Suppression of Rous Sarcoma Virus Growth in Tissue Cultures by *Mycoplasma orale*

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ABSTRACT

SOMERSON, NORMAN L. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), AND M. K. COOK. Suppression of Rous sarcoma virus growth in tissue cultures by *Mycoplasma orale*. J. Bacteriol. **90**:534-540. 1965.—An agent which produced cell destruction in human diploid and chick-embryo fibroblasts was isolated from WI-26 strain of human diploid fibroblasts and shown to be a mycoplasma. The multiplication of Rous sarcoma virus (RSV) and Rous associated virus (RAV) was inhibited in WI-26, WI-38, and chick-embryo fibroblasts infected with this mycoplasma. The mycoplasma isolate, designated strain 941, reacted strongly in the complement-fixation test with antiserum to *Mycoplasma orale* CH19299, an isolate obtained from the human oral cavity. The cytopathic effect of mycoplasma strain 941 could be eliminated by growing the mycoplasma on an artificial agar medium before inoculation into chick-embryo fibroblasts. Serial passage in chick-embryo fibroblasts restored the cytopathogenicity of the agar-grown mycoplasma. However, growth of RSV and RAV was inhibited by both the tissue culture-grown and the agar-grown 941 strain, and also by the CH19299 strain which did not produce any cytopathic effect.

The difficulty in obtaining leukosis-free chick-embryo materials has limited in vitro studies with Rous sarcoma virus (RSV). For this reason, many tissue-culture systems, including human embryonic diploid fibroblasts WI-26 and WI-38 (Hayflick, 1965a), have been examined as substitutes for chick-embryo fibroblast (CEF) cultures (Burgman and Jonsson, 1962; Doljanski and Pikovski, 1942; Jensen et al., 1964; Manaker and Groupé, 1956; Svoboda and Chyle, 1963). The sensitivity of WI-26 for isolation of avian leukosis viruses from chickens showing visceral lymphomatosis was reduced by mycoplasma contamination. This report will describe the inhibition of RSV and Rous associated virus (RAV) in WI-26, WI-38, and CEF by a mycoplasma commonly found in the human oropharynx. This mycoplasma was serologically identical with *Mycoplasma orale* and was shown to produce a cytopathic effect (CPE) in all three tissue cultures.

MATERIALS AND METHODS

Tissue culture. The method of Rubin (1960) was used to prepare CEF from 10- to 11-day-old embryonated hen's eggs (Kimber, strain 13) that were free from resistance-inducing factor (RIF). Eggs from trap-nested, known RIF-negative hens (Truslow Farms, Chestertown, Md.) were also used. The CEF cultures were maintained on a mixture of 49% medium 199, 49% Eagle's medium, and

2% inactivated gamma globulin calf serum. The medium contained 100 units of penicillin and 1 μ g of streptomycin per milliliter of medium. The human diploid strains WI-26 and WI-38 were obtained from Microbiological Associates, Inc., Bethesda, Md., and were maintained on a similar medium.

Virus and assay methods. Partially purified RSV (Bryan strain) was obtained from W. R. Bryan. All pools contained 100 to 1,000 times more RAV than RSV. Infectivity titrations of RSV were performed in stationary tubes of RIF-free CEF. Cultures were observed microscopically for typical RSV cell transformation for a 14-day period. RAV was assayed for infectivity by the COFAL technique (Sarma, Turner, and Huebner, 1964). Briefly, complement-fixing (CF) antigens were prepared from CEF cells, and culture supernatant fluids were collected over a 1- to 21-day period. Such antigens contained 450,000 CEF cells per 0.1 ml of culture supernatant fluid. After three cycles of freezing at -60°C and thawing at 37°C , the antigens were tested for their ability to fix complement in the presence of hamster serum containing RSV Schmidt-Ruppin (S-R) strain antibodies.

Complement-fixation tests. All antigen detection tests were done by use of the micro CF method (Sever, 1962; Huebner et al., 1963) with 4 units of antibody and 2 units of complement. Production of hamster RSV, S-R antiserum, and antigens, provided by R. J. Huebner, has been described elsewhere (Huebner et al., 1964; Sarma et al., 1964).

