GROWTH AND DEVELOPMENT OF T STRAIN PLEUROPNEUMONIA-LIKE ORGANISMS IN HUMAN EPIDERMOID CARCINOMA CELLS (HELA)¹

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Previous investigations have demonstrated the existence of basophilic, intracytoplasmic inclusions in epithelial cells obtained from the anterior urethra of nongonococcal urethritis (NGU) patients (Johnston and McEwin, 1945; Harkness, 1945, 1950; Williams, 1946; Harkness and Henderson-Begg, 1948). Pleuropneumonia-like organisms (PPLO) were frequently recovered in culture from these NGU patients in whom intracytoplasmic inclusions were demonstrated. This associative relationship was interpreted as suggesting that the inclusions may have been an intracellular phase of the pleuropneumonia-like organisms isolated in culture from such patients.

Highly characteristic, minute-colony T strains of pleuropneumonia-like organisms have recently been described (Shepard, 1956) which were recovered in primary culture from approximately 70 per cent of patients with nongonococcal urethritis. Furthermore, a majority of the NGU patients from whom T strain PPLO were recovered in culture also showed characteristic, basophilic intracytoplasmic inclusions in epithelial cells obtained from the anterior urethra. These inclusions exhibited structures that were consistent in size, morphology, and staining reaction to those of the pleuropneumonia group as a whole, and were interpreted as the tissue-phase of the T strain pleuropneumonia-like organism (Shepard, 1957). The demonstration of characteristic, basophilic inclusions in the cytoplasm of experimentally infected epithelial type cells by purified, cultured T strain PPL organisms would provide additional evidence in support of this interpretation. The results of such a study comprise the subject of this report.

METHODS

Test organism. A T strain pleuropneumonia-like organism, designated in this laboratory as strain 211B2, originally isolated from the urethral scrapings of an NGU patient in March, 1956, was selected for use in the experiment. This T strain was initially propagated in 7-day chick embryos by yolk sac route. After 4 passages by yolk sac route, successful passage could be maintained by allantoic route of inoculation. Following 15 passages in chick embryo, the T strain PPL organism was successfully propagated in minced chick embryo medium (Nelson, 1940). Transfers were made at 48 to 72 hr intervals. Sufficient adaptation of the organism had occurred by the 14th passage in minced embryo medium to allow successful propagation in an enriched fluid medium devoid of fresh cellular extracts. This medium (trypic heart infusion-ascitic broth) has been employed previously in our laboratory for the cultivation of pleuropneumonia-like organisms (Shepard, 1956). Two sublines of the T strain organism were employed. Subline A consisted of culture fluid from the 4th passage in trypic heart infusion-ascitic broth, which was quick-frozen and stored at −60 C. Subline B consisted of culture fluid from the then current 37th passage in trypic heart infusion-ascitic broth, quick-frozen and stored at −60 C. Transfers in 2 ml volumes of sterile fluid medium were made at 48 hr intervals and incubated at 36 C. Culture fluids were frozen 48 hr post inoculation. This T strain organism evidenced no detectable turbidity in fluid medium even after 37 consecutive transfers.

Cell culture. HeLa strain human epidermoid carcinoma cells (Scherer et al., 1953) were grown on flying cover slips in Leighton tissue culture tubes. They were cultivated in a nutrient medium consisting of 5 per cent human serum, 20 per cent filtered chick embryo extract (Bryant et al., 1953) and 75 per cent Earle’s balanced salt solution (Earle, 1943). A HeLa cell suspension was transferred to Leighton culture tubes in 1.0 ml volumes, gassed with CO₂, and incubated stationary at 37 C. By 72 hr a cell sheet of sufficient area and cell density had developed on the flying
Figure 1–6. HeLa cells experimentally infected with T strain pleuropneumonia-like organisms.

Figure 1. Early infection of HeLa cells by T strain pleuropneumonia-like organisms. The discrete, elementary body-like T strain particles are visible in moderate numbers scattered throughout the cytoplasm. Azure B eosin. 1600 X.

Figure 2. Early intermediate stage, showing large numbers of T strain particles visible in the cytoplasm. Giemsa. 1600 X.

