SHORT ARTICLES

STAPHYLOCOCCAL PERSISTERS GROWN FROM EMPYEMA FLUID ON L-FORM MEDIUM

S. W. B. NEWSOM

Sims Woodhead Memorial Laboratory, Papworth Hospital, nr Cambridge

PLATE LXVIII

PROTOPLASTS, spheroplasts and L-forms are bacteria with defective cell walls. The defect may be caused by exposure to penicillin or to other antibiotics that prevent the synthesis of cell-wall constituents, or to lysozyme or antibody. Spheroplasts and protoplasts are non-replicating osmotically fragile forms, but L-forms will grow on suitable laboratory media with an increased electrolyte content, and may eventually become adapted to life on media of a lower osmolarity. The term persister is used to describe organisms that survive treatment with an antibiotic of the penicillin-cephalosporin group, although the original strain was sensitive to the antibiotic. Spheroplasts, protoplasts and L-forms are persisters because they lack a fully formed cell wall; another type of persister is a cell in a non-replicating phase on which the antibiotic cannot act because of the absence of cell-wall synthesis.

There have been reports of the isolation of L-forms from human infections, including endocarditis (Neu and Goldreyer, 1968); this subject has been reviewed recently by Feingold (1969). Although this type of organism appears to be unable to initiate infection it may possibly cause its persistence during apparently adequate chemotherapy, for example in the urinary tract (Guze and Kalmanson, 1964; Gutman, *et al.* 1965). This article describes the history of a patient with empyema from whom, at one stage of the disease, *Staphylococcus aureus* was grown on L-form medium but not on normal bacteriological media.

CASE REPORT

A 54-yr-old housewife had a mitral valvotomy for rheumatic heart disease on 7 Jan. 1969. The operation was complicated by the presence of pleural adhesions. At the time of the operation and for 5 days afterwards she received 4×10^6 units of penicillin G daily. Fever began on the 2nd post-operative day (fig. 1) and reached 102°F (38.8°C). There were at first no localising signs, and cultures of the blood, urine and sputum yielded no pathogens. The total WBC was 17,200 per μ l on the 3rd post-operative day and there was a marked increase of "early" neutrophils. The penicillin G was discontinued on 12 Jan. to avoid masking an infection, and 2 days later a left-sided empyema was diagnosed; 900 ml of pus was evacuated and this was found to contain large numbers of *Staph. aureus*. The patient was treated with cloxacillin 3 g daily by injection and 1 g of antibiotic was instilled into the pleural cavity after chest aspirations, which were initially on alternate days but later daily. The temperature and the WBC rapidly returned to normal and the patient felt better. Good levels of cloxacillin activity were demonstrated in the serum and pleural fluid. However, the numbers of organisms grown from the pleural exudate decreased only slowly. Eight days after starting treatment she was still producing 200 ml of pus daily, although a Gram-stained film of this revealed only a few atypical cocci inside the polymorphs, and routine cultures grew very few staphylococcal colonies. In view of these findings a drain was inserted into the empyema, cultures for L-forms were set up and treatment with fucidin 1.5 g daily was begun. Three days later (25 Jan.) the patient was covered with a macular rash. A sinogram revealed

Received 7 May 1970; accepted 9 May 1970.

a track passing from the drain to the wound with several branches at the top. On 27 Jan., treatment with cloxacillin and fucidin was stopped and the patient was given erythromycin 1.5 g and novobiocin 1 g daily by mouth, and in addition 300 mg of erythromycin was instilled into the chest although this was rather painful. Two days later she had a raised temperature, which persisted although the amount drained from the empyema had become negligible; however, on 4 Feb. her wound broke down and discharged pus containing large numbers of normal staphylococci. Cephaloridine was added to her regimen, but she immediately developed a second drug rash so this drug and the novobiocin were discontinued. The wound gradually healed, the temperature fell and cultures became negative. A decortication of the left lung was performed on 18 Feb. A fibrotically thickened parietal pleura 0.8 cm thick with a haemorrhagic and fibrinous inner surface was removed; the empyema cavity measured 3.5 × 1.5 × 3.5 cm. No evidence of active infection was found. Erythromycin was stopped on 27 Feb. and the patient was discharged home. She has remained well since.

FIG. 1.—Progress chart of the patient's illness.

4 = confluent growth; 3 = semi-confluent growth; 2 = 20–200 colonies; 1 = < 20 colonies.

