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1. Hildreth, E. A., Ann. Int. Med., 1963, v58, 883.
2. Farrar, W. E., Warner, A. R., Vivona, S., Military Med., 1964, v129, 960.
3. CDC Veterinary Public Health Notes, 1963, June.
4. Koprowski, H., Bull. W.H.O., 1954, v10, 709.
5. Fox, J. P., Koprowski, H., Conwell, D. P., Black, J., Gelfand, H. M., ibid., 1957, v17, 869.
6. Schwab, M. P., Fox, J. P., Conwell, D. P., Robinson, T. A., ibid., 1954, v10, 823.
7. Atanasiu, P., Bahmanyar, M., Baltazard, M., Fox, J. P., Habel, K., Kaplan, M., Kissling, R. E., Komorov, A., Koprowski, H., Lepine, P., Perez Gallarde, F., Schaeffer, M., ibid., 1956, v14, 593.
8. Koprowski, H., Black, J., J. Immunol., 1954, v72, 503.
9. ———, Proc. Soc. Exp. Biol. and Med., 1952, v80, 410.
10. Dean, D. J., Evans, W. M., Thompson, W. R., Am. J. Vet. Res., 1964, v25, 756.
11. Koprowski, H., Black, J., Johnson, W. P., J.A.V.M.A., 1955, v127, 363.
12. Carneiro, V., Black, J., Koprowski, H., ibid., 1955, v127, 366.
13. Wiktor, T. J., Fernandes, M. V., Koprowski, H., J. Immunol., 1964, v93, 353.
14. Peck, F. B., Powell, H. M., Culbertson, C. G., J. Lab. Clin. Med., 1955, v45, 679.
15. Laboratory Techniques in Rabies, Monograph Series No. 23, W.H.O., 1954.
16. Kissling, R. E., Reese, D. R., J. Immunol., 1963, v91, 362.
17. Fenje, P., Can. J. Microbiol., 1960, v6, 605.
18. Ott, G. L., Heyke, B., Vet. Med., 1962, v57, 158.
19. Abelseth, M. K., Can. Vet. J., 1964, v5, 84.
20. ———, ibid., 1964, v5, 279.
21. Hayflick, L., Am. Rev. Resp. Dis., 1963, v88, 387.
22. Bernhard, W., Tournier, P., Ann. Inst. Pasteur, 1964, v107, 447.
23. Hayflick, L., Indust. Med. & Surg., 1965, in press.
24. Svedmyr, A., Proc. Symp. Charact. and Uses of Human Diploid Cell Strains, Opatija, 1963, 235.

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Significance of Antibody to *Mycoplasma hominis* Type 1 as Measured by Indirect Hemagglutination. (30049)

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Mycoplasma pneumoniae has been established as the etiologic agent of naturally occurring cold agglutinin-positive primary atypical pneumonia, and this mycoplasma may also cause febrile upper respiratory illness(1). In addition, experimentally infected volunteers have been observed to develop bullous myringitis(2). Although *M. pneumoniae* is the only mycoplasma species that has been conclusively shown to be a human pathogen, recent evidence suggests that another species may be capable of causing human disease. In this laboratory, *M. hominis* type 1 has been isolated more frequently from persons with respiratory illness than from healthy persons, and we have demonstrated that *M. hominis* type 1 is capable of producing exudative pharyngitis in experimentally infected volunteers(3). The detection of antibody and measurement of antibody rises in these volunteers was accomplished only by means of the indirect hemagglutination (IHA) technique, due to its greater sensitivity in comparison with other serological methods. Information concerning the sensitivity and specificity of the IHA technique and the speed of development, persistence and protective effect of IHA anti-

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body in the volunteers inoculated with *M. hominis* type 1 is presented here. In addition, information is presented concerning the prevalence of antibody in healthy persons of different ages in the general population.

Materials and methods. Media. The PPLO agar and broth media used for isolation, propagation and maintenance of mycoplasma organisms have been described in detail(4).

Volunteer inoculum and inoculation procedure. Isolation of the DC63 oral strain of *M. hominis* type 1 and preparation of the volunteer inoculum has been described(3). Each volunteer received 10^7 to 10^8 colony-forming units of strain DC63 in its second broth passage; one ml of this material was sprayed into the oropharynx and one ml pipetted into the nose.

Volunteers at National Institutes of Health (NIH) Clinical Center and at Lorton Reformatory. The facilities for conducting volunteer studies at the NIH Clinical Center and at the District of Columbia Department of Corrections, Lorton, Va., have been described(2,3,5). At the Clinical Center, male volunteers, 21 to 40 years of age, were selected who were free of detectable complement-fixing and fluorescent-stainable antibody. This was before the IHA technique had been developed. The volunteers were isolated 3 in a room for a period of 14 to 26 days after inoculation. At Lorton Reformatory, sera from male volunteers, 20 to 62 years of age, were pretested for antibody to *M. hominis* type 1 (strain DC63) by the IHA technique and volunteers selected on the basis of their antibody level. The volunteers were isolated in a dormitory for a period of 18 days following inoculation.

