Immunopathology of Early Pregnancy

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One of the important discoveries in reproduction is that the reproductive process uses for its own purpose lymphokines and cytokines which were initially defined as being of immunologic nature, first traced within the immune system. This is not very surprising, since most of these molecules are more signaling factors and communication molecules between cells of various lineages than molecules strictly restricted to the immune system, such as immunoglobulins (Ig) and T-cell receptors. The field has now evolved from the idea of a host–graft relationship to the concept of an integrated dialogue between the mother and the conceptus, more akin, for some, to a host–tumour relationship. Yet, in fact, the paradigm of acceptance and/or rejection of the foetus by its mother is still heavily embedded in the thinking of many reproductive immunologists.

This is not surprising, since the trend of research that led to modern reproductive immunology was initiated by Sir Peter Medawar's disquisition on "the riddle of the foetal allograft."[1] It is therefore not all disconcerting that, in 1997, one of Medawar's colleagues, Leslie Brent, in his recent book *A History of Transplantation Immunology,* still depicts the materno-fetal relationship as "Nature's (almost) perfect allograft,"[2] nor that pioneering work in the field is due to another collaborator of Leslie Brent and Peter Medawar, Rupert E. Billingham.[3]

However, the paradigms have evolved. As you will see in this brief presentation, there is still room for discussing, and re-envisaging, with the use of murine models and data in humans, the concept and mechanisms of "rejection" of the foetal allograft.

But the question of the tolerance of the conceptus has evolved. First, we have known for a long time that we *can* and *must* dissociate local and systemic events, but this idea has not yet fully penetrated the thinking of the general public.

It is possible to observe rejection of paternal strain tissues after preimmunisation of the mother without compromising pregnancy[4,5] (in fact, the babies born in such situations are in most circumstances heavier), and we have personally verified that.[6]

It ensues that systemic immune responses (which we do not want at all to negate, having taken a rather indisputable part in their discovery[7]) are, at best, a testimony of maternal recognition of the foeto-placental unit.

Indeed, pregnancy is perfectly possible in the absence of humoral (antibody) response.[8,9] Indeed, if tolerance to the foetus was alloantibody mediated, it would be an exception, because tolerance is MHC restricted, and the Nobel prize awarded to Zinkernagel and Doherty recognizes the consequences of that phenomenon. With rare exceptions, antibodies are *not* MHC restricted, and attempts to say that tolerance was antibody mediated should be in immunology history. This is not completely true, since there is not yet a satisfactory explanation of the enhancement phenomenon, possibly because of the controversy over IgG1/IgG2 in mice, which uselessly shifted the debate–enhancing antibodies in rats, *are* part cytotoxic.

Similarly, we do not negate, of course, the existence of systemic suppresser cells,[7] but pregnancy is perfectly viable in animals experimentally depleted of these.[10]

The repeated enunciation in France that pregnancy depends on an alloantibody and suppresser

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T-cell-mediated immune response is simply outdated since around 1979–1981, and claims that unfortunately are expressed in mainstream immunology meetings and in print that "reproductive immunology has to teach the mainstream immunologists how the immune system works" as a "facilitation reaction" with "important role for enhancing antibodies . . . and suppresser cells" have disastrous effects:

- First, mainstream immunologists repeatedly dismiss, in a more and more irritated fashion, such preposterous advances, and, as such, would consider as a backlash the whole field as not serious;
- Second, those claims have led some clinicians to exacerbate the role of maternal antibodies, clouding unconsciously the issue of early pregnancy loss.
- Third, they distract from the main advances that have indeed been going on, leading to *molecular* understanding of the field.

For indeed, there has been progress, such as Tom Wegmann's seminal idea that possibly we should re-envisage our view of the immune system in pregnancy: instead of seeing the foetus as confronted by a threat of rejection by its mother—the "immunotrophic" concept postulated from a discovery made with the murine abortion model (the CBA × DBA/2 system),[11,12] we recognize that immune reaction could be in some cases *beneficial* for fetal survival.[13]

Then, it ensued the demonstration in vitro, then in vivo, that indeed T-cell derived cytokines were growth factors for the placental trophoblasts,[14] followed by in vivo demonstration of T-cell control of placental growth[15,16] and treatment of a murine abortion model (CBA × DBA/2) by purified or recombinant cytokines.[17]

In the very same period, David Clark had made the discovery that this precise model (CBA × DBA/2) was deficient in local, decidual associated suppresser factor,[18] and later on showed that this factor was molecularly related to TGF-b2.[19]

We are now faced with events occurring *mostly locally*, and with the concept of a cytokine network at the foeto-placental interface. The events are in fact *different* whether one looks at parturition (where there is good evidence for involvement of interleukin [IL]-1, IL-6, IL-8, and tumor necrosis factor [TNF][20–23,25]) or the established pregnancy

which is indeed immunologically characterised by transient acceptance of relatively weakly immunogenic tumor grafts of parental strain origin in the first pregnancy, linked to a T-cell anergy state and suppresser T-cell mediated multi-pregnancy induced tolerance to paternal alloantigens.[26–29]

In the latter case, the close examination by polymerase chain reaction (PCR) of cytokine profiles shows that it is more complicated than initially thought, with variations throughout pregnancy of which the physiological significance is yet unclear,[30] and which we will not detail here in the human. Similarly, the profiles are not simple in mice.[131]

And we now come to the topic of this communication, early pregnancy. Nowhere is the understanding of the cytokine network more interesting, and nowhere has it led to more surprising discoveries, opposed to the paradigm of the acceptance of the foetal allograft, and so far away from the initial framework (which *was* indeed *once* useful) of the facilitation reaction.

Early implantation requires "inflammatory" molecules, the consequence, in part, of a quasi-inflammatory reaction locally, followed by an immediate return to non-inflammatory conditions. It is probable, from what we know in animal models, that each of these steps are *absolutely* required for further successful pregnancy. But before that, a word is required about the antigenicity of the embryo in mice and humans.

AN ALLOGRAFT?

