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EXPERIMENTAL PYELONEPHRITIS.
X. THE DIRECT INJECTION OF *E. COLI* PROTOPLASTS INTO THE MEDULLA OF THE RABBIT KIDNEY†

Previous studies have demonstrated that the renal medulla is more susceptible to bacterial infection than the cortex.[1] Although more than 100,000 organisms are usually required to produce infection when injected directly into the normal renal cortex, fewer than 10 are necessary in the medulla. Many factors contribute to the remarkable ease with which infection is initiated in the renal medulla. Among these are the anti-complementary effect of ammonia[2,3] and the interference with phagocytosis which occurs in a hypertonic environment.[4]

A feature which has received little consideration is that the renal medulla is theoretically well suited for the survival of protoplast forms of bacteria. A protoplast, or L-form, is a differently shaped bacterium. Its peculiar shape results from inhibition of cell wall synthesis which may be affected by the action of certain antibiotics or antibody, complement and lysozyme systems.[5-7] The spherical protoplast will inbibe water, swell, and finally explode in media isotonic to plasma, whereas it may persist and even multiply in hypertonic media. Removal of the inhibitor of cell wall synthesis permits the reversion of the protoplast to normal bacterial form. The formation of protoplasts has been demonstrated *in vitro* and *in vivo*[5,8] and undoubtedly represents one means by which bacteria are destroyed in host tissues which are isotonic to plasma. The persistence of protoplasts in hypertonic media *in vitro* prompted the suggestion that these forms might also be able to survive *in vivo* in a hypertonic environment. The renal medulla has, in recent years, been found to provide such a hypertonic environment.

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The experiments described herein were designed to test whether protoplasts can survive and produce infection in the hypertonic environment of the renal medulla.

MATERIALS AND METHODS

Bacteriological media. E. coli were stored, throughout the experimental period, in 18 sealed solid Difco nutrient agar slants at 0 to 10° C. Difco Penassay broth was used as the liquid nutrient medium for normal *E. coli.*

Desoxycholate agar, Kliger's iron agar (Difco), and Simmon's citrate agar (Difco) were used to identify organisms. Sucrose broth was prepared according to the method of Lederberg[9] and was used wherever a hypertonic medium was indicated. It contained 0.3 M. sucrose, M/125 mg.++, and a nutrient base which included:

Casein digest	10 grams
Sucrose	100 grams
Meat extract	10 grams
Sodium chloride	3.5 grams
Glucose	1 gram

After autoclaving for 20 minutes, 10 ml. of 20% mg. SO_4: H_2O were added. This medium was prepared every 10 days.

Penicillin V. aqueous, 200,000 units per 125 mg., was added to the sucrose media. 1,000 units per ml., for the production of protoplasts.

Bacteria. All experiments were performed with Wild Type K-12 *E. coli* obtained from Dr. Philip Cowles (Department of Microbiology, Yale School of Medicine). These bacteria were stored in 18 agar slants. A fresh slant was opened each month. Each week a new broth culture was prepared from the slant, plated on desoxycholate agar, and stored at 0 to 10° C. after 16 hours' incubation at 37° C. A single colony was selected from the desoxycholate plate for each experiment.

Protoplast production. Protoplasts were produced by the three hour incubation of 0.5 ml. of Penassay medium containing 10^8 *E. coli* per ml. added to 9.5 ml. of sucrose and penicillin broth.

Enumeration of protoplasts. Protoplasts were enumerated by reconverting them to rod form by incubation of an aliquot of the sucrose and penicillin culture in pure sucrose broth at 37° C. for one hour.[10] The bacterial colonies detected in the pour plates prepared from this sucrose broth were assumed to represent the total number of protoplasts reconverted to viable rods, plus any rod forms which had survived the exposure to penicillin and sucrose. The number of rod forms not converted to protoplasts was determined by incubating an equal aliquot of the sucrose and penicillin broth in isotonic saline for one hour, thus lysing the protoplasts present, and making appropriate agar pour plates. The lysis of protoplasts in saline, water and Penassay broth was found to be the same. No significant difference could be found in the results of "control" medullary injection of animals that had received the products of lysis in either of these three media.

