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Effect of immunoglobulin therapy on phagocytosis by polymorphonuclear leucocytes in whole blood of neonates

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SUMMARY

There has been some disagreement as to the clinical effect of intravenous immunoglobulin (IVIG) therapy on neonatal bacterial infections. We therefore evaluated the effect of IVIG therapy on neonatal polymorphonuclear leucocyte (PMN) functions by monitoring phagocytosis and hydrogen peroxide (H_2O_2) production. Subjects were 10 mature neonates who had normal plasma levels (i.e. equal to adult plasma levels) of IgG and nine premature neonates who had lower plasma levels of IgG. Phagocytosis by PMN was measured using flow cytometric analysis of whole blood. Addition of γ-globulin to the whole blood of mature neonates increased phagocytosis, but not significantly. Higher doses of added γ-globulin (> 2·0 mg/ml, the concentration was expressed as that in the final reaction volume) decreased phagocytosis to under baseline level. In premature neonates addition of γ-globulin increased phagocytosis and the significant maximum effect was observed with 0·5 mg/ml of the additional γ-globulin. Higher doses of additional γ-globulin (2·5 mg/ml) decreased phagocytosis to baseline level. Phagocytosis in four mature and four premature neonates was compared before and after 1 g/kg of IVIG therapy. Phagocytosis in mature neonates after IVIG therapy did not change compared with the pretreatment level. On the other hand, phagocytosis in premature neonates after IVIG therapy significantly increased compared with its pretreatment level. In both mature and premature neonates H_2O_2 production following phagocytosis varied in parallel with changes of phagocytosis. The patterns of H_2O_2 production following phagocytosis were essentially similar to those observed with phagocytosis. The above results are expected to form the basis for a rational indication for IVIG therapy against bacterial infections in neonates with low plasma IgG levels.

Keywords polymorphonuclear leucocytes neonates phagocytosis hydrogen peroxide production immunoglobulin therapy

INTRODUCTION

Serum IgG is one of the essential humoral factors for efficient opsonization of bacteria to be ingested by polymorphonuclear leucocytes (PMN) [1]. The fetus receives IgG from the mother via the placenta and most of the IgG is transferred after 34 weeks of gestation [2], and therefore preterm neonates have lower levels of plasma IgG than term neonates [3]. Low concentrations of plasma IgG in premature neonates are widely speculated to be a crucial contributory factor to the increased susceptibility to infections seen in the neonatal period [4–8].

Immunoglobulin preparations contain variable amounts of opsonic antibody for several bacterial pathogens [9,10] and their clinical efficacy has been well established in replacement therapy for patients with hypogammaglobulinaemia [11,12]. Intravenous immunoglobulin (IVIG) has been widely used prophylactically in premature neonates at risk for septicaemia [13–18], and actually has been incorporated into the treatment regimen of bacterial infections of neonates [19–23]. IVIG has been shown to yield a favourable clinical outcome in certain neonates [13–15,19–21], but several groups have suggested that IVIG has neither prophylactic nor therapeutic effect on such neonates [16–18,22,23]. Although bacterial clearance by IVIG therapy has been investigated *in vivo* [24,25], there has been no study of PMN functions associated with IVIG therapy *in vivo* and *in vitro*.

By a two-colour-flow cytometric assay, we can simultaneously measure opsonic activity of plasma, capacity of phagocytosis and hydrogen peroxide (H_2O_2) production at a single-cell level. In this study we attempted to determine whether or not *in vivo* and *in vitro* IVIG therapy could be effective in neonates by examining PMN phagocytosis and H_2O_2 production.

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SUBJECTS AND METHODS

Subjects

The subjects were 10 mature neonates (gestational age 39·3 ± 0·5 weeks (mean ± s.d.); birthweight 3147 ± 361 g; IgG level 1267 ± 122 mg/dl; number of PMN 5732 ± 1270/μl with a normal differential) and nine premature neonates (gestational age 33·8 ± 1·4 weeks; birthweight 1689 ± 310 g; IgG level 612 ± 88 mg/dl; number of PMN 5284 ± 1456/μl with a normal differential). After informed consent was obtained from parents, heparinized (100 U/ml) venous blood was collected within the first 3 days of life. All neonates were products of uncomplicated pregnancy, labour, and delivery, during which no general or i.v. anaesthesia was administered. Their Apgar scores at 1 or 5 min were ≧8, and their physical examinations were normal. Four neonates from each group, who developed jaundice 4–5 days after birth, were infused with IVIG for 6–8 h (1 g/kg, once) for hyperbilirubinaemia [26]. The specimen of venous blood was collected before and within 6 h after the infusion, respectively.