It is a tautology, a "Lapalissade" to say that to be alloantigenic, an embryo *must* express foreign tissues antigens. This is not totally true. Failure to express major histocompatibilty antigens has *another* counterpart. It *activates* cells which are what Charlie Loke calls the most primitive immune system,[31] the Natural Killer cells (NKs). That it was so is a relatively recent discovery, which owes a lot to the theory of the "missing self" originally proposed by Klas Karre.[32] Therefore, it ensues that the trophoblasts cannot theoretically be completely neutral, otherwise there would be an NK-mediated rejection reaction: the NKs express two sorts of receptors, which are not yet fully understood, albeit an impressive body of evidence is accumulating (see Immunological Reviews 158). Killer Activating Receptors (KARs), whose engagement, in a

neoclassical fashion, triggers NK lytic pathways (and possibly others, such as cytokine secretion, especially when the Killer Inhibitory Receptors [KIRs] are engaged). The second, and most important, are KIRs. The KIRs are activated when engaged by the reconnaissance of the presence of MHC molecules (most often in a *given minor histocompatibility antigen context*), which explains "easily" some "oddities" which we published for the sake of honesty on the CBA xDBA/2 system and which were then rejected by proponents of the MHC-T-cell duo theory (proponents for whom we, incidentally, have the greatest respect), triggered so as to *inhibit* NK lytic pathway.

An added level of complexity for immunologists and clinicians is that while MHC is (relatively) conserved through evolution as a member of the super Ig family, KIRs and KARs are *not at all* related between mice and humans. *Why?*

Somehow, the trophoblast faces a dilemma: express MHC, which might trigger a classical T-cell-derived rejection reaction, or repress *completely* MHC, which will defuse the KIRs and trigger NK-mediated rejection.

This dilemma is partly solved in the human. The trophoblast expresses, as far as its extravillous component is concerned, a specific antigen, HLA-G, with no variations (or a few amino acids) from one individual to the other, but it is now known that it has some variability.[35] HLA-G has little peptide presenting capacity (*but* it does have it!), and, as a truncated molecule, it is not recognised as "polymorphic." (It has few antigenic disparities between individuals, if any, that can be "seen" by T-cell receptors, whereas it certainly inhibits NK function. Recent experiments by Strominger, Lopez Bottet et al, Ramensee, and Le Bouteiller confirm the earlier ones of Kovatts and Loke.[36,37,38])

The situation would thus seem simple in humans: the extravillous trophoblast is where interaction with decidual lymphocytes takes place and therefore where NK and NK-like cells accumulate and are defused. The lack of classical MHC molecules would render the trophoblast a non-target and a non-inductor of a classical T-cell-mediated response. In addition, there is no bioactive IL-2 in decidua, albeit an IL-2-like material has been traced.[39]

The situation is more complex than that, even

for those who would like to make an exception in the case of primates: in humans, the expression of HLA-G is restricted to extravillous cytotrophoblasts, villous cyto-, and syncytiotrophoblasts that are uniformly MHC-negative, but a mirror situation is observed in baboons, and in the rhesus monkey, HLA-G homologue is a pseudo gene (although its function could be exerted by a yet uncharacterised protein).[40,41] So, this is already a snag.

Two further snags are to be discussed: First, how could the *totally* MHC-negative human syncytiotrophoblast, a fact not dismissed since its description by Faulk,[42,43] not trigger NK-mediated lysis? This is as yet unexplained by proponents of the HLA-G paradigm. Second, we now know that HLA-C is expressed by extravillous trophoblast.[37] It has a restricted polymorphism and interacts with NKs, but the theory must somehow be twisted to make it act as purported for the monomorphic HLA-G, for despite a certain paucity of reports, there are T-cell-mediated responses to HLA-C.

Finally, in *all* species other than the primates, there is expression of classical, *polymorphic* class I molecules, generally *precisely* on the layers of the placenta that confront the maternal immune system (44,45,46). In mice K, D, and L are expressed on the spongiotrophoblast, perfectly accessible to antibodies and cells. The same is true in horses for equine leukocyte antigens, in pigs for swine leukocyte antigens, and, according to Twink Allen, in elephants.

There are three alternative hypotheses for the problem:

- One is that man is man, or, as Peter Johnson wrote (The Immunologist, 1996:4;p172), "differences in placentation between humans, rodents, and other species can make direct comparisons largely meaningless."
- The second one is, as Leslie Brent says when discovering the status of HLA-G in other species, that HLA-G suppresses as would any proper MHC-ligand in the proper cells (that is, by the sort of experiments in which the missing self was discovered to be true) and that HLA-G has likely *another* function, since there is no equivalent in mice, etc, that warrants its evolutionary appearance (a specific peptide or hormone intracellular carrier, for example, yet uncovered).

• The third one is that *there are* in other species *functional* equivalents of HLA-G, which *could* reside in the already discovered monomorphic or poorly polymorphic MHC class I molecules.

None of these theories, incidentally, is mutually exclusive.

However, for class II (I-a in mice, HLA-DR in humans), the situation is more simple. In no species do the trophoblast layers express class II alloantigens, and expression of class II on trophoblasts induced by azacytidine results in regular abortion in mice.[47]

Why? The question is unsettled, since the mechanisms have not been explored, but one should recall here in the same vein that expression of class I in mice is restricted to spongiotrophoblast, with no expression being found on labyrinth. Indeed, derepression of class I expression in the labyrinth is found in interferon γ induced abortion, but one does not know if it is a consequence or a cause of abortion.[48] So, we are in quicksand about the "allograft status of the embryo."

As far as the preimplantation embryo is concerned, it is simpler to say *that there is no class I* expression (nor class II) whatsoever on the gametes, blastocysts, extracellular cell mass or ectoplacental cell cone (EPC) in *any* species, including, when studies could be done, humans.[49] This is somehow troublesome for some aspects of the recent HLA-G theories: the negative EPC cells certainly are facing a very important NK accumulation.[50] And yet, the EPC resists perfectly well NK-mediated lysis. Conversely, aborted embryos in many species at that early stage display MHC expression on EPC cells.

