

THE THIRD K. PRATHAP MEMORIAL LECTURE

EFFECTS OF ANTIBIOTICS ON BACTERIAL PATHOGENICITY

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INTRODUCTION

Pathogenicity is a complex process which results from the interaction between bacteria and their host. Its definition is: "the competence of an infectious agent to produce pathologic effects, as indicated by case fatality rates and/or the ability to invade the tissues of the host."

A successful pathogen must exhibit all of the following properties: Enter the host, multiply in host tissue, resist or not stimulate host defences, and most of all produce damage to the host. Bacterial factors which affect or contribute directly to the pathogenicity of bacteria are: their morphology, rate of replication (growth rate), biochemical products of metabolism (toxins, enzymes, antibiotic-degrading substances) and products of bacterial death.

While pathogenicity of a certain bacterial species or bacterial strain can be evaluated *in vitro*, an accurate determination of this pathogenicity must be carried out *in vivo*. When evaluating bacterial pathogenicity *in vitro* it is of utmost importance to be aware of the three main differences between bacteria grown *in vitro* and bacteria as they are *in vivo* producing disease: a) bacteria *in vivo* multiply much slower than the same organism grown *in vitro* in a nutritionally balanced medium, b) bacteria *in vivo* grow on surfaces, usually relatively hard surfaces in contrast to bacteria grown *in vitro* that usually are tested in a liquid medium, and finally c) what counts *in vivo* is the mass rather than the number of bacteria as determined *in vitro* by the count of colony forming units.¹ During the course of an infection bacterial generation time increases progressively. It might become as long as 20 hours such as in *Staphylococcus aureus* osteomyelitis.² The slow rate of bacterial division *in vivo* is in part due to *in vivo* nutrient depletion, such as the iron restricted conditions seen in certain infections.³ In contrast,

intravenous administration of iron to infected mice promoted growth of *Salmonella typhimurium* indicating an increase in pathogenicity. A factor to be taken into account for the *in vitro* investigation of bacteria, is the physical state of the medium. Colonization of the surfaces of materials and of most tissues is a common and important feature of microbial growth in animals and man. Colonizing bacteria adhere to the surfaces upon which they grow and when they are virulent, penetrate. *In vitro* systems that provide a solid support surface for the growth of bacteria, therefore, should reflect more accurately than liquid media the growth conditions of the *in vivo* stage. The morphology and ultrastructure of bacteria grown by the membrane technique⁴ that is, grown on a relatively hard surface, differ from that of the same organisms grown in broth. This difference is most apparent when *S. aureus* is incubated with beta-lactam antibiotics. On a solid surface or *in vivo*, staphylococci grow into large cells 2–3 μm in diameter and displayed multiple thick cross walls while under the same nutrient conditions in broth, staphylococci grew only to 1.6 μm in diameter and showed only 1 or 2 thick cross walls⁵ (Figs. 1 a and b). Interestingly, the commonly accepted characteristics of staphylococci are those of organisms grown in broth. But, under those circumstances the morphology of the organism does not appear to be that of organisms that infect the host. Hence, the morphology of organisms grown in broth may surprisingly be considered artifactual. When gram negative bacilli are exposed to low concentrations of beta-lactam antibiotics they grow into filaments (Figs. 2a and b) while gram positive cocci under the same condition increase in size and produce cells 2–3 μm in diameter.⁶ In fact, *Legionella pneumophila* converted to filaments (with low pathogenicity) even in the absence of antibiotics.⁷ Giant