BACTERIOLOGICAL METHODS

Routine cultures of pleural fluid and pus were made on Lemco-peptone agar enriched with 7 per cent. horse blood. The L-form medium was Brain-Heart Infusion (Oxoid) with 1.5 per cent. agar, stabilised with 0.6M sucrose and 0.008M magnesium sulphate. The osmotic stabilisers were omitted from the control medium. Cultures were made by adding 0.04 ml of pleural fluid to 20 ml of cooled melted L-form medium, and to a similar volume of control medium, and pouring the mixtures into petri dishes.

L-forms were induced *in vitro* by adding 0.04 ml of a 4-hr broth culture of staphylococci to L-form medium containing 1000 µg cloxacillin per ml.

Measurements of blood levels of antibiotic and of the minimum inhibitory concentration of antibiotics for the patient's organism were made in tubes of peptone water to which had been added 25 per cent. (v/v) serum, 1 per cent. (w/v) glucose and phenol red. Doubling dilutions of patient's serum or of antibiotic were made in this medium, and the Oxford staphylococcus (NCTC6571) was used as a control. Inocula consisted of 0.02 ml of a 1 in 200 dilution of a 4-hr culture, and incubation was overnight at 37°C. Antibiotic activity was demonstrated in the pleural fluid by spreading a heavy inoculum of it over an agar plate and adding 0.1 ml of it into a 9-mm well in the centre. After incubation for 18 hr a replica plate was made from this with a velvet pad (Elek and Hilson, 1954). The production of penicillinase by the staphylococci was demonstrated on a plate by the iodine-starch method (Perret, 1954). The organism was typed with the standard set of bacteriophages, obtained from the Central Public Health Laboratory, Colindale. Osmolarities were measured in the Knauer osmometer.

BACTERIOLOGICAL RESULTS

Cultures of the pus from the empyema on 15 Jan. yielded large numbers of colonies of a coagulase-positive staphylococcus with the phage-typing pattern 29/52/79/80. A 1.5 µg penicillin disk on the primary plate showed narrowing of the zone of inhibition with evidence of an inoculum effect. Tube-dilution sensitivity tests showed that the minimum inhibitory concentration for the organism of penicillin was 5 µg per ml and of cloxacillin was 0.12 µg per ml. The production of penicillinase was subsequently confirmed by the iodine test on a starch agar plate. A further isolate on the 12th day of treatment was inhibited by 0.6 µg of cloxacillin per ml. All the isolates were sensitive to cloxacillin, tetracycline, cephaloridine and fucidin and resistant to penicillin G, when tested by the disk-diffusion technique. Some isolates were also tested against erythromycin, novobiocin, chloramphenicol and the trimethoprim-sulphonamide combination, to which they were all sensitive.

A sample of serum collected on the 3rd day of cloxacillin therapy inhibited the patient's organism at a dilution of 1 in 32. Diffusion tests using the empyema fluid tested against itself repeatedly gave large (6 cm) zones of inhibition of the infecting organisms, but on one occasion three colonies of "persisters" were grown on the replica plate.

Cultures of the pleural fluid in L-form medium were set up on 21 Jan. and 24 Jan. (i.e., after cloxacillin had been given for 7 and 10 days respectively). On both occasions large numbers of staphylococcal colonies appeared after 48 hours' incubation in the L-form medium, but there was no growth in the control medium without osmotic stabiliser (fig. 2). A more heavily seeded culture on blood agar showed a few colonies of *Staph. aureus*.

Heavy inocula of the patient's staphylococcus and the Oxford staphylococcus were made in L-form medium containing cloxacillin. Both organisms produced minute colonies with the characteristics of L-form growth after 3 days. However, after 7 days' incubation the patient's organism showed colonies on the surface of the medium that were larger than L-form colonies, and opaque. A Gram-stained film of these colonies revealed normal Gram-positive cocci in addition to Gram-negative ones. They thus appeared to consist mainly of normal staphylococci, though they were in many cases kidney-shaped (fig. 3); the colonies of the Oxford staphylococcus remained in the L-form after continued incubation.

The osmolality of the empyema pus was 318 m.osmoles and that of the patient's uncoagulated whole blood 280 m.osmoles. The osmolality of empyema fluid from another patient was 320 m.osmoles. These values are well below that of the L-form medium, which was 1000 m.osmoles.

