/–	
Brother	+	+/–	
Sister-in-law	–	+	High
Brother	+	+/–	
Sister-in-law	–	–	Low

HLA = human leukocyte antigen.
[a]HLA-DR1 and/or -DR3.
[b]No HLA typing carried out in these individuals.

Conclusion on immunological causes of RM

In conclusion, the in-vitro experiments concerning T_h1 and T_h2 immunity, the immunogenetic findings and the finding of an increased prevalence of autoantibodies point to mechanisms resembling organ-specific autoimmunity as causing a significant number of RM cases, especially among those who have experienced four or more miscarriages. The evidence for an autoimmunity-like aetiology is summarized in Table II (model C). It is possible that both an HLA class II-determined T_h1/T_h2 imbalance in the response against trophoblast antigens (model C) and NK cell-mediated immunity against trophoblast (model B) may play a role in the pathophysiology of RM, and that these two mechanisms may interact (Figure 4). It is tempting to assume that miscarriage in these patients is initiated by a maternal T_h1-dominated response to trophoblast antigens, which includes the secretion of IL-2, interferon-γ, etc. (Figure 4). This may secondarily trigger the formation of IL-2-activated CD56+ decidual LGL which, in conjunction with the direct harmful effect of T_h1 cytokines, destroy or inhibit the growth of trophoblast cells. The qualitative or quantitative expression of target antigens for T_h1 cells and NK cells on the trophoblast may also play a role in the immunological interactions believed to take place.

Treatment

A poor prognosis in RM patients was reported by Malpas (1938), who found a 73% risk of subsequent miscarriage in untreated women with three previous miscarriages. This report influenced the thinking of gynaecologists for half a century. Any intervention in RM patients followed by a livebirth rate of 50–70% (which might reflect the spontaneous outcome) was considered efficacious and was

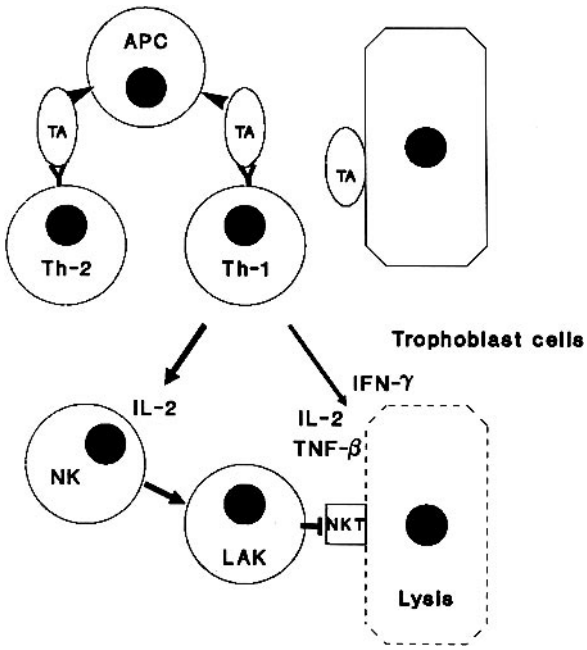

Figure 4. Scenario at the feto–maternal interface illustrating the possible pathophysiological events resulting in immunologically mediated recurrent miscarriage. The patient's human leukocyte antigen class II alleles constitute the genetic background for the predominant type 1 T helper lymphocyte (T_h1) response against the trophoblast-expressed target antigen for T_h1 lymphocytes (TA) resulting in lysis of the trophoblast cells (see text). APC = antigen-presenting cell; NK = natural killer; NKT = trophoblast-expressed target antigen for LAK cells; LAK = lymphokine (interleukin-2)-activated NK cells; TNF-β = tumour necrosis factor-β; IFN-γ = interferon-γ.

promptly applied as general treatment without being tested in prospective placebo-controlled trials. This prevented a search for interventions with proven treatment effects that might help the minority of patients with a poor spontaneous prognosis and favoured the widespread use of interventions (such as diethylstilbestrol) which later proved to be harmful.

A considerable number of studies (Workshop Reports, 1991; Cauchi *et al.*, 1995) have now indicated that the spontaneous prognosis of the average RM patient is much better than stated by Malpas. This is partly because of the statistical phenomenon 'regression to the mean' (James, 1973). A consequence of this phenomenon is that the outcome of future pregnancies of RM patients whatever the treatment will always be much better than the outcome in their previous pregnancies (Christiansen, 1996). There also seem to be indications that psychotherapy in the form of 'tender love and care' (Stray-Pedersen and Stray-Pedersen, 1984) may improve the prognosis. This insight clearly renders uncontrolled trials of the therapy of RM patients of no value.

