Intravenous Immunoglobulin Modulates Human Mononuclear Phagocyte Tumor Necrosis Factor- α Production *In Vitro*¹

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ABSTRACT. Mononuclear phagocytes (MO) secrete tumor necrosis factor- α (TNF) in response to inflammatory stimuli, most notably the bacterial product lipopolysaccharide (LPS). Cross-linking of MO Fc receptors also induces TNF release. Immunoglobulin for i.v. use is currently being investigated for the treatment and prophylaxis of neonatal sepsis and for the treatment of various syndromes of autoimmune dysfunction in children and adults. We examined the *in vitro* effect of immunoglobulin- γ (IgG) on neonatal (cord blood) monocyte and adult MO TNF production. Kinetic studies were performed on MO incubated with IgG alone and on MO preincubated with IgG and stimulated with interferon- γ /LPS. Incubation of MO in IgG (1–25 g/L) for 2, 6, and 24 h did not stimulate TNF secretion or production. However, enhanced TNF secretion was detected in MO preincubated in IgG and subsequently stimulated with interferon- γ /LPS. TNF secretion by cord blood monocytes was increasingly enhanced by preincubation for 6 h with 1, 10, and 25 g/L IgG (2413.1 ± 1389.4 , $p < 0.05$; 4070.4 ± 3069.2 , $p < 0.005$; and 6383.7 ± 2982.2 , $p < 0.005$ versus 1215 ± 575.9 ng/L, respectively, in cells preincubated in medium alone). Significant enhancement was also detected in cord blood monocytes preincubated in IgG for 2 h. TNF secretion by adult MO was similarly enhanced (6082.0 ± 1732.8 , $p < 0.05$; 7158.8 ± 3938.2 , $p < 0.05$; and 7302.7 ± 3451.4 , $p < 0.05$ versus 3353.2 ± 2946.7 ng/L for 1, 10, and 25 g/L IgG, respectively, versus preincubation in medium alone). In additional experiments performed with Fc, Fab, and F(ab')₂ fragments, only F(ab')₂ fragments yielded positive results. Northern analyses revealed increased levels of mRNA for TNF only when 25 g/L IgG were used for preincubation. Preincubation in the lower concentrations of IgG did not result in increased accumulations of TNF mRNA. Thus, IgG acts primarily posttranscriptionally to enhance interferon- γ /LPS-induced TNF release *in vitro*. (*Pediatr Res* 35: 397–403, 1994)

Abbreviations

MO, mononuclear phagocyte
TNF, tumor necrosis factor- α
LPS, lipopolysaccharide
IVIG, immunoglobulin for i.v. use

Received July 19, 1993; accepted December 2, 1993.

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Supported by a Pediatric Infectious Diseases Society Fellowship Award provided by Bristol-Meyers Squibb Company, by the University of Arkansas for Medical Sciences MCPG/CUMG Research Development Fund, and by a donation from Rosalyn and Harry Hastings.

¹Presented in part at the 1993 annual meeting of the Society for Pediatric Research, Washington, DC.

CBMO, cord blood monocyte
IFN- γ , interferon- γ

TNF/cachectin constitutes one of the major inflammatory mediators secreted by activated monocytes and macrophages (1); it is implicated as a primary and proximal mediator of endotoxic shock (2) and plays a physiologic role in inflammation. Receptors for the Fc domain of immunoglobulins are involved in the regulation of various monocyte/macrophage immune functions, including phagocytosis (3), antibody-dependent cell-mediated cytotoxicity (4), the clearance of immune complexes (5), and the secretion of inflammatory mediators (6). Among the secretory products induced by Fc interactions are leukotrienes (7), prostaglandins (8), various hydrolytic enzymes (9), and multiple cytokines, including IL-1 (10), IL-6 (11), and TNF (12). Debets *et al.* (12) showed that when monocyte Fc receptors bound with cytophilic human IgG were cross-linked by the addition of anti-human IgG, rapid secretion of TNF was induced. These experiments were conducted with adherent adult human monocytes; soluble IgG alone failed to induce a response. In subsequent work, Debets *et al.* (13) demonstrated that cross-linking of FcR was not sufficient for the induction of TNF but that high affinity Fc-FcR interactions are required.

Kuhnert *et al.* (14) demonstrated the induction of TNF and IL-1 secretion by adult human mononuclear cells cultured in suspension in the presence of soluble IgG and 10% autologous serum. Moreover, suspension cultures of mononuclear cells exposed to IgG were primed for enhanced production of TNF and IL-1 upon subsequent stimulation with LPS. Although F(ab')₂ fragments exhibited priming capacity similar to intact IgG, they were unable to induce cytokine secretion by themselves, again suggesting that the Fc portion of IgG is important in inducing a cytokine response.

