Mycoplasma Deoxyribonuclease Activity in Virus-infected L-Cell Cultures

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Cell-free extracts of *Mycoplasma hominis* and medium from 72-hr broth cultures had deoxyribonuclease activity like that of deoxyribonuclease I. Mg^{++} stimulated activity, and the pH optimum was between 8.0 and 9.0. Double-stranded or heat-denatured deoxyribonucleic acid (DNA) served as a substrate, and oligonucleotides were produced. Cell-free extracts of L cells infected with *M. hominis* or *M. hominis* plus equine abortion virus (equine herpes virus, EAV) had greatly increased activity over that of extracts of L cells or of L cells infected with EAV alone. In the absence of *M. hominis*, however, extracts had little activity, most of which was in virus-infected cell cultures. Activity was found in the culture medium only in those systems in which *M. hominis* was present. It is concluded that *M. hominis* can contribute significant deoxyribonuclease activity to virus-infected as well as virus-free cell cultures. Perhaps the most interesting question arising concerns the ability of EAV, a DNA virus, to replicate successfully despite the presence of deoxyribonuclease activity at the site of replication (the nucleus).

Misinterpretation of data caused by the unsuspected presence of *Mycoplasma* is a risk well recognized by those who work with cell cultures. Many of the alterations reported can be traced to one of three important enzymatic activities contributed by the *Mycoplasma*. The best known of these is the ability of many species to deplete the cell culture medium of arginine by converting it to ornithine (31). This in turn has been responsible for causing low values in adenovirus titrations (29), altered cell metabolism (4, 17, 34), altered cell morphology (24), and, most recently, chromosomal changes in deliberately infected cell cultures (1). That *Mycoplasma* could produce such changes had been noted earlier, but the mechanism was unknown (6, 22). A second enzymatic activity contributed by *Mycoplasma* is nucleoside phosphorylase (9) which, by converting nucleosides to bases, may reduce their incorporation into nucleic acids by cell cultures. Such activity may have caused the inhibition of incorporation of thymidine and uridine by L cells noted by Nardone et al. (19). It may have also been responsible for some of the changes in nucleic acid synthesis, especially the decreased utilization of ¹⁴C-uridine by human cells infected with a variety of *Mycoplasma* species reported by Levine et al. (14). Holland et al. (12) developed an inhibitor of this

step (5-allyl-2'-deoxyuridine) which is effective in HeLa cultures containing *Mycoplasma*.

Another enzyme, deoxyribonuclease, is also present in many species of *Mycoplasma* (20, 23, 27, 28). Several unusual findings, however, preceded the realization that *Mycoplasma* could contribute this activity to cell cultures.

Randall and Walker (26) found that, during infection of L cells with equine abortion virus (EAV), a large part of the host cell deoxyribonucleic acid (DNA) was degraded to acid-soluble components. This suggested the presence of a viral-induced deoxyribonuclease. It was then found (7) that this same virus multiplied readily in cell cultures inhibited with 5-fluoro-2'-deoxyuridine (FUdR), even though it is a DNA virus. It was possible to explain this unusual finding when the strain of virus used was found to be contaminated with *M. hominis* (25). By contributing deoxyribonuclease activity to the system, the *Mycoplasma* could cause the degradation of host DNA and thus provide thymine nucleotides from that source for viral replication. Later experiments with virus freed from *M. hominis* support this hypothesis; in the absence of *M. hominis*, virus multiplication is sensitive to FUdR (21).

The present study provides further support of this explanation by documenting the ability of *M. hominis* to contribute deoxyribonuclease activity to virus-infected as well as virus-free mammalian cell cultures.

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In addition, it is clear that whenever virus inocula are contaminated with *Mycoplasma* it is very easy to misinterpret the data. This is especially true of studies which show increased deoxyribonuclease activity upon virus infection. If the inoculum and the host cell have not been tested for the presence of *Mycoplasma*, it would be almost impossible to decide whether increases in deoxyribonuclease activity should be attributed to the virus or to a contaminating *Mycoplasma*.

