Decreased Heat-Labile Opsonic Activity and Complement Levels Associated with Evidence of C3 Breakdown Products in Infected Pleural Effusions

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ABSTRACT Heat-labile opsonic activity was measured simultaneously in serum and pleural fluid of patients with transudates, infectious exudates (with positive or negative bacterial culture) and neoplastic exudates, using two different complement-dependent phagocytic tests: the killing of *Staphylococcus aureus* Wood 46 variant strain (K50 opsonic titers) and the assessment of ingestion rate of endotoxin-coated paraffin particles (Oil Red 0 uptake test). K50 opsonic titers were lower in culture-positive pleural effusions as compared to culture-negative ($P < 0.002$) or neoplastic effusions ($P < 0.002$). These results were corroborated by the Oil Red 0 uptake test. The data obtained with the two assays showed a significant correlation ($P < 0.001$).

The hemolytic activity of complement (CH50) as well as the levels of C3 breakdown product, C3d, were measured in the same sera and pleural fluid samples and in an additional group of patients with pleural effusions of the same etiology. Effusions with positive cultures showed lower CH50 values ($P < 0.01$) and higher C3d values ($P < 0.05$) when compared to culture-negative pleural fluids. Finally, evidence for immune complexes in pleural effusions and sera was looked for by determination of Clq binding activity. Levels were higher in culture-positive effusions when compared to culture-negative fluids ($P = 0.005$).

K50 opsonic titers showed a positive correlation with CH50 values ($P < 0.001$) for all fluids tested. Similarly Clq binding activity correlated with C3d levels in effusions of infectious origin ($P = 0.05$). Recovery experiments using the various bacterial species isolated from culture-positive pleural effusions showed evidence of complement inactivation upon incubation with pooled sera at concentrations of 10^7–10^8 microorganisms/ml.

These results indicate that one important reason for bacterial persistence in empyema may be decreased opsonization secondary to local consumption of complement.

INTRODUCTION

Empyema thoracis has always been recognized as a disease entity requiring most often incision and drainage for cure, and the advent of antibiotics has unfortunately not changed this basic rule (1). The persistence of the infectious agent in a pleural cavity despite the presence of abundant polymorphonuclear leukocytes (PMN-s)[1] has been ascribed to deficient surface phagocytosis, a biological process that, in turn, has not yet been fully explained (2).

Extensive knowledge has accumulated in recent years regarding one of the crucial mechanisms regulating phagocytosis by PMN-s, the opsonization of microorganisms. The important role of specific antibodies has been well established in a variety of models. More recently, new information has been provided as to the mechanisms of opsonization mediated by complement activated by either the main or the alternate pathway and leading to the coating of microorganisms with opsonically effective C3 (3, 4). The role of complement has been clearly established experimentally (5, 6) and

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[1] *Abbreviations used in this paper*: ClqBA, I-Clq binding activity; CH50, hemolytic activity of complement; ORO, Oil Red 0; PMN-s, polymorphonuclear leukocytes; rs, Spearman's correlation coefficient.

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indirectly confirmed in clinical situations by the demonstration of decreased opsonic activity in patients with C3 deficiency, and concomitant increased susceptibility to bacterial infections (7). Several clinical studies have also shown that serum complement levels and(or) opsonic activity could be depressed during acute septicemia (8–10). It could be therefore hypothesized that persistence of microorganisms in presence of PMN-s, as encountered in infected pleural effusions, might be a result of inadequate, complement-dependent opsonization of the infectious agent. The present study addresses itself to this question.

Using two different experimental approaches, infected pleural effusions were studied and compared with transudates, neoplastic effusions, and autologous sera. First, heat-labile opsonic activity was measured by a phagocytic bactericidal assay and by assessing ingestion rates of endotoxin-coated, oil-paraffin particles, using normal PMN-s as effectors. Second, complement activity, levels of various complement components, and of C3 breakdown product C3d were measured, and the ^{125}I-Clq binding activity (ClqBA) was evaluated.

The correlations found allow to conclude that one of the factors contributing to the persistence of microorganisms in an infected pleural effusion is local consumption of complement components, leading to decreased opsonization and phagocytosis by PMN-s.

METHODS

Patients, definition of transudates and exudates. Patients undergoing diagnostic thoracenteses at the Department of Medicine of the Geneva University Hospital, Geneva, Switzerland were included prospectively in this study. Pleural fluids were classified as exudates or transudates using well-known criteria of fluid to serum protein and lactate dehydrogenase ratios (11). The 17 transudates were obtained from 10 patients with heart failure, from 4 with liver cirrhosis, and from 3 with hypoproteinemia secondary to renal disease. Their mean age was 70. Patients with exudates included a first group of 32 patients with clinical, bacteriological, and radiological evidence of pulmonary infection, 18 of whom had a positive bacterial culture of their pleural fluid at the time of thoracentesis (culture-positive group, Table I), and 14 whose pleural fluid culture yielded no bacterial growth on the day of the study (culture-negative group). Their mean age was 51. The last group consisted of 34 patients with clinical and pathological evidence of pleural malignancies and included 15 with bronchogenic cancer and 19 with metastatic disease of various origins. Their mean age was 70.

