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Induction of L Forms of *Haemophilus influenzae* in Culture and Their Demonstration in Human Bronchial Secretions

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Transitional forms and round bodies of *Haemophilus influenzae* were identified in sputa from patients with chronic bronchitis who were receiving penicillin therapy for *H. influenzae* infections. In vitro growth of L forms of this organism was induced by penicillin and glycine and was studied for comparison with development in vivo. Variant forms demonstrated in sputum were similar to variant forms observed in penicillin-induced L colonies. Recurrence of infection after cessation of therapy was related to reversion of persisting L forms to bacillary forms. That these forms were derived from *H. influenzae* was established by direct staining with fluorescein-labeled specific antibody. This demonstration that transitional forms and round bodies of *H. influenzae* occurred in vivo suggests that L forms of bacteria may be significant in chronic or recurrent infections.

The in vivo production of L forms of bacteria has been suggested as a factor predisposing towards the development of recurrent or chronic infection (4, 10, 16); however, few direct demonstrations of the occurrence or persistence of these bacterial variants in vivo have been made. Wittler (18) described the conversion of *Bordetella pertussis* to L forms in the lungs and peritoneal cavities of mice; Carey, Muschel, and Baron (1), the formation of protoplasts of *Salmonella* and *Shigella* in peritoneal cavities of experimental animals; Guze and Kalmanson (5), the persistence of *Streptococcus faecalis* protoplasts in the kidneys of rats with enterococcal pyelonephritis; and Mortimer (14), the isolation of L forms of group A streptococci from mice killed by streptococcal infections. Recently, Gutman et al. (4), studying patients with chronic renal infections and pyelonephritis, isolated L forms or protoplasts from filtered urine specimens. They observed variant forms most often in patients on antibiotic therapy and found that bacteriuria resulting from the parent strain recurred within 1 week after cessation of therapy.

Haemophilus influenzae is the organism most frequently associated with persistent infections of the bronchial mucosa in patients with chronic bronchitis, and recurrence of infection is common after seemingly successful antibiotic therapy (15). *H. influenzae* is prone to pleomorphism in an unfavorable environment and can readily be converted in vitro to L-form growth by inducers such as glycine or penicillin (2). Since penicillin is used often in treatment of chronic bronchitis, it is possible that conversion of the organism from bacillary forms to L forms or protoplasts could occur in the lungs of patients under such therapy.

The studies presented here were undertaken to determine whether L forms or protoplasts of *H. influenzae* are induced in the respiratory tracts of patients with bronchitis during treatment with penicillin. The conversion by penicillin and glycine in vitro was also studied to identify stages in conversion from and reversion to bacillary form for comparison with development in vivo.

MATERIALS AND METHODS

Organisms. All strains of *H. influenzae* used in these studies were isolated from sputa of patients with chronic bronchitis who were hospitalized at the Veterans Administration Hospital, Madison, Wis. Bacillary inocula for induction media consisted of 6-hr Levinthal broth cultures of the test strains.

L-form induction media. Trypticase Soy Agar (BBL) plus Levinthal broth (2:1) produced a soft agar which proved suitable for development of *Haemophilus* L colonies without additional salt or sucrose, and was used as the base for all induction media. Penicillin was added in concentrations ranging from 15 to 50 units per ml before pouring media. Penicillin gradient plates were prepared by adding either 1,000 or 10,000

units of penicillin per ml to grooves cut across the center of hardened base medium in 5-cm petri dishes. Glycine was added to the base medium at a concentration of 1.5% (w/v).

Observation of cultures. Induction media were spread with 1 drop of bacillary inoculum and incubated at 37 C in a CO_2 incubator for periods from 7 to 30 days. Microscopic observations of plates (10 × objective) were made at frequent intervals during the growth period. Periodically during the incubation period, the agar block transfer technique described for pleuropneumonia-like organisms (PPLO) by Klieneberger-Nobel (8) was used to subculture L-form growth to fresh induction medium for continued propagation of L colonies, to base agar plus 10% sucrose or to Levinthal broth plus 20% sucrose as stabilization media, and to chocolate-agar or blood-agar (both made from Trypticase Soy Agar plus 10% sheeps' blood) with a *Staphylococcus* streak for determination of reversion to bacillary form. Agar blocks were stained with azure II-methylene blue for detailed microscopic examination, and slide impressions of L-form growth were stained with Gram stain and with specific fluorescein-labeled antibody.

