PART VII. ISOLATION OF MYCOPLASMA FROM HUMAN MALIGNANT AND OTHER TISSUES

CHARACTERIZATION OF MYCOPLASMA STRAINS AND ANTIBODY STUDIES FROM PATIENTS WITH RHEUMATOID ARTHRITIS*

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Previously reported studies[1] have described the isolation methods and initial characterization of mycoplasma from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Reiter's syndrome. These strains were isolated in cell culture using two primary cell lines, green monkey kidney and diploid human embryonic lung fibroblasts. Inocula included synovial fluid, kidney tissue, serum, and bone marrow. All control tubes, and those inoculated with material from patients with osteoarthritis, traumatic arthritis, and gout remained free of mycoplasma. Growth of mycoplasma in cell-free medium occurred after three to eight passages in cell culture.

TABLE 1 summarizes the number of isolations and cell lines used. Not all specimens were inoculated into each cell line. In one case, mycoplasma were isolated from the same specimen in both cell lines. Mycoplasma have been isolated from 10 patients with RA, four from SLE, and two from Reiter's syndrome. This represents an approximate recovery rate of 20%.

Initial studies of antibiotic sensitivity to the RA-SLE strains showed that they were relatively resistant to streptomycin and sensitive to tetracycline. However, sensitivity to chloramphenicol and kanamycin varied considerably. They fermented glucose and caused an alpha type hemolysis of sheep red cells.

The present report describes the antigenic characterization of these strains using growth-inhibition, complement-fixation, and gel diffusion methods. A study of complement-fixing antibody in serum and synovial fluid from patients with RA is included.

Materials and Methods

Media. The medium used for isolation and propagation of the organisms was similar to that described by Taylor-Robinson *et al.*[2] The six strains of mycoplasma studied in detail were each cloned twice[3] and subcultured four times in medium containing rabbit serum, instead of horse serum, before growing antigens for rabbit immunization.

Production of Rabbit Antisera. Antisera to the mycoplasma strains were prepared as described in detail previously.[1] Immunizing antigen represented a 20-fold concentration of washed organisms of six-day flask cultures. Complete Freund's adjuvant was used to enhance antibody production, and multiple intradermal and subcutaneous sites were inoculated. Control rabbits received complete medium with adjuvant processed in the same manner as the organisms.

Complement-fixation Tests. Antigens were grown in standard PPLO medium, washed three times and concentrated 20-fold by centrifugation as previously described.[1] The one-fifth volume Kolmer complement-fixation test was used.[4] Antigen was titrated by the block technique, and one unit of antigen was used.

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TABLE 1

MYCOPLASMA ISOLATION CELL LINE

	AGMK*	HEL†
Rheumatoid arthritis		
synovial fluid	4	1
serum		1
Systemic lupus		
kidney	3	
marrow	1	
Reiter's syndrome		
synovial fluid	2	

* Green monkey kidney.
† Human embryonic lung.

This usually represented the most concentrated dilution which was not anticomplementary. Phenol was routinely added to the growing cultures on day five as described by Chanock.[5] Occasionally, complement was added to decrease anticomplementary antigen. This was inactivated by zymosan[6] rather than heat to preserve all the antigens.

Gel Diffusion and Immunoelectrophoresis. Antigens for gel diffusion and immunoelectrophoresis were grown in medium with rabbit serum. Organisms were washed three times in phosphate-buffered saline (0.15M, pH 7.4) and centrifuged at 17,300 × g for 30 minutes. The final pellet was suspended in distilled water representing a 200-fold concentration. Antigens were frozen and thawed five times.