Figure 3. Intermediate stage cell infection, showing partial loss of cytoplasmic boundary on the right. T strain PPL organisms are exhibiting a tendency toward formation of colony-like aggregates. Azure B eosin. 1600 X.

Figure 4. Late intermediate stage, illustrating characteristic appearance of large colony-like aggregates of T strain particles in addition to large numbers of individual organisms in the cytoplasm. Complete loss of cytoplasmic boundary has occurred. Azure B eosin. 1600 X.
cover slips to permit inoculation with a suspension of T strain pleuropneumonialike organisms.

Frozen culture fluids of sublines A and B (T strain no. 211B2) were thawed at room temperature, thoroughly mixed and 1.0 ml samples diluted $10^{-1}$ in Earle’s balanced salt solution. Three series of HeLa cell cultures were set up. Series I and II cell cultures received 0.1 ml of respective diluted culture fluids (sublines A and B). Series III cell cultures received 0.1 ml of sterile culture fluid (trypsin heart infusion-ascitic broth) diluted $10^{-1}$ in Earle’s balanced salt solution and served as uninoculated controls. The inoculated HeLa cell cultures and controls were incubated at 37°C. Flying cover slips from each of the three series were harvested 1, 2, 3, and 4 days postinoculation. They were washed in balanced salt solution 10 min (room temperature), fixed in absolute methanol 5 min, and stained 2 hr in Giemsa diluted 1:50 in pH 7 phosphate buffer, or buffered azure B eosin (Shepard, 1957).

**RESULTS**

HeLa cells were found to be highly susceptible to infection by the T strain pleuropneumonialike organism employed and to undergo progressive degeneration within 72 hr. Multiplication of the T strain organism was observed to be initially intracellular in position and to occur at a rapid rate. The organisms failed to multiply in HeLa cell nutrient medium in the absence of cells.

In stained flying cover slip preparations, the T strain PPL organisms appeared as monomorphic, round to ovoid, basophilic bodies estimated to be of approximately 330 μm particle size. They occurred mostly singly, frequently in pairs and occasionally in short chains of 3 to 4 elements in the cytoplasm of infected cells. Elongated, bacillary or rickettsia-like forms were observed only rarely (figure 5). The classic bipolar forms and annular forms (ring structures), characteristic of pleuropneumonia group organisms, were not observed in any of our preparations. The intracellular particles observed in the experimentally infected HeLa cells were indistinguishable in
morphology, size, and staining reaction from the elementary body-like cell inclusions (cocoid forms) seen in stained epithelial scrapings from NGU patients from whom T strain PPLO were isolated in primary culture.

Infection of the HeLa cells appeared to be well established by 24 hr postinoculation. Early infection was characterized by the scattered appearance in the cell cytoplasm of scant to moderate numbers of minute, discrete basophilic particles (figure 1). Their development in intracytoplasmic position was indicated by their occurrence at different focal planes within the confines of the same cell. Following early cell infection, multiplication of the T strain PPL organism proceeded at a rapid rate (figures 2 and 3). Large numbers of organisms were detected in the cytoplasm of intact cells and within interconnecting cytoplasmic bridges. A tendency of the organisms toward the formation of colony-like aggregates was apparent at this time (figure 3). By 48 hr, overwhelming infection of the HeLa cells had occurred, and T strain organisms could be observed both intracellularly and in extracellular position, involving large areas of cell sheet. Large, well-defined colony-like aggregates of T strain particles were frequently observed in the cytoplasm of infected cells at this time (figure 4). The period beyond 48 hr (and for many cells by 48 hr) was characterized by a period of progressive cell degeneration, accompanied by loss of cytoplasmic outline and necrotic changes in the nucleus. The organisms appeared to be predominantly extracellular, and to occur in intimate association with degenerating cells and cell fragments. The terminal phase, which appeared to be nearly complete by 72 to 96 hr for most of the infected cells, was characterized by a period of rapid cellular disintegration. Preparations harvested at this time showed principally cellular fragments (nuclear), small to large aggregates of organisms (figure 5) and large numbers of free T strain organisms (figure 6). Their identity was established by subculture from the cell cultures to appropriate PLO medium. Characteristic T strain PPL organisms were recovered in high log titer from all inoculated cell cultures. Pleuropneumonialike organisms failed to be detected microscopically or by subculture from un inoculated control cell cultures.