Multiple clinical trials have evaluated IVIG as adjunctive therapy for neonatal sepsis (15–19), and although the results suggest benefit, the small numbers of patients studied prevent any conclusions regarding efficacy. IVIG is also being investigated for the prophylaxis of neonatal sepsis (20–26). These studies have not revealed any significant toxic effects associated with IVIG administration. Because of the potential for IgG-FcR interactions to occur *in vivo* when IVIG is administered, we sought to examine the *in vitro* effects of IVIG on neonatal MO TNF production. Studies have shown that neonatal monocytes are as competent as adult monocytes in the processing and presentation of antigen (27) and phagocytosis and killing of bacteria (28). However, certain functions are diminished compared with adults, such as chemotaxis (29, 30) and antibody-dependent cellular cytotoxicity to herpes simplex virus (31). Kohl *et al.* (31) found decreased expression of the high-affinity IgG FcR on neonatal mononuclear cells and proposed that defective

FcR expression may partly explain low neonatal mononuclear cell antibody-dependent cellular cytotoxicity. To determine whether the regulatory effects of IVIG regarding MO TNF production are specific to neonates, we paralleled select experiments in adult MO. In initial experiments, we examined the ability of soluble human IgG, in the form of IVIG, to induce TNF production. We then investigated the immunomodulating effects of IVIG by examining its effect on the MO TNF response induced by the known stimulants IFN- γ and LPS.

MATERIALS AND METHODS

Cell isolation and culture. Blood samples were obtained from the umbilical cords of normal, full-term, nonstressed neonates by using sterile blood collection tubes containing dry Na₂-EDTA or from healthy adult volunteers by using sterile heparinized syringes (10^3 U heparin/L blood). After collection, the blood was diluted 1:1 with sterile PBS. The cell suspension was overlaid on Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) and the mononuclear cells were separated by density-gradient centrifugation. These cells were washed once with sterile PBS and then incubated for 10 min at 37°C in 0.01 M Tris-0.83% NH₄Cl (pH 7.2–7.4) to lyse contaminating red blood cells. After centrifugation, cells were washed twice more with sterile PBS before they were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) containing 2 mM L-glutamine, 10^4 U/L penicillin G, 10^4 μ g/L streptomycin, and 10% pooled heat-inactivated human type AB serum (kindly provided by the local blood bank). Enumerated cells were greater than 95% viable by trypan blue exclusion. Monocytes were isolated from mononuclear cells by adherence to plastic. For this purpose, 1×10^5 mononuclear cells were allowed to adhere 1.5 to 2 h at 37°C in 0.3 mL medium/well in 96-well flat-bottomed tissue culture trays (Costar, Cambridge, MA). When cells were prepared for RNA isolation, monolayers were established in 100×20 -mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ). All other conditions were the same. Nonadherent cells were removed by washing five times with warm sterile PBS, and fresh culture medium was added. Monolayers were then cultured for 5 d at 37°C in 5% CO₂, before experimentation. The adherent cell population consisted of more than 90% monocytes, as determined by staining with α -naphthyl esterase (Sigma Diagnostics, St. Louis, MO). Cultures of adherent cells were monitored periodically by inverted microscopy; monocytes routinely took on morphologic characteristics of macrophages after 5 d in culture. All reagents had undetectable levels of endotoxin, as verified by the Limulus amoebocyte assay (Cape Cod Association, Woods Hole, MA) (sensitivity of 20 ng/L).

Production of TNF. After 5 d in culture, monocyte-derived macrophages were replated with culture medium alone or with culture medium containing one of the following: pooled human IgG for i.v. infusion (Gammagard, Baxter-Healthcare Corp., Glendale, CA) in concentrations of 6.3×10^{-6} M, 6.3×10^{-5} M, or 1.6×10^{-4} M (1, 10, or 25 g/L, respectively); human IgG F(ab')₂ fragments in concentrations of 9.1×10^{-7} M or 9.1×10^{-6} M (0.1 g/L or 1 g/L); human IgG Fab fragments in concentrations of 2.0×10^{-6} M or 2.0×10^{-5} M (0.1 g/L or 1 g/L); or human Fc pieces in concentrations of 3.7×10^{-4} M or 3.7×10^{-3} M (0.1 g/L, or 1 g/L). All IgG fragments were obtained from Jackson ImmunoResearch Laboratories, West Grove, PA. Monolayers were incubated for 2, 6, or 24 h at 37°C in 5% CO₂. In early experiments, 50 or 100 g/L of IgG were also used. Monolayer cell counts revealed that such high concentrations of IgG induced significant cell loss (85–95% compared with 40–75% loss of cells incubated in lower concentrations of IgG). After incubation, the plates were centrifuged at $300 \times g$ for 5 min and the cell-free supernatants were harvested from each well. Supernatants from each of three wells that had been treated the same were pooled and stored at -70°C until used for the TNF assays.

In a second series of experiments, monolayers were treated as above; after this initial incubation in medium or medium with

IgG or IgG fragments, supernatants were removed, and 0.25 mL of either culture medium or culture medium with 10^5 U/L (100 U/mL) of human IFN- γ (Genzyme Corp., Cambridge, MA) and 1 mg/L (1 μ g/mL) of *Escherichia coli* LPS (Sigma) were added to each well. After 3 h of incubation at 37°C in 5% CO₂, plates were centrifuged at $300 \times g$ for 5 min, and the cell-free supernatants were harvested and stored at -70°C until used for the TNF assays. Additional experiments performed in this series used lower concentrations of IgG: 6.3×10^{-8} M (0.01 g/L) or 6.3×10^{-7} M (0.1 g/L).