Demonstration of L forms induced in vivo. Sputa from patients at the Veterans Administration Hospital, who were treated either intramuscularly with methicillin or orally with ampicillin for *H. influenzae* infections, were obtained frequently during and after the course of therapy. All specimens were cultured directly and after serial saline washing according to a technique previously described (9). Duplicate smears of each specimen were made and stained with Gram stain and with specific fluorescein-labeled antibody. During the course of this study it was found that, by attaching a narrow strip of masking tape down the center of a slide, half the smear could be stained with Gram stain and the remainder with fluorescent antibody, thus allowing a single smear to be examined by both methods.

Fluorescent-antibody (FA) studies. Immune serum for FA studies was prepared in rabbits by intravenous injections of *H. influenzae* vaccine. This vaccine consisted of a suspension in 0.6% Formol-saline of 18-hr chocolate agar cultures of 10 strains of *H. influenzae* isolated from patients with bronchitis. The γ globulin fraction of the antiserum was separated on columns of Sephadex G-25 and diethylaminoethyl (DEAE) cellulose, conjugated with fluorescein isothiocyanate, and freed from unconjugated fluorescein on a Sephadex column by use of procedures described by Smithies and Olson (17).

This fluorescein-labeled γ globulin stained *H. influenzae* in smears of cultures and in smears of sputum specimens with 4+ fluorescence at dilutions of 1:10 or 1:2, and showed essentially no nonspecific staining. The 1:2 dilution proved best for staining transitional and L forms, probably because they do not react as avidly as do parent forms with antisera prepared against whole cells (3).

Appropriate controls were performed to demonstrate the specific avidity of the conjugate for *H. influenzae*. In indirect staining procedures in which unlabeled rabbit antiserum and fluorescein-labeled anti-rabbit γ globulin (BBL) were used, *H. influenzae* was stained when unlabeled specific antiserum, but not when normal rabbit serum, was used in the first step of the staining procedure. The staining of *H. influenzae* by the specific conjugate was blocked by prior application of unlabeled specific antiserum. α Streptococci, *Neisseria*, and fusiforms, all commonly found in sputum, were not stained by the specific conjugate in either culture or sputum smears.

All smears for FA staining were air-dried and heat-fixed before staining and were examined with a Zeiss GFL fluorescence microscope. Photomicrographs were taken by time exposure on Kodak high-speed Ektachrome film.

RESULTS

Development of penicillin-induced L forms in vitro. At 18 to 24 hr after inoculation of penicillin gradient plates, visible growth had occurred to within 5 to 8 mm of the penicillin groove. Direct microscopic examination revealed confluent bacterial growth along the edges of the plate. Nearer to the groove the growth became very pleomorphic and filamentous and contained a scattering of the round bodies described by Dienes (2). Numerous round bodies were visible within the 5- to 8-mm zone of inhibition, but the number decreased considerably 2 to 3 mm from the groove. Subcultures from all areas to chocolate-agar or blood-agar showed reversion to bacillary growth. Gram and FA stains of slide impressions showed small bacillary forms at the periphery intermingled with a few elongated, bizarre forms. Nearer the groove, serpentine forms, elongated bacilli with large swellings (transitional forms), and round bodies were predominant (Fig. 1a); within the zone of inhibition, only round bodies were evident (Fig. 1c). All forms showed specific staining with FA (Fig. 1b and d).

Between the 3rd and 7th day after inoculation, typical L-form colonies with a dense center and lighter periphery developed from some of the round bodies and were most abundant within 5 mm of the penicillin groove. A few colonies, however, did not appear until after 2 to 3 weeks of incubation. Figure 2a shows L colonies and isolated round bodies in the zone of inhibition merging with an area containing numerous round bodies. Examination of agar blocks stained with Azure II-methylene blue showed the typical L-form morphology of large and small round bodies growing into the agar (Fig. 2b and c). Gram-stained slide impressions of the L-form colonies showed many bizarre forms and amorphous debris, although some of the fragile round forms did survive the staining procedure. FA staining seemed less disruptive, since more intact large and small round bodies were observed in these preparations.