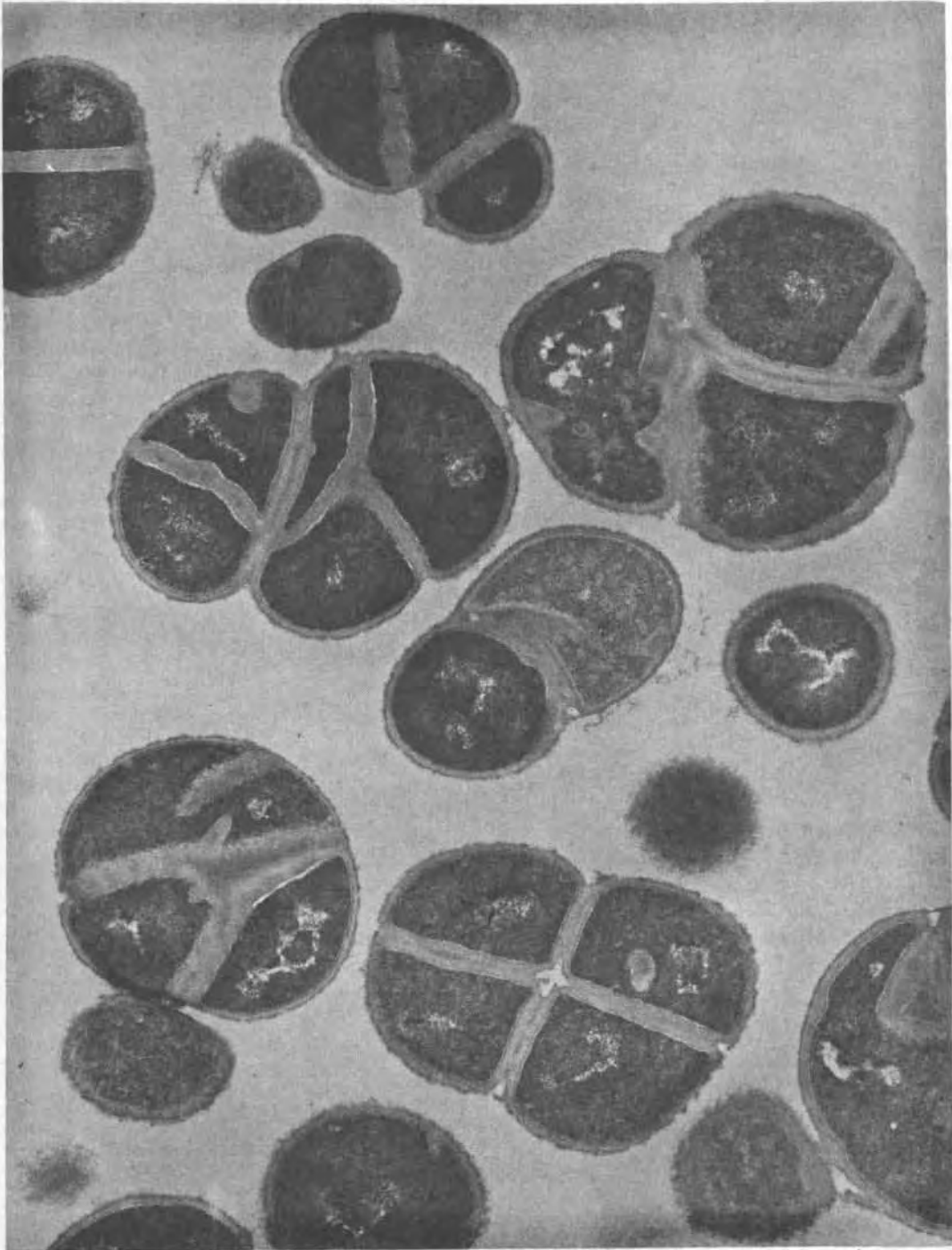


FIG. 1a. *Staphylococcus aureus* grown on membrane placed on agar containing cloxacillin at a concentration equal to 1/3 MIC of this staphylococcus.

