MYCOPLASMA STUDIES OF HUMAN LEUKEMIA*

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To determine whether viruses or mycoplasma have either an inductive or provocative effect on the pathogenesis of the human leukemia, it is first necessary to isolate candidate agents. Next, the agents isolated must be characterized to determine whether they are known human viruses or mycoplasma. Finally, evidence must be accumulated to indicate whether the isolated agents can be implicated in the cause or pathogenesis of human leukemia. Although our long term studies[1] have provided a number of interesting findings concerning the isolation of viruses from bone marrow specimens obtained from leukemic and nonleukemic patients, this report will summarize results on the isolation and characterization of mycoplasma.

Isolation of Mycoplasma

Previous studies indicated[1,2,3] that it is possible to isolate mycoplasma from bone marrow specimens from leukemic patients by cell culture technique when direct cultural methods yielded negative results. Even when cell cultures were used for the isolation of PPLO, it became evident (TABLE 1) that agents were not isolated frequently enough to evaluate whether they could be implicated etiologically in human leukemia. However, these experiments made it possible to classify cell lines according to their relative susceptibility to infection (TABLE 2) and, consequently, to use the sensitive cell lines to improve the isolation technique. Recent improvements in technique have increased the sensitivity of the assays so that it now appears possible to evaluate, on the basis of isolation studies, whether PPLO can play a significant role in the clinical course of leukemia. The methods used to isolate mycoplasma are outlined in TABLE 3. The key points of technique include the use of specimens that are less than three hours old, to make passages only from the positive tubes in a passage sequence, to keep the pH of cell culture fluid less than 7.6, and to avoid freezing either the clinical specimen or passage material. Results are positive most often with specimens obtained from leukemic patients in relapse or who have just had the disease diagnosed.

Representative results of the PPLO isolation studies on bone marrow specimens obtained from leukemic children are presented in TABLE 4. Only children with leukemia in relapse or just diagnosed were studied. Isolations were done principally by the use of the Michigan sublines of Chang-liver and human amnion cells since they are most sensitive to infection.[1] Representative results of isolation studies done on bone marrow specimens obtained from adults with leukemia are tabulated in TABLE 5. Often specimens from a single patient were positive simultaneously in more than one cell line (TABLE 6).

Long-term studies have been carried out in an attempt to define a medium and procedure suitable for the direct cultivation of PPLO from bone marrow specimens.[1] During the period 1961–1965, more than 1000 marrow specimens were cultured for PPLO. A wide variety of media and procedures were used.[1] Beginning in August, 1965, it has been possible to repeatedly isolate mycoplasma from

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TABLE 1

RECOVERY OF AGENTS: 1961–1966

Patient group	Leukemic	Lympho-or-myelo-proliferative diseases	Normal
Children	40/226 = 18%	7/27 = 26%	2/13 = 15%
Adults	12/40 = 30%	2/47 = 4%	0/2 = 0%

TABLE 2

DIFFERENTIAL SUSCEPTIBILITY OF CELL LINES TO INFECTION BY BONE MARROW AGENTS

Very Sensitive	Partially Sensitive	Resistant
Chang-liver	HeLa (Gey)	Rhesus monkey kidney
Human amnion	HeLa (S3)	Cynomolgus monkey kidney
Human embryonic lung	Detroit-6	Mouse fibroblasts
Human embryonic kidney	Dog kidney	Chimpanzee liver cells
Green monkey kidney	Patas monkey kidney	HeLa (CaS)