Level of antibiotic was important in deter-

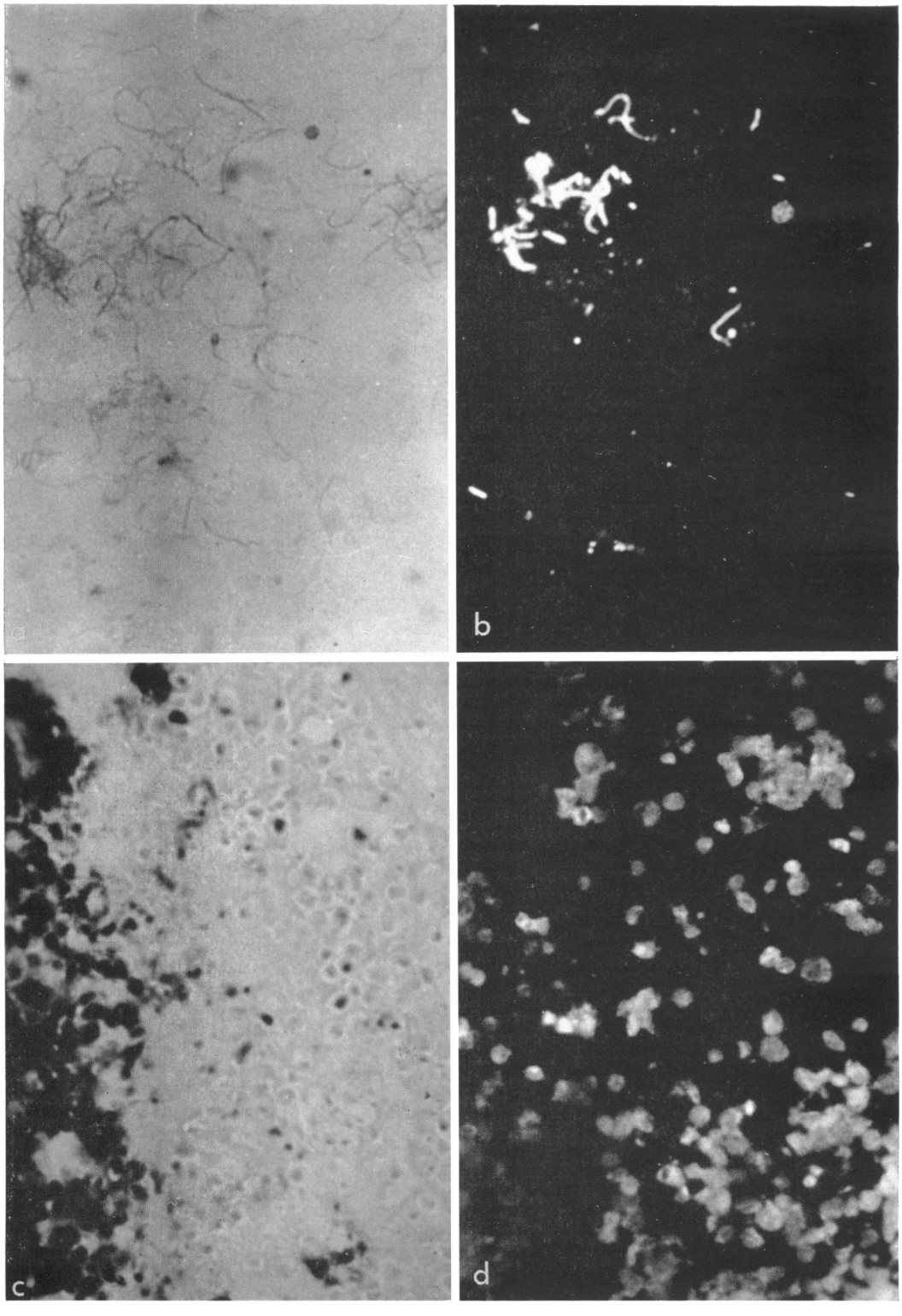

FIG. 1. *Morphological variants of Haemophilus influenzae at 24 hr on penicillin gradient plates. (a) Serpentine forms, transitional forms, and a few round bodies near the zone of inhibition. Gram stain, × 440. (b) FA stain of forms in (a). × 440. (c) Round bodies within the zone of inhibition. Gram stain, × 440. (d) FA stain of (c). × 440.*

