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Complement activity in middle ear effusions

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SUMMARY

Evidence for complement utilization in middle ear fluids (MEF) from patients with otitis media with effusion was sought. It was found that cleavage products of C3, C4 and Factor B could be demonstrated immunochemically in MEF, and that native C3 was present in much lower concentrations than other proteins, relative to their serum concentrations. Haemolytic assays for C1-C5 showed that early complement components are inactivated in MEF. Potential mechanisms for complement utilization in MEF are discussed.

INTRODUCTION

Otitis media is the most common cause of acquired hearing loss in school children. The aetiology of this condition is considered to be eustachian tube dysfunction or the inability of the eustachian tube to ventilate the middle ear (Bluestone, Paradise & Beery, 1972). When the eustachian tube does not ventilate the middle ear, it becomes essentially a closed space. A negative middle-ear pressure occurs initially and is followed by a transudate of serum proteins into the middle ear cleft (Paparella *et al.*, 1970). If this ventilatory obstruction is not relieved, the middle ear mucosa becomes metaplastic and the appearance of goblet cells results in the production of a thick mucoid middle-ear effusion (Sade, 1960; Bernstein & Hayes, 1971; Senturia, Carr & Ahlvin, 1962). The sediments of these middle ear fluids (MEF) contain many lymphocytes and macrophages which may be responsible for the release of lysosomal enzymes resulting in further tissue destruction (Bryan & Bryan, 1976). If the fluid is not removed, it remains for long periods of time; irreversible damage may occur to the mucosa of the middle ear cleft with subsequent chronic otitis media with mastoiditis (Proud & Buff, 1976).

The aim of the present study was to explore the possibility that the complement system may play a role in the pathogenesis of otitis media. We have provided evidence that complement cleavage products occur in the MEF, and that early complement components are present at reduced levels.

MATERIALS AND METHODS

Middle ear fluid (MEF) and serum. Twenty MEF's were removed at the time of surgery from patients with otitis media with either a serous or seromucinous effusion. The MEF's were collected undiluted in a sterile bronch trap or Senturia apparatus. Blood samples were collected at the same time and both the MEF and serum were stored at −70°C until assayed One aliquot of serum was aged at 37°C for 4 days prior to freezing. All assays could not be performed on all MEF samples due to the limited amount of MEF available.

Immunochemical methods. Quantification of individual serum protein levels was accomplished by the method of single radial immunodiffusion (Mancini, Carbonera & Heremans, 1965). Commercially obtained antisera to α-2 HS glycoprotein (Behring Diagnostics, Sommerville, New Jersey), α-1 antitrypsin, transferrin, IgA, IgG, IgM, α-2 macroglobulin, C4 (Meloy Laboratories, Inc., Springfield, Virginia), Factor B, C3 and C3d (Netherlands Red Cross Blood Transfusion Service) and corresponding serum protein standards for all but C3 and C3d were utilized. The anti-C3 was specific for the B determinant.

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Specificity of all antisera was determined in gel diffusion and immunoelectrophoresis studies. Each patient's fresh serum was used as a standard to determine relative native C3 concentrations in MEF's, while aged autologous serum served as a standard for relative C3d content. For the assay of C3d, sera and MEF's were diluted such that the cross-reactivity of anti-C3d with C3 could not be detected in fresh serum.

Immunoelectrophoresis was performed on matched, undiluted samples of serum and MEF in veronal-buffered agarose, pH = 8·7, μ = 0·1, containing 0·01 M Na_2 EDTA. Electrophoresis was carried out for 2 hr at 6 V/cm. Antisera to C3 (a-β_1C/β_1A) and Factor B were utilized.

Antigen-antibody crossed-immunoelectrophoresis was carried out as described by Sjöholm & Laurell (1973). Sera and MEF's were diluted in phosphate buffered saline (0·15 M NaCl, 0·02 M sodium phosphate, pH = 7·4) containing 0·01 M Na_2 EDTA. The first-dimension electrophoresis was carried out in veronal buffered agarose, pH = 8·6 at 6 V/cm and 18°C until bromphenol blue labelled albumin migrated a standard distance. Second-dimension electrophoresis was accomplished in the same buffer in agarose containing a specific antiserum to C4 at 2 V/cm and 18°C for 18 hr. Matching serum-MEF pairs were always electrophoresed in the same gel in the first dimension.