Gel diffusion was performed by a modification of the Ouchterlony technique.[7] A thin layer of 1% Ionagar in distilled water was dried on 3¼ × 4 inch glass plates at 80°C. Then an overlay of 12 ml of 1% Ionagar in phosphate-buffered saline (pH 7.4) was allowed to spread evenly. The plates were placed at 4°C overnight before use. Six holes and a center well were cut in the agar with a Feinberg agar cutter. Antigen was usually placed in the center well, with rabbit antisera in the side wells. Plates were incubated for three days at 37°C in a humidified chamber, washed for 24 hours in normal saline, rinsed in distilled water and air-dried. Precipitin lines were drawn as they appeared and the dried plates were stained with Amidoschwarz stain.[8]

TABLE 2

COMPLEMENT-FIXATION TESTS
ISOLATES FROM RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS

Antigen	Rabbit Antiserum Titers			
	H-EM*	A-MK*	A-AJ†	A-DM†
H-EM	10,240‡	5120	1280	1280
A-MK	2560	2560	2560	1280
A-AJ	5120	5120	10,240	2560
A-DM	5120	5120	5120	5120

* Rheumatoid arthritis.
† Systemic lupus erythematosus.
‡ Reciprocal titer.

TABLE 3

COMPLEMENT-FIXATION TESTS
RELATIONSHIP OF ISOLATED STRAINS TO OTHER STRAINS

	Rabbit Antiserum Titers							
Antigen	*M.hom.* type 1	*M.hom.* type 2	*M.sal.*	*M.orale*	*M.ferm.*	*M.pneu.* (FH)	GDL*	H-EM†
M. hominis type 1	2,560‡	80	<20	40	80	320	160	80
M. hominis type 2	<20	1,280	<20	40	80	160	160	40
M. salivarium	<20	80	20,480	40	80	320	160	20
M. orale	160	80	160	20,480	40	320	160	80
M. fermentans	160	20	<20	<40	5,120	160	80	20
M. pneumoniae (FH)	160	320	80	320	160	2,560	80	320
GDL	320	320	160	320	80	640	10,240	5,120
H-EM†	80	160	320	20	160	320	5,120	5,120

* Strain of Butler and Leach.
† Rheumatoid arthritis.
‡ Reciprocal titer.

Microimmunoelectrophoresis was performed using techniques similar to those described by Campbell *et al.*[9] Microscope slides (1 × 3 inches) were covered with a thin layer of 1% Ionagar in barbital buffer at pH 8.2. Two antigens were electrophoresed on each slide using similar buffer at a constant current of 5 MA for 90 minutes in a Shandon apparatus. Rabbit antisera was introduced into the center slit and the plates were incubated for 24 hours at room temperature in a humidified chamber. Precipitin lines were drawn as they appeared. The slides were washed, dried and stained as described above.

When testing antigens for heat lability, aliquots were heated in a constant temperature water bath at 56° and 60°C for 30 minutes. Better separation of precipitin lines occurred when antigen rather than antibody was electrophoresed. On each slide, both a control untreated antigen and a treated antigen were electrophoresed.

Growth-inhibition Tests. The method as described by Clyde[10] was also used for identification of the isolated strains. Zones of inhibition of growth around 6mm filter paper discs containing 0.025 ml of rabbit antisera were measured in millimeters.

TABLE 4

COMPLEMENT-FIXATION TESTS
ISOLATES FROM REITER'S SYNDROME

	Rabbit Antiserum Titers			
Antigen	A-DS*	*M. hominis* type 1	A-DF*	*M. hominis* type 2
A–DS	5120†	320	40	160
M. hominis type 1	1280	2560	40	80
A–DF	320	80	2560	2560
M. hominis type 2	40	<20	320	1280

* Reiter's syndrome.
† Reciprocal titer.

TABLE 5

GROWTH-INHIBITION TESTS

Strain	Rabbit Antiserum									
	M. hom. type 1	A-DS*	*M. hom.* type 2	A-DF*	*M. sal.*	*M. orale*	*M. ferm.*	*M. pneu.*	GDL§	H-EM†
M. hominis type 1	3.5‡	3.5	0	0	0	0	0	0	0	0
A–DS*	3.0	3.5	0	0	0	0	0	0	0	0
M. hominis type 2	0	0	3.5	2.5	0	0	0	0	0	0
A–DF*	0	0	4.0	3.0	0	0	0	0	0	0
M. salivarium	0	0	0	0	9.0	0	0	0	0	0
M. orale	0	0	0	0	0	4.0	0	0	0	0
M. fermentans	0	0	0	0	0	0	7.0	0	0	0
M. pneumoniae (FH)	0	0	0	0	0	0	0	5.0	0	0
GDL§	0	0	0	0	0	0	0	0	2.0	0
H–EM†	0	0	0	0	0	0	0	0	0	2.5

* Reiter's syndrome.
† Rheumatoid arthritis.
‡ Zone of growth inhibition in millimeters.
§ Strain of Butler and Leach.