Collection and storage of pleural fluids, serum, and plasma samples. All the samples of pleural fluids were immediately mixed with EDTA (20 mM, final concentration), centrifuged at 1,500 *g* for 15 min and stored at −70°C in portions of 0.5 ml. Serum and plasma samples were obtained from all patients with transudates, from all patients with exudates of infectious origin as well as from 15 patients with neoplastic exudates. To obtain serum, blood was allowed to clot at room temperature for 60 min, and serum was separated by centrifugation at 1,500 *g* for 15 min and stored at −70°C. To obtain plasma, blood was collected in tubes containing EDTA (20 mM, final concentration), centrifuged, and the supernate immediately stored at −70°C.

TABLE I

Pleural Effusions of Infectious Etiology (Patients with Positive Cultures): Bacteriological Results

Case	Sex	Age	Bacteria
		yr	
1 F.N.	M	55	*Klebsiella pneumoniae*
2 B.K.	M	33	*Bacteroides species*
3 E.E.	M	54	*Streptococcus pneumoniae* plus *S. aureus*
4, 5* I.H.	M	72	*S. aureus*
6, 7* G.P.	M	69	*Peptococcus magnus* plus *Fusobacterium varium*
8, 9* P.R.	M	42	*E. coli*
10 L.E.	F	45	*Streptococcus pneumoniae*
11 A.M.	F	45	*Streptococcus pneumoniae*
12 R.A.	M	45	*Pseudomonas aeruginosa*
13 A.J.	M	55	*Bacteroides melaninogenicus*
14 B.M.	F	50	*Bacteroides fragilis, Bacteroides melaninogenicus, Peptostreptococcus intermedius*
15 P.L.	M	60	*Fusobacterium nucleatum, Peptostreptococcus anaerobius*
16 M.S.	M	55	*Fusobacterium nucleatum, Peptostreptococcus anaerobius, Propionibacterium acnes*
17 B.F.	M	48	*Peptococcus magnus* plus *Propionibacterium acnes*
18 G.A.	F	78	*Streptococcus viridans, Fusobacterium nucleatum, Bacteroides melaninogenicus*

* After a 1-wk evolution.

Biochemical and hematological values of pleural effusion. Protein, glucose, and lactate dehydrogenase concentrations were measured according to well-established methods. Erythrocyte, leukocyte, and differential counts were performed immediately on freshly obtained pleural effusion. Bacteriological cultures of all pleural effusion were performed on standard and anaerobic media; microorganisms were identified in the microbiology laboratory according to standard methods. Lysozyme levels were determined by the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, N. J.) measured by the decrease of absorbancy at 450 nm (12).

Measurement of opsonic activity

Preparation of leukocytes. Leukocytes used for phagocytosis experiments were collected from peripheral blood obtained by venipuncture from healthy volunteers. Leukocytes were separated from citrated blood (1 ml citrate for 4 ml of blood) by dextran sedimentation and ammonium chloride erythrocyte lysis. They were washed twice and suspended in Krebs-Ringer phosphate medium, pH 7.4.

Phagocytic bactericidal assay. The assay developed for this study was derived from the well-known method devised by Hirsch and Strauss using a variant of *S. aureus* Wood 46 (variant of La656 from the International Center for Information on and Distribution of Type Cultures, Lausanne, Switzer-

land) (13, 14). This strain was kept lyophilized in aliquots for periodic renewal, and was regularly subcultured when used on Mueller Hinton agar. For each assay 0.05 ml of bacteria (2×10^6) was added to a mixture of 0.4 ml PMN-s (3×10^6) in Krebs-Ringer phosphate medium and 0.05-ml portions of EDTA-treated plasma or EDTA-treated pleural fluid that had been serially diluted in Krebs-Ringer phosphate medium. The cell suspensions were incubated at 37°C in a shaking waterbath, and samples of the test suspensions were taken at time 0 and after 30 min incubation. The samples were diluted and osmotically lysed with distilled, sterile water, and plated on Mueller Hinton agar; the number of residual bacterial colonies was counted after 48 h of incubation. Each test was run with several controls including (*a*) heat-inactivated plasma or exudate with PMN-s; (*b*) plasma and exudate without PMN-s (None of these controls was observed to exhibit any bactericidal activity on the test strain. If patients had received penicillin, the only antibiotic used at the time of thoracentesis, their plasma and fluid were preincubated with penicillinase [Institut Pasteur, Paris, France] before testing. Preincubation of plasma or pleural fluid with penicillinase was not found to modify the normal control values); (*c*) finally, each test was run with a serially diluted plasma of 20 normal volunteers in the presence of PMN-s.

