Antibiotic resistance preventive properties of green tea catechins.

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Summary

Numerous laboratory studies point to a central role of reactive oxygen species in the causation of a variety of complex human disease situations including cancer, atherosclerosis, sugar diabetes, cardiovascular disease and, perhaps, aging as well. ROS and reactive nitrogen species are strongly mutagenic as well. Green tea, its extracts, and its catechins are powerful antioxidants and perhaps also possess relevant additional useful properties contributing to their healthy properties. More recently multiple resistance development by a variety of microorganisms has developed worldwide threatening the continued clinical efficacy of major antibiotics. Laboratory experiments are described here demonstrating that the antimutagenic action of green tea catechins can interfere with and even prevent resistance development by several microorganisms against a wide variety of antibiotics and antimicrobial agents. These experiments also suggest a plausible mode-of-action.

Keywords

Antibiotics, resistance, antimutagenesis, green tea catechins

Introduction

From the earliest days of antimicrobial chemotherapy, resistance by some species was noted but this represented a regular characteristic that was easy to ignore. In recent years, however, the incidence of resistance, even simultaneously to multiple antibiotics, has become much more common and is growing explosively in incidence so that today some infectious disease experts express the fear that he age of useful chemotherapy may soon end. The public is alarmed and scientists are working feverishly to prevent this.

Resistance is either intrinsic or acquired. Acquired resistance is the more severe problem as it may develop suddenly during treatment and then spread rapidly to other bacteria of the same species or even to other species. This resistance is often found to be due to one or more of the following: a) failure of the drug to penetrate into the target bacterium or to remain inside in realistic concentrations following administration; b) alterations in the target macromolecule so that the drug fails to interfere with its function; c) elaboration of enzymes that alter the structure of the antibiotic thus inactivating it; or d) biosynthesis of multiple copies of the target macromolecule so that interference by achievable concentrations of the drug is no longer sufficient for antibiosis bacterium.

Currently scientists attempt to cope with this problem in a variety of ways. These include: a) finding novel antibiotics; b) reworking the structures of existing antibiotics; c) uncovering novel targets for chemotherapy; d) inhibiting drug modifying enzymes; e) inhibiting drug export pumps; f) use of combinations of antibiotics; g) use of existing antibiotics more appropriately and h) paying better attention to public health measures. While all of these stratagems are useful and work for a time, the

microorganisms ultimately develop resistance to each of them in turn. Clearly a novel and effective approach would be welcome.

It is clear that with resistance one is witnessing evolution in action. The development of antibiotics was an ecological disaster for bacteria and mankind had the upper hand for a few decades. Resistance is the bacterial counter thrust and they are apparently regaining the upper hand. In this view, this is a struggle that will never end. The best that mankind can hope for is to reset the equilibrium to a more tolerable point.

Bacteria reproduce incredibly rapidly and a single surviving cell is theoretically capable of producing trillions of progeny in a single day. Furthermore, they possess many mechanisms for acquisition and exchange of genes (spontaneous mutation, transfection, transduction, plasmid transfer, gene segment exchanges, and so on). The presence of an insufficiently lethal concentration of antibiotic exerts a positive selecting pressure on such a population leading to the predominance of resistant cells and fixation of this trait in the surviving progeny. The classic view is that the genes are present from the outset and that careless use of antibiotics gives cells possessing them an opportunity to thrive. This does not address the question of their origin in the first place. It is our thesis that the origin of the resistance genes lies in intrinsic/spontaneous bacterial mutability. Most of these mutations are either repaired or are irrelevant to this question. Some, however, convey a competitive advantage n the presence of a positive selecting pressure of insufficient levels of antibiotic to kill all of the cells. Under these circumstances, rapid growth facilitated by the death of their normal competitors leads to widespread resistance within the colony. If this is so, then the use of antimutagenic agents should inhibit this phenomenon. We, therefore, set out to test this hypothesis.

Materials and methods.

Antibiotically naïve microorganisms (*Staphylococcus aureus* ATCC 13709 and *Escherichia coli* ATCC 9637) were grown on Oxoid Nutrient Broth No. 2 for 14 days in 96-well ELISA plates containing various concentrations of test compounds. Growth was measured by absorbency at 570 nm using a Cambridge Technology Inc., Plate Solver Version 4.0. In other experiments (to measure single-strand breaks), 0.2 μg of puc 18 DNA was incubated at 37° C for 5 mins. in a medium (final volume 20 μl) containing 10 μM $FeSO_4$ in de-aerated 10 mM sodium phosphate buffer at pH 7.4 and various concentrations of antibiotic and green tea catechins were added as needed. Separation of the different forms of DNA was performed using 0.7% agarose gel electrophoresis in the usual way. The DNA bands were stained with ethidium bromide and quantitated by scanning using the MACROS program for gel reading.

Also, cells of *S. aureus* RN7044 harboring a plasmid encoding for ethidium bromide efflux (a gift of Dr. Steven Projan of American Cyanamid) were cultured as above to an OD_{600} of 1.8, pelletted and washed twice with 20 mM Hepes/NaOH (pH 7.0) buffer, resuspended in 1 ml of 20 mM Hepes buffer with protonophoric carbonylcyanide m-chlorophenylhydrazone at 10 mM to prevent ethidium bromide efflux. Then 10 mg/ml of ethidium bromide was added and the whole incubated at 37° for 30 min. following which the cells were pelleted and resuspended in 20 mM Hepes buffer. A volume of 100 μM of resuspended cells was pipetted into wells of a Costar 96-well black plate with catechins dissolved in 20 mM Hepes buffer to a final concentration of 10 μg/ml. Fluorescence was measured using a Biotik fluorescence plate reader with excitation at 530/5 nm and emission at 590/35 mm for 1 hour. Influx

of the dye was measured under similar conditions but in the absence of the protonophore.