Results

The two cloned strains from RA and two from SLE cross-reacted closely with each other by complement-fixation tests (TABLE 2). When compared to other characterized mycoplasma strains, the prototype strain H-EM reacted only with the GDL strain of Butler and Leach[11] (TABLE 3). TABLE 4 shows that by complement-fixation tests, the two isolated strains from Reiter's syndrome cross-reacted with *M. hominis* types 1 and 2, respectively. However, both strains reacted more strongly in one direction, indicating some antigenic difference. By the method of growth-inhibition (TABLE 5), the two Reiter's strains were similarly identified as *M. hominis* type 1 and type 2. However strains H-EM (prototype RA strain) and the GDL did not inhibit each other.

By gel diffusion, the results agreed with the complement-fixation tests. An example in FIGURE 1 shows the lines of identity among several RA and SLE strains and the GDL strain. FIGURE 2 shows the line of identity between *M. hominis* type 2 and A-DF from Reiter's syndrome.

The other isolates tested from RA and SLE were identified by growth-inhibition using a polyvalent antiserum composed of equal amounts of antisera to cloned strains A-MK, H-EM, A-AJ, and A-DM. TABLE 6 shows that all of these isolates (both cloned and uncloned) are closely related.

Control studies showed no precipitin lines between control rabbit antiserum and any of the gel diffusion antigens except for *M. pneumoniae*. This strain would grow only in broth medium containing horse serum. A control antigen of broth did not react with any of the rabbit antisera. Complement-fixation tests using all strains and control rabbit antiserum were less than 1:40 except for *M. pneumoniae* which reacted at 1:320. No growth-inhibition occurred to any strain with the control rabbit antiserum.

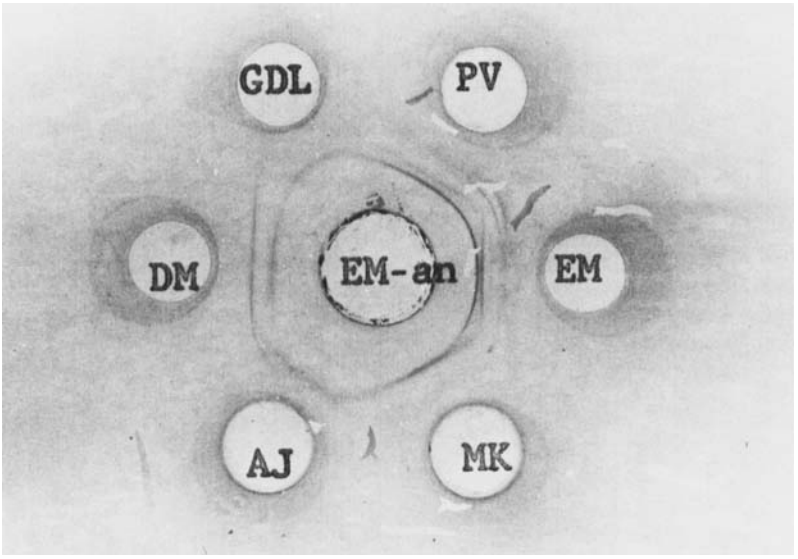

FIGURE 1. Gel diffusion showing lines of identity among the rheumatoid arthritis-systemic lupus erythematosus strains and the GDL strain. PV-polyvalent antiserum.

