Recovery, Identification, and Neurotoxicity of Sabin's Type A and C Mouse Mycoplasma (PPLO) from Lyophilized Cultures. (28966)

JOSEPH G. TULLY AND ISAAC RUCHMAN

Laboratory of Bacterial Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, PHS, Bethesda, Md., and Department of Microbiology, University of Kentucky, Lexington

In 1938, Sabin(1,2) and Findlay *et al*(3) almost simultaneously reported the recovery of pleuropneumonia-like organisms (PPLO) from brains of mice undergoing intracerebral passage of toxoplasma and lymphocytic choriomeningitis virus, respectively. The isolated PPLO, when passed intracerebrally in mice, induced a characteristic turning on the long axis of the body which led to the term "rolling disease"(3). The American strain was called type A by Sabin while the British strain was designated L_5 in Klieneberger's series. Sabin and co-workers(4-6) subsequently recovered type A and 4 other serologically distinct strains of PPLO, types B, C, D and E, from the brain, nose, conjunctival sac, and respiratory tract of normal mice. All 5 types produced arthritis in mice following intravenous inoculation of fluid cultures. In addition, the type A strain was also shown to elaborate, *in vitro*, a potent neurotropic exotoxin for mice. When 0.5-ml amounts of a 24-48 hour fluid culture of type A were introduced intravenously into 3-week-old mice, the rolling syndrome was observed after 1-2 hours and death usually occurred shortly thereafter. The mice that survived with persistent neurological signs showed neurolytic lesions in the cerebellum after 18 hours. The L_5 organism was found to be serologically identical to the type A strain but differed in being less virulent for mice and in not forming exotoxin *in vitro* (3,6). Following these reports, other PPLO isolations from mice were recorded(7-11).

The original neurotoxic type A strain, as well as the other 4 American types, were apparently lost and have not been available for serological comparisons with more recent isolates from mice(11). The original L_5 strain was also lost but a strain with similar properties was subsequently recovered(3) and, although it lost its virulence after prolonged subcultivation, sufficient biochemical and serological characteristics were determined to establish this strain in current classification schemes(10,12,13). This strain (PG-28) is now the prototype of L_5 and type A strains recovered earlier and is known as *Mycoplasma neurolyticum*(12).

We recently secured cultures of Sabin's type A and C strains that were lyophilized in February 1943. This report describes the recovery of mycoplasma from these cultures and the results of virulence and serological tests.

Materials and methods. Mycoplasma isolation. The 2 vials of lyophilized mycoplasma were wiped with gauze soaked in 5% phenol and allowed to air dry. Upon opening the vials, 0.5 ml of sterile brain heart-infusion broth was injected into each with separate sterile needles and syringes. Approximately 0.1 ml from each vial was transferred to agar plates (60 × 15 mm) and liquid cultures (5 ml) of the following media; (A) brain heart-infusion broth (BBL) plus 1% peptone (Parke-Davis) and adjusted to pH 8.0. After sterilization, sterile, fresh 25% yeast extract (14) and sterile horse serum (Cappel) were added to give 10% and 20% concentrations, respectively; (B) PPLO broth (Difco), also fortified with the same concentrations of yeast extract and horse serum. The second broth passage of each strain in this medium was also transferred to: (C) fluid and agar cultures of PPLO broth (Difco), containing yeast extract, horse serum, and 500 units of penicillin and 5 μg amphotericin per ml.

All cultures were incubated aerobically at 37°C, the agar plates being sealed with cellophane tape to prevent media dehydration. Subcultivations were made every other day to the same broth and agar cultures by transferring 0.5 ml or by cutting centimeter square agar blocks from solid cultures and spread-

ing the inverted blocks over fresh medium.

PPLO strains. In addition to type A and C strains, the following mycoplasma were also employed for comparison: *Mycoplasma neurolyticum* (PG-28) (L_5), obtained from Dr. R. M. Chanock, NIAID, NIH; KSA strain of *M. neurolyticum,* obtained from Dr. Ruth Wittler, Walter Reed Inst.; and *M. arthritidis* (JR-3) (L_4), received from Dr. M. F. Barile, DBS, NIH.