According to this technique, 90 to 99 per cent of the organisms in the penicillin and sucrose broth were present in the protoplast form. The total number of these protoplasts varied from 10^6 to 10^8. These figures are in agreement with those obtained by

others.[10] The presence of osmotically resistant forms (ORF) (probably representing rods which had not been converted to protoplasts) may have several explanations. First, penicillin is known to affect only actively dividing micro-organisms.[11] Secondly, since the conversion of rods to protoplasts occurs asynchronously,[6] some rod forms were probably not prepared to convert during the time of exposure to penicillin. It is also possible that ORF are osmotically stable L-forms. Stable protoplasts can be selected by gradual reduction of osmolarity over many bacterial generations.[12] It seems unlikely, however, that there was an opportunity for this adaptation to occur.

It is important to note that only 50 per cent of the protoplasts are thought capable of reverting to rods on exposure to hypertonic media.[10] Thus, the procedure utilized for counting protoplasts results in an underestimation of the number of protoplasts actually present. Although the enumeration of protoplasts was only approximate, the significant aspect of this study was to document the precise number of ORF persisting in the protoplast inoculum. There is no reason to believe there was significant error in the enumeration of these bacterial forms.

Preparation of inocula: 1) *Medullary*—One ("experimental") kidney of each animal was injected with the protoplast culture diluted in sucrose-penicillin broth to contain fewer than 10 residual forms. The final protocol included only those animals receiving no more than 10 ORF.

The remaining ("control") kidney received the same inoculum as the "experimental" kidney diluted and incubated in saline. Thus, this kidney received ORF along with fragments of protoplasts. As a result, the experimental kidney was injected with protoplasts and the control was not. Both kidneys, however, received the same number of ORF.

2) *Intracutaneous*—Protoplasts were prepared in the same fashion. Inocula of rods for control injections were incubated in sucrose broth rather than Penassay.

3) *Non-viable protoplasts*—An aliquot of media containing protoplasts was boiled for five minutes. The inoculum contained 10^1 to 10^3 normal rods and 10^5 to 10^6 heat killed protoplasts. Non-viability of the boiled protoplasts was established by incubation in sucrose for three hours after which agar pour plate cultures were prepared.

Animals. White New Zealand male rabbits weighing 5 to 8 pounds were housed individually and fed standard Purina Chow.®

Abdominal operations. Rabbits were anesthetized by the intravenous administration of 30 to 50 mg. of sodium pentobarbital in sterile saline. Supplemental inhalation ether was employed when additional anesthesia was required. After removing abdominal fur, the skin was scrubbed with 70 per cent alcohol and merthiolate. Bilateral renal exposure was accomplished by a midline abdominal incision, while unilateral exploration was accomplished by a subcostal flank incision. Continuous silk sutures were used to close the wound in two layers.

Injection of renal medulla. A 27 gauge 2.0 cm. hypodermic needle with a sterile syringe calibrated to 0.01 ml. was used for all injections. Inocula of 0.05 ml. of the bacterial suspension were introduced into the medulla. The needle was inserted midway between the upper and lower pole, perpendicular to the kidney surface, 1.3 cm. medial to the lateral margin to a depth of 1 to 1.2 cm. This technique results in the placement

of the needle point in the papilla or low medulla of the rabbit kidney. After removal of the needle, gentle pressure was applied at the injection site to minimize leakage.[1]

Intracutaneous injections. In addition to electric clipping, depilitation was carried out with Nair® (Carter Products, Inc., N.Y.C.) and the abdomen cleansed with alcohol. Inocula of 0.1 ml. were injected intracutaneously in ten sites, each of which was at least 3.0 cm. from any others.

Enumeration of bacteria in organs and tissues. The animals were reanesthetized 4-5 days after they had been injected. Using sterile technique, both kidneys were removed, examined, and placed in Petri dishes. Each kidney was cut into small pieces and placed in a test tube containing 5.0 ml. of saline to make a total volume of 10 ml. and ground in a "Precision" homogenizer. Tenfold dilutions were made in saline and appropriate agar pour plates prepared. The plates were incubated at 37° C. for 36 to 48 hours.

