CHAPTER I

INTRODUCTION

Filaments and other aberrant forms of bacteria have been observed in the blood cultures[1] and spinal fluid[2] of patients treated with antibiotics.

Many patients admitted to hospitals have received prior therapy with antibacterial agents, frequently at low dosages. Typically about a third of all cultures processed in a hospital laboratory have been found to be from patients already receiving such drugs[3]. Subinhibitory concentrations of antibiotics might thus be expected to be present in the blood or tissue of a large number of patients. Pus from the thigh of a patient treated with gentamicin showed bipolar stained bacilli which were proved to be *Klebsiella pneumoniae*[4]. In the laboratory of the Division of Microbiology and Epidemiology, Department of Pathology, The Bronx-Lebanon Hospital Center, the following phenomena were found: filaments of enterobacteria in blood cultures and urine, filaments of bacteroids and giant staphylococci in wounds, and rod-like pneumococci in sputum specimens. In most cases, prior antibacterial therapy were documented[5].

Considerable variations in the morphology of bacteria could be caused by factors other than exposure to antibiotics. A rich culture medium would accelerate the growth rate and increased the

length of Gram-negative bacilli[6], as well as the number of ribosome[7]. Antibacterial agents remained, however, a main cause of abnormal forms of bacteria seen in specimens submitted to a clinical laboratory. Such forms could occasionally simulate the appearance of quite different species, and they might indicate the presence at the site of infection of a subinhibitory concentration that resulted from prior, possibly unsuspected antibiotic therapy[5].

Attempts in the present study have been made to demonstrate abnormal form and growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* produced *in vitro* by the exposure to subinhibitory concentrations of ampicillin and gentamicin, and to determine the sensitivity of growing bacteria after exposure to antibiotics. Electron microscopy was performed to reveal details which might help to explain abnormalities observed in Gram stain.

1. Abnormal forms of bacteria produced by antibacterial agents.

Shortly after the discovery of penicillin, Gardner[8] described abnormal and filamentous forms of Gram-negative bacilli produced when grown in the presence of subinhibitory concentrations of penicillin. Others have since reported the occurrence of abnormal forms when Gram-negative bacilli were grown in the presence of various other antibacterial agents[9-14]. Abnormal froms and lossing ability of Gram staining have been found in staphylococci and streptococci cultured in media containing antibiotics[15,16].

Staphylococcus aureus formed large cells after exposure to various antibiotics[5,16-22], excepted for nitrofurantoin in which they produced abnormal dividing to form small daugther cells and abnormal septum formation at subinhibitory concentration[23]. Exposure to concentration equivalent to one-tenth the minimal inhibitory concentration (MIC) of cephalothin[16,17] or penicillin[17] resulted in the appearance of small bleb-like structure on the surface of occasional cells; irregular spherical structure lying free or appearing to extrude from cells was also observed. After exposure to the minimal inhibitory concentration of penicillin or cephalothin, cells had more frequent appearance of the above defects by one-tenth the MIC as well as occasional large cells of 3 morphologic types: (1) cells with a "cobblestone" or "raspberry" appearance; (2) cells appearing more symmetrical than (1) and exhibiting a mosaic-like surface texture; and (3) large smooth cells. After exposure to ten times the MIC of penicillin or cephalothin, individual cell exhibited each of the above defects as well as large irregular forms resembling known cell-wall-defective staphylococci[16,17]. Exposure to one-third the MIC of penicillin resulted in the much larger cells which had more irregular shape, retained more crystal violet than control cells after Gram staining and had many wide septa without central dense layer[5,18]. Exposure to one-third the MIC of oxacillin or cephaloridine resulted in three to seven times larger cells which had numerous cross walls that were two to four times thicker than normal, crisscrossed the cells and had no central dense layer.

