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Killing of Cells in Bacterial Colonies

DALE E. HUNT AND ROBERT F. PITTILLO

Kettering-Meyer Laboratory, Southern Research Institute (affiliated with Sloan-Kettering Institute for Cancer Research), Birmingham, Alabama

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Bacterial colonies grown on membrane filters and transferred to plates of inhibitor-containing medium decrease in number of total viable colony-forming cells. The decrease in viable cell count occurs in direct proportion to the concentration of inhibitor present and the length of time the colonies are exposed to the inhibitor, suggesting that the kinetics of cell kill approximate a first-order chemical reaction.

The kinetics of bacterial cell kill by antibiotics and certain antineoplastic compounds, which have antibacterial activity, have been investigated. The results indicate that the kinetics of inactivation by both types of inhibitors are essentially identical to that of classical antimicrobial agents (such as phenol and mercuric bichloride) in the following regard: (i) constant fractional cell kill by a fixed concentration of inhibitor, regardless of number of bacteria; (ii) temperature dependence; and (iii), in some instances, increase in the length of lag phase of surviving cells without apparent changes in generation time (4). These studies, like most classical antimicrobial investigations, were conducted with proliferating bacterial populations in liquid medium and with nonproliferating bacterial populations suspended in physiological saline. There are virtually no data available in the literature on the inhibition of bacteria growing in colonies. The present investigation was undertaken to provide such data.

MATERIALS AND METHODS

Organisms. The bacteria used were *Escherichia coli* ATCC 9637 and *Streptococcus faecalis* ATCC 8043. *E. coli* was maintained on agar slants containing 1.5% agar, 0.1% NH_4Cl, 0.73% K_2HPO_4, 0.3% KH_2PO_4, 0.012% $MgSO_4$, and 0.4% glucose (autoclaved separately and added aseptically). *S. faecalis* was maintained on agar slants of the medium described by Flynn et al. (2).

Growth and preparation of inocula and colonies. Stationary cultures of *E. coli* and *S. faecalis* were grown overnight at 37 C in the respective media described above. The cells were collected and washed by centrifuging in physiological saline (0.85% NaCl), resuspended in saline, and adjusted to 0.7 optical density units in a Bausch & Lomb Spectronic-20 colorimeter at 660 mμ. These suspensions contained approximately 10^8 viable cells per milliliter. Suitable dilutions of these inocula were spread on the surface of membrane filters (DAWP 04700; pore size diameter, 0.65 μ; Millipore Corp., Bedford, Mass.) that had been placed on the surface of agar plates containing the appropriate medium for the respective organisms. After incubating for 24 hr at 37 C, the filters, supporting the isolated colonies, were aseptically cut into small pieces with sterile scissors so that each sectioned piece of filter supported one isolated colony. These sections were then placed on the surface of agar plates containing various concentrations of the inhibitors. Subsequently, at various time intervals, the individual colonies on the filter sections were removed, suspended in saline, and disrupted by vigorous manual agitation. The resulting cell suspensions were suitably diluted in saline, and samples were plated in Trypticase Soy Agar (BBL) and incubated for 18 hr at 37 C for the determination of viable cells.

Inhibitors. The following antibiotics and antineoplastic compounds were used: penicillin G (Eli Lilly and Co., Indianapolis, Ind.); chloramphenicol (Parke, Davis and Co., Detroit, Mich.); 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; supplied by John Montgomery, Southern Research Institute, Birmingham, Ala.); 5-fluorouracil; azaserine (diazoacetyl-L-serine); and amethopterin (glutamic acid, *N*-[*p*([2,4-diamino-6-pteridinyl)methyl]methyl - amino)-benzoyl]-) (obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.).

For the determination of median generation times of cells in control and inhibitor-treated colonies, colonies were picked and suspended in Trypticase Soy Broth (BBL). Samples were taken immediately and at 30-min intervals subsequently, and were plated, following suitable dilutions, in Trypticase Soy Agar. Data obtained from these experiments allowed the measurement of the median generation times of colonial populations.