The methodological evaluation of treatment trials of RM

There are many methodological pitfalls that should be acknowledged by readers and researchers before evaluating the outcome of controlled trials of interventions in RM because a number of non-treatment-related factors often display a greater impact on pregnancy outcome than the actual intervention.

The definition of pregnancy and the time of ascertainment of the pregnant patients

A significant proportion of miscarriages among patients with a history of first trimester RM happen before the occurrence of ultrasonic signs of fetal heart action in week 6 of gestation (Stern and Coulam, 1992). Consequently, trials that include pregnant RM patients before this time will end up with a significantly lower spontaneous success rate in both allocation groups than trials including patients in weeks 6–8 of gestation after the demonstration of fetal heart action.

If RM patients are included in weeks 4–5 of gestation, the cut-off value for HCG used as a marker for pregnancy will be crucial for the rate of subsequent miscarriages (Ellish *et al.*, 1996).

The definition of pregnancy success

In several papers of clinical trials, the primary outcome measure (the pregnancy success rate) has not been clearly defined. Authors have calculated the success rate from the number of ongoing pregnancies (without further specification), the number of pregnancies that have proceeded until week 20 of pregnancy or the number of liveborn infants. In the present author's opinion, the best outcome measure is the 'take-home baby rate' per pregnancy, defined by a positive pregnancy test 5 weeks after the last menstrual period.

Background variables of prognostic importance

A number of clinical and laboratory parameters are acknowledged as having a negative impact on the pregnancy prognosis in RM patients. It has been estimated that the chance of having a successful pregnancy declines by 15% for each fetal loss after the third (RMITG, 1994; Cauchi *et al.*, 1995). Other variables with a probable negative impact on pregnancy outcome are the presence of second trimester miscarriages (Cowchock and Smith, 1992; Goldenberg *et al.*, 1993), age, a history of subfertility (Cauchi *et al.*, 1995), the presence of autoantibodies in significant titres (Pattison *et al.*, 1993; RMITG, 1994) and the presence of specified HLA-DR antigens (Christiansen *et al.*, 1993). It is important that data on the majority of these background variables be provided in reports of treatments of RM patients because this allows the reader to evaluate whether bias in baseline data between treated patients and controls could explain a difference in pregnancy outcomes. It will also facilitate comparisons between studies and allow the performance of meta-analyses.

Immunological interventions

Interventions aimed at correcting abnormal maternal immunological reactions against pregnancy are discussed below. Non-immunological interventions have been briefly discussed previously.

Aspirin and heparin

The use of anticoagulation therapy in women with RM was founded on reports that APL-positive women contract an increased risk of thrombosis in placental endothelial cells (Carreras and Vermylen, 1982; De Castellarnau *et al.*, 1983). As discussed previously, this hypothesis has been documented only weakly so far. The administration of low-dose aspirin generally inhibits thromboxane production without affecting prostacyclin metabolism, and has a recognized therapeutic effect in patients predisposed to thrombosis. Therefore treatment of RM patients with low-dose aspirin became quite widespread. RM patients positive (and often also negative) for APL antibodies were subjected to this 'treatment' either as monotherapy or in conjunction with heparin and/or prednisone (Gleicher *et al.*, 1993).

No randomized placebo-controlled trials concerning low-dose aspirin treatment of patients with RM have been published. Out *et al.* (1992) found, in a prospective non-randomized trial of women with APL (and half of them also with previous adverse pregnancy outcomes), a 78% pregnancy success rate in patients treated with low-dose aspirin (and some of them also with heparin) and a 79% success rate in patients who received no treatment. Christiansen (1995) reported results from six prospective studies of women with RM and ACL who were treated with neither anticoagulation therapy nor prednisone. The summarized success rate in untreated patients was 66% (95% CI = 55–75%), which was not different from the summarized 59% success rate in two studies of low-dose aspirin-treated ACL-positive patients.

