Comparison of Mycoplasmas Associated with Human Tumors, Leukemia, and Tissue Cultures

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ABSTRACT

LEACH, R. H. (Wellcome Research Laboratories, Beckenham, Kent, England), AND M. BUTLER. Comparison of mycoplasmas associated with human tumors, leukemia, and tissue cultures. *J. Bacteriol.* 91:934-941. 1966.—Mycoplasmas originally isolated by various workers from tissue cultures prepared from or inoculated with tumor or leukemic cells fell into four groups; each related to existing species or serotypes. These were *Mycoplasma pulmonis*, *M. fermentans*, *M. hominis*, and the GDL serotype, the last two being well known as contaminants of uninoculated cell lines. All the test strains were able to grow well in certain tissue cultures, and some caused cytopathic effects and acidity. These observations are discussed in terms of the relationship of these strains to the malignant tissues with which they were originally associated. The variable results obtained in certain biological tests on these strains emphasized the need for standardization of the conditions under which such tests may be employed to assist in identification of *Mycoplasma* species.

There have recently been several reports of mycoplasmas isolated during the course of investigations of human tumors and leukemia. Although in some instances (17) they have been cultivated directly in artificial medium, most cases have involved prior passage in tissue cultures. Cytopathic mycoplasmas have been isolated in this way from tissue cultures inoculated with material from leukemic patients (14, 15, 22, 23; Horoszewicz, *personal communication*). Other strains have been isolated from cell cultures of solid tumors, for example by Sabin (*personal communication*) and Armstrong et al. (1), both of whom noted, furthermore, that their isolates were related to the cytopathic mycoplasma strain GDL, originally found in uninoculated HEp-2 tissue in this laboratory (2).

This report concerns further comparisons of GDL with several of the above tumor-associated mycoplasmas. Knowledge of the relationships of these organisms may be relevant to the question of their true origin and possible etiological significance.

MATERIALS AND METHODS

The mycoplasmas used in this investigation are listed in Table 1. Most strains were received as pure cultures in artificial medium, but the Negroni myco-

plasmas were isolated in this laboratory from tissue culture pools provided by G. Negroni, thus confirming the observations of Grist and Fallon (15) and Girardi et al. (14). The two strains thus isolated, designated L and KH, were used interchangeably throughout the investigation, and are not recorded separately in the results, as they were known to be representative of a series of nine serologically indistinguishable strains associated with human leukemias (23). Similarly, 880 is 1 of 10 serologically related mycoplasmas associated with leukemia or other neoplasms (Horoszewicz, *personal communication*). K1, K7, K10, and F3 are mycoplasma isolates chosen as prototypes from a larger series of cytopathogenic agents associated with leukemic and other abnormal tissues (22).

All cultures were cloned in this laboratory at least once by subculture from a single colony before the investigation was begun.

Cultural methods and biochemical tests. Mycoplasmas were cultivated at 37 C in Edward fluid, solid or semisolid (0.1% agar) medium, modified as described in a previous report (2), or in the equivalent Hayflick media based on Difco PPLO broth or agar (3).

For determination of sugar reactions, phenol red indicator and 1% (w/v) glucose were added to the fluid or semisolid forms of the media. Hemolysis of horse red cells was tested by adding to heavily streaked growth on agar medium an overlay (3 to 5 ml) of agar (1%, w/v) containing 5% (v/v) washed oxalated

TABLE 1. *Details of strains examined*

Category	Strain	Source (and reference)
Isolated from human tumor cell cultures	T7 F11 F12	Thymoma (Sabin, <i>personal communication</i>) Hemangioma (1) Pharyngeal fibroma (1)
Isolated from tissue cultures inoculated with human malignant material	Negroni (L & KH) 880 K1 } K7 } K10 F3	Bone marrow, leukemias (23) Spleen, leukemia (16) Bone marrow, leukemias (22) Bone marrow, sarcoma (22) Bone marrow, lupus erythematosus (22)
Stock strains*		
<i>Mycoplasma hominis</i>	H50 (PG21)	(10)
<i>M. hominis</i>	"GPEK"	Guinea pig embryo kidney cell line (Leach, unpublished data)
<i>M. fermentans</i>	"G" (PG18)	(10)
<i>M. pulmonis</i>	"Ash" (PG34)	(10)
<i>M. pulmonis</i>	M50 (PG22)	(7, 28)
"Acid-inducing" mycoplasma	GDL	(2)