Collection of specimens. Pharyngeal cultures for mycoplasma isolation were obtained before inoculation with mycoplasma strain DC63, and at 2-day intervals thereafter throughout the period of observation, as described previously(3). Cultures were also obtained from some volunteers 16 weeks after inoculation, *i.e.*, after the period of isolation and clinical observation.

Acute phase serum was obtained from volunteers on the day of, or one to two days before inoculation with strain DC63. In all cases, convalescent sera were taken 18 days and 4 and 5 weeks later. In some instances, serum was obtained 5 and 10 days and 16 weeks after inoculation.

The study of IHA antibody in persons of different ages was carried out with sera obtained between 1959 and 1964 from children and adults without respiratory disease. The children, 3 months to 17 years old, were either hospitalized or attending clinics at Children's Hospital, Washington, D. C. The majority of sera from adults were from patients aged 20 to 60 years and over, with non-respiratory illnesses at D. C. General Hospital, Washington, D. C. These patients were subjects who served as "controls" in a study of pneumonia at this hospital (Mufson, unpublished). Sera were also obtained from young adults on the secretarial staff of the Laboratory of Infectious Diseases. All sera were stored at —20°C.

Mycoplasma isolation and identification procedures. These have been described in detail(3). Briefly, PPLO agar plates, inoculated with pharyngeal swabs, were incubated at 37°C in an aerobic atmosphere, although a few plates were also incubated in a mixture of 5% carbon dioxide and 95% nitrogen. Colonies from the second passage on agar were used to inoculate PPLO broth and these cultures were then used for subsequent identification of the mycoplasma organisms by the growth inhibition technique(6). Hyperimmune rabbit serum, impregnated in a filter disc placed on the surface of a PPLO agar plate, inhibited the growth of the homologous mycoplasma strain in a zone surrounding the disc.

Hyperimmune rabbit sera. Rabbit antisera to *M. hominis* type 1 (strain DC63 and prototype strain PG21) and the other prototype mycoplasma strains were prepared as described previously(4). Briefly, all mycoplasma organisms, except *M. pneumoniae,* were grown in rabbit infusion broth supplemented with rabbit serum and 20-fold concentrates of this material were used to inoculate rabbits; *M. pneumoniae* was grown in chick embryo lung and the homogenized suspension of lung was used to inoculate rabbits.

Complement-fixation (CF) and immunofluorescence techniques. The preparation of

CF antigens and the technique employed have been reported(3,4,7). Eight units of antigen were used and the tests were performed by overnight fixation at 4°C in micro CF plates. Horse anti-human gamma globulin, conjugated with fluorescein isothiocyanate, was used in the indirect fluorescent antibody procedure(8).

Indirect hemagglutination (IHA) technique. The method employed was that described previously(7) but modified by the use of sensitized sheep erythrocytes stored at —70°C. Sonically disrupted mycoplasma organisms were adsorbed onto tanned sheep erythrocytes; sera were then tested for their capacity to agglutinate a 1% suspension of erythrocytes. Tests were performed in disposable plastic microtitration plates (Cooke Engineering Co., Alexandria, Va.) which were incubated at 34°C for 2 hours. Seventy-five per cent agglutination of sensitized erythrocytes was considered the end point for determination of serum titer, and not 50% agglutination as described previously(7). To obtain reproducible IHA tests, a large quantity of sensitized sheep erythrocytes was preserved by a modification of the method described by Hubert *et al*(9). A suspension of one per cent sensitized tanned erythrocytes was centrifuged and resuspended in heat-inactivated normal rabbit serum, diluted 1:200 in buffered saline (pH 7.2), to produce a 4% erythrocyte suspension. These cells were then mixed with an equal volume of dextrose-lactose solution; the final concentrations of dextrose and lactose in this solution were 5% and 7.5%, respectively. Aliquots of the erythrocyte-sugar mixture were sealed in glass ampoules, shell-frozen in dry ice and alcohol and stored at —70°C. When required, the contents of an ampoule were thawed rapidly by agitation in a water-bath at 37°C and the cells centrifuged at 1500 rpm for 10 minutes. The amount of hemolysis in the supernatant fluid was determined using a Bausch and Lomb "Spectronic 20" colorimeter (590μ). The erythrocytes were then washed once in 1:200 normal rabbit serum and resuspended in the same solution to yield at 1% concentration of sensitized sheep erythrocytes.

Results. Indirect hemagglutination (IHA) technique. In a preliminary experiment, erythrocytes from different sheep were examined. Erythrocytes from 13 sheep were tanned and sensitized with various dilutions of *M. hominis* type 1 (strain DC63) antigen and then tested against an homologous rabbit antiserum. The results in Table I show that erythrocytes from 7 different sheep, sensitized with a 1/64 dilution of antigen, varied in their ability to react with the homologous rabbit antiserum. Sensitized cells from several sheep reacted to high titer; however, sheep #48 cells were found to be the most sensitive and were used in subsequent IHA tests.

TABLE I. Effect of Different Sheep Erythrocytes on IHA Test with *M. Hominis* Type 1 (Strain DC63).

