Reversion of a Pleuropneumonia-like Organism to a *Corynebacterium* during Tissue Culture Passage

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SUMMARY: A strain of a pleuropneumonia-like organism (PPLO) isolated from urethral exudate from a case of non-specific urethritis was studied in HeLa cell tissue cultures. Although the organisms entered the cell cytoplasm, they did not produce marked damage or proliferate luxuriantly until filtrate from a broth culture of *Staphylococcus pyogenes* or yeast extract was added to the infected tissue cultures. The organisms subsequently isolated from tissue cultures initially inoculated with PPLO and yeast extract showed conversion from PPLO form to L form of growth. Further culture of the L form, especially with the aid of mucin, resulted in conversion of the L form to a corynebacterium. This corynebacterium was indistinguishable culturally, biochemically and serologically from a corynebacterium isolated on rabbit blood agar plates which had been inoculated with a portion of the original urethral exudate from the same case of non-specific urethritis. The view is expressed that other human genital strains regarded at present as PPLO may be found to be L forms of *Corynebacterium* spp. The criteria for identification of PPLO and L forms are discussed.

During recent years pleuropneumonia-like organisms (PPLO) have been isolated with increasing frequency from the human urethra in the presence and in the absence of various inflammatory processes (Harkness, 1950; Melén & Linnros, 1952; Nicol & Edward, 1953). The pathogenic significance of the PPLO in genital infections, however, is controversial, and most investigators emphasize the need for more information about the organisms themselves before an aetiological role in genital diseases can be attributed to them. Keller & Morton (1954) reported that several human genital strains of PPLO produced no pathological manifestations in the developing chick embryo. No reports have come to our attention on the action of human PPLO on human cells in tissue culture, although Edward (1952) mentioned that tissue cultures might be useful tools for investigating the pathogenicity of PPLO. This investigation was undertaken, therefore, to obtain basic morphological data on the behaviour of a human urethral strain of PPLO in cultures of human cells. The manifestation of any pathogenic effects by the organisms upon the cells was sought. For comparison, the behaviour in tissue culture of a presumably non-pathogenic corynebacterium from the same source as the PPLO was observed. The role of secondary factors which might possibly alter the behaviour of the PPLO in the tissue cell environment was also investigated. The secondary factors chosen for

the study were (a) the filtrate from cultures of *Staphylococcus pyogenes*, another presumably non-pathogenic organism isolated from the same source as the PPLO, and (b) yeast extract as a substitute for *S. pyogenes* filtrate. The results of these studies brought to light an unsuspected relationship between the PPLO and the corynebacterium.

METHODS

Source and collection of organisms. The organisms used in this investigation were freshly isolated from urethral exudate from a patient with non-specific urethritis. Since this disease itself remains ill-defined, choice of the case was based on the criteria for differential diagnosis of non-specific urethritis outlined by Graham (1954). Although the patient had a past history of gonorrhoea, he had been successfully treated with penicillin. Cultures and smears of the exudate were negative for gonococci at the time of this study. Hanging-drop examination of the urethral specimen excluded *Trichomonas* infection, and serological examination of the patient's blood gave negative results in tests for syphilis.

The urethral exudate was collected on two cotton swabs. One swab was placed in a small amount of ascitic fluid+veal infusion broth containing 150 units penicillin/ml. and was cultured for PPLO and for L forms, i.e., the pleuropneumonia-like growth phase of ordinary bacterial genera. The other swab was kept moist with physiological saline solution and was cultured for bacterial species other than PPLO and L forms. The exudate was cultured within 3 hr. of its collection.

Species of organisms used. The three organisms recovered from the urethral exudate and used in this study were: PPLO, a *Corynebacterium* sp., and *Staphylococcus pyogenes* var. *albus*. The PPLO was a strain which did not ferment glucose and grew poorly if at all on media enriched with horse serum. It was identified as a PPLO rather than an L form on the basis of the cultural and morphological characteristics listed in Table 1. The *Corynebacterium* sp. was a strain non-toxicogenic for guinea-pigs and rabbits; it fermented glucose but not maltose or sucrose, was catalase-positive, reduced nitrate to nitrite, and did not hydrolyse urea or produce change in litmus milk. On tellurite agar it formed small, black, shiny, raised, smooth colonies.