* Antisera to the following strains were also used: *M. salivarium* (PG21; 10); *M. orale* ("H" and CH19299; 18, 25), the human oral serotype 2 (CH20247; 26), *M. arthritidis* ("Campo"; 11), and *M. pneumoniae* (FH; 3), but these strains were not tested as antigens.

blood. In some cases, a similar test was carried out with guinea pig cells, but with the use of an overlay containing a 5% (v/v) suspension of washed packed cells.

Serology. Antisera were prepared in rabbits from well-washed organisms grown for at least two subcultures in mycoplasma medium containing 20% (v/v) rabbit serum and dialyzed yeast extract. Exceptions to this were Negroni and 880, which were grown on the same medium but with horse serum in place of rabbit serum. Successful growth of these two strains was subsequently obtained on media with rabbit serum supplemented with cholesterol (8), and antigens used for complement-fixation (CF) tests were prepared from such cultures so as to avoid the possibility of reactions with antibody against horse serum components.

The procedure for antiserum production was as described previously (2). An alternative procedure, used for *Mycoplasma hominis* type 1, *M. salivarium*, *M. orale*, *M. fermentans*, *M. pneumoniae*, and strain GDL was rather similar to that of Taylor-Robinson et al. (27), and comprised a denser suspension combined with incomplete Freund-type adjuvant. With the exception of *M. pneumoniae*, these latter antigens were grown in media based on ox heart infusion broth which had been ultrafiltered to remove potentially antigenic fractions.

CF tests were carried out as described previously (2), except that the antigen dose chosen for cross-tests with other sera was the highest dilution giving full activity when tested in twofold dilutions against the homologous serum (i.e., one full unit of antigen). This was usually at least a 0.5 dilution of a standard suspension having an opacity equivalent to Brown's tube no. 5. Growth inhibition tests (9) were carried

out by the modification described by Clyde (4). This involved the assessment of zones of inhibition which developed around filter paper discs impregnated with 0.02 ml of antiserum and laid onto agar plates seeded with various dilutions of mycoplasma suspension.

Since heat inactivation had no beneficial effect on results of CF or other serological reactions, unheated sera were used for all these tests.

Viable counts. The number of viable mycoplasma particles in artificial media or tissue cultures was measured by the colony count or tissue culture infective dose methods described previously (2).

Tissue cultures. Monolayer cultures of monkey kidney (*Erythrocebus patas*; primary tissue), HEP-2, and the Wistar 38 (WI-38) strain of human diploid cells were used. Cultures for experiments were grown in test tubes (15.2 by 1.6 cm), each seeded with 200,000 cells in 2 ml of medium. The growth medium was replaced by 1.5 ml of maintenance medium when the cultures were confluent, and the tubes were then rolled.

The media for growth and maintenance of HEP-2 and monkey kidney were described previously (2). For growth of WI-38, Eagle's medium (5) containing 10% (v/v) unheated calf serum, 0.1% (w/v) sodium bicarbonate, 100 units per ml of penicillin, and 0.01% (w/v) neomycin was used; the maintenance medium was the same as that used for monkey kidney cultures.

Tissue cultures were inoculated with decimal dilutions (10^{-2} to 10^{-8}) of cultures of the test mycoplasmas grown in Hayflick fluid medium for 3 days, deposited by centrifugation, and resuspended in half the original volume of medium 199 containing 0.2% (w/v) sodium bicarbonate. The same dilutions were used for a colony count. After 1 week, the fluid from the infected tissue cultures was passed at a 0.25 dilution to homologous

tissue, and a colony count was made. The cultures selected for subculture were those inoculated with the highest dilution causing cytopathic effects or acidity, or both, together with the tubes corresponding to the dilution 10-fold greater and less. Where no cytopathic effects were detected, the set of tubes inoculated with the first three levels of dilution (10^{-2} to 10^{-4}) were passaged. At the end of the 2nd week, a final colony count was made on the infected tissue culture fluids.

