Chapter 1
History of Drug-Resistant Microbes

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Resistance to antimicrobial agents has been recognized since the dawn of the antibiotic era. Paul Ehrlich, the father of modern chemotherapy, observed that, during treatment of trypanosome infections, organisms sometimes emerged that were resistant to the agent being used. Resistance was specific in the sense that a fuchsin dye-resistant strain was still susceptible to an arsenic compound, while a strain resistant to the arsenic compound retained sensitivity to the dye. He showed that resistance, once acquired, was stably inherited and in 1908 proposed that resistance was due to "reduced avidity of the chemoreceptors so that they are no longer able to take up" the drug (1). Substitute "target" for "chemoreceptor" and one of the major mechanisms for antimicrobial resistance was revealed as was its specificity for particular compounds. Drug inactivation was discovered early as well. In 1919 Neuschlosz reported that *Paramecium caudatum* resistant to quinine and to certain dyes acquired the ability to destroy the toxic agents (2).

Early on, resistance was categorized as either natural or acquired. For example, natural resistance to gentian violet was a property of Gram-negative as compared to Gram-positive organisms. Some agents (sulfonamides, aminoglycosides, chloramphenicol, rifampin, and others) were recognized to have a broad spectrum, while other agents had a narrower focus (vancomycin, macrolides, and isoniazid). The less susceptible organisms were said to be naturally resistant. The natural resistance of Gram-negative bacteria to dyes and many other agents was attributed to an outer membrane barrier, which with our now increased appreciation of efflux pumps is understood to be only part of the story (3). Acquired resistance properly involved reduced susceptibility of an organism that was previously more sensitive to the drug, and was to be distinguished, if possible, from replacement of a susceptible organism by more resistant but unrelated ones, a process soon appreciated to occur all too readily in hospitals, which became the breeding ground for increasingly resistant flora.

An early concern was whether acquired resistance represented an adaptive response to the drug, which persisted for many generations after the drug was removed, or a selection from the initial population of rare preexisting resistant mutants. The adaptation hypothesis was championed by Hinshelwood who argued that, if a culture was grown in the presence of an inhibitor, the concentration of the substrate for the blocked reaction would accumulate and reverse the inhibition. Serial culturing in successively higher concentrations of a drug was interpreted, thus, as "training" the culture to tolerate the inhibition (4). The issue was settled in favor of mutation by demonstrations that resistance could emerge in the absence of an antibiotic and by the transfer of resistance with DNA. For example, the Lederbergs showed by replica plating that streptomycin-resistant colonies of *Escherichia coli* were present in a culture never exposed to the drug (5), while Hotchkiss demonstrated that penicillin resistance could be transferred to a susceptible pneumococcus by the DNA of a resistant one (6). Adaptation returned later, however, in the form of adaptive mutations, i.e. mutations that are formed in response to the environment in which the mutants are selected (7). Such mutants occur in nondividing or slowly dividing cells and are specific for events that allow growth in that environment, as, for example, the emergence of ciprofloxacin-resistant mutants in nondividing cultures of *E. coli* exposed for a week to ciprofloxacin in agar (8).

Until penicillin became available, sulfonamides were widely used for both treatment and prophylaxis, and before long resistance began to appear in several pathogens. Daily administration of sulfadiazine to prevent upper respiratory infections at military bases during World War II was followed by the emergence of resistant β-hemolytic streptococci. The question was whether the resistance was acquired or preexisting. Since the resistant organisms mainly belonged to only a few serotypes, selection of naturally resistant strains was favored, although the possibility that only particular serotypes could readily acquire resistance seems not to have been considered (9, 10). Use of sulfonamides for treatment of gonorrhea

