Interleukin-8 and Chemotactic Activity of Middle Ear Effusions

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The importance of interleukin (IL)-8 in the chemotactic activity of middle ear effusions (MEEs) was evaluated. There was a significantly higher IL-8 concentration in MEEs of children with acute otitis media (AOM) than in children with otitis media with effusion (OME). The IL-8 concentration in MEEs with bacteria was significantly higher than in MEEs without bacteria. MEEs from children with AOM and OME had equally higher chemotactic activity than the diluent alone. The chemotactic activity was not altered by the presence of bacteria nor did it correlate with IL-8 concentration.

Fractionation of MEEs by gel chromatography demonstrated that the main chemotactic activity could be separated from the IL-8 activity, thus excluding IL-8 as a main chemotactic component in MEEs.

Otitis media with effusion (OME) is a frequent infection in children, and there is growing evidence that bacteria may play a role in the initiation of the inflammatory process leading to OME. It has been suggested that different types of otitis media represent parts of a continuum of a single diagnosis [1]. Neutrophils are the predominant cells involved in the early host responses elicited during bacterial infection in the tympanic cavity [2]. Consequently, neutrophils are numerous in middle ear effusion (MEE) samples from children with acute otitis media (AOM) but may be found in all types of otitis media [3]. To reach the middle ear, the neutrophils first must pass endothelial cells in blood vessels, cross the extracellular matrix, and finally pass the mucous membrane into the tympanic cavity. Previous reports have emphasized the role of bacterial proteins [4] and complement factors [5] as major chemotactic factors in otitis media.

The cytokine interleukin (IL)-8 has chemotactic activity for leukocytes, including neutrophils [6]. Maxwell et al. [7] found IL-8 in 92% of MEEs from children with OME, and Takeuchi et al. [8] detected IL-8 transcripts in 75% of both pediatric and adult MEEs. We evaluated the importance of IL-8 and the chemotactic activity of MEEs from children with AOM and OME.

Materials and Methods

Subjects. From June 1994 to March 1995, we collected 65 MEEs from 45 children (30 boys, 15 girls). None of the children had other chronic diseases or immunodeficiencies, and none had received antibiotic treatment within the prior 14 days. The clinical diagnoses AOM and OME were defined by the presence of fluid in the middle ear with or without signs or symptoms of acute infection. The clinical diagnoses were AOM in 17 children (22 MEEs) and OME in 28 (43 MEEs). The samples were obtained at paracentesis or placement of tympanostomy tubes for treatment of AOM and OME, respectively. Middle ear fluid was aspirated into a sterile syringe, and bacteriologic cultures were done. Samples were sent to a laboratory and stored at -80°C. Before testing, samples were diluted in equal amounts of RPMI 1640 without L-glutamine (Life Technologies GIBCO BRL, Paisley, UK).

The mean ages of children with AOM and OME, respectively, were 16 months (range, 2–34) and 50 months (range, 12–103). The mean durations of illness in children with AOM and OME, respectively, were 3.5 days (range, 0.5–14) and 5.3 months (range, 1–12).

Thirteen (59%) of the 22 MEE samples from children with AOM were culture-positive. Haemophilus influenzae was isolated from 7 samples, Streptococcus pneumoniae from 5, and Staphylococcus aureus from 1. Eight (18%) of the 43 MEE samples from children with OME were culture-positive. H. influenzae was isolated from 7 samples and Serratia liquefaciens from 1.

Isolation of neutrophils. Neutrophils were isolated from healthy donor venous blood in heparin. Briefly, erythrocytes were sedimented in the presence of dextran T-110. Neutrophils were separated by centrifugation (15 min, 1000 g) through Ficoll-Paque (Pharmacia, Uppsala, Sweden). Residual erythrocytes were lysed in distilled water. The neutrophils were washed three times and finally suspended at 10^6 cells in RPMI 1640 medium with 0.5% bovine serum albumin (Sigma, St. Louis). Viability was >97% by Trypan blue extraction.

Chemotaxis assay. Chemotaxis was done in 48 blind-well chemotaxis chambers (Neuro Probe, Cabin John, MD) using 3-μm pore polycarbonate filters (Nuclepore, Pleasanton, CA). We added diluted MEE (28 μL) to the lower compartment and neutrophils (40 μL) to the top compartment. MEEs were tested in triplicate. The fractions of MEE from gel filtration were assayed undiluted. The chambers were incubated for 30 min at 37°C in humidified
air (80%) containing 5% CO₂. Neutrophils on the top side of the filter were removed by washing three times in Hanks’ balanced salt solution. Filters were fixed with methanol (70%), stained with Coomassie blue, and mounted on a microscope slide. The slide was placed in a microscope (×100) equipped with a computer-controlled stepping motor, allowing unbiased selection of microscopic fields in which neutrophils were counted. A well-defined fraction of the migrated neutrophils was counted, and total migrated cells ($N_m$) calculated [9]. Chemotaxis was expressed as the fraction (percentage) as follows: $100 \times N_m/N_t$, where $N_t$ is the number of cells added to the upper compartment. In all experiments, the chemotaxant $N$-formyl-methionyl-leucyl-phenylalanine ($10^{-7}$M; Sigma) was used as a control.

**IL-8 assay.** The IL-8 concentration was measured by ELISA (Quantikine; R&D Systems, Abingdon, UK). Prepared samples were diluted 100 or, if necessary, 1000 times in RPMI 1640. MEE fractions from gel filtration were assayed undiluted. All samples were tested in duplicate. The detection limit was 3 pg/mL.