In an additional series of experiments, CBMO were incubated for 3 h in IFN- γ (10^5 U/L) and LPS (1 mg/L) or in IFN- γ /LPS and IgG (1, 10, or 25 g/L) simultaneously. After a 3-h incubation at 37°C in 5% CO₂, plates were centrifuged at $300 \times g$ for 5 min, and the cell-free supernatants were harvested and stored at -70°C until analyzed for TNF by ELISA.

In a final series of experiments, monolayers were incubated in IFN- γ (10^5 U/L)/LPS (1 mg/L) overnight (18 h), and after centrifugation, the cell-free supernatants were harvested for TNF assays. These cells were then replated with media alone or media with IgG in a concentration of 10 g/L and incubated for 6 h at 37°C in 5% CO₂. After incubation, supernatants were harvested and stored at -70°C until analyzed for TNF by ELISA.

Cell counts were made of each group using the pronase-cetrimide procedure for counting cell nuclei (32). Briefly, to count cells, 0.5 mL of freshly filtered dilute pronase (Sigma) was equally divided among the three wells of a group, mixed, and allowed to incubate for 15 min at 37°C. After this incubation, the pronase suspension was pulled from the wells and put into 12.5 mL of filtered cetrimide (Sigma) solution in an Accuvette II vial (Coulter, Hialeah, FL). The suspension of cell nuclei was then promptly counted in an electronic particle counter (Coulter).

TNF bioassay. TNF activity was determined by using an adaptation of the lytic assay of Carswell *et al.* (33) as modified by Aggarwal *et al.* (34). Briefly, mouse L929 fibroblast cells were plated in 96-well plates at a concentration of 4×10^4 cells/well. After an overnight incubation, supernatants were added to the plates and serially diluted in horizontal rows. To determine the specificity of the assay, appropriate dilutions of selected supernatants or standard human recombinant TNF (sp act 5×10^7 U/mg) (Genentech, South San Francisco, CA) were preincubated with 5 neutralizing U of TNF MAb (sp act 6×10^6 neutralizing U/mg) (Genentech) for 4 h at 4°C before addition to the plates. Actinomycin D (8 mg/L) (Sigma) was added to each well and plates were incubated overnight. After overnight incubation, samples were removed and plates washed with warm PBS to remove dead cells. The plates were stained with 0.05% crystal violet in 20% ethanol for 10 min. Plates were then washed with cold tap water and allowed to dry for 24 h. After drying, stain was eluted with 100 μ L of 100% methanol per well. Plates were read within 10 min of methanol addition at 562 nm on a microplate reader. OD of L929 cells incubated with medium alone represented 0% lysis, and cells treated with 3 M guanidine hydrochloride represented 100% lysis. One unit of TNF is defined as that amount of TNF required to produce 50% lysis. Lysis was normalized to a standard of human recombinant TNF. The lower limit of detectability was 50 ng/L (2.5 U/mL).

TNF-ELISA. TNF concentrations of cell-free supernatants were determined with a TNF-specific ELISA kit (Genzyme) according to the manufacturer's instructions. The lower limit of detectability for this assay is 10 ng/L.

RNA isolation and Northern blot analysis. At the indicated times, total cellular RNA was isolated by the guanidium isothiocyanate-CsCl method (35). Total RNA samples were electrophoresed in 1.2% agarose/formaldehyde gels, and the integrity of the RNA was confirmed by ethidium bromide staining. After electrophoresis, RNA was transferred overnight to Hybond-N filters (Amersham, Arlington Heights, IL), baked at 80°C for 2 h, and prehybridized for 18 h at 42°C in 50% formamide, 5 $\times$

sodium chloride, sodium citrate (SSC), $1 \times$ Denhardt's, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 0.250 g/L salmon sperm DNA, and 10% dextran sulfate. Blots were hybridized for 18 h in the same buffer containing 10^6 cpm/mL of cDNA probe. The probes used were a human TNF cDNA fragment, the 800-bp *EcoRI* cDNA fragment (36), and a 2-kb human β -actin cDNA (Clontech, Palo Alto, CA) that were randomly labeled (Boehringer Mannheim Corp., Indianapolis, IN) overnight at 25°C; unincorporated nucleotides were removed by push column (Stratagene Cloning Systems, La Jolla, CA). After hybridization, blots were washed once with $2 \times$ SSC, 0.1% SDS for 30 min at 25°C, followed by two washes with $0.1 \times$ SSC, 0.1% SDS for 20 min at 55°C, and exposed to Kodak XAR-5 film at -70°C with intensifying screens. For RNA quantification, filters were dehybridized and then rehybridized with the β -actin probe and exposed for 5 d. Stripping was confirmed in each case by autoradiography before a second probe was used. Multiple autoradiographic exposures of multiple blots were quantitated by scanning on a laser densitometer (Hoefer Scientific Instruments, San Francisco, CA). Calculated ratios of TNF to β -actin mRNA levels were used for comparisons within groups.

Statistics. The two-sample *t* test for unpaired data was used for statistical analysis. Differences were considered significant at the two-tailed $p < 0.05$ level.

