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Complement activation in pediatric patients with recurrent acute otitis media

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Abstract

Objective—Otitis media (OM) is one of the most common childhood diseases. The relative contribution of complement activation in protection and pathogenesis during OM remains largely unknown. The purpose of this study was to investigate the beneficial and pathogenic contributions of complement activation in the middle ear of pediatric patients with recurrent acute otitis media (rAOM), and therefore to provide a rational approach to prevent sequelae of OM such as hearing loss.

Methods—Twenty children undergoing pressure equalization tube placement with or without adenoidectomy for rAOM were enrolled in the study. Bacterial cultures, enzyme-linked immunosorbent assay (ELISA) for complement components and cytokines and western blot for complement activation were performed on middle ear effusion (MEE) and serum samples. The levels of complement C3a, C5a and sC5-b9 in MEEs and serum samples were compared. The levels of these factors were also examined in regards to length of episode. Pearson's correlation coefficients were calculated on variables between C5a and IL-6 or IL-8. Complement gene expression in human middle ear epithelial (HMEE) cells induced by otopathogens was evaluated. Data were analyzed with Student's t test or the Mann-Whitney rank sum test. In all cases, a P value of <0.05 was set as the measure of significance.

Results—Our data demonstrated that the complement classical/lectin, alternative and terminal pathways were activated in the middle ear of children with rAOM. Increased complement components of C3a, C5a and sC5-b9 in MEEs were detected in patients with the episode lasting more than six weeks. There was a strong correlation between C5a and IL-6 or IL-8 in the MEEs. Additionally, otopathogens induced enhanced gene expression of factor B and C3 in HMEE cells, which is beneficial for host defense against invading pathogens.

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Conclusion—Our studies provided important new insights on how complement activation contributes to inflammatory process during rAOM. Knowledge of the activity of the complement pathway in patients with rAOM may stimulate the development of new strategies to prevent middle ear inflammatory tissue destruction by directing treatment to specific pathways within the complement cascade.

Keywords

otitis media; complement activation; factor B; C3; C5a; cytokine

Introduction

Otitis media (OM) is one of the most common diseases in infants and young children in the United States and other developed countries. The incidence of acute OM is 10.85% (709 million cases each year) with 51% of these occurring in children under 5 years of age [1]. OM-related hearing *impairment* has a prevalence of 30.82 per ten-thousand [1]. *Streptococcus pneumoniae* (Spn) and nontypable *Haemophilus influenzae* (NTHi) are the two major pathogens to cause OM. In addition, the synergistic effect between Influenza A virus (IAV) and Spn plays a significant role in the pathogenesis of OM [2,3].

The complement system is one of the major components of host innate immune systems in the defense against invading pathogens. Complement activation through the classical, alternative and lectin pathway results in the proteolytic cleavage of C3 to C3a and C3b. Once C3 is cleaved, the terminal pathway is activated, leading to the generation of the anaphylatoxin C5a and the assembly of the membrane attack complex (MAC) on the target surface or complexed with S-protein in plasma (sC5b-9) [4]. Complement proteins have previously been detected in human middle ear effusions (MEEs), and intense complement activation has been demonstrated in chronic otitis media with effusion (OME); maintaining ongoing inflammation in the middle ear [5,6,7]. In addition, the same authors observed strong staining for complement fragments iC3b/C3c and weaker labeling for C3d and C9, on the surface of the middle ear mucosa from chronic OME patients [6]. These findings suggest that intense complement activation could contribute to chronic inflammation resulting in effects on the tympanic membrane and middle ear such as development of myringosclerosis, middle ear adhesions, thinning and retraction of the tympanic membrane.

Our recent studies have demonstrated that the expression and production of alternative pathway components factor B and C3 in the middle ear epithelium are upregulated during the early stage of acute pneumococcal OM in mice [8]. Activation of the alternative pathway in the middle ear is a local phenomenon, which is essential for middle ear defense in bacterial clearance through the opsonophagocytosis mechanism [8]. Although our knowledge of the roles of the complement during OM has increased considerably during recent years, the beneficial and detrimental contributions of the complement system to this complex process remain largely unknown. To extend our findings in experimental animal studies into the human model, we analyzed the complement components of the classical/lectin and alternative and terminal pathways and cytokine levels in MEEs from pediatric patients with recurrent acute OM (rAOM) undergoing tympanostomy tube insertion. In addition, to examine whether complement factor B and C3 are produced locally by middle ear epithelial cells upon infection, we investigated the complement gene expression in primary culture of human middle ear epithelial (HMEE) cells infected with otopathogens. We showed that activation of the complement system significantly contributes to the pathogenesis of rAOM although it is inducted by otopathgens for protection.