RESULTS

The effects of penicillin, chloramphenicol, azaserine, and BCNU on colonial growth of *E. coli* and of 5-fluorouracil and amethopterin on colonial growth of *S. faecalis* are illustrated in

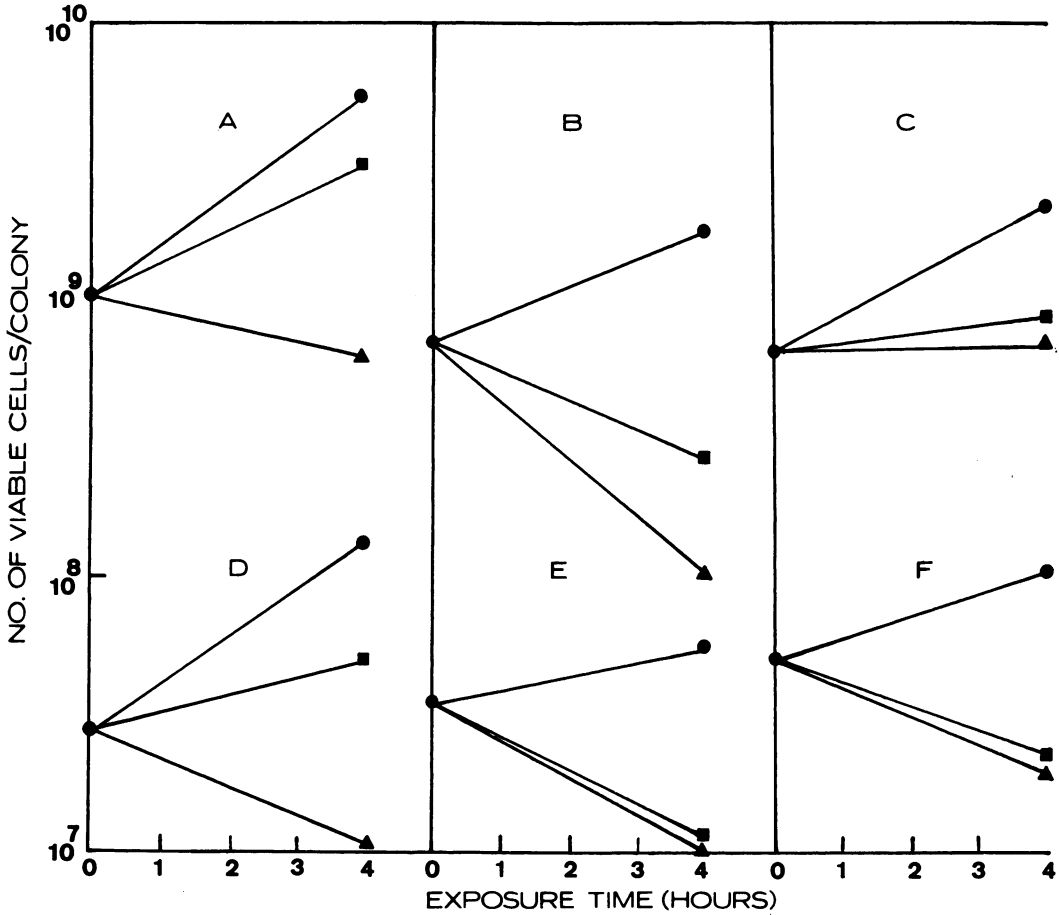

FIG. 1. *Inhibition of cells in bacterial colonies by diverse types of inhibitors. Each point represents the median of 20 colonies. (A) Escherichia coli ATCC 9637; inhibitor, azaserine; ● = no inhibitor, ■ = 10 μg/ml, ▲ = 30 μg/ml. (B) E. coli ATCC 9637; inhibitor, chloramphenicol; ● = no inhibitor, ■ = 50 μg/ml, ▲ = 100 μg/ml. (C) E. coli ATCC 9637; inhibitor, penicillin; ● = no inhibitor, ■ = 20,000 units/ml, ▲ = 50,000 units/ml. (D) E. coli ATCC 9637; inhibitor, 1,3-bis(2-chloroethyl)-1-nitrosourea; ● = no inhibitor, ■ = 200 μg/ml, ▲ = 400 μg/ml. (E) Streptococcus faecalis ATCC 8043; inhibitor, 5-fluorouracil; ● = no inhibitor, ■ = 50 μg/ml, ▲ = 100 μg/ml. (F) S. faecalis ATCC 8043; inhibitor, amethopterin; ● = no inhibitor, ■ = 10 μg/ml, ▲ = 20 μg/ml.*