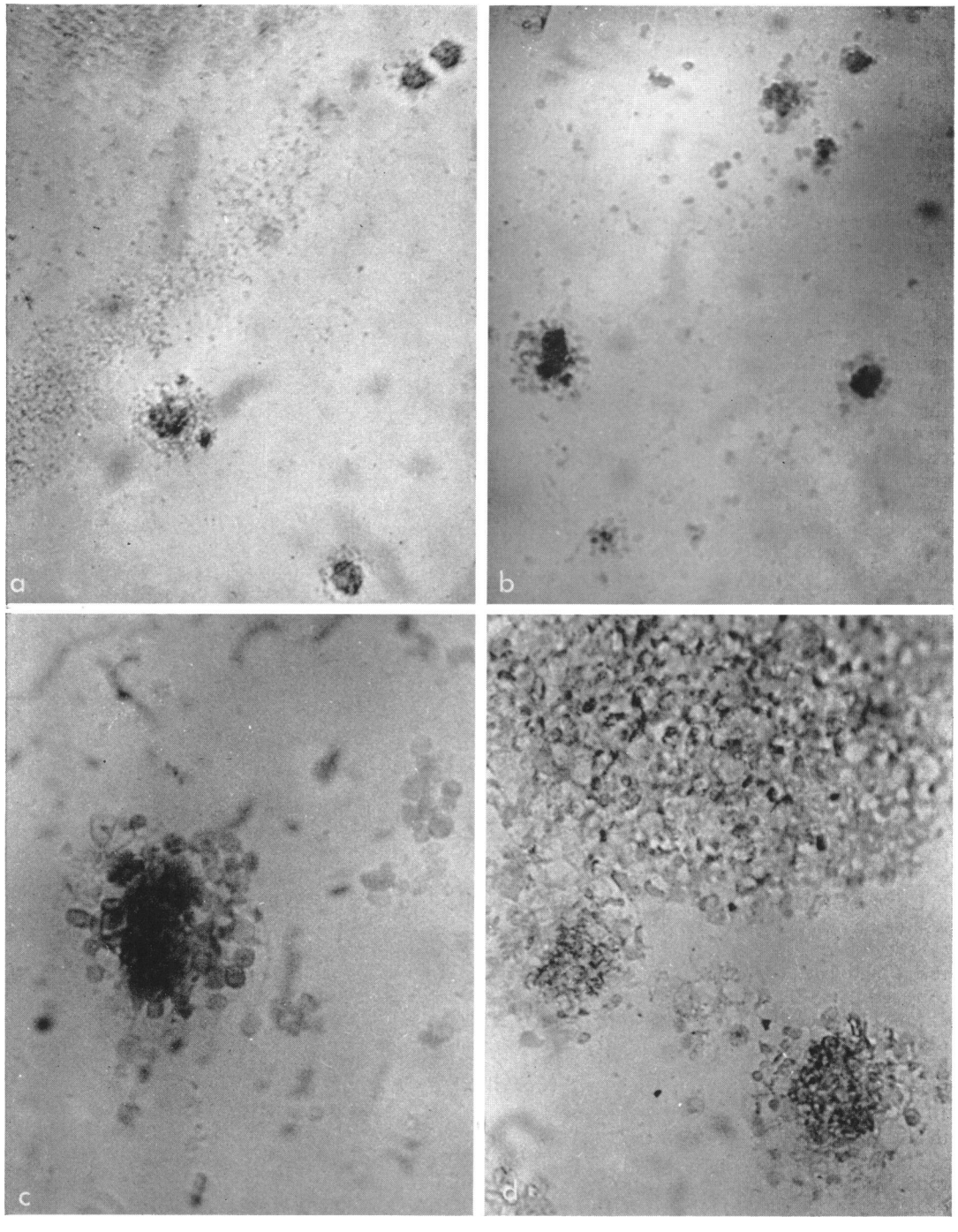

FIG. 2. *(a) Zone of inhibition on a penicillin gradient plate at 72 hr showing typical L colonies and isolated round bodies of Haemophilus influenzae merging with numerous round bodies. Direct microscopic observation, × 100. (b) Azure II-methylene blue stained agar block from within the zone of inhibition at 72 hr. × 160. (c) Higher magnification of (b) showing greater detail. × 440. (d) Stained-agar block of glycine-induced L colonies at 7 days showing a portion of a large colony. Note greater pleomorphism of form than in (c). × 250.*

mining the degree of conversion to L forms. L-form colonies did not develop on induction medium containing 10 or 15 units of penicillin per ml, but pleomorphic growth consisting of long and swollen bacilli was observed. At levels of 20 to 35 units per ml, numerous round bodies developed in 24 hr and scattered L-form colonies were identified after 3 to 21 days of incubation. These results correlated with tube dilution penicillin sensitivity determinations; though most strains were inhibited by concentrations of 6 to 12 units per ml, 100 to 200 units per ml was necessary for bactericidal activity.

On glycine induction medium the development of L-form growth was similar to that induced by penicillin. Within 24 hr the inoculum converted to round bodies which were generally larger than those induced by penicillin and which lost viability more rapidly. L-form colonies, many very large, developed from a relatively small proportion of these round bodies in 5 to 7 days. Stained-agar blocks showed the dense centers of large and small round bodies but more pleomorphism of form than was apparent in penicillin colonies (Fig. 2d).

L-form colonies were successfully retained in the L form when transferred to penicillin (greater than 20 units per ml) or glycine induction medium. The only exceptions were transfers of glycine blocks to glycine medium, and these failed consistently, undoubtedly because of the toxicity of the increased glycine concentration. Addition of a penicillin groove to glycine medium did not increase L-form colony yield when inoculated with bacillary inoculum, nor did it promote stabilization of the L form when inoculated with penicillin-induced L colonies.