Materials and Methods

Patient population, clinical samples collection and processing

Children, between 5 and 38 months of age, undergoing pressure equalization (PE) tube placement with or without adenoidectomy for rAOM were enrolled in the study. rAOM is defined as more than six episodes during the first 2 years of life, or more than four episodes during the first year of life or more than three episodes in 6 months. A disease free interval of at least 30 days separates individual episodes of otitis media. Children with a known immunological disorders (including IgG subclass deficiency), craniofacial malformations or obstructive adenoid hypertrophy were excluded. To collect MEEs, a myringotomy was made in the standard fashion for tympanostomy tube placement. If a middle ear effusion was present it was suctioned with a JUHN TYMP-TAP Middle Ear Fluid Aspirator/Collector™ (Medtronic). Samples were placed on ice as soon as they were collected. Blood samples for sera were obtained prior to tympanostomy. Single use aliquots of the sera were stored at −70°C. Nasopharyngeal samples were then taken from the patients with BBL Culture Plus™ swabs (BD). The swabs were passed along the floor of the nose into the nasopharynx and kept there for 5 seconds and then withdrawn. MEE and NP samples were cultured overnight at 37°C on Chocolate agar and Columbia CNA agar plates in an incubator supplemented with humidity and 5% CO2. A standard dilution assay and plate counting determined the number of CFU per milliliter. The remaining MEE and NP samples were centrifuged and single use aliquots of these samples were stored at −80C. Nationwide Children's Hospital Institutional Review Board approved this study.

Bacteria and virus

Spn serotype 6A (EF3114) with predominant transparent phenotype was kindly provided by Dr B. Anderson, Department of Clinical Immunology, University of Goteborg. NTHi strain 2019 was obtained from Michael A. Apicella, University of Iowa College of Medicine. **I**nfluenza virus A (IAV)/Alaska (6/77) (H3N2) was propagated and its titer was determined by a plaque assay as previously described previously [9]. All these pathogens have been previously described and extensively used for OM studies [10,11,12].

Infection of HMEE cells

The primary culture of HMEE cells had been established from middle ear biopsy specimens taken near the orifice of the eustachian tube from a 19-year-old patient and was reported previously [11]. HMEE cells were infected with formalin-killed NTHi at a multiplicity of infection (MOI) of 10, with IAV at a MOI of 1, and live or ethanol-killed Spn with or without a prior IAV at a MOI of 10 as previously described [12]. Control cell cultures were incubated with medium alone. The experiment included an unstimulated negative control flask and a positive control flask incubated with TNF-α (20 ng/ml; Sigma-Aldrich), as described for a previous study that used cytokines in positive controls [11]. The cell culture supernatants were collected at each time point prior to harvesting cells, centrifuged at 500g, and frozen at −70 °C

Quantitation of complement component transcripts from the HMEE cells by real-time PCR

Real-time PCR assays were performed to quantitate C3, C4, C5, factor B, factor H and factor I transcripts. Total RNA from middle ear lysate sample pooled from five mice was reverse transcribed with random hexamers by using the Superscript preamplification system (Invitrogen, Carlsbad, CA). Real-time PCR primers were selected for each gene by using PRIMER EXPRESS software (Version 2.0, Applied Biosystems, Foster City, CA). Primer sets for the following genes were synthesized by Invitrogen: C3 (sense, 5′-GAACCAGCTTGGCGTCTTG-3′; antisense, 5′-TGGCCCATGTTGACGAGTT-3′), C4