The results were expressed in terms of K50 opsonic titer, defined as the reciprocal value of plasma or pleural fluid dilution achieving a 50% killing of the bacterial population after a 30-min incubation. This value was found to be most suitable for the expression of the results in preliminary experiments where the killing of bacteria was correlated with the dilution of normal plasma added (Fig. 1). There was a good correlation over a wide range of plasma dilutions.

The opsonic requirements of the phagocytic test could be defined as follows (Table II): (*a*) Phagocytosis was equally effective when normal, C4-deficient human plasma (kindly provided by R. Hauptman, Strassbourg, France) or Mg EGTA-treated sera was used. (*b*) It was abolished when normal or C4-deficient plasma was heated at 50°C for 40 min. (*c*) Activity of C4-deficient plasma heated at 50°C for 40 min could be almost completely restored by the addition of purified Factor B (15). (*d*) Phagocytosis of the test strain was similarly effective when either agammaglobulemic plasma (kindly provided by A. Cruchaud, Geneva, Switzerland) or plasma in which antibody had been previously absorbed was used as opsonic source.

Similarly, to exclude that the variations in opsonic titers obtained in pleural effusions might be related to the amount of antibody present, control experiments were performed using pleural fluid specimens of the different groups in which antibody had been previously absorbed. No significant change was seen in the opsonic titers before and after this procedure.

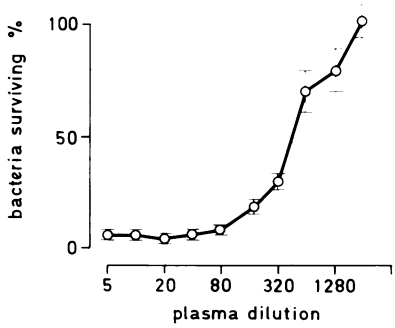

FIGURE 1 Titration of heat-labile opsonic activity of a normal plasma pool by the phagocytic bactericidal assay. The results are expressed as percent bacteria surviving in presence of normal polymorphonuclear leukocytes and plasma at various dilutions after 30 min incubation, compared to the total number of bacteria added initially to the test system. These are mean values ± SE of 20 assays with two different plasma pools.

TABLE II
S. Aureus Wood 46 Variant Phagocytic Bactericidal Assay: Opsonic Requirements

Human plasma 5%	Bacteria killed by normal PMN-s in 30 min*	K50 opsonic titer* (log_2)‡
	%	
Normal	94.4±3.5	6.3±1.2
C4 deficient	95.2±4.1	6.1±1.3
Mg EGTA-treated sera§	90.0±3.1	N.D.‖
Normal heated 50°C, 40 min	3.1±5.2	<0
C4 deficient heated 50°C, 40 min	3.3±5.1	<0
C4 deficient heated 50°C, 40 min, reconstituted with purified Factor B¶	70.2±16.3	N.D.
Normal after absorption of antibody	93.0±4.2	5.1±1.1
Agammaglobulemic**	86.0±10.0	5.1±0.9

* Mean values (±1 SD) of three experiments.
‡ Results represent log_2 of inverse titer leading to 50% bacterial killing in the phagocytic bactericidal assay (log_2, 0 represents 1/5 diluted plasma of pleural fluid).
§ In these experiments bacteria were opsonized in 10 mM EGTA + 1 mM $MgCl_2$-treated sera at 37°C for 30 min, saline washed, and then added to a PMN suspension.
‖ N.D. = Not done.
¶ Factor B concentration was 1 mg/ml.
** No measurable immunoglobulin (Ig)A or IgM and <50 mg of IgG per 100 ml by radial immunodiffusion.

For antibody absorption, EDTA-treated plasma or EDTA-treated pleural fluids were absorbed with the *S. aureus* Wood 46 variant strain obtained from heavily streaked plates that had been incubated overnight (⅓ vol bacteria and ⅔ vol fluid). The absorption was carried in an ice bath for 1 h with frequent stirring of the suspension followed by centrifugation and passage through a Millipore filter (pore size, 0.45 mM; Millipore Corp., Bedford, Mass.).

Oil Red 0 (ORO) uptake test. This method has been previously described in detail by Stossel (16). In brief, di-iso-decyl phtalate particles containing ORO and stabilized with *Escherichia coli* 026:B6 were prepared by sonication, and 0.2 ml sera or 0.3 ml of native pleural fluid were added to 0.2 ml of ORO particles suspension and opsonized by preincubation at 37°C for 15 min. To assay the rate of ingestion of such serum or fluid-treated particles, 0.2 ml of a serum particle mixture or 0.25 ml of a pleural fluid particle mixture were added to 0.8 ml of a leukocyte suspension that had been previously warmed to 37°C in 15 ml siliconized glass test tubes. After incubation at 37°C in a shaking waterbath for 5 min, the experiment was terminated by the addition of 6 ml of ice-cold 1 mM *N*-ethylmaleimide in 0.15 M sodium chloride.

