Ex Vivo Cytokine Responses Against Parvovirus B19 Antigens in Previously Infected Pregnant Women

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Parvovirus B19 infection is a significant cause of fetal death. The aim of this study was to investigate the role of maternal immune status in modulating susceptibility to fetal B19 infection. Peripheral blood was obtained from pregnant women (n = 199) with no clinical evidence of recent B19 infection. Evaluation of ex vivo T cell responses from 149/199 individuals showed significantly higher interferon-γ levels for seropositive individuals following VP1 (268 ± 36 versus 103 ± 19 pg/ml; $P = 0.003$) and VP2 (242 ± 42 versus 91 ± 16 pg/ml; $P = 0.01$) antigen stimulation. Significantly higher levels of interleukin-2 were also observed in seropositive individuals following both VP1 ($P = 0.0003$) and VP2 ($P = 0.0005$) stimulation. The observed Th1 cellular response is lower than that documented previously for non-pregnant individuals and strongly suggests that diminution of the maternal antiviral immune response may increase susceptibility to fetal B19 infection. *J. Med. Virol.* **70:475–480, 2003.** © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Human parvovirus B19 infection can cause serious complications in the immunocompromised host including pregnant women and individuals with underlying blood disorders such as sickle cell disease [Woolf et al., 1989; Serjeant et al., 1993; Heegaard and Hornsleth, 1995; Tarantino and Shahidi, 1995]. Exposure to, and infection with, parvovirus B19 during pregnancy can lead to fetal loss by either spontaneous abortion or non-immune fetal hydrops occurring in 8–10% of infections [Brown, 1989; Jordan, 1996; Kinney et al., 1988]. B19 is transmitted across the placenta during maternal infection and subsequent fetal infection can lead to severe anaemia. A vertical transmission rate of 33–51% has been reported [PHLS, 1990; Yaegashi, 2000]. B19 infection during pregnancy often goes undetected as many pregnant women are asymptomatic while other individuals experience symptoms such as exanthema and arthralgia [Komischke et al., 1997].

Fetal loss as a consequence of intrauterine B19 infection is highest in, but not restricted to, the first 20 weeks of gestation [PHLS, 1990]. This susceptibility could be attributed, at least in part, to the predilection of B19 for rapidly dividing erythroid progenitor cells. In the second trimester, the fetal red cell mass increases three- to fourfold and the effects of B19 may be further compounded by the reduced life span of fetal red blood cells. The relative immaturity of the fetal immune response at this stage may also be a contributory factor. Cases of late second and third trimester non-hydropic fetal loss, caused by an acute B19 infection, have only been reported recently [Skjöldebrand-Sparre et al., 2000; Tolfvenstam et al., 2001]. In the present study, following an examination of incidences of intra-uterine fetal death it was found that 7.5% (7/93) of third trimester and 15% (7/47) of late second and third trimester cases contained B19 DNA in the placental tissue. These cases were not associated with hydrops and no other explanations for fetal death were evident. Unusually, none of the pregnant women showed clinical symptoms of B19 infection and many had delayed or absent humoral responses.

Specific anti-viral antibody is considered the major mechanism of immune protection [Kurtzman et al., 1989; Schwarz et al., 1990], however, more recently the