RESULTS

Evaluation of TNF response in adherent MO exposed to IgG. Adherent CBMO were exposed to increasing concentrations of IgG, and at specific time intervals (2, 6, and 24 h) supernatants were harvested and examined by ELISA for TNF. As shown in Table 1, incubation in concentrations of IgG from 6.3×10^{-6} M to 1.6×10^{-4} M (1–25 g/L) for 6 h did not induce secretion of TNF more than adherence alone. Although low levels of TNF were detected in the supernatants of some of the cultures, the majority had levels that were barely detectable. Comparable results were seen in monolayers incubated for 2 and 24 h (data not shown).

Northern blot analysis of MO RNA levels paralleled these results. Depicted in Figure 1 is a representative autoradiogram from CBMO preparations. After 5 d in culture, a subsequent 6-h incubation in medium alone, 6.3×10^{-6} M IgG, or 6.3×10^{-5} M (1 or 10 g/L) IgG resulted in similar accumulations of TNF mRNA. Bands were quantitated by densitometry, and when ratios of TNF mRNA to β -actin were compared, no alteration in the levels of TNF message was found. Incubation in concentrations of 1.6×10^{-4} M (25 g/L) IgG also had no effect (data not shown). These data indicate that soluble IgG alone does not induce significant production of TNF by adherent CBMO.

To determine whether a lack of response in our system was specific for neonatal MO, we analyzed supernatants from adult MO monolayers incubated with IgG. Again, we found that soluble IgG was unable to induce secretion of TNF greater than that caused by adherence (Table 1).

Effect of IgG on MO IFN- γ /LPS-induced TNF response. We investigated the immunomodulating effects of IVIG by examin-

Fig. 1. Northern blot analysis of CBMO RNA. CBMO were prepared as described in Materials and Methods. Total RNA was extracted and electrophoresed after a 6-h incubation in medium alone (lane 1), medium and 1 g/L IgG (lane 2), or medium and 10 g/L IgG (lane 3). All exposure times were the same.

ing its effect on the MO TNF response induced by IFN- γ /LPS. Preincubation of CBMO in 1, 10, or 25 g/L IgG for 2 or 6 h caused enhanced secretion of TNF upon subsequent stimulation (Table 2). Despite the considerable subject-to-subject variation, the trend for enhancement was consistent. A 6-h preincubation with 25 g/L IgG resulted in the greatest enhancement of TNF secretion (~5-fold, compared with a 3-fold increase at 2 h and only a 2-fold increase when monolayers were preincubated with IgG for 24 h). This decline in enhancement with the 24-h preincubation was not caused by increased cell loss; cell loss varied from 40 to 75% for all three preincubation times as determined by monolayer cell counts. In addition, the time of preincubation did not significantly alter the TNF secretion detected in those cells pretreated with medium alone, in which cell loss also varied between 40 and 75%. In preliminary experiments, concentrations of 50 g/L IgG and greater led to a marked decrease in TNF secretion, yet this concentration resulted in >80% cell loss. Therefore, 25 g/L IgG became the maximum concentration used in our experiments.

Preincubation of adult MO with IgG for 6 h before stimulation with IFN- γ /LPS also resulted in enhanced secretion of TNF (Table 2). Compared with CBMO, adult MO preincubated in medium alone produced significantly greater amounts of TNF upon stimulation with IFN- γ /LPS (3353.2 ± 2946.7 versus 1215.0 ± 575.9 ng/L). Therefore, the relative increase in TNF production by adult MO preincubated with IgG was less than that of CBMO (~2-fold increase in adult MO, compared with ~5-fold increase in CBMO). Concentrations of 10 and 25 g/L IgG were able to induce CBMO to secrete levels of TNF approaching those detected with adult MO (4070.4 ± 3069.2 versus 7158.8 ± 3938.2 ng/L and 6383.7 ± 2982.2 versus 7302.7 ± 3451.4 ng/L, for 10 and 25 g/L, respectively, in CBMO versus adult MO).

Results of an L929 cytotoxicity assay on supernatants from CBMO preincubated for 2 and 6 h with IgG (1–25 g/L) confirmed the enhancement of TNF secretion and indicated that the TNF was functional (Table 3). Once again, preincubation with 25 g/L IgG for 6 h resulted in the greatest secretion of TNF upon subsequent stimulation with IFN- γ /LPS.

To explore the dose-response paradigm more completely, we performed experiments with CBMO using the lower concentrations of 6.3×10^{-6} M (0.01 g/L) and 6.3×10^{-7} M (0.1 g/L) IgG for preincubation. Preincubation in these lower concentrations of IgG for 2 or 6 h did not enhance the TNF response seen upon subsequent stimulation with IFN- γ /LPS (Table 3). Thus, the ability of IgG to enhance IFN- γ /LPS-induced TNF secretion by MO *in vitro* is dose dependent with a threshold concentration [6.3×10^{-6} M (1 g/L)] below which an enhanced response is not induced.

Northern analyses were performed on MO treated in the same manner to determine at what molecular level the TNF response was being increased. Depicted in Figure 2 is a representative

Table 1. TNF secretion by CBMO and adult monocytes exposed to IgG*

Cultured in	TNF (ng/L)	
	CBMO, 6 h	Adult MO, 6 h
Medium only	35.2 ± 20.7	72.8 ± 131.2
6.3×10^{-6} M (1 g/L) IgG	12.8 ± 17.4	15.3 ± 37.4
6.3×10^{-5} M (10 g/L) IgG	99.9 ± 179.8	88.6 ± 158.4
1.6×10^{-4} M (25 g/L) IgG	91.8 ± 192.3	88.4 ± 23.6

* Monocytes isolated as described in Materials and Methods were incubated with 1, 10, or 25 g/L IgG. After a 6-h incubation period, TNF concentration in the supernatant was determined by ELISA. Results are means $\pm$ SD of 10 experiments each.