The initial rate of uptake of di-iso-decyl-phtalate was calculated from the optical density of Oil Red 0 in the dioxane extracts. Because of some variability of ingestion rates by dif-

ferent leukocyte preparations, the results were expressed as percent uptake as compared to the uptake of particles opsonized with pooled adult serum. Heated pleural effusions or sera used as controls exhibited null ingestion rates.

Complement studies. These were carried out on EDTA plasma or EDTA pleural fluids. Hemolytic activity (CH50) was measured in a continuous flow system (17). Clq, C4, Factor B, C3 levels were quantitated by single radial immunodiffusion as previously described (18). The C3 breakdown product, C3d, levels were quantitated in a two-step procedure. In the first step, native C3 and the high molecular weight fragments C3b and C3c were precipitated with polyethylene glycol. In the second step, the C3d was measured in the polyethylene glycol supernate by single radial immunodiffusion with anti-C3d antiserum (19). The values for CH50, Clq and C4, Factor B and C3 were expressed in percent of a normal plasma pool as well as in milligrams per gram of total protein. The value of C3d was expressed in milligrams per 100 ml as well as in milligrams per gram of total protein. Immunoelectrophoresis of culture-positive and culture-negative infectious pleural effusions was performed with an antiserum reacting with the different antigen determinants of C3 as previously described (19).

^{125}I-C1q binding test. The ^{125}I-Clq binding test was used for detection of soluble immune complexes in patients' sera and pleural fluid (20). Briefly, Clq was isolated from normal human serum and labeled with ^{125}I-Clq. This preparation was then mixed with unheated test serum or EDTA pleural fluids from the patients. Free ^{125}I-Clq was separated from ^{125}I-Clq bound to complexes by precipitation with polyethylene glycol. Results were expressed as percent ^{125}I-Clq precipitated when compared with the total protein bound radioactivity obtained by 20% (wt/vol) trichloroacetic acid precipitation of 50 ml of normal human serum mixed with 50 ml of ^{125}I-Clq. To avoid variations resulting from the use of different batches of ^{125}I-Clq, the results were corrected, using a modified Farr's formula, for the precipitation of radiolabeled Clq observed in presence of normal serum (21).

DNA. DNA levels were measured by a radioimmunoassay as previously described (22).

In vitro activation of complement by bacterial strains isolated from empyema. These experiments were conducted as previously reported (23). In brief, 18-h, saline-washed cultures of several bacterial species isolated from empyemas were diluted in 0.5 ml saline over several tenfold dilutions to provide a wide range of challenge doses. The actual number of viable organisms was determined by standard residual colony counts after plating of the initial bacterial concentration. 0.5 ml of serum, 0.5 ml of serum chelated with EGTA (10 mM) plus $MgCl_2$ (1 mM) or EDTA (10 mM) were then added to each tube containing saline-diluted bacteria; control tubes consisted of serum, EDTA or Mg EGTA-treated serum added to saline. The tubes were incubated in a 37°C water bath for 60 min with agitation; at the end of the incubation the tubes were cooled and centrifuged at 2,500 rpm for 10 min. The supernate was withdrawn and tested for residual complement hemolytic activity (CH50) or C3d levels. Control experiments were performed to demonstrate that small quantities of Mueller Hinton broth which might have been carried over from the preincubation did not significantly activate complement breakdown.

Statistical evaluation. To compare the distributions of results among different patient groups, the unidirectional Wilcoxon test was applied. For correlation analysis, the Spearman's correlation coefficients (rs) were used.

RESULTS

Heat-labile opsonic activity in pleural fluids. The heat-labile opsonic activity of plasma and pleural fluid samples was first evaluated by a phagocytic-bacterici-

TABLE III

Opsonic Activity in Pleural Fluids and Sera/Plasma from Patients with Pleural Transudates, Infectious Exudates with Either Positive or Negative Pleural Fluid Bacterial Culture, and Neoplastic Exudates