cellular response to parvovirus B19 infection has been explored. B19 specific T-cell proliferative responses of recently infected and healthy individuals to the capsid antigens [von Poblotzki et al., 1996; Corcoran et al., 2000; Franssila et al., 2001] and to the non-structural protein, NS1 [von Poblotzki et al., 1996; Mitchell et al., 2001] have been observed. T helper cells secrete cytokines, which have an important role in determining the outcome of a pregnancy [Chaouat et al., 1990; Hill, 1991; Tangri and Raghupathy, 1993]. Th1 and Th2 cells are the major subsets of fully differentiated $CD4^+$ T cells and normal pregnancy is dominated by T helper cells that secrete a Th2 type pattern of cytokines including interleukin-4 (IL-4) and interleukin-5 (IL-5) [Wegmann et al., 1993]. Th1 cytokines, including interferon-gamma (IFN-γ) and interleukin 2 (IL-2), are associated with a poor pregnancy outcome and are suppressed by placental products such as progesterone, prostaglandin E2 and cytokines such as IL-4 and IL-10 [Wegmann et al., 1993; Kelly and Critchley, 1997]. During pregnancy, elimination of a viral infection via a Th1 response may result in endangering the developing conceptus. Cytokines typical of a Th1 response, including IFN-γ, IL-2, IL-6, and IL-1β have been reported upon stimulation of T cells of healthy adults with parvovirus B19 [Wagner et al., 1995; Moffatt et al., 1996; Corcoran et al., 2000; Jordan et al., 2001]. Furthermore, Corcoran et al. [2000] have demonstrated that PBMCs isolated from normal healthy individuals (n = 7, comprising 3 females) produced IFN-γ levels of 610 ± 348 pg/ml and 1765 ± 825 pg/ml when stimulated with VP1 and VP2, respectively.

In this study, we investigated if pregnancy, and the associated suppression of Th1-type responses, could modulate ex vivo B19-specific cytokine production from T cells isolated from pregnant women to elucidate the role of host factors in determining susceptibility to B19 infection.

MATERIALS AND METHODS

Study Population

Heparinised blood specimens (n = 199) were obtained from pregnant women with no evidence of recent parvovirus B19 exposure or symptoms (trimester 1 (n = 78), trimester 2 (n = 80) and trimester 3 (n = 41)). The age range of the volunteers was 15–50 and the mean age was 28 years. The gestation period varied from 4 to 42 weeks. No further clinical data was available on the volunteers. Ethical permission for this study was obtained from the National Maternity Hospital, Dublin, Ireland and written consent was obtained from all volunteers prior to specimen collection.

Antigen Expression and Purification

Parvovirus B19 recombinant VP1 and VP2 capsids and NS1 were expressed in the baculovirus expression system using *Spodoptera frugipera* cells [Brown et al., 1990; Brown et al., 1991; Ennis et al., 2001]. Capsid and NS1 purification have been described previously [Kerr et al., 1999; Ennis et al., 2001].

Parvovirus B19 IgG Enzyme Immunoassays

Plasma specimens were analysed for B19 IgG specific for the capsid antigens VP1 and VP2 in both their native (N) and denatured (D) conformations as previously described [Corcoran et al., 2000]. An enzyme immunoassay was used to measure the level of reactivity against B19 NS1. Results are expressed as index values (I.V.) representing specimen absorbance divided by cut-off absorbance. The cut-off was established as the absorbance +2 SD greater than the mean absorbance obtained from a panel of B19 negative samples. An IV of <1.0 was considered seronegative. B19 IgG reactivity to conformationally intact VP1 (VP1-N) was determined by a commercial qualitative immunofluorescent assay (IFA). The degree of fluorescence was graded on a scale of 0-4 according to manufacturers' instructions (Biotrin, Dublin, Ireland).

T cell proliferation assays. Isolated peripheral blood mononuclear cells (PBMCs) were cultured in triplicate with purified recombinant VP1 (10 µg/ml), VP2 (10 µg/ml) and NS1 (10 µg/ml) for 72 hr. Cells cultured with medium alone or with phytohaemagglutinin (PHA: 2 µg/ml) served as negative and positive controls, respectively. The level of B19-specific T cell proliferation was measured depending on the amount of [^{3}H]-thymidine incorporation during the final 4 hr of culture [Corcoran et al., 2000].

