per square inch absolute (15 p.s.i.a.) or to oxygen at 30 p.s.i.a. resulted in a marked and equivalent bactericidal effect. Four hour cultures were more susceptible to the bactericidal action of oxygen than 24 hour cultures. The rate of inactivation of *C. perfringens* by oxygen was decreased by the presence of blood or muscle and was less at 25° than at 37°C. Exposure to oxygen of *C. perfringens* on the surfaces of agar plates resulted in inhibition of growth on subsequent incubation in an anaerobic environment. Four hour cultures were more susceptible to the inhibitory activity of oxygen than 24 hour cultures and presence of blood in the agar inhibited the antibacterial action of oxygen. Exposure of broth cultures of *C. tetani* to air or oxygen at 15 p.s.i.a, or to oxygen at 30 p.s.i.a., resulted in a bactericidal effect similar to that observed with *C. perfringens*.

Mice injected intramuscularly in normal muscle with *C. perfringens* could be protected against mortality with treatment with oxygen at 45 p.s.i.a. Oxygen also protected against death in experiments in which infusorial earth was injected with *C. perfringens* into crushed muscle. Variations in the experimental model such as injecting calcium chloride instead of infusorial earth with *C. perfringens* into normal or crushed muscle eliminated the protective effect of oxygen.

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Antibodies to Mycoplasma in Sera of Leukemic Patients.* (31744)

W. H. MURPHY, I. J. ERTEL, C. BULLIS, AND C. J. D. ZARAFONETIS
(Introduced by W. J. Nungester)
Departments of Microbiology, Pediatrics, and Internal Medicine, Simpson Memorial Institute, University of Michigan School of Medicine, Ann Arbor

The experiments reported were done to determine whether virus neutralization technics were useful for testing patients' sera for antibodies that neutralized the cytopathic effect (CPE) and growth of mycoplasma (PPLO) isolated from bone marrow specimens obtained from leukemic and non-leukemic patients. Results also are presented from pilot studies carried out to test whether a correlative pattern existed between leukemia and the occurrence of antibodies to such mycoplasma. The findings are of interest because evidence is presented that a complement dependent normal antibody is present in serum that neutralizes PPLO CPE and growth. The problem of interpreting results is discussed when serum specimens are obtained from patients with a disease that causes marked pathologic changes in antibody forming tissues.

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Materials and methods. Cell lines. The