(sense, 5′-GCGCAACCCTGTACGACTACT -3′; antisense, 5′-TACTTGGTGCCCCGTAAAACA -3′), C5 (sense, 5′-GCGAGCTGCACGGATTAGTT-3′; antisense, 5′-TGCGACGACACAACATTCAGT-3′), factor B (sense, 5′-GCCAGACTATCAGGCCCATTT-3′; antisense, 5′-AGCCTCAAAGCTCGAGTTGTTC-3′), factor H(sense, 5′-CCATCCTGGCTACGCTCTTC-3′; antisense, 5′-GGAGTAGGAGACCAGCCATTCTC-3′), and GAPDH (sense, 5′-ATGGAAATCCCATCACCATCTT -3′; antisense, 5′-CGCCCCACTTGATTTTGG -3′). Reactions were performed in a 50-μl volume that included diluted cDNA sample, primers, and SYBR Green PCR Master mix (Applied Biosystems) according to the manufacturer's protocol. Real time PCR amplifications were performed on an Applied Biosystem Prism 7900 HT Sequence Detector according to the manufacturer's instructions. All data were normalized to the GAPDH mRNA. Relative changes in gene expression were determined using the $2^{-\Delta\Delta CT2}$ method as previously described [8] and expressed as the n-fold difference relative to a cDNA from normal control mice prepared in parallel with the experimental cDNAs.

Immunofluorescent staining

The cells were cultured on four-chamber microscope slides. After Spn treatment, the cells were fixed in 4% paraformaldehyde solution and permeabilized with DPBS containing 0. 5% TritonX-100, and then blocked with DPBS (pH7.2) containing 1% bovine serum albumin (BSA). The cells were incubated with primary antibodies: goat anti-human factor B polyclonal antibody (1:500, Quidel), goat anti-human C3 (1:100, MP Biomedicals, Solon, OH) at 4°C overnight, and then incubated with secondary antibodies: DyLight 488 donkey anti-goat IgG or DyLight 594 donkey anti-goat IgG (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1h, and then incubated with DAPI (1:10,000, Invitrogen, Carlsbad, CA) at room temperature for 2 min. The slides were mounted with Vectashield Mounting Medium (VectorLaboratories, Burlingame, CA). The samples were viewed and photographed using an Olympus Flowview 1000 laser scanning confocal microscope.

Western blots

For comparison of complement activation in MEEs and HMEE cell samples, equal amounts of protein (30μg) from MEEs and HMEE cell lysate samples were loaded on an SDS-PAGE gel. Samples were transferred to PVDF membranes. The membranes were blocked, and then incubated with goat anti human factor B polyclonal antibody (1:1000, Quidel) or HRP conjugated goat anti- human C3 monoclonal antibody (1:1,000, MP Biomedicals in TBST containing 5% dry milk at 4°C overnight. Blots were then washed and incubated with HRP-conjugated rabbit-anti goat IgG (1:10,000, Calbiochem, San Diego, CA) diluted in TBST containing 2% dry milk. The membranes were developed with a chemiluminescent detection system (ECL; GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol.

ELISA

The cell culture supernatants from infected or control groups were collected prior to harvesting cells and then concentrated using centrifugal filter units (Millipore, Billerica, MA), and stored at −70°C until use. Human complement factor C3a, C4a, C5a, sC5b-9, and cytokine IL-6 and IL-8 were measured with commercial ELISA kits (Quidel, San Diego, CA; BD Biosciences, San Diego, CA; eBiocsience, San Diego, CA), according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± the standard error of the mean (S.E.M.) or standard deviation of the mean (S.D.) as indicated. Data were analyzed using SigmaStat (SPSS Inc., Chicago, IL). Student's t test or the Mann-Whitney rank sum test was used for comparison of gene expression between experimental and control cohorts as well as for comparison of complement components measured in MEEs and serum samples. Pearson's correlation coefficients were calculated on the variability between C5a and IL-6 or IL-8. In all cases, a *P* value of <0.05 was set as the measure of significance.

Results

Patients

The study group consisted of 24 MEEs obtained from 20 patients with a gender distribution of 16 males and 4 females, whose ages ranged from 5 to 38 months (mean 18 months). Bacterial pathogens were identified in 5 MEEs (20.8%). Spn were present in 2 MEEs and 10 nasopharyngeal (NP) samples, NTHi in 3 MEEs and 6 NP samples, and mixed flora in 4 NP samples.

The classical/lectin, alternative and terminal pathways are activated in MEEs

In the alternative pathway of complement activation, complement factor B is cut into Bb and Ba, therefore the presence of Bb and Ba is indicative of the complement alternative pathway activation. To examine whether Bb and Ba exist in the middle ears of patients with rAOM, MEE samples were evaluated by western blot. We found that Bb and Ba fragments were prominent in the MEE samples, suggesting the complement alternative pathway is activated during rAOM. A representative western blot of seven samples is shown in Fig. 1A.