Table 2. IgG modulation of IFN- γ /LPS-induced monocyte TNF secretion*

	Pretreated with	TNF (ng/L)		
		2-h Preincubation	6-h Preincubation	24-h Preincubation
CBMO	Medium only	1133.9 $\pm$ 618.7	1215.0 $\pm$ 575.9	1333.8 $\pm$ 422.9
	6.3 $\times$ 10 ⁻⁶ M (1 g/L) IgG	2547.6 $\pm$ 1217.7†	2413.1 $\pm$ 1389.4‡	1788.1 $\pm$ 816.3
	6.3 $\times$ 10 ⁻⁵ M (10 g/L) IgG	3542.6 $\pm$ 3402.5‡	4070.4 $\pm$ 3069.2†	2078.8 $\pm$ 1254.8
	1.6 $\times$ 10 ⁻⁴ M (25 g/L) IgG	3733.5 $\pm$ 2420.1†	6383.7 $\pm$ 2982.2†	2724.0 $\pm$ 1366.7†
Adult MO	Medium only	ND	3353.2 $\pm$ 2946.7	ND
	6.3 $\times$ 10 ⁻⁶ M (1 g/L) IgG	ND	6082.0 $\pm$ 1732.8‡	ND
	6.3 $\times$ 10 ⁻⁵ M (10 g/L) IgG	ND	7158.8 $\pm$ 3938.2‡	ND
	1.6 $\times$ 10 ⁻⁴ M (25 g/L) IgG	ND	7302.7 $\pm$ 3451.4‡	ND

* Monocytes isolated as described in Materials and Methods were preincubated with 1, 10, or 25 g/L IgG. After 2, 6, or 24 h, monocytes were replated with IFN- γ [10⁵ U/L (100 U/mL)] and LPS [1 mg/L (1 μ g/mL)]. After a 3-h incubation in these stimulants, TNF concentration in the supernatant was determined by ELISA. Results are means $\pm$ SD of 10 experiments. ND, Not determined.

† $p < 0.005$.

‡ $p < 0.05$.

Table 3. L929 assay for TNF production by CBMO*

Pretreated with	TNF ($\times 10^3$ U/L)	
	CBMO, 2 h	CBMO, 6 h
Medium only	1503.0 $\pm$ 872.7	2072.0 $\pm$ 1043.7
6.3 $\times 10^{-8}$ M (0.01 g/L) IgG	1460.3 $\pm$ 823.0	2269.9 $\pm$ 1157.0
6.3 $\times 10^{-7}$ M (0.1 g/L) IgG	1962.0 $\pm$ 1703.7	2340.3 $\pm$ 1164.4
6.3 $\times 10^{-6}$ M (1 g/L) IgG	4362.0 $\pm$ 4137.6†	5621.1 $\pm$ 2862.0‡
6.3 $\times 10^{-5}$ M (10 g/L) IgG	4379.4 $\pm$ 4521.5	5431.2 $\pm$ 3706.1†
1.6 $\times 10^{-4}$ M (25 g/L) IgG	5225.0 $\pm$ 2164.1‡	6084.3 $\pm$ 4007.0†

* Monocytes isolated as described in Materials and Methods were preincubated with 1, 10, 25 g/L IgG. After 2 h or 6 h, monolayers were replated with IFN- γ (10⁵ U/L) and LPS (1 mg/L). After a 3-h incubation in these stimulants, TNF activity in the supernatant was determined by bioassay. Results are means $\pm$ SD of 10 experiments.

† $p < 0.05$.

‡ $p < 0.005$.

Fig. 2. Northern blot analysis of CBMO RNA. CBMO were prepared as described in Materials and Methods. After 6 h of preincubation in medium (lane 1), medium and 1 g/L IgG (lane 2), or medium and 10 g/L IgG (lane 3), CBMO were stimulated with IFN- γ /LPS for 3 h and total RNA was extracted and electrophoresed. All exposure times were the same.

autoradiogram of TNF mRNA levels in CBMO preincubated for 6 h in medium alone or medium plus 1 or 10 g/L IgG and subsequently stimulated with IFN- γ /LPS. When ratios of TNF mRNA to β -actin were compared, no alterations in the accumulations of TNF message were detected. Similar results were seen in cells preincubated for 2 and 24 h with these same concentrations of IgG (data not shown). But, when monolayers were preincubated in 25 g/L of IgG, increased accumulations of mRNA for TNF were seen (Fig. 3). TNF mRNA was detectable

Fig. 3. Northern blot analysis of CBMO RNA. CBMO were prepared as described in Materials and Methods. Lane 1 represents CBMO cultured for 6 h in medium alone. Lane 2 represents CBMO preincubated in medium for 6 h and then stimulated with IFN- γ /LPS for 3 h. Lane 3 represents CBMO preincubated in 25 g/L IgG for 6 h and then stimulated with IFN- γ /LPS for 3 h. All exposure times were the same.

in unstimulated monocytes, and the absolute amount varied between individuals (compare Fig. 1, lane 1 with Fig. 3, lane 1). IFN- γ /LPS stimulation led to greatly increased accumulations of TNF mRNA. Preincubation of CBMO in 25 g/L IgG for 6 h increased the levels of TNF message induced by IFN- γ /LPS by ~ 2 -fold (by densitometry). Northern analyses performed with adult MO yielded similar results; increased levels of mRNA for TNF were detected only when the higher concentration of 25 g/L IgG was used for preincubation (data not shown).