RESULTS

Serology. Growth inhibition and CF tests indicated that the organisms fell clearly into four serological groups (Tables 2 and 3).

Despite low values for GDL complement-fixing antibodies (also obtained with other batches of antiserum to this agent), the combined results of CF and growth inhibition results indicated that GDL, F7, and F12 were interrelated, and formed a distinct serological group. Organisms in the other three groups were related respectively to *M. pulmonis*, *M. fermentans*, and *M. hominis*.

With regard to F11, the identification of this strain as *M. hominis* has already been recorded by Armstrong et al. (1), but we failed to detect the secondary mycoplasma reported by these

workers, even in the original uncloned culture. Subcultures of individual "breakthrough" colonies which appeared in some inhibition tests against *M. hominis* antiserum, or of colonies showing differences in size or form from the majority, also proved to be *M. hominis*. Furthermore, the uncloned cultures failed to ferment glucose or to induce cytopathic changes in HEp-2 cultures.

Growth and biological tests. The test organisms were facultative aerobes, although they tended to grow best in the gaseous conditions in which they had been originally passaged. Most were grown routinely in 5% CO₂ in nitrogen on Hayflick-type medium, but GDL and Negroni, having been isolated in this laboratory, were usually grown on modified Edward medium. Because of the ability of mycoplasmas to adapt to particular media and gaseous conditions, these growth requirements were not regarded as significant and are not included in Table 4, which lists some other biological properties of the test strains and certain other strains. In general, the organisms within each serological group had similar biological properties.

TABLE 2. Results^a of growth inhibition tests

Antigen	Rabbit antisera														
	GDL	T7	F12	Negroni	880	<i>Mycoplasma pulmonis</i> (M50)	K1	K7	K10	F3	<i>M. fermentans</i> ("G")	F11	<i>M. hominis</i> (H50)	Other species ^b	Preimmune ^c
GDL.....	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0
T7.....	+	+	TR	0	0	0	0	0	0	0	0	0	0	0 ^d	0
F12.....	+	±	+	0	0	0	0	0	0	0	0	0	0	0	0
Negroni.....	0	0	0	+	+	+	0	0	0	0	0	0	0	0	0
880.....	0	0	0	+	+	±	0	0	0	0	0	0	0	0	0
<i>M. pulmonis</i> ("Ash")...	0	0	0	+	+	+	0	0	0	0	0	0	0	0	0
<i>M. pulmonis</i> (M50)....				+	+	+									0
K1.....	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0
K7.....	0	0	0	0	0	0	+	+	TR	+	+	0	0	0	0
K10.....	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0
F3.....	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0
F11.....	0	0	0	0	0	0	0	0	0	0	0	+	+	0	0
<i>M. hominis</i> (H50).....	0	0	0									+	+	0	0

^a Zones of inhibition: +, definite zone (>1 mm); ±, very small (<1 mm), variable, or otherwise indeterminate zone; 0, no zone, or not different from test with normal (preimmune) serum.

^b As for complement-fixation tests (see Table 3).

^c Serum taken from rabbit before immunization with the stated antigen.

^d Antigen T7 reacted in some tests with our standard *M. orale* antiserum, but tests with other batches of antiserum, including one (CH 19299) supplied by D. Taylor-Robinson, were negative.