Cytokine enzyme immunoassays. Supernatants were removed from PBMCs cultures, at optimal timepoints, to determine the concentration of IL-2 (24 hr) and IFN-γ, IL-4 and IL-5 concentrations (72 hr). Levels of cytokine secreted were determined by EIA using commercially available antibody pairs (PharMingen, San Diego, California, USA). Concentrations were determined by comparing the absorbance at 405 nm for test samples with a standard curve for recombinant cytokines of known concentration. Levels of IL-2 are expressed in terms of index values (specimen IL-2 (U/ml) divided by the mean negative control IL-2 level (U/ml) plus 2 standard deviations) to compensate for differences in control IL-2 levels between experiments.

Statistical Methods

Cytokine secretion data from different treatment groups were compared by use of Student's t-test.

RESULTS

Seroreactivity of Study Population

Parvovirus B19 seroprevalence in the adult population has been reported to be 60–70%. In this study, where B19 seropositivity was defined by the presence of antibody against VP2-N (capsid VP2), all trimester groups had expected levels of reactivity against VP2-N (63–71%) (Table I), thus giving a representative sample population in terms of past B19 infection for each of the trimesters. Levels of reactivity against VP1 ranged from 59–68% (Table I). The low level of antibody reactivity observed against linear epitopes of VP1 (35–52%) and

TABLE I. Antibody Response in Plasma Obtained From Pregnant Women (n = 199) Against the B19 Capsid Proteins VP1 and VP2*

Detection antigen	Trimester		
	1 Positive	2 B19 IgG status (%) positive	3 Positive
VP1-N	59	65	68
VP2-N	63	65	71
VP1-D	52	51	35
VP2-D	15	6	3
NS1-D	2	4	0

*In conformationally intact (N) and denatured (D) forms, and to the B19 nonstructural protein, NS1. B19 seropositivity was defined by the presence of antibody against VP2-N (capsid VP2).

VP2 (3–15%), in the seropositive group, is indicative of past infection of healthy individuals and also emphasises the importance of using capsid-based detection systems for accurate diagnosis of past exposure to B19 in pregnant women. It is known that antibodies against linear epitopes on VP2 are undetectable 6 months post-infection [Söderlund et al., 1995]. As specimens from this cohort exhibited the expected low level of reactivity against linear VP2 (Table I), it can be concluded that 92% of seropositive pregnant women in this study did not have an acute B19 infection.

Strong IFN-γ Responses Dominate the Cytokine Profile

Not all PBMCs isolated from whole blood specimens taken from pregnant women exhibited reactivity when stimulated with positive control (PHA) in the T cell proliferation assay. Thus, subsequent data evaluation involved the study of T cell responses from the 147/199 individuals with PHA-induced responses. Higher levels of the inflammatory cytokine IFN-γ were secreted by PBMCs obtained from seropositive individuals (n = 99) than in those obtained from seronegative donors (n = 48). Most significant were levels of IFN-γ (268 ± 36 pg/ml) when T cells were stimulated with the immunodominant capsid protein, VP1 ($P = 0.003$). Levels of IFN-γ were also significant when cells were stimulated with the capsid protein VP2 ($P = 0.01$) and to a lesser extent with NS1 ($P = 0.09$) (Table II).

In order to determine if there was a trimester-dependent pattern in the ex vivo cytokine response, specimens were re-classified based upon the stage of

TABLE II. Ex Vivo IFN-γ Secretion by PBMCs Isolated From Seropositive and Seronegative Pregnant Women After Stimulation With Purified VP1, VP2, or NS1 (10 μg/ml)

Specimen seroreactivity	B19-specific IFN-γ production (pg/ml + SE)		
	VP1	VP2	NS1
Positive (n = 99)	268 ± 36	242 ± 42	185 ± 34
Negative (n = 48)	103 ± 19	91 ± 16	100 ± 17