Erythrocytes* from sheep No.	% agglutination in antigen control	Reciprocal of antibody titer with rabbit antiserum
37	100	?†
54	75	?
42	50	?
44	25	320
75	—	160
70	—	5120
48	—	10240

* Erythrocytes sensitized with a 1/64 dilution of antigen.

† No end point because of agglutination in antigen control.

Even with the use of erthrocytes from the same sheep it was difficult to maintain reproducibility in consecutive IHA tests. Furthermore, the process of tanning and sensitizing erythrocytes for individual tests was laborious and introduced an additional variable. Therefore, in order to make the technique less tedious and increase reproducibility, a large volume of erythrocytes, sensitized with mycoplasma strain DC63, was shell-frozen, stored in aliquots at —70°C and used when required over a period of several months. As shown in Table II, freezing the 4% sensitized erythrocytes and thawing 4 days later resulted in 22% hemolysis. The amount of hemolysis increased a further 2-fold over a storage period of 3 months but was not associated with a decrease in sensitivity of the test. Further, the titer of a standard human serum did not alter more than 2-fold over the 3 months, so that the test was reproducible during this period.

TABLE II. Reproducibility of IHA Test Over a 3 Month Period.

Time of testing DC63-sentitized sheep erythorcytes after freezing	% hemolysis after thawing	Reciprocal of antibody titer with standard convalescent human serum (Pinckney)
Pre-freezing	—	160
4 days	22	160
5 wk	32	320
9 "	39	320
10 "	35	320
11 "	40	320
12 "	40	320

The IHA titers of sera were the same whether the sera and sensitized erythrocytes were incubated at 4°C, 23°C, 34°C or 39°C, except that at 39°C the titers of some sera were lower than those obtained at the other incubation temperatures. A temperature of 34°C was arbitrarily chosen for performance of the test. After partial, or complete settling of erythrocytes which required about 2 hours, agitation and reincubation did not alter the serum titers. It was not necessary to read tests immediately after settling of the cells since the patterns did not change over a period of at least one week if the plates were placed at 4°C. After this time, lysis of erythrocytes occurred.

Relationship of DC63 and PG21 (prototype) strains of M. hominis *type 1 by IHA.* The close relationship between the DC63 oral strain and the prototype PG21 strain of *M. hominis* type 1 was demonstrated previously with hyperimmune rabbit sera by both CF (4) and growth inhibition(3) techniques. In the current study, the 2 mycoplasma strains were found to be closely related when tested by IHA using specific hyperimmune rabbit sera (Table III). However, previous gel-diffusion studies with rabbit antisera indicated that the DC63 and PG21 strains of *M. hominis* type 1 were closely related although not identical since the PG21 strain was deficient in an antigenic component which was prominent in the DC63 strain(4). The relationship of the 2 strains was further investigated in IHA tests with paired sera from 18 volunteers infected with the DC63 strain. All 18 volunteers developed a 4-fold or greater rise in IHA antibody to strain DC63 within 3 to 4 weeks following inoculation, whereas only 9 of the volunteers developed a similar rise in IHA antibody to strain PG21 (see Table VI). The titer of the standard DC63 convalescent human serum (Pinckney) was 8-fold lower in the test with PG21-sensitized erythrocytes than in the test with the DC63-sensitized cells. However, it seems unlikely that the PG21-sensitized cells were deficient in antigen since the titers of 3 of the DC63 volunteer convalescent sera were the same whether they were tested with PG21- or DC63-sensitized cells, and the PG21-sensitized erythrocytes reacted to high titer with both PG21 and DC63 rabbit antisera (Table III). The failure to detect as many antibody rises with the PG21 antigen is another reflection of a minor antigenic difference between mycoplasma strains PG21 and DC63. This difference is more readily demonstrable with sera from infected volunteers than with sera from hyperimmunized rabbits which develop broadly reactive antibodies.

Antibody to M. hominis *type 1 (strain DC63) in sera from persons of different ages without respiratory illness as measured by the IHA and CF techniques.* Two hundred and fifty-six sera from persons of different ages, ranging from 3 months to 60 years and

TABLE III. Relationship Between DC63 and PG21 (Prototype) Strains of *M. Hominis* Type 1 in IHA Tests with Rabbit Antisera.

Rabbit serum prepared against	Reciprocal of serum titer with sheep erythrocytes sensitized with indicated antigen						
	M. hominis Type 1		*M. hominis*	*M.*	*M.*	*M.*	*M.*
	Strain DC63	Strain PG21	Type 2	*salivarium*	*fermentans*	*orale*	*pneumoniae*
M. hominis Type 1							
Strain DC63	10240	1280	80	<10	<10	40	ND*
" PG21	5120	10240	40	160	80	640	80

* Not done.

TABLE IV. A Comparison of IHA and CF Tests for Determination of Antibody to *M. Hominis* Type 1 in Sera from Children and Adults Without Respiratory Illness.

CF antibody titer (reciprocal)	No. of persons with IHA antibody titer (reciprocal) of								
	<10	10	20	40	80	160	320	640 or>	Totals
<4	107	11	18	33	27	17	16	10	239
4	1	1	2	1	1		1		7
8	1	1		1			1		4
16					2	1			3
Totals	109	13	20	35	30	18	18	10	253

TABLE V. Protective Effect of IHA Antibody Against Experimentally Induced *M. Hominis* Type 1 Illness.