TABLE 3

METHOD FOR ISOLATION OF MYCOPLASMA

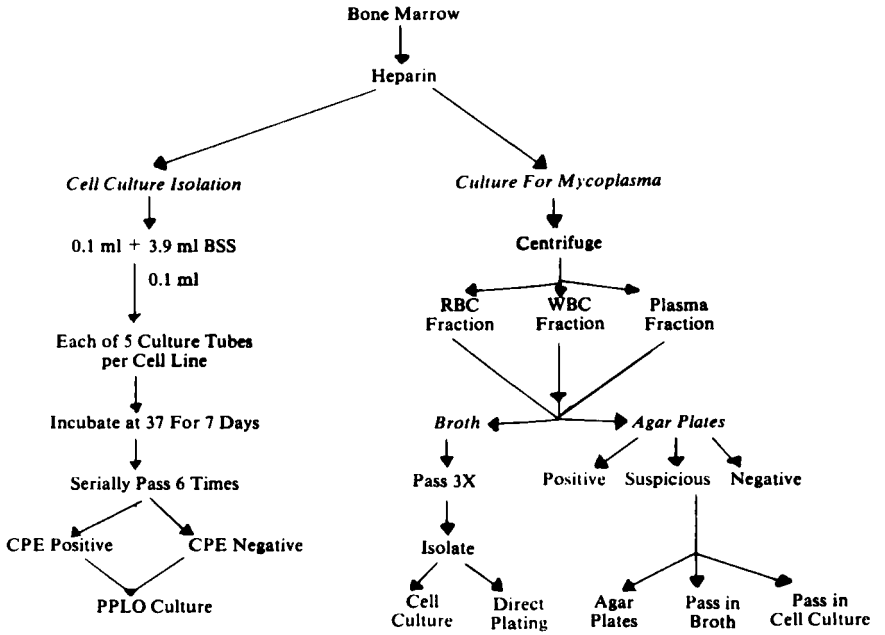

TABLE 4

ISOLATION OF AGENTS FROM LEUKEMIC CHILDREN

Agents recovered from bone marrow specimen:			
None	Unidentified*	Viruses	Mycoplasma
6/13	4/13	1/13	2/13

* Most of the unidentified agents appear to be PPLO. The virus isolated was advenovirus, type 12. Both specimens that yielded PPLO in cell culture also were positive when plated directly on agar.

TABLE 5

ISOLATION OF AGENTS FROM LEUKEMIC ADULTS

Agents recovered from bone marrow specimens:			
None	Unidentified*	Viruses	Mycoplasma
0/10	4/10	1/10	5/10*

* Three specimens yielded PPLO on direct plating. The virus isolated was herpes. Most of the unidentified agents appear to be mycoplasma.

TABLE 6

SIMULTANEOUS RECOVERY OF PPLO IN MULTIPLE CELL LINES

Leukemic bone marrow specimen		Mycoplasma isolated in:				
		Chang	Amnion	HeLa-S3	Detroit-6	Hep-2
Adults	Z135	+	+	+	+	+
	Z86	+	+			
Children	E768	+	+			
	E645		+		+	

TABLE 7

MEDIUM USED FOR DIRECT CULTIVATION OF PPLO

Solid Medium		Cultural Conditions
Difco PPLO agar	3.4 g	Final pH 7.4 to 7.6; 5% CO_2–95%
Difco Brain-Heart Infusion without agar	3.7 g	N_2, 37°C; water saturated gas phase.
Difco Yeast Extract	0.2 g	Subculture positive or suspicious
Distilled water	70.0 ml	plates weekly for three weeks by agar
AGVD supplement*	3.0 ml	block transfer technique
Human serum	20.0 ml	
Fresh yeast extract	10.0 ml	

* Each 100 ml contained l-arginine HCL, 2.1 g; l-glutamine, 3.0 g; Eagle 100× basal vitamin solution, 10 ml; dextrose 5.0 g; and distilled water.[1] Fresh human serum was inactivated at 56°C for 30 min before use. Fresh yeast extract was prepared as described by Hayflick *et al.*[4] Broth medium contained Difco PPLO broth in place of Difco PPLO agar.

TABLE 8

DIRECT ISOLATION OF MYCOPLASMA FROM BONE MARROW SPECIMENS

Source of specimen	Type of leukemia	Specimens* positive	PPLO isolated on	
			Plates	Broth
Children	ASL	13	1	12
Adults	AGL; AMML+ CLL, HML	4	3	1

* Total number positive to date.

+ ASL—Acute stem-cell leukemia; AMML—Acute myelomonocytic leukemia; AGL—Acute granulocytic leukemia; CLL—Chronic lymphocytic leukemia; HML—Histiomonocytic leukemia.