DISCUSSION

This case is of interest because infection persisted despite seemingly adequate antibiotic therapy and relapsed clinically when treatment with cloxacillin had to be stopped because of the appearance of a rash. Although the patient's fever subsided during cloxacillin treatment and the infecting strain of *Staph. aureus* became more and more difficult to isolate by ordinary cultural methods, large numbers of almost normal staphylococcal colonies appeared after little delay on L-form medium. The absence of antibiotic in this medium, other than the small amount carried over in the inoculum, makes it probable that the cell-wall-deficient organisms were not induced on the plate, but were actually present in the pus. These organisms may have been true L-forms that were capable of multiplying in the tissues, but were unstable and had reverted to the bacterial form in culture before typical L-form colonies could be seen; or they may have been merely persisting in the tissue as protoplasts or spheroplasts. The observation that L-form colonies in cultures of the infecting organism on cloxacillin plates showed a similar reversion when incubated for long enough for the cloxacillin to be destroyed favours the former view. The kidney shape of the staphylococcal colonies seen on these plates is similar to that described for reverting colonies of *Streptococcus faecalis* (Gooder, 1968).

In any event, organisms capable of becoming normal staphylococci if provided with osmotic support for a short time had survived in large numbers in the empyema fluid in the presence of a high concentration of cloxacillin and this may well have accounted for the

persistence of the infection and its relapse when cloxacillin treatment had to be stopped. Reversion of L-forms to pathogenic bacteria has been observed *in vivo* as well as *in vitro*. Proteus L-forms reverted in the case of urinary infection described by Gutman, Schaller and Wedgwood (1967), and staphylococcal L-forms injected into eggs caused no damage when penicillin was given, but when this was discontinued reversion took place and the eggs were destroyed by staphylococcal growth (Brier, Ellis and Godzeski, 1963).

The osmolarity of the pus was well below that required for the in-vivo culture of L-forms. Some other stabilising influence must be sought to explain this finding. Streptococcal L-forms have been recovered from the peritoneal exudate formed in mice in response to the injection of normal streptococci (Mortimer, 1968) and an "exudate factor" has been suggested.

A few atypical cocci were seen in heat-fixed Gram-stained films of the pleural exudate, but no true L-forms were seen. According to Charache (1968), air-dried films are required for the proper visualisation of L-forms.

Other factors may have contributed to the slow recovery of the patient. Exceedingly large numbers of staphylococci were present in the pleural exudate from the start; the patient received cloxacillin, which is significantly less resistant to penicillinase than is methicillin (Barber, 1962; Newsom, 1967); and the presence of protein reduces the activity of cloxacillin to a much greater extent than that of methicillin. There was also evidence of loculation of pus within the empyema cavity, probably as a result of pre-existing pleural adhesions. This may explain why fever persisted despite systemic treatment with erythromycin and novobiocin, and the instillation of large amounts of erythromycin into the empyema cavity. The temperature did not settle and the empyema did not cease to discharge until the pocket of pus had burst out through the wound.

Two main conclusions can be drawn from this case. Firstly L-forms should be looked for more often in staphylococcal or suspected staphylococcal disease, by means of appropriately stained air-dried films, and by cultures on L-form medium. A recent experience reinforces this point. A patient suffered from influenza, complicated by what was thought to be pneumonia. Cloxacillin therapy was given, although no sputum was sent for laboratory examination. On admission to hospital 4 days later, an empyema was diagnosed. The pus aspirated from this showed Gram-variable cocci inside the polymorphs. Cultures on routine media were sterile, but again on two occasions small numbers of normal staphylococcal colonies grew on L-form medium. Erythromycin therapy was begun, fucidin was added later, and the patient made an uneventful recovery. Charache found L-forms in 41 patients (including three with empyemas) during 8 mth of routine screening, but L-forms have rarely been found in osteomyelitis, and cannot be assumed to be the cause of all chronic or relapsing infections.

Secondly, the treatment of severe staphylococcal infections should be framed with the possibility of L-forms in mind. L-forms are immune to further action by antibiotics acting on the cell wall, including penicillins, cephalosporins, vancomycin and bacitracin, but they are as susceptible as the parent bacteria to the aminoglycosides and fucidin and more susceptible to erythromycin, tetracycline and chloramphenicol (Williams, 1963; Kagan *et al.*, 1964). Thus combined treatment with the appropriate penicillin and erythromycin or fucidin might be of value in serious staphylococcal infections if the organism responsible was sensitive to these antibiotics. This approach may explain the good results obtained by Jensen and Lassen (1969) with a combination of methicillin and fucidin. Recurrent boils have been cleared by treatment with oxacillin followed by tetracycline (Kagan, 1968); the two antibiotics were not given together to avoid the possibility of antagonism between them.

