INFECTION AND IMMUNITY, Dec. 2000, p. 6917–6923 Vol. 68, No. 12
0019-9567/00/$04.00+0

Gene Expression and Production of Tumor Necrosis Factor Alpha, Interleukin-1β (IL-1β), IL-8, Macrophage Inflammatory Protein 1α (MIP-1α), MIP-1β, and Gamma Interferon-Inducible Protein 10 by Human Neutrophils Stimulated with Group B Meningococcal Outer Membrane Vesicles

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Received 26 July 2000/Accepted 8 September 2000

Accumulation of polymorphonuclear neutrophils (PMN) into the subarachnoidal space is one of the hallmarks of *Neisseria meningitidis* infection. In this study, we evaluated the ability of outer membrane vesicles (OMV) from *N. meningitidis* B to stimulate cytokine production by neutrophils. We found that PMN stimulated in vitro by OMV produce proinflammatory cytokines and chemokines including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-8, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β. A considerable induction of gamma interferon (IFN-γ)-inducible protein 10 (IP-10) mRNA transcripts, as well as extracellular IP-10 release, was also observed when neutrophils were stimulated by OMV in combination with IFN-γ. Furthermore, PMN stimulated by OMV in the presence of IFN-γ demonstrated an enhanced capacity to release TNF-α, IL-1β, IL-8, and MIP-1β compared to stimulation with OMV alone. In line with its downregulatory effects on neutrophil-derived proinflammatory cytokines, IL-10 potently inhibited TNF-α, IL-1β, IL-8, and MIP-1β production triggered by OMV. Finally, a neutralizing anti-TNF-α monoclonal antibody (MAb) did not influence the release of IL-8 and MIP-1β induced by OMV, therefore excluding a role for endogenous TNF-α in mediating the induction of chemokine release by OMV. In contrast, the ability of lipopolysaccharide from *N. meningitidis* B to induce the production of IL-8 and MIP-1β was significantly inhibited by anti-TNF-α MAb. Our results establish that, in response to OMV, neutrophils produce a proinflammatory profile of cytokines and chemokines which may not only play a role in the pathogenesis of meningitis but may also contribute to the development of protective immunity to serogroup B meningococci.

Polymorphonuclear neutrophils (PMN) are natural effector cells mediating antimicrobial defense via the release of toxic oxygen intermediates and lytic enzymes (15a). Nevertheless, the studies conducted in many laboratories during the last decade have clearly established that the release of cytokines constitutes another important aspect of the biology of PMN (reviewed in reference 11). The cytokines that PMN produce include, for instance, interleukin-1α/β (IL-1α/β), IL-1 receptor antagonist, IL-12, tumor necrosis factor-alpha (TNF-α), transforming growth factor β, vascular endothelial growth factor, and chemokines such as IL-8, macrophage inflammatory protein 1α/β (MIP-1α/β), and gamma interferon (IFN-γ)-inducible protein 10 (IP-10) (11). Broadly speaking, these mediators exert not only pro- or anti-inflammatory activities but also important immunoregulatory actions (8). Since PMN usually represent the first cell type encountering, and interacting with, the etiological agent in an inflammatory context, the fact that they can synthesize and release a wide array of cytokines should lead to a reconsideration of their role in immunoregulation and physiopathology.

Bacterial meningitis is among the most dangerous infections of children and young adults because of the rapidity of onset, the high mortality rate, devastating sequelae, and its tendency to spread and cause outbreaks (13). *Neisseria meningitidis*, commonly known as meningococcus, is the most important cause of purulent meningitis and septicemia worldwide (38). Meningocci are classified into different serogroups, serotypes, and immunotypes according to their capsular polysaccharides, outer membrane proteins, and lipopolysaccharides (LPS), respectively (1, 15, 24). The virulent strains of *N. meningitidis* responsible for 90% of meningoccoccal diseases correspond to serogroups A, B, and C, with serogroup B meningococci being the most common cause of meningococcal diseases in many countries (30). The hallmark of bacterial meningitis is the entry of an enormous number of leukocytes into the subarachnoid space, with a clear neutrophil predominance during the initial phases, followed by a monocyte increase later in the course of the disease (25). Leukocytes are considered to initiate and propagate brain injury through the release of reactive oxygen and nitrogen metabolites, proteases, and/or toxic cytokines. Interestingly, very little is known regarding the capacity of human neutrophils to generate cytokines in response to *N. meningitidis*. To address this issue, we investigated whether human PMN produce proinflammatory cytokines and chemokines in response to bacterial components derived from serogroup B meningococci. In particular, we used lipo-oligosaccharide-reduced outer membrane vesicles (OMV) from an epidemic Cuban strain of *N. meningitidis* (CU385, B:4:P1,19,15), which is the major component of the Cuban vaccine called VA-MENGOC-BC (16, 17, 35). OMV consists of defined amounts

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of purified OMP from serogroup B *N. meningitidis* enriched with proteins from the high-molecular-mass protein complex (65 to 95 kDa), containing also a controlled proportion of LPS and phospholipids. In addition to OMV, the vaccine contains purified capsular polysaccharide of serogroup C meningococcus, and both are adsorbed on aluminum hydroxide (16, 17, 35). Indeed, although polysaccharide-based vaccines are available and offer a high protection against *N. meningitidis* serogroups A and C in children older than 2 years, this principle cannot be applied to the B serogroup because of the poor immunogenicity of polysaccharide B in humans (42). A randomized double-blind placebo controlled trial and observational studies carried out with VA-MENGOC-BC have demonstrated an efficacy and effectiveness of >83% against serogroup B meningococci (35, 36).