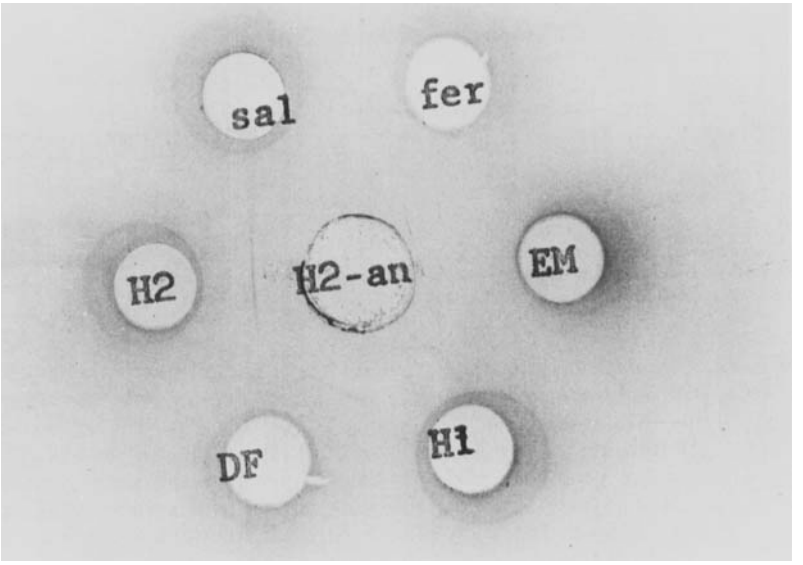

FIGURE 2. Gel diffusion showing line of identity between *M. hominis* type 2 and isolate A-DF from Reiter's syndrome.

TABLE 6

GROWTH-INHIBITION TESTS

Isolates	Polyvalent Antiserum*
A–DM[L]	4.5†
A–AJ[L]	3.0
A–HN[L]	3.5
A–DB[L]	3.5
H–EM[R]	4.0
A–MK[R]	2.5
H–MG$_2$[R]	5.0
H–MG[R]	3.0
H–LH[R]	6.0
A–CR[R]	3.0
A–WH[R]	2.5

* Antisera to strains A–AJ, A–DM, H–EM, and A–MK.
† Zone of growth inhibition in millimeters.
[L] Systemic lupus erythematosus.
[R] Rheumatoid arthritis.
Underlined isolates cloned twice.

TABLE 7

COMPLEMENT-FIXATION TESTS

	Polyvalent Antigen Titer				
	0	1:8-1:32	1:64-1:256	>1:256	Total
Rheumatoid Arthritis* Def. or class.	5	22	23	2	50
Controls†	48	2	0	0	50

* Fifteen synovial fluid and 35 sera.
† Ten synovial fluids and 40 sera.

Studies of Circulating Antibody. Earlier studies of complement-fixing antibody in patients with RA indicated that titers varied considerably with the different isolated strains. Minor antigenic differences among the RA-SLE strains were noted with gel diffusion and complement-fixation tests. Antibiotic sensitivity also varied among these strains especially to chloramphenicol and kanamycin. For these reasons, a polyvalent complement-fixing antigen was composed of equal amounts of cloned strains H-EM, A-MK, A-AJ, and A-DM, two each from SLE and RA.

Serum and synovial fluid from patients with definite or classical RA were tested for complement-fixing antibody using the polyvalent antigen and *M. hominis* type 1 for comparison. An equal number of control specimens were studied. This group consisted of patients on general medical wards with unrelated diseases, but including serum and synovial fluid from gout and osteoarthritis patients. The other half of the control group were from university faculty members undergoing routine periodic health examinations.

TABLE 7 shows the results of complement-fixation tests in patients with RA and the control group using the polyvalent antigen. 90% of the rheumatoid patients were positive in titers of 1:8 or higher, and 46% were positive at 1:64 or greater. Only 4% of the control specimens reacted; these were all in low titer.

Complement-fixation titers to *M. hominis* type 1 are shown in TABLE 8. 26% of the RA specimens and 16% of the control group reacted, all in low titers of 1:32 or less. Both groups were tested with a control antigen, and these were negative.

During the study of complement-fixation testing for antibody in human specimens, it became apparent that the antigens were in part heat-labile at 56°C.

TABLE 8

COMPLEMENT-FIXATION TESTS

	M. hominis type 1 Titer				
	0	1:8-1:32	1:64-1:256	>1:256	Total
Rheumatoid Arthritis* Def. or class.	37	13	0	0	50
Controls†	42	8	0	0	50

* Fifteen synovial fluid and 35 sera.
† Ten synovial fluids and 40 sera.