Transfer of L colonies, none of which had been in L phase for longer than two transfers on induction medium, to sucrose stabilization medium or to Chocolate Agar or blood-agar resulted in reversion to bacillary growth. Viability and ability to revert persisted in some of the L colonies for as long as 30 days. The large round bodies which did not develop into L colonies reverted to bacillary growth in sucrose medium and chocolate agar but seemed to lose viability after 5 to 10 days, glycine round bodies being less hardy than those induced by penicillin.

The facility of *H. influenzae* for conversion to round bodies and L colonies in the presence of penicillin or glycine was confirmed. Of 14 strains investigated, all produced round bodies and L colonies on glycine medium and 13 produced L colonies on penicillin medium, the 14th producing round bodies only.

Demonstration of transitional forms and round bodies in vivo. Pleomorphic and transitional forms (elongated forms with large swellings which develop into round bodies) and round bodies of *H. influenzae* similar to those seen during the development of L colonies in vitro (Fig. 3a) were identified in Gram- and FA-stained (Fig. 3b) smears of sputa from seven patients being treated with ampicillin or methicillin for *H. influenzae* bronchial infections. Three patients were carefully followed before, during, and after penicillin therapy. Before therapy, these patients were producing purulent sputa which showed numerous polymorphonuclear leukocytes and tiny gram-negative bacilli on microscopic examinations. Saline-washed samples of these sputa yielded pure cultures of *H. influenzae*. By the 2nd day after initiation of therapy, numerous pleomorphic gram-negative bacilli (Fig. 4a), identified as *H. influenzae* by FA staining (Fig. 4b), were seen in the sputum smears. Sputum at this time was still copious and purulent, and cultures yielded numerous colonies of *H. influenzae*.