Results.

From figure 1 it can be seen that resistance emergence develops after a time at low but inhibitory doses of antibiotic. This phenomenon is seen with most antibiotics examined but particularly with tetracyclines and fluoroquinolones. When recultured, the resistant colonies obtained are highly resistant to the antibiotic in question. Green tea catechins were chosen as antimutagenic agents and added to analogous cultures in graded doses chosen so that they by themselves did not interfere with growth. This generally suppressed or, mainly, prevented emergence of resistance colonies (figure 2).

Tetracyclines and fluoroquinolones can be shown in this way to cause strand breaks in pUC18 DNA at analogous concentrations. This effect was suppressed or prevented by inclusion of graded concentrations of green tea catechins in the media. Furthermore, incorporation of ferrous iron in the media led to an enhanced incidence of resistance emergence and emergence at a shorter time than seen in its absence (figure 3). As both tetracyclines and fluoroquinolones are well known chelating agents this is not surprising. In this they behave rather like the Fenton reagent.

That the effects observed were not due to simple membrane damage that would allow an excess of antibiotic to enter the target cells, exclusion ability of the cells to entrance of ethidium bromide was measured using *Staphylococcus aureus*. The catechins did not allow a significant ingress of the dye into the cells. To examine the question of whether an efflux pump might be involved actively excluding the antibiotics, a culture permeable to ethidium bromide and not known to have an operating efflux pump was similarly treated. There was a normal loss of dye eliminating the possibility that these cells had a pump induced by the catechins.

Antibiotics for which resistance emergence was measured in this study and for which green tea catechins exerted a preventative effect were: quinolones [nalidixic acid, oxolinic acid, cinnoxacin, ofloxacin, trovafloxacin, piromidic acid, lomefloxacin, ciprofloxacin, norfloxacin, pipemidic acid, sparfloxacin, and clinafloxacin], beta-lactams [imipenem, amoxicillin, cefoxitin], tetracyclines [tetracycline, doxycycline] and a lincosaminide [clindamycin].

Discussion

Multiple resistance to antibiotic therapy by infectious microorganisms is a widespread contemporary phenomenon. Many laboratories have attempted to deal with this problem in a variety of ways but the usual result is that success is temporary and resistance emergence occurs all too rapidly. We posited that the phenomenon is the simple result of evolutionary pressures exacerbated by careless habits and inappropriate application of existing antibiotics. In this work we demonstrate that resistance emergence is easily demonstrated in the laboratory when doses of antibiotic sufficient to inhibit but insufficient to kill are employed. The rate of resistance development is enhanced by the use of DNA damaging agents (OH radical generated by Fenton chemistry) and it has been shown that certain chelating antibiotics (tetracyclines and fluoroquinolones) are themselves capable of causing DNA strain breakage.

Resistance generated in these ways is suppressed or prevented by application of low doses of antimutagenic agents of which the green tea catechins are particularly effective. These same agents are also shown to inhibit strand breakage of DNA. These results strongly imply that an antimutagenic mechanism is involved. It seems plausible

that co-administration of green tea beverage or catechins and antibiotics would decrease the rate and extent of resistance emergence in clinical practice. The broad spectrum of antibiotics against which this phenomenon has been demonstrated *in vitro* suggests that this device could be applicable generally. The widespread and venerable dietary acceptance of green tea and its catechins suggest that people would accept this.

If so, it is possible that presently existing resistance could fade in time due to lack of recruitment of fresh genes but this is more problematic. Several studies suggest that additional mutations in time increase the vitality of resistant microorganisms. Certainly, however, it seems prudent to investigate *in* vivo the use of antimutgenic agents under conditions paralleling those existing in the clinic.

Interested readers are encouraged to consult S. R. Pillai, C. A. Pillai, D. M. Shankel and L. A. Mitscher, Mutation Research (2001) 496: 61-73 for published further details of this work.

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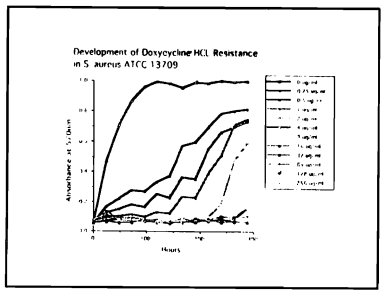

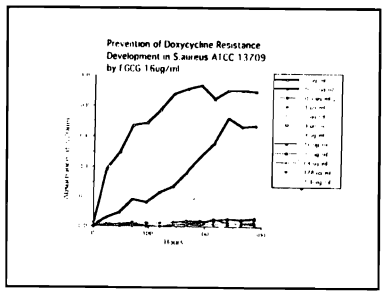

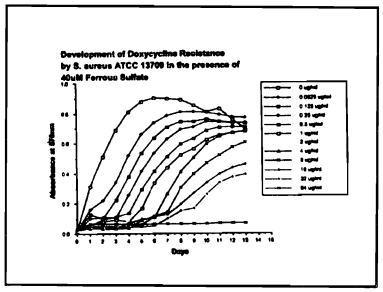

Figures

Figure 1 (upper left) represents the pattern of resistance development over time by *S. aureus* to doxycycline. Figure 2 (upper right) represents the inhibition of resistance development to doxycycline by graded doses of EGCg. Figure 3 (lower right) represents enhanced resistance to doxycycline mediated by ferrous sulfate.