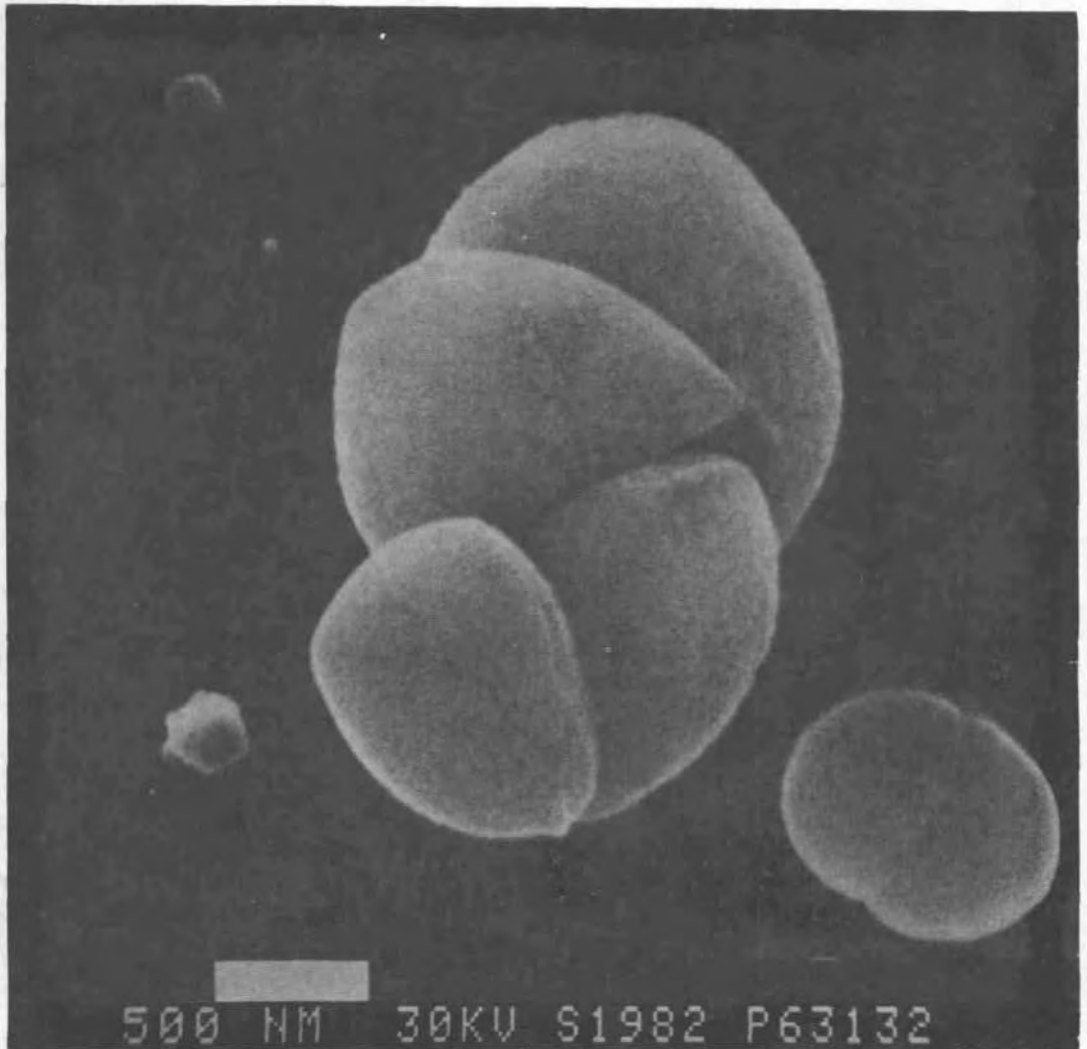


FIG. 1b. (Same as 1a). This staphylococcus has a mass of approximately 50 picograms.

forms of bacteria produced under the effects of low concentrations of antibiotics represent divisions without separation; the antibacterial agent did not inhibit genetic replication but inhibited cross wall formation and its lysis. These giant organisms planted on agar will produce each, one colony forming unit, when in reality each unit contains multiple genomes. While one staphylococcus has a mass of 1.5 picogram (pg) and an *Escherichia coli* has a mass of 3–4 pg, the large staphylococci produced with sub-MICs of antibiotics weigh 18–20 pg while filaments of *E coli*, up to 100 pg. Since these large forms of organisms have been shown to exist *in vivo* when the host

was treated with a low dose of antibiotics, it must be recognized that bacterial mass could be more relevant than the bacterial number during the investigation of pathogenic effects to a host. Antibiotics at concentrations equal to the MBC eliminate the pathogenicity of any given bacteria inasmuch as these concentrations kill the offending organism. At concentrations equal to the MIC, the antibiotic completely inhibits the growth of the bacteria and, therefore, eliminates their pathogenicity. If antibiotics are successful to influence certain factors, such as bacterial morphology and/or the synthesis and release of substances which contribute to bacterial virulence/pathogenicity