Heparin has been used in the treatment of RM patients either as monotherapy (Rossove *et al.*, 1990) or in conjunction with low-dose aspirin (Christiansen *et al.*, 1988b) or aspirin and prednisone (Kwak *et al.*, 1992b; Out *et al.*, 1992). There are no published placebo-controlled studies of the efficacy of heparin in the treatment of RM patients. P.V.Bagger *et al.* (personal communication) carried out a controlled trial of heparin/aspirin treatment versus no treatment in ACL-positive women with previous adverse pregnancy outcomes or RM. They found no difference in success rates in the two groups. Christiansen *et al.* (1992a) found a 50% success rate in 18 RM patients treated with heparin and/or low-dose aspirin, selected for this treatment primarily because of the presence of ANA, a-ds-DNA or second trimester intrauterine deaths. This success rate was similar to the 56% success rate in RM patients with a comparable prevalence of ACL antibodies who received placebo in a trial of active immunization.

There is an urgent need for trials comparing the efficacy of heparin and possibly low-dose aspirin with placebo.

Glucocorticoid treatment

Prednisone was the first therapeutic approach studied in women with RM and APL (Lubbe *et al.*, 1984). Prednisone treatment using large doses (40 mg/day) usually depresses the increased LAC and APTT to normal values, and it was initially suggested that this suppression was mandatory for successful pregnancy outcome. However, Branch *et al.* (1985) and Silver *et al.* (1993) found that suppression of LAC or APL through prednisone therapy was not associated with pregnancy outcome.

No prospective, placebo-controlled trial has been carried out comparing prednisone therapy with placebo. In almost all trials comparing prednisone therapy with other treatment modalities, prednisone-treated women had also received low-dose aspirin and/or heparin.

Lockshin *et al.* (1989) found that prednisone treatment may diminish the pregnancy success rate among SLE patients with high titre ACL and previous pregnancy losses. Out *et al.* (1992) found a 36% pregnancy loss rate in 11 APL-positive patients treated with prednisone (and the majority of them also with anticoagulation therapy) compared with a 22% pregnancy loss rate in APL-positive patients treated with anticoagulation therapy only. Cowchock *et al.* (1992) compared treatments with heparin and low-dose aspirin with prednisone and low-dose aspirin in 20 APL-positive RM women. The pregnancy loss rate was 25% in both groups.

Out *et al.* (1992), Cowchock *et al.* (1992b), Kwak *et al.* (1992b) and Silver *et al.* (1993) agreed that prednisone-treated patients have a significantly increased risk of preterm delivery; bearing infants with a low birthweight, maternal morbidity is high and there is no evidence that treatment improves fetal survival. Therefore most authors agree that it should be abandoned as a therapeutic option.

Active immunization with allogenic blood cells

Taylor and Faulk (1981) were the first to report the use of active immunization of women with RM with allogenic blood cells. The rationale for this treatment was to create antipaternal cytotoxic or blocking antibodies thought to prevent miscarriage (see above). The belief that paternal lymphocyte infusions would be beneficial in the prevention of fetal death was supported by findings from studies in mice (Chaouat *et al.*, 1983) and from transplantation immunology, where it was found that pre-transplant blood transfusions improved kidney graft survival (Opelz *et al.*, 1973).

Many subsequent studies adopted eligibility criteria inspired by the initial studies by Taylor and Faulk (1981) and Beer *et al.* (1981), and exclusively included women sharing several HLA with the spouse (McIntyre *et al.*, 1986; Smith and Cowchock, 1988); women negative for complement-dependent cytotoxic antibodies (Beer *et al.*, 1985; Mowbray *et al.*, 1985; Smith and Cowchock, 1988; Cauchi *et al.*, 1991; Carp *et al.*, 1992; Gatenby *et al.*, 1993; Christiansen *et al.*, 1994a); and women negative for blocking antibodies (Takakuwa *et al.*, 1986; Unander and Lindholm, 1986; Smith and Cowchock, 1988). In the majority of studies, immunization was offered exclusively to patients with first trimester RM and, in some of them, only to women with primary RM and/or patients negative for autoantibodies. Immunization was usually performed using the husbands' lymphocytes separated and administered intradermally, s.c. and i.v., as reported by Mowbray *et al.* (1985). A few studies used buffycoat or leukocyte-rich erythrocyte concentrates (Taylor and Faulk, 1981; Unander and Lindholm, 1986; Ho *et al.*, 1991b; Christiansen *et al.*,

1994a). The rates of successful pregnancies in immunized women were reported to range from 50 (Smith and Cowchock, 1988) to 88% (Unander and Lindholm, 1986).