	Infectious							
	Pleural fluid				Plasma-sera			
	Trans*	Cult pos*	Cult neg*	Neopl*	Trans	Cult pos	Cult neg	Neopl
Total protein concentration, *g/liter*	18‡ ±4	40 ±11	42 ±8	44 ±8	60 ±8	66 ±3	61 ±10	72 ±10
K50 opsonic titer, $\log_2$§	1.6 ±1.1	1.0 ±1.54	3.9 ±0.99	5.0 ±1.4	6.2 ±1.3	5.6 ±0.8	6.5 ±1.5	6.8 ±1.4
K50 titer/total protein concentration	0.079 ±0.052	0.030 ±0.04	0.09 ±0.04	0.099 ±0.034	0.106 ±0.024	0.088 ±0.012	0.103 ±0.021	0.098 ±0.020
No. of samples tested	5	15	10	13	5	6	6	8
ORO-uptake, %‖	8.40 ±8.79	3.75 ±10.61	45.73 ±60.03	42.75 ±49.38	133.00 ±68.47	160.00 ±51.05	191.2 ±51.01	183.13 ±81.29
No. of samples tested	5	8	11	13	5	5	5	13

* Trans, transudates; cult pos, culture-positive infectious exudates; cult neg, culture-negative infectious exudates; neopl, neoplastic exudates.

‡ These are mean values (±1 SD).

§ Results represent $\log_2$ of reciprocal titer leading to 50% bacteria killing ($\log_2$, 0 represents 1/5 diluted plasma or pleural fluid).

‖ Results represent percent of values found with normal sera pool with the ORO-uptake test.

dal assay using normal PMN-s. This assay was performed in 5 patients with pleural transudates associated with cardiac failure or hypoproteinemia; in 25 patients with infectious pleural exudates, either with a positive bacterial culture or a negative bacterial culture of pleural fluid at the time of the study; and in 13 patients with neoplastic pleural exudates. The pleural fluid or plasma dilutions leading to a killing of 50% of the bacteria in the phagocytic-bactericidal assay (K50 opsonic titers) are summarized in Table III (upper panel). The following findings were made: the K50 opsonic titers were significantly lower in culture-positive, infectious, pleural effusions as compared to culture-negative exudates ($P < 0.002$) or neoplastic exudates ($P < 0.002$). K50 opsonic titers were significantly lower in culture-positive fluids whether expressed as absolute values or adjusted to protein concentrations, i.e., as the ratio of K50 opsonic titer per gram of protein. Transudates had also low K50 opsonic titers, but the ratio of K50 opsonic titer per gram of protein was not different from that of culture-negative or neoplastic fluids. When compared to the plasma values from all patient groups, the various effusions exhibited decreased K50 opsonic titers, whatever their etiologies. Finally, no significant differences were found in the opsonic titers of the various plasma sample groups.

The heat-labile opsonic activity of the various effusions and sera was also evaluated by the ingestion rates of endotoxin-coated paraffin particles by normal PMN-s (ORO-uptake test). The values obtained are summarized in Table III (lower panel). As already demonstrated with the phagocytic-bactericidal test, low values of ORO-uptake test were found in culture-positive infectious exudates when compared to culture-negative infectious exudates ($P < 0.05$). For transudates and culture-positive infectious pleural fluids, the values obtained with the ORO-uptake were mostly below the sensitivity range of the assay. Patients with culture-negative or neoplastic exudates showed ORO-uptake results distributed over a wide range from zero up to serum values. Finally, the serum samples of the four patient groups showed an increased ORO-uptake when compared to normal pool sera, but no differences could

TABLE IV

Complement Levels and C1qBA in Pleural Fluid and Plasma from Patients with Pleural Effusions

	Normal plasma pool values	Infectious							
		Pleural fluid				Plasma			
		Trans*	Cult pos*	Cult neg*	Neopl*	Trans	Cult pos	Cult neg	Neopl
No. tested		12	15	11	13	12	15	11	13
CH50									
Normal pool, %‡	100	19±10	16±20	48±10	45±16	102±16	108±11	114±5	106±15
Protein, *% per g*	1.53	1.05	0.40	1.14	1.02	1.70	1.63	1.86	1.47
C1q									
Normal pool, %	100	20±12	45±35	37±15	55±14	94±11	118±28	95±39	119±25
Protein, *mg/g*	2.76	2.00	2.02	1.58	2.25	2.82	3.21	2.80	2.97
C4									
Normal pool, %	100	15±15	41±22	28±15	40±35	105±12	115±40	102±43	116±37
Protein, *mg/g*	6.92	3.75	7.53	3.00	4.09	7.87	7.84	7.52	7.25
Factor B									
Normal pool, %	100	15±12	67±49	34±21	53±34	105±22	156±33	132±35	134±32
Protein, *mg/g*	3.84	2.08	4.18	2.02	3.01	3.58	5.90	5.40	4.65
C3, *mg/100 ml*	150	24±12	77±35	51±30	55±24	135±20	180±24	161±49	165±39
Protein, *mg/g*	23.000	20.00	28.87	18.21	18.75	33.75	40.90	39.59	34.37
C3d, *mg/100 ml*	<1.9	<1.9	7.2±6.3	3.3±3.3	3.4±2.7	<1.9	2.7±2.8	<1.9	2.5±2.2
Protein, *mg/g*	<0.29	0.71	1.80	0.78	0.77	0.22	0.40	0.28	0.34
C1qBA§	0.1	1.6±3	13±10	7±5.1	12.1±13	1±2.8	12.9±19.5	9.9±11.7	3±4.6
Protein, *% per g*	0.002	0.08	0.32	0.16	0.27	0.016	0.19	0.16	0.04
Total protein concentration, *g/liter*		18±4	40±11	42±8	44±8	60±8	66±3	61±10	72±10