Mycoplasma CF antigens were prepared from

FIG. 1. Photomicrographs showing development of CPE in human embryonic diploid fibroblasts (WI-26). (1) Ten-day-old noninoculated WI-26; Giemsa stained; 450 $\times$. (2) WI-26 inoculated with eighth WI-26 cell passage of a chicken carcinoma suspension; day 3; Giemsa stained; 450 $\times$. (3) WI-26 infected as in (2), but 8 days after inoculation; Giemsa stained; 450 $\times$. (4) WI-26 infected as in (2), but 4 months after inoculation; unfixed; unstained; 100 $\times$. (5) Eighth WI-26 passage of noninoculated control culture; unfixed; unstained; 100 $\times$.

TABLE 1. CPE production by agent found in cell cultures of human diploid fibroblasts

Assay tissue	Day of appearance of CPE*		CPE end point
	Earliest	Complete	
WI-26	8	14	10 ⁹
CEF†	2	9	>10 ⁸

* From date of tissue-culture preparation.

† Initial diploid suspension passed into CEF.

organisms grown for 10 days in mycoplasma medium (Hayflick, 1965b). The organisms were concentrated 10-fold by methods detailed elsewhere (Chanock et al., 1962). Anticomplementary activity was reduced by adding guinea pig complement (final 10% concentration) to the concentrates and then incubating the mixtures at 37 C for 1 hr, followed by 56 C for 30 min. If necessary, the treatment was repeated. Such antigens were tested with 4 units of each of the following six rabbit antisera prepared against the human mycoplasma species: *M. hominis* type 1, strain V2785; *M. hominis* type 2, strain Campo; *M. pneumoniae* FH; *M. fermentans* G; *M. salivarium* buccal; *M. orale* CH19299.

Mycoplasmas. The tissue-culture mycoplasma strain 941 was isolated from WI-26 cultures supplied by a commercial organization. The CH19299 strain was isolated from a child at the Children's Hospital, Washington, D.C., and was shown to be serologically identical with other human oral mycoplasmas classified as *Mycoplasma orale* (Taylor-Robinson et al., 1964) or as PATT strain (Clyde, 1964).

Mycoplasma growth medium. The medium used for isolation, propagation, and maintenance of mycoplasma was the same as the formula of Hayflick (Chanock, Hayflick, and Barile, 1962; Hayflick, 1965b). The medium was supplemented with penicillin (final concentration, 1,000 units per milliliter), and thallium acetate and amphotericin B (final concentration, 2 mg/ml). Since evidence has accumulated that amphotericin B is capable of inhibiting some mycoplasmas (Lampen et al., 1963; Chanock, unpublished data), it was omitted from the mycoplasma medium used for detection of mycoplasmas in tissue culture. The broth medium was prepared similarly, except that PPLO broth (Difco) was substituted for PPLO agar (Difco).

The mycoplasmas recovered on agar from WI-26 fibroblasts were prepared for storage by ejecting the agar through a syringe into a measured amount of mycoplasma broth. Samples were sealed in ampules and stored at -70 C. To grow large quantities of organisms for inoculation into tissue cultures, 1 ml from an ampule was added to 100 ml of mycoplasma broth medium. The number of colony-forming units (CFU) per milliliter of broth was determined by decimal dilutions and inoculation

onto agar medium. After 5 days of incubation, the inoculated mycoplasma broth contained at least 6×10^7 CFU/ml. Mycoplasma suspensions intended for tissue-culture inoculation were grown in mycoplasma broth without antibiotics or thallium acetate. Since toxic effects were observed in tubes receiving undiluted mycoplasma broth medium, the mycoplasma cultures were diluted at least 1:10 before addition to the tissue cultures. Control cultures received no additions or 0.2 ml of mycoplasma broth medium.

Isolation and detection of mycoplasma from tissue cultures. The WI-26 or CEF cells were frozen and thawed three times, and 0.1 ml of cell suspension was plated onto mycoplasma agar medium. Two plates were used to detect mycoplasmas, one plate being incubated aerobically and the other under reduced oxygen tension (95% nitrogen and 5% carbon dioxide). Agar plates were examined daily for mycoplasma colonies.