Herein, we report that human neutrophils, upon incubation with OMV, release both proinflammatory cytokines, such as TNF-α and IL-1β, and chemokines, such as IL-8, MIP-1β, and IP-10. These findings suggest that neutrophil-derived cytokines and chemokines might represent an early event during the course of meningitis.

MATERIALS AND METHODS

Cell purification and culture. Highly purified granulocytes (>98% purity) and peripheral blood mononuclear cells (PBMC) were isolated under endotoxin-free conditions from the buffy coats of healthy donors, as previously described (4). The granulocyte populations contained usually <4% eosinophils ($n = 42$), as revealed by May-Grunwald-Giemsa staining. Immediately after purification, cells were usually suspended in RPMI 1640 medium supplemented with 10% low-endotoxin fetal calf serum (FCS; <0.009 ng/ml; Seromed; Biochrom KG, Berlin, Germany) and stimulated with OMV. The OMV were prepared as previously described (16, 17). Briefly, *N. meningitidis* strain B:4:P1,19,15 was grown until early stationary phase, and OMV were extracted by using 0.1 M Tris-HCl (pH 8.6), 10 mM EDTA, and 0.5% (wt/vol) deoxycholate. The OMV were purified by sequential centrifugation steps at 20,000 × *g* for 30 min. Following ultracentrifugation at 125,000 × *g* for 2 h, the OMV were pelleted and homogenized in phosphate-buffered saline (PBS; pH 7.2) with 3% (wt/vol) sucrose (16, 17). The concentration of LPS (from *N. meningitidis* B:4:P1,19,15; L3,7,9) inserted into the lipid bilayer of OMV preparation ranges from 1 to 4% (35). Preliminary dose-response experiments established that 5-μg/ml concentration of OMV represented the optimal concentration to stimulate cytokine release by PMN. In selected experiments, cells were costimulated with OMV or 200 ng of LPS per ml from *N. meningitidis* (strain B:4:P1,19,15; L3,7,9; prepared at the Finlay Institute, Havana, Cuba) in combination with either 100 U of IFN-γ (Hoffman-La Roche, Basel, Switzerland) (27) per ml, or 10 ng of IL-10 (Peprotech Inch, Nutley, N.J. (3) per ml. In other experiments, neutrophils were pretreated for 30 min with either 10 μg of polymyxin B sulfate (PMX) (Sigma) per ml or neutralizing monoclonal antibodies (MAbs) against TNF-α (B.154.2) and isotype-matched control MAb for B.154.2, as previously described (6). Cells were then plated at 5×10^6/ml either in 12-well tissue culture plates (BioWhittaker) or in polystyrene flasks (Greiner, Nurtingen, Germany) at 5×10^6/ml and cultured at 37°C in a 5% CO_2 atmosphere. At the times indicated, cell supernatants were harvested and stored at −20°C, whereas the pellets were used for RNA extraction. All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use (3, 4, 6, 27, 33).

RNA isolation, Northern blot analysis, and RPA. Total RNA from PMN and PBMC was extracted, by the guanidinium isothiocyanate method, usually from 6×10^7 to 7×10^7 PMN and 2×10^7 to 3×10^7 PBMC per condition, and then analyzed by either Northern blotting (4, 6) or by RNase protection assay (RPA) (33). For Northern blot experiments, the filters were hybridized using TNF-α and actin cDNA fragments, previously ^{32}P labeled using a Ready-To-Go DNA labeling kit (Pharmacia, Uppsala, Sweden). For the RPA experiments, the RiboQuantTM hcK-2 and hcK-5 Human Multi-Probe Template Sets were used according to the manufacturer's instructions (PharMingen International, San Diego, Calif.). The extent of hybridization was quantitatively analyzed in an InstantImager (Packard Instruments, Meriden, Conn.) and plotted after actin normalization.

Cytokine measurements. Antigenic IL-8 was measured in the cell supernatants by using a double-ligand enzyme-linked immunosorbent assay (ELISA) method (with a 20-pg/ml detection limit) (6). The levels of TNF-α and IL-1β were determined in the cell supernatants by using ELISA kits purchased from Endogen (Woburn, Mass.; 5-pg/ml detection limit) and Diaclone (Cedex, France; 15-pg/ml detection limit), respectively. Antigenic MIP-1β and IP-10 were measured in the cell supernatants by using specific ELISAs developed with antibodies purchased from R&D Systems (Minneapolis, Minn.). Briefly, flat-bottomed 96-well plates (MaxiSorp 439454; Nunc) were coated with 50 μl of a 1-μg/ml concentration of polyclonal anti-human MIP-1β or IP-10 antibodies (MAb271-NA and MAb266-NA, respectively) per well in PBS (pH 7.4) and then incubated overnight. After four washings of the plates with PBS–0.05% Tween 20 (pH. 7.4) (washing buffer), 150 μl of blocking buffer (PBS containing 1% bovine serum albumin [BSA], 5% sucrose, and 0.05% NaN_3) was added per well, followed by a 1-h incubation. Plates were then rinsed with washing buffer before the addition of 50 μl of either MIP-1β and IP-10 standards (30 to 960 pg/ml, diluted in RPMI 1640 with 10% FCS) or cell supernatants per well and subsequently incubated for 2 h. Biotinylated anti-human MIP-1β antibody (BAF271) or anti-human IP-10 antibody (BAF266) at 0.5 μg/ml (50 μl/well) in Tris-buffered saline solution (20 mM Trizma base, 150 mM NaCl) containing 0.1% BSA–0.05% Tween 20 (pH 7.3) was added after washing the plates, and the mixtures were then incubated for 2 h. Plates were washed before the addition of 50 μl of 1:10,000 streptavidin-horseradish peroxidase (Zymed, San Francisco, Calif.) per well in TBS and incubated for 20 min. After a washing with TBS–0.05% Tween 20 (pH 7.4), 20- to 30-min chromogenic reaction was performed using 1:1 mixture of H_2O_2 and tetramethylbenzidine (Medix Biotech, San Carlos, Calif.). The reaction was stopped with 0.5 M H_2SO_4, and the absorbance at 450 nm was measured. All of the incubation steps were performed at room temperature. The detection limit of these MIP-1β and IP-10 ELISAs were 30 and 60 pg/ml, respectively.