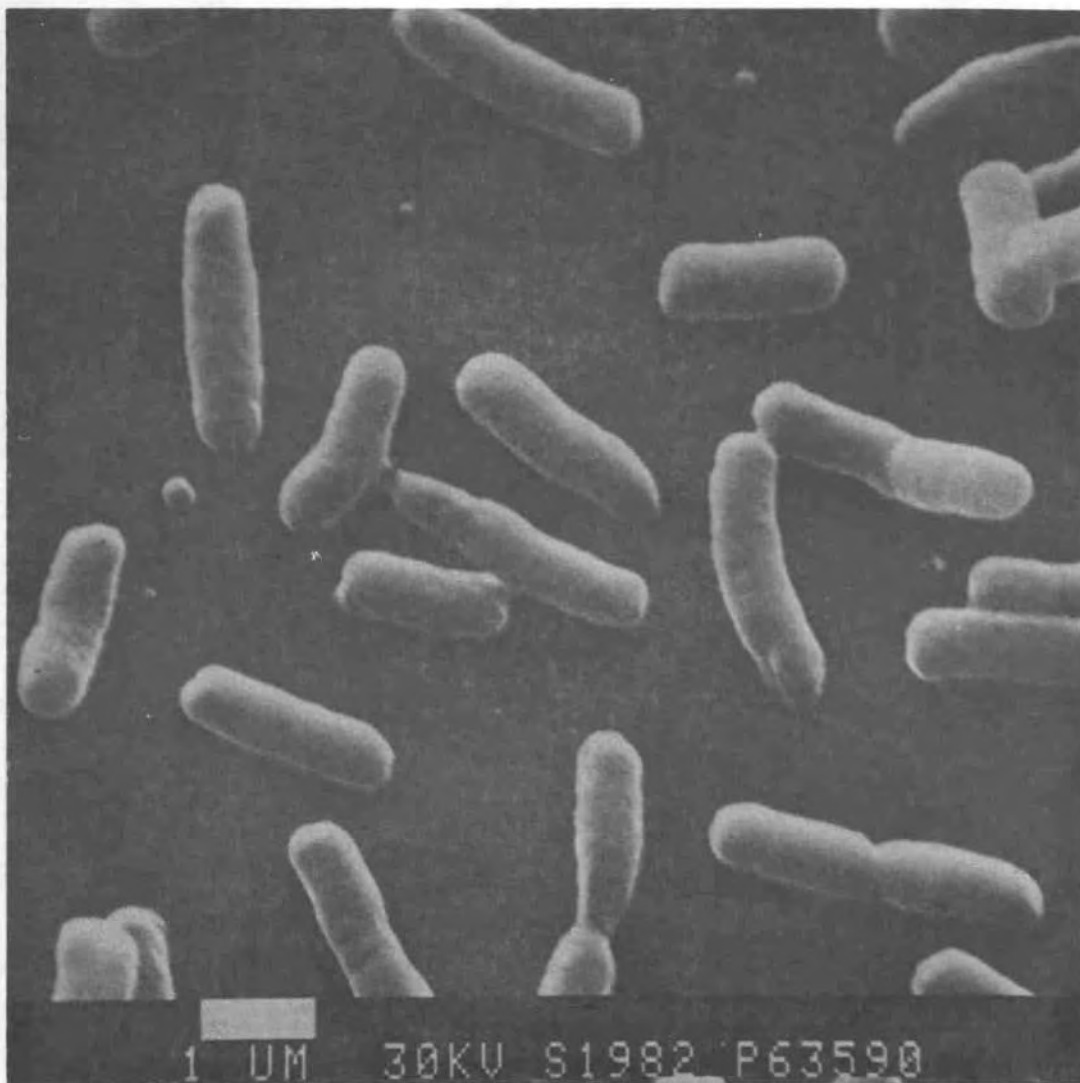


FIG. 2a. *Escherichia coli* grown on a membrane placed on agar. Bacilli have a mass of 2–4 picograms.

the concentration of antibiotic or time exposure to it must be below that which prevents growth or kills the bacteria. Concentrations below the MIC (**sub-MICs**) are known to promote a variety of bacterial properties, most of which affect the pathogenicity of a bacterial population, either enhancing or diminishing it. The earliest observation that low concentrations of antibiotics have a biological effect was in 1949 when Jukes described the growth promotion effect in young animals.⁵ The term antibiotic growth effect refers to the promotion of additional growth in young animals that is obtained by adding small amounts of certain antibiotics

to a complete diet. It has been shown that the low concentration of antibiotic added to animal feed prevents bacterial destruction of protein in the gut; it inhibits toxin production by certain organisms and prevents thickening of the gut wall, thus permitting better absorption of amino acids and probably also of nutrients. The low concentrations of antibiotics in the gut also prevent bacterial destruction of vitamins and favor certain bacterial species that synthesize vitamins. The use of antibiotics in animal feeds had a tremendous impact in the meat production industry and with few exceptions it is practiced all over the world. **Sub-MICs** of antibiotics at the site of infection