The solution may be simple: at that stage, the cells of the EPC are *intrinsically* resistant to cell-mediated lysis,[51–53] most probably because cell-mediated death involves target cell participation and the pathways of apoptosis. Like other cells secreting high amounts of steroids, trophoblasts are resistant to steroid-induced apoptosis and thus to cell-mediated death. However, they are sensitive to LAKCs mediated cell death,[54] but for that we will see that there is in addition a lot of immuno-suppressive material in the vicinity, and that abortion might *not* be mediated by trophoblast death. Such an immunosuppression takes place after implantation, which on the contrary calls for inflammation, and is a Th1 response.

INFLAMMATION AND IMPLANTATION

In France, the early stages of pregnancy are difficult to study in humans, despite advances in medically assisted reproduction. The Loi Huriet requests full informed consent before taking a biopsy sample, and in the very "sensitive" area of reproductive tract organs, this is quite a deterrent, as opposed to the ease with which blood samples are obtained.

In the implantation period, where some events are very transiently associated with blastocyst adhesion and initial invasion of uterine walls, it is, of course, inconceivable and unlawful to deliberately transfer an in vitro fertilisation (IVF) for the sole purpose of aspiration or, even worse, (total) removal of uterine tissues for immunological investigations. As stated, animal models are not necessarily fully pertinent, and thus Y.W. Loke said at the 15th World Congress on Fertility and Sterility in Montpellier that the only valid model for human pregnancy is "the human species itself."

But, this does not solve fully the problem of the periimplantation period. Macaques are not fully relevant, because of antigenic disparities, like baboons, at the trophoblast level with human situation, and chimpanzees are an endangered and protected species of extremely limited availability. For these reasons, in vitro alternative models have been developed, which may or may not be totally relevant, complemented by studies in animals, mostly in mice.

Animal Studies

The studies performed in animals have revealed a salient and totally unexpected aspect contrasting with the established pregnancy: the preimplantation uterus undergoes an "inflammatory-like" reaction, with transient influx, after mating in mice and rats, there of lymphocytes and macrophages, and seminal fluid is required, since this is not seen in females with fallopian tubal ligation or in vasectomised males.[55–57] As expected, an increased secretion of IL-1 α, IL-1 β, TNF α, IL-6, and mRNA is detected in the uterus, paralleled by the pregnancy-associated continuous rise in production of CSF-1 which starts by then.[58,59] Except for CSF-1, those inflammatory cytokines return to basal levels

by days 3 and 4, but CSF-1 secretion continues to increase throughout pregnancy.[58,59] In rats, local injection of the PAF-acether antagonist BN 50081 completely prevents implantation, whereas the untreated contralateral horn is fully receptive.[55,56]

Three cytokines are, in our opinion, crucial, as established in mutant or "knock-out" animals: CSFs, IL-1, and LIF.

CSFs (CSF-1, S-CSF, GM-CSF)

CSF-1 deficient oSP (osteopetrotic, or op/op) mutant mice have a profound pregnancy defect, and males have also reproductive functions. CSF-1 receptor, c-fms, is expressed after the 2–4 cell stage until the EPC outgrowth and later the spongiotrophoblast and is also found at very high levels in the maternal then the primary decidual zone and finally in the sole decidua basalis.[62] S-CSF and its receptor (c-kit, which maps in the dominant white spotting W locus in the mouse), is also important, since mutations in the W locus t result in sterility in mice, often associated with abnormal embryonic development.[62]

GM-CSF was the first cytokine with IL-3 involved in the immunotrophic theory[13–16] and is found in the preimplantation uterus. Tartakowsky has shown that culture of CBA × DBA/2 embryos (a murine abortion model[11,12]) in GM-CSF- or CSF-1-containing medium partly corrects the deficient implantation rate seen when transferring these embryos in CBA /J mothers.[63] GM-CSF receptor is expressed in the EPC and later spongiotrophoblast cells and in the early implantation uterus.[64,65] Recently, in an elegant series of experiments, Robertson implied the inflammatory response and more peculiarly the GM-CSF. In agreement with studies conducted to prove the immunotrophic theory,[15,16,66] she found that GM-CSF-deficient mice have smaller litter sizes and placental weights than controls and implies GM-CSF and components in the semen ejaculate in hyporesponsiveness to paternal alloantigens.[67]

IL-1

IL-1 might play a mandatory role in pregnancy. According to Simon and Polan,[68] IL-1 receptor antagonist completely prevents successful implantation in mice. However, Colin Stewart et al. (Stewart, personal communication and answers at many meetings) have examined at the Roche Institute

for Molecular Biology IL-1 deficient mice with perfectly normal reproductive function, nor did we obtain implantation failure in mice by in vivo injection of neutralising anti-IL-1 antiserum.

LIF

By gene knock-out technology, Stewart has elegantly shown that maternal production of LIF is mandatory for successful implantation: LIF-deficient mice, obtained by "gene knockout" are fertile, *but sterile*. LIF-/LIF- embryos implant in normal CD1 recipient mice, but LIF +/LIF + embryos do not implant in LIF-/LIF- mice. The defect is corrected by recombinant HILDA LIF via an osmotic pump.[69]

Human Studies
IL-1

IL-1 α, β expression is found in human endometrial epithelium throughout the menstrual cycle, reaching a peak in the late luteal phase.[70,71] Immunohistochemistry shows that IL-1 receptor antagonist in human endometrial cells is at higher levels during the follicular phase than during the early and mid luteal phases.[72] IL-1 is also found in trophoblasts and IL-1 receptor type I on syncytiotrophoblasts, suggesting an autocrine and paracrine role of IL-1 in human implantation.[73] Indeed, it has been reported that IL-1 is a modulator of the decidualisation itself,[74] and it is also controlling hCG production by human trophoblasts in culture.[75] The production and role of IL-1 by early human embryos is controversial; some authors made it a predictor of successful implantation in humans,[76,77] while others did not detect it at all.[34,78] Differences in culture media, conditions, etc. obviously could be invoked. In a co-culture of endometrial epithelial cells and or stromal cells with embryos, IL-1, IL-1 receptor antagonist, and IL-1 receptor type 1 were found in about 56% of cells from embryos cultured with epithelial cells but not stromal cells, apparently correlated with pregnancy success.[79]

LIF

LIF is present in human endometrium, with consensus for its expression in the second part of the cycle, and during pregnancy, this in variance with mice.[80–82,84] Optimal uterine secretion coincides with the implantation window and expression of