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D.L. Mayers (ed.), *Antimicrobial Drug Resistance*,
DOI 10.1007/978-1-59745-180-2_1, © Humana Press, a part of Springer Science+Business Media, LLC 2009

was followed by increasing failure rates and the proliferation of sulfonamide-resistant strains of *Neisseria gonorrhoeae* (11). Increasing sulfonamide resistance was also noted in *Neisseria meningitidis* with corresponding clinical failure (12). Whether the neisseria truly acquired resistance was unclear since sulfonamide-resistant strains were discovered in cultures of *N. gonorrhoeae* or *N. meningitidis* from the pre-sulfonamide era (12, 13). Sulfonamide treatment of bacillary dysentery became complicated as well by the isolation of resistant strains, especially of resistant *Shigella sonnei* (14). Isolated instances were also reported of sulfadiazine resistance in pneumococci recovered after therapy of either pneumococcal pneumonia (15) or pneumococcal meningitis (16). Knowledge of bacterial biochemistry and metabolism had advanced after the empirical discovery of sulfonamides so that in 1940 *p*-aminobenzoic acid (PABA) was discovered to block the action of sulfonamide. PABA was proposed to be an essential metabolite for bacteria. Sulfonamide was hypothesized to mimic the chemical structure of PABA and to impede bacterial growth by competing with PABA to prevent its utilization (17). Extracts of resistant pneumococci were soon found to contain increased amounts of a sulfonamide inhibitor (18), which was identified as PABA in extracts of other sulfonamide-resistant bacteria (19), so all seemed consistent with resistance as a result of PABA overproduction. The story took another twist, however, when sulfonamide-resistant *E. coli* were found to make not excess PABA but a sulfonamide-resistant enzyme that utilizes PABA in an early step of folic acid biosynthesis (20). Such target enzyme insensitivity is now thought to be the main, if not the sole, mechanism for sulfonamide resistance (21).

The major mechanism for resistance to penicillin was identified much more quickly. The dramatic increase in penicillin resistance in *Staphylococcus aureus* that took place in the first decade of the antibiotic's use resulted from the selective advantage provided by an enzyme that inactivated penicillin, which was present initially in only a few isolates. The enzyme, penicillinase, was first described, not in *S. aureus*, but in *E. coli*, in 1940, and in the same year clinical studies with penicillin began (22). By 1942 increased resistance was reported in *S. aureus* from patients receiving penicillin (23), and in 1944 penicillinase was extracted from resistant strains of *S. aureus* obtained from patients who had not even been exposed to the drug (24). At Hammersmith Hospital in London the fraction of *S. aureus* isolates that were penicillin resistant increased rapidly from 14% in 1946, to 38% in 1947, and to 59% in 1948 (25) eventually stabilizing at the 90% resistance seen today and inspiring the development of semisynthetic β-lactamase-resistant penicillins, which were the first antibiotics specifically designed to overcome a characterized resistance mechanism (26). Unfortunately, methicillin-resistant *S. aureus* appeared within a few years and were found to make not a methicillin-degrading enzyme but rather a novel methicillin-resistant protein involved in cell wall biosynthesis (27, 28). The battle between bacteria and pharmaceutical chemists synthesizing improved β-lactam antibiotics had been joined and would continue (29).

The basis of resistance to streptomycin remained a puzzle for a long time. Streptomycin-resistant mutations arose at low frequency in many kinds of bacteria, including, unfortunately, *Mycobacterium tuberculosis* when the agent was used alone for treatment. Mutation produced not only high-level resistance but also bacteria dependent on streptomycin for growth, a curious type that could even be recovered from patients treated with the drug (30). A variety of biochemical changes followed exposure to streptomycin, including damage to the cell membrane (31), but it was the observation that the growth of a streptomycin-dependent mutant of *E. coli* in a suboptimal concentration of streptomycin resulted in decreased concentrations of protein and increased amounts of RNA led Spotts and Stanier to propose that streptomycin blocked protein synthesis in susceptible cells but was required for proper mRNA attachment to the ribosome in dependent ones (32). Direct demonstration that streptomycin impaired amino acid incorporation in a cell-free system soon followed (33). Streptomycin at a concentration as low as 10^{-6} M could inhibit polyuridylate-directed incorporation of phenylalanine, but a 1,000-fold higher concentration was required if the cell-free system was derived from a streptomycin-resistant organism. Furthermore, streptomycin was found to cause misreading of the genetic code, so that in its presence, polyuridylate catalyzed the misincorporation of isoleucine and other amino acids (34). So much was learned in studying the interaction of streptomycin and other drugs with the bacterial ribosome (35) that it came as something of a surprise that clinical isolates resistant to streptomycin relied on quite a different strategy, namely modification by adenylation, phosphorylation, and, for other aminoglycosides, acetylation as well (36). The lesson that resistance selected in the laboratory could be different from that selected in the clinic had to be learned.