RESULTS

CPE in human fibroblasts. Thirty-second passage WI-26 cell cultures (Fig. 1, no. 1) were inoculated with 20% cell suspensions of a carcinoma removed from the neck of an 8-month-old chicken. These cultures were maintained at 35 C, and the medium was changed twice weekly. Degeneration of both inoculated and noninoculated WI-26 was observed after 3 to 8 days (Fig. 1, no. 2 and 3; Table 1). The cell sheets were torn and, in time, appeared vacuolated. After 3 to 4 months, the vacuolization was very marked, and the cultures were unrecognizable as WI-26 (Fig. 1, no. 4 and 5). Attempts to subcultivate these 3- to 4-month-old cultures failed. Uninoculated WI-26 cultures and similar cultures inoculated with tumor material were carried in parallel through seven serial passages. The CPE was present in all cultures. Similar results were obtained with the WI-38 strain of human diploid fibroblasts.

Avian leukosis CF antigens could not be demonstrated in 50% suspensions of cells obtained from any of these cultures. Chickens inoculated with such cells did not develop RSV-like tumors at the site of inoculation. Furthermore, clinical visceral lymphomatosis or avian leukosis neutralizing antibodies did not appear over an 8-month period. These results contrasted with previous positive findings with the same tumor suspension in mycoplasma-free WI-26 cells (Cook and Gross, unpublished data).

CPE in CEF. The WI-26 cultures inoculated with chicken sarcoma tissue were transferred into CEF. Suspensions of WI-26 cells were also transferred into CEF (Fig. 2, no. 1) to serve as control cultures. All cultures inoculated with suspensions of WI-26 cells showed a granular-type CPE after 2 to 4 days (Fig. 2, no. 2). The cell sheets appeared

FIG. 2. Development of CPE in CEF cultures. (1) Noninoculated culture, day 6, 100 X; (2) culture infected with tissue culture-passaged mycoplasma strain 941, (3) mycoplasma-infected CEF, day 4. Phase contrast, 450 X.

TABLE 2. CPE production after passage of *Mycoplasma orale* 941 on artificial medium and reinoculation into tissue culture

Passage level of inoculum	Tissue culture inoculated	CFU/ml in PPLO broth*	Day of CPE appearance		CPE end point in tissue culture
			Earliest	Complete	
1st agar passage	WI-26	10 ⁷	6	18	10 ^{7.5}
12th agar passage		10 ⁷	No CPE	—	—
12th agar passage	CEF	10 ⁷	13	Incomplete at 25	10 ⁴ †
3rd CEF passage after agar		≥10 ⁶	5	Not recorded	10 ⁸

* Titer of mycoplasma broth culture; only 0.2 ml of broth was added to each tissue culture.
† CPE in cultures inoculated with serial decimal dilutions of the 12th agar passage of mycoplasma.

to be torn and frayed, and, when viewed by phase-contrast microscopy, numerous particles were observed in the cytoplasm (Fig. 2, no. 3). The entire cell sheet became detached from the glass on about the 9th day. Inoculation with as little as 0.2 ml of 10⁻⁸ dilution of WI-26 suspension produced the CPE and the subsequent cell destruction. Frozen and thawed WI-26 cell suspensions were passed serially in CEF. CPE was produced through nine serial passages in CEF. Noninoculated control CEF did not show CPE.

Isolation and identification of a mycoplasma as the cause of CPE. Four or five tubes from each of six different shipments of WI-26 cell cultures received from a commercial laboratory were tested for mycoplasma contamination. This sampling represented approximately 5% of the total number of WI-26 cell cultures received. Mycoplasmas were isolated from every tube tested. Typical mycoplasma colonies were observed on mycoplasma agar medium incubated

for 4 days at 34 C under reduced oxygen tension. By the 11th day of incubation, some mycoplasma colonies were also found on plates incubated aerobically.

Noninoculated WI-26 suspensions were diluted in serial 10-fold steps and added to CEF. Mycoplasma colonies were grown from the CEF inoculated with WI-26 suspensions, but not from control cultures inoculated with culture medium. A CPE was observed in all tissue cultures inoculated with the 10⁻¹ through 10⁻⁸ dilutions, but not with the 10⁻⁹. Thus, the production of CPE by mycoplasmas present in WI-26 cultures was associated with the cell destruction observed in CEF.

