Ultrastructure of a Mycoplasma (Negroni) Isolated from Human Leukemia

KLAUS HUMMELER,¹ NATALE TOMASSINI, AND LEONARD HAYFLICK²

Virus Laboratories at the Children's Hospital of Philadelphia, School of Medicine, University of Pennsylvania, and The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania

Received for publication 5 April 1965

ABSTRACT

HUMMELER, KLAUS (The Children's Hospital of Philadelphia, Philadelphia, Pa.), NATALE TOMASSINI, AND LEONARD HAYFLICK. Ultrastructure of a mycoplasma (Negroni) isolated from human leukemia. *J. Bacteriol.* **90**:517-523. 1965.—A strain of mycoplasma isolated in tissue cultures from human leukemic bone marrow was cultivated in cell-free media for 23 passages. Concentrates of broth cultures were investigated under the electron microscope in thin sections and negative contrast. The ultrastructure of the mature mycoplasma cells and their elementary bodies is described. It was found that the elementary bodies, particularly in the negative contrast preparations, bear a close resemblance to viruslike particles seen in tissues and plasmas of leukemic patients.

Efforts to determine the cause of human leukemia have been directed in recent years largely towards the isolation of cytopathic agents from leukemic tissues and visualization of virus particles in such materials. The validity of this approach is based upon the expected similarities between human leukemia, on the one hand, and avian and murine leukemias of known viral etiology, on the other. Several reports have appeared recently which describe the isolation of cytopathic agents in tissue cultures inoculated with human leukemic bone marrow (Hakala, Holland, and Horoszewicz, 1963; Negroni, 1964; Murphy, Furtado, and Plata, 1965) and the visualization of viruslike particles in tissues and plasmas of leukemic patients (Dmochowski, 1958; Beard, 1959; Braunsteiner, Fellingner, and Pakesch, 1960; Almeida, Hasselback, and Ham, 1963; Burger et al., 1964; Porter et al., 1964; Smith, Benyesh-Melnick, and Fernbach, 1964). Most of the isolates have been identified subsequently as mycoplasmas (Hakala et al., 1963; Murphy et al., 1965; Grist and Fallon, 1964; Girardi et al., 1965).

Some mycoplasmas share a number of properties with certain viruses, especially those belonging to the myxovirus group, i.e., their size, sensitivity to ether, ability to hemagglutinate and cause hemadsorption, lack of inhibition by many antibiotics, interference with virus replication in vitro, and production of cytopathic

effects in cell cultures (Hayflick and Chanock, 1965).

The frequent isolation of mycoplasmas from cell cultures inoculated with leukemic material and the recent isolation of mycoplasmas from leukemic bone marrow directly in cell free media (Hayflick and Koprowski, 1965) stresses the need of examining the ultrastructure of mycoplasmas found in association with this disease. Although preclusion of a virus as the causative agent of human leukemia cannot be inferred from these findings, it is important to compare the ultrastructure of known mycoplasmas with those viruslike particles seen in human leukemic cells.

This communication reports on an electron microscope study of an agent isolated in cell cultures from human leukemic tissues by Negroni (1964) and subsequently determined to be a mycoplasma (Grist and Fallon, 1964; Girardi et al., 1965). The results demonstrate that caution must be exercised in describing structures in human leukemic materials as viruses, or as viruslike, in the absence of biological tests for mycoplasmas.

MATERIALS AND METHODS

Propagation of mycoplasmas. The mycoplasma strain used was isolated from material received from G. Negroni (Girardi et al., 1965). This strain had undergone 23 consecutive passages on agar and in broth prior to examination under the electron microscope. This represents a dilution of approximately 10^{-31} of the original sample. The agar and broth formulas used were those previously de-

¹ Career Development Award K3-HD-22707.

² Career Development Award K3-CA-5938.

scribed (Hayflick, 1965). To remove particulate matter from the broth used for cultivation of the Negroni mycoplasma, the fluid was filtered through a Millipore filter (ADP, 220 μ) before inoculation with the organism. The addition of 1:2,000 thallium acetate was included to prevent contamination with airborne bacteria. Broth-grown cultures were used after approximately 5 days of incubation at 37 C, at which time 5×10^7 to 7×10^7 colony-forming units per milliliter could be found.

Electron microscopy. Samples of broth cultures of the mycoplasmas were centrifuged at $25,000 \times g$ for 40 min. The sediments were reconstituted to one-tenth the original volume with phosphate-buffered saline solution (PBS), and the concentrates were prepared for sectioning and negative contrast staining as described below. All preparations were examined in a Siemens Elmiskop 1 electron microscope.