Antigen and antibody preparation. Two liter amounts of medium C were inoculated with approximately 10% of 2-day broth cultures of the mycoplasma strains and each was incubated aerobically for 3 days at 37°C. Cultures were harvested in a Servall refrigerated centrifuge at 10,000 rpm for 1 hour and the packed cells washed twice in sterile buffered (pH 7.0) isotonic saline. Washed mycoplasma cells were resuspended in buffered saline containing 1:10,000 merthiolate to a 50% light transmittance at 650 mμ in a model B Beckman Spectrophotometer. These suspensions were utilized as antigen in agglutination and fluorescent antibody (FA) tests as well as for preparation of hyperimmune rabbit antisera. Immune rabbit antisera to mycoplasma strains were prepared by the procedure of Bailey *et al*(15).

Serologic tests. Agglutination and indirect FA tests were performed as previously described(15,16). Appropriate pre-immunization rabbit sera and antigen controls were included in all tests.

Virulence tests. Twenty-four hour broth cultures of PPLO were prepared in medium B containing 0.5% glucose. Fluid cultures or Swinny Seitz EK filtrates were inoculated into 3-week-old (8-10 g) NIH general purpose mice by intravenous (0.5 ml), intraperitoneal (0.5 ml), or intracerebral (0.03 ml) routes. Control mice received the same quantity of uninoculated broth.

Results. The recovery of mycoplasma from lyophilized type A and C cultures was evident when the first broth passages on media A and B were transferred to agar plates. Small colonies appeared in 2 days and were successfully passed to new media. The fluid cultures did not appear turbid at the first passage but by the third passage a noticeable turbidity developed which reached a peak at about 48 hours' incubation. After several subcultivations on agar, the growth of both A and C strains became evident in 24 hours and the colonies enlarged to approximately 50 μ in 3-4 days. The typical "fried egg" appearance, with a central, raised, and darker area of growth into the agar surrounded by a lighter, circular zone of surface growth, was usually observed with the type A strain after 2-4 days' incubation (Fig. 1). The type C strain appeared to possess less tendency to grow into the agar and the central part of the colony was not well defined (Fig. 2). These characteristics are still evident with both strains after more than 30 passages on solid medium. Cultures on medium A showed the same amount and type of growth as on the 2 other media and subcultivation of the type A and C strains on this medium was stopped after the twentieth passage. The colonial appearance of the A and C strains at the twentieth passage level on solid medium B (Fig. 1 and 2) was compared to those of other mouse mycoplasma strains, PG-28 and KSA (Fig. 3 and 4). Agar subculture of the type A and C strains maintained in broth revealed less distinct colonial characteristics (Fig. 5 and 6).

The serological characteristics of the 2 recovered mycoplasma are shown in Table I. Both indirect FA and agglutination tests confirmed the close serological relationship of type A to the PG-28 and KSA strains, as well as demonstrating the distinct immunological characteristics of the type C strain. None of the mouse mycoplasma strains appeared related to the *M. arthritidis* L_4 strain of rats by the procedures employed here.

In addition to serological tests with antisera prepared against the recovered agents, indirect FA procedures were also performed with a rabbit anti-type A serum prepared in Dr. Sabin's laboratory in 1939. This antiserum, held at about —70°C during the intervening years with only intermittent thawing, had an FA titer of 1:512 against the newly recovered type A strain and titers of 1:512 and 1:256 against the PG-28 and KSA strains, respectively. No significant titer was observed against the type C strain.