Fig. 1. The median number of viable cells per colony was significantly less in those colonies exposed to the inhibitor-containing medium compared to the median number of viable cells per colony in colonies placed on inhibitor-free medium.

More detailed data (summarized in Fig. 2) have been collected regarding the inhibition of *E. coli* colonies by penicillin; these data show that the decrease in the number of viable cells per colony was dependent on concentration of, and length of time colonies were exposed to, the antibiotic.

Histograms giving the distribution of median generation time of cells that survived exposure to penicillin or azaserine are presented in Fig. 3. The progeny of cells that survived drug treatment appears to have increased median generation time. This observation, on the basis of preliminary experiments, may also apply to bacterial populations arising from cells that survived treatment with other inhibitors. The increase in generation time appears to be related to the concentration of inhibitor to which the original colonies were exposed; i.e., the higher the concentration of inhibitor, the longer the median generation time of the surviving progeny. Slight differences in the generation times of untreated

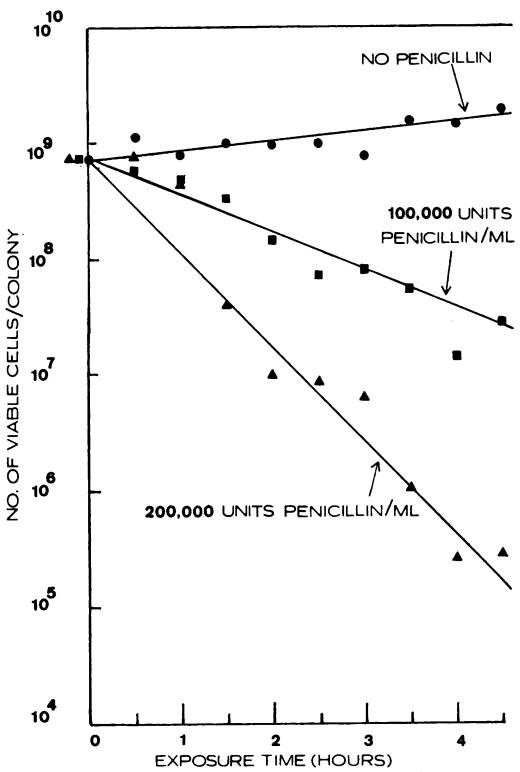

FIG. 2. *Inhibition of colonies of Escherichia coli ATCC 9637 by penicillin. Each point represents the median of 10 colonies.*

control cells were noted in different experiments; however, within each experiment, a trend toward longer generation time with increasing concentrations of inhibitor seems to have occurred. In some instances (Fig. 3), every viable cell in an inhibitor-treated colony was killed. Additionally, the number of viable cells decreased in some antibiotic-treated colonies after these colonies were resuspended in growth medium.

DISCUSSION

The relationship between the concentration of a disinfectant and the time required to kill a bacterial population has been expressed mathematically by Watson (6), who proposed the formula: $C^n \cdot t = k$(a constant), or, in logarithmic form, $n \log C + \log t = \log k$, where C = disinfectant concentration; n = dilution coefficient, concentration exponent, or a constant that may vary with each disinfectant or, in other words, the power to which C must be elevated so that when multiplied by t the product will be a constant; and t = time required for the killing process to occur.