Between the 3rd and 5th day of therapy, sputum purulency decreased, cultures showed reduced numbers of *H. influenzae* colonies, and sputum smears revealed transitional forms and round bodies (Fig. 4c), the origin of which was established by FA staining (Fig. 4d). By the 7th or 10th day, when therapy was usually terminated, sputum purulency had markedly decreased and only occasional round bodies which stained specifically with FA were identified in smears (Fig. 4e). Cultures showed few colonies of *H. influenzae* in two cases and were negative in the third.

Relapse with return of copious purulent sputum containing numerous *H. influenzae* organisms occurred in all patients whose sputa showed transitional forms and round bodies during therapy. In one of the three patients followed closely, this occurred 2 days after cessation of therapy, in another, 20 days after, and in the third, 6 days after. In each case, the antibiograms of the organisms before therapy and at relapse were identical.

DISCUSSION

The demonstration that transitional forms and round bodies of *H. influenzae* occur in vivo suggests that L forms of bacteria may be significant in chronic or recurrent infections. The forms induced by penicillin in vivo were similar to forms in L colonies induced by the same agent in vitro. Round bodies in L colonies remained viable and could revert to bacillary growth after as long as 3 weeks. It is, therefore, not unreasonable to suggest that the round bodies induced in vivo remained viable during the time after cessa-

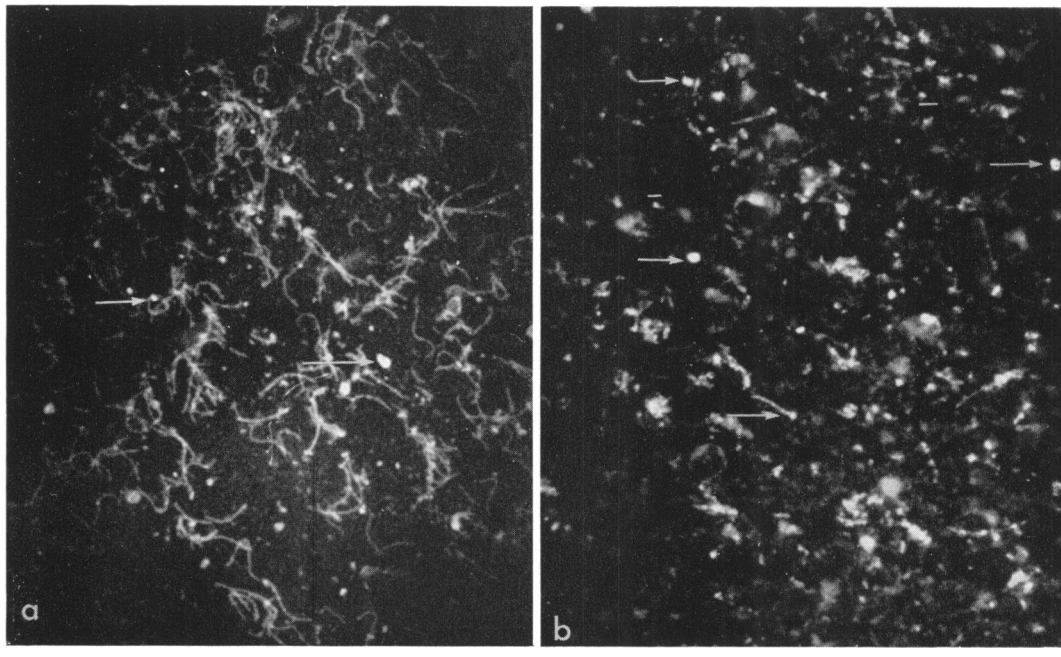

FIG. 3. *(a) FA-stained impression of in vitro penicillin-induced pleomorphic forms. × 250. (b) FA-stained smear of sputum showing similar forms induced in vivo. Arrows point out representative transitional forms and round bodies. × 250.*

tion of therapy when the patients were apparently free from *Haemophilus*, and that they reverted to bacillary form when the respiratory-tract environment was favorable, thus allowing infection to recur.