Reciprocal of pre-challenge IHA titer	No. volunteers in group	No. with 4-fold or greater rise	No. volunteers with following signs or symptoms				
			Exudative pharyngitis	Pharyngeal erythema or tonsillar enlargement	Cervical adenopathy	Total	Sore throat
<5	20	19	13	3	11	16	7
5–40	17	14	6	0	3	6	2
80 or >	13	3	2	1	1	3	0

cal adenopathy occurred significantly more frequently in volunteers free of pre-inoculation antibody than in volunteers with antibody (pharyngitis: $X^2 = 8.4$, 2 d.f., $p = 0.02$; cervical adenopathy: $X^2 = 10.3$, 2 d.f., $p < 0.01$). Tonsillar enlargement occurred too infrequently for statistical analysis but an analysis of the summation of signs at each antibody level indicated that they occurred significantly more frequently in the volunteers without pre-inoculation antibody ($X^2 = 12.4$, 2 d.f., $p < 0.01$). In addition, sore throat occurred significantly more frequently in volunteers without IHA antibody ($X^2 = 7.2$, 2 d.f., $p = 0.03$). Therefore, there is evidence of a protective effect of IHA antibody, although this antibody does not afford complete protection since 2 of 13 volunteers with pre-inoculation IHA antibody levels of 80 or greater developed pharyngitis.

Specificity of IHA antibody response in volunteers infected with mycoplasma strain DC63. Eight paired sera were obtained from volunteers in the NIH Clinical Center before and 18 days or 4 weeks after inoculation with the DC63 strain of *M. hominis* type 1. Each of these paired sera showed a 4-fold or greater rise in IHA antibody to strain DC63 but no rises were detected in CF and immunofluorescence tests. When these sera were tested against sheep erythrocytes sensitized with *M. penumoniae* antigen, no antibody rises were detected except in one instance where there was a 16-fold rise. Experiments with *M. pneumoniae* were taking place concurrently in other isolated volunteers but this organism was not isolated from the volunteer inoculated with strain DC63. It is possible that the antibody rise was heterotypic since an antigenic relationship is known to exist between the human mycoplasma species(4). This problem was investigated more fully with paired sera obtained before and 18 days to 4 weeks after inoculation of 18 volunteers at Lorton Reformatory with mycoplasma strain DC63. Each volunteer had a 4-fold or greater homologous antibody rise in IHA tests but no rise in CF tests. The paired sera were tested against sheep erythrocytes sensitized with antigens prepared from *M. hominis* type 1 (prototype strain, PG21), *M. hominis* type 2, *M. salivarium, M. fermentans, M. orale* and *M. pneumoniae.* The results are shown in Table VI. Antibody rises to the PG21 strain of *M. hominis* type 1 occurred in 9 instances. As indicated previously, the failure to demonstrate antibody rises with all of the paired sera probably reflects an antigenic difference between the PG21 and DC63 strains of *M. hominis* type 1 rather than an inability of the PG21-sensitized erythrocytes to react

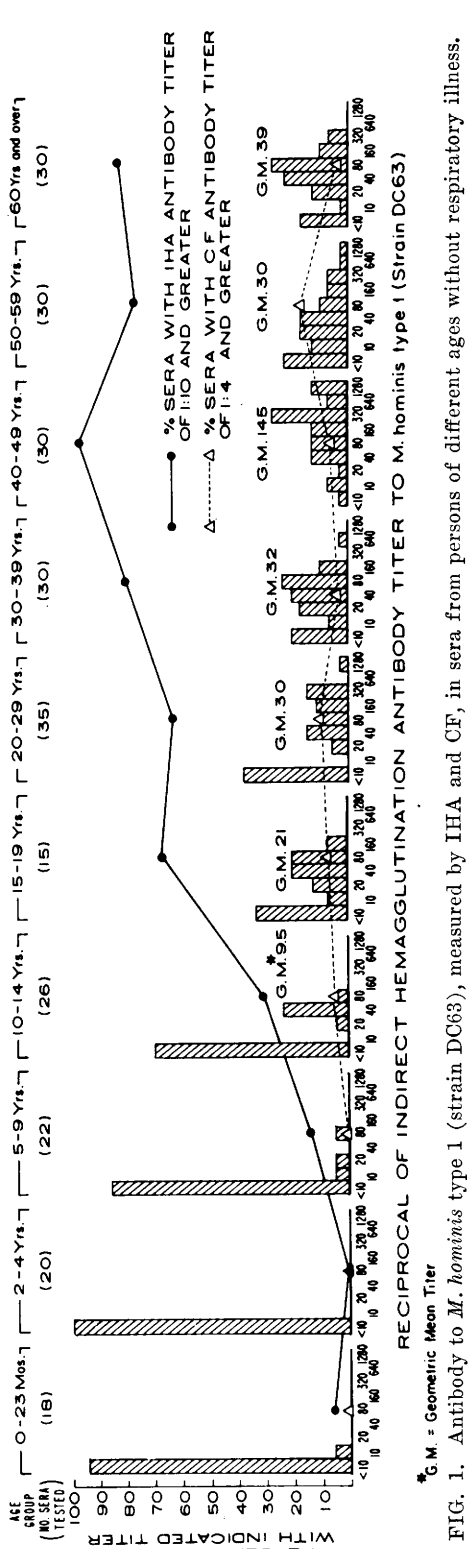

FIG. 1. Antibody to *M. hominis* type 1 (strain DC63), measured by IHA and CF, in sera from persons of different ages without respiratory illness.