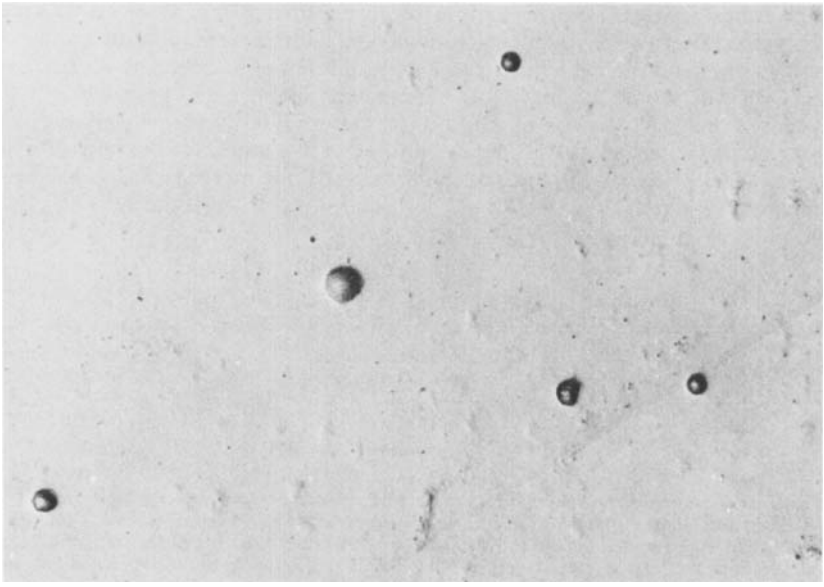

FIGURE 1. When bone marrow specimens were plated, often colonies were observed that appeared to be PPLO. They could not be propagated on artificial medium. Frequently they could be grown if transferred to susceptible cell cultures (see TABLE 3) and passed serially.

fresh marrow specimens by use of the media described in TABLE 7. The specimens that have yielded PPLO to date are listed in TABLE 8. Many specimens yielded abortive colonies that could not be serially propagated (FIGURE 1).

Some guidelines evolved from these studies. First, it appears that PPLO can be isolated regularly from bone marrow specimens obtained from leukemic patients. Our results suggest that, under ideal conditions, patients with leukemia in relapse or with initially diagnosed disease most frequently yield PPLO. Perhaps 0.1 to 1.0% of normal persons have transient or latent PPLO infection. It is particularly clear that the PPLO isolated are extraordinarily fastidious in their requirements for growth in vitro.

Serologic Analysis of Isolated Mycoplasma

Because of pitfalls[1,4–7] inherent in the use of cell cultures for the isolation of PPLO, it was essential to determine whether the mycoplasma isolated were known strains. It also was necessary to antigenically group the isolated mycoplasma to carry out comparative antibody studies on leukemic and nonleukemic patients. Fermentation and Clyde tests, carried out to make a preliminary identification of the isolated mycoplasma, disclosed that many of them were related to *M. fermentans*. The results of such pilot studies provided the basis for an analysis of their antigenic relationships. Cloned lines of PPLO grown in rabbit serum medium were used to immunize rabbits.[8]

Because the PPLO isolated were cytopathic, it was possible to compare them according to the effect of antisera on their cytopathic effect in cell culture (CPE neutralization tests) and, at the same time, measure the inhibition of growth in terms of colony-forming units (CFU inhibition tests). Antiserum, (0.1 ml) and PPLO (100 $TCID_{50}$-Tissue Culture Infectious Doses) was added to each of five culture tubes per test. Control cultures received either PPLO alone, PPLO plus homotypic antiserum, or immune serum alone. Cultures were incubated at 37° C for seven days and the pH adjusted to 7.4 by addition of bicarbonate on alternate days. CPE was scored 1^+ to 4^+; 4^+ designated complete cell destruction. CPE was scored daily. At the end of seven days, culture fluids were pooled and plate counts made. Experiments were repeated three to five times. On the basis of such tests (TABLES 9 and 10), it was possible to classify the mycoplasma as *identical, related,* or *different.*

TABLE 9

CPE NEUTRALIZATION TESTS WITH CHANG-LIVER CELLS

Mycoplasma	CPE in cultures containing anti-K10 serum	Degree of inhibition of CPE*
K10	$1–2^+$	>75%
K1	None	100%
Z62	$3–4^+$	0%

* Strains were scored *identical* if CPE was inhibited by 50% or more, *related* if inhibition was less than 50%, and *different* if CPE was not inhibited. K10 = PPLO from undifferentiated sarcoma with bone metastases; K1 = PPLO from acute granulocytic leukemia; and Z62 = PPLO from acute myelomonocytic leukemia.