TABLE 9

EFFECT OF HEAT-TREATED ANTIGEN (56°C–30 MIN) UPON COMPLEMENT-FIXATION TITERS

Untreated Antigen	Treated Antigen
1:256	1:8
1:256	1:32
1:256	1:32
1:256	1:32
1:128	1:32
1:128	1:8
1:128	1:8
1:128	0
1:128	0
1:64	1:8
1:32	1:8
1:16	0
1:8	0
1:8	0
1:8	0

TABLE 9 shows the results of testing 15 specimens of synovial fluid and serum from patients with RA when the polyvalent antigen was heated at 56°C for 30 minutes. In each case, antibody titer decreased, often to less than 1:8—the lowest dilution tested. Further studies of heat lability of mycoplasma antigens were undertaken using gel diffusion and immunoelectrophoresis. The latter method gave better definition of antigen-antibody reactions.

FIGURE 3 shows that *M. hominis* type 1 antigen is quite heat stabile even at

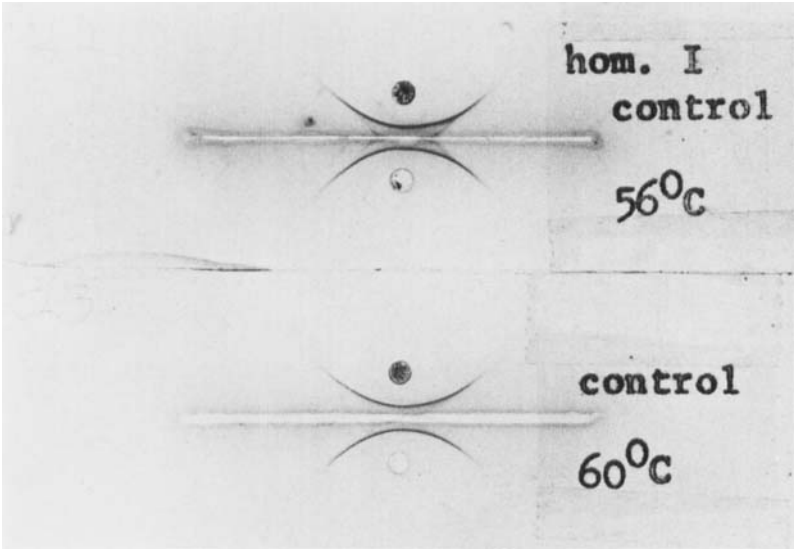

FIGURE 3. Immunoelectrophoresis of *M. hominis*, type 1, showing no loss of precipitin lines at 56° and 60°C.

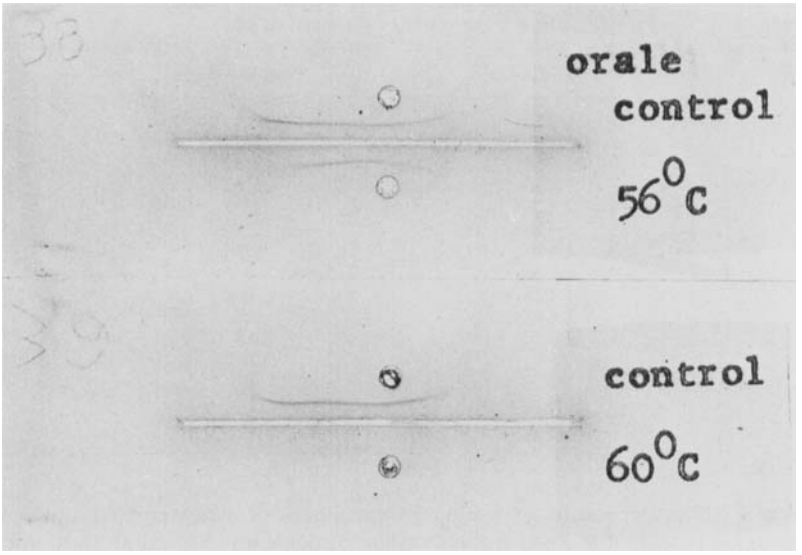

FIGURE 4. Immunoelectrophoresis of *M. orale*, type 1, showing partial loss of precipitin lines at 56° and complete loss at 60°C.