origin of the Chang-liver cell line was described(1). The strain of "transformed" human amnion cells (HAT-human amnion transformed) was derived from normal human amnion in 1961 by Dr. P. Ludovici(2) and has been maintained in continuous serial culture. Both cell lines are PPLO free(3). Cell lines were propagated by standard technics(1) except that antibiotics were not used in growth medium (Eagle basal medium 80%, human serum 20%). *Viruses.* Stock laboratory strains of poliovirus, type 2, vaccinia, herpes, and adenovirus-type 4, were grown and titrated in HeLa-S3 cells. Adenovirus-type 12 was grown and titrated in HAT cells. *Mycoplasma.* All PPLO strains were isolated from bone marrow specimens(3) obtained from children: K1 = acute granulocytic leukemia; K7 = acute stem cell leukemia; K10 = undifferentiated sarcoma with bone marrow mestastases; and F3 = lupus erythematosus. *Mycoplasma growth media.* Each 100 ml of *solid* medium contained: Difco PPLO agar 3.5 g; Difco brain heart infusion *(without agar),* 3.7 g; Difco yeast extract 0.2 g; fresh yeast extract(4) 10 ml; AGVD solution 3 ml; and calf serum 20 ml. Final pH was adjusted to 7.8. AGVD solution contained per 100 ml: l-arginine HCL, 2.1 g; l-glutamine, 3.0 g; dextrose 5.0 g; Eagle 100 × basal vitamin solution 10 ml (Microbiological Associates); and distilled water, 90 ml. AGVD solution was sterilized by filtration and stored in screw cap vials at —20C. Broth contained the same ingredients as solid medium except that Difco PPLO broth was substituted for Difco PPLO agar. *Sera:* Sera were collected aseptically by venipuncture, allowed to clot 4 to 8 hours, and usually tested for antibody to PPLO within 24 hours. Sera were stored at —20 or —70C; no preservative was added. *Neutralization technic.* Log phase cells (100,000/ml growth medium) of the Chang-liver or human amnion mammalian cell lines were inoculated (1 ml) into screw cap (16 × 125 mm) culture tubes, incubated at 37C for 24 hours, and cell monolayers washed 3 times with Ca^{++} and Mg^{++} free buffered salt solution (1). Each culture received in sequence 0.8 ml of maintenance solution(1), 0.1 ml of antiserum diluted 1:10 in Hanks' solution(1), and 0.1 ml of virus or PPLO inoculum. Five culture tubes per serum specimen were used for each virus or PPLO neutralization test. Each 0.1 ml of virus contained, respectively: herpes-50 $TCID_{50}$; vaccinia or poliovirus-10 $TCID_{50}$; adenovirus, type 4-a 1:4 dilution of cell culture fluid; and adenovirus, type 12-a 1:2 dilution of cell culture fluid. Each 0.1 ml of PPLO inoculum contained 100 $TCID_{50}$. Higher PPLO concentrations gave false negative results; lower amounts were not regularly cytopathic. Generally 100 $TCID_{50}$ of PPLO contained from 10^2 to 10^3 colony forming units (CFU). Prior incubation of sera with viruses or PPLO did not enhance neutralization, except for herpes virus. Controls (5 tube cultures) used with each test included uninoculated cells and cells infected with each virus or mycoplasma. Cultures were incubated at 37°C and read daily for CPE(3). Neutralization tests were repeated from 3 to 5 times per serum specimen. *Clyde tests:* The method used was essentially the same as that described by Clyde(5) except that an inoculum of from 300 to 500 CFU of PPLO was used per plate. Antisera to the test PPLO were prepared in rabbits essentially by the methods reported by Taylor-Robinson *et al*(6).

Results. Inhibition by antisera of PPLO growth and cytopathology. This experiment was done to determine whether a standard cell culture virus neutralization technic could be used to test patients' sera for antibodies that neutralized CPE (cytopathic effect) of mycoplasma (PPLO) isolated from leukemic or nonleukemic patients(3). To establish the validity of results, neutralization by patients' sera was compared with neutralization by specific rabbit antisera. Because pilot studies showed that the CPE neutralizing serum component (designated "antibody" hereafter) of patients' sera was labile, serum specimens were not heat inactivated and were tested usually within 24 hours after they were drawn. To increase the sensitivity of the neutralization test, sera (1:10 dilution) were added directly (0.1 ml) to culture tubes that next immediately received 0.1 ml of PPLO titrated to contain 100 $TCID_{50}$. Cell cul-

TABLE I. Inhibition of PPLO Growth and Cytopathic Effect.

Strain of PPLO*	Inhibition by rabbit serum of CPE	Growth†	Inhibition by human sera of CPE	Growth
K1	+	10^4 to 10^6	+	10^3 to 10^5
K7	+	10^4 to 10^5	+	10^1 to 10^2‡
K10	+	10^4 to 10^8	+	10^6 to 10^7
F3	+	10^5 to 10^6	+	Not done

* See text for origin and designation of PPLO strains.

† Reduction in plate count relative to control containing serum free of antibody. Results are representative of antisera prepared to 11 strains of mycoplasma isolated from leukemic and nonleukemic patients. Serum was pooled from each of 2 rabbits used to prepare an antiserum to a PPLO strain.