Culture media. The medium used for isolation and maintenance of the corynebacterium and *Staphylococcus pyogenes* strains was trypticase soy agar containing 5 % (v/v) rabbit blood. Incubation of the *Corynebacterium* sp. was carried out in a candle jar.

The medium used for the PPLO was veal infusion agar (pH 7·6–7·7) containing 20 % (v/v) human ascitic fluid. For primary isolation (but not thereafter) 1 drop of a penicillin solution (10,000 units/ml.) was placed in a cup on the agar plate after inoculation of the specimen to prevent overgrowth by bacterial colonies. Veal infusion broth containing 20 % (v/v) human ascitic fluid was used whenever fluid cultures were desired.

Tissue cultures. The human epidermoid carcinoma cells (strain HeLa, from

Microbiological Associates, Bethesda, Maryland, U.S.A.; Scherer, Syverton & Gey, 1953) were cultured and maintained as described by Warren, Wittler & Vincent (1955). Before inoculation with any of the strains of organisms, the tissues were washed repeatedly with maintenance fluid to remove antibiotics originally incorporated in the growth fluids. Antibiotics were never added thereafter to the tissue cultures.

Staining methods. Conventional bacterial forms were stained with methylene blue and by the Gram method.

PPLO and L forms were stained by the method of Klieneberger-Nobel (1950) using impression preparations and Giemsa stain. The method of Dienes (1939, 1942), using direct staining of colonies on agar with methylene blue and azure stain previously dried on a coverslip, was combined with examination by phase contrast using a microscope with a long working distance condenser and an oil-immersion objective.

Tissue cells were stained *in situ* after removal of the maintenance fluids. The cells were rinsed rapidly with cold M/150-phosphate buffer (pH 7·2), fixed in cold methanol for 4 min., then rinsed again with buffer. When dry, the cells were stained in a 2% (v/v) solution of Giemsa for 30 min., rinsed once with buffer, and allowed to dry.

Serological methods. The *corynebacterium* strain used for rabbit immunization was grown in veal infusion broth containing 5% (v/v) rabbit serum. Growth was sedimented by centrifugation, resuspended in 2% (v/v) formolized saline, and allowed to stand at 5° for 24 hr. The organisms were then washed three times in saline, resuspended in 0·2% (v/v) formolized saline, and adjusted to match a no. 5 McFarland density standard. Rabbits were injected with increasing doses of the antigens on 4 successive days each week for 3 weeks. Agglutination tests were incubated at 52° for 18 hr. and then read.

The PPLO used for rabbit immunization was grown in ascitic fluid + infusion broth. The growth was sedimented by centrifugation, washed and resuspended in saline, and adjusted to match a no. 3 McFarland density standard. Rabbits were injected with 2 ml. of freshly prepared living antigen on 3 successive days each week for 3 weeks. Agglutination tests were carried out by the method of Edward (1950).

RESULTS

Behaviour of PPLO in HeLa cultures

A detailed study of the behaviour and appearance of the PPLO strain in HeLa cells was made, and evidence for all pathological changes in the infected tissue cells was noted. Multiplication of the PPLO was determined by subculture to agar plates. The appearance in tissue culture of the PPLO and of the HeLa cells was examined microscopically after staining with Giemsa solution.