Resistance to other antimicrobial agents emerged and was studied, but the next major conceptual advance was the appreciation of the importance of R-plasmids, which led not only to a better understanding of resistance acquisition and dissemination but ultimately to recombinant DNA and the biotechnology revolution. The demonstration of transferable resistance in Japan dated from 1959 but took several more years to attract attention and be accepted (37, 38). An explosion of discoveries followed. R-plasmids were found around the world not only in *Enterobacteriaceae* but also in pseudomonas, acinetobacter, staphylococci, enterococci, bacteroides, clostridia, and in virtually every bacterial species examined. Some had remarkably wide host ranges, while others were limited to Gram-positive, Gram-negative, anaerobic, or even smaller bacterial subsets. Techniques were developed

for plasmid transfer, isolation, and classification (39, 40). Transposons that allowed resistance genes to jump from one DNA site to another were discovered (41), as were integrons that allowed resistance gene cassettes to be captured on plasmids and efficiently expressed (42). Restriction enzymes, often plasmid mediated, facilitated analysis of plasmid structure and permitted DNA cloning. The genetics of antibiotic resistance became as tractable as its biochemistry and contributed much to the emerging discipline of molecular biology.

The finding that a β-lactamase (designated TEM) from a clinical isolate of *E. coli* was carried on an R-plasmid (43) led to the realization that this resistance mechanism could spread, not only to other *E. coli* but also to other genera. Before long, TEM β-lactamase was found in ampicillin-resistant *Haemophilus influenzae* (44) and in penicillin-resistant *N. gonorrhoeae* (45). Enzymes more active on cephalosporins than penicillins were discovered, functional classification of the growing body of β-lactamases began (46), the technique of isoelectric focusing was added to the repertoire of β-lactamase biochemists (47), introduction of cefamandole led to the recognition that β-lactamase derepression could provide resistance in some organisms (48), and clinical use of expanded-spectrum cephalosporins was followed by an explosion of extended-spectrum and other β-lactamases (29, 49).

Plasmids carry genes for resistance to many other antimicrobial agents. Some genes code for enzymes that modify or inactivate the agents, others for enzymes that alter drug targets in the cell or provide alternate biosynthetic pathways. Genes for antibiotic efflux (chloramphenicol, tetracycline) were also found to be plasmid determined, but efflux-mediated resistance occurred also from chromosomal mutations that altered control circuits involved in expression of outer membrane proteins that form porin channels for antibiotic uptake. Study of bacteria collected in the preantibiotic era indicated that the plasmids that organize, express, and transmit resistance predated the clinical use of antibiotics (50). R-plasmids resulted from the insertion of resistance genes into previously existing plasmids. The resistance genes themselves probably had a diverse origin. Some could have come from organisms producing antibiotics since those organisms needed a mechanism for self-protection (51, 52). Others may have originally had another function in the cell that could be adapted for antibiotic protection. Given the degree of horizontal gene exchange occurring between bacteria, the donor could be a quite distant relative.