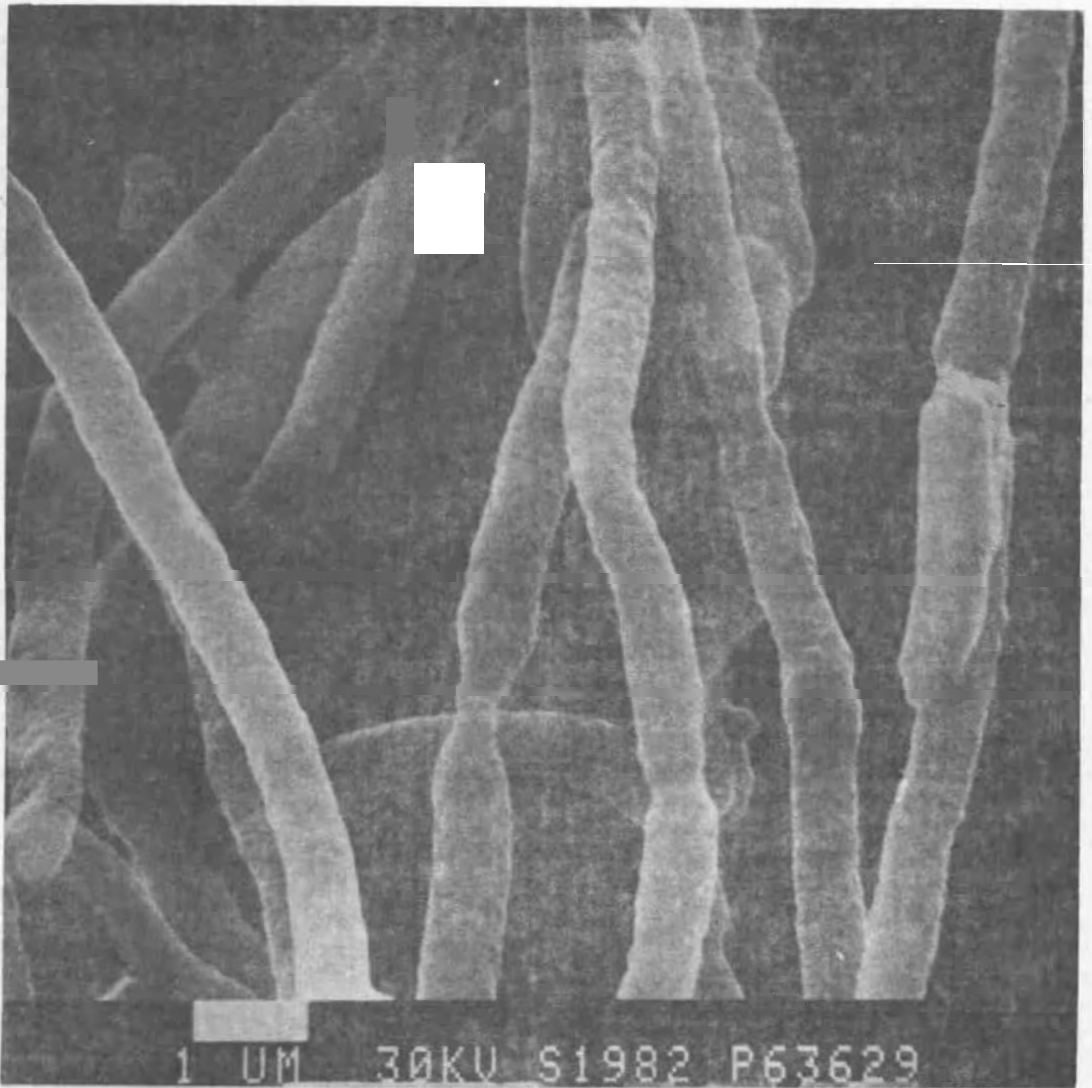


FIG. 2b. *Escherichia coli* grown on a membrane placed on agar containing ampicillin at a concentration equal to $1/3$ MIC of this *E. coli*. Each of these filaments has a mass of 30–100 picograms.

have been shown in experimental animals to have therapeutic efficacy. Zak and Kradolfer produced peritonitis in rabbits with *E. coli*.⁹ They showed that a $1/3$ MIC of gentamicin reduced mortality in a proportion of 80% as compared to a concentration above the MIC. A group of 120 patients with gram negative bacteremia were treated with gentamicin or tobramycin. It was later identified that 22 patients had aminoglycoside concentrations in the serum that were less than the MIC of the organism producing their infection. In fact, half of them had less than $1/4$ of the MIC of aminoglycosides. The overall

mortality for the entire study groups was 37% while the mortality rate among the group of patients with sub-MICs in their serum was 36%.¹⁰ Another example that antibiotics administered at very low dose and producing sub-MICs at the site of infection can result in lasting therapeutic effects is shown in a prospective study with a low dose of ampicillin in urinary infections produced with *E. coli*.¹¹

A total of 20 patients with symptomatic urinary tract infections produced by *E. coli* (10^5 cfu/ml of urine) and pyuria received 10 mg of ampicillin and 2 liters of fluid daily for three days. The ampicillin concentration in

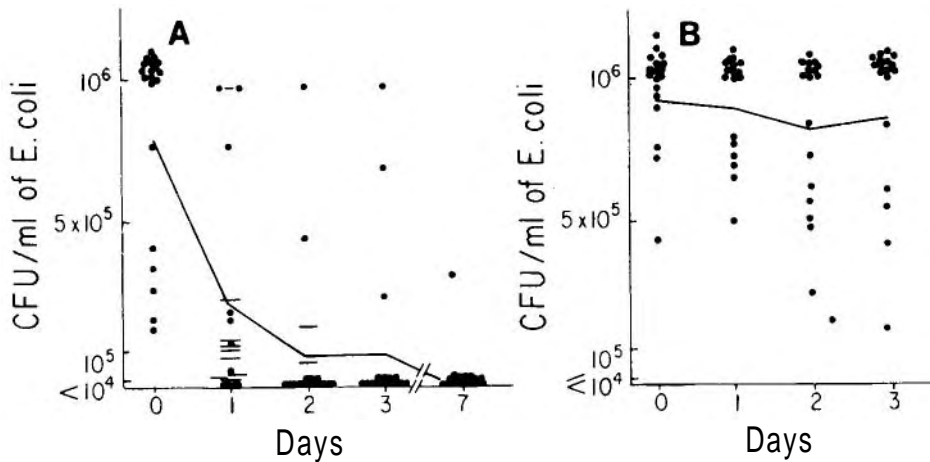


FIG. 3. Number of CFU of *Escherichia coli* in the urine of treated patients (A) and untreated (control) patients (B). Dashes represent urine samples in which filaments were observed.

the urine at its peak was equal to 0.8 of the MIC and at its trough it was 0.12 of the MIC of the respective *E. coli* strains. After two days of treatment, 16 patients had culture-negative urine without pyuria which remained negative for at least one week (Fig. 3). Another group of 18 similar patients received only 2 liters of fluid and after four days, all had more than 10^5 cfu of *E. coli* per ml of urine and 10^4 leukocytes per mm^3 of urine. These therapeutic results are attributed in part to the reduction in the number of organisms in the urine. Another factor to be considered is the effect of sub-MICs of ampicillin on the adherence of the *E. coli* (filaments) to the urinary epithelial cells. The non-adhering *E. coli* may simply have been washed away by the large volume of liquid excreted.