**DISCUSSION**

The above observations on experimentally infected HeLa cells indicate that T strain pleuropneumonialike organisms are capable of intracytoplasmic growth and rapid multiplication in HeLa cells. Following infection, the HeLa cells evidenced a progressive cytopathogenic reaction characterized by (1) an early stage without detectable cellular changes, but numerous T strain particles visible in the cytoplasm: (2) an intermediate stage in which the loss of cytoplasmic boundary (cell membrane) was the prominent feature, accompanied by a large increase in number of intracytoplasmic T strain particles; and (3) a terminal stage indicated by advanced necrotic changes and rapid, progressive disintegration, accompanied by the release of large numbers of free organisms. Certain infected cells had reached terminal stage by 24 hr. This observation serves to explain the appearance of extracellular organisms in the early infection stage. Our findings are not in agreement with the observations of Wittler et al. (1956) who found that a classic-type pleuropneumonialike organism (isolated from the urethral exudate of an NGU patient) produced little cellular damage when cultivated in HeLa cells, although organisms were found in the cytoplasm of infected cells.

As herein reported, the intracytoplasmic structures seen in the experimentally infected HeLa cells were morphologically indistinguishable from the intracytoplasmic inclusions observed in stained epithelial scrapings obtained from the NGU patient from whom T strain no. 211B2 was originally isolated. This observation provides additional evidence in support of the interpretation (Shepard, 1957) that the intracytoplasmic inclusions seen in urethral epithelial cells from nongonococcal urethritis patients are the tissue phase of T strain pleuropneumonialike organisms.

To date, over 400 cases of primary and recurrent NGU have been cultured in our laboratory for pleuropneumonialike organisms. Approximately 70 per cent of these patients have yielded T strain organisms in primary culture. Moreover, characteristic, basophilic intracytoplasmic inclusions in urethral epithelial cells have been demonstrated in stained urethral scrapings from a high proportion of these NGU patients. An associated picture of this magnitude is suggestive evidence that the T strain pleuropneumonialike organism may be one of the etiologic agents of nongonococcal urethritis in human males.

The findings with both the early and late passage sublines (4th and 37th, fluid-medium adapted) were identical. It was of interest to note that the principal morphologic form of this T
strain organism in HeLa cells was the elementary body-like coccoid form (Shepard, 1957). This observation is interpreted as suggesting that rapidly multiplying T strain organisms may, under certain conditions, give rise principally to coccoid forms. Pleomorphic, bacillariform, elongated or rickettsia-like elements were rarely observed and occurred only in material harvested during the terminal phase (72 to 96 hr). Annular forms and the classic bipolar forms were not seen. In stained epithelial scrapings obtained from the NGU patient from whom T strain No. 211B2 was isolated, both coccoid (monomorphic) and pleomorphic PPL forms were observed, with pleomorphic forms predominant. Had we employed a human epithelial cell line in which multiplication of the T strain organism developed more slowly than occurred in HeLa cells, a continuing trend toward pleomorphism might have been observed. Such a developmental trend is suggested by the appearance of pleomorphic structures adjacent to cells which were in the terminal stage. By 96 hr so few intact cells remained in the inoculated cell cultures that further observation on morphologic changes of the T strain organism was not possible.

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SUMMARY

A T strain pleuropneumonialike organism isolated from the urethra of a nongonococcal urethritis patient, propagated in 7-day chick embryo, and later adapted to serial propagation in artificial medium, was successfully grown in experimentally infected epithelial type cells (HeLa). Cell infection produced a progressive, degenerative cytopathogenic reaction. Rapid intracytoplasmic multiplication of the organism occurred, and was demonstrated by the technique described. This T strain PPL organism, as visualized in experimentally infected HeLa cells, exhibited intracytoplasmic structures that were indistinguishable in morphology, size, and staining reaction from the monomorphic inclusion bodies observed in parasitized epithelial cells from the urethral mucosa of nongonococcal urethritis patients. This evidence supports the premise that the intracytoplasmic inclusions observed in parasitized epithelial cells from the urethral mucosa of nongonococcal urethritis patients are the tissue-phase of T strain pleuropneumonialike organisms. The etiologic implications of these findings are discussed.

REFERENCES


