Conversion of Pleuropneumonialike Organisms to Bacteria.* (23536)

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The relationship of pleuropneumonialike organisms (PPLO) to L forms and to bacteria is unknown. By definition the difference between L forms and PPLO is that the L forms are known to be associated with some bacterial species while the PPLO are not. Although there are innumerable instances of the production of L forms from bacteria and the reversion of the L forms to bacteria, no definitive proof of such a conversion of PPLO to a bacterium has been reported. Minck(1) reported that 5 PPLO isolates from the female genital tract developed into diphtheroids when inoculated into broth. McKay and Taylor(2) described the reversion of poultry strains of PPLO to bacteria similar to *Hemophilus gallinarum* when inoculated from fowl or chick embryo onto solid medium in the presence of added carbon dioxide and partial vacuum. They concluded that these organisms were not PPLO but L forms. Peoples *et al.*(3) reported on the association of *Corynebacterium* and the Campo strain of PPLO. Recently Wittler *et al.*(4) described the reversion of a strain of PPLO isolated from the human urethra to *Corynebacterium* following the inoculation of tissue cultures. This *Corynebacterium* was indistinguishable from another *Corynebacterium* isolated from the same urethral specimen.

A crucial test to establish the relation of PPLO to bacteria consists of causing the conversion of a well established strain of PPLO to a bacterium, reliably establishing the relationship of the two, and finally bringing about the production of the strain of PPLO from the derived bacterium. This report relates the findings of such an experimental attempt.

Materials and methods. The strain of PPLO employed for most of this study was Campo "L", obtained in 1949 from Dr. Dienes who isolated it from the human urethra and characterized it as a typical PPLO. It has been maintained on artificial solid medium for more than 10 years without evidence of reversion to a bacterium. In our laboratory it has been subcultured weekly on solid medium for several years. Two other strains, V73, isolated in our laboratory from the human urethra(5), and SF29, isolated by Carter(6) from the lung of a calf with bronchopneumonia, were examined with respect to association with bacteria. Statistical tests were designed and calculations performed by Dr. Max Woodbury, New York University. The tests involved were of the chi square method modified to account for (1) the probability that contamination of cultures occurred, (2) the probability that the contaminant could be a diphtheroid and (3) the probability that the contaminant could be a nondiphtheroid. Antigens for PPLO were prepared by growing the organisms in large quantities as previously described(7), with the exception that penicillin was added at a level of 100 u/ml to prevent the formation of diphtheroids. Antigens for the diphtheroids were prepared by harvesting 18-24 hour cultures grown in heart infusion broth. Both types of organisms were sedimented under sterile conditions in a Servall angle centrifuge, repeatedly washed in saline and resuspended in saline with "Falba" added as adjuvant. Considerably better titers of antisera were obtained with the use of the adjuvant. Duplicate albino rabbits were injected intramuscularly with 2 ml of the "Falba" adjuvant antigen, weekly, until each animal had received approximately 2.0 mg of cellular nitrogen. The animals were rested for one week and bled by cardiac puncture. Antigens for agglutination tests consisted of saline suspensions of organisms adjusted to give a Klett reading of 100. Standard tube agglutination tests were found to be satisfactory and therefore were used for all serological tests. Fermentation tests were run in either bromcresol purple or phenol red broth with added one per cent carbohydrate. Tubes were ob-

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served for acid production and growth over a period of 2 weeks.

Results. Considerable variation in the growth of the Campo strain of PPLO has been noted over the past 5 years. In addition to loss of the requirement for the lipoprotein growth factor(8), it has been observed that when large volume broth cultures of this strain were grown for use in metabolic studies bacteria were encountered very frequently in the absence of thallium acetate. The bacteria never were observed to appear on agar cultures of the Campo strain of PPLO and rarely in small numbers of 10 ml broth cultures. The high frequency of these bacteria in large volume cultures prompted an investigation of the incriminating organisms. Upon subculture onto solid medium, the bacteria grew as very minute colonies, approximately 5x the diameter of the Campo colonies. They were slightly raised with irregularly shaped, dense centers, could be transferred with an inoculating needle and grew without the added PPLO serum fraction. However, added 5% yeast extract greatly stimulated growth. Morphologically, they were Gram-positive, short, club-shaped rods and occasionally beaded. Fermentation tests revealed that like the Campo strain of PPLO, they were inert to all the carbohydrates tested. On the basis of these findings, the bacterium was identified as belonging to the genus *Corynebacterium*. Similar results were obtained with strains V73 and SF29.

