Isolation of L-Forms in a Clinical Microbiology Laboratory

JOHN A. SWIERCZEWSKI AND PERLA REYES

Department of Microbiology, St. Mary Mercy Hospital, Gary, Indiana 46402

Received for publication 3 March 1970

Previous studies have demonstrated that L-forms of bacteria may play a role in persistent, chronic, or recurrent urinary-tract infections. A 2-year program was initiated to determine the feasibility of culturing for L-forms on a routine basis, and to determine the effectiveness of such a program. In relation to the total number of specimens, few L-forms were actually isolated. In comparison with the amount of equipment and technician time required, the return was negligible; only 0.5% of all urine specimens were positive for L-forms. An increase to only 1.2% was noted when culturing for L-forms was limited to patients with a diagnosis of bacteriuria or pyelonephritis. It is recommended that this technique be reserved for those patients with a long history of recurrent urinary-tract infections, after other attempts to cure the patient have met with failure.

Recent studies (4, 6) have demonstrated that L-forms of bacteria, including protoplasts, spheroplasts, and bacterial variants, may play a significant role in persistent, chronic, or recurrent urinary-tract infections, especially in chronic pyelonephritis. In a study of urine cultures from patients with chronic urinary-tract infections, Gutman and associates (5) demonstrated L-forms in 19%, and Conner et al. (2) in 23%, of the patients studied.

To determine the feasibility of establishing such a program in a general hospital laboratory, on a routine basis, this study was initiated. If the projected isolation rate (20 to 23%) was realized, particularly from patients with recurrent urinary-tract infections, the routine culture of urine specimens for L-forms could be justified.

The study was divided into two distinct phases. During the first year, all urine specimens received were cultured for L-forms of bacteria, without regard to the patient's past history or present illness. During the second year, urine specimens were cultured automatically only if they were obtained by cystoscopy by a urologist. Otherwise, the patient's chart was evaluated, to decide whether the urine should be cultured for L-forms.

MATERIALS AND METHODS

Carefully collected clean-voided or catheterized urine samples were collected in 5 ml of sterile 20% sucrose solution. L-forms were separated from classical bacteria according to the technique described by Gutman et al. (5).

The L-form agar medium and biphasic tube medium utilized during the program were those proposed by Gutman et al. (5). Although penicillin and amphotericin B are not required if filtration techniques are properly followed, the technique was adhered to in this respect.

L-form plates and tube media were incubated aerobically for 7 days at 37°C and checked for growth of typical L-form colonies. If plates were negative at the end of 7 days of incubation, fresh L-form agar plates were inoculated from the biphasic medium. This process was repeated at 14 and 21 days of incubation. If there was no evidence of growth in the biphasic medium, or on the agar plates, the specimen was reported as negative for L-forms.

Colonies of L-form isolates were inoculated into the reversion medium suggested by Gutman et al. (5), and the procedure described for reversion of L-forms was followed. Those L-forms which reverted to the parent bacteria were identified by classical bacteriological methods. Isolates which failed to revert to the parent bacteria were listed as stable L-forms.

RESULTS

During the first year, a total of 988 urine specimens were received and screened for the presence of L-forms. Only 0.6%, a total of six specimens, were positive for L-forms of bacteria.

A total of eight urine specimens were received from the six patients positive for L-forms (Table 1). Colony counts varied from no growth to $10^4$ organisms/ml; however, the counts tended to be
low in most instances. Only patients 1, 2, and 4 had counts of 50,000 organisms or more per ml.

Four patients, 1, 2, 5, and 6, had L-forms which were stable and could not be reverted to the parent bacteria. The L-form from patient 3 reverted to an organism not isolated in the original specimen, and patient 4 had the same bacterium isolated in both the routine and L-form culture.

During the second year, we were more selective as to which urine specimens were cultured for L-forms. All urine specimens submitted by urologists from cystoscopy were automatically cultured. Cultures of all other urine specimens were delayed until a review of the patient's chart was made. (An effort was made not to exceed a hold of 24 hr on any specimen.) After evaluation, a decision was made to culture for L-forms, or to discard the urine. Routine cultures were not affected by this evaluation. Points taken into consideration were: the tentative diagnosis, past history of urinary-tract infections, routine urinalysis report, repeated catheterization, antibiotics received, and the results of previous urine cultures.

