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The promoter polymorphism of the interleukin-6 gene regulates interleukin-6 production in neonates but not in adults.

Abstract

In the promoter region of the IL-6 gene there is a single base exchange (G Æ C) polymorphism at position -174. Recent findings suggest that this polymorphism may affect the transcription rate of the IL-6 gene and IL-6 plasma levels. To analyse its biological significance, we examined IL-6 plasma levels in cord blood and IL-6 production by neonatal cells after LPS-stimulation in relation to the

presence of the IL-6G and IL-6C alleles. We hypothesized that since healthy neonates lack a previous exposure to exogenous antigens, their cytokine production could be genetically regulated. We also assumed that the normal labour-related stress could provide a physiological stimulus for IL-6 production. Cord blood was collected from 50 healthy, full-term neonates after normal vaginal delivery (VD) and from 42 healthy, full-term neonates after elective caesarean section (ECS). Adult samples were obtained from 450 healthy adult controls. The -174 polymorphism was analysed using PCR. IL-6 plasma levels and in vitro IL-6 production were measured using an ELISA method. Generally, IL-6 plasma levels in neonates were significantly higher than those in adults (neonates born by VD versus adults $p < 0.001$ and neonates born by ECS versus adults $p < 0.001$); the median value for neonates born by VD was 11.4 pg/ml (4.5-45.9), for neonates born by ECS 2.9 pg/ml (1.9-6.4) and for adults, 1.2 pg/ml (0.7-2.0). Surprisingly, cord blood IL-6 levels after VD differed significantly from those after ECS ($p < 0.001$). An analysis was carried out to ascertain if there was a genetic association between different IL-6 genotypes and IL-6 plasma levels in neonates. In the group of VD neonates with the CC genotype, non-carriers of the G allele, secreted significantly more IL-6 than carriers of the G allele ($p < 0.03$); 21.4 pg/ml (9.5-81.3) and 9.6 pg/ml (3.5-36.2) respectively. In line with this, ECS newborns with the CC genotype had higher IL-6 plasma levels than carriers of the G allele ($p < 0.02$); respective median values were 6.3 pg/ml (2.2-12.9) and 2.7 pg/ml (1.7-4.1). These findings were also supported when in vitro IL-6 production by neonatal mononuclear cells was compared carriers of the G allele and non-carriers of the G allele. IL-6 levels were significantly lower in carriers of the G allele than in non-carriers ($p < 0.04$); respective median values were 6,980 pg/ml (4,175-16,800) and 17,425 pg/ml (11,400-33,900). In vivo or in vitro production of IL-6 of adult controls was not associated with the IL-6 -174 polymorphism. The difference between cord blood IL-6 levels after VD and after ECS suggests that normal labour-related stress induces IL-6 production. Our data also suggest that the -174 polymorphism of the IL-6 gene participates in the regulation of IL-6 responses in both groups of neonates. Furthermore, the naive IL-6 response of stimulated neonatal cells is associated with the -174 polymorphism of the IL-6 gene. In healthy adults, the regulation of IL-6 responses differs from that of healthy neonates, since baseline and inducible IL-6 levels in adults were not associated with this polymorphism. This indicates that the genetic regulation of IL-6 production can be observed in naive cells, while in adult cells previous contact with exogenous antigens probably modifies their responses.

Full text

INTRODUCTION

IL-6 is a 26-kDa, multifunctional cytokine inducing diverse biological responses from various hemopoietic and nonhemopoietic cells. It has been shown that IL-6-deficient mice develop normally, but they have reduced T-lymphocyte numbers, and their antiviral and antibacterial immune responses, as well as acute phase responses to infection and tissue damage, are severely impaired [1].

During embryogenesis, pregnancy and finally at term, IL-6 also has an important role. Human IL-6 stimulates early differentiation of murine embryonic stem cells *in vitro* [2] and several studies have shown its role in early embryonic hematopoietic development [3, 4]. Research on different neuronal populations has revealed that IL-6 also has some neurotrophic activity [5]. It has various actions that may be relevant to tissue remodeling and activity of leukocytes in the uterus during pregnancy [6]. Furthermore, it has been suggested that uterine IL-6 could act as an important mediator of normal term labour; together with IL-1beta and TNF-alpha, IL-6 induces prostaglandin release from uterine tissues leading to myometrial contractions and delivery [7]. This is in line with the previous finding that in pregnant women serum levels of IL-6 increase with gestational age [8].