TABLE 10

GROWTH (CFU) INHIBITION TESTS WITH CHANG-LIVER CELLS

Mycoplasma	Inhibition of growth* in cultures containing anti-K10 serum
K10	6 logs
K1	4 logs
Z62	0 logs

* Growth in control cultures containing normal rabbit serum usually reached a density of 10^7 to 10^8 CFU/ml. Strains were scored *identical* if growth was reduced 5–8 logs, *related* for 3–4 logs, and *different* if reduction was two logs or less. Experiments usually were repeated two to four times.

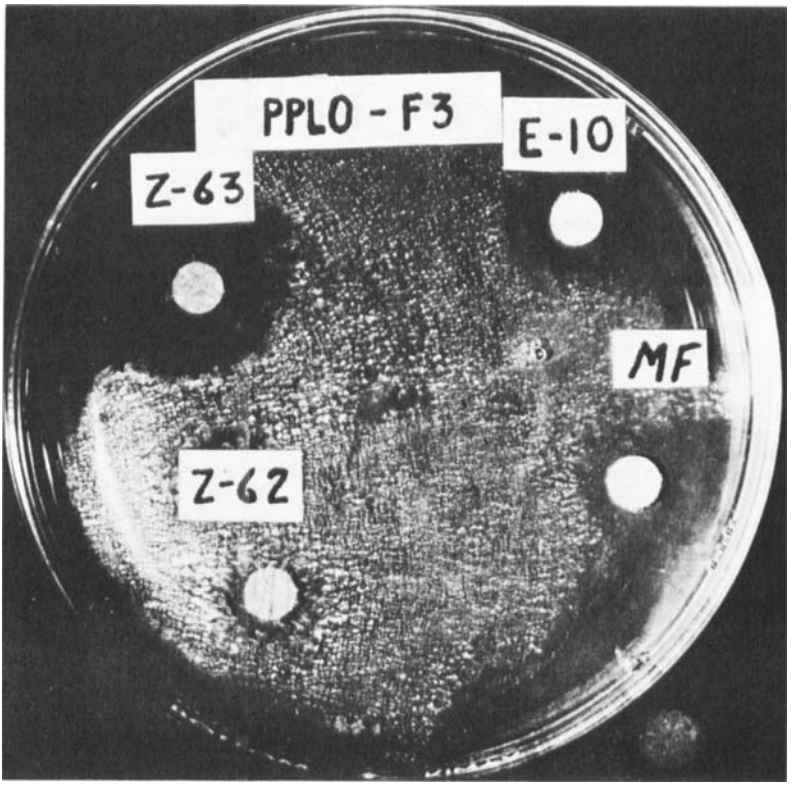

FIGURE 2. Mycoplasma strain F3 was spread over the agar surface. Antisera to the indicated strains (MF = *M. fermentans*) were tested against strain F3. Control tests with homotypic antisera were done in parallel. The results illustrate that strain Z62 was different from F3.

TABLE 11

CLASSIFICATION OF MYCOPLASMA BY THE CLYDE GROWTH INHIBITION TEST

Mycoplasma	Inhibition: Diameter in MM			Degree of* inhibition
	Confluent	100–500	10–100	
K10	6	10		100%
K1	15	20	20	200%
Z62	0	4	7	<50%

* Strains were scored *identical* if the zone of inhibition at the 100 to 500 dose of inoculum was 5–15 MM, *related* if less than 5 MM, and *different* if there was no inhibition of growth. Anti-K10 serum was used for tests.

A study of the Clyde technique revealed that an inoculum that gave from 100 to 500 colonies per plate gave results that were accurate and the most sensitive (FIGURE 2). From the representative results presented in TABLE 11, it was possible to classify the bone marrow PPLO as *identical, related* or *different.*

In general, the procedures described by Taylor-Robinson *et al.*[8] were used for complement-fixation (CF) tests. Most of the strains of PPLO tested were not

TABLE 12

CLASSIFICATION OF MYCOPLASMA BY COMPLEMENT-FIXATION TESTS

Mycoplasma	Reciprocal of CF Titer with anti-K10 serum	Relationship of Strains
K10	800	I
K1	800	I
F21	100	D

* Strains were scored either *identical* or different by the CF test. Strains were scored different only if their titer was three tubes different from control.