Many cells exposed to oxacillin showed a thick central cross wall dividing the large cells into two hemispheres. These were subdivided by cross walls arranged at angles to the central cross wall. In many cells that exposed to cephaloridine, the cross walls were rather irregularly oriented. The peripheral cell wall appeared normal except for some thick area. Exposure to one-fourth the MIC of oxacillin resulted in larger cells held together by thick and irregular cross walls. Exposure to one-fourth the MIC of lincomycin resulted in 1.5 to 2 times larger cells, many cells had two to four cross walls which were two to three times thicker than those of controls and had a central dense layer, the peripheral cell wall was twice as thick as that of controls[19]. Exposure to the minimal inhibitory concentration of penicillin resulted in large cells with the septum that showed loss in density and irregularity of shape, and the peripheral cell wall was markedly thin[20].

In the presence of physiological concentration 1 microgram (μg)/milliliter (ml) of tetracycline, *Staphylococcus aureus* which had no transverse septa, were larger, had a greater electron density, and thicker cell walls[22].

Pseudomonas aeruginosa became filamentous form after exposure to concentration equivalent to one-tenth the MIC to ten times the MIC of carbenicillin or ticarcillin[17,24], sublethal concentration of benzylpenicillin[25], and cephalosporin which treated cells tended to coil[26]. However, at higher concentration of carbenicillin or ticarcillin, the cells became sphaero-

plast[24]. At ten times the MIC of carbenicillin, the cells also resulted in elongation as well as saccular outpouchings from the cell surface[16,17].

2. Characteristics of *Staphylococcus aureus*

2.1 Taxonomy

Staphylococcus aureus was classified according to Bergey[27] as:

Kingdom Procaryotic

Division II The Bacteria

Family Micrococcaceae

Genus Staphylococcus

2.2 Microscopic morphology

Staphylococcus aureus is spherical, 0.8 to 1.0 micrometer (μm) in diameter. Cells occur singly, in pairs, or in short chain, and divide in more than one plane to form irregular clusters. They are non-motile and Gram-positive. Some strains possess a capsule or slime layer[27,28].

2.3 Pathogenicity

The most important and widespread agent involved in hospital infection is *Staphylococcus aureus*[29]. The organisms are normally present on skin, and their entrance into cut or scratch may lead to infection. They are the cause of boils, furuncles, abscesses, and suppuration in wounds. Pus consists largely of an accumulation of bacteria and polymorphonuclear

leucocytes in the infected area.

Staphylococcus aureus and *Pseudomonas aeruginosa* are frequently found together in pyogenic infection. The organisms rarely produce septicemia but may be a secondary invader in peritonitis, pyemia, cystitis, osteomyelitis, meningitis, lung abscess, and brain abscess[28].

Certain strains of *Staphylococcus aureus*, of unusual virulence and frequently resistant to common antibiotics, have been so widely associated with hospital infections that they are sometimes designated "hospital staphylococci". The habitat of these staphylococci is the upper respiratory tract, usually the nasal passages, and they often become established as "normal flora" in hospital personnel. In such healthy personnel the organism may cause no disease, but these symptomless carriers may be a source of infection for susceptible patients[29].

2.4 Drug resistance

The staphylococcal variation of by far the greatest practical importance is that in susceptibility to the antimicrobial activity of the antibiotics, and has been considered elsewhere as an outstanding example of acquired resistance. The staphylococci appeared to become drug-resistant more readily than most other bacteria. They might be made resistant to antibiotics by culture in the presence of successively increasing concentrations of these substances, and they might also become drug resistant under natural conditions. The appearance of drug resistance in strains

isolated from pathologic processes had followed the introduction of the various antibiotics into general use, and the percentage of resistant strains found has continuously increased[30].

3. Characteristic of *Pseudomonas aeruginosa*

3.1 Taxonomy

Pseudomonas aeruginosa was classified according to Bergey[27] as:

Kingdom Procaryotic

Division II The Bacteria

Family Pseudomonadaceae

Genus Pseudomonas

3.2 Microscopic morphology

The cells of *Pseudomonas aeruginosa* vary considerably in size and proportion but appear usually as small, slender rod, 1.5 to 3.0 μm long and 0.5-0.8 μm broad, frequently unite in pairs and short chains. There are one to three polar flagella, and the bacterium is actively motile. Neither capsules nor spores are formed. The bacilli stain readily with the usual aniline dyes and are Gram-negative[30].