A total of 623 urine specimens were received from cystoscopy during the second period; these specimens were automatically cultured for L-forms. An additional 318 urine specimens were evaluated prior to screening for L-forms; 172 were subsequently cultured, and 146 were discarded.

This total of 795 urine specimens cultured (Table 2) consisted of 183 from patients with bacteriuria or pyelonephritis, 65 from patients with diagnoses of renal disease other than the preceding, and 547 from patients with diagnoses other than bacteriuria, pyelonephritis, or various related renal diseases. Of these 795 specimens, 3 were shown to have L-forms present, two from patients with bacteriuria or pyelonephritis and one from a patient with renal disease other than the preceding. There were no isolates from the 547 patients with diagnoses other than bacteriuria, pyelonephritis, or renal disease other than the preceding.

One specimen per patient was received for each positive isolate during the second part of the program (Table 3). Patient 9 had a colony count of over 100,000 organisms/ml, whereas patients 7 and 8 had counts of 100 organisms/ml. In one instance, patient 8, the identical organism was isolated in the original urine culture and the L-form culture. L-form isolates for patients 7 and 9 could not be reverted to the parent bacteria.

A summary of the data on patients with L-form isolates is presented in Table 4. Only one patient, no. 1, was known to have histological evidence of pyelonephritis. This information was either negative or unknown for the majority of patients positive for L-forms. Two patients, 4 and 9, had more than $10^6$ organisms/ml present in urine cultured. Among the five patients with a history of chronic long-term urinary-tract infection, only one had a sterile urine culture. All others had urine specimens with bacteria present.

Information on the utilization of antimicrobial agents during hospitalization was partially available and is documented in Table 5. Three patients, 1, 7, and 9, received no antibiotics. Six patients,
VOL. 20, 1970

CLINICAL ISOLATION OF L-FORMS

325

TABLE 4. Summary of data on patients with L-forms isolated

| Patient classification                  | Patients with L-forms | Patients with sterile urine | Patients with bacteria |
|----------------------------------------|------------------------|----------------------------|------------------------|
| Known histological evidence of pyelonephritis | 1                      | 0                          | 1                      |
| More than 10⁶ organisms/ml in urine     | 2                      | 0                          | 2                      |
| History of previous infection          | 5                      | 1                          | 4                      |
| Prior chemotherapy                     | 2                      | 0                          | 2                      |

TABLE 5. Antibiotics and chemotherapeutics utilized

| Patient | In-patient antimicrobials | Duration | Any prior treatment |
|---------|---------------------------|----------|---------------------|
| 1       | None                      | —        | Yes                 |
| 2       | Nitrofurantoin            | 7        | ?                   |
| 3       | Oxytetracycline           | 6        | ?                   |
| 4       | Sulfoxazole               | 9        | ?                   |
| 5       | Tetracycline              | 13       | ?                   |
| 6       | Chloramphenicol           | 6        | ?                   |
| 7       | Lincomycin                | 6        | ?                   |
| 8       | Varied                    | —        | Yes                 |
| 9       | None                      | —        | ?                   |

2, 3, 4, 5, 6, and 8, received a variety of antibiotics and chemotherapeutics. In only two instances, patients 1 and 6, was any information on prior treatment documented. A list for both patients was not available, but patient 6 was known to have been treated over a period of 10 years with various antibiotics.

DISCUSSION

The existence of L-forms of bacteria has been known for many years; however, only recently have techniques for their isolation been available. Our primary objectives were to adopt these techniques on a routine hospital basis, establish a protocol for the isolation of L-forms of bacteria, and evaluate the results obtained during the program. From these parameters, a judgment could be made as to the practicality of the adoption of these techniques by a routine clinical microbiology laboratory.

Establishing a protocol for the isolation of L-forms of bacteria presented problems not encountered in previous programs. Technically, difficulties were met in obtaining urine samples in 20% sucrose solution and in the production of the synthetic medium utilized. Contamination of plates by saprophytic fungi and the presence of inordinate amounts of proteinaceous material in urine samples posed additional difficulties.