TABLE 13

CLASSIFICATION OF MYCOPLASMA BY THE INDIRECT HEMAGGLUTINATION TEST

Mycoplasma	Reciprocal of IHA Titer with anti-K10 serum	Relationship of Strains*
K10	500	Identical
K1	200	Identical
F21	100	Different

* Strains were scored different only if the IHA titer was two tubes greater or less than control. F21 was isolated from a child with acute stem-cell leukemia.

TABLE 14

ANTIGENIC RELATIONSHIP OF MYCOPLASMA TO GDL STRAIN

Mycoplasma strain	Isolated from patient with	Results of tests with anti-GDL serum:			
		CFU	CLYDE	CF	IHA
GDL*		I	I	I	I
F21	ASL	D	D	I	I
F7	ES	D	D	D	I
Z62	AMML	D	D	D	I
Z86	AGL	D	D		D
Z63	CLL	D	D	D	D

* GDL = Contaminant of human embryonic lung cells.[7]
ASL = Acute stem cell leukemia.
ES = Ewing sarcoma.
AMML = Acute myelomonocytic leukemia.
AGL = Acute granulocytic leukemia.
CLL = Chronic lymphocytic leukemia.

TABLE 15

ANTIGENIC DIFFERENCES IN THE MYCOPLASMA ISOLATED FROM BONE MARROW SPECIMENS

Mycoplasma	Antiserum Prepared Against:											
	M. fermentans				E-10				Z-62			
	CFU	CLYDE	CF	IHA	CFU	CLYDE	CF	IHA	CFU	CLYDE	CF	IHA
M. fermentans	I*	I	I	I	R†	I	I	I	D‡	R	I	I
Z63	I	I	D	I	I	I	I	D	R	I	I	I
K1	I	I	I	I	D	I	I	I	D	D	I	D
F21	D	D	I	D	D	D	D	D	D	D	D	D

* I = Identical.
† R = Related.
‡ D = Different.

anticomplementary. Strains were scored as either *identical* or *different* when tested against a single antiserum (TABLE 12).

When the representative strains of mycoplasma were compared[8] by use of the indirect hemagglutination technique (IHA), it also was possible to classify them as *identical* or *different* (TABLE 13).

A number of serologically distinct PPLO have been isolated from cell lines[7]—*viz.*, strains F11, F12, T7 and GDL. Results of tests with anti-GDL sera (TABLE 14) showed that our strains were different from the GDL group. In TABLE 14, the term F designates a bone marrow specimen used by us for isolation studies.[1]

A comparison of the results (TABLE 15) obtained for the representative mycoplasma provided a basis for a number of generalizations: The CFU growth-inhibition test was more reliable than the CPE neutralization technique and more sensitive than the Clyde test. The results of the Clyde and other growth-inhibition tests agreed in general. The CF and IHA tests gave results that were not related to each other or to the growth-inhibition tests. The isolated mycoplasma comprised three distinct groups. One group was very similar to *M. fermentans:* for convenience, members of the group were scored *identical* (I = identical). The second group included strains that were either closely or distantly related (R = related) to *M. fermentans.* The third group was different from *M. fermentans* (D = different). Antiserum to *M. fermentans* failed to distinguish among the groups because *M. fermentans* appears to have antigens in common with organisms in all three groups. By the use of appropriate antisera, it is possible to further subdivide each of the major groups. Thus, like the *Salmonella* and *Shigella,* each species of mycoplasma can be expected to encompass a mosaic of antigens that differ from each other to various degrees. Major methodological problems exist in arriving at a serologic analysis of mycoplasma strains.[9]

Induction of Leukemoid Disease in Mice by Isolated Mycoplasma

To further characterize the strains of mycoplasma isolated, they were cloned, grown in medium containing horse serum, centrifuged (15,000 × G–30 min), resuspended in growth medium to a concentration of approximately 10^8 CFU (colony-forming units) per ml, and injected IP (intraperitoneally) into 3–4-weeks-old BALB female mice. In from two to eight weeks, mice developed a