Haemolytic assay. Complement components C1-C5 were determined in MEF's and matching sera, using appropriate cellular intermediates EAC 4 human (hu), EAC 1 guinea-pig (gp) and EAC 1 gp, 4 hu and the respective purified complement components. The cellular intermediates, functionally purified complement components and guinea-pig serum, were all obtained from Cordis Laboratories (Miami, Florida). The procedures used have been described for C1 through C5 and modified slightly for the macrotube titration (Mayer, 1961; Nelson *et al.*, 1966). A reference human serum pool of at least thirty donors was titrated each time a complement component assay was performed.

The CH_{50} units for C1–C5 for each MEF specimen and serum specimen were determined on equivalently diluted material, and the functional activity of C1–C5 in MEF is expressed as a percentage of the functional activity found in the patient's serum following correction for protein concentration. The individual complement components and total protein content in MEF's and sera were compared using the Student's *t*-test.

RESULTS

Protein concentrations in MEF

The concentrations of various plasma proteins in the MEF and in corresponding sera from six children with otitis media with either a serious or seromucinous effusion (as compared in Fig. 1). Protein concentrations in serum were in all cases within accepted normal limits. IgG, IgA and several other proteins of lower molecular weights were found at levels two to three times those found in serum. Proteins of higher

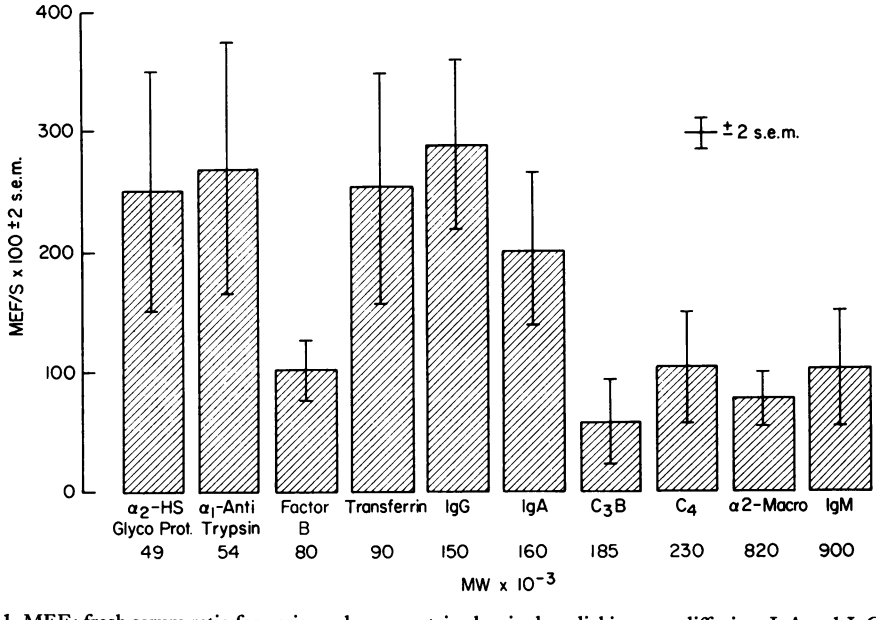

FIG. 1. MEF: fresh serum ratio for various plasma proteins by single radial immunodiffusion. IgA and IgG are significantly elevated ($P < 0.01$ and $P < 0.005$ respectively, Student's *t*-test) in comparison to the serum. C3 is significantly depressed in relation to the serum ($P < 0.05$).

molecular weights, such as IgM and α-2 macroglobulin are present at levels comparable to serum. Complement components C4 and Factor B were detected at levels comparable to those in serum, and native C3 as detected by measurement of the B determinant was found at about half the level of that in serum.

Fig. 2 demonstrates that C3d antigen is detectible in MEF at dilutions such that C3 in equivalently diluted fresh serum is not detectible. Native C3 is present at lower levels in MEF than fresh serum and it is likely that the C3 cleavage products C3d and possibly C3b detected in MEF are derived from MEF C3 (Molenaar, *et al.*, 1974).