An ascitic fluid + infusion broth culture of the PPLO was inoculated and passed serially every 6 days in HeLa cell cultures. Ordinarily 0·2 ml. of undiluted inoculum was used for each tissue culture plate during these and all following experiments. The PPLO were found in the cytoplasm but appeared

to cause little damage to the HeLa cells. By the second or third serial passage, the PPLO could no longer be recovered from the tissue cultures by subculture to agar. Small red granules and bodies similar to those described by Harkness (1950) were found in the Giemsa-stained HeLa cells infected with PPLO. These inclusions persisted during several further serial passages, but then were no longer discernible. Under these experimental conditions the PPLO itself did not induce gross pathological changes in the tissue cells, but seemed instead to have been destroyed or possibly lost during continued passage in tissue cultures.

Behaviour of Corynebacterium sp. in HeLa cultures

The ability of the corynebacterium to produce gross damage in the tissue cells was then studied for comparison with the PPLO. The procedures followed were similar to those previously used for observation of the PPLO strain.

The corynebacterium also appeared in the cytoplasm of the HeLa cells without producing marked damage. Agar subculture from the second or third tissue culture passage yielded no growth of the corynebacterium. By the fourth or fifth tissue passage, the infected Giemsa-stained HeLa cells showed only very few highly pleomorphic rods. It was, however, noted that inclusions resembling L forms of bacteria (Harkness, 1950; Wittler, 1952) were present in these tissue cells. These experiments indicated that the corynebacterium itself did not induce gross pathological changes in the tissue cells. There was, however, definite evidence that the bacterium was not at once destroyed or lost, but that it underwent a morphological change while in the tissue cultures. This type of change in the morphology of an organism during its sojourn in living cells had previously been described for *Haemophilus pertussis* in the mouse lung (Wittler, 1952).

Effect of the addition of Staphylococcus pyogenes filtrate to PPLO-infected HeLa cells

Klieneberger-Noble (1954) pointed out that it might '...be profitable to search for a second factor or second infective agent in natural diseases associated with PPLO, when a causal relationship between organisms and the disease has not been established.' In view of the lack of evidence in previous experiments for direct destructive action by the PPLO on the HeLa cells, the possibility of enhancing the pathogenicity or altering the behaviour of the PPLO in the cells in the presence of a secondary factor was investigated.

A bacteria-free infusion broth filtrate, made from 24 to 48 hr. cultures of the *Staphylococcus pyogenes* strain, was tested for its ability to supply a possible secondary factor. *S. pyogenes* was chosen since it was present together with the PPLO in the urethral exudate, and since members of this genus are quite commonly found in such exudates. The filtrate was added in 0.2 ml. amounts to HeLa cultures infected with second or third serial tissue culture passage PPLO. Within 48 hr. after addition of the *S. pyogenes* filtrate, the HeLa cells were laden with structures resembling PPLO, and PPLO grew luxuriantly

when such cells were subcultured on agar. Such HeLa cells, inoculated with PPLO together with *S. pyogenes* filtrate, showed very extensive damage. Relatively moderate damage was produced in control cells treated with the filtrate alone. Veal infusion broth alone, tested in other sets of control cells, did not produce cell destruction. Infusion broth added to PPLO-infected cells did, however, enhance the growth of the PPLO sufficiently to permit recovery of at least a few organisms on the agar subcultures. These results indicated that factors in *S. pyogenes* broth filtrate were capable of altering the behaviour of PPLO in HeLa cultures, so that luxuriant growth of the PPLO occurred and concomitantly the tissue cells were destroyed.

Effect of the addition of yeast extract to PPLO-infected HeLa cells

Edward (1947) reported that yeast extract, as well as staphylococcal culture filtrate, added to artificial culture media enhanced the growth of certain strains of PPLO. The ability of yeast extract to replace *Staphylococcus pyogenes* filtrate in PPLO-infected tissue cultures was, therefore, tested. It was found that the addition of 0.5% (v/v, expressed as final concentration throughout this report) yeast extract caused an increase in growth of the PPLO comparable to that which occurred when *S. pyogenes* filtrate was used. Furthermore, partial destruction of the HeLa cells also occurred under these conditions, although this amount of yeast extract caused only slight damage to uninfected HeLa cells. Increasing the yeast extract to 4% resulted in total destruction of the HeLa cells but still allowed a great increase in growth of the PPLO to take place.