Statistical analysis. The data are expressed as the means ± the standard error of the mean (SEM). Statistical evaluation was performed by using the Student's t test for paired data and was considered significant if the P values were <0.05.

RESULTS

TNF-α mRNA expression and production by PMN stimulated with OMV. In the first series of experiments, we examined the capacity of neutrophils to produce TNF-α in response to OMV. For this purpose, purified populations of PMN were cultured in the absence or the presence of OMV. At the indicated times, total RNA was isolated for Northern blot analysis, whereas cell supernatants were harvested for TNF-α detection by ELISA. Autologous PBMC were also isolated and stimulated as the PMN. Figure 1A shows that treatment of PMN with OMV resulted in a time-dependent induction of TNF-α mRNA that reached maximum levels at 2 h and then declined. Interestingly, the levels of TNF-α mRNA accumulated in PMN stimulated for 2 h with OMV were higher than those observed in autologous PBMC treated under identical conditions (Fig. 1A). The latter observation argues against the possibility that the TNF-α mRNA found in stimulated neutrophils is attributable to the minimal contamination by PBMC. In accord with the Northern blot data, culture of PMN with OMV stimulated a detectable extracellular production of TNF-α protein that began as early as after 2 h ($P < 0.001$, $n = 5$) and continued to progressively rise for up to 21 h ($P < 0.005$, $n = 6$) (Fig. 1B). Cell-associated TNF-α was found only in OMV-stimulated PMN and not in freshly isolated or medium-treated cells, thus excluding the possibility of a TNF-α release from preformed stores (not shown). Ability of OMV to induce the release of TNF-α was dose dependent (being already evident at 1 μg/ml) and was not inhibited by PMX, either at the level of mRNA or at the level of protein production (data not shown).

Effect of IFN-γ and IL-10 on the production of TNF-α by OMV-stimulated PMN. Since IFN-γ and IL-10 were previously shown to modulate cytokine gene expression and release in LPS-treated PMN (5, 7, 10), we examined their effects on the release of TNF-α by OMV-stimulated PMN. Neither IFN-γ nor IL-10 used alone stimulated TNF-α mRNA expression, nor did they influence TNF-α secretion by PMN (6, 27). In contrast, PMN treated with IFN-γ plus OMV for 21 h secreted approximately ninefold more TNF-α protein than PMN stimulated with OMV alone ($P < 0.001$, $n = 6$), whereas PMN treated with IL-10 were dramatically inhibited in their capacity to release TNF-α (by 85 ± 13%, $P < 0.005$, $n = 4$).

IL-1β production by PMN stimulated with OMV. Subsequently, we decided to investigate whether neutrophils synthesize and release IL-1β protein in response to OMV. Preliminary experiments indicated in fact that, in the presence of

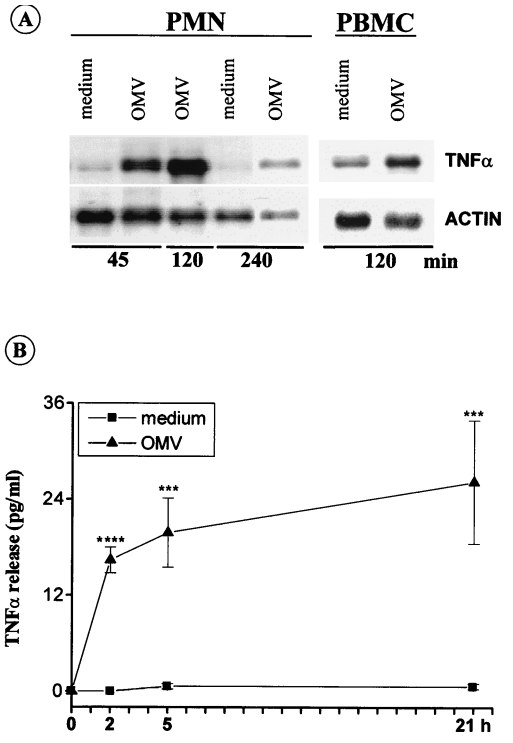

FIG. 1. Effect of OMV on the induction of TNF-α production by human neutrophils. (A) Comparative ability of neutrophils and PBMC to express TNF-α mRNA in response to OMV. Samples of 7×10^7 PMN were cultured in the presence or absence of 5 μg of OMV per ml. After the times indicated, total RNA was extracted and Northern blot analysis for TNF-α and actin transcripts was performed. Then, 10 μg of total RNA was loaded per each gel lane. The figure shows the results of one representative experiment out of two performed with identical results. (B) Time course of TNF-α release by OMV-stimulated neutrophils. PMN (5×10^6/ml) were stimulated with OMV for the times indicated before determining the levels of TNF-α in the cell supernatants by ELISA. The figure shows the mean values ± the SEM for each time point, which were obtained from at least five experiments performed under the same conditions. The asterisks represent significant differences between OMV-treated and control PMN: ***, $P < 0.005$; ****, $P < 0.001$.