TABLE I. Endometrial HILDA/LIF production index in fertile women

HILDA/LIF production index[a]	Cycle day	Age (years)	Obstetrical status	
			Number of successful pregnancies	Last pregnancy (years)
1.05	20	50	2	18
1.66	6	36	2	4
1.94	14	41	2	20
2.21	10	37	4	5 weeks
2.22	20	41	2	1
2.28	7	32	2	9
2.35	22	34	2	10
3.16	10	36	1	12
3.27	18	32	1	1
3.39	10	41	5	14
3.85	19	35	2[b]	6 weeks
4.06	19	36	2	12
4.51	22	32	2	4
4.57	37	30	1	5
6.48	11	38	2	8
9.95	24	41	1[b]	1

[a]Median = 3.22.
[b]Plus one pregnancy loss before successful pregnancy.
From: Delage G, Moreau J-F, Taupin J-L, et al.: In vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. Hum Reprod 10:2483–2488, 1995.

LIF receptor by human embryos.[85,86] We have found women with quantitative defects in LIF production;[87,88] we have observed such a deficiency in women with successful IVF but confirmed sterility.[87,88] We have now confirmed those data, obtained in a culture system, by both immunohistochemistry, using polyclonal antibodies, and evaluation of LIF production by isolated uterine epithelial cells.

Tables 1, 2a, 2b, and 3 show typical LIF production indexes in fertile and infertile women, as defined, in an in vitro assay system, by the index of LIF production by explant cultures in culture supernatants from Day 5 and Day 1. It is interesting to note that in women whose most recent reproductive event was a pregnancy loss, there are a great number with a low LIF production index (Table 3), the indexes being *very* low for women with recurrent implantation failure or recurrent unexplained sterility (Tables 4 and 2b). The existence of a low producer of LIF has been confirmed ex vitro by uterine flushing and ELISA assays.[89]

As stated above, we wanted to have a totally objective measure of LIF production in situ by the human endometrium. We therefore set up the technique of selective separation digestion of glandular and stromal endometrium, after a Cornier of Frydman pipette biopsy sample, and using polyclonal antibodies raised in cooperation with J Mar-

tal (INRA, France), E. Boosmans (Eurogenetics, Belgium), and J F. Moreau and J L. Taupin (CNRS, Bordeaux). Data show that there is always a specific staining in the glandular cells, though contaminating cells of this origin can account for the low number of positive cells in the "stromal" compartment, which is in fact "negatively selected." One observes, as a result of trypsin digestion, that dead cells are already permeated, thus accounting for the positive staining seen in the non-permeated populations. We will give two experiments as examples, values being expressed as mean fluorescence intensity (which cannot be compared from one experiment to another, of course, but in the future we will always refer to the positive clone 8 values). On May 16, 1997, we found a mean value of 52.75 in an infertile woman, vs. 112.26 and 137.43 in two fertile ones, and on April 10, 1997, 108.75 in the fertile woman, vs. greater than 90.39 in infertile woman number 1 and greater than 37.27 in infertile woman number 2. On May 20, 1997, we similarly found 401.11 in the fertile woman, vs. greater than 158.25 in the infertile woman. Though this technique is somewhat limited by the restricted availability of the rabbit antiserum used (not all the rabbit anti sera were useable for FACS analysis, and both an excellent neutralising goat antiserum that we raised as well as commercial monoclonals proved unreliable in that respect), we

TABLE 2A. Endometrial HILDA/LIF production index in infertile women with recurrent implantation failure

HILDA/LIF production index[a]	Cycle day	Age (years)	Number of IVF with embryos	Number of pregnancies	Last pregnancy (years)
0.96	10	34	3	I (EUP)	15
0.97	5	29	20	3 (2 PL, I EUP)	5
0.97	21	34	8	2 (I EPT, I EUP)	4
1.01	6	34	2[b]	I (PL)	9
1.22	15	35	3	0	
1.41	10	39	I[b]	0	
1.49	18	33	2	0	
1.68	9	36	6	0	
1.69	21	36	2	0	
1.70	7	34	4	0	
1.74	14	33	4	I (EUP)	10
2.45	18	38	6	0	

[a]Median = 1.45.
[b]Plus GIFT.
EUP = extrauterine pregnancy; PL = pregnancy loss; EPT = elective pregnancy termination.
From: Delage G, Moreau J-F, Taupin J-L, et al.: In vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. Hum Reprod 10:2483–2488, 1995.

TABLE 2B. Endometrial HILDA/LIF production index in infertile women with unexplained primary sterility

HILDA/LIF production index[a]	Cycle day	Age (years)
1.22	12	41
1.28	6	30
1.39	8	30
1.48	6	33
2.70	13	34

[a]Median = 1.39.
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believe we have an objective assay that can complement immunohistochemistry, analysis on cytofuge smears, and in vitro production in explant culture.

It is important, in this context, to emphasize that recombinant human HILDA LIF is available, and women could benefit from substitutive therapy. While we feel confident enough to go forward with such a procedure, the clinicians would prefer we first correct a defect outside pregnancy before going further on.

This leads to questions about the mechanisms of action of LIF and, indeed, the inflammatory response. It is our working hypothesis that the local inflammation is required for optimal expression on placental trophoblast and uterine decidual cells of adhesion molecules, which are necessary for implantation to occur, such as trophoblast laminin and

fibronectin receptors, and specifically the α 1 β 1, α 5 β 1, α 6 β 1, and α 6 β 4 integrin heterodimers, and expression of their receptor molecules (laminin and fibronectin) in the periimplantation as well as first trimester uterus with specific trophoblast down-regulation of expression of the β 4 integrin and up-regulation of the β 1, α 5, α 1 subunits during the invasion process.[91–93]

We believe that an important part of early pregnancy loss is due to abnormal processes of this early reaction. It is noticeable that dysregulations are already known, such as in preeclampsia, where trophoblasts do not down-regulate β 4, nor is α 1 up-regulated.[94] In addition, the effects of cytokines on the matrix degrading protease are also to be encompassed in that respect.[31]