3.3 Pathogenicity

Pseudomonas aeruginosa, which is a normal habitat of soil and water, is a common hospital pathogen and is usually found in urinary tract infections, wounds, and burns. The organism is especially troublesome in postoperative wounds[29,31]. It also

causes septicemia, abscess, and meningitis. Bronchopneumonia and subacute endocarditis are also increasing in frequency by *P. aeruginosa* infections[32].

The organism forms its characteristic blue pus by the production of two water soluble pigments, a blue-green pyocyanin and a greenish yellow fluorescein. In addition to its habitation of wound infections, *Pseudomonas aeruginosa* occurs in cases of otitis media and in infection of the genitourinary tract, the respiratory tract, and the eye or joints. Meningitis may follow the accidental infection which contaminated needles are used for spinal puncture. Pneumonia, burns, and urinary and respiratory infections may be caused by the organism, particularly after antibiotic therapy that has eliminated other flora. Postoperative wound infected by this organism is also troublesome[31].

3.4 Drug resistance

Pseudomonas aeruginosa is a bacteria that is naturally resistant to many antibiotics and is adaptable to a wide variety of human habitat[29]. Antibiotic resistance has proved troublesome, especially in burns, where the organism produces its blue-green or blackish pigmented exudate[31].

4. Ampicillin

Ampicillin, a semisynthetic penicillin derived from 6-aminopenicillanic acid, is acid stable and has the same general spectrum of activity as penicillin G against Gram-positive organisms and is more effective against some Gram-negative organisms[33]. Since

ampicillin is a zwitterion and a poor candidate for effective penetration of the Gram-negative outer envelope, the early disruption of murein biosynthesis brought about by the ampicillin may cause some disorganization of the outer layers and lead to an increased rate of penetration of ampicillin[34]. The penicillins tended to be irreversible inhibitors of cell wall synthesis and they were usually bactericidal at concentration close to their bacteriostatic level. As a consequence, the penicillins were used widely for treating bacterial infections and were regarded as highly effective antibiotics with low toxicity[35].

4.1 Mode of Action

The penicillins inhibited the biosynthesis of bacterial cell wall. The cell wall contained a matrix of complex macromolecules that provide rigidity and mechanical stability by way of cross-linked latticework-like structures. One such structure was the peptidoglycan layer, apparently present in both Gram-positive and Gram-negative bacteria[35]. The first step in cell wall synthesis took place in the cytoplasm of the cell, the pentapeptide building blocks of the cell wall were put together. These pentapeptides, which were attached to a sugar group (N-acetyl-muramic acid), consisted of five amino acid molecules: one each of L-alanine, D-glutamine, and L-lysine and two of D-alanine[35,36].

The second step in synthesis of the bacterial cell wall consisted of the joining of the pentapeptide building blocks (in the form of N-acetyl-muramyl pentapeptide) with another molecule,

an aminosugar called N-acetyl glucosamine, to form linear strands of cell wall material known as peptidoglycans. During this step the peptidoglycan strands had to be passed through the cell membrane and put in place on the growing cell wall outside the cell membrane[35,36].

The final step in cell wall synthesis occurred outside the cell membrane. It involved a cross-linking of the linear peptidoglycan strands to form a structure with increased rigidity, resembling a three dimensional fish net or a child's jungle gym. The cross-linking step was mediated by the enzyme transpeptidase[35,36].

The penicillins acted as inhibitor of the transpeptidase and carboxypeptidase enzymed involved in the final cross-linking of the peptidoglycan chains. The transpeptidase was irreversibly inhibited; the carboxypeptidase, although more sensitive to these antibiotics, was reversibly inhibited[35,36]. Strominger[37] has extensively reviewed the specific mechanism of cell wall inhibition and has proposed that the penicillins were structural analogues of the terminal D-alanyl-D-alanine residue of the peptidoglycan strands that was operated on by these enzymes. However, the sensitivity of the enzymes to the penicillins and cephalosporins did not seem to fully explain the killing of the bacterial cell since bactericidal activities of different β-lactam antibiotics did not correlate well with the enzyme inhibition data. In addition, radioactivity labeled β-lactam antibiotics bound to multiple sites on the cell wall and different

antibiotic displayed different reactivities towards these binding sites[38]. The nature and specific relationship to the killing of the cell of these other binding sites have not been completely resolved.