Materials

2′7′-dichlorofluorescein diacetate (DCFH-DA; Eastman Kodak, Rochester, NY) was dissolved in ethanol at a concentration of 25 mM and stored in the dark at 4°C. Propidium iodide (PI; Sigma, St Louis, MO) was dissolved in normal saline at a concentration of 5%. Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS, ethylene diamine tetraacetic acid (EDTA) and sodium azide were from Wako Chemicals (Osaka, Japan). PBS containing 5 mM glucose and 0·1% gelatin (PBSg) was used for suspension. Phorbol myristate acetate (PMA; Sigma) was dissolved in ethanol at a concentration of 25 μg/ml.

We used preparations of human IVIG (Venoglobulin IH; Green Cross Co., Osaka, Japan). Fc and Fab fragments of IgG were obtained from Seikagaku Co. (Tokyo, Japan). Purified whole molecule of IgG and the vehicle for Venoglobulin IH were kindly supplied by Green Cross Co.

Staphylococcus aureus, strain ATCC 25923 (kindly supplied by Shionogi Pharmaceutical Co., Osaka, Japan), was cultured for 18 h in tryptic soy broth (Difco, Detroit, MI) at 37°C. Subsequently the bacteria were heat-killed at 60°C for 30 min, washed three times in normal saline, and labelled with PI for 30 min at room temperature in the dark. The fluorescent bacteria were washed three times in normal saline and suspended in PBSg. Bacterial density was adjusted to an absorbance value of 2·50 at 620 nm with a UV-Visible Recording Spectrophotometer 240 (Shimadzu, Kyoto, Japan). The number of bacteria at this density was about $2·4 \times 10^9$ colony-forming units (CFU)/ml. Aliquots were stored at −80°C and thawed immediately before use.

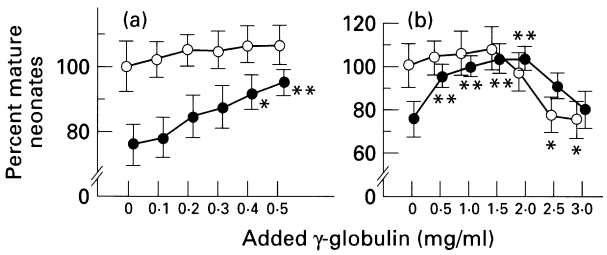

Fig. 1. Effect of adding γ-globulin on phagocytosis by polymorphonuclear leucocytes (PMN). Bars represent mean ± s.d. Numbers on the ordinate indicate percentage against the baseline of the mature neonates. Numbers on the abscissa indicate added γ-globulin concentration: (a) 0–0·5 mg/ml; (b) 0–3·0 mg/ml. ○, Mature neonates; ●, premature neonates. *$P < 0·05$; **$P < 0·01$ *versus* each baseline.

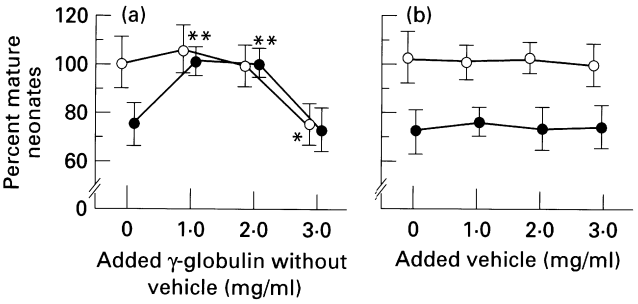

Fig. 2. Effect of purified whole IgG molecule and the vehicle on phagocytosis by polymorphonuclear leucocytes (PMN). Bars represent mean ± s.d. (a) Purified whole IgG molecule. (b) The vehicle. ○, Mature neonates; ●, premature neonates. *$P < 0·05$; **$P < 0·01$ *versus* each baseline.