Various cell types are reported to contribute to IL-6 production in the uterus; epithelial and stromal cells, placental trophoblast cells [6] and amnion cells [9]. It is not clear when a human fetus is able to secrete
IL-6, but at least first-trimester human chorionic villi are capable of a moderate expression of IL-6 mRNA [10]. In mice, IL-6 has been reported to be expressed as early as at the blastocyst-stage embryo and in the thymus from fetal day 14 [11, 12]. Elevated levels of IL-6 in amniotic fluid have been measured in preterm labours with and without infection [13, 14].

Previous studies in adults and in neonates have demonstrated the important role of IL-6 in the pathogenesis of the sepsis syndrome [15-17]. Results from *in vitro* and *in vivo* studies of the capacity of neonates to produce IL-6 have been contradictory. Reduced or similar levels of IL-6 have been observed in stimulated neonatal cells as compared to stimulated adult cells [18-20]. However, plasma

levels of IL-6 in septic neonates seem to be greatly elevated as found in septic adults [15-17]. To date, there are only a few studies involving both *in vivo* and *in vitro* production of IL-6 by neonates [21].

IL-6 is a single-copy gene located on chromosome 7p21. Within the regulatory region of this gene occur polymorphic sites and at least one of these is associated with an altered rate of expression of the IL-6 gene. Fishman *et al.* [22] reported that a G/C polymorphism at position -174 in the promoter region could affect the transcription rate of a reporter gene in transient transfection studies, and that this could be associated with the different IL-6 responses to stressful stimuli.

To analyse the role of the G/C polymorphism at position -174 in the function of the IL-6 gene, we examined the cord blood plasma IL-6 levels in neonates in relation to the presence of the IL-6G and IL-6C alleles. We assumed that the normal labour-related stress provides a physiological stimulus for cytokine production. We also considered that the cytokine plasma levels from cord blood are worth studying, since healthy neonates lack previous exposure to exogenous antigens, and their native cytokine production could be primarily driven by their genetic information.

MATERIALS AND METHODS

Subjects

Cord blood was collected from umbilical veins of 50 healthy, full-term newborns after normal vaginal delivery and from umbilical veins of 42 healthy, full-term neonates after elective caesarean section (collection was kindly performed by the staff of the Department of Obstetrics, Tampere University Hospital, Finland). Previous delivery by caesarean section, poor presentation, disproportion, fear of delivery and placenta praevia were indications for elective caesarean sections, in order of frequency. Collected cord blood samples were analysed only if they met evaluation criteria. Evaluation consisted of health and the medication of mothers, the nationality of parents and a gestational age and Apgar scores of neonates. All 450 adult blood samples were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere. The donors were adults (18-60 years old) and, according to the information on questionnaires, they had had no blood-transmitted diseases or any signs of other infections during a 2-week period

prior to the blood donation. Samples were collected into plastic tubes treated with citrate. From the blood samples, plasma was separated, aliquoted and stored at - 20° C until further use. From the remaining blood, the mononuclear cells were isolated by Ficoll-Isopaque centrifugation (Pharmacia, Uppsala, Sweden). The approval for human blood use was given by the ethics board of the Finnish Red Cross Blood Transfusion Centre.

Analysis of IL-6 gene polymorphism

Genomic DNA was isolated from the mononuclear cells using the salting out method [23]. The IL-6 promoter polymorphism at position -174 was analyzed as previously described [22]. Briefly, oligonucleotides 5'TGACTTCAGCTTTACTCTTGT3' and 5'CTGATTGGAAACCTTATTAAG3' were used as primers in the polymerase chain reaction (PCR). Conditions used were: five cycles of 96° C for 9 min, 55° C for 1 min and 72° C for 3 min, followed by 30 cycles of 95° C for 1 min, 55° C for 1 min and 72° C for 1 min, with a final incubation at 72° C for 10 min. The PCR products were digested with 5 units of *Nla*III at 37° C for 24 hours and analyzed on a 9% polyacrylamide gel stained with ethidium bromide. This generated fragments of 119 bp + 49 bp for allele 2 and a single fragment of 168 bp for allele 1.

Measurement of IL-6 plasma levels

Cord blood IL-6 plasma levels after normal vaginal delivery (N = 50), after elective caesarean section (N = 42) and adult IL-6 plasma levels (N = 400) were measured using an enzyme-linked immunosorbent assay (ELISA; CLB, Pelikine Compact human IL-6 ELISA kit, Amsterdam, The Netherlands). The sensitivity of the assay was 0.2-0.4 pg/ml and the assay was performed according to the manufacturer's instructions.