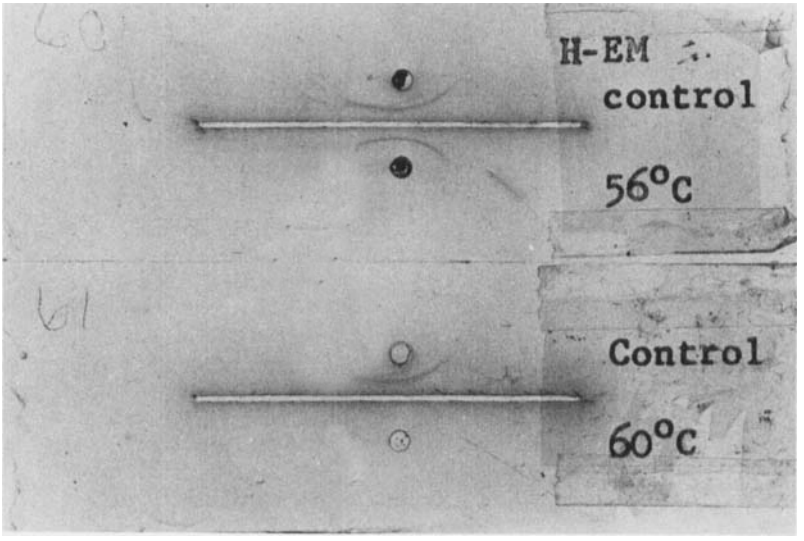

FIGURE 5. Immunoelectrophoresis of strain H-EM from rheumatoid arthritis, showing loss of one precipitin line at 56°C, and complete loss at 60°C.

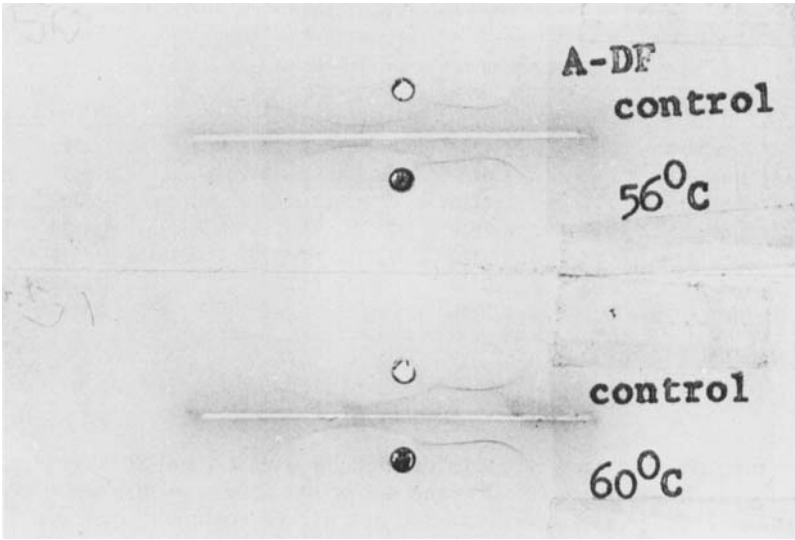

FIGURE 6. Immunoelectrophoresis of strain A-DF from Reiter's syndrome showing no loss of precipitin lines at 56° or 60° C.

TABLE 10

IMMUNOELECTROPHORESIS
EFFECT OF TEMPERATURE ON PRECIPITIN LINES

Strain	Number of Precipitin Lines			
	Untreated Antigen	50°C	55°C	60°C
M. hominis 1	1	1	1	1
M. hominis 2	5	5	5	5
M. salivarium	4	1	1	1
M. orale	2	1	1	0
GDL	3	2	1	0
A–JH*	4	4	3	1
H–EM*	2	2	1	0

* Rheumatoid arthritis.

60°C. Mycoplasma orale type 1 (FIGURE 4) shows partial loss of antigen at 56° and complete loss at 60°C. Strain H-EM (RA strain) shows loss of one precipitin line at 56° and both at 60°C (FIGURE 5). Strain A-DF (related to *M. hominis* type 2) is stabile at these temperatures, (FIGURE 6). TABLE 10 summarizes the results in the strains tested. *Mycoplasma hominis* types 1 and 2 showed no loss of precipitin lines with heating up to 60°C. The other strains tested all showed loss of precipitin lines including the GDL and two RA strains.