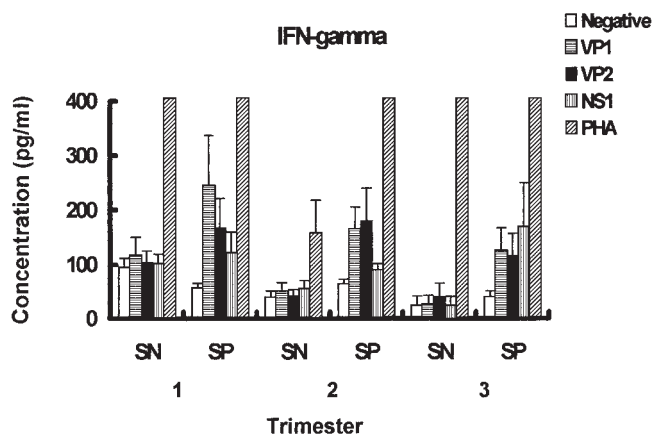IFN, interferon; PBMCs, peripheral blood mononuclear cells.

Fig. 1. Trimester dependence of ex vivo interferon-γ production following PBMCs stimulation with B19 antigens. Isolated PBMCs were individually stimulated with purified B19 antigens, VP1, VP2 and NS1 or either medium alone or PHA as a negative or positive control, respectively. Seronegative (SN) and seropositive (SP) specimens were categorised according to trimester 1 (SP (n = 39); SN (n = 20)), 2 (SP (n = 41); SN (n = 17)) and 3 (SP (n = 19); SN (n = 11)). Results represent the mean concentration of interferon-γ produced from triplicate wells (±SE).

gestation. IFN-γ was detected in specimens obtained across all three trimesters, however no significant difference was observed in the amounts of IFN-γ secreted ex vivo, in response to all B19 antigens tested, across the trimesters of pregnancy (Fig. 1).

B19-Specific IL-2 Production by Pregnant Women

Although significantly high levels of IL-2 were produced by PBMCs when stimulated with B19 antigens, initially there was no statistically significant difference between levels produced by seropositive (n = 99) and seronegative (n = 48) specimens due to the fact that three seronegative specimens produced high levels of IL-2 upon stimulation. Exclusion of these three specimens (see Discussion) results in the observed levels of IL-2 secretion being significantly higher in seropositive specimens for both VP1 ($P = 0.0003$) and VP2 ($P = 0.0005$) stimulation. Strong IL-2 production was observed following ex vivo stimulation of PBMCs from seropositive pregnant women (n = 99) with VP1, VP2 and NS1, resulting in IL-2 index values of 1.43 ± 0.9, 1.36 ± 0.7 and 1.27 ± 0.9, respectively (Fig. 2). In comparison, IL-2 index values of 0.85 ± 0.25, 0.89 ± 0.25 and 0.83 ± 0.29 were evident following analysis of T cells from seronegative individuals when stimulated with VP1, VP2 and NS1 (Fig. 2). In fact, 44/99 seropositive samples produced significantly higher levels of IL-2 compared to seronegative samples ($P = 0.004$ for VP1, $P = 0.003$ for VP2) despite the inclusion of the 3 seronegative/high IL-2 specimens in the analysis (see above). Mean values of IL-2 for these 44 samples were 0.407 ± 0.049 U/ml for VP1 and 0.306 ± 0.029 U/ml for VP2. No difference was observed in the extent of ex vivo IL-2 secretion from stimulated PBMCs between trimesters. Despite the bias towards Th2 cytokine production
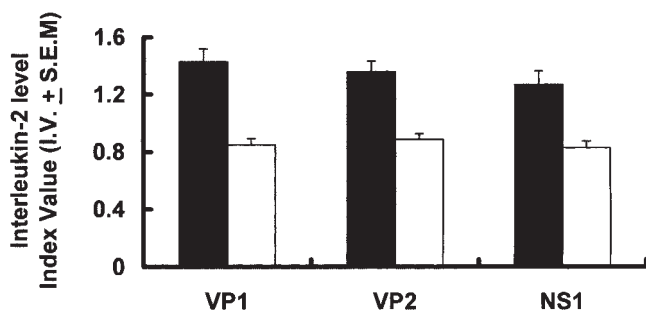
Fig. 2. Ex vivo IL-2 production following stimulation of isolated PBMC, from seropositive (black bars) and seronegative (white bars) individuals, with parvovirus B19 VP1, VP2 and NS1 antigens, respectively. IL-2 results are expressed as index values (I.V. ± S.E.M.) representing the amount of IL-2 produced above the background level for each individual (I.V. = mean IL-2 concentration (U/ml)/mean IL-2 concentration of the negative control +2 standard deviations).