Until recently, the question as to whether immunotherapy is efficient in the prevention of RM was unsolved and controversial because only few and contradictory placebo-controlled trials had been published. Only one of these showed that treatment with paternal lymphocytes was significantly better than injections with autologous lymphocytes (Mowbray *et al.*, 1985). A meta-analysis of all published and ongoing placebo-controlled trials of allogenic lymphocyte immunization was carried out by the American Society for Reproductive Immunology (RMITG, 1994). A total of 430 cases from eight trials was found to be eligible for analysis.

The success rate in patients receiving allogenic cells compared with those who received placebo (percentage livebirth ratio) was calculated after adjustment for age and the number of previous miscarriages. The combined percentage livebirth ratio was increased significantly in the total patient group (1.16, 95% CI = 1.01–1.36, $P < 0.02$), but this increase was exclusively because of a significantly increased success rate in patients with primary RM, whereas there was no therapeutic effect in women with secondary RM. The therapeutic gain of immunization treatment in the total patient groups was 8%.

Daya *et al.* (1994) performed a meta-analysis of data concerning 285 patients from the above-mentioned study with no previous pregnancy beyond the 20th gestational week, no HLA antibodies against the partner and no ANA and ACL. It showed that immunotherapy in this subgroup significantly improved the probability of a live birth, and the therapeutic gain was 16.3% (95% CI = +4.8 to +27.8%). The relative effect of the treatment became greater with the number of previous miscarriages.

The results are in agreement with those from the present author's group. In a blinded placebo-controlled trial (Christiansen *et al.*, 1994a), in which the patients were randomized to receive either active immunization using i.v. infusions of leukocyte-rich blood concentrates from third-party donors or autologous blood, the therapeutic gain was 23% in all 62 patients enrolled (not significant). Among patients with primary RM, the therapeutic gain was 38% (95% CI = +7 to +68%; $P < 0.02$), whereas there was no therapeutic effect of immunization in patients with secondary RM. In conclusion, allogenic lymphocyte immunization seems to be efficacious for a subset of women with primary RM without autoantibodies and cytotoxic antibodies, but the effect is quite limited.

The present author suggests that suitable RM patients can be offered allogenic leukocyte immunization after they have been adequately informed of the fair prognosis of achieving a successful pregnancy without any treatment at all and after being informed of the established and potential risks associated with the treatment (Blumberg and Heal, 1989). No immunization should be given to patients with significant titres of autoantibodies (Christiansen *et al.*, 1988a) or to patients who do not carry the HPA-1a thrombocyte allotype (risk of formation of thrombocyte antibodies). The lymphocyte donor should, of course, display negative tests for human immunodeficiency virus, hepatitis B and hepatitis C.

The immunological events causing the therapeutic effect of allogenic lymphocyte immunization must, at the moment, be considered unknown because previous theories (Taylor and Faulk, 1981) are not supported by the bulk of studies. Changes in the level of IL receptors (Kilpatrick, 1992), the suppression of NK cell activity (Higuchi *et al.*, 1995) or changes in the numbers of $CD8^+$ T cells (Takakuwa *et al.*, 1991) are theoretical possibilities.

Intravenous immunoglobulin

The recognition that allogenic leukocyte immunization seems to display a limited therapeutic effect emphasizes the demand for more efficient and safer treatments. Pooled i.v. immunoglobulin (Ig) has a proven effect in several well-established immunological disorders (Dwyer, 1992; Ronda *et al.*, 1993). If we accept that anti-trophoblast immunity plays a part in the RM syndrome (Hill *et al.*, 1995b), it is an obvious conclusion that i.v. Ig might also prove beneficial in the treatment of RM.

Mueller-Eckhardt *et al.* (1989) and Christiansen *et al.* (1992b) were the first to report larger series of RM patients treated with i.v. Ig. Both groups found an 82% success rate in treated patients. In the study by Christiansen *et al.* (1992b), the patients included had a median of 6.0 previous miscarriages and the majority had experienced several pregnancy losses in the second trimester (both these baseline parameters are prognostically negative).

So far three placebo-controlled trials of i.v. Ig treatment of RM have been published (German RSA/IVIG Group, 1994; Christiansen *et al.*, 1995b; Coulam *et al.*, 1995b) (Table VI). In the placebo-controlled trial by Christiansen *et al.* (1995b), only patients with secondary RM or RM including at least one fetal loss beyond the 14th gestational week were eligible. These criteria were mainly adopted because these patient subsets were identical to those who displayed no benefit from allogenic leukocyte immunization (Christiansen *et al.*, 1994a; RMITG, 1994). The amount of i.v. Ig given to each patient during a successful pregnancy ranged from 380 to 550 g provided in 17 infusions. This dose was deduced from the experience of the

pilot study (Christiansen *et al.*, 1992b) and was dependent on the patients' pre-pregnancy weight. The dose was considerably higher than that provided in the other controlled trials. Infusions were started in week 5 of pregnancy prior to ultrasound examinations and continued until week 34.