When the heat-treated complement-fixing antigen was tested against specific rabbit antisera, very little loss in titer occurred, except for strain H-EM where a three tube decrease was noted (TABLE 11).

TABLE 11

EFFECT OF TEMPERATURE UPON COMPLEMENT-FIXATION TITER

	Rabbit Antiserum Complement-fixation Titer		
Antigen	Untreated	56°C	60°C
M. hominis 1	1:1280	1:1280	1:1280
M. hominis 2	1:640	1:640	1:640
M. salivarium	1:1280	1:640	1:640
M. orale	1:2560	1:1280	1:1280
GDL	1:5120	1:5120	1:5120
A-JH*	1:2560	1:2560	1:1280
H-EM*	1:20480	1:5120	1:2560

* Rheumatoid arthritis.

Discussion

The mycoplasma strains isolated from patients with RA and SLE are closely related to each other antigenically, and are of the same type first reported by Butler and Leach.[11] This was discovered in a HEp-2 continuous cell line. The origin of this strain is unknown. Girardi *et al.*[2] have reported its isolation in several primary cell lines, including chick embryo and green monkey kidney. The only direct isolation of this strain is by Hayflick,[13] who reported isolation in a human bladder tumor. This strain has also been found in other cell cultures inoculated with material from solid tumor and leukemia.[14] As yet, there is no evidence that it is an animal strain. It is conceivable that this strain is of human origin, which usually requires cell culture passage before it will grow in cell-free medium.

Despite the fact that all control cell culture tubes and those inoculated with specimens from other "control diseases" remained free of mycoplasma, the possibility of contaminating mycoplasma exists.

However, the results of complement-fixation tests suggest that there is circulating antibody against these strains in cases of established RA. A nonspecific effect of rheumatoid sera or synovial fluid is unlikely, as the results using *M. hominis* type 1 antigen were quite different and agreed with the control series. There was no correlation between the complement-fixing titer and the presence of rheumatoid factor as measured by the latex fixation test. Patients with very early RA or those in complete remission had negative complement-fixation tests.

These preliminary studies indicate that certain antigens of mycoplasma are heat-labile. Such antigens have been described in certain viral species as psittacosis,[15] influenza,[16] and foot-and-mouth disease.[17] Further study of these characteristics is indicated, as it may affect the results of serologic testing.

The fact that rabbit antisera titers drop very little with the heated antigen indicates that, in this host, antibody is probably directed against all antigens including those which are heat-stabile. As rabbits do not develop overt disease to these strains, a direct correlation to antibody formed as a result of actual infection can not be made.

Initial studies of growth-inhibiting antibody in RA sera against these strains using metabolic inhibition methods have been negative.

The results of typing the two isolates from Reiter's syndrome are inconclusive. *Mycoplasma hominis* type 1 is the most common tissue culture contaminant.

Mycoplasma hominis type 2 has been identified in human material from the genitourinary tract, but there is evidence that it is related to *M. arthritidis* in rats.[18] It has not been a common cell culture contaminant.[19] It is unlikely that rat contamination occurred, as there was no contact with them at the time this isolate was discovered. Furthermore, this strain (A-DF) has been inoculated into 20 young white rats intraperitoneally and no disease occurred, nor could the strain be reisolated from the animals after 24 hours. Study of circulating antibody of this strain are in progress.

Summary and Conclusions

Mycoplasma strains from synovial fluid, serum, bone marrow aspirate, and kidney tissue of patients with RA and SLE have been antigenically characterized as related to the GDL strain of Butler and Leach. Two strains from synovial fluid of Reiter's syndrome are strains of *M. hominis* types 1 and 2.

Complement-fixing antibody has been demonstrated in 90% of patients with established RA against these strains isolated. The antigen responsible for this response appears to be heat-labile at 56°C. Heat-labile antigens occur in several human mycoplasma strains, as well as the GDL strain.

The GDL strain has now been identified by several laboratories as M. hyorhinis – isolated from swine and causing arthritis and polyserositis.

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