OMV, IL-1β mRNA transcripts markedly increased in PMN by 2 to 3 h and then declined to almost disappear over the next 18 h (data not shown). Figure 2A shows that neutrophils did not spontaneously release IL-1β into the culture supernatants but they did secrete IL-1β following treatment with OMV in a time-dependent manner (Fig. 2A). Similarly to what we observed for TNF-α, the release of IL-1β by OMV-stimulated PMN was significantly enhanced by IFN-γ and inhibited by IL-10 (Fig. 2B).

Chemokine gene expression and production by PMN stimulated with OMV. Because it is well established that, in addition to TNF-α, PMN are able to express and secrete a number of chemokines (8, 11), we investigated whether PMN challenged by OMV could produce IL-8, MIP-1α/β, and IP-10. Initially, we evaluated by RPA chemokine mRNA expression in PMN and PBMC stimulated for 3 h with OMV in the presence or absence of IFN-γ. Figure 3A shows that IL-8, MIP-1α, and MIP-1β mRNA levels were dramatically increased by OMV in either PMN or PBMC. Time course analyses in OMV-treated neutrophils revealed very similar patterns for IL-8, MIP-1α, and MIP-1β mRNA expression, all of which displayed maximum levels at 3 h after stimulation and a gradual decrease thereafter (Fig. 3B). IP-10 mRNA was also induced in PMN, but only if neutrophils were stimulated with OMV in combination with IFN-γ (Fig. 3A). The latter cytokine was per se ineffective (Fig. 3A), as previously described (9). In contrast, IFN-γ alone represented a very potent stimulus for IP-10 mRNA accumulation in PBMC (Fig. 3A), its effect being partially suppressed by the presence of OMV (Fig. 3A). Furthermore, unlike neutrophils, PBMC also accumulated RANTES transcripts, even though their levels did not change under any conditions (Fig. 3A). Again, these observations indicate that the chemokine mRNA found in PMN is not attributable to their minimal contamination with PBMC or eosinophils.

Next, we investigated whether OMV-treated PMN could also release IL-8, MIP-1β, and IP-10 proteins by utilizing specific ELISAs. Kinetic experiments demonstrated that in OMV-treated PMN, significant levels of IL-8 appeared in the super-

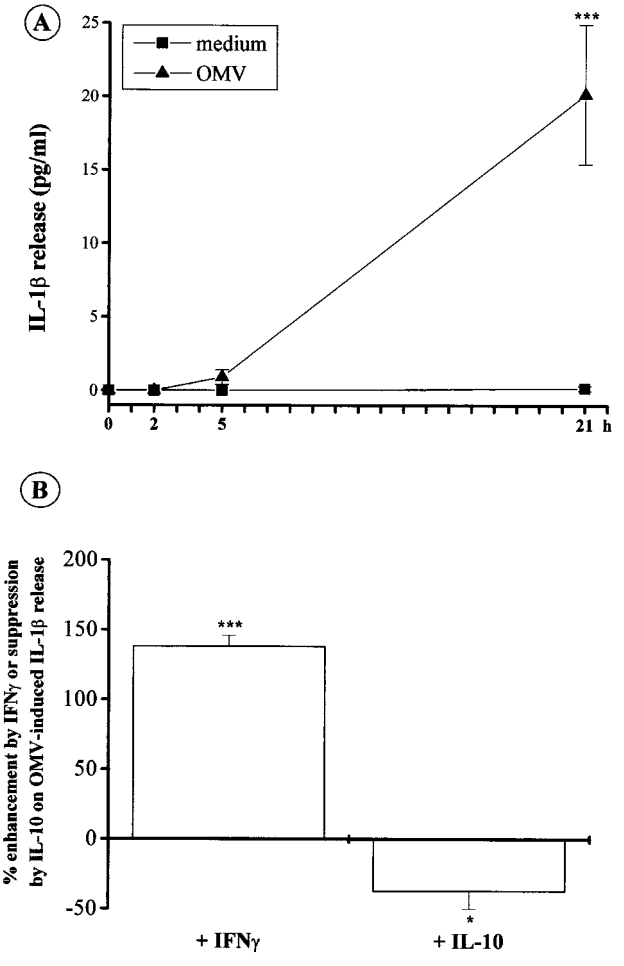

FIG. 2. Effect of OMV on the induction of IL-1β production by human neutrophils. (A) Time course release of IL-1β by neutrophils stimulated with OMV. PMN were incubated with or without 5 μg of OMV per ml for up to 21 h at 37°C, and the resulting cell supernatants were processed for the determination of IL-1β levels by ELISA. The results are expressed as the mean ± the SEM for each time point of duplicated determinations from five independent experiments. The asterisks represent significant differences between OMV-treated and control PMN, i.e., "***" indicates $P < 0.005$. (B) Effect of IFN-γ and IL-10 on the production of IL-1β in OMV-simulated PMN. PMN were preincubated with or without 100 U of IFN-γ or 10 ng of IL-10 per ml for 30 min and then cultured for 21 h after the addition of 5 μg of OMV per ml. The bars report the mean values ± the SEM of the percentage of enhancement or inhibition of IL-1β release by OMV-stimulated PMN, as exerted by IFNγ or IL-10, respectively, calculated from five experiments. The percent values were calculated from the difference in the amount of IL-1β produced in the absence or presence of IFN-γ or IL-10. *, $P < 0.05$; ***, $P < 0.005$.