‡ In general anti-K7 sera more effectively neutralized CPE than PPLO growth.

tures incubated at 37°C were read daily for 7 days; bicarbonate was added to cultures to maintain pH at about 7.4. CPE was scored as indicated below. At the end of the incubation period, or when CPE was complete, cultures were frozen (—20°C) and thawed (37°C) 3 times and PPLO plate counts made on cell homogenates. In parallel experiments sera and mycoplasma were added to *broth* cultures, also incubated at 37°C for 7 days, and again plate counts done to test for inhibition of PPLO growth. Clyde tests were done concurrently to provide a common basis for comparing results. All assays were repeated 3 to 5 times.

The results of the neutralization tests (Table I) showed that sera from leukemic patients prevented the growth and CPE of the test PPLO in cultures of Chang liver and human amnion cells. Such results have been consistent during the last 3 years(7) and apply to a variety of cell lines and human strains of cytopathic mycoplasma isolated from bone marrow specimens. The usefulness of the CPE inhibition test is limited by the failure of test PPLO to always be cytopathic. This problem is accentuated when the morphology of uninoculated control cells is not optimal. Thus, it is most effective(3) to grade CPE simply as no change, 1 to 2^+ CPE (<50% of cells affected), or 3 to 4^+ CPE (*ca* 75% or more of cells affected). The PPLO growth inhibition tests done by use of cell cultures provided clear cut results, always were confirmed when experiments were repeated a second or third time, and were not liable to subjective errors of interpretation. The Clyde growth inhibition test confirmed both the CPE and PPLO growth inhibition cell culture tests. The growth inhibition test carried out by addition of antisera and PPLO to *broth* cultures was unsatisfactory because antisera reduced PPLO counts only 1 to 2 log values.

Effect of complement on neutralizing activity of sera. Although mycoplasma have been considered unique because they ordinarily are not lysed by complement in the presence of specific antibody, more recent studies(8,9) suggest an effect of complement on these organisms. Because preliminary results(3) suggested that fresh human sera had the greatest CPE neutralizing activity, additional pilot experiments were done to test whether neutralization was caused by a labile, complement-dependent, serum component.

Complement (normal guinea pig serum) was used at a dilution of 1:80 because titrations disclosed that it was somewhat cytotoxic to Chang-liver and human amnion cells at higher concentrations. Additional titrations established that a dilution of complement of 1:40 or greater did not affect the CPE of the test dose of the PPLO strains used. Experiments also were done to determine how long positive sera retained their neutralizing activity when stored at —20 or —70°C. Such information was necessary before a characterization of the CPE neutralizing serum component could be undertaken.

When sera were heat inactivated (56°C-30 min) they lost (Table II) their CPE neutralizing activity; it was restored when complement was added. The results presented in Table III show that most sera rapidly lost their neutralizing activity when stored at either —20 or —70°C. Additional tests revealed that the process of freezing sera was not responsible for its loss in activity and that storage of sera at —70°C was not superior to —20°C for preserving its neutralizing effect. Rabbit sera also exhibited a decrease in CPE neutralizing activity when frozen and stored. The results of these pilot

TABLE II. Effect of Normal Guinea Pig Serum on Neutralizing Activity of Human Sera.

Serum specimen	Inhibition of CPE* by: Fresh serum	Fresh serum + C'†	Inactivated serum	Inactivated serum + C'
E399	+	+	—	+
E430	+	+	—	+
P61	+	+	—	+
P62	+	+	—	+
P225	—	—	—	—
P230	—	—	—	—

* PPLO strain K7 was used for these tests.
† Normal guinea pig serum was used as complement (C').

experiments suggest that the CPE neutralizing substance detected in human sera is a heat-labile complement-dependent "normal serum antibody." Possibly sera that retained their activity at —20°C also contained classical stable antibody.

Neutralizing activity of sera from leukemic and nonleukemic patients. Neutralization tests were carried out to test whether a correlation existed between leukemia and the mycoplasma isolated from leukemic and nonleukemic children. Experiments also were done to test whether serial serum specimens obtained from individual patients were positive or negative for antibody in relation to stage of disease and chemotherapy. Serum purchased from adult male prisoners (20 pints of blood per pool) was used to represent normal adults. Individual adult sera were obtained from laboratory personnel and from nonleukemic adults with hematologic diseases other than leukemia. Leukemic and nonleukemic children were age and sex matched.