We performed an additional set of experiments to examine the ability of IgG to potentiate an IFN- γ /LPS-induced TNF response. CBMO were incubated in IFN- γ /LPS for 18 h, after which their supernatants were removed for TNF assay. A group of cells was replated in medium and a second group in 10 g/L IgG. After a 6-h incubation, supernatants were again harvested for analysis. TNF secretion as detected by ELISA was 641.7 $\pm$ 145.3 ng/L for cells incubated in IFN- γ /LPS for 18 h. When the cells were replated in medium for 6 h, the amount of TNF detected decreased to 59.9 $\pm$ 34.6 ng/L, but in cells replated with 10 g/L of IgG for 6 h, the levels of TNF remained high (602.2 $\pm$ 243.7 ng/L). Values are means $\pm$ SD of 10 experiments each. Detection of TNF in the last group of cells indicates the ability of IgG to augment a prior stimulus for TNF release. Although these data support and confirm our earlier results, they do not increase our knowledge of the mechanism for this increased response.

The potential exists for antibodies present in the IVIG to bind LPS, creating LPS/IgG immune complexes, which could act as a potent stimulus *in vitro*. We explored this possibility by adding IgG, IFN- γ , and LPS simultaneously. CBMO were incubated for 3 h in the stimulants IFN- γ and LPS or in IFN- γ /LPS in combination with 1, 10, or 25 g/L IgG. Although a trend toward increased TNF secretion was detected, the increases were not statistically significant (1812 ± 2362.0 , $p = 0.72$; 2087.5 ± 1916.9 , $p = 0.46$; and 3260.9 ± 4472.8 , $p = 0.29$ in cells incubated in IFN- γ /LPS combined with 1, 10, or 25 g/L of IgG, respectively, versus 1444.9 ± 1489.4 ng/L in cells incubated in IFN- γ /LPS alone). TNF levels were measured by ELISA ($n = 8$). IgG-MO interaction causes increased TNF production in MO subsequently stimulated with IFN- γ /LPS or in MO previously exposed to these stimulants. However, if IgG-MO interactions occur simultaneously with IFN- γ /LPS stimulation, no increase in TNF secretion is observed.

Modulation of IFN- γ /LPS-induced TNF secretion by Fc, Fab, or F(ab')₂ fragments. To determine whether the entire molecule of IgG is required for regulation of TNF production, experiments were performed in which either Fc, Fab, or F(ab')₂ fragments of human IgG were used for preincubation. Preincubation of CBMO in Fc fragments in concentrations of 3.7×10^{-4} M (0.1 g/L) or 3.7×10^{-3} M (1 g/L) or Fab fragments in concentrations of 2.0×10^{-6} M (0.1 g/L) or 2.0×10^{-5} M (1 g/L) for 6 h had no effect on subsequent IFN- γ /LPS-induced TNF secretion (Table 4). However, when CBMO were preincubated in 9.1×10^{-7} M (0.1 g/L) or 9.1×10^{-6} M (1 g/L) F(ab')₂ fragments, enhanced TNF secretion was detected. This enhancement was not as great as that previously seen with whole IgG molecules (maximum increase seen was ~3-fold) (Table 4). Experiments were also performed in which CBMO were incubated in these reagents without subsequent stimulation with IFN- γ /LPS. Negligible amounts of TNF were induced by these reagents alone (data not shown).

DISCUSSION

In previous works by Kuhnert *et al.* (14), the addition of soluble IgG to human mononuclear cells in suspension induced the secretion of TNF and IL-1. In contrast, Debets *et al.* (12), found that solid-phase antibodies specific for binding Fc γ RI or Fc γ RII induced TNF secretion by adherent human monocytes, whereas the same antibodies in the fluid phase failed to induce a response. An additional study by Shimozato *et al.* (37) demonstrated that i.v. infusion of IgG resulted in a reduction of TNF serum levels in rabbits subsequently given LPS and that IgG suppressed the production of TNF by rabbit macrophages stimulated *in vitro* with LPS.