over, were tested for antibody to mycoplasma strain DC63 by IHA and CF. In the IHA test, 145 of the sera (57%) were found to contain antibody at a titer of 1:10 or greater. Only one infant, 3 months old, of 18 infants below 2 years of age, had a detectable amount of antibody; this antibody was presumably maternal in origin. After the age of 5 years, an increasing proportion of sera contained IHA antibody and the geometric mean (G.M.) titer increased with age (Fig. 1). Thus, in the age group 15-19 years, 67% had antibody (G.M. 1:21) and in the age group 40-49 years, 97% possessed antibody (G.M. 1:145). In the CF test, only 14 (5%) of 253 sera (3 were anticomplementary) contained CF antibody at a titer of 1:4 or greater (Table IV). These results demonstrate the much greater sensitivity of the IHA technique compared to the CF technique for measurement of antibody to *M. hominis* type 1. Although statistical analysis of the data in Table IV showed that there was some association between the presence of antibody as measured by IHA and as measured by CF ($X^2 = 3.85$; $p = 0.05$), there was no correlation between the absolute titers of sera as determined by IHA and as determined by CF (Spearman rank correlation, 2-sided, $r = 0.341$; $p = 0.28$). Thus, there is certainly no more than a suggestion that the IHA and CF tests measure the same type of antibody.

One hundred and fourteen of 172 sera (66%), from volunteers aged 20 years and older, at Lorton Reformatory, contained IHA antibody at a titer of 1:10 or greater. This finding was consistent with results obtained with sera collected from persons of different ages in Washington.

Protective effect of IHA antibody against experimentally induced M. hominis *type 1 illness.* Fifteen volunteers at the NIH Clinical Center and 35 volunteers at Lorton Reformatory were inoculated *via* the nasopharynx with mycoplasma strain DC63. Table V is a summary of the data from these studies, the clinical features of which have been presented in detail(3). The occurrence of exudative pharyngitis, cervical adenopathy and sore throat was found to be inversely related to the level of pre-inoculation IHA antibody. Thus, both pharyngitis and cervi-

TABLE VI. IHA Antibody Response of Lorton Volunteers to Prototype Mycoplasma Strains After Inoculation of the DC63 Strain of *M. Hominis* Type 1.

	Reciprocal of paired serum titers with sheep erythrocytes sensitized with indicated antigen															
Volunteer No.	*M. hominis* Type 1 Strain DC63		*M. hominis* Type 1 Strain PG21		*M. hominis* Type 2		*M. salivarium*		*M. fermantans*		*M. orale*		*M. pneumoniae*	
131636	[<5	160]*	<10	<10	<10	10	10	10	<10	<10	<10	<10	40	40
125045	[<5	80]	10	10	10	10	20	10	<10	<10	80	80	20	20
120869	[<5	80]	[<10	20]	<10	<10	<10	<10	<10	<10	<10	<10	10	10
85670	[10	160]	[10	80]	10	10	40	40	<10	<10	20	10	10	10
137458	[<5	320]	320	640	<10	10	>1280	>1280	<10	<10	1280	1280	10	20
113312	[<5	160]	[<10	160]	<10	<10	20	20	<10	<10	<10	10	10	10
128736	[<5	80]	<10	<10	<10	10	10	<10	<10	<10	20	10	20	20
133607	[20	320]	[10	80]	10	10	<10	<10	<10	<10	10	10	10	20
96868	[20	160]	<10	10	40	40	20	10	<10	<10	40	20	80	40
116168	[5	320]	[<10	20]	<10	<10	<10	<10	10	10	10	10	80	160
111298	[<5	160]	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	20	20
136226	[<5	320]	[<10	40]	10	10	80	40	<10	<10	20	20	40	40
46573	[<5	80]	[10	80]	<10	10	20	20	<10	<10	10	20	<10	10 or <
94664	[5	320]	[10	40]	40	40	40	40	20	40	[160	640]	[80	1280]
133305	[<5	80]	40	40	20	40	160	160	<10	<10	160	80	<10	<10
94042	[<5	160]	<10	10	<10	<10	40	40	<10	<10	20	20	160	160
93889	[<5	40]	<10	<10	10	10	20	20	<10	<10	20	20	10	20
132103	[80	320]	[80	320]	<10	<10	40	40	20	40	40	40	80	160
Standard serum	320†		40†		5120‡		20480‡		160‡		5120‡		640§	

* Four-fold or greater rise in antibody indicated by brackets.

† Antibody titers with *M. hominis* Type 1 (Strain DC63) convalescent human serum (Pinckney).

‡ Antibody titers with homologous rabbit antiserum.