MATERIALS AND METHODS

L-cell cultures and EAV stocks were maintained and propagated as described (26). Trypsin, a possible source of unwanted nucleases, was not used in any part of the procedures. Freedom from covert contamination with *Mycoplasma* was controlled by regular inoculation of both cell suspensions and samples of virus preparations onto a modified solid medium. This medium consisted of 3.5 g of tryptose-blood-agar base and 1 g of powdered yeast extract dissolved in 90 ml water and autoclaved. Before cooling, 2.7 ml was dispensed to each plate. After the agar had hardened, 0.3 ml of sterile microgamma calf serum (Microbiological Associates) was added. The plates were incubated at 37 C overnight to allow excess moisture to evaporate and then were ready for use. Inoculated plates were incubated at 37 C, approximately 100% relative humidity, and in an atmosphere of 5% CO₂-95% N₂. No contamination was observed; by this procedure *M. hominis* could be grown out, within 24 to 48 hr, from broth culture, from deliberately infected cell cultures, and from known contaminated virus passage material.

The strain of *M. hominis* used was isolated from EAV stocks in this laboratory in 1965 (25) and was identified by Wallace Clyde of the University of North Carolina, Chapel Hill. The organism was propagated at 37 C in broth (11) consisting of 70% Difco PPLO Broth (without crystal violet), 20% microgamma calf serum (Microbiological Associates), and 10% fresh yeast extract (Robbin Laboratories, Chapel Hill, N.C.). The medium included, in addition, 1% D-glucose, 0.004% phenol red, 2 units of penicillin G per ml, and 0.4 µg of streptomycin sulfate per ml. The concentration of *M. hominis*, expressed as colony-forming units (CFU) per milliliter, was measured with the solid medium described above.

Infection of L-cell cultures. Infection of L-cell suspension cultures was initiated by the addition of *M. hominis* from broth culture, EAV from the supernatant fluid of 48-hr infected stationary L-cell cultures, or both. The final concentrations, at zero-time in the infected cultures, were as follows: L cells, 7.5×10^5 per ml; EAV (when used), 10^5 plaque-forming units (PFU)/ml; *M. hominis*, 10^5 CFU/ml. Uninfected control cultures contained 7.5×10^5 L cells per ml. All infected and control cultures were incubated at 37 C on a rotary incubator.

When harvested, the cultures were centrifuged at low speed for 10 min. The supernatant fluids were stored at -20 C and were later assayed for deoxyribonuclease activity. The cell pellets were washed

twice with 0.15 M NaCl, resuspended in one-twentieth of the starting volume of distilled water, then frozen and thawed three times at -40 C. The lysed cells were centrifuged at $27,000 \times g$ for 50 min, and the supernatant fluids were assayed for deoxyribonuclease activity. Cell-free extracts from broth cultures of *M. hominis* were prepared in the same manner, except that the cells were sedimented initially at $27,000 \times g$ for 50 min.

Deoxyribonuclease and protein assays. The incubation mixture (0.6 ml total volume) included 0.4 ml of Tris buffer [0.1 M tris(hydroxymethyl)amino-methane, pH 8.0, plus 0.005 M MgSO₄] containing 15 µg of highly polymerized L-cell DNA ($E_{260} = 0.75$) labeled with thymidine-2-¹⁴C (2,000 to 3,000 counts per min per µg) and 0.2 ml of enzyme preparation. In some experiments Mg⁺⁺ was omitted, and in others the pH was varied. Potassium phosphate buffers (0.1 M) were used at pH values below 7.0. The DNA was extracted from L cells prelabeled with 10⁻⁶ M thymidine-2-¹⁴C (0.1 µc/ml, final concentration) by the method of Soehner et al. (32). The reaction mixture was incubated at 37 C for 30 min. The enzyme reaction was stopped, the excess DNA was precipitated with 1.2 ml of 100% ethyl alcohol, and the mixture was allowed to stand for 12 to 18 hr at 4 C. The precipitate was centrifuged at 300 to $500 \times g$ for 20 min, and 0.1 ml of the supernatant fluid was counted in a liquid scintillation counter (8). Two standards were used, each containing 0.2 ml of buffer instead of the enzyme preparation. The DNA was precipitated in one; the other served as a control. Protein content of the enzyme preparations was determined by the method of Lowry et al. (15).