The antibiotic's effect on the various components of pathogenicity including adherence, resistance to immunodefences, local proliferation, tissue damage, invasion, and dissemination will be analyzed.

DIRECT EFFECTS OF ANTIBIOTICS ON BACTERIAL PATHOGENICITY

1. Adherence.

The infectious process is considered to be a chain of multiple steps initiated in most cases by bacterial colonization of mucosal surfaces.^{1,2} The first step in tissue invasion or damage most likely begins with adherence of bacteria to epithelial cells of the host mucosa.^{1,3} The ability of organisms

to adhere to epithelial cells has been found to be associated with surface structures called fimbriae or pili in gram-negative bacteria and fibrillae in gram-positive bacteria.^{1,4} Bacterial adherence is influenced not only by net surface charge and/or specific binding considerations but also by host factors and by variations in a particular bacterial strain. Sub-MICs of various antibiotics modify the surface of the bacteria, thus changing the ability of the bacteria to adhere to the surface of host cells. Sub-MICs of antibiotics exert their antiadhesive effects in three different ways: first, the antibiotic may induce the leakage of the performed adhesion from the bacterial cells as was seen when penicillin induced a loss of lipoteichoic acid from streptococci.^{1,5} Second, it may suppress the formation and expression of the surface adhesion in growing organisms. Finally, the antibiotic may induce the formation of functionally aberrant ligands.

2. Effect of Serum

Complement is a lytic agent for some gram-negative bacilli. The direct bactericidal effect of complement seems in general to be far less important *in vivo* than the opsonins and chemotaxis factors. Only a few species among the gram-negative bacilli which produce infections in man are sensitive to the bactericidal effect of serum. While *E. coli* is the gram-negative bacillus most commonly isolated from infections, only about $\frac{1}{2}$ of the strains are serum-sensitive.

3. Phagocytosis.

Root and his associates¹⁶ incubated a strain of *S. aureus* in trypticase soy broth (TSB) for 2 hours. At this point penicillin was added to obtain a final concentration of 1/4 – 1/32 of the MIC of the strain, and the TSB – staphylococcus-penicillin broth was incubated for an additional 2 hours, planted on agar, and CFU counts were done. Staphylococci grown in the presence of penicillin at 1/4 the MIC are more susceptible to killing by normal polymorphonuclear cells (PMNs) than untreated bacteria (survival was 0.17% as against 1.5%, respectively, at 35 minutes in 14 experiments: $P < 0.01$ by t test). Furthermore, susceptibility to killing was enhanced by penicillins at sub-MICs to the point that the organisms were killed without phagosome formation.

In another study, Lorian and Atkinson¹⁷ grew staphylococci on filter membranes placed on trypticase soy agar (TSA) for 90 minutes. The membranes containing growing staphylococci were then transferred to TSA containing oxacillin, at a concentration equal to 1/4 the MIC for the staphylococci tested, and were incubated for 3 hours. These staphylococci grew into large cells which consisted of numerous staphylococci held together by thick cross walls. After 30 minutes of incubation with PMN cells, the killing of oxacillin-exposed staphylococci as determined by CFU counts was 51 + 3.2% as against 71 + 1.9%, and after 1 hour the oxacillin-exposed cells showed 65% killing as against 85% for the control. The mass as well as the size of the large staphylococci is, however, 7–10 times larger than that of a normal staphylococcus. Therefore at equal CFU numbers, the mass of large staphylococci phagocytized, is significantly larger than the mass of phagocytized normal staphylococci. It appears, therefore, that the conclusion regarding susceptibility of staphylococci exposed or not exposed to oxacillin based on CFU counts could be misleading. In terms of staphylococci mass phagocytized, the large staphylococci are significantly more susceptible to the bactericidal effect of PMNs than the normal staphylococci.

Phagocytic activity is commonly inferred from the number of CFU remaining after exposure of bacteria to leukocytes. The number of CFU is used for quantitation of the number of viable cells. However, since the sizes (masses) of bacilli and filaments are so different, a better basis for comparison of phagocytic activity is cell mass.¹⁸ The use

of mass as a basis for comparison leads to a conclusion that differs from that obtained with CFUs. The mean mass of *E. coli* bacillus is 3.8 pg; that of a *P. aeruginosa* bacillus is 4.4 pg. The mean mass of a filament of *E. coli* obtained with ampicillin is 45.5 pg; that of an *E. coli* filament obtained with mezlocillin is 29.3 pg. The mean mass of a filament of *P. aeruginosa* obtained with azlocillin is 27.2 pg. Filaments produced from phagocytosis-resistant strains of *E. coli* were killed at a higher rate than their respective bacilli. After incubation for 3 hours, 75% of filaments and 21% of bacilli had been killed (Figs. 4a, b, and c). It appears, therefore, that one reason for the higher susceptibility of filaments to phagocytosis is that the PMNs detect filaments faster than an equal mass of dispersed individual bacilli.