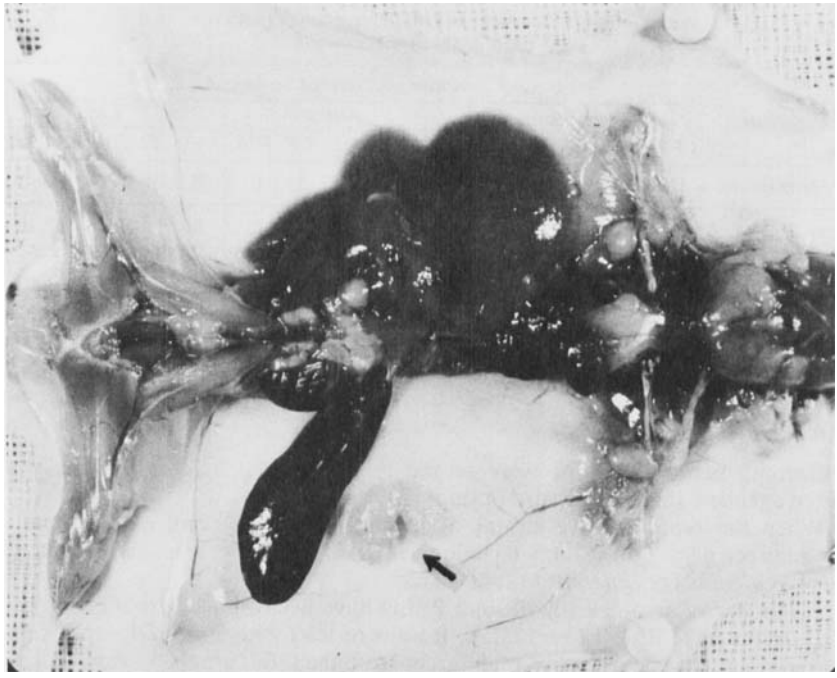

FIGURE 3. Mouse with fatal leukemoid disease. Note enlarged spleen, axillary and inguinal nodes, mesenteric nodes (arrow), and thymus.

leukemoid disease[1,2,3] that was either chronic or acute. The gross pathology of the disease simulated transplanted or spontaneous virus leukemia (FIGURE 3). The peripheral white blood count in mice with leukemoid disease ranged from three to ten times normal, and was characterized by a marked increase in segmented neutrophiles. The spleen, liver, inguinal and axillary nodes were moderately to greatly enlarged. The microscopic pathology was characterized by a marked follicular hyperplasia of the spleen and nodes. In chronic disease, the initial follicular hyperplasia involving the spleen was followed by a progressive loss in its architecture (FIGURE 4). Bone marrow, like the spleen and lymph nodes, was very hyperplastic with a predominance of mature-appearing segmented neutrophiles (FIGURE 5). However, there was no infiltration of malignant cells into organs that was characteristic of leukemia. Anemia occurred in diseased mice but was not prominent. Mice with leukemoid disease had their growth stunted and were ill throughout their life. The mycoplasma injected could be isolated from mice with leukemoid disease, particularly if they were treated with cortisone.

When the dose of mycoplasma injected IP was 10^9 CFU/ml or greater, mice died in from 24–48 hours (TABLE 16). No signs of CNS disease were observed that resembled those caused by the toxin of *M. neurolyticum*. Various inbred strains of mice were susceptible to infection. The injected mycoplasma could be recovered from mice in pure culture and were identified by serologic and other tests as the PPLO injected originally.

When the bone marrow strains of mycoplasma (10^9 CFU per ml) were injected IV (intravenously-1.0 ml/mouse), mice died in from two to five minutes. The mycoplasma when injected IV also killed rabbits within 24–48 hours. When strains of *M. fermentans, M. hominis,* types 1 and 2, *M. orale, M. salivarium,* or *M. pneumoniae* were grown and injected into mice by the methods used for the bone marrow strains of PPLO, they were essentially nonpathogenic. Filtrates of the broth in which the bone marrow PPLO were grown likewise were not pathogenic when tested throughout the growth cycle of the organisms.