Assay for products of deoxyribonuclease activity. A deoxyribonuclease assay of cell-free extracts of *M. hominis* was performed as described above, except that nonradioactive L-cell DNA was used as the substrate. After the ethyl alcohol precipitate was sedimented, the optical density (OD) of the supernatant fluid was read at 260 nm. A sample (1.5 ml) of this supernatant fluid was lyophilized and redissolved in 0.8 ml of 0.1 M Tris (pH 9.0) plus 0.003 M MgSO₄, and was incubated with 0.08 ml of venom phosphodiesterase (1 µg/ml; Sigma Chemical Co., St. Louis, Mo.) for 5 hr at 37 C. The reaction was stopped with 0.7 ml of 0.3 N HCl. The OD was read again at 260 nm. Control samples incubated without added DNA showed that acid-soluble materials derived from the crude enzyme did not contribute significant OD either before or after the diesterase treatment. Therefore, hyperchromicity observed after phosphodiesterase treatment would indicate that oligonucleotides were formed by the deoxyribonuclease activity.

RESULTS

Several fractions from different systems were assayed for deoxyribonuclease activity. Broth cultures of *M. hominis* were used; L-cell cultures, either control cultures (free from virus and *M. hominis*) or cultures infected with EAV, with *M. hominis*, or with both, were also used. For each

system, the supernatant medium was assayed, as well as the cell-free extract.

Deoxyribonuclease activity of broth culture of *M. hominis*. Significant deoxyribonuclease activity was found in cell-free extracts of *M. hominis* and in the broth in which the organisms were grown, but not in uninoculated control broth. The activity was optimal between pH 8 and 9; when Mg^{++} was omitted, the activity was reduced by about 80% and the pH optimum shifted to between pH 7 and 8 (Table 1). Although native DNA served well as a substrate and was used in the standard assay, heat-denatured DNA gave values several-fold higher than native DNA (Table 2). The nature of the products of the action of deoxyribonuclease on native DNA was examined (Table 3). If the deoxyribonuclease were an endonuclease, then the products (which because of the way the assay was done would be alcohol-soluble) should be primarily oligonucleotides, which on further enzymatic degradation to mononucleotides would show a marked hyperchromic effect. If the deoxyribonuclease were an exonuclease, the products should be mono-

TABLE 1. Effect of Mg^{++} and pH on mycoplasmal deoxyribonuclease activity^a

pH	0.005 M Mg^{++}	Control
5.0	18.3	8.3
6.0	18.3	16.7
7.0	88.3	48.3
8.0	204.9	41.6
9.0	198.4	25.0

^a Activity is measured in micrograms of DNA made ethyl alcohol-soluble in 30 min by 1 mg of protein. Enzyme was from cell-free extracts of *M. hominis* propagated in broth.

TABLE 2. Mycoplasmal deoxyribonuclease activity with native and heat-denatured DNA

Sample	Activity ^a	
	Native DNA	Heat-denatured DNA ^b
1	835.2	3,961.2
2	749.7	1,610.3
3	705.2	1,630.3
4	855.0	10,344.0

^a Activity is measured in micrograms of DNA made ethyl alcohol-soluble in 30 min by 1 mg of protein. Enzyme was from cell-free extracts of *M. hominis* propagated in broth.

^b Heated at 100 C for 10 min and chilled rapidly. This procedure did not render the DNA alcohol-soluble.

TABLE 3. Products from deoxyribonuclease reaction^a

Sample	OD(deoxyribonuclease)/mg of protein ^b	OD(diesterase)/mg of protein ^c	Hyperchromicity
			%
1	2.04	3.57	75
2	1.89	3.18	68
3	1.70	3.20	88

^a Nonradioactive DNA from L-cell cultures was used. Enzyme was from cell-free extracts of *M. hominis* grown in broth.

^b OD of supernatant fluid after deoxyribonuclease reaction.

^c OD of supernatant fluid after deoxyribonuclease reaction and venom phosphodiesterase treatment.

nucleotides which would not show marked hyperchromicity. When tested after digestion with venom diesterase, the products showed increases in absorbance at 260 nm of a minimum of 70 to 80%, which is consistent with the hypothesis that the products were oligonucleotide fragments produced by endonuclease activity.

Because of the pH optimum (8 to 9), the stimulation by Mg^{++} , the greater activity with denatured DNA, and the apparent endonuclease activity, the deoxyribonuclease of *M. hominis* appears to be a deoxyribonuclease I.