Statistical tests. Initially, attempts were made to establish on a statistical basis whether the diphtheroids were appearing as contaminants or whether a relationship existed between them and the PPLO strain. Consequently, 2 statistical tests were devised. In the first experiment, 100 tubes, each containing 10 ml of the PPLO medium, were inoculated with agar blocks containing colonies of the Campo strain. These agar blocks did not contain any bacterial colonies upon observation at 100X magnification. An additional like 100 tubes were inoculated with sterile agar blocks. The investigator performing the inoculation was unaware of the type of agar block used for inoculum. Following 3 through 7 days of incubation at 37°C all the tubes were subcultured onto solid medium and those appearing to contain bacteria were examined by Gram staining. PPLO was found to be growing in all tubes inoculated with the Campo strain. Table I shows the results of this first experiment. Statistical analyses revealed that the probability of the diphtheroids being a contaminant was less than 1 in 10,000.

TABLE I. Frequency and Type of Isolations after Inoculation of 100 Tubes of Broth.

	1st exp.		2nd exp.	
	Inoc. with Campo PPLO	Control, inoc. with sterile agar blocks	Inoc. with Campo PPLO	Control, inoc. with sterile broth
Campo only	79		93	
Sterile		96		98
Diphtheroids	18	0	5	1
G-positive cocci	1	0	2	0
G-negative rods	2	4	0	1
Total	100	100	100	100
X^2	20.162		2.886	
P	1:10,000		1:10	

Since there was a chance that microscopic bacterial colonies were present on the agar inoculum and since the diphtheroids appeared only in liquid medium, a test was devised to select against the occurrence of the bacteria. The second experiment was identical to the first except that the inoculum used was 0.1 ml of pooled broth cultures of the Campo strain free from bacterial growth and of sterile broth. Broth cultures of the Campo strain prepared for inoculum which exhibited diphtheroids were discarded. The results of this second experiment are shown in Table I. As would be expected when employing an inoculum selected against the formation of diphtheroids, the probability that the diphtheroids were contaminants increased. However, it was still within the limits of 10%.

Repeated experiments of these 2 types by different investigators gave approximately the same results over a period of 3 years. However, it was found that the addition of 5% yeast extract aided in the production of the diphtheroids.

TABLE II. Cross Agglutination between PPLO Strains Expressed as Reciprocal Titers.

Antisera	Antigen: Campo PPLO	O7 PPLO	J PPLO
Campo PPLO	1024	32	1024
O7 "	"	1024	"
J "	0	0	2048

Biochemical tests. Examination of the fermentative ability of 15 diphtheroids isolated from Campo PPLO cultures and the Campo strain of PPLO showed that neither of these 2 types of organisms produced acid in the presence of glucose, maltose, sucrose, lactose, fructose, mannitol, inositol and salicin. The organisms multiplied in these test cultures. All diphtheroids isolated as air contaminants and from the oral cavity of the investigators were shown to be capable of producing acid from some of the above mentioned carbohydrates.

Serological tests. Although statistical tests indicated that the diphtheroids were not contaminants and that the diphtheroids isolated from Campo cultures differed from diphtheroids isolated from the air and the oral cavity in their fermentative capacity toward carbohydrates, a more reliable relationship was sought by the use of serological tests.

Table II shows that a relationship exists between 2 human urethral strains, Campo and 07, and a poultry strain, J, of the pleuropneumonialike group. The inability of the J antiserum to agglutinate Campo and 07 antigens can be explained by interference of antibody production to a minor antigen by a major antigen(9).

The cross agglutination reactions of the Campo PPLO, the Campo diphtheroid (Campo D) and the V73 diphtheroid (V73D) are shown in Table III. PPLO strains, 07 and J, failed to react significantly with the diphtheroids, although there is a cross reaction between

TABLE III. Cross Agglutination between PPLO and Diphtheroids.