5. Gentamicin

Gentamicin is a broad-spectrum aminoglycosidic aminocyclitol antibiotic. It was first studied and described by Weinstein and co-workers[39,40] in 1963. It was then isolated, purified, and characterized by Rosselot and colleagues[41] in 1964. It was currently of great value in the therapy of severe infections due to Gram-negative bacteria, and it was the most important aminoglycosidic aminocyclitol[42]. As naturally formed gentamicin contained several components of which those designated C_1, C_{1a} and C_2 were required by USA regulations to be presented in the commercial product in certain flexible proportions[43].

The most significant efficiency of gentamicin was its activity against *Pseudomonas aeruginosa* which has been shown to be 5-10 times greater than that of neomycin or kanamycin[43]. Gentamicin was a drug of choice for septicemia and serious infections of the central nervous system, urinary tract, respiratory tract, gastrointestinal tract, skin, bone and soft tissue (including burns) caused by sensitive Gram-negative organisms[44]. When *Pseudomonas aeruginosa* was the infectious organism involved, carbenicillin was often administered concomitantly, since synergism of these two antibiotics has been widely reported[44]. Aminoglycosidic aminocyclitols showed a useful synergy with peni-

cillins against Enterobacteriaceae and penicillin-aminoglycosidic aminocyclitol combination were the therapy of choice for serious enterococcal infections, especially endocarditis[44].

5.1 Mode of action

The aminoglycosidic aminocyclitols acted by inhibiting protein biosynthesis. Ribosomes were the sites of protein synthesis in the cell, they served as the supporting structure for messenger ribonucleic acid (mRNA) that has been formed by using chromosomal deoxyribonucleic acid (DNA) as a template. The mRNA molecules contained a series of nucleic acid triplets, each of which coded for a specific amino acid. Messenger RNA thus served as the blueprint for the incorporation of amino acid into protein. A number of ribosomes might be associated with a strand of mRNA (so-called polyribosomes). In turn, the ribosomes also served as the point of attachment for aminoacyl transfer RNA (tRNA). These molecules of tRNA, each containing a specific amino acid, were attached to the ribosome in the order for which the amino acid they carried was coded by mRNA. Thus the amino acids were transferred in proper order to the growing polypeptide or protein chain on the ribosomes. Bacterial ribosomes might be dissociated into 50 Svedberg units (S) and 30S subunits by placing them in low concentrations of magnesium[36].

The aminoglycosidic aminocyclitols also bound to the 30S subunit of the ribosome and inhibited protein synthesis (Fig. 1) page 13[45]. The mechanism of their actions was unique. Unlike

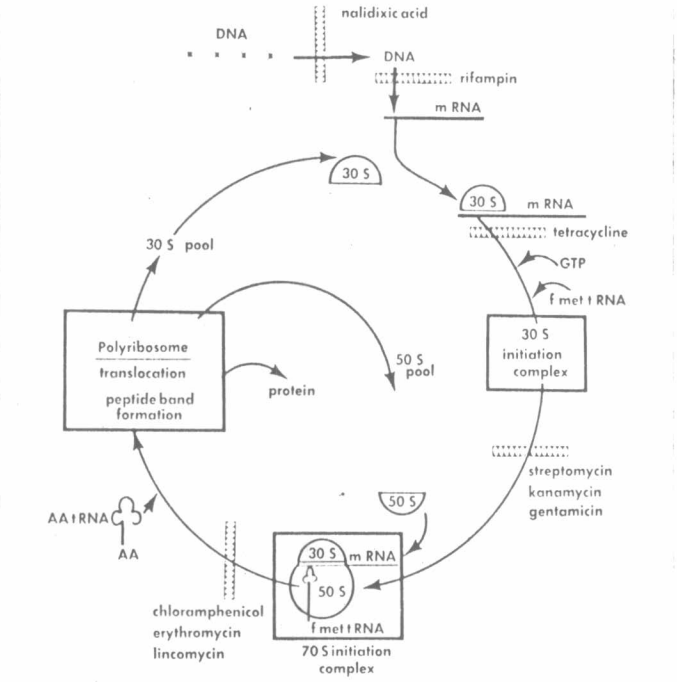

Fig. 1 Protein biosynthesis illustrating sites of action of agents that inhibit protein synthesis.