TNF

There is early and temporary expression of relatively high levels of TNF by invading extravillous trophoblasts[95] which could, as seen in other systems, up-regulate adhesion molecules and selectin.[96] This inflammatory reaction, however, has to be then very quickly down-regulated, since during the early post implantation phase it is abortogenic. There are many other cytokines that we will not discuss here, for sake of space, but we cannot *not* mention here CSF-1 and its receptor, whose programme is very parallel to what is seen in rodents and which has a similar role,[97–101] mainly as placental trophoblast growth factor acts as the other immunotrophic cytokine, IL-3[102] In this commu-

TABLE 3. Endometrial HILDA/LIF production index in women whose last reproductive event was a pregnancy loss

HILDA/LIF production index[a]	Cycle day	Age (years)	Number of successful pregnancies	Last successful pregnancy (years)	Number of pregnancy losses	Last pregnancy loss (years)
0.98	15	38	0		1	12
1.12	15	31	0		1	4
1.20	19	34	2	11	2	3 and 2
1.79	9	35	0		2	2
1.86	6	33	0		1	16
1.89	20	34	0		4	1
1.98	13	36	3	3	1	1
2.33	19	38	2	6	1	4
2.38	19	41	2	13	1	1
2.43	22	32	0		2	3
2.45	12	35	0		>20	0.5
2.79	14	41	1	5	1	3

[a]Median = 1.94.
From: Delage G, Moreau J-F, Taupin J-L, et al.: In vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. Hum Reprod 10:2483–2488, 1995.

TABLE 4A. Endometrial HILDA/LIF production index in infertile women with recurrent implantation failure

HILDA/LIF production index[a]	Cycle day	Age (years)	Number of IVF with embryos	Number of pregnancies	Last pregnancy (years)
0.96	10	34	3	1 (EUP)	15
0.97	5	29	20	3 (2 PL, 1 EUP)	5
0.97	21	34	8	2 (1 EPT, 1 EUP)	4
1.01	6	34	2[b]	1 (PL)	9
1.22	15	35	3	0	
1.41	10	39	1[b]	0	
1.49	18	33	2	0	
1.68	9	36	6	0	
1.69	21	36	2	0	
1.70	7	34	4	0	
1.74	14	33	4	1 (EUP)	10
2.45	18	38	6	0	

[a]Median = 1.45.
[b]Plus GIFT.
EUP = extrauterine pregnancy; PL = pregnancy loss; EPT = elective pregnancy termination.
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nication, however, we would like to mention tau and placental interferons.

Placental Interferons

Corpus luteum maintenance in ovine does not depend on non-chorionic gonadotropin, but on trophoblastin whose trophoblast secretion occurs in the periimplantation, also named ovine trophoblast protein (oTP). It has pleiotropic activities.[103,104] It exerts a wide variety of effects, such as local antiviral properties, preparation of the uterus for optimal implantation by promoting 2 5 A synthetase activity, and local cytostatic properties, making it an ideal candidate for early immune suppression. It is likely to be involved in control of the Th1/Th2 balance.[105–108] It is in fact an interferon of the new

TABLE 4B. Endometrial HILDA/LIF production index in infertile women with unexplained primary sterility

HILDA/LIF production index[a]	Cycle day	Age (years)
1.22	12	41
1.28	6	30
1.39	8	30
1.48	6	33
2.70	13	34

[a]Median = 1.39.
From: Delage G, Moreau J-F, Taupin J-L, et al.: In vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women. Hum Reprod 10:2483–2488, 1995.

tau interferon family. When such trophoblastins exist, they are constitutively secreted at very high doses locally by periimplantation trophoblast only during the periimplantation phase. It is non abortogenic (of course, *it maintains* pregnancy) and non toxic/cytostatic for trophoblast, which secretes it, while ? interferon at high doses is an abortifacient in a variety of animal models. The quest is still ongoing for human equivalents, but not conclusively, despite suggestive evidence[109], so one speculates that its functions could in fact be exerted by omega interferons.

Th1/Th2 BALANCE AND LOCAL IMMUNOSUPPRESSION

As soon as one completes the implantation period, in humans and in mice, an abnormal Th1 reaction to trophoblast causes early pregnancy loss, recurrent spontaneous abortion, and infertility. Several mechanisms are involved. For example, the HELLP syndrome (hemolysis, elevated liver function tests, and low platelet counts) is linked to an elevated IL-12 serum level and possibly an enhanced IL-2 level in the serum[110,111] of unclear origin, since despite recent molecular evidence for the contrary[112], no IL-2 is assumed to reside in decidua and placenta. This is especially important, since in a variety of animal systems, activated NKs (by IL-2 or IL-4, e. g, LAKCS), are abortifacient, and indeed LAKCS can lyse trophoblast cells in vitro,[54] but not normal NKs or CTls. Thus, there has been a quest as indeed "built-in" in the "allograft model" for local immunosuppression for the mechanisms of early pregnancy loss and its control.

The Th1 cytokines, be they IL-2, interferon γ, or TNF, are abortifacient in vivo, the latter at *any* stage of pregnancy, in abortion-prone and non-abortion-prone systems[17,113,114,116,117] Interferon γ and TNF act in synergy. Their control is exerted by a variety of non-cytokinic mediators (prostaglandins, 1-25 OH dihydroxyl cholecalciferol in the decidua, placental low MW suppresser factors), as well as by suppressive cytokines such as TJ6 (the "pregnancy-associated cytokine") which has been cloned initially in mice. Monoclonals against TJ6 are abortifacient at early stages, and deficient expression of TJ6 is reported to be associated with pregnancy loss in human, especially if the defect is on CD 19- and CD 56-positive peripheral and decidual lymphocytes. Such a defect could be predictive of risk of pregnancy loss.[118–121] Another regulator which is, like TJ6, progesterone dependent is the progesterone induced blocking factor (PIBF), secreted by activated T lymphocytes.[122] PIBF alters the Th1/Th2 balance towards Th2 and corrects activators of NKK (poly IC) and natural (CBA × DBA/2) induced abortion.[123–125] Its expression by maternal lymphocytes has been shown to correlate with (perhaps even require) implantation.[126] The decidua-associated transforming growth factor β-2 analogue[19] is secreted by uterine non-T, non-B cells in mice and decidual transforming growth factor β-2 secreting cells in humans.[126] It is a potent immunosuppressive factor, and it has been reported that its production was defective in women with repeated miscarriages.[126] Finally IL-10 is clearly important, since in animal models pregnancy is very obviously a Th2 phenomenon.[127] In humans, it is secreted by placental trophoblasts and choriocarcinoma,[128,129] and, interestingly, its secretion is enhanced by GM-CSF. IL-10 is also produced by isolated decidual cells.[130] In mice, its secretion is more controversial.[131,132] But, in murine models, IL-10 with IL-3 suppress local action of IL-2 and gamma interferon, biasing the maternal immune response towards a Th2 profile. Deneys (UCL, Bruxelles, Belgium) has shown that in pregnant women, TNF was down-regulated and IL-4 up-regulated when measured in the serum, a pattern not seen in recurrent aborters (Deneys, personal communication). Further proof of the role of IL-10 is the effect observed in infections. Leish-