TABLE 3. Antibody titers^a obtained in complement-fixation tests

Antigen	Rabbit antisera														
	GDL	T7	F12	Negroni	880	<i>Mycoplasma pulmonis</i> (M50)	K1	K7	K10	F3	<i>M. fermentans</i> ("G")	F11	<i>M. hominis</i> (H50)	Other species ^b	Preimmune ^c
GDL.....	80	40	160	0	0	0	0	0	0	0	0	0	0	0	0
T7.....	30	240	20	0	0	0	0	0	0	0	0	0	0	0	0
F12.....	30	160	80	0	0	0	0	0	0	0	0	0	0	0	0
Negroni.....	0	0	0	40 (160) ^d	80 (320)	60	0	0	0	0	0	0	0	0	0
880.....	0	0	0	20	160	0	0	0	0	0	0	0	0	0	0
<i>M. pulmonis</i> ("Ash")..	0	0	0	40 (20)	80 (320)	40	0	0	0	0	0	0	0	0	0
K1.....	0	0	0	0	0	0	240	40	240	160	60	0	0	0	0
K10.....	0	0	0	0	0	0	160	0	60	80	40	0	0	0	0
F3.....	0	0	0	0	0	0	160	20	40	60	80	0	0	0	0
<i>M. fermentans</i> ("G")							40	80	80	80	80		0	0	0
F11.....	0	0	0	0	0	0	0	0	0	0	0	320	40	0	0
<i>M. hominis</i> (H50).....	0	0	0	0	0	0						80	160	0	0

^a Recorded reciprocally. Titers of less than 20 regarded as negative (0).^b *M. salivarium*, *M. orale*, *M. pneumoniae*, *M. arthritidis* ("Campo"). Some antigens (GDL, T7, F12, F11, and *M. hominis*) were also tested with negative results against serum to a new human oral serotype (provided by D. Taylor-Robinson).^c Serum taken from rabbit before immunization with the stated antigen.^d Titers in parentheses refer to tests with rabbit antisera provided by G. Negroni and J. Horoszewicz (strain 880), respectively.TABLE 4. Biological characters of test strains of *Mycoplasma*

Strain	"Film and spots" in horse serum-agar	Growth on rabbit serum-agar	Hemolysis of blood-agar		Glucose fermentation	
			Horse blood	Guinea pig blood	Semisolid medium*	Fluid medium†
GDL.....	—	Moderate	± or —	+	—	±
T7.....	—	Moderate	±	+	—	—
F12.....	—	Moderate	+	+	—	+
Negroni.....	—	Initially negative	± or +	+	± or —	+
880.....	—	Initially negative	+	+	—	+
<i>M. pulmonis</i> ("Ash")..	—	Very good	+	+	Trace	±
K1 } K7 } K10 } F3 }	+	Good	— (or trace)	—	—	+
<i>M. fermentans</i> ("G")..	+	Good	—	—	+	+
F11.....	—	Moderate	—	—	—	—
<i>M. hominis</i> (H50).....	—	Moderate	—	—	—	—

* Edward medium.

† Hayflick medium.

With regard to colonial morphology, striking similarities were observed between Negroni and 880, both producing within 2 or 3 days large (up to 0.5 mm) but fairly typical "fried-egg" colonies. After more prolonged incubation, the colonies usually became even larger (up to 1 mm) with an opaque and roughened surface and heavy pigmentation which obscured the central "nipple." The degree of pigmentation varied between individual colonies, even taking the form of transparent or pigmented segments or outgrowths. In the case of 880, these latter variants were rare, but could be selectively induced by passage in monkey kidney tissue culture. A nonpigmented variant of 880 was cultivated which retained its serological identity with the parent culture. Standard *M. pulmonis* strains, which were antigenically related to 880 and Negroni, also formed large, opaque colonies with somewhat rough surfaces (20, 28), but were less granular and without pigmentation. All the other mycoplasmas formed fairly small (<0.2 mm) characteristic colonies.

K1, K7, K10, F3, and *M. fermentans* were distinguished from all others by their production of "film and spots" on solid medium (6).

Negroni and 880 differed from *M. pulmonis* "Ash" and all other test organisms in their poor growth on rabbit serum-agar.

Few of the organisms caused fermentation in Edward semisolid medium, but in Hayflick fluid medium pH changes were more common. However, in this medium appreciable pH change occurred in inoculated medium without added glu-

cose. We confirmed reports by other workers (14; Sabin, *personal communication*; Hayflick, *personal communication*) that GDL fermented glucose in Hayflick medium, whereas this organism, as shown previously (2), failed to cause detectable fermentation in Edward semisolid medium. Nevertheless, all test organisms except F11 and T7 caused some degree of specific fermentation of glucose in this medium.