Mycoplasma CF antigens were prepared with organisms isolated from WI-26 and from CEF inoculated with WI-26. In CF tests, these isolates gave antigens which reacted to a high level with antiserum to *M. orale* CH19299. Little or no CF reaction was observed when the antigens were

TABLE 3. *RSV (Bryan strain) in vitro transforming and complement-fixing antigen titers in Mycoplasma orale*-infected and mycoplasma-free chicken-embryo monolayers*

Reciprocal of log ₁₀ virus dilution	<i>M. orale</i> -infected chick-embryo monolayers		Mycoplasma-free chick-embryo monolayers†								
	RSV transformation†	Avian leukosis CF antigen titer on days 1 to 21	RSV transformation†	Avian leukosis CF antigen reciprocal titer on indicated day							
				1-3	4	6	8	10	12	14	21
3	0	0	+	0	2	4	4	4	4	4	4
4	0	0	+	0	0	2	4	4	4	4	4
5	0	0	+	0	0	2	2	2	4	4	4
6	0	0	0	0	0	0	2	0	2	2	2
7	0	0	0	0	0	0	0	0	0	0	2
8	0	0	0	0	0	0	0	0	0	0	0
Noninoculated control	0	0	0	0	0	0	0	0	0	0	0

* Results from one experiment. All experiments showed at least 1 to 2 log reductions by transformation assays.

† Transformation of CEF into typical sarcoma cells (Manaker and Groupé, 1956).

‡ Mycoplasmas were recovered on days 1, 6, 12, and occasionally on day 21 from tissue cultures inoculated with *M. orale*, and were not grown from tissue cultures which were not inoculated with mycoplasma.

tested with the five other antisera against mycoplasmas of human origin. The isolates appeared to be identical, and one of them, designated strain 941, was selected for reinoculation into tissue culture. Serial 10-fold dilutions of the mycoplasma broth suspension were added to CEF, and the typical CPE described earlier was apparent in as little as 2 days. The CPE was observed by the fifth day in cultures inoculated with a 10^{-6} dilution. The 10^{-1} dilution of uninoculated mycoplasma broth did not produce morphological changes in these cultures.

Elimination of CPE by passage of organisms on artificial medium. *M. orale* 941 was subcultured every 5 to 7 days on mycoplasma agar medium. After the 12th agar passage, the organisms were grown in mycoplasma broth; a sample of the culture was frozen, and serial 10-fold dilutions were inoculated into CEF. The organisms were recovered from the 10^{-1} through 10^{-6} mycoplasma broth dilutions, but there was no evidence of CPE over a 21-day period in any of the CEF which received these dilutions (Table 2).

Reappearance of CPE after passage in tissue culture. With *M. orale* 941, the ability to produce CPE was regained through serial subpassage in CEF. This phenomenon is illustrated in the following experiment. The CEF cells infected with the 12th agar passage of mycoplasma strain 941 (no evidence of CPE) were harvested on the 10th and 12th days. Serial 10-fold dilutions were inoculated into fresh CEF. The CEF cell-supernatant mixture received 0.2 ml of mycoplasma suspension containing 10^7 CFU/ml, about the same number found in the infected WI-26. The

newly infected cultures showed CPE after 13 days, but the cell sheet was not completely destroyed as late as the 25th day. In contrast (Table 1), *M. orale* 941, originally found in WI-26 and not passed in an artificial medium, showed CPE in 2 days and complete cell destruction in 9 days. The CPE end point in the CEF was obtained at the 10^{-4} dilution; the end point in infected WI-26 was over 10^{-7} (Table 2). Mycoplasmas were recovered from CEF cultures which had received 10^{-1} dilutions of the first repassage of mycoplasma in CEF.

Additional serial subpassages through tissue culture increased the potential of *M. orale* 941 to produce CPE. After three serial passages through CEF, inoculation with strain 941 produced CPE in the tissue cultures as early as 5 days, and the CPE end point was 10^{-8} .