The Complement system activation in response to invading pathogens is mediated through the classical, alternative and lectin pathways. The center of all three pathways is C3 cleavage into C3a, iC3b C3b and C3d. To examine C3 breakdown products in the middle ear, MEE samples were analyzed by western blot. C3d and iC3b were present in the MEEs as showed in Fig. 1B, indicating C3 activation during rAOM. The classical pathway component C4a was also detected in MEEs, which could result from complement C4 activation through either the classical or lectin pathway (Fig. 1C). In addition, C3a in MEEs was significantly higher than that in serum samples (Fig. 1D), which suggested that C3 activation in the middle ear is a local phenomenon.

The levels of the components of terminal pathway C5a and sC5b-9 in the MEEs were also significantly higher than that in serum samples (Fig. 2A, 2B), indicating the terminal pathway is specifically activated in the middle ear during rAOM, Moreover, concentration of C5a correlated significantly with lL-8 ($p < 0.05$) (Fig. 2C) and IL-6 ($p < 0.05$) (Fig. 2D). Furthermore, sub-group analysis revealed that levels of C3a, C5a and SC5b-9 levels were significantly elevated in patients with the length of current episode lasting for 6 weeks or more compared to the group with the length of less than 6 weeks (Table 1). However, the concentrations of C3a, C5a and sC5b-9 in serum samples between these two groups were not significant (Table 1). These data suggest that levels of C3a, C5a and sC5b-9 in the middle ear are informative biomarkers for persistent inflammation during rAOM.

Taken together, our findings strong suggest that the activation of the complement classical/lectin, alternative, and terminal pathways in the middle ear enhances the host innate immune defense. However, breakdown products of C3 and C5 may also contribute to an active inflammatory process during rAOM. Targeting these molecules such as using anti C-5 antibody to reduce C5a level may provide a potential therapeutic for rAOM.

Otopathgens induced an increase in gene and protein expression of factor B and C3 in primary cultures of human middle ear epithelial cells

It is well known that recurrent episodes of AOM are associated with bacteria and/or viral infections. Complement factor B and C3 are produced mainly in the liver and circulating in blood and are also produced by other cells such as epithelial cells. We previously reported that factor B and C3 produced by mouse middle ear epithelium during acute pneumococcal OM contribute to complement activation during the disease course [8]. To investigate how human middle epithelial cells respond to a variety of otopathogens in complement gene expression and production, we examined the changes in gene expression for several complement components. Expression of classical pathway components (C4), alternative pathway (factor B), C3, terminal pathway C5 and the inhibitory regulators factor H in HMEE cells were evaluated. There was no significant alteration in gene expression of C4, C5, factor H in HMEE cells upon infection, suggesting HMEE cells may not be a source of these components presented in the middle ear during OM. Stimulation of HMEE cells with Spn, NTHi, IAV, TNFα resulted in induction of factor B and C3 genes examined in this study (Fig. 3). Furthermore, we investigated the production of C3, and factor B protein in primary cultures of HMEE cells. HMEE cells maintained with culture medium only (control group) constitutively produce low levels of C3 in its supernatant. Exposure of HMEE cells to Spn, IAV, NTHi or TNFα demonstrated increased secretion of C3 in their supernatants (Fig. 4A). In contrast, factor B was only secreted upon otopathogen stimulation in HMEE cells and a significant increase of factor B was evident in the supernatants in infected HMEE cells (Fig. 4B). In addition, we found that factor B and Bb fragments were prominent in HMEE cell lysate infected with Spn or with dual infection of IAV and Spn (Fig. 4B). Intensive immunofluorescence staining of C3 (Fig. 4C) and factor B proteins (Fig. 4D) was noted in HMEE cells infected with Spn. These results correlated with induction of C3 and factor B gene expressions as detected by real time PCR. Our results suggest that HMEE cells may be one of the major sources of local complement production upon infection, which could play a key role in enhanced complement activation during rAOM. A certain level of complement activation may be beneficial for the clearance of otopathgens from the middle ear. However, continuous activation of the complement system via the alternative pathway by otopathogens may lead to persistent inflammation in the middle ear. Our findings implicate that effective elimination of otopathogens or their products is important to prevent uncontrolled complement activation during rAOM.