the other inhibitors of ribosomal function, these were bactericidal agents which caused rapid cessation of polypeptide chain elongation in protein synthesis and breaking down of polyribosomes, but neither of these effects accounted fully for their lethality to bacteria. It was also known that they were capable of causing misreading of the genetic code and that this ability varied among the different aminoglycosidic aminocyclitols. Gentamicin produced much higher levels of misreading of the messenger RNA than streptomycin. Thus an amino acid other than that coded for by messenger RNA might be incorporated into the growing polypeptide chain, resulting in formation of the so-called nonsense

proteins. The exact mechanism by which the aminoglycosidic aminocyclitols caused cell death was not known, although it might be related to the fact that their binding to ribosome was essentially irreversible[34,36,46,47,48].

6. Morphologic observation of microorganisms

In the studies of this project, two procedures for observation of bacterial morphology were employed namely the Gram staining and the electron microscopy.

6.1 Gram staining

Gram staining was developed empirically by the Danish bacteriologist Christian Gram in 1884[49]. The cells were first fixed to the slide by heat and stained with a basic dye (e.g. crystal violet), which was taken up in similar amounts by all bacterial cells. The slides were then treated with an iodine-potassium iodide mixture to fix (mordant) the stain and washed with acetone or alcohol. Finally they were counterstained with a polar dye of different color (e.g. safranin). Gram-positive organisms retained the initial violet stain, while Gram-negative organisms were decolorized by the organic solvent and hence showed the counterstain[32].

6.2 Electron microscopy

6.2.1 Electron microscope

The electron microscope was possible to examine cells and tissues at magnifications far beyond the range

of the light microscopes. It has revealed much of the ultra-structure of cells and modified the knowledge and concepts about cell structure[50,51].

By using an electron beam instead of light ray, the electron microscope gave much better resolution. Electron microscope was therefore constructed on the same optical principles as the light microscope. The ordinary light source was replaced by electrons that emitted by a tungsten filament. Their effective wavelength was determined by the voltage by which they were accelerated. Magnetic fields took the place of glass lenses, and a fluorescent screen replaced the human eye for direct viewing[50,51].

The instrument consisted of a source of electrons coupled with a series of magnetic lenses which bent or focussed the electron beam. The electron beam was obtained from a heated tungsten filament which was surrounded by a metal cylinder known as the Wehnelt cylinder or Wehnelt cap. This cap served to stabilize the beam current, tended to focus the electron beam towards the hole in the anode, which created an accelerating electrostatic field between gun assembly and itself to propel the electron down the column. A high voltage current was applied between the cathode (the tungsten filament) and the anode[50,51].

The electron beam first passed through the condenser lens. As in the light microscope this lens served to focus the beam on the object, and so provide "illustration".

The magnetic lens of an electron microscope could have different powers depending on the amount of current flowed in the electrical coils. In the electron microscope, all of the lens were rigidly fixed, and their focal points were variable by adjusting the lens currents. The illumination of the object was achieved by varying the current in the condenser lens[50,51].

The imaging system of the electron microscope usually consisted of three lens, the objective, the intermediate, and the projector lens. This gave three stages of magnification and made it possible to achieve high magnification in a reasonable amount of space. The objective lens was placed with its focal point close to the object. Intermediate images were formed between each lens. The projector threw its image on to a fluorescent screen which might be substituted by a photographic plate to make a permanent record for interpretation of the fine structural components of the cells[50,51].

The entire illuminating and imaging system was usually referred to as the microscope column. The column was very rigidly constructed and was maintained in a high vacuum since air molecules would defect the electron beam. The specimen had to be placed inside the vacuum, it was impossible to examine living material in the electron microscope[50,51].