Isolation of a few colonies of *H. influenzae* from sputum in which only a few round bodies but no bacillary forms were identified microscopically may have been the result of reversion of these forms in vitro. That recurrence of infection was due to bacillary forms persisting in bronchial epithelium rather than to reversion of the round bodies cannot be discounted without thorough examination by culture and FA techniques of tissue obtained by bronchial biopsy in a similar study. Such specimens would obviate the difficulties encountered in thorough microscopic and cultural studies of sputum which is grossly contaminated with the flora of the nasopharynx. Specific FA staining is of critical importance in identification of the variant forms, because Gram-stained smears of spinal fluid, sputum, or other body secretions often show round bodies and bizarre forms that are cellular fragments or other artifacts.

May and Delves (13) suggest that *H. influenzae* is able to survive extended ampicillin therapy and to cause relapse when it is stopped because the ampicillin level in bronchial secretions decreases as inflammation subsides and before all organisms are killed. The organisms thus survive in the sputum, inaccessible to the antibiotic, and reinvade the bronchial mucosa when therapy is stopped and antibiotic has disappeared from the tissues. May and Delves did not consider the possibility of L forms maintaining infection. However, the techniques used by these authors involve homogenization of sputum with pancreatin and shaking before bacteriological evaluation. We have found that so rigorous a procedure destroys the integrity of the fragile transitional forms and round bodies.

The protoplasmic forms discussed here represent not the stable L forms but transitional or unstable L forms. Apparently, the pathogenicity of these unstable L forms depends upon the ability to persist and revert, and, thus, to initiate infection. Sputum purulency decreased with disappearance of bacillary organisms and was negligible when only round bodies were evident, indicating lack of intrinsic pathogenicity of the *Haemophilus* L forms. This is in accord with earlier reports on pathogenicity of L forms of other bacteria reviewed by Klieneberger-Nobel (8). Stable L forms of pathogenic bacteria are not pathogenic unless the organism produces potent endotoxin, whereas unstable L forms are able to initiate infections by reverting to bac-