SUMMARY

A case of staphylococcal empyema is presented in which resolution was slow in spite of apparently adequate therapy with cloxacillin. A relapse occurred when the drug was stopped after 2 wk because of the appearance of a rash. On two occasions while the patient was being treated with cloxacillin, large numbers of colonies of the bacterial form of *Staphylococcus aureus* appeared on plates of L-form medium but not on control plates without osmotic

STAPHYLOCOCCAL PERSISTERS FROM EMPYEMA FLUID

FIG. 2.—Culture of pleural fluid after 48 hours' incubation in pour plate of (L) L-form medium and (R) control medium without osmotic stabiliser. $\times c. \frac{1}{2}$.

FIG. 3.—Patient's organism after incubation for 7 days on L-form medium containing cloxacillin, showing reversion of L-form colony to kidney-shaped staphylococcal colonies. Small L-form colonies are just visible in the background. $\times 4$.

stabilisers. Relapse was associated with the re-emergence of staphylococci that could be isolated on conventional media, and a similar reversion of L-colonies made *in vitro* from the patient's organism could be demonstrated on prolonged incubation. The infection was finally eradicated by erythromycin, novobiocin and drainage. It is suggested that cultures on L-form medium should be set up in any severe staphylococcal infection that fails to respond to chemotherapy and drainage, and that combined therapy with methicillin and an antibiotic that does not act on the cell wall should be considered.

I would like to thank Mr Christopher Parish for his encouragement and permission to publish this case, also Dr E. M. Cheffins for permission to publish details of the second case.

REFERENCES

- BARBER, MARY 1962. *In* Resistance of bacteria to the penicillins. *Ciba Fdn Study Grp* no. 13, p. 91.
- BRIER, G., ELLIS, L., AND GODZESKI, G. W. 1963. *In* Antimicrobial agents and chemotherapy, 1962, ed. by J. C. Sylvester, *Ann Arbor*, p. 854.
- CHARACHE, PATRICIA 1968. *In* Microbial protoplasts, spheroplasts, and L-forms, ed. by L. B. Guze, *Baltimore*, p. 484.
- ELEK, S. D., AND HILSON, G. R. F. . . 1954. *J. Clin. Path.*, **7**, 37.
- FEINGOLD, D. S. 1969. *New Engl. J. Med.*, **281**, 1159.
- GOODER, H. 1968. *In* Microbial protoplasts, spheroplasts, and L-forms, ed. by L. B. Guze, *Baltimore*, p. 46.
- GUTMAN, LAURA T., SCHALLER, JANE, AND WEDGWOOD, R. J. 1967. *Lancet*, **1**, 464.
- GUTMAN, LAURA T., TURCK, M., PETERSDORF, R. G., AND WEDGWOOD, R. J. 1965. *J. Clin. Invest.*, **44**, 1945.
- GUZE, L. B., AND KALMANSON, G. M. . 1964. *Science, N. Y.*, **143**, 1340.
- JENSEN, K., AND LASSEN, H. C. A. . . 1969. *Q. J. Med.*, **38**, 91.
- KAGAN, B. M. 1968. *In* Microbial protoplasts, spheroplasts, and L-forms, ed. by L. B. Guze, *Baltimore*, p. 372.
- KAGAN, B. M., MOLANDER, C. W., ZOLLA, SUSAN, HEIMLICH, E. M., WEINBERGER, H. J., BUSSE, R., AND LIEPNIKS, S. 1964. *In* Antimicrobial agents and chemotherapy, 1963, ed. by J. C. Sylvester, *Ann Arbor*, p. 517.
- MORTIMER, E. 1968. *In* Microbial protoplasts, spheroplasts, and L-forms, ed. by L. B. Guze, *Baltimore*, p. 345.
- NEU, H. C., AND GOLDRAYER, B. . . 1968. *Amer. J. Med.*, **45**, 784.
- NEWSOM, S. W. B. 1967. *Br. Med. J.*, **3**, 678.
- PERRET, C. J. 1954. *Nature, Lond.*, **174**, 1012.
- WILLIAMS, R. E. O. 1963. *J. Gen. Microbiol.*, **33**, 325.