during pregnancy, PBMC stimulation with B19 antigens did not produce any significant levels of interleukin-4 and -5 (data not shown).

DISCUSSION

This study represents the first simultaneous evaluation of both humoral and cellular immunity against parvovirus B19 in pregnant women. The population cohort exhibited the expected high level of seropositivity (63–71%) against B19 and a significant ex vivo Th-1 response against B19 antigens, though reduced compared to non-pregnant individuals. Furthermore, evidence is presented for the presence of a cellular response to past B19 infection in the absence of a detectable humoral response, which has implications for current B19 diagnostic practices.

Overall the level of parvovirus B19 seroprevalence amongst pregnant women in Ireland was 66% (132/199). This evaluation compares favourably with that reported previously in both German and US (73.2%) populations using identical test methodology [Searle et al., 1997; US Food and Drug Administration, 1999]. Significantly, a lower level of IgG antibody positivity is observed against both linearised B19 VP1 and VP2, confirming the necessity for B19 IgG detection systems to utilise native B19 antigens to facilitate optimal test sensitivity [Kerr et al., 1999]. Söderlund et al. [1995] have shown previously that antibodies against linear epitopes are lost within 6 months following B19 infection, thus the low level of antibody positivity against these epitopes, observed in the present study, confirms further that the majority of seropositive individuals were infected at least 6 months prior to specimen donation.

Cases of maternal infection and subsequent fetal death due to B19 infection have been reported in all three trimesters [Wright et al., 1996; Yaegashi et al., 1998; Skjöldebrand-Sparre et al., 2000]. Although the direct pathogenic effects of B19 on fetal development are a major cause of mortality, the role of the maternal immune status in either protecting against or mediating parvovirus B19–induced fetal loss is unknown. It is

generally accepted that a successful pregnancy requires a Th2 response and that strong Th1 responses are associated with pregnancy rejection [Raghupathy, 1997; Raghupathy, 2001]. Interestingly, recent work by Luppi et al. [2002] presents evidence of an increase in CD8^{+} lymphocyte frequency in peripheral blood specimens taken during the third trimester of pregnancy compared to a control group. In this present study we show that ex vivo stimulation with B19 antigens results in significant IL-2 and IFN-γ production from PBMC despite the proposed Th2 bias of pregnancy. Comparative information on ex vivo cytokine secretion in response to B19 antigen stimulation is limited. However, in the present study, the level of ex vivo IFN-γ secretion was reduced substantially from that of normal healthy individuals stimulated with the same antigens [Corcoran et al., 2000] where B19 seropositive adults produced IFN-γ levels of 610 ± 348 pg/ml and 1765 ± 825 pg/ml when stimulated with VP1 and VP2, respectively. Interferon-γ production by PBMCs from pregnant women (present study) was 205 ± 27 pg/ml with VP1 and 176 ± 26 pg/ml with VP2 stimulation. This is suggestive of a pregnancy-associated diminution of Th1 mediated immunity. In fact, a transient down-regulation in the Th1 response is seen during pregnancy in rheumatoid arthritis patients and this reduction is associated with a state of remission as pro-inflammatory cytokines cause the pathological damage of joints evident during rheumatoid arthritis [Russell et al., 1997].