With regard to hemolysis tests, the third and fourth groups consistently gave negative reactions, whereas the other two groups usually gave positive results, though with marked quantitative and qualitative variations.

Growth in tissue culture. The results are again assembled in terms of the four serological groups, and are expressed in a simplified form to avoid the complexity of figures showing tissue culture infective dose or colony counts (Table 5). Mycoplasmas in the first group caused the HEp-2 cells to become spindle-shaped, then rounded and granular, and to fall off the glass. These effects closely paralleled the development of acid. WI-38 cultures were unaffected by F12, but both GDL and T7 caused a slight drop in pH, accompanied by development of granularity and coarsening of the cells, and some cell rounding at the edges of the culture. In *Erythrocebus patas* cultures, GDL and T7 caused a generalized degeneration into spindly fibroblastic cells, with some granular rounded cells formed mainly at the edges of the cultures.

In the second group, 880 induced within a few days a severe degeneration of WI-38 into granu-

TABLE 5. Growth of test mycoplasmas in tissue cultures

Organism	HEp-2			WI-38			<i>Erythrocebus patas</i>		
	Growth*	CPE*	Acid*	Growth	CPE	Acid	Growth	CPE	Acid
GDL.....	4	2	4	4	TR	2	4	1	0
T7.....	4	3	4	4	TR	2	3	1	0
F12.....	4	2	4	3	0	0	2	0	0
880.....	4	2	4	4	4	2	3	2	0
Negroni.....	4	2	4	4	1	0	2	TR	0
<i>M. pulmonis</i> ("Ash").....	1	0	0	2	0	0	1	0	0
K10.....	4	TR	2	2	0	0	2	0	0
F3.....	4	0	TR	1	0	0	2	0	0
<i>M. fermentans</i> ("G").....	2	0	0	1	0	0	TR	0	0
F11.....	4	0	0	3	0	0	1	0	0
<i>M. hominis</i> (GPEK).....	4	0	0	3	0	0	2	0	0
<i>M. hominis</i> (H50).....	3	0	0	1	0	0	2	0	0

* Growth measured by viable counts made at the end of the second passage of the test organism and graded 1 to 4. Cytopathic effects (CPE) and degree of acid formation recorded at the same time were similarly graded. Trace (TR) represents slight, variable values.

lar rounded cells within a matrix of granular debris and fibrous strands; this phenomenon was associated with some degree of acidity. The effects caused by Negroni were much less marked and did not become at all generalized until after subculture for 9 days, while no fall in pH was detected. *E. patas* cultures infected with 880 were also more grossly affected than with Negroni. The infected cultures developed a network of spindle-like cells which tended to become rounded.

In the third and fourth groups, although negligible cytopathic effects were observed, the test organisms grew well particularly in HEp-2.

DISCUSSION

On the basis of their serological properties, these mycoplasmas can be classified into four distinct groups, each related to an existing species or serotype, i.e., GDL, *M. pulmonis*, *M. fermentans*, and *M. hominis*.

With regard to the first group, the present work confirmed and extended earlier observations (2) that GDL is serologically distinct from known mycoplasmas of human origin. It also confirmed that F12 and GDL are serologically related (1). Armstrong et al. (1) found a similar organism mixed with the *M. hominis* serotype in cultures of F11. Furthermore, they refer to work (Girardi, Hamparian, Somerson, and Hayflick, *in press*) on the isolation of similar organisms from uninoculated tissue cultures. Although the exact relationship of GDL to these mycoplasmas has yet to be fully defined, it seems probable that GDL lies naturally within a separate species which would include F12 and T7. However, since its original habitat and its possible relationship to mycoplasma species from various animal sources are still unknown, it would seem premature to designate this group as a new named species.