The PPLO which were subcultured to agar from the yeast extract-treated tissue cells showed a striking change in colonial form. Originally the PPLO colonies were small and discreet, grew deeply into the agar, showed a typical dense centre with lighter periphery, and were composed mainly of tiny granule-like particles and small vacuoles. Pl. 1, fig. 1, illustrates the typical morphological features of a stock transfer of the PPLO strain on agar before passage in tissue culture. The altered PPLO colonies which developed after tissue culture passage in the presence of yeast extract were quite large and spreading. They did not grow as deeply into the agar and often did not show a well-defined centre. They were composed of large globules and vacuoles, large and small bodies, and masses of amorphous material in addition to the granule-like particles. These colonies resembled L colonies (similar to large L colony in Pl. 1, fig. 2) rather than classical PPLO colonies (Table 1). Furthermore, the infected Giemsa-stained HeLa cells treated with yeast extract showed, in addition to the small red granules and bodies, various inclusions resembling L forms and occasionally small rods morphologically similar to the corynebacterium. Pl. 1, figs. 3 and 4, illustrate infected Giemsa-stained HeLa cells treated with yeast extract. The cells were photographed before maximum cytoplasmic destruction had occurred. At this stage the PPLO had grown luxuriantly, and large numbers of the organisms were packed in the cell cytoplasm. The small individual granules and the pleomorphic elements resembling L forms and rod forms all of which composed the clusters may be best

distinguished lying free of the cytoplasm or near the edges of the cells. Yeast extract and *Staphylococcus pyogenes* filtrate appeared to be equally capable of enhancing the growth of PPLO in tissue culture and of causing tissue cell damage. The yeast extract, however, brought about a striking morphological change in the PPLO. Whether the factor responsible for this change in the PPLO is present also in *S. pyogenes* filtrate remains to be investigated.

Table 1. *Cultural and morphological characters used for distinguishing between PPLO and L forms of bacteria*

The distinctions tabulated here are based on published data from Klieneberger-Nobel (1954), Edward (1954), and on personal observations.

Criteria	PPLO	L forms
Growth in broth	Very faint turbidity; very fine colony clumps visible only with magnification	Light turbidity; larger colony clumps, 0.5–1.0 mm., easily visible
Growth on agar	Colonies small (approximately 0.1–0.3 mm.), round, circumscribed, transparent, with fine surface markings Distinct central 'button' Grow deeply into the agar	Colonies larger (approximately 0.5–1.0 mm.), frequent irregularity in shape, more opaque, with coarser surface markings Central 'button' not always well defined May not grow as deeply into the agar
Relative stability upon initial isolation	Cultures established easily on agar and retain typical colonial form after subculture	Cultures established with difficulty on agar and especially in broth because of frequent instability during early passages; may die off or revert to bacterial form
Microscopic appearance of elements composing colonies	Minimal reproductive units regularly arranged and lying more or less in one plane Bodies frequently fairly homogeneous in shape and size Relatively little amorphous material present 'Myelin' structures not abundant or conspicuous by darkground microscopy	Minimal reproductive units irregularly arranged, often in clumps, and lying in various planes Bodies frequently highly pleomorphic in shape with large variation in size Much amorphous material frequently present 'Myelin' structures frequently abundant and conspicuous by darkground microscopy

Reversion of PPLO to a corynebacterium

The foregoing experiments showed that under certain conditions in tissue culture both the corynebacterium and the PPLO could assume the L form, and that the PPLO could possibly appear in a bacillary form. It was, therefore, necessary to ascertain whether the PPLO was, in fact, related to, or perhaps was the L form of the *Corynebacterium* sp.