Lack of CPE with a serologically related mycoplasma. To determine the effect in tissue culture of a human oral isolate serologically related to strain 941, *M. orale* CH19299 was subcultured for at least four passages in CEF. A suspension of $10^{7.5}$ CFU/ml failed to produce CPE in CEF. This strain was easily recovered from infected tissue cultures, although there was no overt cell pathology.

Inhibition of RSV-RAV by M. orale. *M. orale* CH19299, or strain 941 after subculture on agar, was inoculated at a multiplicity of 1.0 into 18-hr CEF. After 24 hr at 35 C, the cultures were washed and refed with maintenance medium. Decimal dilutions of 10^{-3} to 10^{-8} RSV-Bryan were inoculated into the CEF previously infected with mycoplasma and into mycoplasma-free

tissue cultures. On days 1 to 21, the cultures were observed for typical RSV transformation. The CF antigens were prepared and tested against 4 units of antibody in the sera of hamsters carrying RSV-S-R transplanted tumors. In every experiment, at least 1 to 3 log₁₀ titer reductions were observed by use of RSV transformation assays in mycoplasma-infected CEF (Table 3).

Suppression of RAV was demonstrated by failure to detect avian leukosis CF antigen in mycoplasma-infected CEF inoculated with RSV. Both *M. orale* CH19299 and the agar-passed strain 941 suppressed the formation of specific CF antigens and foci formation by RSV.

Levels of arginine which had been reported by others to reverse mycoplasma suppression of viral multiplication could not be used. In one experiment, the addition of as little as 0.005 M arginine HCl reduced the amount of avian leukosis CF antigen formed.

The inhibition of RSV did not appear to be a nonspecific suppression of virus, since CEF infected with *M. orale* CH19299 supported the multiplication of influenza B Taiwan strain.

DISCUSSION

The inhibition of adenovirus and measles virus multiplication by mycoplasma has been reported by others (Rouse, Bonifas, and Schlesinger, 1963; Butler and Leach, 1964). The present report describing the suppression of RSV and RAV by *M. orale* adds further evidence that the presence of mycoplasmas in tissue cultures restricts the growth of viruses. In the case of limited inocula (i.e., in clinical specimens), tissue cultures may fail to give evidence of the presence of virus.

There are numerous reports of tissue-culture contamination by mycoplasmas (Barile, Malizia, and Riggs, 1962; Herderschee, Ruys, and van Rhijn, 1963; Pollock, Treadwell, and Kenny, 1963) and accumulating evidence that a number of mycoplasmas can produce CPE in a variety of different cultures (Kramer et al., 1963; Girardi et al., 1965; Grace et al., 1963; Rovozzo, Luginbuhl, and Helmboldt, 1963). *M. orale* 941 produces CPE in at least two different tissue-culture systems. Tissue-culture contamination by mycoplasma may originate from the oral cavity (O'Connell, Wittler, and Faber, 1964; Hayflick, 1965b), and *M. orale* is a common oral isolate (Taylor-Robinson et al., 1964). Thus, the finding of an *M. orale* strain in WI-26 is not surprising. The deleterious effect in tissue cultures may not be discernible, since even serologically identical isolates (strains 941 and CH19299) vary in their ability to produce CPE.

The CPE produced by *M. orale* 941 is apparently related to its passage history. The capacity to produce CPE is lost after agar subcultivation. The same concentration of attenuated organisms which produced CPE before the series of agar subcultures did not show cytological evidence of mycoplasma infection after subcultures on artificial medium. Thus, the agar-passed *M. orale* 941 gave results which might be obtained with strain CH19299 and with many other mycoplasmas—the inapparent mycoplasma contamination of tissue culture.

Since the CPE is a variable depending on (i) the type of tissue culture, (ii) the serological strain of mycoplasma, and on (iii) the passage history of the mycoplasma, there is no certainty of mycoplasma-free tissue cultures when cells appear morphologically normal. Furthermore, addition of antibiotics may mask the presence of mycoplasmas by suppressing growth of the organisms in the tissue-culture medium. Therefore, all tissue cultures used for isolation and growth of avian leukosis virus and other viral agents should be continuously tested for mycoplasma by culturing on artificial medium.

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