The decrease in the cellular immune response to B19 observed may be relevant to the adverse outcomes associated with B19 infection during pregnancy. We propose that this diminished Th1 response may decrease the rate of B19 clearance, thus giving the virus the opportunity to exhibit greater infectivity. A similar phenomemon has been reported with *Leishmania major* parasitic infections of genetically resistant pregnant mice whereby cellular responses against *L. major* were weakened due to reduced IFN-γ production during pregnancy and was associated with diminished clearance of the parasite [Krishnan et al., 1996]. In addition, *L. major* infection in these mice caused an increase in the frequency of fetal resorptions. Although a systemic Th1 response was beneficial in fighting infection, it was also detrimental to gestation, even at a decreased level.

IL-2 is not present normally in substantial levels during pregnancy around the trophoblast [King et al., 1995], however, in the present study, ex vivo stimulation with B19 antigens elicited production of this pro-inflammatory cytokine. IL-2 production has been observed previously in women who were infected with B19 during pregnancy [Jordan et al., 2001]. Using immunohistochemical techniques on placental tissue sections (n = 25), IL-2 was detected at the maternal-fetal interface of all women in the study who seroconverted during pregnancy despite the outcome of the pregnancy. However, it was proposed that in pregnancies with a poor outcome IL-2 is detectable on the fetal side of the interface, whereas those with a positive outcome tend to

have the IL-2 on the maternal side. It is well established that some cytokines have adverse effects on the outcome of pregnancy, particularly IFN-γ, IL-2 and TNF-α. These cytokines activate cytotoxic cells such as natural killer (NK) and lymphokine activated killer (LAK) cells which can kill trophoblast cells [Drake and Head, 1989] and when these cytokines are administered to mice they cause abortions [Kinsky et al., 1990]. In pregnant women with a history of recurrent spontaneous abortions significantly higher levels of NK cells [Kwak et al., 1995], IFN-γ and IL-2 expression [Tangri and Raghupathy, 1993] have been found when compared to normal pregnant women. IFN-γ production by B19-specific T cells may also be detrimental to the conceptus by inhibiting granulocyte-macrophage colony-stimulating factor (GM-CSF), which is involved in trophoblast growth and differentiation [Robertson et al., 1994].

Although Th1 cytokines are important during implantation and parturition of pregnant women [Haimovici and Anderson, 1993; Aboagye-Mathiesen et al., 1996], there may be times during pregnancy when the fetus is more sensitive to the level of pro-inflammatory cytokines. Our study shows that the levels of Th1 cytokines did not significantly differ over the three trimesters but the level of IFN-γ produced was highest in trimester 1. Previous studies have shown that pro-inflammatory cytokines such as IFN-γ can have deleterious effects on the fetus during pregnancy [Raghupathy, 1997]. This observation, combined with the relative immaturity of the fetus during trimester 1, suggests that an increased Th1 response to B19 may be detrimental to the fetus. Other factors to be considered when determining the effect of cytokines caused by B19 infection during pregnancy are the gestational period of the infection, the stage of differentiation of the conceptus, the level of soluble cytokine receptors and the ratio of Th2 cytokines.

Interestingly, three of the seronegative donors produced high levels of IL-2 upon ex vivo B19 antigen stimulation. The reason for this is unclear but recent evidence suggests that certain individuals with past B19 infection may possess an antigen-specific cell mediated immune response in the absence of a humoral response to the virus. Tolfvenstam et al. [2001] observed four B19 antibody negative cases that had specific T cell memory proven by either ELISpot assay or tetramer binding. These observations have significant consequences for the design of an efficacious vaccine for parvovirus B19 in that future subunit vaccines should contain both B19 T cell and B cell epitopes.

Previous studies on B19 infection during pregnancy have focussed on both the direct effects of B19 on the fetus and the maternal humoral response as the major mechanism of protection against B19-induced fetal death. Our results, which show a significant diminution in ex vivo interferon-γ secretion in response to B19 antigen stimulation, demonstrate that cellular immunity against B19 is attenuated during pregnancy which may have an adverse effect on anti-viral Th1-type responses thereby increasing fetal susceptibility to infection.

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