Approximately 1 ml. of blood, obtained by cardiac puncture, was added to melted agar, poured into a Petri dish, allowed to harden, and incubated as above. The bladder was aspirated to procure urine which was streaked on desoxycholate agar.

Results of the intracutaneous injections of bacteria were determined by measuring the greatest diameter of the zone of induration 36 hours later. The same observer measured all sites, and different observers were able to obtain reproducible results accurate to 0.5 cm.

EXPERIMENTAL RESULTS

Medullary injections. Protoplasts were injected directly into the renal medulla of a group of rabbits. The opposite kidney of the same animal was inoculated with bacteria derived from the same protoplast culture diluted, however, with an isotonic solution, thus lysing the protoplasts and leaving only the osmotically resistant bacterial forms (ORF) intact. It is known that the inoculation of more than 10 bacilli into the renal medulla of the normal animal will frequently result in infection.[1] Since there were always bacteria in the protoplast culture which resisted lysis in normal saline, the experiments with inocula diluted to contain no more than 10 ORF are presented. The number of protoplasts detected in the inocula varied from 2.5 to 500. It must be emphasized, however, that this is undoubtedly an underestimate of the true number of protoplasts present in the bacterial suspension.

In Table 1, it can be seen that of 17 animals, 7 were given protoplasts without detectable numbers of ORF. After 4-5 days, 5 of these 7 animals had $>10^3$ *E. coli* in the kidney receiving L-forms. The control kidneys were sterile in 5 instances. Only one of the "control" kidneys contained as many as 10^3 *E. coli* and the "experimental" kidney of this animal contained greater numbers of bacteria than the "control."

In all, 10^3 or more *E. coli* were recovered from the protoplast injected kidney in 11 of 17 animals. Only 2 control kidneys contained this number

of bacteria. In those 2 animals, the opposite kidneys (injected with protoplasts), also contained large numbers of bacteria. No control kidney (receiving ORF only) contained a greater number of bacteria than did the experimental kidney.

In two animals (Nos. 9 and 12) there were gross wedge-shaped lesions characteristic of pyelonephritis in the experimental kidney. The cortical

TABLE 1. INJECTIONS DIRECTLY INTO THE RENAL MEDULLA

	Experimental kidney		*Control kidney*	
	Protoplasts injected*	E. Coli *recovered*	*Rods injected*	E. Coli *recovered*
1	2.5	10^4	0	0
2	2.5	10^4	0	10^8
3	3	0	0	0
4	3	10^5	0	0
5	6.5	10^8	0	10^1
6	4.5	10^8	0	0
7	500	0	0	0
8	350	0	1	0
9	350	10^4	1	10^4
10	65	10^8	1	0
11	150	10^8	1	10^2
12	8	10^5	2.5	10^2
13	35	0	5	0
14	35	0	5	0
15	40	10^5	5	10^2
16	40	0	5	0
17	200	10^4	10	0

* It must be emphasized that the actual number of protoplasts present undoubtedly exceeds the number determined by the techniques used in this study (see MATERIALS and METHODS).

surface of these lesions was studded with abscesses. Large numbers of bacteria were recovered from both of these kidneys.

In four animals (Nos. 1, 4, 12 and 17), *E. coli* were cultured from the bladder urine at autopsy. In all of these animals, large numbers of bacteria were also present in the experimental kidney. Blood cultures were sterile in all animals.

Intracutaneous injections. It was evident that protoplasts could survive and even multiply and produce infection in the hypertonic renal medulla. It was decided, therefore, to test their ability to produce visible inflam-

mation in an isotonic tissue, the abdominal dermis. Five animals were injected intradermally at 10 different sites with inocula containing either rod forms or mixtures of ORF and protoplasts. As can be seen in Table 2, all five sites injected with 10^7 rods had areas of induration at least 1 cm. in diameter. Only one site of six receiving 10^7 or more protoplasts had induration greater than 0.5 cm. in diameter. Thus, protoplasts do not seem to be effective in producing visible inflammation of the skin.