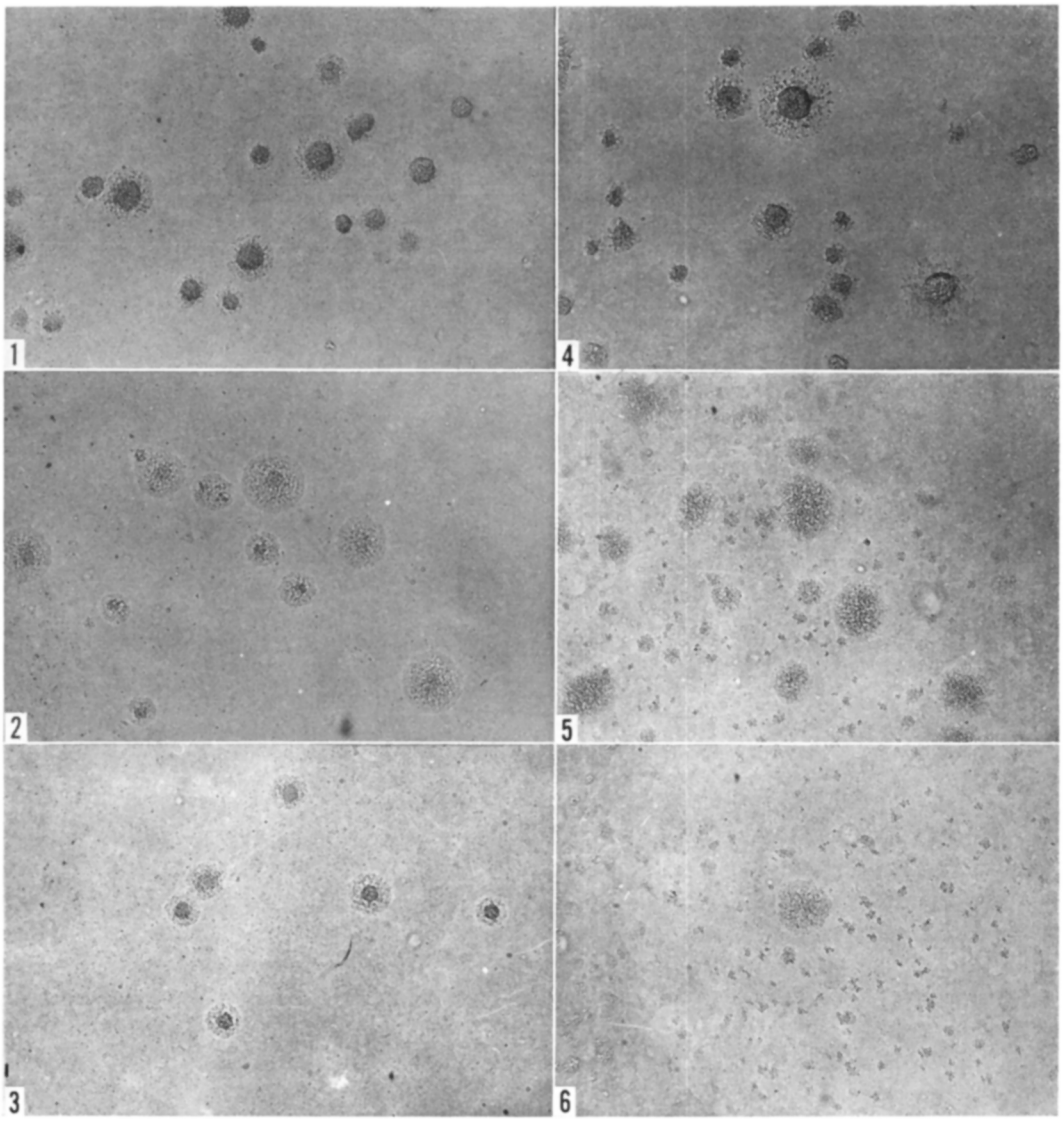

FIG. 1. Type A mycoplasma. Colonies at 2 days' incubation of 20th agar passage. × 21.
FIG. 2. Type C mycoplasma. Colonies at 2 days' incubation of 20th agar passage. × 21.
FIG. 3. PG-28 mycoplasma. Colonies at 2 days' incubation of 21st agar passage. × 21.
FIG. 4. KSA mycoplasma. Colonies at 2 days' incubation of 5th agar passage. × 21.
FIG. 5. Type A mycoplasma. Colonies at 2 days' incubation of 20th broth passage. × 21.
FIG. 6. Type C mycoplasma. Colonies at 2 days' incubation of 20th broth passage. × 21.