With respect to curves of concentration-action, Davis (1) indicated that the concentration coefficient (n) for most chemical inhibitors is approximately 1. Nine antibacterial compounds of diverse chemical structure have been shown experimentally to have n values near unity (4). Since the equation appears to be valid, the killing reaction in bacteria obeys first-order kinetics. Hence, the number of bacterial cells killed in a unit of time is proportional to the number present, regardless of the values of C and n, provided these two values remain constant. Since data presented in this paper have shown that the decrease in the number of viable cells per colony was dependent on concentration of inhibitor and length of time colonies were exposed to inhibitor (as described in the equation), the cell-killing reaction in a bacterial colony also obeys first-order kinetics. However, this may apply only to those cells that have relatively the same generation time, since the chemical composition of bacterial cells is profoundly influenced by their growth rate (3). An understanding of the kinetics of inactivation of cells in large and compact populations is important, since in some instances (e.g., in experimental cancer chemotherapy) considerable emphasis is placed on the kinetics of inactivation of a large mass of malignant cells as is encountered in solid neoplasms (4, 5). Two important similarities exist between a typical bacterial colony and certain solid tumors: large numbers of cells are compacted to form a stationary cell mass, and individual cells within such a mass are increasing in size and dividing by geometric progression with the passage of time. Although some types of solid tumors possess a vascular system and undergo differentiation, most highly malignant tumor cells are not differentiated, and the same physicochemical mechanisms, which control the exchange of materials into and out of cells and cell masses, apply regardless of the presence or absence of a vascular system. Therefore, it appears possible that the kinetics of inactivation of cells in a bacterial colony may also apply to the inactivation of cells in a solid tumor.

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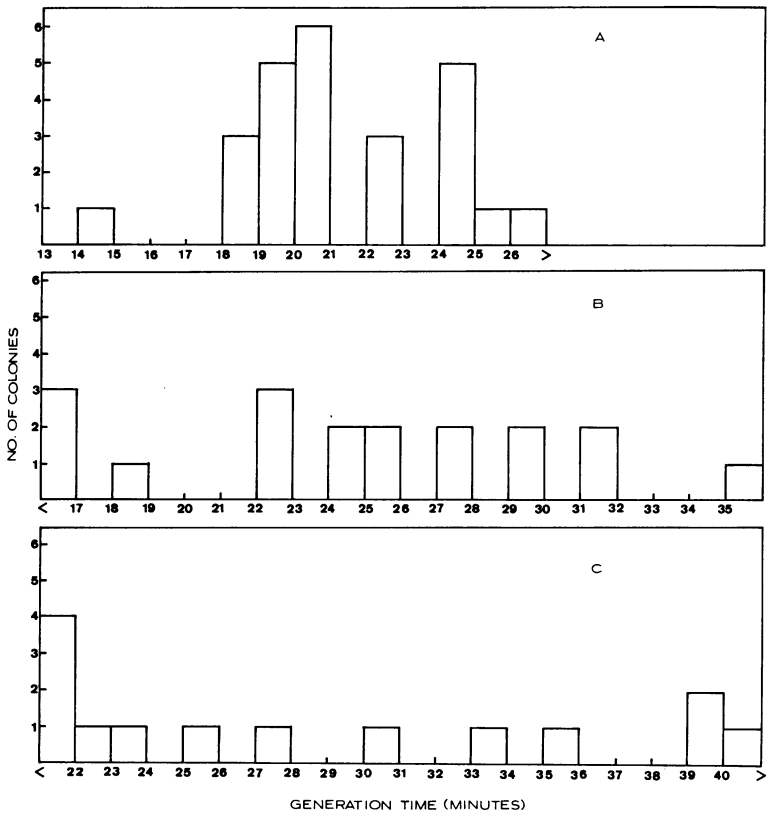