4. Agglutination.

Although agglutination has not been proven to be an effective immunodefence mechanism *in vivo*, it nevertheless serves to illustrate antigen-antibody compatibility.

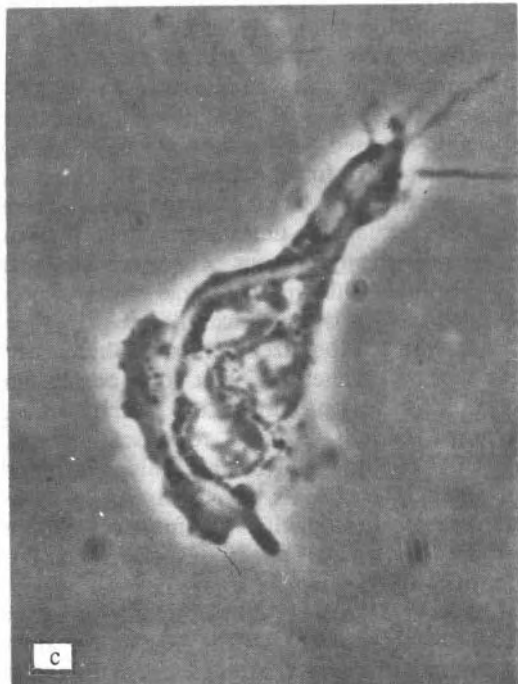
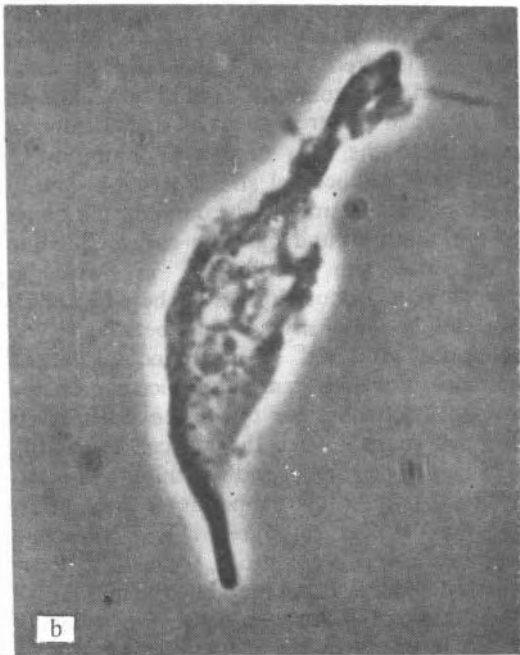
PATHOGENIC SUBSTANCES PRODUCED BY BACTERIA

Pneumococci characteristically produce alpha-hemolysis of red cells on agar; when disks containing penicillin were placed on blood agar inoculated with pneumococci and incubated anaerobically and then exposed to air at 6°C, large zones of beta-hemolysis surrounded the area of growth inhibition.¹⁹ All 100 pneumococcal strains tested produced rings of beta-hemolysis surrounding the zones of inhibition around oxacillin and methicillin. All beta-lactam antibiotics as well as vancomycin produced rings of beta-hemolysis of various intensities. The beta-hemolytic substance produced by pneumococci in the presence of beta-lactam antibiotics is probably not an enzyme, because the degree of hemolysis dropped sharply when the red cell concentration was increased, and because hemolysis occurred only at low temperatures. In contrast to the effects produced by beta-lactam antibiotics, other drugs, mostly those interfering with ribosome activity, inhibit the production of hemolysins and other enzymes which staphylococci produce without significantly affecting the rate of growth. Exposure of *P. aeruginosa* to sub-MIC of aminoglycosides inhibited the production of most enzymes including protease and elastase.²⁰



FIGS. 4a, b, and c.

Phagocytosis of a filament of *Escherichia coli*.



Clostridium difficile has been implicated as the etiological agent in pseudomembranous colitis in humans. The organism produces an exotoxin within the gastrointestinal tract which may be detectable *in vitro* by cytopathic activity. Six clinical isolates of *C. difficile* from patients with proven pseudomembranous colitis were grown in trypticase nitrate broth (TNB) for 4 days in the presence of $\frac{1}{2}$ - $\frac{1}{4}$ the MIC of clindamycin; whereas the number of CFU remained unaffected, the *C. difficile* strains, actively produced high titers of toxin.^{21,22} A strain which could produce toxin *in vivo* but not *in vitro* could be induced to elaborate cytotoxin in culture when clindamycin was added to the medium.