mania biases the responses towards Th1, and there is less IL-10 but more pregnancy failure.[133,134]

EFFECTOR MECHANISMS

What are the exact mechanisms of early pregnancy loss? It is clear that interferon and gamma interferon are involved, very often as a consequence of either abnormal maternal recognition of the conceptus/semen, or local, undetected *"a très bas bruit"* infection, with peculiar insistence on mycoplasma.[17,108,133–136] The data of Hill strongly suggest it is also the case in humans, e.g., Th1 immunity causes recurrent abortions and sterility, with emphasis on gamma interferon.[137,138] But what are the exact mechanisms?

First, the cells and the "immunological events" need to be reconsidered, even for immunotrophism; interest is leaning now toward NKs rather than T cells. Anne Croy has shown that placentae of NK-deficient mice are grossly hypotrophic, leading to premature foetal death,[139] shifting the "immunotrophic" paradigm from T cells to NK cells. Thus, the production of immunotrophic cytokines might be in fact *mostly* (but not exclusively) under direct control of NK cells more than T cells, and "allorecognition" of pregnancy might, in fact, be exerted by these NKs rather than in a classical fashion by T cells. In consequence, a hitherto little suspected role could be envisaged for HLA-G: the control of the production of immunotrophic cytokines and not solely the local defusing of NK mediated cytolytic functions (a role that was forecasted by Y W Loke[31]).

In the CBA × DBA/2 model of natural immune abortion, together with the poly IC 12 U model, (an NK activator),[142] we explored the role of NKs in cooperation with P. Kourilsky.[140,143,144] Anti MHC H-2d (BALB/c) alloimmunisation prevents the effect of Poly IC or Poly I Poly C12 U as well as CBA × DBA/2 fetal loss. *But, not all* selective alloimmunisation prevents the CBA × DBA/2 foetal loss, and the genetic patterns of the effective immunising splenocytes (MHC restricted, minor loci dependent), albeit apparently representing a single trait,[12,141] are so odd to comprehend in terms of "classical" T-cell recognition (BALB/c works in CBA ×DBA/2, B10,D2 does *not–both* are H-2d) that some T-cell and MHC proponents preferred to dismiss the data as non reproducible (which they were not, being reproduced at present by more than 20

labs, with significant developments) or (we quote) "absolute bullshit."

But, we then demonstrated that immunisation with Kd or Ld transfected L cells prevented resorptions and used that system to study the effect of amino acid mutations on the border of the pocket of the MHC molecule in that system. These were shown to affect recognition by maternal cells promoting optimal foetal survival when immunisation was performed using H-2^d mutant transfected L cells. We then used various mutations that were artificially induced and localized on the border of the molecule's groove or in the peptide binding site. Some H-2d mutations on the helixes are recognized as foreign to H-2d and afford foetal protection against Poly IC. With P. Kourilsky, we wondered whether NK recognition of discrete determinants on the MHC molecule was not involved and, with help of Sylvie Delassus, Jean Pierre Abastado, Claude Roth, and Jos Even, we immunised CBA/J with L cells transfected with H-2d molecules mutant on positions 65, 69, which do not affect peptide presentation and 114 as a control. These protected against CBA × DBA/2 fetal loss, and, more important, enhanced IL-10 production in the supernatant of placental and decidual explants as already described using a commercial ELISA of high sensitivity, while treatment with asialo GM1 abolishes this effect.

At this stage, we propose the following working hypothesis: the NK cell repertoire is heteroclitic and consists of the recognition of selected altered domains of MHC molecules, whether that alteration results from mutations or allosteric conformational changes induced by (in the context of) background genes. Such a recognition either switches lytic/cytostatic pathway (macrophage activation, NK activation, TNF secretion, and balance of the CD4 system towards a Th1 profile) to IL-4 and IL-12 secretion by the NKs or pushes the system towards IL-10 secretion, and secretion by NKs or under the influence of NKs of the so-called "immunotrophic" cytokines. Thus, the concept would integrate *both* immunotrophism and the Th1/Th2 balance.

As far as the effector mechanisms are involved, it is already well known that TNF and interferon are causative and act in synergy,[108,145,146] but other mediators have been implied, such as nitric oxide released by activated macrophages.[147–150] There is

TABLE 5. Role of asialo GM1 + NK cells and macrophages in abortions

Expt	Day 6.5 treatment	Day 7.5 treatment	Day 13.5 assay	N^a resorptions/total	% abortions
1	PBS	PBS	8	23/56	41%
	PBS	1000 υ TNF-α	8	43/60	72%[b]
	PBS	2000 υ TNF-α	8	57/64	89%[c]
	anti-asialo GM1	PBS	8	10/59	19%[d]
	anti-asialo GM1	1000 υ TNF-α	8	12/63	16%[e]
	anti-asialo GM1	2000 υ TNF-α	8	12/55	22%[e]
2	PBS	PBS	16[f]	43/101	43%
	PBS	1000 υ γ-IFN[g]	16	79/93	85%[g]
	PBS	1000 υ γ-IFN + TNF-α[h]	16	74/89	83%[h]
	anti-asialo GM1	PBS	16	11/71	15%[d]
	anti-asialo GM1	1000 υ γ-IFN	16	12/98	12%[e]
	anti-asialo GM1	1000 υ γ-IFN + TNF-α[h]	16	89/104	86%[h]
3. ctrl[i]		PBS	8	36/88	41%
ctrl		γ-IFN + TNF-α[j]	8	65/80	81%[j]
SiO_2[k]		PBS	8	14/55	25%[k]
SiO_2		γ-IFN + TNF-α[j]	8	52/65	80%[j]