Discussion

Bacterial or microbial fragments such as virus envelopes activate the complement pathways. Activation of the complement system through the alternative and the classical/lectin pathways was observed in MEEs of rAOM in the present study. Activation of the alternative pathway cleaves factor B into Ba and Bb and generates the C3 convertase of the alternative pathway, C3bBb, to further cleaves C3 into C3a and C3b. In addition, the alternative pathway plays an amplification role for complement activation of the classical/lectin pathway leading to increased C3 activation [4,13]. Considering our previous repot demonstrating an important role for the alternative complement pathway in the innate immune defense against pneumococcal infection during the early stage of acute OM in mice [8], it is likely that complement activation in the middle ear of patients with rAOM may be, at least in part, initiated via the alternative pathway by invading otopathogens.

We demonstrated that the levels of the complement activation markers C3a, C5a and SC5b-9 are significantly higher in MEEs than that in serum samples. These findings would suggest that complement activation makers in the middle ear may not be derived from vascular transudation of serum complement components. They are generated from the local expression and synthesis of complement components through complement activation in the

middle ear. Moreover, increased levels of these markers are associated with the persistence of rAOM, suggesting abnormal regulation of complement activation. Activation of the complement cascade may significantly contribute to serious sequelae of rAOM. There was a strong correlation between C5a concentration and the levels of IL-6 and IL-8 in MEEs. C3a and C5a, known as anaphylotoxins, are generated during the process of complement activation and play a critical role in the chemoattraction of inflammatory cells and induction of the "cytokine storm" in the inflammation process. C5a has been shown to promote inflammation by causing a massive influx of neutrophils and protein into the middle ear [14]. C5a plays a significant role in both inflammatory and immune effects by binding to the G-protein coupled receptor, C5aR (CD88) and a second receptor, the C5a-like receptor 2(C5L2). Expression of C5aR has been reported in many cell types [15]. Interaction of C5a/C5aR on several myeloid cells (e.g., neutrophils, monocytes/macrophages, and mast cells) leads to the induction of local inflammation through a process of cellular degranulation, increased vascular permeability, and leukocyte recruitment to the site of injury/infection, whereas engagement of the receptor on parenchymal cells leads to cell activation and functional modulation [15, 16, 17]. The soluble complement terminal complex sC5b-9 may directly activate neutrophils [18]. In addition, C3a and C5a can be directly cleaved by proteases such as kallikrein and thrombin in the absence of C3 activation [19, 20]. Both the kallikrein-kinint and fibrinolytic systems appear to be present in human MEEs [21, 22] and are capable of activating complement. Molecular interactions among these systems may have a profound effect on middle ear inflammation. There is growing evidence that activation of the C5-C5aR axis is important in human diseases and C5a-C5aR axis represents an attractive target for drug development [23]. A recent study demonstrated that a C5 deficiency and C5a blockade protects mice against pneumococcal meningitis [24]. Knowledge of the C5 system activity in the context of acute otitis media may provide promising insights for adjunctive immunomodulatory treatment of rAOM and thus warrants further investigation.

The middle ear epithelium plays a crucial role and serves as a first line of defense in the interaction with invasive pathogens. These epithelial cells are not simply a passive target for infection but may actively participate in the innate immune response. HMEE cells retain many features of respiratory epithelial cells and have been previously shown to be susceptible to Spn, NTHi and IAV and have the capacity to produce chemotactic cytokines upon infection [11,12]. In this report, we have demonstrated that HMEE cells are one of the major sources of complement C3 and factor B and is involved in complement activation during OM. HMEE cells are able to secrete C3 constitutively without stimulation but they also secrete factor B upon infection or stimulation. Our observation of the complement gene expression profile on HMEE cells is in agreement with a previous report that showed factor B and C3 genes are expressed following intra-tracheal instillation of lipopolysaccharide in the mouse lung [25]. The mechanism of regulation of factor B or C3 expression has not been completely elucidated. HMEE cells may be directly activated by otopathogens or indirectly through secreted cytokines such as TNFα as shown in this study. Our findings also suggest that the middle ear microenvironment, particularly the inflammatory mediators during OM, may play a critical role in controlling middle ear epithelium complement gene expression. Although antibacterial therapy for rAOM in children remains controversial [26], our results implicate that cleansing of otopathgens from the middle ear by antibiotics or antiviral agents may be an effect way to reduce excessive complement activation leading to persistent inflammation. On the other hand, targeted down regulation of the complement system has been identified as a virulence factor in the pathogenesis of NTHi [27].