Methods

Measurement of phagocytosis and H_2O_2 production has been described previously [27]. Briefly, a mixture consisting of the following ingredients was prepared in a plastic tube, the final reaction volume being 1000 μl: 100 μl heparinized whole blood, 150 μl DCFH-DA in PBSg, 650 μl PI-labelled *S. aureus* suspension and 100 μl normal saline or IgG preparation. The amount of each IgG concentration in the Figures was expressed as that in the final reaction volume of 1000 μl. The tube was incubated with rotational agitation for 30 min at 37°C in a shaking water bath, and then 2·0 ml of 3 mM EDTA were added to terminate phagocytosis and prevent bacterial adherence to the PMN membrane. Erythrocytes were afterwards removed by hypotonic lysis for 20 s. After centrifugation, each cell pellet was finally resuspended in 1 ml of 3 mM EDTA in PBSg and was applied to flow cytometry.

A mixture of 100 μl heparinized whole blood, 1·8 ml DCFH-DA in PBSg containing 5 mM sodium azide, 10 μl PMA and 100 μl normal saline or IgG preparation was incubated with rotational agitation for 30 min at 37°C in a shaking water bath. Residual erythrocytes were afterwards removed by hypotonic lysis for 20 s. After centrifugation, each cell pellet was finally resuspended in 1 ml PBSg and was applied to flow cytometry.

Intracellular PI and DCF fluorescence of PMN were determined by flow cytometry (EPICS PROFILE; Coulter Co, Hialeah, FL). Quantification of phagocytic activity and H_2O_2 production were estimated by mean PI and DCF fluorescence per cell, respectively. They were determined using the following formula:

$$\frac{\text{Sum of (PMN count on each channel} \times \text{channel number)}}{\text{Total PMN count}}$$

At least 5000 PMN were examined in each sample.

Data were presented as mean ± s.d. Statistical analysis was performed by Dunnett's *post-hoc* procedure for comparisons of multiple means and two-group *t*-test for comparison of means for paired samples.

RESULTS

There was a good positive correlation between phagocytosis and plasma IgG levels in both groups ($r = 0·784$, $P < 0·01$).

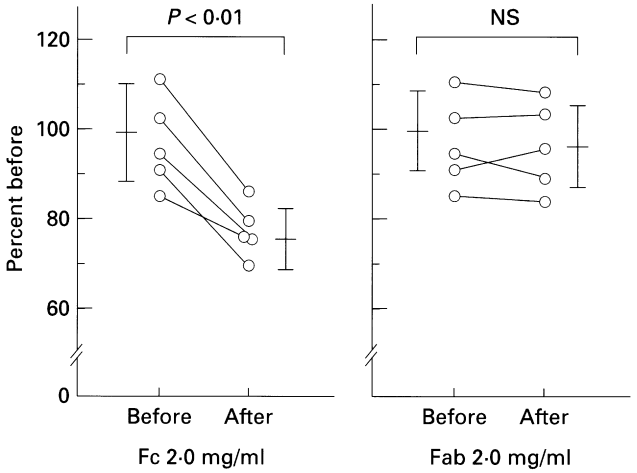

Fig. 3. Comparison of phagocytosis before and after addition of each fragment. Each 2·0 mg/ml of either Fc or Fab fragment was prepared so as to give >3·0 mg/ml of IgG molecule. Numbers on the ordinate indicate percentage against that before addition. Bars represent mean ± s.d.

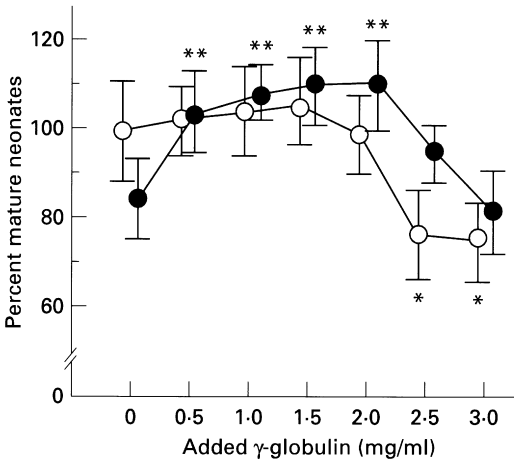

Fig. 5. Effect of adding γ-globulin on H_2O_2 production following phagocytosis. Bars represent mean ± s.d. ○, Mature neonates; ●, premature neonates. *$P < 0·05$; **$P < 0·01$ *versus* each baseline.