The Growth Rate as a Pathogenicity Factor. Sub-MICs of various antibiotics have been shown to inhibit the rate of growth *in vitro*.²³ The number of infecting organisms is extremely important to the clinical outcome of urinary, lung and wound infections; therefore, a 90% reduction in bacterial populations by sub-MICs of antibiotics could have clinical consequences. Since the minimum antibiotic concentration (MAC) reflects the lowest-acting concentration of an antibiotic, the ratio MIC/MAC can be used to indicate the magnitude of the concentration range for effective activity.

INDIRECT EFFECTS OF ANTIBIOTICS ON PATHOGENICITY

Antibiotic resistant bacteria are equal or less pathogenic than the sensitive organism, never more virulent. A study of 188 clinical isolates of *S. aureus* showed that the strains resistant to five to seven antibiotics differed from those resistant to a smaller number of drugs. They were shown to produce less lecithinase, hyaluronidase and hemolytic activity.²⁴ Exposure of a large inoculum of *S. aureus in vitro* to progressive concentrations of gentamicin which exceeded the MIC resulted in aminoglycoside-resistant bacteria which grew as very small nonhemolytic colonies. Most were coagulase, deoxyribonuclease and mannitol negative. When injected into rats, they produced less extensive disease than the parent strain. Gentamicin-resistant staphylococci were also shown to be less virulent to mice since they produced a milder degree of pyelonephritis than the gentamicin-sensitive parental strains.²⁵ Gentamicin-resistant colonies of *S. aureus* were selected from clinical isolates and their infectivity and pathogenicity were compared to those of the sensitive variant of the same strain. In a rabbit model of

endocarditis it was shown that the mortality rate was 10% for the rabbits injected with gentamicin-resistant staphylococci but 75% for the animals injected with the sensitive parent strain.²⁶ A study of 43 newborn infants infected with *S. aureus* showed no difference in pathogenicity between gentamicin-sensitive and gentamicin-resistant strains. A 6-year study at the Mayo Clinic showed that, of 144 infections with *P. aeruginosa*, 21% of strains were resistant to gentamicin. The pathogenicity of these gentamicin-resistant isolates appeared to be less than that of susceptible organisms. The number of bacteremias produced by resistant organisms was significantly smaller than that produced by the sensitive strain.²⁷ In a rat model, gentamicin-sensitive *P. aeruginosa* (MIC less than 10 ug/ml) were shown to produce a significantly higher mortality than medium-sensitive and highly resistant *P. aeruginosa* strains (MIC 12.5-312 ug/ml). A slower rate of growth in gentamicin-resistant strains of *P. aeruginosa* was observed in both the lag and log phase than in sensitive strains. The decreased pathogenicity of gentamicin-resistant strains was attributed also to the decreased ability of the organism to produce heat-labile toxic components. Streptomycin-resistant mutants of *Pseudomonas phaseolytica* were also less virulent. In a mouse model, the pathogenicity of fosfomycin-resistant *Salmonella enteritides* was found to be 1000 times less than that of sensitive strains. Resistance to erythromycin also reduced pathogenicity.

Clostridium perfringens resistant to clindamycin showed changes in morphology and biochemical activity, a decrease in the lecithinase and a decrease in pathogenicity for animals. INH-resistant *Mycobacterium tuberculosis* is usually catalase-negative. It is also less virulent or completely avirulent for guinea pigs. The loss of pathogenicity is related to the loss of catalase which would otherwise inactivate the bactericidal effects of peroxides in the guinea pig tissues.

Gonococci isolated from urogenital lesions have developed some drug resistance, but gonococci causing disseminated infection have retained exquisite sensitivity to penicillin. The most virulent strains have been found to be the most sensitive.²⁸ One of the mechanisms of resistance is an alteration of bacterial permeability. In some cases antibacterial agents are prevented from gaining access to their sites of action or are excreted from the bacteria at a significantly higher rate. Such alterations in the permeability of resistant bacteria also affect the penetration

of nutrients and growth factors, which eventually results in an overall slowing down of bacterial activities.

The studies presented above clearly show that bacteria resistant to an antibacterial agent are different in many ways from sensitive strains. Their growth rate is slower, their output of pathogenic enzymes is smaller, their biochemical profile is different and more time is required for the modification of biochemical substrates, which is probably also due to the slower growth rate. All of these changes result usually in lower pathogenicity. The stationary phase of bacteria is in itself a form of resistance to the effect of antibacterial agents. This has changed lately. A new beta-lactam antibiotic (CGP-17520) has been discovered. It kills most gram-negative bacilli even in the stationary phase.^{2,9} A new quinolone, ciprofloxacin has also been shown to have a significant antibacterial activity on *E. coli* during stationary phase.³⁰

As has been shown³¹ bacterial resistance of the older antibiotics is not on the increase; what is most important is the fact that industry is providing each year original new concepts in antibacterial activity which will ultimately result in the ideal antibiotic.

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