Thin sectioning. Mycoplasma suspensions were drawn into a syringe and squirted into an equal volume of buffered osmium tetroxide. The mixture was centrifuged at $8,000 \times g$ for 15 min, and the resulting osmium-fixed pellet was removed, cut into small fragments, dehydrated in graded ethyl alcohol, and embedded in epoxy resin. Thin sections were cut with a diamond knife on a Porter-Blum microtome. The sections were stained with uranyl acetate and photographed at $10,000 \times$.

Negative contrast. Carbon-coated Formvar grids were used as specimen supports. Samples of mycoplasma, concentrated as above, were examined immediately at magnifications varying from 8,000 to $40,000 \times$. The negative staining procedure, with use of 4% phosphotungstic acid, has been described previously (Hummeler, Anderson, and Brown, 1962).

RESULTS

The mycoplasma preparations showed particles of different sizes and morphology when sectioned as well as when negatively stained. Despite a considerable amount of pleomorphism, two types of particles could be discerned: (i) large, presumably mature forms of mycoplasma which ultrastructurally did not differ to any great extent from those described by others in studies of different mycoplasma strains derived from agar colonies (Domermuth et al., 1964), and (ii) considerably smaller particles which seemed to correspond to previously described elementary bodies (see Hayflick and Chanock, 1965). The aspects of these forms in the Negroni mycoplasma are described in detail below.

Thin sections. The mature forms showed an average diameter of 540 m μ . The estimated diameter of the individual particle depends on the plane of sectioning, and, thus, it was not possible to obtain a more accurate determination. The

fine structure of the mature Negroni agent consisted of an apparently flexible double membrane and an internal arrangement of dense material adjacent to the limiting membrane which was connected by a network of fine fibrils (Fig. 1).

The elementary bodies showed a narrower size range, with an average diameter of 90 m μ . Their fine structure was less distinct. Beyond a double membrane, only a dense core was obvious, except that some of the elementary bodies had tail-like appendices (Fig. 1). The interspace between the two limiting membranes was usually narrower than that found in appropriate animal viruses (Fig. 2), but frequently elementary bodies were seen that closely resembled such viruses. An example of such a particle is presented in Fig. 3. In some preparations, the elementary bodies appeared to have a hexagonal outline (Fig. 4), and they were found to bud from mature mycoplasma cells (Fig. 5).

Negative contrast. The mature mycoplasmas were easily recognized. They showed a lack of detail owing to the inability of the contrast material to penetrate. In general, they appeared large, approaching 1 μ or more in diameter. This increase in size, as compared with the thin sections, was presumably due to the surface tension exerted upon them by the drying of the material on the grid. Thus, the application of the negative-contrast technique is limited when used to examine the structure of the mature mycoplasmas.

The elementary bodies, which exhibited a degree of uniformity in thin sections, showed a greater array of detail in negative-contrast preparations. They were either round or ovoid, resembling myxoviruses, or presented "tailed" structures. Occasionally, filaments were also seen. The various manifestations can be seen in Fig. 6 to 15.

However, the myxoviruslike particles, with a dense membrane, surface spikes, and internal detail, differed from myxoviruses in some respects. The spikes were not regularly spaced and appeared to be less rigid. This material may correspond to the "amorphous or floccular substance" consistently found in close connection with the surface of mycoplasma membranes in thin sections of agar colonies (Domermuth et al., 1964). Furthermore, the internal structure did not show any regular helical formation of ribonucleoprotein as in myxoviruses. The surface of elementary bodies frequently showed distinct "subunits" not seen in myxoviruses (Fig. 9).

The tailed structures closely resembled those described as virus particles in human leukemic

FIG. 1. Thin section of a mycoplasma pellet. Mature cells and elementary bodies, one with tail-like appendix. 50,000 $\times$. All bars on figures represent 0.1 μ .

FIG. 2. Thin section of an elementary body with narrow interspace between two limiting membranes. 50,000 $\times$.

FIG. 3. Thin section of an elementary body with wide viruslike interspace between two limiting membranes. 50,000 $\times$.

FIG. 4. Thin section of an elementary body showing hexagonal shape. 50,000 $\times$.

FIG. 5. Thin section of an elementary body "budding" from mature cell. 50,000 $\times$.