Measurement of IL-6 production

IL-6 production *in vitro* was analyzed in 50 cord blood and 50 adult blood samples. Unstimulated and LPS-stimulated mononuclear cells (10^6 cells/ml and 1 ml/well) were incubated at 37° C for 24 hours. LPS was used at a final concentration of 1 mug/ml. The cells were then harvested and the supernatants were collected and stored at - 20° C. IL-6 levels were measured as described above.

Data analysis

Statistical methods appropriate for non-parametric data were used throughout. The Mann-Whitney U-test and the Kruskal-Wallis test were used to test for significant associations between IL-6 production and IL-6 genotypes, and a test value of $p < 0.05$ was considered significant. Statistical calculations were performed using SPSS for Windows, version 6.1 (SPSS Inc., Chicago, Illinois, USA). The Hardy-Weinberg equations were calculated using the Arlequin program, version 1.1 (Genetics and Biometry Lab, University of Geneva, Switzerland).

RESULTS

C allele frequency

The IL-6 genotype frequencies were in Hardy-Weinberg equilibrium. The frequencies of the C allele in neonates born by vaginal delivery (N = 50) and in neonates born by elective caesarean section (N = 42) were comparable with the frequencies found in the two adult groups (N = 400 and N = 50); respective values were 0.52, 0.61, 0.55 and 0.54. There is a previous study on a G/C polymorphism at position -174 of the IL-6 gene where the authors found a C allele frequency of 0.40 in a group of 383 healthy males and females in the United Kingdom [22].

IL-6 plasma levels

Our results demonstrated that IL-6 plasma levels in neonates were significantly higher than those in adults (neonates born by VD *versus* adults $p < 0.001$ and neonates born by ECS *versus* adults $p < 0.001$); the median value for neonates born by VD was 11.4 pg/ml (4.5-45.9), for neonates born by ECS it was 2.9 pg/ml (1.9-6.4) and for adults, 1.2 pg/ml (0.7-2.0) (Figure 1). It was also observed that newborns born by VD had significantly higher IL-6 plasma levels than those born by ECS ($p < 0.001$) (Figure 1). This finding suggests that normal labour-related stress could induce IL-6 production in newborns.

An analysis was carried out to ascertain if there was a genetic association between different IL-6 genotypes and IL-6 plasma levels in neonates. It was noticed in the group of VD, that CC genotypes had increased IL-6 plasma levels compared with GC or GG genotypes ($p = 0.08$); respective

median values were 21.4 pg/ml (9.5-81.3), 9.7 pg/ml (3.4-46.3) and 6.0 pg/ml (3.5-24.5). This finding was also confirmed when the comparison was made according to the carrier status. Non-carriers of the G allele secreted significantly more IL-6 than carriers of the G allele ($p < 0.03$) (Figure 2); 21.4 pg/ml (9.5-81.3) and 9.6 pg/ml (3.5-36.2). Furthermore, section newborns with genotype CC had higher IL-6 plasma levels than carriers of the G allele ($p < 0.02$); respective median values were 6.3 pg/ml (2.2-12.9) and 2.7 pg/ml (1.7-4.1). No association was found between IL-6 plasma levels and different IL-6 genotypes in the adult controls (Figure 3).

IL-6 production after LPS stimulation

Next, our *in vivo* results were confirmed with *in vitro* experiments. The results again suggested that after LPS stimulation, the mononuclear cells of neonates of the CC genotype secreted more IL-6 than those of the GC or GG genotype ($p = 0.05$); the median value for CC genotypes was 17,425 pg/ml (11,400-33,900), for GC genotypes 14,000 pg/ml (3,260-22,725), and for GG genotypes 5,850 pg/ml (4,535-11,365). This finding was also supported when IL-6 production was compared between carriers of the G allele and non-carriers of the G allele. IL-6 levels were significantly lower in carriers of the G allele than in non-carriers ($p < 0.04$); respective median values were 6,980 pg/ml (4,175-16,800) and 17,425 pg/ml (11,400-33,900) (Figure 4). There was no difference in IL-6 production by adult cells between carriers of the G allele G and non-carriers of the G allele ($p = 0.68$) (Figure 5). IL-6 production by neonatal or unstimulated adult cells was not associated with the IL-6 -174 polymorphism.