Antibody Studies

To learn whether the mycoplasma isolated could be implicated in the pathogenesis of leukemia, serum neutralization tests were done. Mycoplasma representative of those isolated from children were used. Sera usually were tested 0 to two days after they were drawn. Pilot studies disclosed[1] that most sera lost their neutralizing activity when stored at either −20 or −70 C. Sera diluted 1:10 in Hanks solution[10] were added (0.1 ml) to each of five culture tubes per test. Each test strain of PPLO was used at a dose of 100 $TCID_{50}$. Cultures were incubated at 37° C for seven days and CPE-scored as noted above. The results (TABLE 17) of the neutralization tests revealed that neutralizing "antibody" to mycoplasma strain F3 was present two to three times more frequently in sera

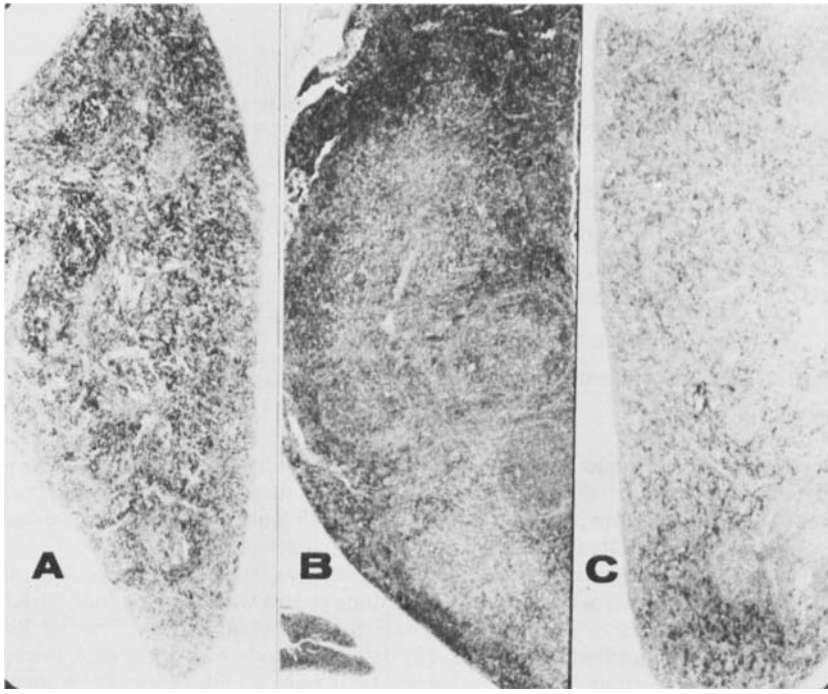

FIGURE 4. Spleens from mice with leukemoid disease showed a marked follicular hyperplasia: Normal mouse spleen (A); spleen from a mouse with acute leukemoid disease (B); spleen from mouse moribund from chronic leukemoid disease (C). In (C) note the loss in architecture. (Magnification 35×; hematoxylin-eosin stain).

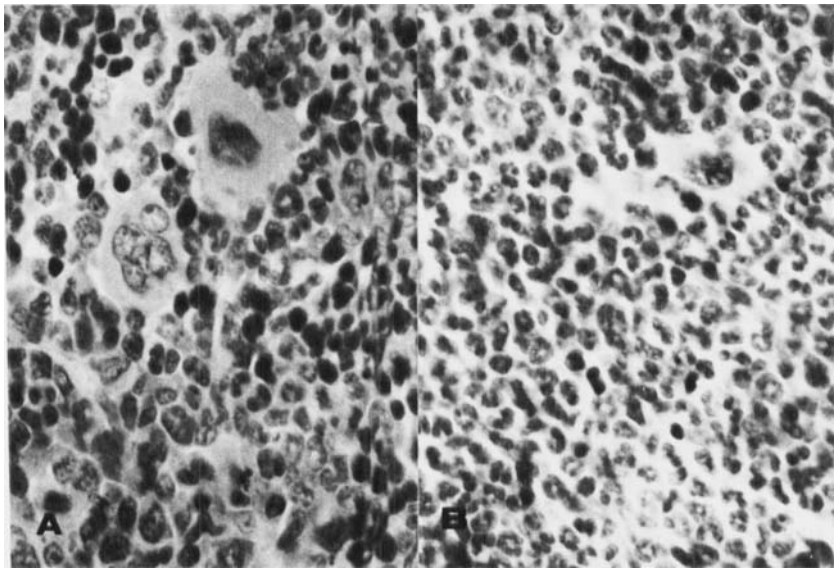

FIGURE 5. Hyperplastic changes in the spleen (A) and bone marrow (B). Note the marked megakaryocytosis and granulopoiesis. (Magnification 432×; hematoxylin-eosin stain).