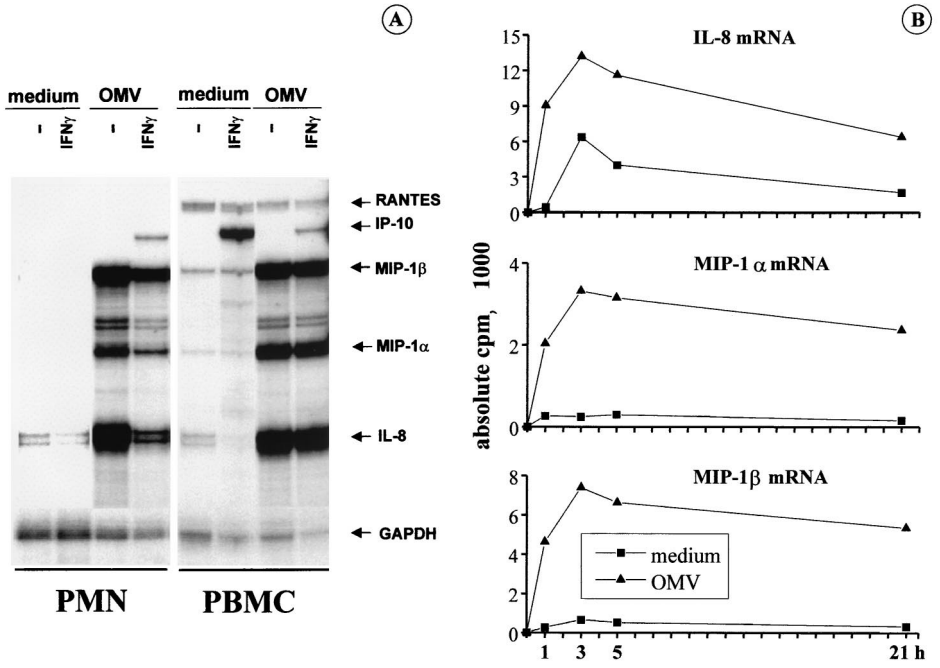

FIG. 3. Chemokine mRNA expression in OMV-stimulated neutrophils. (A) Purified populations of PMN and PBMC isolated from the same donor were cultured with or without 5 μg of OMV per ml. Total RNA was then extracted, and chemokine mRNA levels were assessed by RPA. Then, 10 μg of total RNA was used for each condition. The autoradiography shown is representative of three different experiments. (B) Time course of chemokine mRNA expression. PMN were incubated with or without 5 μg of OMV per ml. At the time points indicated, the total RNA was extracted and analyzed for IL-8, MIP-1α, and MIP-1β mRNA expression by RPA. Hybridization signals were quantitatively analyzed in an InstantImager, as described in Materials and Methods. The experiment depicted in this figure is representative of two.

natants as early as 2 h after of stimulation ($P < 0.05$, $n = 5$), before progressively accumulating for up to 21 h ($P < 0.005$, $n = 5$) (Fig. 4A). In contrast, MIP-1β production in response to OMV, although significant already after 2 h ($P < 0.05$, $n = 5$), rapidly increased at later time points (Fig. 4B). As in the case of TNF-α release, the ability of OMV to trigger the production of IL-8 and MIP-1β was not inhibited by PMX (data not shown). Surprisingly, the yields of IL-8 and MIP-1β produced in response to 200 ng of LPS per ml from serogroup B *N. meningitidis* was not inhibited by PMX (data not shown). However, the latter observation is in line with the notion that the PMX-mediated inhibition of LPS-induced cytokine secretion depends on the origin of LPS (12).

Neutrophils were also found to release considerable amounts of IP-10, but only when they were cultured with OMV in association with IFN-γ for 21 h ($P < 0.005$, $n = 5$) (Fig. 5A). Interestingly, the production of IP-10 by PMN was delayed in comparison with those of IL-8 or MIP-1β, since >80% of the total antigenic IP-10 was released between 5 and 21 h (Fig. 5A). In contrast to PMN, PBMC treated with IFN-γ alone released amounts of IP-10 higher than those detected in IFN-γ plus OMV-treated cells (Fig. 5B), which is in line with the pattern of IP-10 mRNA expression shown in Fig. 3A.

Effects of IFN-γ and IL-10 on chemokine production by OMV-stimulated PMN. We then determined whether IFN-γ and IL-10 had any effect on the production of IL-8 and MIP-1β by OMV-stimulated PMN. Neither IFN-γ nor IL-10, used alone, stimulated any secretion of IL-8 and MIP-1β by PMN (6, 18, 19, 27). The yields of IL-8 and MIP-1β recovered after 21 h from PMN stimulated with IFN-γ plus OMV were higher than those recovered from PMN treated with OMV alone (by 382 ± 197%, $P < 0.01$, $n = 8$ for IL-8, and by 45 ± 19%, $P < 0.05$, $n = 8$ for MIP-1β) (Fig. 6A). In contrast, the yields of IL-8 and MIP-1β detected after 21 h of OMV stimulation were markedly suppressed by IL-10 (by 52 ± 13%, $P < 0.01$, $n = 8$ for IL-8; by 50 ± 8%, $P < 0.01$, $n = 8$ for MIP-1β) (Fig. 6B).