The PPLO subculture used in this particular test was one which had never been exposed to a tissue culture environment but had been transferred regularly on agar for many months. A broth subculture of the PPLO was inoculated

into HeLa cultures, and the fluids were harvested from the tissue cultures after 6 days. A second serial passage was performed and again harvested on the sixth day. Agar subcultures at this point yielded a light growth of typical PPLO colonies. The harvest material was passed a third time in HeLa cultures, but this time yeast extract was added to a final concentration of 4 % in broth. Growth was harvested after 3 days, since the tissue cells were by then almost completely destroyed by the high concentration of the yeast extract. Rod forms, as well as the usual granular and pleomorphic forms, could be observed in the cytoplasm of Giemsa-stained tissue cells, but agar subcultures yielded only large L form colonies. Bacillary colonies were not obtained at this point. A fourth serial passage of the harvest fluids was made in HeLa cells, this time using only 0.5 % yeast extract in broth. Pl. 1, fig. 5, illustrates the appearance of some of the organisms in these HeLa cells 3 days after inoculation. In this Giemsa-stained preparation, lying at the edge of the destroyed tissue cells, is a cluster of large L bodies, small granular forms, and long filamentous forms in which the purple rod forms were taking shape. The rod forms could easily be distinguished in the stained preparation by their colour and shape, but are difficult to identify in the photograph. Fluids from these HeLa cultures were harvested on the third day and cultured on rabbit blood agar plates which were incubated in a candle jar. Duplicate cultures were made on ascitic fluid + veal infusion agar plates. After 5 days L colonies had appeared over the whole inoculated surface of the blood agar plates. In addition, there were one or two small corynebacterium colonies on each of these blood agar plates. To encourage if possible a conversion of the L form to a corynebacterium form, sterile hog gastric mucin, which has a growth-promoting effect on corynebacteria (Maccabe & King, 1951), was spread carefully over a part of the surface of the grown plates where only the L form colonies were present. A wide area around the corynebacterium colonies was left undisturbed. After re-incubation for another 2 days, the areas coated with mucin yielded pure cultures of corynebacterium colonies, whereas the areas without mucin showed no further development of corynebacterium colonies nor any change in the L colonies. The ascitic fluid plates which received no mucin yielded only a mixture of PPLO type and L type colonies. Pl. 1, fig. 6, illustrates the mixture of L and bacillary colonies which formed along the edge of the mucin-coated areas on the blood agar plates. The L colonies, composed of scattered granules and pleomorphic elements, were lying deeper in the agar than the dark bacillary colonies. The white forms in the bacillary colonies are rods which are lying above the level of focus. On the basis of the observed morphological changes, it appeared that conversion from PPLO through L form to a corynebacterium had taken place, but further evidence was needed to rule out the possibility of chance contamination having occurred.

Biochemical and serological studies of the derived corynebacterium strains

The two corynebacterium strains derived from PPLO on the blood agar plates, one in the presence of mucin, the other in the absence of mucin, were compared biochemically and serologically to determine their relationship to

the corynebacterium strain originally isolated from the urethral exudate. Culturally and biochemically these three strains were identical. All three fermented glucose but not maltose or sucrose, were catalase-positive, reduced nitrate to nitrite, did not hydrolyse urea or produce change in litmus milk. They produced small, black, shiny, raised, smooth colonies on tellurite agar.

Serologically the original strain and the two derived strains of corynebacterium were also identical. All three were agglutinated by a 1/1280 dilution of rabbit antiserum prepared against the original *Corynebacterium* sp. at the time of its isolation from the patient (Table 2). Moreover, the PPLO strain

Table 2. *Serological reactions of corynebacterium strains recovered, respectively, from initial urethral culture and from PPLO culture following tissue culture passage*

Strains	Agglutination titre of rabbit serum*	
	Anti-corynebacterium	Anti-PPLO
Original <i>Corynebacterium</i> sp.	1/1280	1/20
Corynebacterium derived from PPLO grown in tissue culture and subcultured on blood agar	1/1280	1/20
Corynebacterium derived from PPLO grown in tissue culture and subcultured on blood agar with mucin	1/1280	1/20
Original PPLO	1/640	1/1280

* Antisera were prepared against original strains of the organisms shortly after their isolation from clinical material.