Additionally, we observed that unstimulated adult cells secreted spontaneously more IL-6 than neonate ($p < 0.03$) (Figure 6); the median value for adult cells was 621 pg/ml (211-1,470) and the median value for newborn cells was 271 pg/ml (93-977). However, after LPS stimulation mononuclear cells from neonates produced amounts of IL-6 comparable with adult cells ($p = 0.15$) (Figure 6); the corresponding median values were 13,100 pg/ml (4,594-22,875) and 10,380 pg/ml (3,740-14,750).

DISCUSSION

The aim of this study was to determine the capacity of newborns to produce IL-6 *in vivo* and *in vitro*. In most of the studies so far, the detection of the IL-6

secretion ability of neonates has been performed either by measuring plasma levels or by measuring the cellular production after stimulation *in vitro*. We also wanted to find out whether this naive IL-6 response of newborns was associated with a promoter polymorphism of the IL-6 gene.

The first finding of this study was the significantly higher cord blood IL-6 plasma levels after vaginal delivery compared with those after elective caesarean section ($p < 0.001$); the median value for neonates born by VD was 11.4 pg/ml (4.5-45.9) and the median value for neonates born by ECS was 2.9 pg/ml (1.9-6.4). Increased IL-6 plasma levels after VD suggest that the normal labour-related stress by itself provides a physiological stimulus for IL-6 production. Various factors during a normal delivery may induce IL-6 secretion in neonates. IL-6 is produced in response to other cytokines, such as TNF-alpha and IL-1. These cytokines are present in the cord blood of healthy, full-term neonates [16] and increased levels of these cytokines have also been found in the circulation and in the amniotic fluid of women in labour [24]. After membrane rupture, a neonate is exposed to a variety of aerobic and anaerobic bacteria in the mother's genital tract. This may be one of those mechanisms stimulating the release of IL-6 in newborns, since bacterial products, for example lipopolysaccharide (LPS) of gram negative bacteria, are potent stimulators of IL-6 production. Actually, this exposure of newborns may occur even earlier, since Romero *et al.* [25] showed that nearly 20% of women at term have subclinical microbial invasion of the amniotic cavity. On the other hand, it has been observed that IL-6 is increased by physical activity [26] and that the physical strain of labour is associated with the IL-6 concentration in maternal serum [27]. Thus, it is not surprising that the physical stress of labour also effects cytokine plasma levels in cord blood. In line with our results, Opsjon *et al.* and Buonocore *et al.* found higher IL-6 plasma levels in mothers and their newborns after normal vaginal delivery than in mothers and babies after elective caesarean section [24, 28].

Since IL-6 is a proinflammatory cytokine that is normally tightly regulated and expressed at low levels except during infection, trauma or other stress [29], low IL-6 levels of adult controls were not surprising. However, ECS newborns without normal labour-related stress had significantly elevated IL-6 levels compared to those of adult controls ($p < 0.001$). Although there are not enough data available on fetal production of IL-6 during advanced pregnancy, the observation may reflect the involvement of IL-6 in an inflammation-like process at a normal term

[7, 8]. Similarly, the increased concentrations of inflammatory cytokines were measured healthy neonates during the perinatal period [30]. The design of the present study does not allow any conclusions as to whether elevated levels of IL-6 in cord blood reflect those found in maternal serum. Present data on the transfer of cytokines through the placenta in both materno-fetal and feto-maternal directions are contradictory [16, 24, 31]. Berner *et al.* [16] have demonstrated that plasma levels of proinflammatory cytokines were significantly higher in the cord blood of septic infants than the corresponding maternal levels. The observation suggests that only very small amounts of cytokines may be transferred through the placenta. Accordingly, no association was observed between the amount of IL-6 in amniotic fluid or maternal or cord sera in data from Opsjon *et al.* [24]. In contrast, De Jongh *et al.* found a significant positive correlation between cord blood IL-6 levels and maternal serum IL-6 levels [31], although this finding does not exclude endogenous IL-6 production by healthy newborns. Our *in vitro* data also suggest that neonates are capable of producing IL-6; neonatal mononuclear cells secreted large amounts of IL-6 after LPS-stimulation (Figure 6). Further studies are required to find out whether this increased endogenous IL-6 production of neonates has some physiological significance.