Electron optics were essentially similar to light optics. One important difference was that the formation of the image was due to scattering of electrons by the molecules of the specimen and this scattering depended solely on the mass densities. Elements of high atomic weight caused marked electron scatter and appeared very dense in the electron image. The lighter elements caused little electron scatter and had poor contrast. In the light microscope, the image was due to absorption of light which depended more on molecular structure than atomic weight[50,51].

In photography, a photographic recording meterial consisted of a plastic plate coated with an emulsion that was made up of a layer of gelatin in which was embedded a photosensitive silver halide (usually bromide). The plates were fed one by one into position by means of a motor-driven plate feeding device. Automatic exposure meter gave correct photographic exposure time. The electron beam acted by the liberation of free silver from the silver halide grains and produced, after conventional development, a photographic negative of the final electron image. A print of the image on the film was known as an electron micrograph. The fine-grained negative contained a more detailed and higher contrast image than that produced on the fluorescent screen. By enlarging the electron micrograph, made it possible to study the detailed information of final visible image.

The comparison of the light microscope and the electron microscope was shown in Table 1.

Table 1 Comparison of the compound microscope and the electron microscope[50,51].

Element	Light microscope	Electron microscope
Source	Incandescent lamp	Heated tungsten filament
Intensity control	Condenser	Condenser
Specimen support	Glass slide	Copper grid
First image	Objective	Objective
Magnification	Eyepiece	Intermediate and Projection
Medium	Air and glass	Vacuum 10^{-5} Torr
Viewing image	Eye	Fluorescent screen
Focus	Change in lens position	Change in objective lens current
Recording	Photographic	Photographic plate

6.2.2 Negative staining

This was probably the simplest and quickest technique used. It was useful in the studying of tiny objects such as virus, bacteria. This method consisted of placing the specimen in a drop of "stain" such as phosphotungstic acid. The metal atoms penetrated between and around the smallest

spaces and structures of the specimens and revealed images not seen by other methods. The space would appear well defined in negative contrast[52]. When viewing in the electron microscope, the particles appeared as light areas due to their low scattering power, surrounded by the electron dense stain. This was the opposite of positive staining which made particles visible by actually combining a heavy metal salt with them[53]. The diagram was shown in Fig. 2, page 20[54].

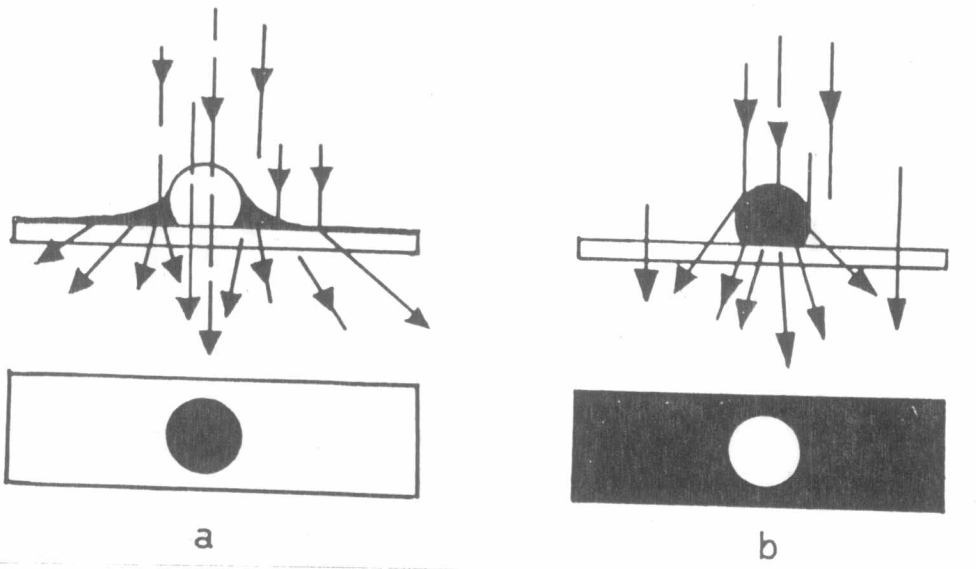

Fig. 2 The diagram illustrated the contrast being produced from a particle negatively stained (A) and positively stained (B). The contrast in the final image is completely reversed.