FIG. 6. *Myxoviruslike elementary body. Internal structure. Dense limiting membrane. Negative contrast. 200,000 X. All bars on figures represent 0.1 μ .*

FIG. 7. *Myxoviruslike elementary body. Thick dense limiting membrane. Diffuse internal structure. Negative contrast. 200,000 X.*

FIG. 8. *Myxoviruslike elementary body. Dense inner core. Negative contrast. 200,000 X.*

FIG. 9. *Myxoviruslike elementary bodies. Particle on left shows dense limiting membrane and surface spikes. Particle on right shows "subunits." Negative contrast. 200,000 X.*

FIG. 10. *Smallest elementary bodies seen. Negative contrast. 200,000 X.*

FIG. 11. *Filamentous structure. Negative contrast. 120,000 X.*

plasma (Burger et al., 1964; Porter et al., 1964). They were often dense and impermeable to the phosphotungstate (Fig. 12). Where penetration occurred, the "head" of the particle showed similar internal material to that seen in the

round or ovoid agents (Fig. 13 to 15). The fine surface structure appeared similar to those of the myxoviruslike particles.

The relationship of these elementary bodies to mature mycoplasma cells can be seen in Fig.

FIG. 12. Tailed elementary body. Little or no penetration of phosphotungstate. Negative contrast. 200,000 $\times$. All bars on figures represent 0.1 μ .

FIG. 13. Tailed elementary body. Diffuse internal structure and surface spikes. Negative contrast. 200,000 $\times$.

FIG. 14. Tailed elementary body. Negative contrast. 200,000 $\times$.

FIG. 15. Two tailed elementary bodies. Negative contrast. 200,000 $\times$.

FIG. 16. Elementary body attached to wall of mature cell. Negative contrast. 50,000 $\times$.

FIG. 17. Tailed elementary body near mature cell. Negative contrast. 30,000 $\times$.

16 and 17. As was evident in thin sections, they may be found attached to, or budding out of (Fig. 16), or in close proximity to, the mature cells. This is evident from Fig. 17 where a tailed particle can be seen next to a mature cell.

DISCUSSION

Investigations of the fine structure of mycoplasmas have shown that these microorganisms exist in an array of morphological forms (Dormer-muth et al., 1964). Nevertheless, two classes of

particles have been found with consistency: mature cells with a diameter ranging from 500 to 700 μ , and elementary bodies with an average size of 100 to 150 μ . Depending on the technique employed, filaments have also been observed (Morton et al., 1954). All of these features were evident in the Negroni mycoplasma preparations. These diverse morphological forms can lead to confusion with certain viruses in interpretation of electron micrographs, especially when only a few mature mycoplasmas are found together with many of the small elementary bodies.

The preponderance of one element of a mycoplasma population over another appears to depend on the strain studied and upon the growth medium used. The composition of the media also seems to influence structural details. The double limiting membrane, for instance, was not evident in experiments in which mycoplasmas were propagated in tissue cultures, but were quite obvious when the strain was grown in cell-free mycoplasma medium (Hummeler, Armstrong, and Tomassini, 1965). Changes in structure and size of mature mycoplasmas are greatly influenced by the method of preparation for electron microscopy. In negative-contrast preparations, the size of mature cells was found to be up to twice as large as those observed in thin sections. This is probably due to the surface-tension forces exerted upon the organisms during the drying process. Thus, thin sectioning appears to be the method of choice for investigation of the ultrastructure of the mycoplasmas.

The difficulties encountered in interpretation of the fine structure of these agents when grown in cell-free media are compounded when they are encountered in vivo. Although the mature cells can be recognized, if only by their size, the elementary bodies are in the size range of viruses. Thus, the visualization of viruslike particles in human leukemic tissue must also include the possibility that mycoplasmas may be present. This is particularly evident in negative-contrast preparations, as can be seen in the appropriate figures. Thin sections are generally less confusing. The elementary bodies of mycoplasmas grown in broth have a fairly uniform size which is well within the size range of viruses. They may have a double membrane reminiscent of some viruses, but the interspace between the two membranes seems to be narrower in most particles, but not all, than that found in such viruses. It remains to be seen whether this is also true for the elementary bodies of mycoplasmas found in infected tissue cultures.

The electron microscopic investigations directed toward elucidation of the role of viruses

in malignant disease are now made even more difficult by the possible presence of mycoplasmas. It should, therefore, be of paramount importance to include tests for the presence of these organisms when "viruslike" particles are seen by electron microscopy. The definition of the order Mycoplasmatales presupposes a demonstration of characteristic growth on agar as a salient requirement. Although structures conforming to known mycoplasma morphology may be observed by electron microscopy, any method of detection circumventing characteristic growth in cell-free media begs the question.