The balance between the benefits of complement activation to protect against microbial invasion and the resulting inflammatory reaction that can lead to permanent damage is a major issue when pursuing any intervention targeting specific complement pathways. If

inflammation in the middle ear is left unchecked it can lead to chronic effusion with conductive hearing loss, damage to tympanic membrane and middle ear structures, facial nerve palsy, vertigo, intracranial spread of infection and possible sensorineural hearing loss. In the last decade, complement-targeted therapies have emerged that effect different points along the cascade. Drugs that inhibit levels of C1, C3, C5, and C5a have been developed and used therapeutically for a wide variety of disease processes including transplant rejection and enterohemorrhagic E. coli infections [28]. Elucidating which components of the complement system are important in both protective and inflammatory roles in the middle ear is thus important to drive development of OM-specific therapies.

In conclusion, our data show evidence for the induction of the complement activation in the middle ear of children with rAOM and suggest an important role of complement factors C3a C5a and sC5-b9 in the pathogenesis of rAOM. Further studies are warranted to confirm the pathophysiological role of these complement factors in rAOM and to stimulate the development of new strategies to prevent middle ear inflammatory tissue destruction by directing treatment to specific pathways within the complement cascade.

Acknowledgments

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Abbreviations

MEEs	middle ear effusions
Spn	*Streptococcus pneumoniae*
NTHi	nontypable *Haemophilus influenza*
IAV	influenza A virus

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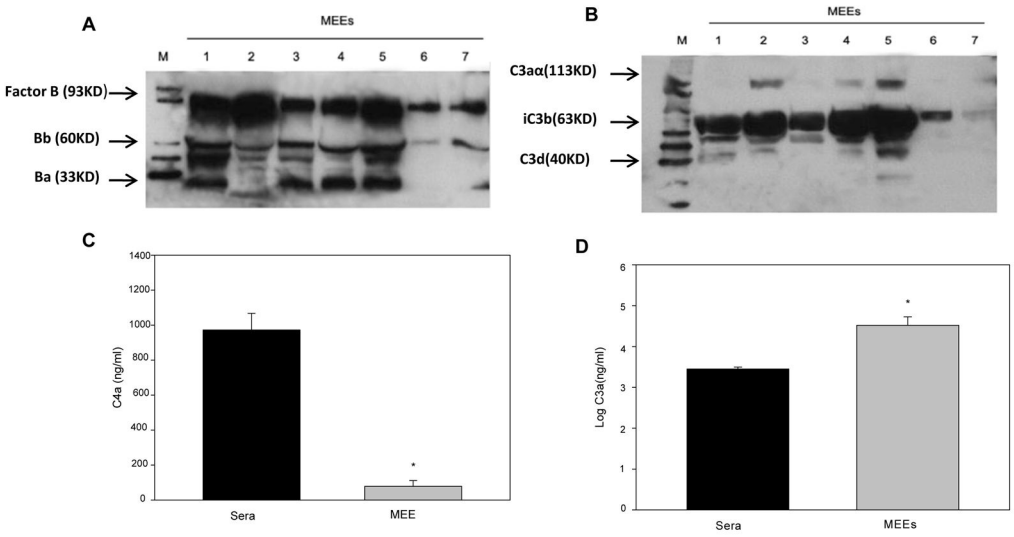

Fig. 1.

Factor B and C3 activation in MEEs collected from seven patients. (A) Factor B of the alternative pathway is activated in MEEs. Factor B fragments Bb and Ba were detected in MEEs by western blot. (B) C3 activation in MEEs. C3 fragments in MEEs detected by western blot. (C) C4a in MEEs (79.1± 16.2 ng/ml) was 1.2 log lower than that in serum samples (972.3± 47.6 ng/ml). (D) C3a in MEEs (33,000 ± 10,220 ng/ml) was 1.1 log higher than that in sera samples (2,800 ± 162 ng/ml), *, $p<0.001$.