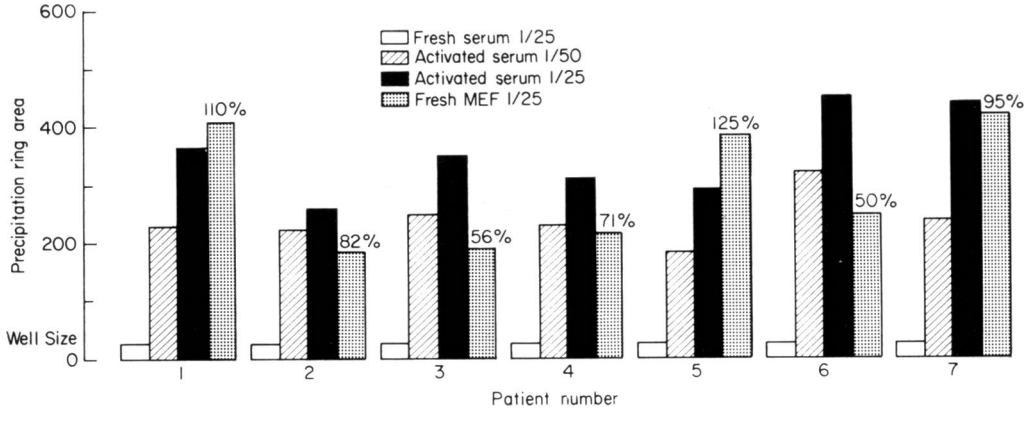

FIG. 2. C3d in MEF and aged (37°C, 96 hr) serum by single radial immunodiffusion in seven patients. C3d antigen is not detected in fresh serum under the assay conditions utilized, but is found in fresh MEF at an equivalent dilution. The quantity of C3d in fresh MEF is expressed as the percentage of C3d in activated serum (1/25).

Demonstration of C3, Factor B and C4 cleavage products

Cleavage of Factor B was demonstrated in each MEF, but never in fresh serum (Fig. 3a). Fig. 3b demonstrates the cleavage of C3 in MEF by immunoelectrophoresis. Cleavage of C3 was demonstrated in each of seven MEF's tested, but never in corresponding serum samples.

Figs 4–6 show results of the crossed-immunoelectrophoresis assay (Laurell, 1965) for C4. Fig. 4 demonstrates a quantitative elevation of C4 in MEF with no evidence for C4 cleavage. On the other hand, Fig. 5 is an example of a MEF in which C4 is depleted relative to serum, and which also demonstrates a change in electrophoretic mobility of C4 from β2 to slow β2. In addition, when serum and MEF are mixed, the resulting pattern shows a major peak with a predominant shoulder and immunochemical identity, indicating that the slow β2 peak is a C4 cleavage product similar in electrophoretic characteristics to C4c. Of the four specimens examined by crossed-immunoelectrophoresis for C4, two showed evidence of a C4 cleavage product. Fig. 6 is an example of an MEF containing less C4 than does serum.

Haemolytic levels of C1-C5

Table 1 demonstrates the haemolytic levels of C1-C5 compared to autologous serum, and Table 2 depicts each patient's serum C1-C5 levels. In each case, MEF complement was significantly depressed ($P < 0{\cdot}0005$) compared to serum, while MEF total protein is significantly greater than serum protein ($P < {\cdot}005$).

DISCUSSION

The present data suggest that complement utilization occurs in MEF, but the mechanism of inactivation of early complement components is not clear. Haemolytic complement titres of C1–C5 are clearly

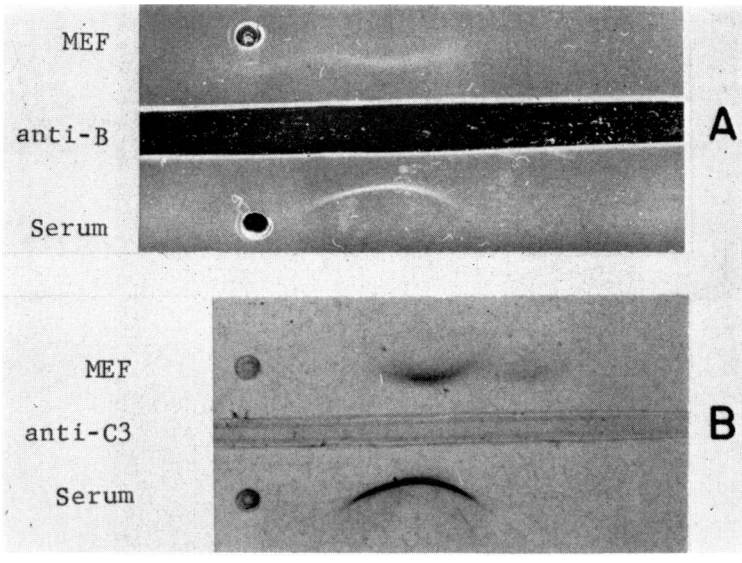

FIG. 3. (a) Factor B cleavage in MEF from a patient with serous otitis media with effusion as demonstrated by immunoelectrophoresis. (b) Cleavage of C3 in MEF from a patient with serous otitis media with effusion as demonstrated by immunoelectrophoresis. Serum is from the same patient and was collected at the time of surgery.