The nursing and medical staff required a period of time to adjust to the methods utilized in collection and transportation of urine specimens. However, once they were accustomed to the new medium, the percentage of urine specimens not received in 20% sucrose decreased perceptibly.

A constant problem was contamination of L-form plates by saprophytic fungi. Because of long periods of incubation, plates must be stored in a moist chamber. This was accomplished in a large glass container by adding a small amount of water in a 25-ml beaker. Unfortunately, if one specimen becomes contaminated, the whole series of plates may be lost. To limit the possibilities for contamination, containers should be sterilized and remain closed until used for incubation purposes.

Prefiltration of urine samples was necessary in some instances. Ordinarily, clear urines will pass through 0.45-µm membrane filters without difficulty. However, cloudy urines containing an inordinate amount of proteinaceous material will block the filter, and may cause leakage around the swirly adapters. To eliminate this problem, prefiltration, with a filter of larger pore size, may be employed. In any case, urine specimens should be filtered and inoculated into suitable media without delay. Collection of specimens from patients receiving excess fluids should be discouraged, as anything but concentrated first morning specimen decreases the chances for isolation of L-forms of bacteria (1).

The most difficult problem encountered was the evaluation of L-form isolates. Education of medical staff personnel was a long and demanding process. Because of questions surrounding the actual role of L-forms in urinary-tract infection, most members of the medical staff doubted the significance of L-form isolates. Most were concerned with the classical bacteria and their susceptibility to specific antimicrobial agents. On certain occasions, patient 6, for example, a relationship could be shown between the presence of an L-form and recurrent urinary-tract infection. However, in the majority of instances, no clear relationship could be established. Fortunately, other studies have demonstrated that there may be a relationship between L-forms and recurrent urinary-tract infection (2, 5, 7). These studies were utilized in educating the medical staff on the possible relationships of L-forms and disease. Since the present study did not segregate patients to the extent that other programs have (5), the problems of L-form isolation and evaluation were more acute. One cause of this was that,
for some unknown reasons, L-forms may be isolated from patients without direct evidence of chronic bacteriuria or pyelonephritis (2, 7). According to Feingold (3), it is not possible to assign L-forms an important role in disease, and evidence indicates that they are rarely pathogens. However, final judgement must be withheld because several studies (4, 5, 7) have been reported in which L-forms have been shown to cause infection of the urinary tract. In those cases where histological evidence of pyelonephritis is present, a history of recurrent urinary-tract infection is noted, and L-forms are isolated, the implication of the L-forms as a possible cause of disease is justified. However, when an L-form is isolated from patients without pyelonephritis and recurrent urinary-tract infection, the significance of such an organism is difficult to explain.

Obviously two things are necessary: a rapid method for isolation and identification of L-forms (9) and conclusive proof that L-forms are a direct cause of disease. The possibility that L-forms may revert to the parent form, which may cause disease, is not enough justification for the establishment of a program for their isolation. In such cases, the clinician would prefer to concentrate on the classical bacteria and leave the unusual forms to the research laboratory. Therefore, there must be unequivocal proof that L-forms cause disease before the clinician will accept the significance of their isolation from urine samples. When this is accomplished, the clinical laboratory will have to find the means for isolation, identification, and evaluation of such isolates. Obviously, the methods now in use are not practical for routine adoption by every clinical laboratory. In any event, a laboratory must produce results on an acceptable level to maintain a program of this type. A projected isolation rate of 20 to 25%, as suggested by Gutman et al. (5) and Conner et al. (2), would fall within acceptable limits. However, our results suggest that the use of such a program must be limited to highly selected patients. A total of 1,783 urine specimens in 2 years produced only nine L-forms (0.5%). Even selection of patients failed to increase the number of isolations. For example, when 248 patients with diagnoses of bacteriuria or pyelonephritis were cultured, the percentage of positive cultures increased to only 1.2%, hardly an acceptable figure. Many factors may have contributed to this low rate of isolation. Among these are the rapid movement of patients through the general hospital, and a lack of control over the individual patient and his particular case. For many patients, admission, with a tentative diagnosis, is quickly followed by discharge. In addition, the number of histories which are completed before discharge is very small. Thus, the minimal amount of information available prevents the laboratory from making an accurate study of each specimen received. Because of this, the laboratory must rely on the physician, who has a complete history of the patient, to seek specific avenues of diagnosis such as the L-form culture. However, until L-forms are implicated as the cause of disease on an impressive scale, the average house physician will not consider them with the degree of interest accorded classical bacteria. For much the same reasons, plus the cost of training personnel and maintaining the program, the hospital pathologist will not consider L-forms important enough for inclusion in his routine bacteriology program.