[a]N represents number of pregnant mice per group.
[b]Significant increase in abortion rate, $P < 0.005$ by χ^2.
[c]Significant increase in abortion rate compared to PBS control, $P < 0.005$ by χ^2; significant difference compared to lower dose of TNF-α, $P < 0.05$.
[d]Significant reduction in abortion rate by anti-asialo GM1 antibody compared to PBS control, $P < 0.005$ by χ^2.
[e]No significant boosting of abortion rate compared to PBS injected anti-asialo GM1-treated group.
[f]Result from two independent experiments giving same result have been pooled.
[g]γ-interferon (γ-IFN) significantly boosted abortion rate, $P < 0.005$ by χ^2.
[h]TNF-α was given at 1000 υ and 2000 υ in separate experiments with γ-IFN and gave similar results; the data have been pooled for ease of presentation. The abortion rate was significantly boosted, $P < 0.005$ by χ^2.
[i]Untreated CBA/J female mice mated to DBA/2 males.
[j]1000 υ γ-IFN + 2000 υ TNF-α significantly boosted abortion rate, $P < 0.005$ by χ^2.
[k]CBA/J mice injected twice a week for 4 weeks with 100 mg/kg silicon dioxide before mating significantly reduced abortion rate, $P < 0.05$ by χ^2.

no doubt that asialo GM1+ cells or cells of the NK lineage are involved, since their transfer, once activated, causes abortion, and, since they do accumulate at the site of embryo resorption, their modulation positively (Poly IC activation) or negatively (anti-asialo GM1 treatment) influences parallel resorption rates.[151–154] We have studied this problem with David Clark, taking into account the fact that TNF and gamma interferon are by themselves abortifacients, and performing cell deletion to learn the cellular mechanism triggered.[155]

In Table 5, intraperitoneal injection of TNF-α enhanced CBA xDBA/2 resorptions, but anti-asialo GM1 antibody both decreased the background rate and prevented the action of TNF-α. When we added γ-interferon, it had as expected an abortifacient effect, but that was not seen in NK-cell-depleted mice, suggesting that the Baines model[149,150] (-interferon (macrophages (activated to produce NO (embryo death was not correct. However, when γ interferon and TNF-α were administered together, more than 80% of the implanted embryos aborted. This confirmed the already observed synergy/codependence and sug-

gested that in fact it might be crucial. Indeed, NK cell depletion suggested that TNF-α could not find enough NK-derived γ-interferon, while γ-interferon alone fails because macrophages which depend on NK-cell-derived γ-interferon have and thus did not produce TNF-α, and the intraperitoneally injected cytokine does not stimulate TNF-α production quickly enough.

The action of NKs or macrophage has been attributed to a direct killing of trophoblast. In silica treated macrophage-depleted mice, the abortion rate was reduced, *but* this treatment was ineffective in TNF-α plus γ-interferon treated animals, whose macrophage depletion was checked by the anti F4/80+ MoAb. Ovarian failure could have happened after silica, but this was ruled out since it should have caused abortions in 100% of cases, and the results were unaltered hormone replacement therapy treated animals.

The most logical target appeared to be the maternal uterine vascular endothelial cell, since the cytokines stimulate surface expression of procoagulant (flg/2-prothrombinase, which is distinct from tissue factor) and clotting. Table 6 shows that an-

TABLE 6. Antibody to flg/2 prothombinase prevents abortions in DBA/2-mated CBA/J mice

Pretreatment group	Day 7.5 treatment	Day 13.5 assay	N resorptions/total	% abortion
Control Rabbit IgG	nil	8	21/56[a]	38%
Control Rabbit IgG	γ-IFN + TNF-α[b]	8	48/55	87%[c]
Rabbit IgG anti-flg/2	nil	9	3/66	4.5%[d]
Rabbit IgG anti-flg/2	γ-IFN + TNF-α[b]	9	9/68	13%[e]

[a]Results from two independent experiments which gave the same result.
[b]1000 υ γ-IFN and 2000 υ TNF-α was injected intraperitoneally.
[c]Significant increase in abortion rate $P < 0.001$ compared to no cytokine control group. Fisher's Exact test.
[d]Significant reduction in spontaneous abortion rate $P < 0.001$ compared to no cytokine control group, Fisher's Exact test.
[e]Significant reduction in abortion rate $P < 0.001$ compared to cytokine-treated controls, Fisher's Exact test. No significant difference compared to anti-flg/2-treated mice which did not receive an injection of cytokines.

tibody to flg/2 prothrombinase reduced the background rate of abortion to 4% and prevented the effects of TNF-α plus γ-interferon. Since granulocytes are observed in resorption sites and lyse of TNF-α plus γ-interferon-activated endothelial cells, we tested the injection of pregnant mice with a monoclonal anti-granulocyte antibody which is known to block granulocyte-mediated tumor rejection in vivo.[156] Anti-granulocyte antibody partially reduced the spontaneous abortion rate and significantly abrogated the effect of TNF-α plus γ-interferon (as control, see effects of rIL-10) (Table 7). Thus, vasculitis, rather than a direct cytotoxic action on fetal trophoblast, leads to abortion.