Additionally we noted that IL-6 levels varied widely between neonates; a range of IL-6 levels of 1.2-625 pg/ml in neonates born by VD, and 0-424 pg/ml in neonates born by ECS were observed. All cord blood samples were collected from mature neonates, who were healthy at the time of delivery and who were delivered without signs of maternal infection or maternal or peripartal risk factors. In a quite similar study involving 35 cord blood samples, a range of IL-6 plasma values 0.4-897 pg/ml [16] was found, which is similar to our range of values. Furthermore, it had been observed previously that a wide range of IL-6 values is found particularly in sepsis patients [15-17, 32] and that the magnitude of the increase of IL-6 levels correlates with mortality [15]. Taken together, it becomes tempting to suggest that this wide variation of IL-6 plasma values in stress conditions could be explained by genetic factors. Austgulen *et al.* also reported a wide variation of soluble IL-6 receptors (IL-6Rs) in the serum of healthy pregnant women, and that these differences of individual IL-6Rs levels remained constant through-out the pregnancy period [8].

The C allele frequencies in our study groups (0.52-0.61) were slightly different from those published earlier [22] in a group of 383 healthy males and females in

the United Kingdom (0.40), probably reflecting the interethnic variation in the frequencies of this polymorphism. Although in the same study Fishman *et al.* [22] demonstrated that IL-6 levels were lower in healthy adults with the CC genotype compared with GC or GG subjects, we could not find an association between IL-6 production and this polymorphism of the IL-6 gene in adult controls.

CONCLUSION

Our results suggest that the -174 polymorphism of the IL-6 gene participates in the regulation of the IL-6 response in neonates; IL-6 plasma levels and IL-6 secretion after stimulation were increased in neonates with the CC genotype. Since this polymorphic site was not associated with the baseline and inducible IL-6 levels of adults, the present study suggests the occurrence of a different regulation mechanism of the IL-6 response in the cells of neonates and in the cells of healthy adults. The underlying mechanism for this difference is not clear. One possible explanation for this phenomenon could be the naive immune system of a neonate. Our *in vitro* data indicated that the repeatedly stimulated adult cells spontaneously secreted more IL-6 than the naive neonatal cells (Figure 6). Cytokine gene expression is usually tightly controlled at the level of transcription by the co-ordinated binding of multiple transcription factors to regulatory elements in the promoter region. Although those factors binding at position -174 of the IL-6 gene are not known, there are probably differences in factors in naive neonatal cells and in repeatedly stimulated adult cells. Additionally, recent results have shown that the relationship between cytokine expression and the single polymorphic site is not as simple as might be expected; rather than a single polymorphic site it may be the combination of base changes at several sites on the promoter, *i.e.* the haplotype, that has effect on IL-6 expression [33]. It also seems obvious that there is a cell type and stimulation-specific regulation of IL-6 expression. Thus, it is highly unlikely that the C allele of the -174 polymorphism alone represents the susceptibility site for increased IL-6 production in neonates and more likely that it may interact with other sites on the IL-6 promoter to produce the increased production of IL-6.

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Figures

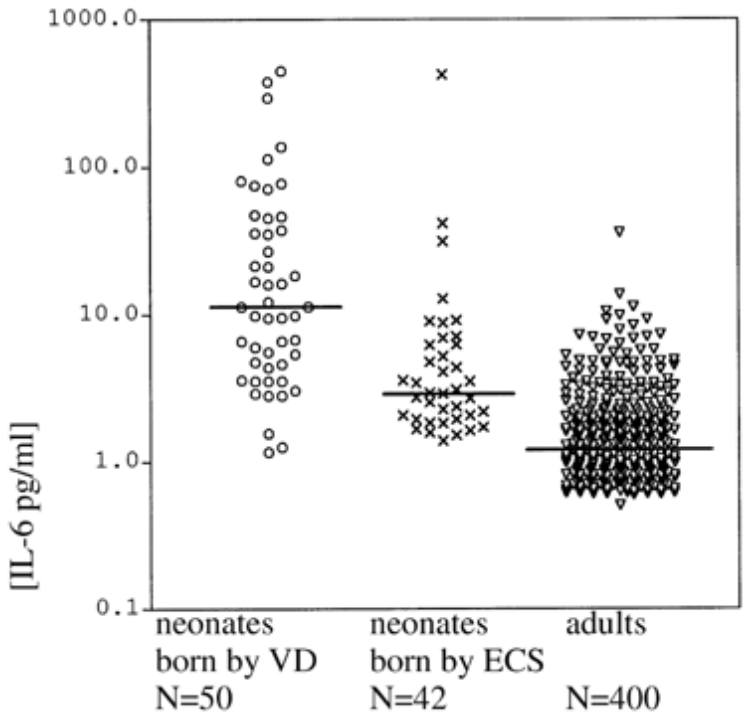


Figure 1

IL-6 plasma levels in neonates born by vaginal delivery, neonates born by elective caesarean section, and adults.