Lack of an autocrine role of TNF-α in the stimulation of IL-8 and MIP-1β production by OMV. TNF-α represents a very efficient stimulus for IL-8 and MIP-1β release by PMN (2, 6), and it has been previously shown to play an autocrine role for IL-8 and MIP-1α/β expression in neutrophils stimulated with LPS from *Escherichia coli* (2, 6, 19, 27). Therefore, we evaluated the possibility that the expression of IL-8 and MIP-1β induced by OMV was driven by the TNF-α induced early after OMV stimulation (Fig. 1B). These experiments revealed that neutralizing anti-TNF-α MAb did not inhibit the yields of IL-8 ($n = 3$) or MIP-1β ($n = 3$) measured after 21 h of stimulation with OMV (Fig. 7). In contrast, the production of IL-8 or MIP-1β induced by LPS from *N. meningitidis* strain B was significantly reduced by the anti-TNF-α MAb (by 21 ± 12%, $P < 0.05$, $n = 3$ for IL-8; by 19 ± 7%, $P < 0.05$, $n = 3$ for MIP-1β) (Fig. 7). Incubation of neutrophils with isotype-matched antibodies had no effect on the production of chemokines by stimulated neutrophils (not shown).

DISCUSSION

The results of this study demonstrate that human PMN respond to stimulation with OMV from serogroup B *N. meningitidis* by producing several cytokines and chemokines, including TNF-α, IL-1β, IL-8, and MIP-1α/β, as well as IP-10, when costimulated with IFN-γ. As previously shown in LPS-treated PMN (2, 6, 10, 18, 27, 41), the production of TNF-α, IL-1β, IL-8, and MIP-1α/β induced by OMV was greatly potentiated by IFN-γ and dramatically suppressed by IL-10. By contrast, anti-TNF-α MAbs had no influence on the capacity of OMV to induce the release of either IL-8 or MIP-1β, whereas they significantly inhibited the stimulation determined by LPS

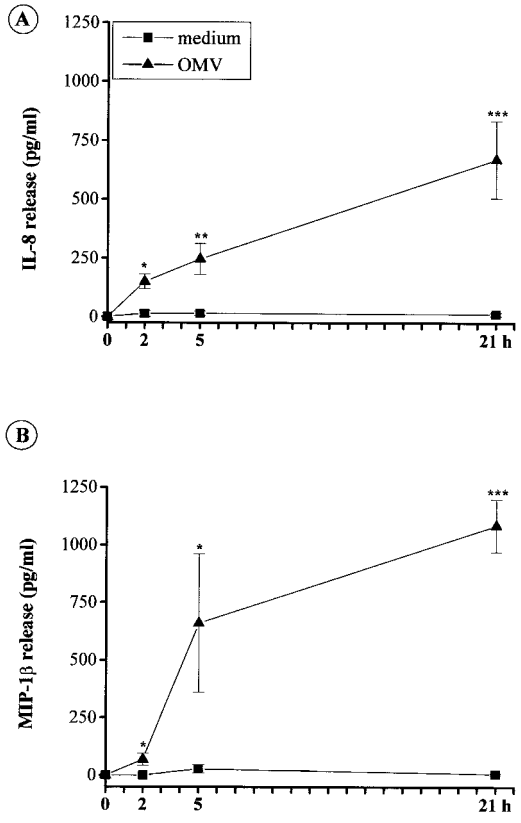

FIG. 4. Kinetics of IL-8 and MIP-1β production by OMV-stimulated neutrophils. PMN were stimulated with 5 μg of OMV per ml for the times indicated. IL-8 (A) and MIP-1β (B) levels were then determined in the cell supernatants by ELISA. For each cytokine, the figure shows the mean value ± the SEM of duplicate assays for each time point, each obtained from five experiments performed under the same conditions. The asterisks represent significant differences between OMV-treated and control PMN: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$.

from *N. meningitidis* serogroup B used at the concentrations present in our OMV preparation or from *E. coli* (6). These findings not only indicate that endogenous TNF-α does not play any autocrine role in inducing IL-8 and MIP-1β in OMV-treated PMN but also demonstrate that the effects attributed to OMV are not caused by the small amounts of LPS inserted into in the OMV complex. Nevertheless, the possibility that LPS contributes to stimulate PMN is not excluded. Although we cannot exclude the role of other endogenous factors produced in response to OMV, for example IL-1α and IL-1β, our study strongly indicate that OMV (containing LPS) and soluble LPS affect PMN cytokine production by distinct pathways. Whether OMV interacts with a specific receptor on neutrophils or is simply internalized by fluid-phase pynocytosis remains to be determined. In this context, it is important to remember that the OMV complex has a diameter of 70 ± 20 nm and that such a particulate structure is likely to be phagocytosed.