Effect of media on results. The special environmental demands of L-forms made the use of unusual media obligatory. A test was conducted

TABLE 2. INTRACUTANEOUS INJECTIONS

	Lesion size in cm.	
Number of micro-organisms injected	*Rods*	Protoplasts*
10^8		$<0.5, 0, <0.5$
10^7	1.5, 1.5, 1.0, 1.0, 1.0	0, 0, 1.0

* Protoplast inocula contained 10^3 to 10^4 rod forms. It must be emphasized that the actual number of protoplasts present undoubtedly exceeds the number determined by the techniques used in this study (see MATERIALS and METHODS).

of the effect of these media on the ability of normal *E. coli* to produce infection. Multiplication of *E. coli,* in sucrose or Penassay broth, using otherwise identical conditions, was similar to that found with beef heart infusion broth. Furthermore, rod forms incubated in Penassay, sucrose, or beef heart infusion broth seemed equally capable of producing medullary infection. An inoculum containing 10^1 *E. coli* cultured in sucrose broth was injected into the medulla of six animals. Three of the inocula were diluted in saline and three in sucrose. Two of the three animals in each group developed infection.

DISCUSSION

The present study indicates that protoplast forms of *E. coli* can initiate infection in the hypertonic environment of the renal medulla. Experiments designed to test the biological behavior of protoplasts are difficult since conversion of *E. coli* to protoplasts is never complete. For this reason, inocula were serially diluted to minimize the number of unconverted bacteria administered. In 17 experiments, protoplasts mixed with 10 or fewer ORF were introduced into the medulla of the kidney. After 4-5 days, 10^3 or more *E. coli* were recovered from 11 of these 17 kidneys.

On the other hand, kidneys injected with the same inocula diluted in saline, thus lysing the protoplasts, contained 10^3 bacteria in only 2 of 17 instances. Furthermore, in these animals there was bacterial multiplication in the experimental kidney, in one case exceeding and the other equaling that in the control kidney, thereby suggesting that transient bacteremia, rather than the original inoculation, might have been responsible for the delivery of bacteria to the control kidney.[1, 18] Looking closely at the single control kidney which contained as many as 10^4 bacteria, it is evident that the kidney which received protoplasts also contained 10^4 micro-organisms but, in addition, displayed gross lesions typical of abscesses.

It is concluded that protoplast forms of *E. coli,* when placed in the proper environment, can produce infection. No effort has been made to compare the ability of rods and protoplasts to produce infection since enumeration of viable protoplasts is so inexact.

It would be expected that protoplasts could not produce infection in tissues isotonic to plasma. The production of infection in isotonic tissues requires large numbers of bacteria, however, and the use of this many protoplasts was frequently complicated by the presence of large numbers of ORF. It was difficult to resolve this issue. The most successful experiments were those in which protoplast inocula containing relatively few ORF were injected into the abdominal dermis. Large numbers of protoplasts produced less visible inflammation than did smaller numbers of rod forms, thus supporting the hypothesis that protoplasts are not able to produce infection in tissues isotonic to plasma.

The renal medulla is particularly susceptible to the initiation of bacterial infection. The present studies emphasize one possible explanation for the unique microbiological properties of this zone of the kidney. A number of previous reports have demonstrated the formation of protoplasts *in vivo* due to the action of antibiotics or naturally occurring host factors. Braude, Siemienski, and Jacobs[8] have demonstrated protoplasts in human urine and in the renal medulla of the rat. The present experiments establish that protoplasts can initiate infection. It is not known as yet whether protoplasts must first convert to rods before they can multiply and produce tissue damage.

The administration of large quantities of fluid has long been advocated in the treatment of urinary tract infections. This therapy appears to have a rational basis since lowering the tonicity of the renal papilla

and urine would produce a condition less favorable to the survival of protoplast forms of bacteria.

SUMMARY

Experiments have been described which demonstrate that protoplast forms of *E. coli* can persist and multiply in the renal medulla of the rabbit. The ability of protoplasts to survive in the peculiar hypertonic environment of the renal medulla provides another explanation for the unique microbiologic properties of this zone of the kidney.

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