Brief mention should be made regarding the differentiation of L-forms of bacteria and mycoplasmas. There are various characteristics which can be employed when confronted by the possibility that mycoplasmas may be present. Basically, the mycoplasmas are a distinct class of microorganisms, whereas L-forms are bacteria which have lost their rigid cell wall, but may revert to the parent bacteria. Mycoplasmas may be distinguished from L-forms by subculture on media without bacterial inhibitors. On this type of medium, L-forms usually give rise to colonies of the parent bacteria. Mycoplasmas have a more amorphous appearance, stain less intensely, and have cell bodies that penetrate the usual bacterial filters. The gross colony characteristics are similar to those of L-forms, but colonies penetrate the medium to a greater extent. It is very difficult to remove a mycoplasma colony from a plate medium, whereas the bacterial colony and L-form colony are easily removed with an inoculating loop. Mycoplasmas, once grown, can be subcultured many times, whereas L-forms are difficult to produce, show great fragility, and tend to revert quickly to the parent bacteria (8).

In conclusion, a comprehensive program of L-form culturing is a long, tedious, and unrewarding program for the routine clinical laboratory. In our study, only a small percentage of urine specimens yielded L-forms; however, in each instance where an L-form was obtained the diagnosis was bacteriuria, pyelonephritis, or some other renal disease. Thus, broad coverage of all patients, without regard to the individual patient's history, should be discouraged. In certain instances of chronic recurrent urinary-tract infection, L-form cultures should be set up. Such a program would have to be established on some formal basis because the production of media and its storage are time-consuming and costly. Until the status of L-forms and their role in recurrent
infections of the urinary tract have been fully determined, culturing for L-forms should be limited to patients with a long history of chronic recurrent infection.

ACKNOWLEDGMENTS

We thank Albert Balows of the National Communicable Disease Center for his comments and for reviewing the article during its preparation. We are also grateful to Earl J. Mason and Rudolfo Jao for their comments and assistance.

LITERATURE CITED

1. Braude, A. I., J. Siemienki, and I. Jacobs. 1961. Protoplast formation in human urine. Trans. Ass. Amer. Physicians Philadelphia 74:234–245.
2. Conner, J. F., S. E. Coleman, J. L. David, and F. S. McGauhey. 1968. Bacterial L-forms from urinary tract infections in a veterans hospital population. J. Amer. Geriat. Soc. 16:893–900.
3. Feingold, D. S. 1969. Biology and pathogenicity of microbial spheroplasts and L-forms. N. Engl. J. Med. 281:1159–1170.
4. Gutman, L. T., J. Schaller, and R. J. Wedgewood. 1967. Bacterial L-forms in relapsing urinary tract infection. Lancet 1: 464–466.
5. Gutman, L. T., M. Turck, R. G. Petersdorf, and R. J. Wedgewood. 1965. Significance of bacterial variants in urine of patients with chronic bacteriuria. J. Clin. Invest. 44:1945–1952.
6. Guze, L. B. (ed.) 1968. Microbial protoplasts, spheroplasts and L-forms. The Williams & Wilkins Co., Baltimore.
7. Kalmanson, G. M., and L. B. Guze. 1968. Pyelonephritis: isolation of protoplasts from human kidney tissue, p. 406–414. In L. B. Guze (ed.), Microbial protoplasts, spheroplasts and L-forms. The Williams & Wilkins Co., Baltimore.
8. Klieneberger-Nobel, E. 1962. Pleuropneumonia-like organisms (PPLO): Mycoplasmataceae. Academic Press Inc., New York.
9. Nimmo, L. N., and D. J. Blazevic, 1969. Selection of media for the isolation of common bacterial L-phase organisms from a clinical specimen. Appl. Microbiol. 18:535–541.