Deoxyribonuclease activity of cell culture medium. Low but significant deoxyribonuclease activity was found in the supernatant medium from L-cell cultures simultaneously infected with *M. hominis* and EAV; considerably higher levels were found in the medium from L-cell cultures infected only with *M. hominis* (Fig. 1). In marked contrast, when *M. hominis* was not present in the cultures (EAV, control; Fig. 1), deoxyribonuclease activity could not be demonstrated in the culture fluids.

Deoxyribonuclease activity of extracts of L-cell cultures. Cell-free extracts of cell cultures always had at least a slight level of deoxyribonuclease activity. The lowest levels were found in control (*Mycoplasma* and virus-free) cultures (Fig. 2). Significantly more activity was found in cultures infected with EAV alone; the level was maximal at 12 hr after addition of virus and declined thereafter. The highest levels, however, were in the extracts of cultures to which *M. hominis* had been added, whether EAV was or was not present (Fig. 2). In both cases, the activity was similar in quantity and continued to rise throughout the period studied.

FIG. 1. Deoxyribonuclease activity of the medium in which L-cell cultures were growing at various times postinfection. (Activity was calculated as micrograms of DNA made ethyl alcohol-soluble in 30 min by the medium from 10^6 L cells.)

DISCUSSION

That *M. hominis* can contribute significant deoxyribonuclease activity to otherwise normal or to virus-infected cell cultures seems clear. In our system, the most reliable indication of *M. hominis* deoxyribonuclease appears to be its excretion into extracellular fluids. In broth cultures, activity was found in cell-free supernatant fluids. In cell cultures, it was always found in the supernatant medium when *M. hominis* was present, and was never found when *M. hominis* was absent. The level of activity observed in the medium of EAV-infected cell cultures was lower than in the medium of cell cultures infected only with *M. hominis*. The reasons for this are not clear, but one possibility is that *M. hominis* might grow principally at the cell surface in the virus-free cultures and more intracellularly in the virus-infected ones. In the latter case, more of the excreted deoxyribonuclease might remain inside the cells.

The pattern of appearance of deoxyribonuclease activity in the extracts of cell cultures is also

quite different when *M. hominis* is present. The relatively lower level seen in the virus-infected cultures in the absence of *M. hominis* reaches a maximum early (12 hr) and then declines, paralleling the gradual decline of cell viability. The deoxyribonuclease activity of extracts of cultures containing *M. hominis*, however, climbed throughout the experiments, and was remarkably similar in pattern between virus-free and virus-infected cell cultures (both with *M. hominis*). This would be compatible with the continued multiplication of the *Mycoplasma* throughout the experiment.

Although the deoxyribonuclease activity noted in EAV-infected cells free from *Mycoplasma* may be either viral or lysosomal, it is obvious that *M. hominis* can contribute considerable activity to the system which can mask the former activity, especially at later intervals.

Our earliest ideas about the deoxyribonuclease activity seen during EAV infection were that it was viral in origin, because we did not know that our virus preparations contained *M. hominis*. *Mycoplasma* contamination of virus preparations can still be a trap for the unwary. Indeed, there is a recent report (2) that commercial avian laryngo-tracheitis virus vaccine is frequently contami-

FIG. 2. Deoxyribonuclease activity of cell-free extracts of L-cell cultures at various times postinfection. (Activity was calculated as micrograms of DNA made ethyl alcohol-soluble in 30 min by 1 mg of protein.)

nated with *M. gallisepticum*, and contamination of smallpox vaccines with *Mycoplasma* has also been reported (33). It is possible that, on further study, some of the reports of viral-induced deoxyribonuclease activity (3, 5, 10, 13, 16, 18, 30) may be explained in part by mycoplasmal contamination of the virus inoculum. Perhaps the foremost question arising from this work, however, concerns the ability of EAV, a DNA virus, to replicate in the presence of deoxyribonuclease activity. During replication the genome is almost certainly naked, and, at the same general location (in the nucleus), the host DNA is being degraded by mycoplasmal deoxyribonuclease. Why does this deoxyribonuclease activity not destroy the replicating viral genome? We cannot answer this question at present, but we feel that study of this problem will result in some interesting findings.

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