Phagocytosis by PMN in vitro

Figure 1a,b shows the effect of added γ-globulin on PMN phagocytosis in both groups of neonates. In the mature neonates (open circles), phagocytic activity gradually increased, but there were no significant differences in phagocytic activity among the concentrations of added γ-globulin. At 1·5 mg/ml phagocytosis showed peak activity, and over 2·5 mg/ml it significantly decreased compared with the control with no addition. In premature neonates (closed circles), the enhancement of phagocytosis was largely in parallel with the added dose of γ-globulin up to 0·5 mg/ml, after which there was a gradual but not significant increase to 2·0 mg/ml. The decline thereafter was similar to the pattern observed in mature neonates.

Patterns of PMN phagocytosis following the addition of the purified whole molecule of IgG from IVIG were similar both in mature and in premature neonates as with the IVIG (Fig. 2a). On the other hand, the addition of the vehicle alone from the IVIG was without any effect on PMN phagocytosis, both in mature and in premature neonates (Fig. 2b).

Each 2·0 mg of an Fc or Fab fragment was added to whole blood of five mature neonates and examined for the effect on phagocytosis. Treatment with the Fc fragment significantly decreased phagocytic activity ($P < 0·01$). On the other hand, the Fab fragment did not influence PMN phagocytosis (Fig. 3).

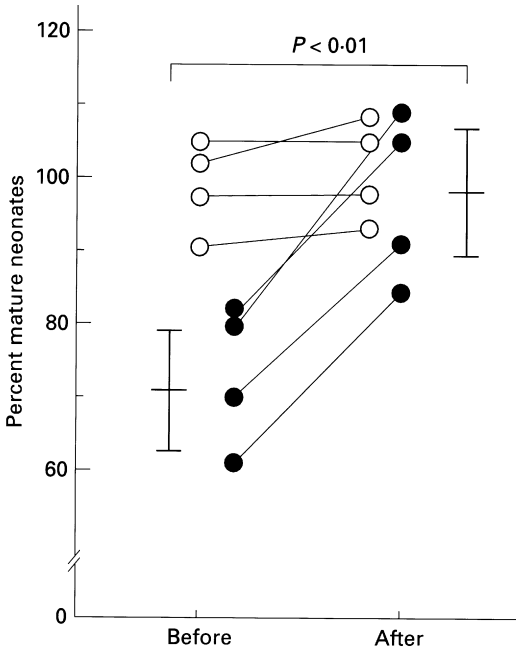

Fig. 4. Effect of intravenous immunoglobulin (IVIG) therapy on phagocytosis by polymorphonuclear leucocytes (PMN). Bars represent mean ± s.d. ○, Mature neonates; ●, premature neonates.

Phagocytosis by PMN after IVIG therapy

By infusing 1 g/kg of IVIG, plasma IgG levels increased from 1141 ± 102 mg/dl to 2003 ± 144 mg/dl in mature neonates and from 583 ± 68 mg/dl to 1398 ± 121 mg/dl in premature neonates.

Phagocytosis was compared before and after IVIG therapy. In mature neonates, phagocytosis after IVIG did not significantly change compared with that before therapy, while in premature neonates it significantly increased ($P < 0·01$), being equivalent to that of mature neonates (Fig. 4).

H_2O_2 production following phagocytosis

H_2O_2 production following phagocytosis by PMN (Fig. 5) varied almost in parallel with changes of phagocytosis (Fig. 1b). The patterns of H_2O_2 production following phagocytosis were essentially similar to those observed with phagocytosis.

H_2O_2 production with PMA stimulation

Added γ-globulin, ranging between 0 and 3·0 mg/ml, did not influence H_2O_2 production with PMA stimulation either in mature or in premature neonates (data not shown).