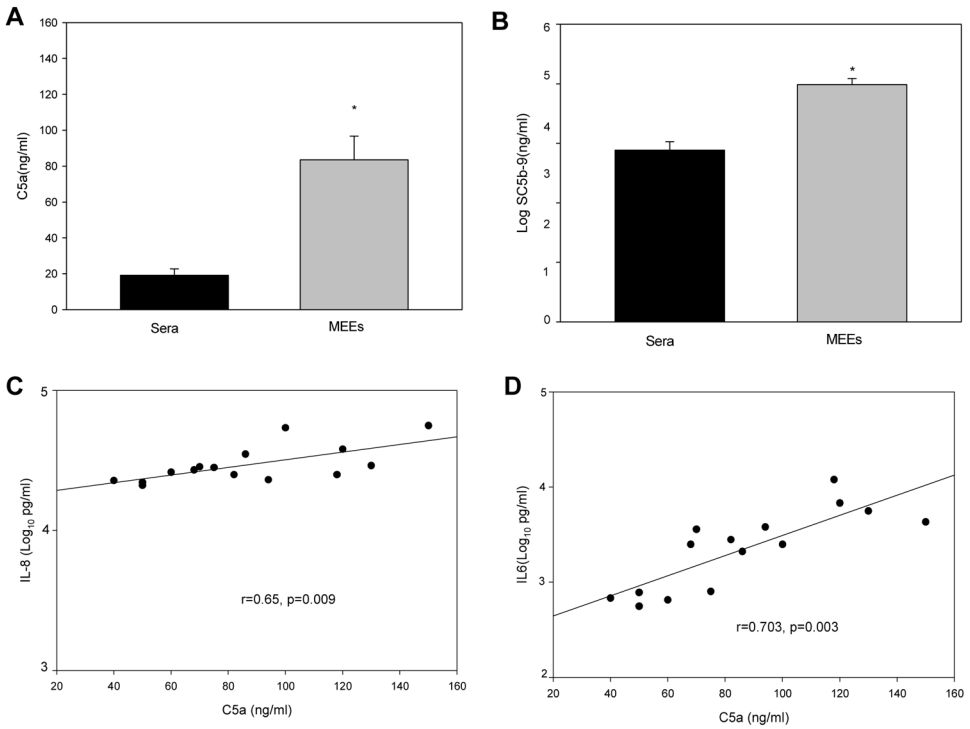

Fig. 2.

The terminal pathway is activated in MEEs. (A) C5a concentration in MEEs (84 ± 6.6 ng/ml) was significantly higher than in sera samples (19 ± 1.8 ng/ml), *, $P < 0.01$. (B) sC5b-9 concentration in MEEs (9600 ± 1,330 ng/ml) was significantly higher than in sera samples (756 ± 148 ng/ml), *, $P < 0.01$. (C and D) Pearson correlation analysis between C5a and IL8 and IL-6.

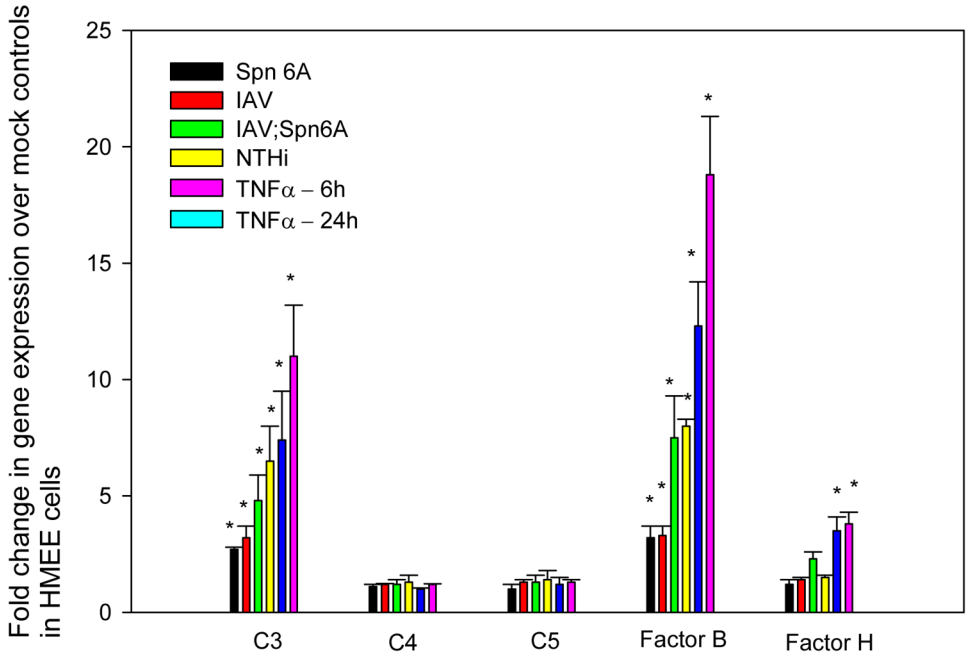

Fig. 3.