Table II presents the results of toxicity and virulence tests when the type A and C strains were in the twenty-second to thirtieth passage. The 4 strains studied were given four 24 hour passages in glucose broth prior to challenge. All mice receiving undiluted type A and KSA cultures by the intravenous route demonstrated rolling disease and died within 24 hours, the majority succumbing 3-5 hours after challenge. The rolling syndrome observed followed closely the original descriptions by Sabin(2) and Findlay *et al* (3). Within an hour the animals began to look ruffled, acted irritable, and many showed a slow backward rotation of the head. The first indication that rolling was about to take place was a raising of the foreleg and an extreme backward tilt of the head. Some ani-

TABLE I. Serological Relationships of Type A and Type C Mycoplasma from Mice with Other Rodent Mycoplasma.

	Indirect fluorescent antibody (FA) and agglutinin (Ag) titers of rabbit antisera to mycoplasma strains									
	Type A		Type C		PG-28(L_5)		KSA		JR-3(L_4)	
Antigen	FA	Ag	FA	Ag	FA	Ag	FA	Ag	FA	Ag
Type A	1024*	320*	16	N †	256	320	256	160	N †	N
" C	32	20	256	160	16	N	N	N	16	N
PG-28(L_5)	1024	160	64	N	1024	320	512	320	16	N
KSA	1024	160	32	N	512	160	1024	640	N	N
JR-3(L_4)	N	N	N	N	N	N	N	N	1024	320

* Reciprocals.
† Less than 1:10 titer in agglutination tests and 1:16 in FA tests.

mals continued to roll for 30-60 seconds and ended the turning motion on their backs with all 4 extremities extended and in a state of tremor. Three hours after inoculation some mice were in various forms of spasticity and still rolling but most appeared quiet and seemed to be paralyzed just prior to death. Dilutions of the type A culture decreased the toxicity but a 1:20 dilution of KSA was still active. These results have not yet been correlated with numbers of mycoplasma. Filtration of undiluted KSA fluid culture through Seitz EK pads removed the neurotoxicity and a large number of the viable cells but growth appeared after 48 hours on plates seeded with filtrate. Type A organisms passed through Seitz pads without apparent reduction in numbers and the filtrates were toxic to mice. One animal each in the groups receiving undiluted type A by the intracerebral and intraperitoneal routes died on the fourth post-challenge day without exhibiting the rolling syndrome. The type C and PG-28 strains were not toxic or pathogenic by any of the routes tested and all mice remained well after challenge. No evidence of arthritis was observed in mice receiving the C strain.

TABLE II. Toxicity and Virulence of Mouse Mycoplasma Strains.

	Mouse mortality (deaths/No. inoculated) by challenge route		
Challenge material	Intrav	Intraper	Intracer
Type A—undil	20/20	1/10	1/10
1:5 dil	1/5	—	—
1:10 "	0/5	—	—
1:20 "	0/5	—	—
Undil Seitz filtrate	5/5	—	—
KSA—undil	20/20	0/10	0/10
1:5 dil	5/5	—	—
1:10 "	5/5	—	—
1:20 "	5/5	—	—
Undil Seitz filtrate	0/10	—	—
Type C—undil	0/15	0/10	0/10
PG-28 — "	0/15	0/10	0/10
Control media	0/10	0/10	0/10