It is well established that in humans, classic proinflammatory cytokines, including TNF-α, IL-1, and IL-12, are present in the cerebrospinal fluid (CSF) during meningitis (20, 21, 32). In addition, CXC and CC chemokines, including IL-8, GRO-α, MIP-1α, and MIP-1β, have also been found in the CSF of such patients, including those infected with *N. meningitidis* (37). Furthermore, a critical pathogenetic role of cytokines and chemokines has been carefully established by using several different experimental models of bacterial meningitis. For example, while injection of TNF-α and IL-1 directly into the CSF results in an inflammatory response, antibodies neutralizing TNF-α and IL-1 are able to mitigate the extent of inflammation in experimental meningitis (29, 31, 40). Other evidence indicates that the recruitment of leukocytes in infectious meningitis involves the intrathecal production of chemokines (22). Interestingly, the majority of cytokines and chemokines are present at high concentrations in the CSF during meningitis, whereas they are undetectable in plasma, suggesting that cytokines are produced locally (26, 28, 40). Potential sources of cytokines and chemokines have been identified during meningeal inflammation, both within the brain parenchyma and in meningeal inflammatory cells. Indeed, endothelial cells, microglial cells, astrocytes, and infiltrating monocytes are considered to be the major origin sites of cytokines and chemokines (39), but only little attention has been devoted to the role of PMN, the hallmark of early events of meningeal infections. For instance, in an experimental model in which mice were intracerebrally infected with *Listeria monocytogenes*, the majority (80%) of the invading cells at 24 h postinfection were PMN (34). However, after 72 h, >50% of the cellular infiltrate consisted of monocytes. As measured by in situ hybridization, it was shown that

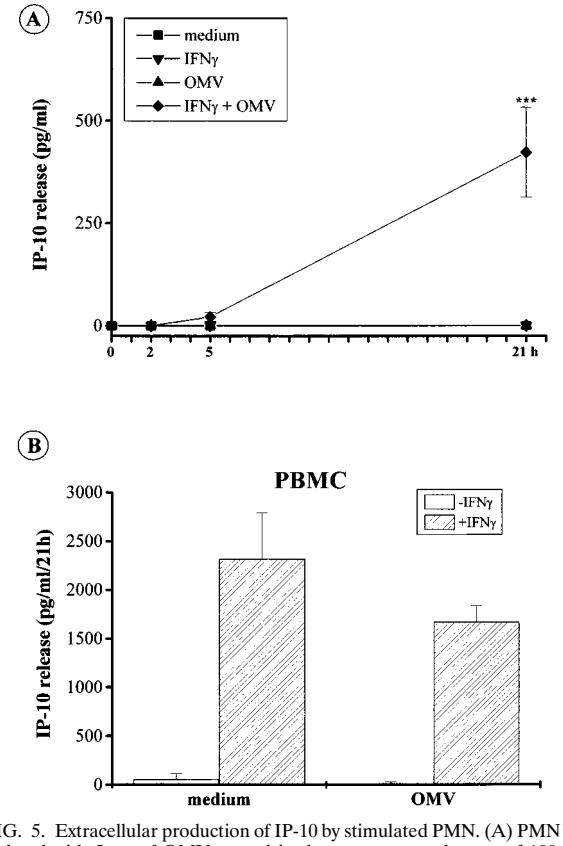

FIG. 5. Extracellular production of IP-10 by stimulated PMN. (A) PMN were stimulated with 5 μg of OMV per ml in the presence or absence of 100 U of IFN-γ per ml. Cell supernatants were collected at the indicated time points, and IP-10 protein levels were measured using a specific ELISA. Values are expressed as the means ± the SEM of averaged duplicate determinations for each time point, each obtained from five experiments. The asterisks represent significant differences between OMV plus IFN-γ-treated and control PMN (***, $P < 0.005$). (B) PBMC (5×10^5/ml) from autologous donors were stimulated with 5 μg of OMV per ml in the presence or absence of 100 U of IFN-γ per ml. Cell supernatants were collected after 21 h, and antigenic IP-10 was then measured by ELISA. The figure shows the mean values ± the SEM of duplicate assays for each condition obtained from three independent experiments.