Plasmids are not the only vehicle for such a gene transfer. Naturally transformable pathogens such as *Streptococcus pneumoniae*, *N. meningitidis*, *N. gonorrhoeae*, and *H. influenzae* were found to exchange chromosomal genes with members of closely related species, including genes for penicillin-binding proteins and topoisomerases that provide resistance to penicillin or quinolones (53–55). Mutation plays an important role in resistance to some antimicrobial agents usually by altering enzyme specificity or reducing binding to a lethal target. The notion that resistance was based on infrequent mutational events also led to the concept that resistance could be prevented by simultaneous administration of two drugs since the product of the likelihood of resistance emerging to each would be greater than the size of any possible infecting inoculum, a thesis best justified by the success of multidrug treatment of tuberculosis. An increased mutation rate eventually exerts a fitness cost, but limited rate increases have been found in organisms with resistance attributable to an altered target (quinolone resistance from *gyrA* mutations) (56) or modified enzyme (expanded-spectrum β-lactam resistance due to extended-spectrum β-lactamases) (57).

Antibiotic resistance has come to be accepted as an inevitable consequence of antibiotic use. The ubiquity of the phenomenon has been amply illustrated with emerging resistance to antiviral and antiparasitic agents as well. On the positive side understanding the mechanisms of antibiotic resistance has often provided important insights into how antibiotics work. Knowledge about R-factors has unfortunately not made a direct attack on the genetic basis of resistance possible, but insight into resistance mechanisms has guided the development of expanded-spectrum β-lactams (cefepime, cefotaxime, ceftazidime, ceftriaxone, aztreonam, and others), aminoglycosides (amikacin), and tetracyclines (tigecycline) as well as such resistance inhibitors as clavulanic acid, sulbactam, and tazobactam. A number of enigmas remain. Some organisms, such as *S. aureus* and *Pseudomonas aeruginosa*, seem particularly adept at acquiring resistance, while others are puzzlingly reluctant with certain drugs. *Treponema pallidum* and *Streptococcus pyogenes*, for example, remain fully susceptible to penicillin G despite decades of exposure to the drug, while other organisms have become progressively more resistant. The tempo at which resistance develops is also remarkably variable (Table 1). Resistance may appear

Table 1 Timetable of Antibiotic Discovery and Resistance

Antibiotic	Discovered or reported	Clinical Use	Resistance identified	Organism
Sulfonamide	1935	1936	1939	*S. pneumoniae*
Penicillin G	1928	1941	1942	*S. aureus*
	1940 (purified)		1965	*S. pneumoniae*
Methicillin	1960	1960	1961	*S. aureus*
Oxyimino-β-lactams	1978	1981	1983	*K. pneumoniae* *E. coli*
Streptomycin	1944	1946	1946	*E. coli*
Tetracycline	1948	1952	1959	*S. dysenteriae*
Erythromycin	1952	1955	1957	*S. aureus*
Vancomycin	1956	1958	1987	*E. faecium*
Gentamicin	1963	1967	1970	*K. pneumoniae* *P. aeruginosa*

soon after a drug is introduced or only after many years. Methicillin-resistant *S. aureus* were isolated in the UK within a few years of the drug being introduced (58, 59), but 20 years elapsed before pneumococci with reduced susceptibility to penicillin were isolated and another 20 years before resistance was recognized as a worldwide problem (60). Vancomycin resistance took even longer to appear (61). The equilibrium level at which resistance becomes stabilized is also curiously variable. β-Lactamase production has reached 10–30% in the gonococcus, 15–35% in *H. influenzae*, 30–40% in *E. coli*, 75% in *Moraxella catarrhalis*, and 90% in *S. aureus*, but what determines these levels is poorly understood. Once it has been acquired, however, resistance is slow to decline (62), and there are few examples of reduced antibiotic use associated with diminished resistance (63) so that prevention of resistance by prudent antibiotic use remains the keystone to control. Appropriate use applies to nonhuman applications as well with restraining antibiotics in animal feed as a prominent example.

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