TABLE III. Loss of CPE Neutralizing Effect of Sera Stored at -20°C.

Serum source	Proportion of sera that inhibited CPE of one or more of test PPLO: 0-2 days after* blood was drawn	Weeks stored at -20°C: 1	2 to 6
Normal adults:			
Individuals	6/23	0/23	0/23
Pools	20/33	12/33	9/33
Normal children	3/19	1/19	1/19
Leukemic children	13/46	6/46	2/46

* Sera were not frozen but stored at 4°C until tested. Mycoplasma strains K1, K7, K10 and F3 were used for tests.

Nonleukemic children were those who were hospitalized for heart disease and had no other detectable illness.

The results of these experiments (Table IV) established that the cell culture neutralization technic could be used for antibody studies but was impractical for large scale epidemiologic surveys. About 40% of children with acute stem cell leukemia (ASL) neutralized the CPE of the K7 strain of PPLO isolated from a child with ASL. About 20% of leukemic children also neutralized CPE by the K10 and F3 strains of PPLO; only about 8% neutralized CPE by mycoplasma K1. Sera from normal children did not neutralize CPE by PPLO strain K7, and

TABLE IV. CPE Neutralizing Effect of Sera from Leukemic and Nonleukemic Patients.

Serum source	Proportion of sera that neutralized CPE of PPLO strain: K1	K7	K10	F3
Pooled sera, normal adults	5/66	7/66	8/66	0/66
Individual sera,* normal adults	0/31	0/31	0/31	1/31
Adults with:				
granulocytic leukemia	1/29	0/27	0/29	7/29
lymphocytic "	2/20	0/20	0/20	9/20
other types of "	0/5	0/5	0/5	1/5
Polycythemic adults	1/16	0/16	1/16	0/16
Normal children	5/39	0/39	2/39	4/39
Leukemic " †	3/39	15/39	7/39	8/39

* Blood specimens from laboratory personnel taken before they worked with test PPLO.
† 37 children had acute stem cell leukemia; 2 had acute granulocytic leukemia.

less frequently neutralized CPE caused by PPLO strains K1, K10 and F3 than sera from leukemic children. Sera from leukemic adults usually had no neutralizing activity to PPLO strains K1, K7, and K10 but frequently neutralized CPE caused by PPLO strain F3. Sera from laboratory personnel did not neutralize CPE caused by the K1, K7, and K10 strains of PPLO; only 1/31 neutralized strain F3 CPE. Sera from normal adults (prisoners) had no "antibody" to mycoplasma strain F3 (in contrast to adults with leukemia) but about 10% had antibody to mycoplasma strains K1, K7, or K10. The results presented in Table V fail to show a correlative pattern between chemotherapy,

clinical stage of leukemia and neutralizing activity of sera for mycoplasma. The results tabulated in Table VI indicate that fresh sera from institutionalized children with Downs syndrome frequently neutralized mycoplasma CPE. The data summarized in Fig. 1 suggest a seasonal distribution in occurrence of serum neutralizing activity.

Antibody distribution to common viruses. Since leukemia involves aberrations of the RE system, one may expect hematologic changes to be accompanied by aberrations in the serum or plasma components of blood (11,12). Thus, the correlative pattern observed between ASL in children and antibody to the K7 strain of PPLO could reflect either a specific immune response to infection or a nonspecific change in serum components related to the pathologic processes that characterize leukemia. This question was investigated by determining whether leukemic patients had a normal pattern of antibody distribution to common viruses.

The neutralization tests (Table VII) revealed that sera from leukemic adults 3 times as frequently were positive for antibody to adenovirus type 4 than leukemic or nonleukemic children or nonleukemic adults. Parallel tests done with adenovirus type 12 and herpes virus, showed no such difference. Both leukemic children and adults less frequently had antibodies to poliovirus type 2 and vac-

TABLE V. Neutralizing Effect of Serial Serum Specimens from Leukemia Patients.