was agglutinated by a 1/640 dilution of the anti-corynebacterium serum. Rabbit antiserum prepared against the original PPLO strain was also tested against the original and the two derived strains of corynebacterium. In each case agglutination occurred at a dilution of 1/20, whereas the PPLO strain was agglutinated by its homologous antiserum at a dilution of 1/1280. The growth inhibition test described by Edward & Fitzgerald (1954) was also employed to obtain additional evidence of antigenic relationship between the several growth forms, PPLO, L and bacillus. Anti-corynebacterium serum and anti-PPLO serum both inhibited growth of the PPLO form and the L form completely at 1/500 and partially at 1/1000. Several pools of normal rabbit serum used as controls did not cause any inhibition of growth of PPLO or L forms. These experiments showed that a definite antigenic relationship existed between the PPLO and the corynebacterium isolated from the original specimen, and strongly suggested the corynebacterium was actually derived from the PPLO.

DISCUSSION

The PPLO strain originally recovered from the urethral exudate and used in this study appeared to be a 'true' PPLO and not an L form; colonial appearance and morphological features were typical of PPLO. Early subcultures of the organism were established with ease in contrast to the difficulty of establishing

L phase cultures in stable form. All colonies appeared upon original isolation in equal distribution both near to and distant from penicillin in a cup on the agar. The strain was in no instance exposed to penicillin or other inhibitory agents after its first appearance on the original plates. Furthermore, this strain of PPLO, which has been carried for about one year in continuous stock passage on agar with frequent transfers to broth, has shown no tendency to revert to a bacillary form or even to that L stage which consists primarily of large bodies.

The corynebacterium strain isolated in a different section of the laboratory from a duplicate original specimen and kept completely separate from the PPLO cultures gave no evidence of being in the more or less labile L stage. In fact efforts to produce PPLO and L form colonies from the corynebacterium by cultivation on agar with penicillin, glycine, salts or by other methods failed to induce this conversion. Only in tissue culture was evidence obtained that this corynebacterium strain was capable of undergoing conversion at least to the L stage.

The experiments reported here have been carried out in detail only on the organisms isolated from a single case of non-specific urethritis. Work now in progress indicates, however, that the same relationship exists between PPLO and *Corynebacterium* sp. isolated by us from other cases of non-specific urethritis. A relationship between PPLO and the genus *Corynebacterium* has also been reported by other investigators. Minck (1953) showed that a number of so-called PPLO strains from the female genital tract were actually the L forms of corynebacteria. Peoples, Smith & Morton (1955) reported on the association of diphtheroids with the 'Campo' strain of PPLO in broth cultures. The question, therefore, arises: are the numerous strains of so-called PPLO isolated so commonly from the mucous membranes of human beings and animals in fact L forms whose bacterial parentage could be demonstrated if adequate efforts were made to reveal the relationship?

There is indeed an increased awareness of the possibility of confusing L forms with 'true PPLO'. Recent reviews by Edward (1954) and Klieneberger-Nobel (1954) and earlier reviews by Dienes & Weinberger (1951) and Tulasne (1951) deal at length with the similarities of and the distinctions between PPLO and L forms. Colonial appearance, cultural characteristics, morphological features, growth requirements, and the like are undoubtedly useful at times in attempting distinction between growth in the PPLO phase and growth in the L phase, but there is at other times sufficient overlapping of characteristics for these criteria to become wholly inadequate for classification purposes. Serological and metabolic properties are at present frequently disregarded when relationship to a parent bacterial form is unknown or unsuspected. The discussion of Dienes & Weinberger (1951) is pertinent in this respect and is here quoted in full: 'Organisms of the pleuropneumonia group usually can be isolated from the human throat and from the female genitals. These organisms were studied by several authors and their classification with the pleuropneumonia group is not questioned. No connexion of these organisms to bacteria is apparent. The reviewers believe that if the

3A L forms of *Salmonella* which do not return to bacillary forms were cultivated from similar sources, they would be regarded also as members of the pleuropneumonia group. There is nothing in the appearance of the colonies or in the morphology of the organisms to differentiate them from this group. Only the study of the serological and metabolic properties would identify them as *Salmonella* L cultures.'