§ Antibody titer with *M. pneumoniae* convalescent human serum (McMillen).

TABLE VII. *M. Hominis* Type 1 (Strain DC63) IHA Antibody Titers in Serial Sera of Volunteers and Isolation of Strain DC63 from Volunteers Inoculated with this Mycoplasma at Lorton Reformatory.

Volunteer No.	Before inoculation	Reciprocal of DC63 IHA antibody titer in sera obtained: At indicated time after inoculation: 5 days	10 days	18 days	4 wks	5 wks	16 wks
46573	< 5	<5(+)*	160(+)	160	80	80	40†
133305	< 5	<5(+)	40(+)	40(+)	40	40(+)	160
94042	< 5	<5(+)	160(+)	80(+)	80(+)	160(+)	160
82777	80	80(+)	320(+)	640(+)	320	160(+)	320
131636	< 5	ND(+)	ND(+)	40(+)	40	40(+)	80
125045	<10	ND(+)	ND(+)	160(+)	160	80	80
85670	10	ND(+)	ND	320(+)	160(+)	160(+)	80†
96868	20	ND(+)	ND(+)	320(+)	320(+)	160	160(+)
116168	< 5	ND(+)	ND	160(+)	160(+)	160	40†
136226	< 5	ND(+)	ND(+)	160	160	160	80
94664	< 5	ND(+)	ND(+)	320(+)	160(+)	160(+)	160
93889	< 5	ND	ND	20	10	10	20

* (+) = isolation of strain DC63; strain not isolated where not indicated.
† Four-fold fall in serum titer compared with maximum titer.
ND = not bled at time indicated.

with antibody due to a failure of PG21 antigen to coat the erythrocyte surfaces. In marked contrast, tests with other mycoplasma antigens demonstrated heterotypic IHA antibody rises with only one of the 18 paired sera; a 4-fold rise to *M. orale* and a 16-fold rise to *M. pneumoniae.*

These results indicate the specificity of the IHA antibody response to mycoplasma strain DC63. It seems likely that the occurrence of IHA antibody to other mycoplasma strains is generally not due to a heterotypic response to infection with *M. hominis* type 1.

Speed of IHA antibody response and persistence of antibody in volunteers infected with mycoplasma strain DC63. Serial serum specimens were obtained from 12 volunteers at Lorton Reformatory (Table VII). All these volunteers had a 4-fold or greater rise in antibody to strain DC63. Sera were obtained from 4 volunteers 5 and 10 days after inoculation; an antibody rise was not detected with the sera taken at 5 days but a 4-fold or greater rise was demonstrated with all the sera obtained at 10 days. In 2 instances, the titer attained at this time was not subsequently exceeded and only one of the 12 volunteers had a serum titer at 18 days which was subsequently exceeded.

At 5 weeks after inoculation a 4-fold fall in antibody titer compared with the maximum IHA antibody titer attained occurred in only one instance. A 4-fold fall in titer 16 weeks after inoculation occurred in only 3 instances. In 8 volunteers the antibody titer remained the same or there was a 2-fold fall or rise. In one instance, there was a 4-fold antibody rise at 16 weeks. The persistence of IHA antibody could not definitely be associated with persistent excretion of mycoplasma strain DC63 from the throat. The mycoplasma was isolated from volunteer #125045 for not more than 4 weeks after inoculation and from volunteer #136226 for no longer than 14 days after inoculation and yet the IHA antibody titers were maintained for at least 16 weeks. It was noteworthy, however, that the mycoplasma was not isolated at 16 weeks from the 3 volunteers who had a 4-fold antibody fall at this time.

Discussion. Several serological techniques have been described for the measurement of antibody to *M. hominis* type 1 in human sera. Each of these techniques may measure a different type of antibody and not all are equally sensitive indicators of the presence of antibody. Gel diffusion and growth inhibition are insensitive for measuring antibody in human sera. The immunofluorescence technique, likewise, is rather insensitive and also laborious. The method most widely used has been complement fixation. Stokes(10) demonstrated antibody rises to *M. hominis* type 1 in 2 patients with puerperal sepsis, one with

pyosalpingitis and one with postoperative empyema, but no antibody in 171 "control" sera obtained from a variety of sources. Melén and Gotthardson(11) found CF antibody in the sera of 28% of gynaecological patients but in only 9% of "normal" persons; the mycoplasma strain used by these workers was presumably *M. hominis* type 1. Card(12) found the highest incidence of antibodies (34%) in patients attending venereal disease clinics and the lowest incidence (2%) in children and blood donors. Oates *et al* (13), on the other hand, found a higher incidence (14%) in male blood donors. Lemcke and Csonka(14) were able to demonstrate that 51% of persons with salpingitis had CF antibody for *M. hominis* type 1 whereas only 4% of control subjects had such antibody.

Indirect hemagglutination for the mycoplasma group of organisms has been described by Tully(15), and by Dowdle and Robinson(16) who demonstrated antibody rises to *M. pneumoniae* in persons infected with this organism. We have used this technique for measurement of antibody to all the prototype human mycoplasma strains(7). A major factor in maintaining reproducibility of the test has been the ability to store a large amount of sensitized sheep erythrocytes in small aliquots in the frozen state, permitting such aliquots of material to be thawed and used as desired. This procedure not only ensures reproducibility from one test to another, so that results may be compared, but also eliminates the laborious procedure of tanning and sensitizing erythrocytes immediately prior to each test.