**Gel chromatography.** Gel chromatography was done using a Superose 12 HR 10/30 column (Pharmacia) equilibrated by RPMI 1640 by fast-pressure liquid chromatography equipment. Three culture-negative MEE samples from children with OME were chromatographed at 0.5 mL/min, and 0.25-mL fractions were collected. Recombinant IL-8 (Quantikine ELISA; 10 µg/mL) was chromatographed under the same conditions. The protein concentration was measured by a UV detector.

**Statistical analyses.** Statistical analyses were done by Mann-Whitney $U$ test or linear regression. Significance was $P < .05$.

**Results**

**IL-8 in MEE samples.** IL-8 was detected in all samples ($n = 63$). The mean concentration in MEEs from children with AOM was 136 ng/mL (range, 13–500), significantly higher than in MEEs from children with OME (mean, 65 ng/mL; range, 8–230; $P = .001$, Mann-Whitney test). The mean IL-8 concentration in the culture-positive MEEs (149 ng/mL) differed significantly from that in the culture-negative MEEs (66 ng/mL; $P = .001$, Mann-Whitney). The presence of bacteria was associated with elevated IL-8 content in MEEs in both groups (OME: 57 vs. 106 ng/mL, $P = .02$ Mann-Whitney; AOM: 102 vs. 165 ng/mL, $P = .04$). The IL-8 content could not be related to the species cultured from the MEEs. The mean IL-8 concentration declined significantly with the duration of illness in children with OME ($P = .03$, linear regression).

**Chemotactic activity of MEEs.** Culture medium was used to test random migration of the neutrophils. The fraction of neutrophils that migrated towards medium was 9.2% (expressed as percentage of total cells used in each test). Samples from children with AOM and OME possessed significantly higher chemotactic activity than medium ($P > .001$, Mann-Whitney). The mean percentage of cells that migrated towards MEEs from children with AOM was 23.3% (range, 5.7%–45.5%), which was similar to that in MEEs from children with OME (25.8%; range, 3.8%–43.8%; $P = .55$, Mann-Whitney). The chemotactic activity in culture-positive and -negative samples, respectively, were 25.3% and 23.8% ($P = .6$, Mann-Whitney). No correlation was found between the chemotactic activity of the MEEs and duration of illness ($P = .9$, linear regression).

**Correlation between chemotactic activity and IL-8 concentration.** No correlation was observed between chemotactic activity and IL-8 concentration in MEEs from children with AOM or OME (figure 1).

**Chromatography of MEEs.** The contribution of IL-8 to the total chemotactic strength of MEE from children with OME was further investigated after fractionation of the sample by gel chromatography. Figure 2 shows the fraction protein profiles, chemotactic activity, and IL-8 concentrations. Fractionation of effusions resulted in similar protein patterns in the 3 samples analyzed, although there were some quantitative differences. A narrow protein peak was found in fractions 32–35. A second, broader protein peak was found in fractions 42–61, which had the main chemotactic activity ($n = 3$). A minor, but reproducible chemotactic activity was observed in initial fractions 27–30. Recombinant IL-8 eluted with a peak in fraction 74. When MEEs were fractionated, IL-8 had two peaks: The first was in the initial fractions, representing aggregation of IL-8 with high-molecular-mass proteins, and the second peak reached its maximum in fraction 74, corresponding to that of recombinant IL-8.

**Discussion**

In this study, we found higher IL-8 concentrations in MEEs with bacteria. This observation suggests that bacterial sub-
<25% of the MEEs. However, endotoxin is almost universally present in MEEs from children with OME, regardless of whether or not the cultured bacteria contained endotoxin [11]. In accordance with our findings, a broad spectrum of microorganisms has been shown to induce IL-8 secretion in patients with urinary tract infection and empyema [12, 13]. It cannot be excluded that the presence of IL-8 and endotoxin in culture-negative effusions was due to the presence of microorganisms not found in routine cultures.

In addition to bacteria, a variety of proinflammatory stimuli can induce IL-8 secretion. These include IL-1, tumor necrosis factor (TNF), and histamine. The presence of IL-8 in MEEs was previously shown to be predicted by IL-1α and to a lesser degree by TNF-α [7].

In contrast to previous studies [4, 14], we could not demonstrate that the presence of bacteria altered the chemotactic activity of the MEEs. The activity of chemotactic proteins is dependent on the concentration, and as with IL-8, they often possess a well-defined narrow maximum after which the chemotactic activity declines. Thus, to obtain reliable results, it is important to keep the concentration of the chemotactic factors as close to those in vivo as possible. Consequently, we diluted the samples in a minimal volume of medium. Previous studies of the chemotactic activity of MEEs used higher dilutions of samples to obtain equal protein concentrations [4, 14].

The hypothesis has been advanced that IL-8 is responsible for the accumulation of leukocytes in the tympanic cavity [7]. The correlation between IL-8 concentration and chemotactic activity should be a bell-shaped curve if IL-8 is the only chemotactic component in MEEs. However, as shown in figure 2, this was not the case. To further address this question, we studied 3 MEE samples from children with culture-negative OME by gel chromatography. With this process, it appeared that several proteins contributed to the chemotactic activity in the MEEs. Essentially, IL-8 was eluted in a peak clearly separated from the peak that included the main chemotactic activity. We therefore conclude that IL-8 is not the main chemotactic component in MEEs. Brennen et al. [15] found no correlation between chemotactic activity and IL-8 concentration in synovial fluids from patients with rheumatoid arthritis. In accordance with our results, they concluded that the mechanism of migration into the inflammatory environment is likely to be due to a number of chemotactic signals in addition to that of IL-8.

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