Gene expression of complement factor B and C3. In HMEE cells. Induction of gene expression as measured by real-time PCR on total RNA samples of HMEE cells stimulated with otopathogens and TNFα. Results are the mean fold changes the transcript levels (± SEM) from two separate experiments. *, $P < 0.05$ compared with the values determined for the medium control cohorts.

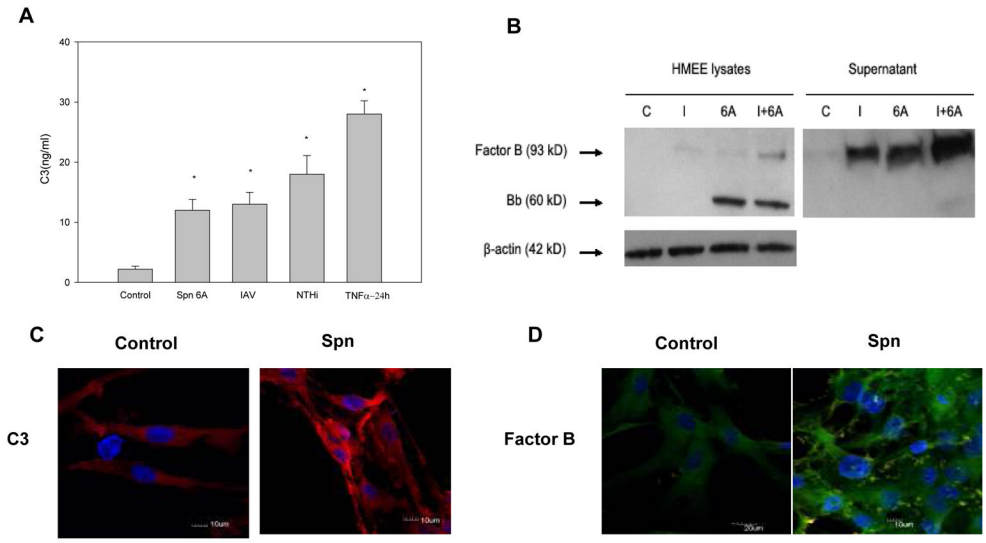

Fig. 4.

Protein production of complement factor B and C3 in HMEE cells. (A) Concentrations of C3 in the HMEE cell culture supernatant. Results are the mean concentration of C3 (± SEM) from two separate experiments. *$P < 0.05$ for the comparison with each medium control cohorts. (B) Levels of factor B protein and factor B fragments in HMEE cells infected with killed Spn, IAV or the combined infection. Approximately 30 μg of protein samples was subjected to SDS-PAGE. Bb and Ba were detected in HMEE cell lysates upon infection. Factor B was secreted into the supernatants upon infection. A representative of two experiments is shown. (C and D) Increased C3 and factor B immunofluorescence staining in HMEE cells at 24 h post infection with killed Spn compared with the sham control cohorts. Images are representative of three independent studies.

Table 1

Comparison of the levels of complement components C3a, C5a and sC5-b9 in middle ear effusions (E) and serum (S) with the length of the current onset of acute otitis media

	Length of the current OM episode		
	< 6 weeks, (n=12)	> or =6weeks, (n=8)	
	Median (range) ng/ml		*p*
C3a (E)	19,200 (12,700–22,230)	29,305 (22,700–32,950)	<0.05
C3a (S)	2,655 (1,780–4,773)	3,058 (2,100–4,043)	0.233
C5a (E)	60 (40–94)	100 (75–150)	<0.01
C5a (S)	18.6(10–36)	19.9(14–30)	0.742
sC5-b9 (E)	5,966 (1,800–8,280)	12,925 (8,260–26,950)	<0.001
sC5-b9 (S)	675.6 (125–1,740)	765.7 (225–1200)	0.665