The conclusion that the IHA technique is more sensitive than the CF technique is based upon several observations. First, in IHA tests, 57% of sera from persons without respiratory illness contained antibody whereas only 5% of the same sera contained antibody detectable by CF; this low incidence of CF antibody is in general agreement with the findings of others, cited previously, who have studied sera from "normal" persons. Second, various levels of antibody were detected by IHA in 30 of 50 volunteers prior to inoculation with *M. hominis* type 1, but antibody was not detected by CF, nor by immunofluorescence. Thirty-six of the volunteers developed a 4-fold or greater rise in antibody to *M. hominis* type 1 as measured by IHA but no rises were observed by CF or by immunofluorescence, again indicating that the IHA test was the most sensitive of the serological methods used. There was only a suggestion that the IHA and CF techniques measure the same antibody, the data being insufficient to establish a definite conclusion; for this, it would be necessary to examine many more sera by both techniques in order to obtain, in particular, sera that were positive in the CF test.

Examination of serial sera obtained from some of the volunteers showed that they developed high IHA antibody titers within 10 days of inoculation of mycoplasma strain DC63 and, in general, this antibody persisted for at least 16 weeks after inoculation. It is interesting that in many instances mycoplasma excretion continued for several weeks even in the presence of high levels of antibody. However, the data were insufficient to attribute the persistence of antibody to continued mycoplasma excretion.

The specificity of the IHA antibody response developed by the volunteers was indicated by the fact that heterotypic responses to sheep erythrocytes sensitized with antigens from the other human mycoplasmas were rare. This specificity of response is supported by studies with hyperimmune rabbit sera in which the IHA test has proved to be more specific than the CF test(7).

The occurrence of pharyngitis, cervical adenopathy and sore throat in volunteers inoculated with mycoplasma strain DC63 was found to be inversely related to the level of pre-inoculation IHA antibody, which indicates the protective effect of antibody measured by the IHA technique. In several myxovirus and rhinovirus diseases in which respiratory mucosal epithelium is involved, a high level of serum neutralizing antibody does not always afford complete protection (17, 5, 18); in part, this may be due to the superficial, rather than the systemic nature of the infections. Similarly, IHA antibody does not afford complete protection since 2 volunteers with high pre-inoculation antibody titers developed pharyngitis. Another pos-

sible explanation for this incomplete protection is that the IHA test presumably measures not only protective antibody but also antibodies to other components of the mycoplasma organism since sheep erythrocytes are coated with antigens from sonically disrupted mycoplasma organisms. On the other hand, it is possible that tanned sheep erythrocytes selectively adsorb certain antigens onto their surfaces; this would account for the specificity of the IHA technique in comparison with CF in which the antigen is likely to contain all the antigenic components of the mycoplasma. It may be possible to increase further the specificity of the IHA technique by coating tanned erythrocytes with purified mycoplasma antigens.

In experimental infections with *M. hominis* type 1, the IHA technique has been invaluable, since only by this method has it been possible to select antibody-free susceptible volunteers and measure antibody responses of those infected. Because of its sensitivity and specificity, it seems clear that the IHA technique may be a valuable tool in studying, not only experimental infections by other human mycoplasma species, but also natural mycoplasma infections.

Summary. Tanned sheep erythrocytes, sensitized with *M. hominis* type 1 antigen, were stored in aliquots at —70°C and used in IHA tests over a period of 3 months. This procedure maintained reproducibility. Fifty-seven per cent of sera from healthy persons of all ages contained antibody when tested by IHA but only 5% contained antibody when tested by CF, indicating the greater sensitivity of the IHA technique. There was an inverse relationship between the presence of IHA antibody in the sera of volunteers before inoculation with the DC63 strain of *M. hominis* type 1 and the occurrence of illness, indicating the protective effect of this antibody. Most volunteers without antibody prior to inoculation developed an illness and a 4-fold or greater rise in antibody as measured by IHA; no rises were demonstrated by CF. Tests on serial sera obtained from some volunteers indicated that 4-fold or greater antibody rises developed 10 days after inoculation and in some instances the antibody persisted for at least 16 weeks. The greater sensitivity of the IHA technique was not accompanied by a loss of specificity, since only one of 18 volunteers with a 4-fold or greater rise to the DC63 strain developed a 4-fold rise to another mycoplasma species. Only 9 of these volunteers developed a 4-fold rise to the PG21 prototype strain of *M. hominis* type 1 which was probably due to a minor antigenic difference between the DC63 and PG21 strains. The IHA technique may be valuable in studying, not only experimental infections by other mycoplasma species, but also natural mycoplasma infections.

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1. Chanock, R. M., Mufson, M. A., Somerson, N. L., Couch, R. B., Am. Rev. Resp. Dis., 1963, v88, 218.