* Trans, transudates; cult pos, culture-positive infectious exudates; cult neg, culture-negative infectious exudates; neopl, neoplastic exudates.
‡ ±1 SD.
§ ^{125}I-Clq precipitated.

be detected when the four groups were compared among themselves.

As suggested by our preliminary experiments (Table II) and by the opsonic requirements demonstrated for the ORO-uptake test (24), a significant positive correlation was found between K50 opsonic titers and ORO-uptake for all four types of effusions (rs = 0.703, $P < 0.001$).

Complement levels. The hemolytic activity of complement (CH50) and the levels of Clq, C4, Factor B, C3, and of the C3d small catabolic fragment of C3 were measured in all plasma and pleural fluid samples in which the opsonic activity was studied, and are summarized in Table IV. The study was completed by the determination of complement components in an additional 39 patients with pleural effusions subdivided in the same four groups. Low values of complement hemolytic activity were observed in pleural fluid samples from patients with culture-positive pleural effusions as compared with culture-negative infectious fluids ($P < 0.01$) or with neoplastic fluids ($P < 0.01$), although the concentrations of protein in these fluids were similar (Table III). This finding contrasts with the absence of significant differences between the concentrations of complement components (Clq, C4, C3, Factor B) in the corresponding sample groups. Transudates showed a lower level of complement activity as well as of complement components, than exudates, but these differences were not significant when the results were corrected for protein concentrations. The mean plasma levels of CH50, Clq, and C4 were within the normal range in all groups studied, whereas the levels of C3 and of Factor B were increased in patients with infectious or neoplastic exudates.

High concentrations of C3d were found in pleural fluids from patients with empyema as compared to the levels observed in culture-negative infectious exudates ($P < 0.05$). All three groups of exudates had increased levels of C3d as compared to the values observed in normal plasma (P always <0.05). Trace amounts of C3d were occasionally detectable in plasma samples of patients with infectious or neoplastic exudates. To obtain direct assessment of C3 conversion, immunoelectrophoreses were carried out on representative samples of culture-positive and culture-negative pleural effusions using an antiserum reacting with the different antigenic determinants of C3. These experiments demonstrated the presence of C3c in culture-positive effusions, and its absence in culture-negative fluids, as shown in Fig. 2.

Correlation between heat-labile opsonic activity and complement levels in pleural fluids. There was a highly significant correlation between K50 opsonic titers and CH50 values (rs = 0.884, $P < 0.001$) (Fig. 3) for all pleural fluids tested. Such a correlation was also found for the infectious group when taken alone (rs = 0.920, $P < 0.001$). K50 opsonic titers were not significantly correlated with C3d, Clq, C3, or Factor B.

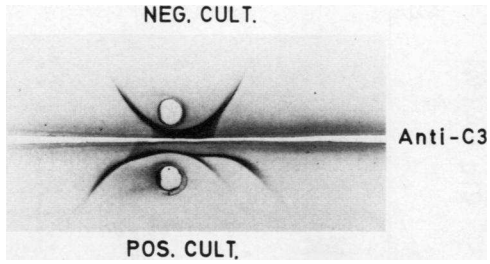

FIGURE 2 Immunoelectrophoresis of culture-positive and culture-negative infectious pleural fluids with an antiserum reacting with the different antigen determinants of C3. The anode is on the left.

Quantification of C1qBA in pleural fluids and sera, and correlation with K50 and CH50 values. The ClqBA values were increased in culture-positive infectious fluids when compared to culture-negative infectious exudates ($P < 0.005$) (Fig. 4). Nevertheless, all three groups of exudates had increased ClqBA values when compared to transudates. Inverse correlations were found for infectious exudates between ClqBA values and CH50 values (rs = 0.425, $P = 0.05$). A positive correlation was also found between ClqBA and C3d levels (rs = 0.464, $P = 0.05$) in infectious fluids. Serum ClqBA values were increased in 38% of patients with malignant and in 53% of patients with infectious pleural effusions whether culture positive or negative. Finally, no statistical correlation was found in paired analyses of ClqBA in pleural effusions and sera for the different groups.

In view of the possible interference between DNA and ClqBA, the DNA concentrations were measured in the pleural effusions and were found to be in the range of 0–100 μg/ml, with higher values in infectious fluids.