In the second group, 880 and Negroni were closely related to each other and were serologically related to strains of *M. pulmonis*, which occurs naturally in rodents. This confirms the relationship between *M. pulmonis* and Negroni reported by Fallon et al. (12) and by Clyde (as quoted in reference 19). The quantitative serological differences indicated by our CF tests were also observed by the former authors. In assessing the importance of such serological differences between mycoplasma strains, it has to be decided which serological test most accurately determines the identity of a particular strain as a member of a *Mycoplasma* species. Agglutination tests tend to emphasize small differences between closely related strains (7, 24). Others, such as CF and agar-gel precipitin tests, may reveal shared antigens between different species (21, 27, 29). The growth

inhibition test, which clearly distinguishes between species without being sensitive to minor antigenic differences between strains (4, 9), would seem to be the most reliable method for identifying species. According to this criterion, 880 and Negroni can be identified as belonging to the species *M. pulmonis*.

It must be admitted that in some cases the test organisms showed differences in cultural or biological properties from the representative strains of the species to which they belonged serologically (Tables 4 and 5). Such differences may result from the fact that the test organisms, in contrast to the representative strains, had all undergone passage in tissue culture.

In other cases, individual organisms gave widely varying results for a given biological test (Table 4). These observations emphasize a need for greater standardization and agreement regarding media and other conditions under which tests to assist in identification of mycoplasmas should be carried out.

The observations reported in this paper provide scant supporting evidence for any specific connection of the test mycoplasmas with tumors or leukemias. Indeed, the problem of their true origins is complicated rather than simplified by the variety of *Mycoplasma* species shown to be involved. In addition to the four serotypes revealed in this investigation, other human species have been implicated (17). It is noteworthy that almost identical organisms should have been isolated from similar sources in widely separated laboratories as in the case of 880 and Negroni associated with leukemic tissue and of F12 and T7 associated with solid tumor cell cultures. On the other hand, K1 and K7, apparently associated with leukemic tissue, have proved to be quite distinct from 880 and Negroni. Furthermore, although associated with different types of leukemia or other diseases, K1, K7, K10, and F3 isolated in the same laboratory were all strains of *M. fermentans* hitherto found only in the human genital tract (13, 24). Finally, mycoplasmas related to F12 and T7 have been found in uninoculated tissue cultures (1, 2), and *M. hominis*, although of human origin, is also a common contaminant of tissue culture.

Tissue cultures readily support the growth of a variety of extraneously introduced mycoplasmas (this paper; 2), and, since all the test organisms were isolated by means of tissue culture, it is necessary to consider the possibility that at least some may have been present as contaminants in the originating laboratories. In this connection, our own experience indicates that it is not always possible to cultivate on artificial media mycoplasmas from tissue cultures known to be con-

taminated with these organisms. It is conceivable that, where such "crypto-infections" exist, alteration in the tissue culture conditions, for example by inoculation with certain materials, might encourage a form of mycoplasma growth susceptible to detection in artificial medium.

It is obvious that, in any consideration of the association of mycoplasmas with human tissues, including malignancies, greater significance must be given to those cases where isolations have been made directly in artificial media (17), and preferably on solid media, since even fluid media may favor enrichment cultivation of laboratory contaminants. In our laboratory, mycoplasmas serologically related to *M. orale* or similar to GDL have recently been isolated on a few occasions from tissue cultures inoculated with material from leukemic patients, or from control uninoculated cultures, or from both. However, as we failed to isolate mycoplasmas directly in artificial media from any of the 25 such samples so far tested, it remains very doubtful that any of these isolates were actually present in the original samples.

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ADDENDUM IN PROOF

Since the preparation of this paper, a detailed description of the isolation of *Mycoplasma* 880 and related strains has been published (J. T. Grace, J. S. Horoszewicz, T. B. Stim, E. A. Mirand, and C. James, *Cancer* 18:1369, 1965).

W. H. Murphy, I. J. Ertel, and C. D. J. Zarafonitis (*Cancer* 18:1329, 1965) have confirmed the identity of their four prototype strains as *M. fermentans* but pointed out that some other strains isolated in their laboratory from tissue cultures inoculated with leukemic material were not *M. fermentans*. Furthermore, these authors reported isolations of mycoplasmas made directly onto agar from leukemic bone marrow.

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