TABLE 16

LETHALITY OF MYCOPLASMA FOR MICE

Strain	Origin	CFU/ml	Deaths*
K1	Acute granulocytic leukemia	10^9 to 10^{10}	22/22
K7	Acute stem-cell leukemia	"	18/18
K10	Undifferentiated sarcoma with bone metastases	"	46/51
F3	Disseminated lupus erythematosus	"	31/33

* Composite results from three or more replicate experiments.

obtained from leukemic patients than in controls. A correlative pattern existed between the neutralizing effect of sera from leukemic children and mycoplasma strain K7, isolated from a child with acute stem cell leukemia. Leukemic children also more frequently had antibody to mycoplasma strain K10.

Because a correlation appeared to exist between leukemia in children and mycoplasma K7, control experiments were done to test whether leukemic patients had a normal pattern of antibody distribution to common known viruses.[1] Both leukemic children and adults (TABLE 18) less frequently had antibodies to polio and vaccinia virus than normal children. Leukemic adults twice as frequently had antibody to adenovirus type 4 compared to normal controls. The results of these studies suggest that a pattern of antibody distribution which appears to relate leukemia to a given virus or mycoplasma infection may be misleading. Since

TABLE 17

SERUM NEUTRALIZATION TESTS ON MYCOPLASMA ISOLATED FROM BONE MARROW SPECIMENS

Serum Group	Proportion of Sera That Neutralized:			
	K1	K7	K10	F3‡
Normal adults*	5/53	5/53	8/53	0/53
Leukemic adults†	3/54	0/52	0/54	17/54
Normal children	5/39	0/39	2/39	4/39
Leukemic children	3/39	15/39	7/39	8/39

* Combined serum specimens from 15 to 20 pints of blood. All antibody titrations were repeated 3 to 5 times.

† All types of leukemia or lymphoma are grouped in this category. Mycoplasma strain F3 was neutralized by 45% (9/20) of serum specimens obtained from adults with lymphocytic leukemia.

‡ K1 = Acute granulocytic leukemia; K7 = Acute stem cell leukemia; K10 = Undifferentiated sarcoma with bone metastases.

TABLE 18

NEUTRALIZATION OF KNOWN VIRUSES BY SERA FROM LEUKEMIC AND NONLEUKEMIC PATIENTS

Serum Group	Proportion of Sera That Neutralized:				
	Poliovirus type 2	Vaccinia virus	Herpes-virus	Adenovirus type 4	Adenovirus type 12
Normal adults	28/40	24/40	19/40	5/20	8/40
Leukemic adults	21/40	20/40	16/40	11/20	6/41
Normal children	33/40	28/40	9/40	5/34	8/40
Leukemic children	28/41	21/41	13/41	3/27	8/41

leukemia markedly affects the reticulo-endothelial system, it is reasonable to assume that it may manifest itself in a variety of ways, including unusual antibody patterns.[11]

Discussion and Summary

Mycoplasma were isolated regularly by cell culture techniques from bone marrow specimens of leukemic patients. Mycloplasma also were isolated directly on agar plates but with a very low (0.1%) frequency. Mycoplasma were isolated most frequently from adults with leukemia. Specimens obtained from patients with leukemia in relapse, or who just had the disease diagnosed, most often were positive.

An antigenic analysis of the isolated mycoplasma disclosed that they comprised three subgroups. Two subgroups shared common antigens and were either closely or distantly related to *M. fermentans*. The third subgroup was very different antigenically from *M. fermentans* but also has some antigens in common with it.

The isolated mycoplasma induce a granulocytic leukemoid disease in mice that resembles a syndrome in man[12] called "Familial Myeloproliferative Disease." High concentrations of PPLO killed mice or rabbits in 24–48 hours.

The results of antibody studies in leukemic and nonleukemic patients, in conjunction with the isolation and mouse pathogenicity experiments, suggest that mycoplasma cause latent infection in both normal and leukemic patients. In leukemic patients, particularly adults, active mycoplasma infection may depend on the stage of disease. Mycoplasma infection presumably becomes active in patients with leukemia because of decreased host resistance during progression of the disease.

Acknowledgments

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