IL-6 levels were measured using a specific enzyme-linked immunoassay and the sensitivity of the assay was 0.2-0.4 pg/ml. Each dot represents the value of one sample, and bars indicate group medians. IL-6 plasma levels in neonates were significantly higher than those of adults (neonates born by VD *versus* adults $p < 0.001$ and neonates born by ECS *versus* adults $p < 0.001$); the median value for neonates born by VD was 11.4 pg/ml (4.5-45.9), for neonates born by ECS 2.9 pg/ml (1.9-6.4) and for adults it was 1.2 pg/ml (0.7-2.0). Also cord blood IL-6 levels after VD differed significantly from those after ECS ($p < 0.001$).

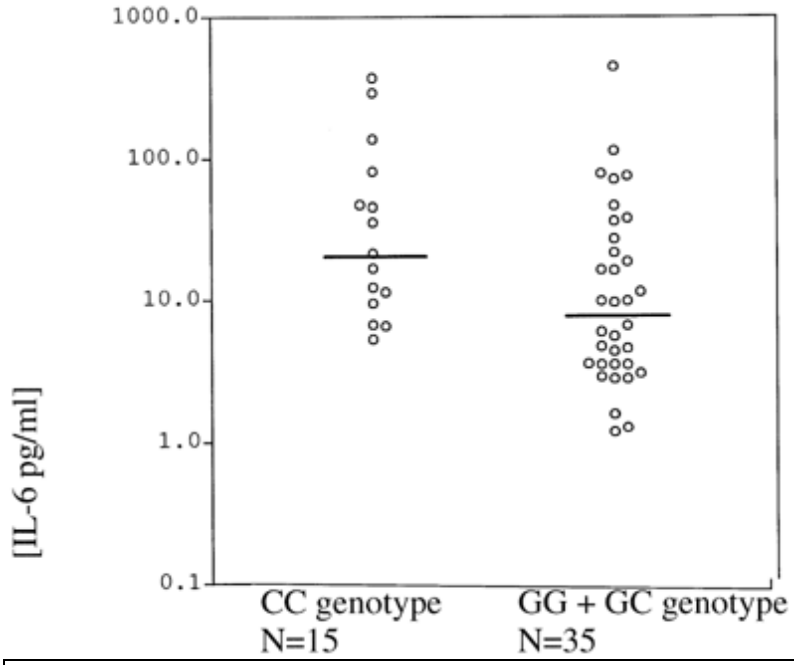


Figure 2

IL-6 plasma levels in neonates born by vaginal delivery grouped according to their IL-6 genotype.

IL-6 levels were measured using a specific enzyme-linked immunoassay and the sensitivity of the assay was 0.2-0.4 pg/ml. The IL-6 promoter polymorphism at position -174 was analysed using PCR. Each dot represents the value of one sample, and bars indicate group medians. Neonates with the CC genotype secreted significantly more IL-6 than carriers of the G allele ($p < 0.03$); respectively 21.4 pg/ml (9.5-81.3) and 9.6 pg/ml (3.5-36.2)

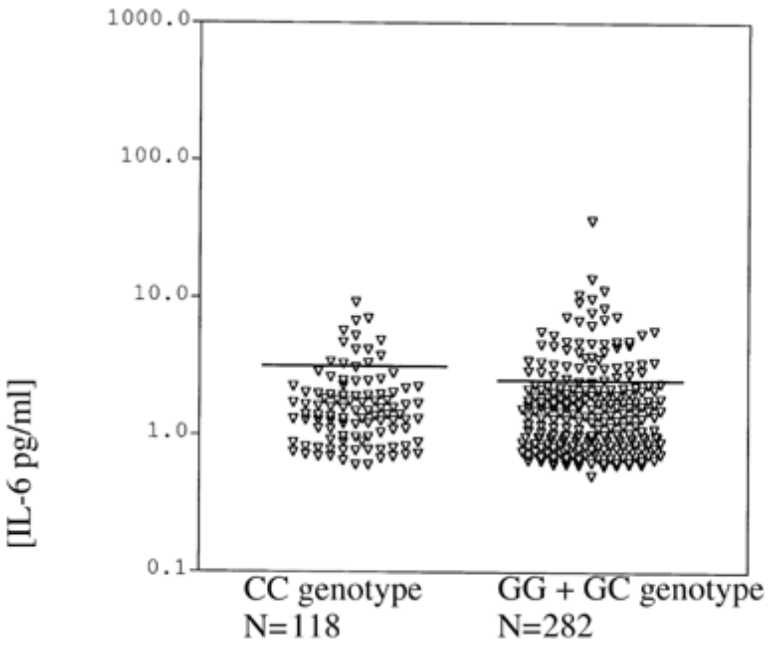


Figure 3

IL-6 plasma levels in adults grouped according to their IL-6 genotype.