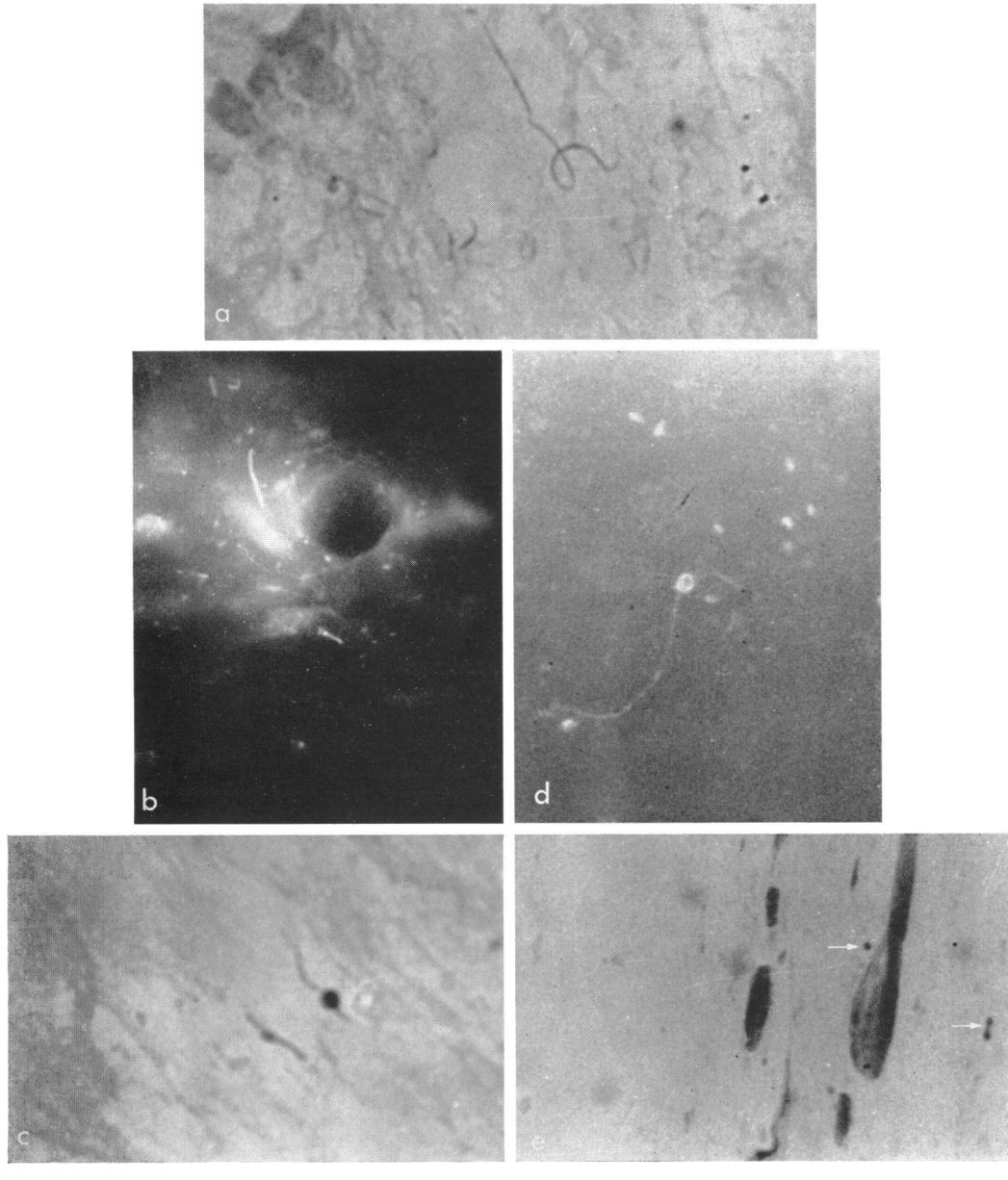

FIG. 4. *Development of Haemophilus influenzae L forms in sputum of patients treated with penicillin. (a) Elongated bacilli seen after 2 days of therapy. Gram stain. × 970. (b) FA stain of sputum seen in (a). × 440. (c) Transitional forms on 3rd day of therapy. Gram stain. × 970. (d) FA stain of transitional forms in sputum as in (c). × 440. (e) Arrows point out representative round bodies present after 10 days of therapy. Gram stain. × 440.*

terial form. However, McKay, Abelseth, and Vandreumel (11) recently reported the production of pneumonia in pigs with protoplasts of *H. parainfluenzae*, and they suggested that protoplasts may have pathogenic significance.

The development of large bodies or protoplasts from bacteria can occur under a variety of conditions (2, 3) that may involve either interference with cell wall synthesis, as with antibiotics such as penicillin and cycloserine, or lysis of the cell wall by agents such as lysozyme, phage, and antibody and complement. Conditions that

do not allow optimal growth, such as low *p*H, lack of a required nutrient, or presence of toxic substances, may also act as inducers with some organisms. In most instances, these round bodies can either revert to bacilli or develop into L forms, depending on the environment to which they are transferred. Since *H. influenzae* readily shows pleomorphism and swelling under adverse conditions and since the human respiratory tract apparently provides an appropriate environment for L-form survival, it is possible that transitional L forms of this organism could be induced in vivo by factors other than the presence of antibiotic. Wittler (18) noted that transformation to L forms in vivo of intranasally administered *Bordetella pertussis* was more rapid and complete in specifically immunized mice than in the nonimmune. Kalmanson and Guze (7) related the persistence of penicillin-induced protoplasts in rat kidney to immune response. May (12) has shown that 85% of patients with purulent sputum due to *H. influenzae* have serum antibody against this organism. Could this antibody promote occurrence of *Haemophilus* L forms in vivo?

Hatten and Sulkin (6) have recently shown that *Brucella* L forms occurred intracellularly in tissue cultures of *Brucella*-infected hamster kidney cells. *H. influenzae* is frequently seen within polymorphonuclear leukocytes and macrophages in stained smears of sputa from infected patients, and these cells are known to contain leukozymes which are capable of inducing protoplasts of gram-negative bacteria (3).

Investigation of the effects of antibody, the influence of the intracellular milieu, etc., on the induction and persistence of *H. influenzae* L forms in vivo, together with a thorough search for L forms within bronchial tissue of chronically infected patients, would provide a greater understanding of the significance of L forms in chronic or recurrent infections.

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