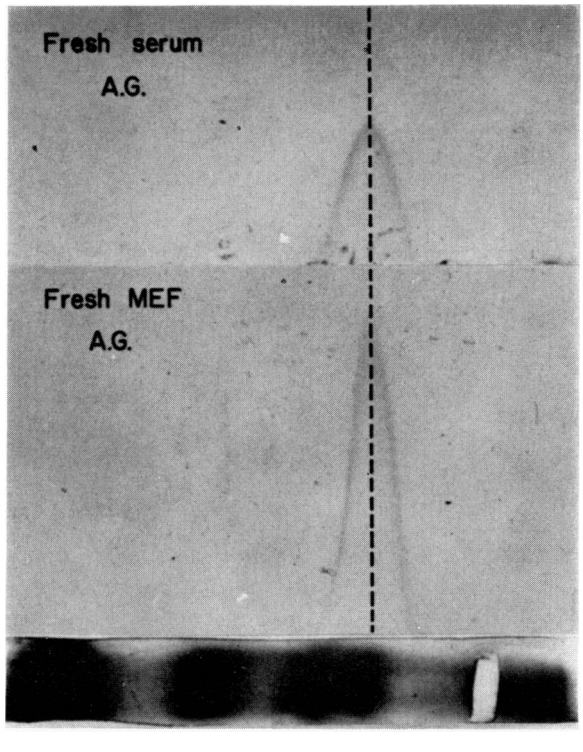

FIG. 4. C4 in fresh serum and MEF from a patient with serous otitis media with effusion. C4 in MEF is in this case elevated as compared to serum, as demonstrated by crossed-immunoelectrophoresis.

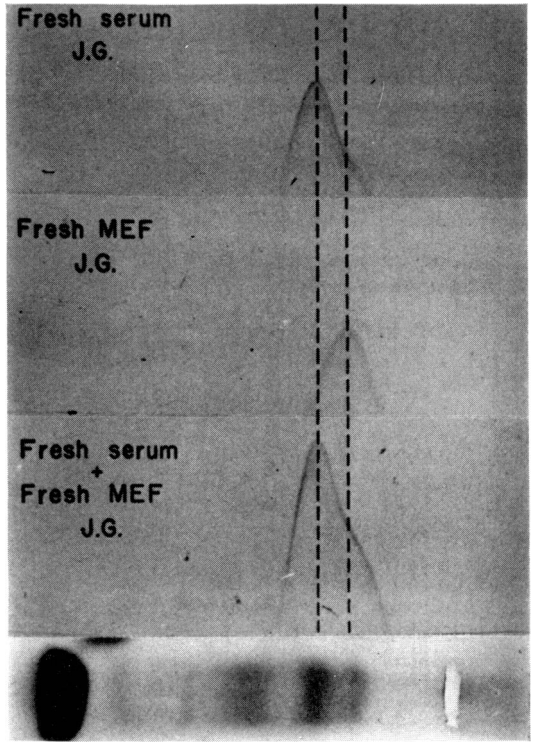

FIG. 5. C4 in fresh serum and MEF from a patient with serous otitis media with effusion. C4 in the MEF appears to be present as a species of slower electrophoretic mobility as compared to C4 in fresh serum. Combining serum plus MEF prior to first dimension electrophoresis indicates the presence of two species demonstrating immunochemical identity. This may indicate *in vivo* cleavage of C4 in the MEF.

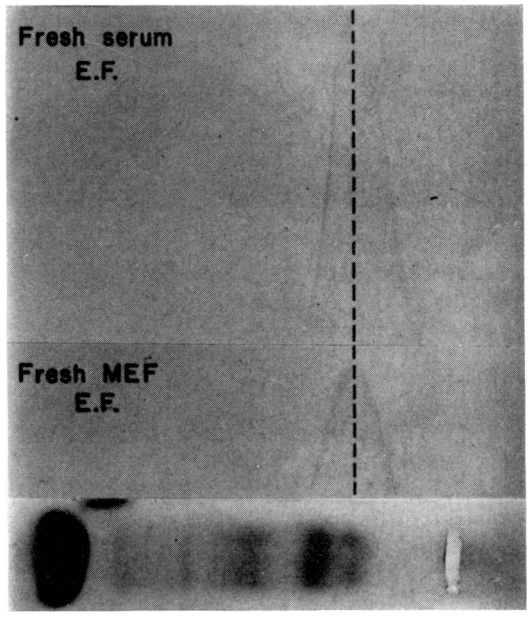

FIG. 6. C4 in fresh serum and MEF from a patient with serous otitis media with effusion. C4 in MEF is in this case diminished as compared to serum.