FIG. 3 part 1

FIG. 3. *Generation times of Escherichia coli ATCC 9637 from untreated colonies and from colonies exposed to penicillin and azaserine. (A) Generation times of E. coli ATCC 9637 from untreated preformed colonies; median generation time of cells from 25 colonies = 20.3 min. (B) Generation times of E. coli ATCC 9637 cells from preformed colonies which survived 1 hr of treatment with 100,000 units of penicillin/ml; median generation time of cells from 18 colonies = 24.9 min; after exposure to drug, 7 colonies never gave rise to logarithmically growing cultures. (C) Generation times of E. coli ATCC 9637 cells from preformed colonies which survived 1 hr of treatment with 200,000 units of penicillin/ml; median generation time of cells from 14 colonies = 26.7 min; after exposure to drug, 11 colonies never gave rise to logarithmically growing cultures. (D) Generation times of E. coli ATCC 9637 cells from untreated preformed colonies; median generation time of 24 colonies = 17.4 min. (E) Generation times of E. Coli ATCC 9637 cells from preformed colonies which survived 1 hr of treatment with 3.12 mμg of azaserine/ml; median generation time of cells from 20 colonies = 18.5 min. (F) Generation times of E. coli ATCC 9637 cells from preformed colonies which survived 1 hr of treatment with 6.25 mμg of azaserine/ ml; median generation time of cells from 16 colonies = 21 min; after exposure to drug, 8 colonies never gave rise to logarithmically growing cultures.*

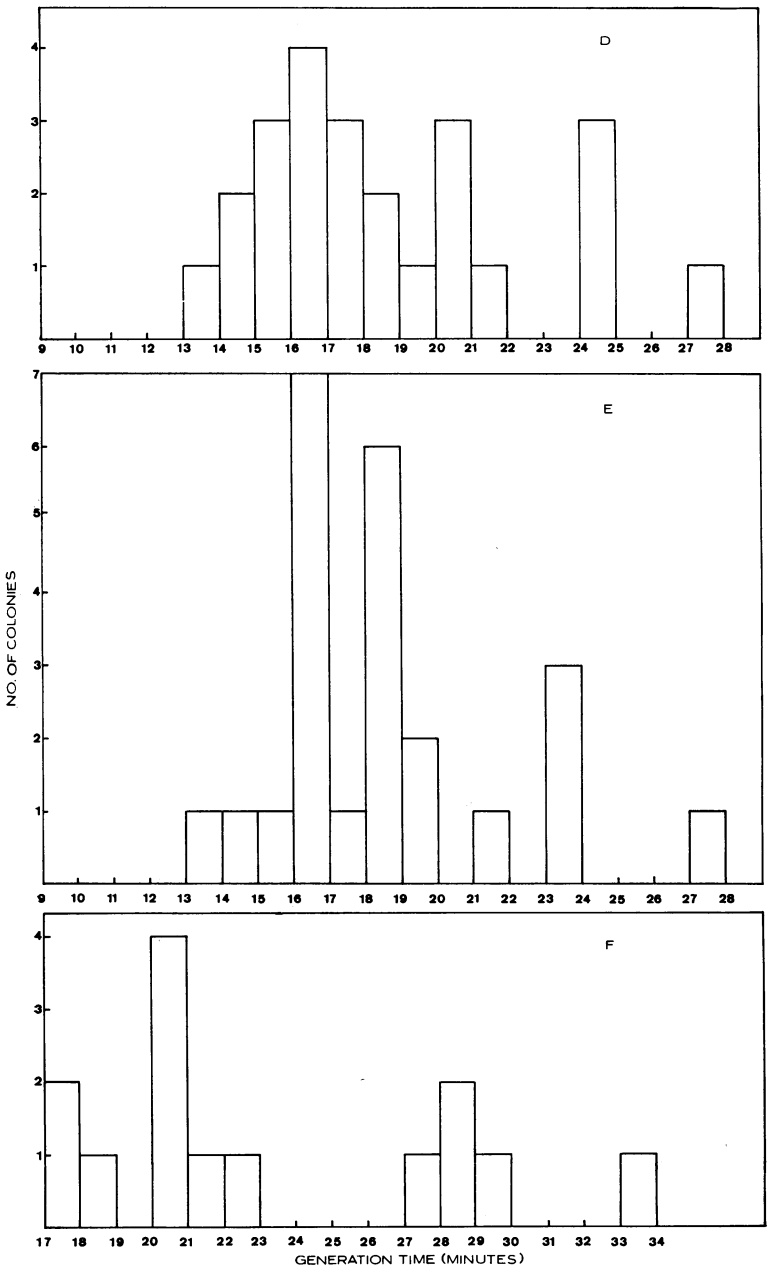

FIG. 3. Part 2

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