Because of the widespread use of IVIG in our neonatal nur-

series for both prophylaxis and treatment of sepsis, we initially sought to address the question of the ability of IgG to induce TNF production by neonatal MO. We demonstrated that the addition of soluble IgG to adherent CBMO is not a sufficient stimulus to generate the production of TNF (Table 1, Fig. 1). This was true for adult MO as well; we demonstrated that the lack of response is not due to a deficiency inherent to the neonate. In addition, all experiments were performed in monocyte-derived macrophages that were cultured for 5 d. Time in culture allows for the development of Fc γ RIII expression, in addition to the constitutive expression of Fc γ RI and Fc γ RII. Fc γ RIII develops on monocytes *in vitro* after 2 d of culture (38), although recently it was discovered that a subpopulation (~10%) of freshly isolated monocytes expresses Fc γ RIII (39). Thus, in our system, the potential exists for IgG to interact with any or all three of the Fc γ R. Kuhnert *et al.* (14) used 10% autologous serum in their experiments and proposed that dimeric IgG generated by idiotype-antiidiotype complexes between donor and host IgG or between IgG molecules from different donors are a possible mechanism whereby IVIG promotes cytokine synthesis. Our experiments used 10% pooled human AB serum and concentrations of IVIG that are comparable to those seen *in vivo* (22, 40). The obvious difference between our experiments and those performed by Kuhnert *et al.* (14) is that we used MO cultured as adherent monolayers rather than as suspended cells. It is possible that the act of adherence inhibited the induction of TNF by IgG. The prolonged contact of monocytes with polystyrene can inhibit other FcR-mediated functions, such as phagocytosis of opsonized erythrocytes (41). Evidence against inhibition of TNF production by adherence lies in the positive results of the studies of Debets *et al.* (12, 13). Furthermore, we have previously shown that the act of adherence turns on various cytokine genes in MO, including the gene for TNF (42).

Kuhnert *et al.* (14) demonstrated that IgG exposure primed suspended mononuclear cells for enhanced cytokine production in a second 24-h culture with LPS. We demonstrated a similar priming phenomenon with adherent MO. Neonatal CBMO and adult MO, when exposed to IgG in concentrations of 1–25 g/L, exhibited enhanced secretion of TNF upon subsequent stimulation with IFN- γ /LPS (Tables 2 and 3). In the absence of IgG preincubation, CBMO secreted significantly less TNF in response to IFN- γ /LPS stimulation when compared with adult MO. Similar findings were reported by Burchett *et al.* (43). They demonstrated that, although freshly isolated CBMO secreted TNF in amounts similar to adults when stimulated with LPS, cultured neonatal monocyte-derived macrophages as well as fetal placental macrophages secreted less TNF than adult cells. In addition, IFN- γ enhanced LPS-induced TNF production by neonatal cells less effectively than it enhanced that by adult cells. Burchett *et al.* (43) proposed that a combined deficiency of neonatal lymphocyte IFN- γ production with a decreased macrophage response to IFN- γ may lead to relatively decreased secretion of TNF by neonatal tissue macrophages. The results of our study indicate that exposure of neonatal MO to IgG may augment their TNF secretion in response to IFN- γ /LPS exposure to levels that approach those seen in adults. The absence of an enhanced response by CBMO when lower concentrations of IgG were used for preincubation [6.3×10^{-8} M and 6.3×10^{-7} M (0.1 and 0.01 g/L)] indicates a dose dependency for the mechanism of augmentation (Table 3).

Northern analyses of mRNA for TNF indicated that the increased TNF response mediated by IgG primarily occurred posttranscriptionally (Fig. 2). Although preincubation of MO in 1 and 10 g/L IgG resulted in significant increases in the secretion of TNF, levels of TNF mRNA were unchanged. These concentrations of IgG may act to enhance translation of TNF mRNA in response to IFN- γ /LPS stimulation or by improving posttranslational processing and secretion of the protein. When MO were preincubated in 25 g/L IgG, slight augmentation of the MO TNF response occurred at the pretranslational level (~2-fold increase

Table 4. Modulation of IFN- γ /LPS-induced CBMO TNF secretion by Fc, Fab, and F(ab')₂ fragments*

Pretreated with	TNF secretion (ng/L) by CBMO (6 h)
Medium only	1224.4 $\pm$ 529.3
3.7×10^{-4} M (0.1 g/L) Fc	1457.0 $\pm$ 957.4
2.0×10^{-6} M (0.1 g/L) Fab	1632.2 $\pm$ 684.8
9.1×10^{-7} M (0.1 g/L) F(ab') ₂	3003.1 $\pm$ 2024.6†
3.7×10^{-3} M (1 g/L) Fc	2134.0 $\pm$ 2432.3
2.0×10^{-5} M (1 g/L) Fab	780.2 $\pm$ 411.4
9.1×10^{-6} M (1 g/L) F(ab') ₂	3520.0 $\pm$ 2534.2†

* Monocytes isolated as described in Materials and Methods were preincubated with 0.1 or 1 g/L Fc, Fab, or F(ab')₂ fragments. After 6 h, monolayers were replated with IFN- γ (10^5 U/L) and LPS (1 mg/L). After a 3-h incubation in these stimulants, TNF concentration in the supernatant was determined by ELISA. Results are means $\pm$ SD of 10 experiments for all except "Medium only" and F(ab')₂, where $n = 15$.

† $p < 0.05$.

in mRNA for TNF). This increase in mRNA for TNF could be caused by enhanced mRNA stability or by an increase in transcription.