2. Rifkind, D., Chanock, R. M., Kravetz, H., Johnson, K. M., Knight, V., ibid., 1962, v85, 479.

3. Mufson, M. A., Ludwig, W. M., Purcell, R. H., Cate, T. R., Taylor-Robinson, D., Chanock, R. M., J. Am. Med. Assn., 1965, in press.

4. Taylor-Robinson, D., Somerson, N. L., Turner, H. C., Chanock, R. M., J. Bact., 1963, v85, 1261.

5. Mufson, M. A., Ludwig, W. M., James, H. D., Jr., Gauld, L. W., Rourke, J. A., Holper, J. C., Chanock, R. M., J. Am. Med. Assn., 1963, v186, 578.

6. Clyde, W., Jr., J. Immunol., 1964, v92, 958.

7. Taylor-Robinson, D., Canchola, J., Fox, H. H., Chanock, R. M., Am. J. Hyg., 1964, v80, 135.

8. Chanock, R. M., Mufson, M. A., Bloom, H. H., James, W. D., Fox, H. H., Kingston, J. R., J. Am. Med. Assn., 1961, v175, 213.

9. Hubert, E. G., Kalmanson, G. M., Guze, L. B., J. Bact., 1963, v86, 569.

10. Stokes, E. J., Lancet, 1955, v1, 276.

11. Melén, B., Gotthardson, A., Acta Path. Microbiol. Scand., 1955, v37, 196.

12. Card, D. H., Brit. J. Vener. Dis., 1959, v35, 27.

13. Oates, J. K., Whittington, M. J., Wilkinson, A. E., ibid., 1959, v35, 184.

14. Lemcke, R., Csonka, G. W., ibid., 1962, v38, 212.

15. Tully, J. G., Proc. Soc. Exp. Biol. and Med., 1963, v114, 704.

16. Dowdle, W. R., Robinson, R. Q., ibid., 1964, v116, 947.

17. Chanock, R. M., Bell, J. A., Parrott, R. H., Perspectives in Virology, M. Pollard, ed., Burgess Publishing Co., Minneapolis, 1961, v2, 126.

18. Taylor-Robinson, D., Bynoe, M. L., Brit. Med. J., 1964, v1, 540.

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Solubilization by Butanol of Rabbit Cell-Antigens Associated with the Rejection of Transferred Lymph Node Cells. (30050)

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When rabbit lymph node cells are incubated *in vitro* with Shigella O antigen, washed and transferred to X-irradiated rabbits, agglutinins to Shigella appear subsequently in the sera of the recipient animals(1). Production of the antibody by the transferred cells can be prevented by prior injection of the prospective recipients with pooled rabbit leucocytes(2), or by incubation of the lymph node cells with rabbit anti-rabbit-leucocyte serum(3). Among the data indicating that the substance in the rabbit anti-rabbit-leucocyte serum causing suppression of transferred lymph node cells is an antibody was the observation that the suppressive antibody could be absorbed out of such sera by suspensions of whole lymph node cells. In further work, directed at purification and characterization of this antibody, it was found that it could also be absorbed from such suppressive sera by fragments of lymph node cells(4), prepared by differential centrifugation of suspensions of disrupted cells or of wash fluids of lymph node cells(5), and that from the cell-fragment-antibody complexes thus obtained the antibody could be eluted(4).

The availability of a specifically purified antibody which causes rejection of allogeneic transferred cells, and of a cell-fragment bearing the rejection-associated antigen, suggested studies on the solubilization of this antigen, in which the suppressive antibody would be used for detection of solubilized antigen. The extraction of such antigens by the use of Triton and snake venom phospholipase, based on a method described by Kandutsch and Stimpfling(6) for solubilization of mouse sarcoma antigens, has been reported elsewhere(7). A report is given here of the solubilization by butanol of rejection-associated antigens from cell fragments of normal rabbit liver or from Triton extracts of fragments of normal rabbit lymph node cells.

Materials and methods. Cells were prepared from lymph nodes and spleens of rabbits, and cell fragments were prepared from suspensions of these cells, as described elsewhere(5). For transfer of lymph node cells, cells prepared from popliteal and axillary nodes of 8 to 10 rabbits were washed, incubated at 37°C for 30 minutes with a Shigella-trypsin filtrate containing approximately 10^{-4} mg of solute per ml, washed again, and finally transferred in units of 2×10^8 to irradiated recipients, as described elsewhere (1). For preparation of rabbit anti-rabbit-leucocyte serum, rabbits were given 2 injections, at 5-week intervals, of 1.5×10^8 washed blood leucocytes pooled from 40 to 50 rabbits. The rabbits were bled 3 times between the 7th and 12th day after the injection, and the sera pooled.

Results and discussion. 1. *Spontaneous release of soluble antigen from normal rabbit liver cell fragments.* In an earlier study(4) cell fragments obtained by differential centrifugation of sonically disrupted lymph node cells had been found to react serologically with suppressive rabbit anti-rabbit-leucocyte sera and to absorb from such sera their effect of suppressing antibody formation by transferred lymph node cells. In the present study cell fragments of approximately similar centrifugal properties were obtained from rabbit liver cells as follows: Livers of normal rabbits