IL-6 levels were measured using a specific enzyme-linked immunoassay and the sensitivity of the assay was 0.2-0.4

pg/ml. The IL-6 promoter polymorphism at position -174 was analysed using PCR. Each dot represents the value of one sample, and bars indicate group medians. IL-6 levels of carriers of the G allele were similar to those of adults with the CC genotype (p = 0.28); respectively 1.1 pg/ml (0.7-2.0) and 1.3 pg/ml (0.8-2.0).

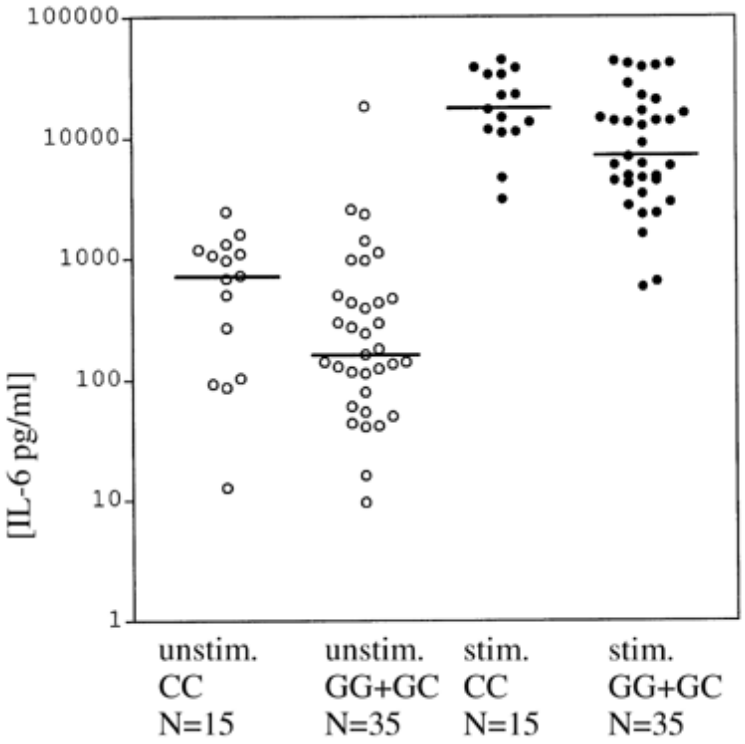


Figure 4

IL-6 production by neonatal mononuclear cells grouped according to their IL-6 genotype.

Unstimulated and LPS-stimulated mononuclear cells were incubated at 37° C for 24 hours. LPS was used at a final concentration of 1 mug/ml. IL-6 levels and the IL-6 -174 polymorphism were analysed as described in Figure 3. Each dot represents the value of one sample, and bars indicate group medians. IL-6 levels were significantly lower in carriers of the G allele (stim. GG + GC) than in

non-carriers (stim. CC) ($p < 0.04$); corresponding median values were 6,980 pg/ml (4,175-16,800) and 17,425 pg/ml (11,400-33,900). IL-6 production of unstimulated cells (unstim. CC and unstim. GG + GC) did not associated with the IL-6 -174 polymorphism.