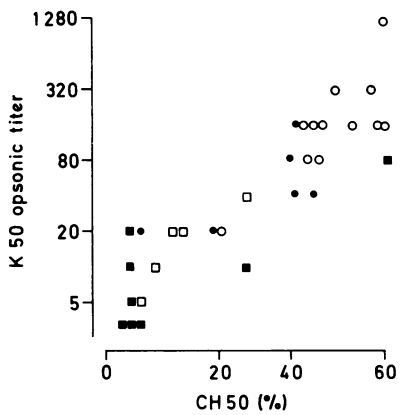

FIGURE 3 Correlation between the K50 opsonic titers and CH50 in pleural fluids. Transudates □, neoplastic exudates ○, culture-positive ■, culture-negative ● infectious exudates. rs = 0.884; $P < 0.001$.

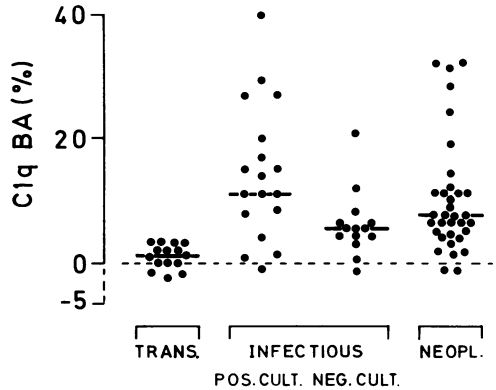

FIGURE 4 ClqBA in pleural fluids from patients with transudates (trans.), with culture-positive (pos. cult.) or culture-negative (neg. cult.) infectious exudates, and with neoplastic exudates (neopl.). These values were corrected for the nonspecific precipitation.

DNA levels, however, did not correlate with ClqBA, K50 opsonic titers, or C3d values.

Finally, lysozyme levels were measured in all fluids and were found in the range of 3–90 μg/ml with higher values in infectious fluids. Leukocyte counts are shown in Table V. No correlation was found between lysozyme levels, leukocyte count, number of polymorphonuclear leukocytes and ClqBA, K50 opsonic titers, CH50, or C3d values.

In vitro inactivation of serum complement by bacteria isolated from culture-positive infectious exudates. To substantiate the inactivation of complement components in empyema by the causative microorganism, recovery experiments were performed in vitro by incubating bacteria isolated from infectious pleural effusions with pooled human serum for 60 min at 37°C. The results of these experiments are shown in Table VI. It was found that all the bacteria tested were able to inactivate complement in vitro. 10^7–10^8 microorganisms/ml consumed 50% of normal serum complement hemolytic activity when incubated for 1 h at 37°C. At concentrations superior to 10^9 microorganisms/ml, >90% complement activity was consumed and there was a significant increase of the C3d content of these serum samples.

To further assess whether complement consumption occurred via the alternate pathway, the same types of recovery experiments were repeated with the causative microorganisms and Mg EGTA-treated sera. All the strains tested were able to inactivate complement when incubated under such conditions; the reaction was suppressed in presence of EDTA.

TABLE V
Leukocyte and Differential Counts in Pleural Fluids of Various Etiologies

	Leukocyte counts in pleural fluids		Number of effusions with more than	
	Range	Median	50% PMN-s	50% lympho-mono-cytes
Transudates	300–3,000*	500	2	13
Neoplastic	500–15,000	2,500	4	27
Culture negative infectious	800–14,400	3,700	10	4
Culture positive infectious	500–150,000	15,000	18	0

* Number of leukocytes per cubic millimeter.

DISCUSSION

Using two different phagocytic assays, we have been able to show in the present study that opsonic activity is almost undetectable in culture-positive, infected pleural effusions when compared to values obtained in pleural effusions of other origins or to autologous serum. These results are substantiated by the demonstration of very low complement activity and evidence of complement catabolism in the same effusions.

There are several lines of evidence showing that both phagocytic assays used in this study represent adequate approaches for measuring complement-mediated opsonic activity. Our investigations of the opsonic requirements of the *S. aureus* Wood 46 variant used in the phagocytic bactericidal assay demonstrate that opsonization of this strain depends on the complement system—in particular on an intact alternate pathway—and that the role played by antibody is minimal in this system. As to the ORO-uptake test, it has been demonstrated by Stossel et al. (24) to be a reproducible quantitative functional assay of the alternate complement pathway.