In this context, and in keeping with the aforementioned rôle of NK cells in cytokine production, concerning protection against abortion, TGF-β2-producing γδ T cells in the uterine lining appear to be important.[157,158] These cells are producing TGF-β2 and IL-10, albeit, by in situ and immunohistochemistry, we find IL-10, IL-3, and IL-4 in murine spongiotrophoblast mainly, with traces in the decidua (Cayol, DEA Paris 7, Sept 1997, and in preparation; See also photos 1,2, and 3), in agreement with what is reported in humans.[128,129]

STRESS-INDUCED ABORTION

In a series of models, we have studied stress-induced abortion. Stress (be it contention, ultrasonic stress, etc.) can induce abortion in mice, and this can be prevented by alloimmunisation.[145] This phenomenon has been studied in detail. It involves pathways which are by some aspects very similar to those discussed above but involving substance P, CD8 T cells, and alterations of mast cells. The discussion of stress-induced pathways could be by itself a review, and readers should refer to other published papers.[158-165]

This model is of importance in discussing early human pregnancy loss. Of interest is the fact that a monoclonal antibody, BA 11, prepared by R. Jalali in James Mowbray laboratory, blocks *both* classical and stress-mediated abortion.[166,167]

To finish, we would like to say we do *not* negate systemic events, we simply relativise them. As stated at the beginning of this paper, we do not negate the existence of allograft enhancement during pregnancy, nor peripheral T-cell hyporesponsiveness.[26-29,67,168-170] We merely believe they reflect local events, for the most part, including the hitherto undiscussed placental suppresser factors. These, that we have suspected since 1980,[171] have proved to be elusive,[172-174] but with a continuous effort in mice,[175-182] we have delineated the active moiety in human and murine placental supernatants, and this induces T-cell anergy[181,182] in a very similar manner to staphyloccocal enterotoxin,[183] explaining, in our view, the T-cell unresponsiveness observed locally[184] whose aforementioned phenomenons (especially the report by Tafuri[27]) are in our opinion a mere reflection. In that respect, since the anergy is *transient* and reversible, it is interesting to note that optimal secretion of the factor in mice is seen by cells of the invasive ECP,[172] and that there is a local deficiency in suppresser materials in the CBA × DBA/2 window of abortion.[173]

CONCLUSIONS

There are *several* causes of early pregnancy loss, which we are only beginning to uncover. Several, as in the murine system, have different etiologies, *but* use a *final* "rejection-like" common pathway, where TNF and gamma interferon are important. But several others are likely to be due to NK failure

TABLE 7. Effect of anti-granulocyte antibody on abortion rate

Group	Day 6.5 treatment	Day 7.5 treatment	Day 13.5 assay	N resorptions/total	% abortion
1	Control rat IgG[a]	PBS	8	17/57	30%
2	Rat anti-granulocyte[b]	PBS	6	7/45	16%[c]
3	Control rat IgG[a]	TNF-α + γ-IFN	9	48/56	86%[d]
4	Rat anti-granulocyte	TNF-α + γ-IFN	7	8/57	14%[e]
5	rIL-10	rIL-10	5	2/42	5%[f]

[a]Monoclonal isotype control IgG 100 mg intraperitoneally as in Materials and Methods.
[b]Low endotoxin rat monoclonal IgG 100 mg intraperitoneally as in Materials and Methods.
[c]Reduction in abortion rate compared to group 1, P = 0.072 by Fisher's Exact test.
[d]TNF-α + γ-IFN given as in Tables 1 and 2. Significant increase in abortion rate compared to group 1, $P < 0.001$ by χ^2 and Fisher's Exact test.
[e]Significant reduction in abortion rate compared to group 3, $P < 0.001$ by χ^2 and Fisher's Exact test. Pooled result from groups 2 and 4, 15/102 = 15% abortion rate, significantly less than 30% rate in group 1, $P < 0.05$ by χ^2.
[f]Significant reduction in abortion rate compared to group 1, $P < 0.0012$ by Fisher's Exact test. No significant reduction compared to group 2 (P = 0.096) or pooled groups 2 and 4 (P = 0.075).
Clark DA, Chaouat G, Arck P, Mittruecker HW, Levy GA: Cytokine-dependent abortion in CBA X DBA/2 mice is mediated by the procoagulant flg/2 prothrombinase. In press.
Stoppacciaro A, Melani C, Parenza M, et al.: Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon γ. J Exp Med 178:151, 1997.

or misrecognition, hence improper cytokine secretion and lack of growth factors.

Several others are due to defects of the local inflammatory reaction (endometriosis, with preexisting *high* levels of TNF, is among these), and it is likely that, as in the LIF system, we will see discrete defects leading to definition of molecular abnormalities, with defects in adhesion molecules being involved.

Thus, early pregnancy loss and recurrent miscarriage, though due to "immunological-like" circuitry, are likely to be split up into different syndromes. It is our opinion in that respect that it is not *at all* surprising that only one woman out of 10 or 11 benefits from purely imunologic treatment. We have to be able to define which women will benefit by rigorous immunological criteria. (Unfortunately, the leukocyte immunisation saga has lead to many charlatan, unconscious, or preposterous theorisations, sometimes with disastrous consequences; women are *not* guinea pigs nor mice! We have always wondered what was the real basis for the MHC linked, disequilibrium antigen, as well as how a system like the CBA xDBA/2–which was described as minor loci dependent, MHC restricted and in which the good father (BALB/c) and the bad father (DBA/2) were *both* H-2d–could have been taken as an example of the proof for the need for absence of HLA homology! This is just an example, but we could—and, in fact, we are due to— write horrid things about anti-paternal antibodies, to illustrate what was said at this paper's onset.)

This has to be said, because otherwise what we

learn from the discoveries of complex cytokine networks at present is that by unfounded treatments we are at risk of altering other useful pathways and induce the lack of remission of transient infertility.

The "stress" saga tells us about the importance of neuroimmunoendocrine pathways, which also should not be a surprise.

To the impatience of clinicians, we recall what H. Metzger said when he was cloning the IgE receptor: "Haste is waste." We believe we have made significant progress in the past five years in understanding, *with the help of animal models*, in unraveling pathways which are operational indeed.

But, we now have to take into account that added level of complexity. We know it sounds difficult for clinicians, because we have a jargon, but we cannot escape the truth. *Yes, a cytokine network is operating in early pregnancy*, and, in certain cases, we might be found guilty if we were treating patients as if ignoring its existence.

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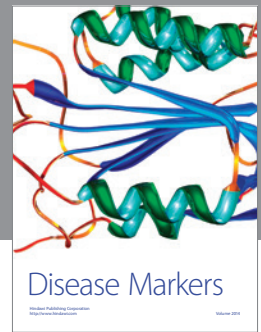
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