It is our opinion that ordinary bacterial strains can exhibit all the characteristics of the PPLO form of growth as well as those of the L form, and furthermore, that the L form probably represents a transitional phase between bacillary and PPLO growth forms. Whereas an organism can be identified as the L form of a bacterium on the basis of serology, metabolism, or demonstration of actual conversion, there are no adequate criteria as yet for positive identification of an organism as a PPLO. Although a strain appears to be a PPLO because its growth form is 'fixed' and stable on artificial media, one should not assume that this will necessarily be so *in vivo*. From our observations, it seems likely that factors which produce changes in the host cells may in turn result in changes in the form or in the type of growth of the infecting organism, yet this may not always be readily apparent when the organism is again cultured *in vitro*. In the absence of actual demonstration of conversion from one to another growth form, the too rigid use of metabolic activities, growth requirements, perhaps even antigenic components as criteria of genetic relationship between growth forms so unlike as PPLO and bacilli may at times be misleading. For instance, the PPLO strain used in this study did not ferment glucose or any sugars tested, whereas the *Corynebacterium* spp. derived from the PPLO did ferment glucose.

The possible pathogenicity of the *Corynebacterium* sp. in its PPLO growth form or otherwise merits further study. There was clear-cut experimental evidence that in the presence of yeast extract or *Staphylococcus pyogenes* filtrate, the PPLO form of the corynebacterium was capable of multiplying rapidly in HeLa cultures and of producing extensive damage to the cells. It remains to be investigated whether or not the organism is capable of exhibiting toxigenic properties during some stage of its conversion from one growth form to another which might account for this cytopathogenic action. The experiments here reported do, in any case, suggest a promising line of approach for studies on the nature and pathogenicity of other organisms of the ill-defined pleuropneumonia group.

The authors wish to thank Dr Aston B. Greaves (District of Columbia Polk Health Center) for supplying the clinical specimens used in these studies; Major D. H. Hunter (Department of Bacteriology, Walter Reed Army Institute of Research) for advice and assistance on the serological tests; Cpl. Esther Steinhilber (Walter Reed Army Institute of Research) for technical assistance in maintaining tissue cultures; and the Medical Illustration Service of the Armed Forces Institute of Pathology for assistance with the photography.

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EXPLANATION OF PLATE 1

Fig. 1. Seventy-two-hour culture of stock transfer of PPLO strain on ascitic fluid + veal infusion agar. Dienes's stain and phase contrast; $\times 360$.

Fig. 2. Seventy-two-hour growth of the L form of a *Corynebacterium* sp. on ascitic fluid + veal infusion agar. Dienes's stain and phase contrast; $\times 900$.

Figs. 3, 4. HeLa cells photographed 72 hr. after inoculation with PPLO and yeast extract. The PPLO forms show as grey or black granules lying both intra- and extra-cytoplasmically. A large cluster of pleomorphic elements composed of L bodies and rod-shaped forms in a mass of amorphous material is lying free of the cells. Giemsa stain; $\times 1350$.

Fig. 5. HeLa cells photographed 72 hr. after inoculation with fourth serial passage PPLO and yeast extract. Large L bodies, small granular forms, and long filamentous forms are seen lying at the edge of the destroyed HeLa cells. The developing rod forms show as slightly darker and thicker portions of the filamentous forms. Giemsa stain; $\times 2700$.

Fig. 6. Seven-day growth of L and bacillary colonies of *Corynebacterium* sp. on rabbit blood agar plate treated with mucin. The bacillary colonies are composed of the large very dark elongated forms and the L colonies of the grey scattered granular and diffuse elements. Dienes's stain and phase contrast; $\times 900$.

(Received 11 January 1956)

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