Several methodological aspects and limitations of the two phagocytic assays used in this study should be discussed. (*a*) Owing to the possible variations in protein concentrations of infectious pleural effusions, control groups had to be included in the study with either high (i.e., carcinomatous) or low (i.e., transudates) protein contents. Infected, culture-positive, pleural effusions had low opsonic titers when compared with pleural effusions with similar protein concentrations but of different origins. Moreover, the same difference in opsonic activity held true when the infected pleural effusions were compared with transudates, the results being expressed as the ratio of heat-labile opsonic titers per gram of protein. The results obtained could therefore not be ascribed to the variations in protein concentrations of the pleural effusions. (*b*) To exclude that the differences in opsonic activity of the different groups of pleural effusions could have been related to similar variations in opsonic activity of the autologous sera, most effusions were tested simultaneously with the same patient's

TABLE VI

In Vitro Inactivation of Serum Complement by Microorganisms Isolated from Empyemas

Microorganisms isolated	Values leading to 50% consumption of CH50		
	Normal sera	Mg EGTA-treated sera	EDTA-treated sera
	microorganisms/ml		
S. aureus	4×10^6*	5×10^7	No consumption at $>1 \times 10^9$‡
E. coli	1.2×10^8	2×10^8	No consumption at $>5 \times 10^9$
Klebsiella pneumoniae	1.9×10^7	2.3×10^7	No consumption at $>3 \times 10^9$
Streptococcus pneumoniae	7.9×10^7	1×10^8	No consumption at $>1 \times 10^9$
Peptostreptococcus magnus plus *Fusobacterium varium*	$<1 \times 10^9$§	$<1 \times 10^9$	No consumption at $>1 \times 10^9$

* CH50 was measured after incubation of microorganisms in pooled human serum for 1 h at 37°C.

‡ No complement consumption was detected at bacterial concentrations indicated.

§ These anaerobic strains were only tested at a concentration of 10^9 per milliliter. CH50 consumption was higher than 95%.

serum obtained on the same day, stocked under identical conditions, and run as an internal control. There was no difference between the four groups of sera in their opsonizing activity, adding evidence for a localized process leading to decreased activity in infected pleural effusions. (*c*) Finally, when compared with the results obtained with the phagocytic assay, opsonic activity measured by the ORO-uptake assay was lower or sometimes even undetectable in infected pleural effusions. This discrepancy is explained by the lower sensitivity of the ORO-uptake assay, a one-fourth dilution of normal serum leading to undetectable ingestion rates (16).

The present data demonstrate a parallel decrease of opsonic activity and of CH50 values in infected pleural effusions. Although one cannot exclude that a common mechanism could simultaneously alter independent effector mechanisms, it is more likely that the decrease in opsonic activity directly reflects a decreased complement activity. In view of the requirement for an intact alternative pathway in the phagocytic bactericidal assay used in this study, the correlation between CH50 and K50 values is probably dependent on the existence in infected pleural fluids of a limiting concentration of the late components of complement C3–C9 or of a parallel decrease of the early components of both the classical pathway and of the alternative pathway. Several possible mechanisms may be involved in the decrease of complement activity in infected pleural effusions. The apparent discrepancy between total complement activity and the levels of complement components measured excludes the hypothesis of a low pleural diffusion or an absence of local synthesis of complement components. The presence of C3 breakdown products in these fluids supports the idea of a local consumption of complement and opposes the hypothesis of the presence of inhibitors of its activation.

Nevertheless, the mechanisms responsible for complement activation in the pleural spaces are difficult to define. As a first possibility, the negative correlation between ClqBA and CH50 values in the infected pleural effusions suggests a possible activation of complement by Clq binding material which could be immune complexes. Thus, it has already been shown for pleural effusions because of other causes, i.e., lupus erythematosus and rheumatoid arthritis, that the decreased complement levels measured were associated with a local increase of immune complexes (25). However, a similar correlation was not found in a large series of neoplastic fluids[2] suggesting that the capacity of the ClqBA material to activate complement differs according to the nature of the fluid or that the ClqBA material is not directly involved in the complement consumption.

Our in vitro studies with bacteria isolated from empyema suggest that microorganisms probably play an important role in complement activation at concentrations similar to those found in localized human infections (26, 27). These results are substantiated by other studies showing that endotoxin and other bacterial cell wall products activate the alternative complement pathway (28, 29). Finally, as an alternate hypothesis, one has to consider the possibility that proteolytic enzymes could be released from bacteria, PMN-s, or macrophages into infected fluids and would induce an enzymatic degradation of some complement components, as suggested by in vitro studies (30, 31) and by the significant differences obtained in our study between culture-negative and culture-positive fluids.

In conclusion, our study suggests that once infection has gained access to a pleural space, activation of complement leads to depletion of complement components, thereby resulting in deficient opsonization and persistence of live microorganisms. Other factors might play an additional role in decreasing opsonization of bacteria in infected pleural effusions, such as low pH and breakdown of immunoglobulins (32).

[2] Manuscript in preparation.

Thus, in view of the difficulties inherent in the medical treatment of empyema, it is not unreasonable to postulate that complement-mediated opsonic activity could be restored in infected pleural effusions by the administration of autologous fresh serum and(or) protease inhibitors. Present data do not permit any conclusion in this regard and will require further study.

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