Patient*	Dates sera collected	State of disease	Medication	Neutralization of CPE by PPLO strains
MS	11/ 5/63	Remission	None	None
	11/19/63	Relapse	None	K7
	1/14/64	Remission	6MP	K7, F3
KK	9/30/63	Diagnosis	Diagnosis	None
	10/29/63	Relapse	Cytoxan	"
	12/ 3/63	"	Prednisone	K7, F3
	12/17/63	"	Vincristine	None
RC	11/11/63	Relapse	Cytoxan	K7
	11/26/63	"	Vincristine	"
	3/ 9/64	"	"	"
	3/16/64	"	"	K1
	11/10/64	"	A8103	None
SW	12/30/63	Relapse	6MP	K7
	2/24/64	"	Cytoxan	K7
	3/ 9/64	"	"	None
BE	1/13/63	Diagnosis	Prednisone and 6MP	K1, K7, K10, F3
	12/15/64	Remission	None	None
	1/ 5/65	"	"	None
RH	11/10/64	Remission	Prednisone and 6MP	None
	12/ 8/64	"	Methotrexate	"
	1/ 5/65	"	Vincristine	"

* Serial specimens from 24 patients were taken over a period ranging from 6 mos to 2 yrs. Only representative results are recorded.

TABLE VI. Neutralization of Viruses and PPLO by Sera from Normal and Mongol Children.

	Proportion of sera that neutralized CPE caused by:					
		Viruses				
Serum source	PPLO*	Poliovirus, type 2	Adenoviruses, type 4	type 12	Herpes	Vaccinia
Normal children	3/9	38/46	8/40	7/44	10/46	33/46
Mongol " †	7/10	11/11	5/11	3/11	7/11	11/11

* Sera neutralized CPE of one or more PPLO strains: K1, K7, K10, or F3.
† Children with Downs syndrome are predisposed to granulocytic leukemia.

TABLE VII. Neutralization of Common Viruses by Sera from Leukemic and Nonleukemic Patients.

Serum group	Fraction of sera with antibodies to: Poliovirus type 2	Adenovirus type 4	Adenovirus type 12	Herpes	Vaccinia
Adults:					
Normal	28/40	8/41	8/40	19/40	24/40
Leukemic	21/40	28/40	6/41	16/40	20/40
Children:					
Normal	33/40	5/40	8/40	9/40	28/40
Leukemic	28/41	5/40	8/41	13/41	21/41

cinia virus than normal controls.

Discussion. The findings presented here apply to 3 related problems: (i) whether mycoplasma isolated from bone marrow specimens obtained from leukemic patients can be etiologically implicated in the disease; (ii) the problem of interpreting results of antibody tests done on sera obtained from patients with a severe disease (leukemia) involving the RE system; and (iii) whether a standard virus neutralization technic could be used for serologic surveys of mycoplasma infection.

The last point can be answered most directly. The CPE neutralization test is useful only as a special laboratory technic. The major difficulty is that even fully adapted cytopathic strains of mycoplasma too often fail to cause CPE under standardized laboratory conditions. Consequently, tests on some sera often must be repeated 2 to 3 times. Clear-cut easily reproducible results were obtained with the PPLO cell culture growth inhibition tests. Results of both the CPE and PPLO cell culture growth inhibition tests were confirmed by the Clyde growth inhibition technic. The *broth* culture PPLO growth inhibition test was unsatisfactory because PPLO growth was not inhibited sufficiently under the conditions employed. The metabolic inhibition tests recently described(8,9) are clearly superior for large scale serologic studies.