Data supporting a posttranscriptional mechanism for augmentation come from experiments in which CBMO were first incubated in the stimulants IFN- γ /LPS for 18 h, then incubated in either medium alone or medium with 10 g/L IgG for 6 h. The addition of IgG allowed for continued detection of TNF (602.2 ± 243.7 ng/L), whereas in cells replated with medium alone the TNF levels were minimal (59.9 ± 34.6 ng/L). IFN- γ combined with LPS, in the concentrations used in our study, has been shown to induce transcription of TNF mRNA at near maximal levels (44). In addition, studies indicate that once the TNF gene is transcriptionally active posttranscriptional mechanisms operate to control TNF gene expression (45, 46). Therefore, the ability of IgG to induce an enhanced response in the face of prior stimulation with IFN- γ /LPS suggests that the entire effect of IgG demonstrated in our study is most likely posttranscriptionally mediated.

Several mechanisms could be acting to produce this effect. The potential exists for antibodies present in the IVIG to bind to LPS and form IgG/LPS immune complexes that could act as a potent stimulus for TNF production *in vitro*. Interestingly, when CBMO were incubated in IgG and IFN- γ /LPS simultaneously for 3 h, no significant increase in TNF secretion was detected compared with cells incubated in IFN- γ /LPS alone. Thus, it seems unlikely that IgG/LPS complexes are the stimulus for enhanced TNF production in our studies. A 2-h preincubation in IgG before a 3-h incubation in IFN- γ /LPS resulted in a significant increase in TNF secretion compared with CBMO preincubated in medium alone. These data indicate that a minimum amount of time must be present for IgG to interact with the MO and produce the signals necessary for an enhanced response.

To further explore the mechanism whereby IgG enhances IFN- γ /LPS-induced TNF production, experiments were performed with the Fc, Fab, and F(ab')₂ fragments of IgG. Only the F(ab')₂ fragments were able to produce an enhanced response, which did not reach the levels seen with whole IgG molecules. These results indicate involvement of the F(ab')₂ portion of IgG. It should be noted that all our experiments were performed in 10% pooled human AB serum containing significant amounts of IgG. The results of our study also indicate a concentration dependence of this enhanced response, with 25 g/L IgG consistently producing the maximum increase in TNF secretion and no increase in TNF secretion being detected when concentrations of IgG below 1 g/L were used with CBMO.

Another potential mechanism is that exposure of MO to IFN- γ leads to up-regulation of Fc γ RI (47), which then binds IgG through its Fc region, leading to high levels of IgG-FcR interaction that stimulate pathways that augment the TNF response. However, this does not explain the positive effects of F(ab')₂ fragments.

Interaction of Fc γ R with immune complexes has been shown to trigger release of a variety of soluble mediators. Debets *et al.* (13) demonstrated that MO FcR cross-linking was necessary but not sufficient for the induction of TNF by IgG; no response was seen with the use of anti-FcR MAbs and bridging antibody. In our system, enumerable IgG idiotopes exist; IgG is present from host, from serum that is used for culture, and from more than 10 000 donors in the IVIG. Perhaps the formation of idiotype-antiidiotype complexes between the multiple sources of IgG, or between the multiple sources of IgG and F(ab')₂ fragments, leads to cross-linking of some FcR and binding of others through the Fc region of IgG. Higher concentrations of IVIG would increase the chance for such processes to occur. These interactions are obviously not sufficient to stimulate TNF release by themselves but are able to augment stimulatory pathways.

A fourth hypothesis is that alloantibodies react with MO surface antigens, thereby promoting cell activation. IL-1 is in-

duced in human monocytes exposed to antibodies against Ia antigens (48). Alloantibodies directed against immunoglobulin allotypes might also function to produce immune complex formation, thereby promoting cell stimulation. It is also possible that no single mechanism is responsible for the increase in TNF production, but that several different IgG-MO interactions combine to produce the observed response.

The absence of a TNF response by MO cultured in the presence of IVIG *in vitro* lends support to the continued use of IVIG as replacement therapy for neonates, as well as for other patients with hypogammaglobulinemia. It has been proposed that the interaction of MO with IVIG dampens the cytokine response to other unrelated stimuli (49). Indeed, IVIG has been shown to be clinically beneficial in patients with Kawasaki disease (40) and infection-associated hemophagocytic syndrome (50), conditions associated with cytokine activation. In addition, multiple treatment studies in neonates with sepsis have not demonstrated any toxic effects associated with IVIG administration (15–19). The enhanced TNF response detected from MO cultured in the presence of IgG and stimulated with IFN- γ /LPS *in vitro* is not consistent with these *in vivo* observations. Several possibilities exist for this discrepancy. Our studies examined isolated populations of MO, whereas *in vivo* multiple inflammatory cells and their products interact in a complex manner to control cytokine production. It is also possible that IgG elicits different responses when interacting with blood monocytes in the peripheral circulation than when interacting with adherent macrophages in the tissues.

In at least two animal models of established infection, infusion of IgG in addition to antibiotics has been found to be detrimental, resulting in increased mortality rates (51, 52). Both these studies used high doses of IVIG in rats, indicating that deleterious effects may not be seen unless high *in vivo* concentrations of IgG are produced. These animal studies support the detection of a lower threshold concentration in our present *in vitro* studies. The immunomodulating effects of IVIG are complex and include Fc receptor blockade, antiidiotype effects, and possibly down-regulation of immunoglobulin synthesis. The data presented here are further indications of the complex network of responses that exist to control the inflammatory response.

Acknowledgment. The authors thank Christopher B. Wilson, M.D., for his critical review of this manuscript.

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