TABLE 1. Complement components of middle ear fluids*

Patient	Total Protein Serum gm/ml	Total Protein MEF gm/ml	C1	C2	C3	C4	C5
1	7·1	8·3	n.d.†	30	19	18	38
2	7·2	12·3	n.d.	29	16	32	32
3	7·2	10·8	16	34	21	34	27
4	5·5	11·6	n.d.	12	16	20	20
5‡	n.d.	n.d.	25	20	2	16	7
6‡	n.d.	n.d.	n.d.	32	17	35	28
7‡	n.d.	n.d.	n.d.	14	17	25	38
8‡	n.d.	n.d.	<7	<6	<7	<2	<8
9‡	n.d.	n.d.	5	15	13	14	9

* The complement components of the MEF's are expressed as a percentage of the complement component of the patients' sera after correction for total protein.

† n.d. = Not done

‡ Total MEF protein for patients 5–9 could not be determined due to lack of material. Complement levels are corrected for the average MEF : serum protein ratio of subjects 1–4.

TABLE 2. Complement components of patients' sera*

Patients	C1 %	C2 %	C3 %	C4 %	C5 %
1	n.d.†	104	110	56	106
2	n.d.	95	87	61	64
3	n.d.	91	92	70	82
4	—	123	124	192	154
5	161	104	120	112	107
6	n.d.	104	110	56	106
7	n.d.	89	120	150	91
8	n.d.	136	122	127	99
9	143	140	132	122	248

* The complement components of patients' sera are expressed as a percentage of the complement components in pooled normal sera.

† n.d. = Not done.

lower in MEF than in serum, and native C3 is found in lower concentrations in MEF than in serum. In addition, anti-C3d reactive material is found in MEF under conditions such that C3 is not detected, indicating the generation of C3 cleavage products. C3 and Factor B cleavage are demonstrated in MEF by immunoelectrophoresis, and C4 cleavage products are found in some MEF.

Veltrie & Sprinkle (1976) have recently reported soluble immune complexes in MEF. The nature of the antigen that may be involved has not yet been defined. Bacteria are present in as many as 50% of MEF in otitis media with effusion (Senturia, 1970). Many of the organisms are not considered to be pathogenic and are not characteristic of acute suppurative otitis media. The most common organisms found in acute suppurative otitis media in infants are *Streptococcus pneumoniae, Streptococcus pyogenes* and *Haemophilus influenza* with the pneumococcus predominating (Howie & Ploussard, 1969). We have been unable to demonstrate anti-pneumococcal antibodies in middle ear effusions and it is not likely that pneumococcal or viral antigens play an important role in generating immune complexes in middle ear

inflammation (Bernstein & Allen, unpublished observations). Additionally, antibodies against nucleic acids have not been demonstrated in MEF (Bernstein & Bartholomew, unpublished observations), suggesting that these antigens are also not involved. The classical pathway may in part account for complement utilization in some MEF, but data concerning the nature of immune complexes are incomplete.

There is no direct evidence that alternative pathway activation occurs in MEF, although this possibility has not been ruled out; particularly in view of the ability of many strains of pneumococci to activate this pathway (Stephens, Williams & Reed, 1977). Factor B cleavage demonstrated in the present study might occur due to recruitment of the C3b-dependent feedback mechanism or proteolysis, as well as by direct activation.

Cleavage products of C3 may be generated via the classical-pathway, the alternative pathway, or by non-complement mediated proteolysis. Tryptic cleavage, for example, produces C3 cleavage products similar to C3b, C3c and C3d (Bokish, Mueller-Eberhard & Cochrane, 1969). Similarly, the tryptic product of C4 in serum (Sjoholm & Laurell, 1973) produces cleavage products immunochemically and electrophoretically similar to C4c and C4d (Shirashai & Stroud, 1975). Proteolytic activity in MEF may likewise account for complement cleavage products and functional inactivation. It is not clear, therefore, what the mechanism for complement utilization might be in MEF, and is subject to further study.

It is clear, however, that early complement components are largely inactivated in MEF, thereby depleting the middle ear of a possibly important defence mechanism. In addition, it is possible that the complement system contributes to the pathogenesis of middle ear disease.

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This paper is dedicated to our teacher and friend, Dr. Felix Milgrom, of the State University of New York, Buffalo, New York, on the occasion of his 30th anniversary of his research activity.

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