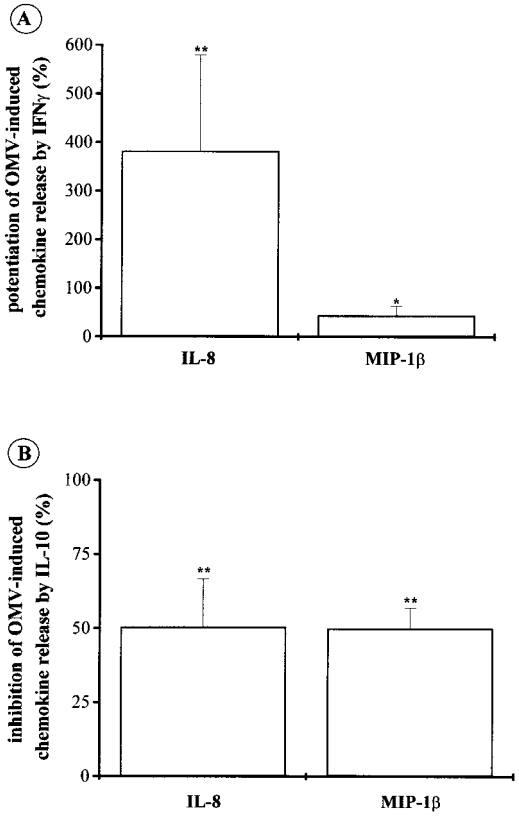

FIG. 6. Effect of IFN-γ and IL-10 on the release of IL-8 and MIP-1β induced by OMV. (A) PMN were preincubated for 30 min with or without 100 U of IFN-γ per ml and then cultured for 21 h after the addition of OMV before determining the chemokine release. The figure shows the mean values ± the SEM of the percentage of enhancement of chemokine release determined by IFN-γ treatment. Percent values were calculated from the difference in the amount of cytokine produced in the absence or presence of IFN-γ calculated from eight experiments. The values of the constitutive cytokine secretion were not subtracted. The asterisk represents significant differences between IFN-γ-treated and control PMN (*, $P < 0.05$; **, $P < 0.01$). (B) PMN were preincubated with or without 10 ng of IL-10 per ml for 30 min and then cultured for up to 21 h after the addition of OMV. The cell supernatants were then collected, and the levels of IL-8 and MIP-1β protein were determined. Values are the mean ± the SEM of the percentage of IL-10 inhibition, as calculated from eight independent experiments for IL-8 and MIP-1β, respectively. The asterisks represent significant differences between IL-10-treated and control PMN (*, $P < 0.05$; **, $P < 0.01$).

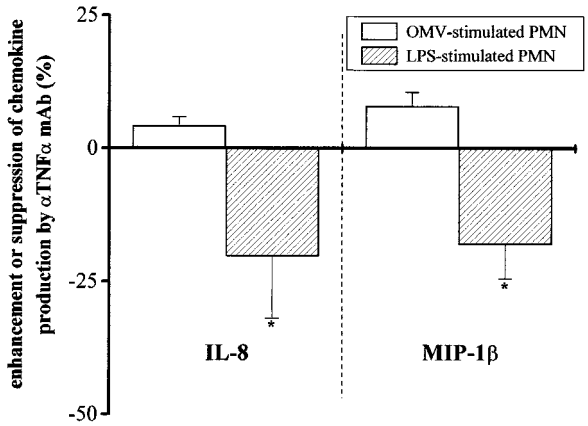

FIG. 7. Effect of neutralizing anti-TNF-α MAb on PMN-derived IL-8 and MIP-1β induced by OMV. PMN were stimulated with 5 μg of OMV or 200 ng of LPS per ml from *N. meningitidis* strain B for 21 h in the presence or absence of anti-TNF-α neutralizing antibodies. Cell supernatants were then analyzed for IL-8 and MIP-1β accumulation into the supernatants. The figure shows the mean values ± the SEM of the percentage of enhancement or suppression of chemokine release as determined by measuring the levels of anti-TNF-α MAb. The percent values were calculated from the difference in the amount of cytokine produced in the absence or presence of anti-TNF-α MAb, as determined from three experiments. The asterisks represent significant differences between anti-TNF-α MAb-treated and control PMN (*, $P < 0.05$).

MIP-1α and MIP-1β genes were expressed in infiltrating cells already after 12 h and that PMN were the main source of the two chemokines in the early phases of the disease (34). On the other hand, in the later phases, both PMN and monocytes were shown to produce MIP-1α and MIP-1β (34). MIP-1α, MIP-1β, and MIP-2 were also found in the CSF of the infected mice and contributed to CSF-mediated chemotaxis on PMN and mononuclear cells in vitro (34). More recently, in an infant rat model of *Haemophilus influenzae* meningitis, elevated mRNA expression for MIP-2, MIP-1α, macrophage chemotactic protein-1 (MCP-1), and RANTES was found, with kinetics paralleling those of inflammatory cells and disease severity (14). PMN and monocytes/macrophages were the main sources of MIP-2 and MIP-1α mRNA in the brains of mice with *H. influenzae* meningitis (14). Neutrophils and monocytes/macrophages were the main sources of MIP-2 and MIP-1α mRNA in the brains of mice with *H. influenzae* type b meningitis (14). Importantly, treatment with neutralizing antibodies against MIP-2, MIP-1α, and MCP-1 significantly reduced the recruitment of leukocytes into the brain in response to *H. influenzae* type b inoculation, suggesting that leukocyte accumulation was dependent, at least in part, on the production of these chemokines (MIP-2, MIP-1α, and MCP-1) (14). The capacity of PMN to express the genes for many chemokines in response to *N. meningitidis* products, documented in our study, not only extend the data described above but also highlights the potential role of PMN in mediating a leukocyte influx through the release of various chemokines.

In conclusion, the results presented here also provide new insights for a better understanding of the cellular mechanisms whereby VA-MENGOC-BC, the Cuban OMV-based vaccine, exerts its immunogenic and protective effects (23, 35). Presumably, all the cytokines and chemokines produced by PMN in response to OMV, as shown here, play a role in the cellular responses induced at the level of both the inductive and effector arms of the immune response. Neutrophils rapidly migrate in large numbers at the infection or immunization sites. The fact that they also serve as a cytokine source may contribute to the generation of the conditions necessary for both the recruitment and activation of monocytes, dendritic cells, and lymphocytes and the development of a protective immunogenic response.

ACKNOWLEDGMENTS

This work was supported by grants from M.U.R.S.T. (40 and 60% funds), The Progetto Sanità Fondazione CARI-VR-VI-BL-AN, and The Consorzio per lo Studio e lo Sviluppo degli Studi Universitari di Verona. José A. Lapinet is a fellow of the International Center for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy. Patrizia Scapini was supported by a fellowship from Fondazione Italiana per la Ricerca sul Cancro (FIRC).

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Editor: S. H. E. Kaufmann