In the trial by Coulam *et al.* (1995b), i.v. Ig infusions were initiated during the months before conception and continued if conception occurred. In this way, patients with very early miscarriages were also included. The demonstrated significant improvement in the success rate suggests that the pre-conceptional treatment regimen may be effective in preventing some very early miscarriages. The therapeutic gain in the trial of Christiansen *et al.* (1995b; 24%) was comparable with the therapeutic gain of 28% in the trial of Coulam *et al.* (1995b), but the therapeutic gain was only statistically significant in the latter trial because of the larger number of patients.

In the German trial, only patients with miscarriages prior to week 16 were included, and most patients were included only after an ultrasound examination had revealed fetal heart action. This is expected to result in a selection of patients with a good prognosis, which is also reflected in the high success rates in both allocation groups.

The protocols for the three trials were thus very different, making comparisons between the results difficult without adjustments for differences in the baseline parameters of prognostic significance. However, when the findings of the placebo-controlled trials were summarized, a significant result was achieved (Table VI). The present author is currently conducting a trial with more patients and using larger doses of i.v. Ig than in the previous trial. It is hoped that the results from this will give a clear picture of the therapeutic efficacy of i.v. Ig in RM. Furthermore, future meta-analyses of placebo-controlled trials will probably provide insights into the efficacy of the treatment and give some clues as to the optimal doses and infusion intervals.

Table VI. Survey of published placebo-controlled trials of i.v. immunoglobulin (Ig) in the treatment of recurrent miscarriage

Reference	Success rate	
	I.v. Ig	Placebo
German RSA/IVIG Group (1994)	20/27 (74)	21/30 (70)
Coulam *et al.* (1995b)	18/29 (62)[a]	11/32 (34)[a]
Christiansen *et al.* (1995b)	9/17 (53)	5/17 (29)
All trials	47/73 (64)[b]	37/79 (47)[b]

[a]$P < 0.05$, therapeutic gain = 28% (95% confidence interval = +4 to +52%).
[b]$P < 0.05$, therapeutic gain = 17% (95% confidence interval = +2 to +33%).

The immunological changes of relevance for the potential therapeutic effect of i.v. Ig in the treatment of RM patients are unclear, but all suggested modes of action of i.v. Ig in autoimmune diseases (Dwyer, 1992; Ronda *et al.*, 1993) may be relevant: blockade of Fc receptors, blockade of complement C3 fragment binding to target cells, action of anti-idiotypic antibodies, increase in the numbers of suppressor cells, etc. (Ronda *et al.*, 1993), and blockage of T_h lymphocyte receptors (Hill *et al.*, 1995b). The trial by Christiansen *et al.* (1995b) suggests that suppression of the most commonly investigated autoantibodies does not take place within a short period after i.v. Ig therapy.

Conclusions and future perspectives of immunotherapy of RM

The lack of placebo-controlled trials of adequate size and quality leave us in a situation where there is no definitive proof for the efficacy of any intervention to prevent RM. This applies to both immunologically and non-immunologically based interventions.

With the current knowledge of the immunological architecture of the feto–maternal interface, research into new and specific therapies of immunologically mediated RM is needed. If the theories that RM is frequently caused by NK cell-mediated cytotoxicity and/or a T_h1 cell-mediated IL response against trophoblast are found to be correct, specific therapies with monoclonal antibodies directed against NK cell target antigens or receptors for IL-2 and interferon-γ on the trophoblast might prove beneficial. Furthermore, the administration of IL typical for a T_h2 lymphocyte response will be presumed to display a positive effect with regard to pregnancy outcome. However, none of these potential treatments has been evaluated so far in clinical trials.

In couples with RM, investigations of polymorphic genes of relevance for immunologically mediated RM (e.g. genes encoding HLA class II antigens and mannan binding protein) might soon allow us to discriminate between couples with good and poor prognoses as soon as the woman has suffered her first or second miscarriage. In the future, this will hopefully allow us to restrict the use of expensive immunological therapies to women with calculated poor spontaneous prognoses at an early stage of their reproductive lives.

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