It must be pointed out that the current investigation was limited to one strain of mycoplasma. It is quite possible that other strains isolated from human leukemia may show elementary bodies of smaller size and different morphology. Thus, each isolate may pose a separate problem.

Attempts at serological identification of the Negroni strain by Girardi et al. (1965) were unsuccessful. Recently, however, the agent was identified as *Mycoplasma pulmonis* by W. A. Clyde (personal communication).

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grants AI-04911 and CA-04534 from the National Institutes of Health.

LITERATURE CITED

- ALMEIDA, J. D., R. C. HASSELBACK, AND A. W. HAM. 1963. Virus-like particles in blood of two acute leukemic patients. *Science* **142**:1487-1489.
- BEARD, J. W. 1959. Virus tumors in cancer research. *Perspectives Virol., Symp.*, New York, p. 197-230.
- BRAUNSTEINER, H., K. FELLINGER, AND F. PAKESCH. 1960. On the occurrence of virus-like bodies in human leukemia. *Blood* **15**:476-479.
- BURGER, C. L., W. W. HARRIS, N. G. ANDERSON, T. W. BARTLETT, AND R. M. KNISELEY. 1964. Virus-like particles in human leukemic plasma. *Proc. Soc. Exptl. Biol. Med.* **115**:151-156.
- DMOCHOWSKI, L. 1958. Studies on submicroscopic structures of leukemias of known or suspected viral etiology: a review. *Blood* **13**:1017-1042.
- DOMERMUTH, C. H., M. H. NIELSEN, E. A. FREUNDT, AND A. BIRCH-ANDERSEN. 1964. Ultrastructure of *Mycoplasma* species. *J. Bacteriol.* **88**:727-744.
- GIRARDI, A. J., L. HAYFLICK, A. M. LEWIS, AND N. L. SOMERSON. 1965. Recovery of mycoplasmas in the study of human leukemia and other malignancies. *Nature* **205**:188-189.
- GRIST, N. R., AND R. J. FALLON. 1964. Isolation of viruses from leukemic patients. *Brit. Med. J.* **2**:1263.
- HAKALA, M. T., J. F. HOLLAND, AND J. S. HOROSZEWICZ. 1963. Change in pyrimidine des-

- oxyribonucleoside metabolism in cell cultures caused by mycoplasma (PPLO) contamination. *Biochem. Biophys. Res. Commun.* **11**:466-471.
- HAYFLICK, L. 1965. Tissue cultures and mycoplasmas. *Texas Rept. Biol. Med.* **23**(Suppl.1):285-303.
- HAYFLICK, L., AND R. M. CHANOCK. 1965. *Mycoplasma* species of man. *Bacteriol. Rev.* **29**:185-221.
- HAYFLICK, L., AND H. KOPROWSKI. 1965. Direct agar isolation of mycoplasmas from human leukemic bone marrow. *Nature* **205**:713-714.
- HUMMELER, K., T. F. ANDERSON, AND R. A. BROWN. 1962. Identification of poliovirus particles of different antigenicity by specific agglutination as seen in the electron microscope. *Virology* **16**:84-90.
- HUMMELER, K., D. ARMSTRONG, AND N. TOMASINI. 1965. Cytopathogenic mycoplasmas associated with two human tumors. II. Morphological aspects. *J. Bacteriol.* **90**:511-516.
- MORTON, H. E., J. E. LEECE, J. J. OSKAY, AND N. H. COY. 1954. Electron microscope studies of pleuropneumonia-like organisms isolated from man and chickens. *J. Bacteriol.* **68**:697-717.
- MURPHY, W. H., D. FURTADO, AND E. PLATA. 1965. Possible association between leukemia in children and virus-like agents. *J. Am. Med. Assoc.* **191**:110-115.
- NEGRONI, G. 1964. Isolation of viruses from leukemic patients. *Brit. Med. J.* **1**:927-929.
- PORTER, G. H., A. J. DALTON, J. B. MOLONEY, AND E. Z. MITCHELL. 1964. Association of electron-dense particles with human acute leukemia. *J. Natl. Cancer Inst.* **33**:547-556.
- SMITH, K. O., M. BENYESH-MELNICK, AND D. J. FERNBACH. 1964. Studies on human leukemia. II. Structure and quantitation of myxovirus-like particles associated with human leukemia. *J. Natl. Cancer Inst.* **33**:557-570.