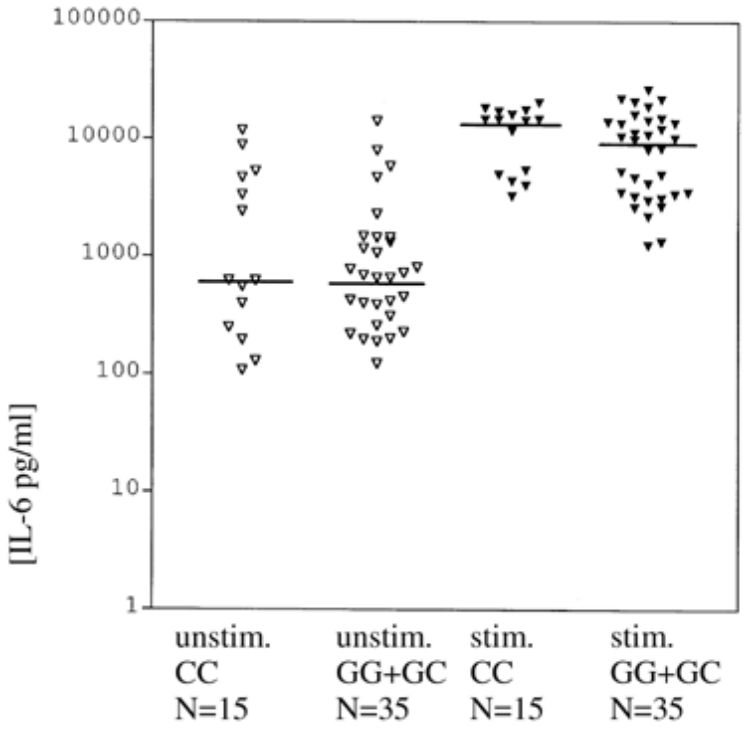


Figure 5

IL-6 production by mononuclear cells of adults grouped according to their IL-6 genotype.

Unstimulated and LPS-stimulated mononuclear cells were incubated at 37° C for 24 hours. LPS was used at a final concentration of 1 mug/ml. IL-6 levels and the IL-6 -174 polymorphism were analysed as described in Figure 3. Each dot represents the value of one sample, and bars indicate group medians. There was no difference in IL-6 production by adult cells between carriers of the G allele and non-carriers of the G allele ($p = 0.68$); respective

median values were 9,065 pg/ml (3,318-14,025) and 14,600 pg/ml (4,960-17,100). IL-6 production by unstimulated cells (unstim. CC and unstim. GG + GC) was not associated with the IL-6 -174 polymorphism.

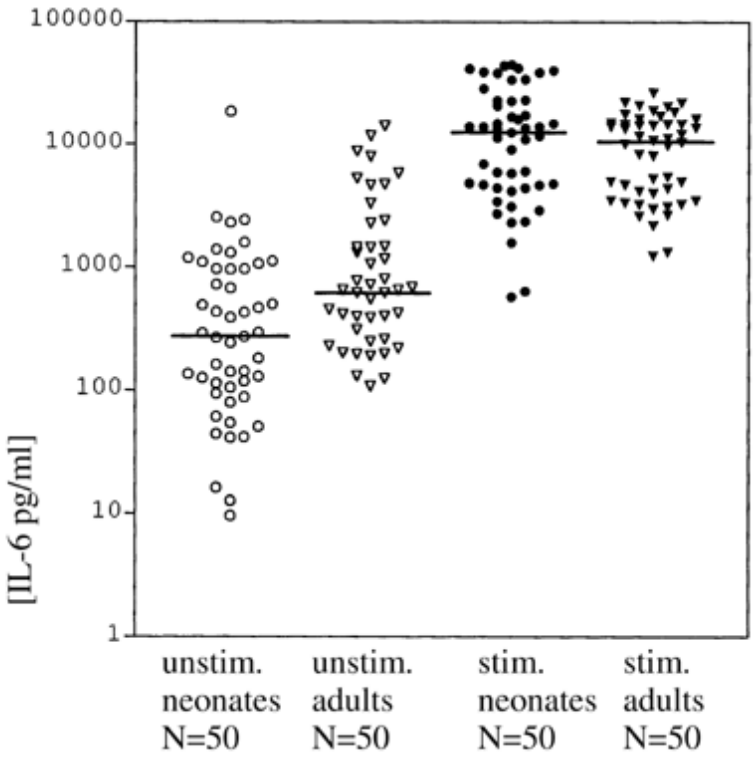


Figure 6

IL-6 production by neonatal and adult mononuclear cells.

Unstimulated and LPS-stimulated mononuclear cells were incubated at 37° C for 24 hours. LPS was used at a final concentration of 1 mug/ml. IL-6 levels were measured using a specific enzyme-linked immunoassay and the sensitivity of the assay was 0.2-0.4 pg/ml. Each dot represents the value of one sample, and bars indicate group medians. Unstimulated cells of adults secreted significantly more IL-6 spontaneously than cells of neonates ($p < 0.03$); respective median values were 621

pg/ml (211-1,470) and 271 pg/ml (93-977). However, after LPS stimulation cells of neonates produced amounts of IL-6 which were comparable to those levels produced by adult cells (p = 0.15); the corresponding median values were 13,100 pg/ml (4,594-22,875) and 10,380 pg/ml (3,740-14,750).

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human, IFN-g, IFN-g receptor, signal transduction, cellular proliferation, apoptosis.

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IL-10, recombinant, rat, cytokines.

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