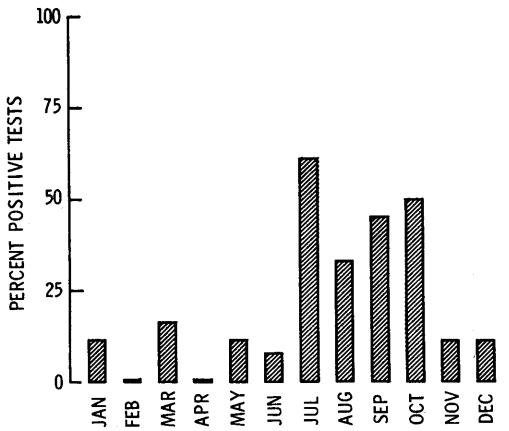

FIG. 1. Inhibition of growth and CPE of PPLO strains K1, K7, K10 and F3 by pooled adult normal sera. Each serum pool was tested for "antibody" to each strain of PPLO. The bars represent proportion of positive tests in each month. From 2 to 5 pools of sera were tested per month. Results are representative of the period 1963-1966.

The significance of the results of the mycoplasma antibody studies are difficult to assess. First, results differ when different technics are used to measure antibodies in human sera to human mycoplasma(8-10), *i.e.*, the metabolic inhibition, CPE neutralization, indirect hemagglutination and complement fixation tests do not appear to measure a common antibody response to a mycoplasma antigen. Since one seeks to measure specifically either immunity or a serologic response to mycoplasma infection, a 4-fold increase in antibody titer in response to infection is an important requisite(8). For antibody studies to mycoplasma infection there also may be unique requirements in the selection of serum donors. For example, in an attempt to match children by age and sex(11) sera for control studies were obtained from children institutionalized with Downs syndrome. Since their sera often neutralized mycoplasma CPE they could not be used as a normal control group; the same difficulties may apply to other population groups. Differences in the seasonal occurrence of "antibody" also are suggested by

the neutralization data. Mycoplasma antibody studies summarized in this report also suggest, like those of other investigators(8-10), that complement (fresh normal guinea pig serum) can affect results of serologic tests on mycoplasma and thus indicate the need to reexamine the effects of complement on mycoplasma growth or on cytopathic effect. Possibly mycoplasma CPE in some cases can be caused by PPLO membrane enzymes or membrane components. Antibody and complement may act jointly to prevent CPE if it is caused in this way.

Antibody studies done on leukemic patients introduce additional complexities because the leukemic patient during the course of disease undergoes a variety of homeostatic changes definable by both clinical and laboratory criteria(11,12). For example, when immunosuppressive drugs are used to cause remission the patient may be immunologically incompetent either from the disease process or/and drug effect. The patient in remission, while not under drug therapy, may or may not have normal immunologic competence(11,12). Moreover, the tissue changes that characterize leukemia may cause alterations in serum constitution analogous to those recognized to occur in Hodgkins disease(12) and multiple myeloma(13). Thus, the findings outlined here suggest that the observed correlation between leukemia and antibodies to PPLO strain K7 or adenovirus type 4, may be more properly related to a more general aberration of the RE system in leukemia which manifests itself in a variety of ways including unusual antibody patterns(14). Such a negative conclusion could be turned to positive advantage if antibody studies could be used to characterize the course of leukemia and to aid in the prognosis of drug therapy. The possibility exists that the serum profile of the leukemic patient may have to be characterized during the course of disease before antibody studies on leukemic patients can be evaluated more satisfactorily.

Summary. Studies were done to test whether virus neutralization technics were useful for detecting antibodies in patients' sera that prevented the growth and cytopathic effect (CPE) of mycoplasma isolated from bone marrow specimens of leukemic and nonleukemic patients. A standard virus neutralization technic was satisfactory for special laboratory studies but unsatisfactory for large-scale serologic surveys. Sera from leukemic and nonleukemic patients prevented mycoplasma CPE and growth. Evidence was presented that the CPE neutralizing serum component was complement-dependent, appears to have a seasonal occurrence, and may be a "normal serum antibody." Sera from children and adults with leukemia about twice as frequently neutralized mycoplasma CPE as sera from normal controls. No correlative pattern was observed between clinical stage of leukemia, chemotherapy, and occurrence of "antibodies" to the test mycoplasma. The problem of interpreting such results was discussed.

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