DISCUSSION

Opsonins and PMN are essential components for efficient phagocytosis to take place [28]. When sufficient amounts of opsonin are being provided, neonatal PMN of both mature and premature

neonates are able to amply ingest foreign particles and to initiate respiratory burst [29]. In general, IgG and activated complement components constitute the opsonic activity [28]. In newborns, however, the complement system is still immature and its role in opsonization is suggested to be less contributory than IgG [1]. Besides, IgM, a potent opsonin for Gram-negative organisms, is not provided by the placental transfer. When these observations are collectively taken into consideration, IgG may therefore be reasonably regarded as a major determinant in evaluating the opsonic activity of newborn neonates. Our current assay system consists of whole blood, supplying PMN and a varying amount of inherent IgG, *S. aureus* as phagocytizable particles, and an exogenous IgG preparation. As the PMN were largely equal in quantity in both groups of neonates with an equal dose of bacteria to be ingested, the overall phagocytic process could be concluded to be largely regulated by the amount of IgG in the reaction system.

A high dose of IVIG is said to block the reticuloendothelial system [30], but its precise mechanism remains to be determined. The C2 domain is assumed to reside in the Fc fragment of the IgG molecule, which binds to reticuloendothelial Fc receptors. As the reticuloendothelial system can be inhibited by anti-Fc receptor antibodies, Atrah & Davidson suggested that its blockade was mediated by that of Fc receptors [31]. Observations on the effect of Fc and Fab fragments on phagocytosis indicate that high doses of Fc fragment significantly suppress phagocytosis, while Fab fragment is without any effect. Inhibition with Fc fragment and its absence with Fab further support the speculation that high doses of IgG reversibly occupy Fc receptors, thereby causing blockade of phagocytosis by PMN. IgG molecules construct immune complexes and the complexes might be rapidly cleared *in vivo*. It can thus be concluded that excess IgG may lack further potentiating effect on phagocytosis both *in vitro* and *in vivo*, but may rather inhibit it *in vitro*.

Hasui *et al.* reported that a positive correlation exists between phagocytosis and H_2O_2 production following ingestion of *S. aureus* [27]. PMA is known to directly activate protein kinase C, bypassing the process of phagocytosis [32]. When stimulated with PMA, addition of IgG did not have any appreciable effect on H_2O_2 production with PMN of both mature and premature neonates. Therefore suppression of H_2O_2 production following phagocytosis in the presence of excess of IgG does not appear to result from reduced potency of H_2O_2 production, but from suppression of phagocytosis by IgG.

Studies of the effect of IVIG on the course of hospitalization of neonates have generated conflicting data [13–23], and discrepancies could be explained by three major items of evidence. First, a number of PMN abnormalities are known on the host defence system in high-risk premature neonates, such as relatively low activity of PMN adherence to vascular endothelia, aggregation and chemotaxis [5,6]. Eisenfeld *et al.* reported that adult fresh frozen plasma enhanced impaired chemotaxis of neonatal PMN owing to supply of complement and fibronectin [33]. IVIG therapy which aims to compensate for the deficit of immunoglobulin could not be expected to restore such impairment of PMN functions to yield favourable clinical outcome and survival in such neonates [17,18]. Second, neonates with birthweights above 1500 g may have nearly normal γ-globulin plasma concentrations, in whom IVIG may not increase phagocytosis [14,15,19]. Third, the administration of IVIG to maintain the expected level of IgG may improve outcome from infections [18], because the half-life of the terminal elimination phase of plasma IgG increased in premature neonates [14]. Clapp *et al.* demonstrated that the incidence of nosocomial sepsis was less frequently observed in neonates whose serum IgG levels were maintained at near 700 mg/dl by the administration of IVIG than in neonates with lower serum IgG levels [13]. Based on our experimental results and previous clinical studies, IVIG treatment for bacterial infections may not be effective in mature neonates, but could be beneficial for premature neonates with low serum IgG concentrations.

Because γ-globulin injections induce an inhibition of proliferation of T and B lymphocytes and maturation of B lymphocytes [34], polyclonal immunoglobulin production is suppressed by γ-globulin [35]. Although other mechanisms may also operate, the disagreement regarding the effect of IVIG therapy on neonatal bacterial infections may partly be ascribed to decreased PMN phagocytosis by an excess of added γ-globulin. From our data and previous studies, IVIG against bacterial infections prophylactically or therapeutically may have an effect in neonates with low plasma IgG levels. Moreover, we suggest that conventional IVIG therapy to neonates with normal plasma IgG levels may be without any favourable effect on the outcome of bacterial infections when we focus on PMN functions.

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