Antisera	Antigen: Campo PPLO	Campo D	V73 D
Campo PPLO	1024	64	16
" D	16	1024	128
V73 D	512	256	1024
PPLO strain, O7	1024	ND	0
" " , J	0	"	4

ND = not done.

these 2 PPLO and the Campo PPLO. No antigen or antiserum was prepared for the V73 PPLO because of the poor capacity of this strain to grow in liquid medium. Table IV shows the results of cross agglutination reactions between the Campo PPLO, the Campo diphtheroid and the V73 diphtheroid after reciprocal adsorptions. As can be seen reciprocal adsorptions removed the common antibodies in all instances.

Conversion of diphtheroids to PPLO. Numerous attempts have been made to derive a PPLO from the diphtheroids isolated from Campo cultures. In no instance was an L type colony reliably identified. Among the methods used in these attempts were the use of high concentrations of penicillin with and without added salts(10), cold shock and the use of glycine(11).

TABLE IV. Cross Agglutination after Adsorption with PPLO and Diphtheroids.

Antisera	Cells used for adsorption	Antigen: Campo PPLO	Campo D	V73 D
Campo, PPLO	Unadsorbed	1024	64	16
	Campo, PPLO	0	0	0
	" D	64	0	ND
	V73 D	64	ND	0
Campo D	Unabsorbed	16	1024	128
	Campo, PPLO	0	512	ND
	" D	0	0	32
	V73 D	ND	8	0
V73 D	Unadsorbed	512	256	1024
	Campo, PPLO	0	ND	16
	" D	ND	0	4
	V73 D	0	16	0

ND = not done.

Discussion. The combined results of the various studies established that a relationship exists between the diphtheroids isolated from the PPLO cultures and certain strains of PPLO. First, statistically designed tests showed that the bacterium found most frequently in the PPLO cultures, a species of *Corynebacterium*, was not a contaminant, even when the inoculum was selected against the presence of PPLO capable of producing diphtheroids. Second, morphological and biochemical studies indicated that the diphtheroids isolated from the PPLO cultures were identical with one another but different from air and oral diphtheroids. Third, a definite serological re-

lationship was found between those PPLO giving rise to diphtheroids and the derived diphtheroids but no serological cross reaction between PPLO strains not giving rise to diphtheroids and the derived diphtheroids. Although it is apparent that the Campo and V73 strains can be converted to diphtheroids, final confirmation of the phenomenon cannot be made until serological tests show the diphtheroids from PPLO cultures are similar or identical with one another but different from contaminant diphtheroids and until the diphtheroids are converted to a PPLO similar or identical to the parent PPLO.

Eventual establishment of a relationship between all PPLO strains and some microbial species is not improbable. The conversion of bacteria to a stable L form can be considered to involve a mutation or mutations, either spontaneous or induced. Conversion of a PPLO, which can be considered a stable L form, although the related bacterium is unknown, to a bacterium can likewise be considered to involve a mutation or mutations. In the case of the 3 strains which exhibited conversion to diphtheroids in this study, spontaneous mutation could be operative. Other strains, which have not shown a tendency to convert may have such a low mutation rate that conversion is not evident. If the proper conditions were employed conversion might occur in all PPLO strains. Presently these conditions are unknown. Additional evidence that PPLO may be related to some microbial species is the widespread ability of bacteria to form L colonies and protoplasts.

Summary. A high frequency of appearance of diphtheroids in liquid culture of 3 established strains of pleuropneumonialike organisms has been noted. Statistical, morphological, biochemical and serological tests have shown that a relationship exists between these diphtheroids and the pleuropneumonialike organisms in which cultures the bacteria appear. All PPLO strains tested are serologically related. PPLO strains not giving rise to diphtheroids do not cross react with diphtheroids. Diphtheroids appearing in PPLO cultures differ from oral and air diphtheroids.

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Chlortetracycline in the Nutrition of Guinea Pigs.*† (23537)

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Numerous reports indicate that chlortetracycline (aureomycin) is harmful to the guinea pig. Roine *et al.*(1) have reviewed the literature and have explained the toxicity of orally administered aureomycin on the basis of a change in the intestinal flora. They found in treated animals an abundance of organisms that belong to the genus *Listeria,* and believed this organism to be the cause of death. Eyssen *et al.*(2) found penicillin, baci-

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