Discussion. The results presented here indicate that mycoplasma possessing characteristics similar to those originally described by Sabin were recovered from 20-year-old lyophilized cultures. The identification of the type A strain has been based on a serological relationship to previously described mycoplasma from mice, reproduction of the rolling disease syndrome in these animals, and a significant FA titer when tested against antisera to the original type A strain. As a consequence of the loss of the original type A strain, the PG-28 (L_5) strain has been established as the prototype of *M. neurolyticum*. However, this strain did not possess the virulence of the original type A strain, either on initial isolation or after rapid intracerebral passage(3), and was capable of producing rolling disease in mice only when administered intracerebrally with agar. Even this property has apparently now been lost(10), and the decreased pathogenicity of PG-28 appears to be supported by observations reported here. Lemcke(11) recently recovered a mycoplasma strain (KSA) from mouse brain that produced rolling disease in 3 of 8 mice inoculated intracerebrally with agar cultures but no serological procedures were performed to definitely establish a relationship between this strain and L_5. However, the re-

sults of this study suggest not only a similarity in serological properties and production of neurotoxic substance of type A and KSA strains but indicate that both these strains are also immunologically related to the PG-28 (L_5) strain.

The original type C strain was recovered from lungs of mice given various materials intranasally under ether anesthesia(4). Sabin later grouped types C, D, and E strains into a single species, *Musculomyces histotropicus,* and indicated that all were pathogenic for mice(6). Type C produced a progressive polyarthritis following intravenous inoculation but differed serologically from types A and B and produced no exotoxin. Since this strain has not been available to investigators, its relation to other mycoplasma strains from other sources has prevented a decision regarding its classification. Thus, the confirmation of the strain recovered here as type C is based solely upon its growth characteristics and its distinct immunological properties. We have not been able to produce arthritis in mice with it, but Sabin(6) had previously indicated a decreased ability of the type B strain to induce arthritis in mice after prolonged cultivation. Whether this property may be reacquired through mouse passage or the introduction of larger quantities of mycoplasma into animals is uncertain but under investigation. Additional studies on the biosynthesis of type A neurotoxic material and the passage of this substance as well as viable particles through filters of various porosity are also in progress.

Large numbers of different strains of mycoplasma have been isolated from mice but their relationship to each other and to strains recovered from rats is still confusing. The recovery of the original type A and C strains described by Sabin may help not only to reestablish these strains as definite species but assist comparative studies with other mycoplasma from mice and rats.

Summary. Mycoplasma possessing characteristics similar to those isolated from mice and described by Sabin have been recovered from lyophilized cultures after 20 years. The type A strain is immunologically related to the L_5 prototype of *Mycoplasma neurolyticum* and produces, *in vitro,* a substance capable of inducing rolling disease in mice. The type C strain is serologically unrelated to the type A and L_5 strains and appears to have lost its pathogenicity for mice.

We wish to acknowledge the valuable assistance of C. Winslow Renshawe.

1. Sabin, A. B., *Science,* 1938, v88, 189.
2. ———, *ibid.,* 575.
3. Findlay, G. M., Klieneberger, E., MacCallum, F. O., MacKenzie, R. D., *Lancet,* 1938, v235, 1511.
4. Sabin, A. B., *Science,* 1939, v90, 18.
5. Sabin, A. B., Johnson, B., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 569.
6. Sabin, A. B., *Bact. Rev.,* 1941, v5, 1.
7. Sullivan, E. R., Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 620.
8. Edward, D. G., ff, *J. Path. Bact.,* 1940, v50, 409.
9. Klieneberger, E., *J. Hyg.,* 1940, v40, 204.
10. Edward, D. G. ff, *J. Gen. Microbiol.,* 1954, v10, 27.
11. Lemcke, R., *J. Hyg.,* 1961, v59, 401.
12. Edward, D. G. ff, Freundt, E. A., *J. Gen. Microbiol.,* 1956, v14, 197.
13. Freundt, E. A., *The Mycoplasmataceae,* Munksgaard, Copenhagen, 1958, p22.
14. Taylor-Robinson, D., Scmerson, N. L., Turner, H. C., Chanock, R. M., *J. Bact.,* 1963, v85, 1261.
15. Bailey, J. S., Clark, H. W., Felts, W. R., Fowler, R. C., Brown, T. McP., *ibid.,* 1961, v82, 542.
16. Tully, J. G., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 704.

Received October 30, 1963. P.S.E.B.M., 1964, v115.