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The Genetic Origin of Drug Resistance in Neoplasms: Implications for Systemic Therapy

James H. Goldie[1,2] and Andrew J. Coldman[1]

Divisions of Advanced Therapeutics [J. H. G.] and Epidemiology and Biometry [A. J. C.], Cancer Control Agency of British Columbia, and Department of Medicine, University of British Columbia [J. H. G.], Vancouver, British Columbia V52 1L3, Canada

Abstract

Drug resistance continues to be a major factor in limiting the effectiveness of cancer chemotherapy. Evidence from a variety of sources implicates a genetic basis for most drug-resistant phenotypes. Assuming a random spontaneous origin for these resistant cells, it is possible to develop mathematical and computer-based models of the drug treatment of tumors. These can provide a more intuitive understanding of the basis of treatment success or failure. This in turn may lead to the development of more rational and effective treatment protocols. Studies of phenomena such as pleiotropic drug resistance are providing insights into how multiple levels of drug resistance occur and are yielding information on how certain types of drug resistance may be prevented or overcome.

Introduction

There has been sufficient progress in the application of clinical cancer chemotherapy that its use is beginning to have a definite impact on overall cancer survival statistics (24, 30). While these trends are encouraging, it is nonetheless evident that there are many major obstacles still present to achieving the goal of having effective therapy for every type of advanced cancer. Generally speaking, the most chemosensitive tumors are characterized by rapid growth rates and a tendency towards early and extensive dissemination. Local treatment is rarely curative, and in the absence of effective systemic treatment these diseases are quickly and uniformly fatal. They constitute the most lethal end of the neoplastic spectrum, are typically seen in children and young adults, and are often of lymphoreticular or embryonal cell origin. The less responsive groups of tumors have a tendency towards long clinical doubling times and late metastatic spread. Locoregional therapies may produce significant cure rates, and the presence of metastatic disease is often still compatible with a fairly long survival, even in the absence of effective treatment. These cancers tend to occur in middle and older life and generally arise in epithelial tissues.

Despite the availability of approximately 40 clinically active antineoplastic agents, eventual treatment failure is the outcome in the chemotherapy of the great majority of types of cancer. Even with the most drug-sensitive classes of tumor, a significant proportion may show poor initial responses or ultimately relapse in a drug-resistant state. Switching to new drugs only infrequently produces clinical responses that are qualitatively and quantitatively equivalent to that which is achievable on first application. When patients ultimately die of metastatic cancer, their disease is characterized by an extreme insensitivity to even the highest tolerated doses of all of the available anticancer drugs.

What can be said about the factors which dictate whether a particular neoplasm will respond satisfactorily to drug treatment and, especially, which factors determine whether the cancer can actually be cured? Why are some classes of tumors significantly less responsive to chemotherapeutic effect from the beginning, and why do even some of the most drug-sensitive neoplasms develop resistance to chemotherapy during the course of treatment? Research in a number of areas in recent years has started to shed light on some of these questions, and from this work more rational and effective chemotherapeutic strategies may be developed.

Possible Mechanisms of Drug Treatment Failure. A number of explanations have been advanced to account for the mechanism of treatment failure during cancer chemotherapy (38, 49, 58, 72). Among the most widely accepted of these has been the hypothesis that it is the overall growth kinetics of the tumor which is most important in dictating response to chemotherapeutic agents. Tumors that are characterized by a low growth fraction, a long volume-doubling time, and (perhaps) a long intermitotic time for component cells might be expected to be less sensitive to chemotherapy by virtue of presenting a smaller "target" per unit time. There does in fact exist a general correlation between clinical doubling time and chemosensitivity. That is, the most rapidly growing neoplasms tend to be the ones that are most sensitive to drug effect and are more likely to be curable when treated at an advanced stage (62).

Some difficulties arise when one attempts to rely solely on kinetic phenomena as being the basis for drug sensitivity. The trend between growth rate and curability is only approximate. Burkitt's lymphoma, generally considered to be the most rapidly growing human cancer, is not the most highly curable (83). The kinetic parameters of a relatively responsive solid tumor such as breast carcinoma tend to significantly overlap much less responsive tumors such as melanoma (62). A slow-growing solid tumor such as adenoid cystic carcinoma may display sustained responses to chemotherapy, and an extremely rapidly growing neoplasm such as blast cell crisis of chronic myelogenous leukemia is generally very refractory to drug treatment. In addition, clinical resistance to chemotherapy in responding tumors frequently occurs at a time when there has been significant regression of measurable disease. One would expect at this point that the kinetic parameters should be becoming more favorable for chemotherapeutic effect, but it is precisely at this time that the tumor becomes resistant. Moreover, tumors of identical histolog-

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ical type and kinetic behavior may show significantly discrepant patterns of response to the same chemotherapy.

In experimental tumors, the change from curability to incurability frequently occurs over a tumor size range in which there are no measurable alterations in kinetic parameters (66, 69). For example, in the L1210 leukemia, incurability by methotrexate occurs at a tumor burden of approximately 10^4 cells, a point at which there has been no significant change in doubling time or growth fraction. As well, the curable burden for this and other experimental tumors varies substantially, depending on the type of chemotherapeutic agent that is being administered. Potent 2-drug regimens may cure burdens up to 10^8 cells, whereas single less effective agents are unable to cure burdens in excess of 10^3 (67). It is therefore difficult to develop a rationale for the use of multiple-agent chemotherapy from purely kinetic considerations. While some correlation between certain kinetic properties of tumors and chemosensitivity exists, the relationship is insufficiently strong to permit attribution of resistance exclusively to these effects.

It has also been suggested that failure to control neoplasms by chemotherapy, especially large solid tumors, can be attributed to an inability to achieve effective tumoricidal concentrations of drug within the bulk of the tumor mass (38). Therefore, the treatment failure comes about because of problems relating to diffusion of the drug into the depths of the tumor, and attempts to overcome this by raising peak drug concentration are limited because of toxicity to normal tissues. There are experimental models that are consistent with this explanation, the best known of these being the multicellular spheroid model (73). In this system, it can be clearly demonstrated that cells near the periphery of the spheroid are much more susceptible to drug-induced cytotoxicity and that there is a progressive diminution in the cytotoxic effect of the drug as one extends deeper within the interior of the spheroid.

If diffusion of drug is the most important factor in generating clinical effects on tumors, then one would expect that treatment protocols incorporating high concentrations of single agents of a highly diffusible type should be the most effective pharmacological approach. Simple diffusion would not argue for the use of combination chemotherapy where peak concentration of individual agents is sacrificed to permit multiple drug administration. Small-molecular-weight, highly diffusible drugs such as the nitrosoureas would be expected to be much more effective than many of the large-molecule heterocyclic compounds, which are in fact among the most generally useful clinical agents. It is also difficult to see why 2 tumor nodules of the same type and of the same size should vary markedly in their response to chemotherapy, a situation which is commonly observed. Diffusion theories run into further difficulties when one tries to account for the nonresponsiveness of microscopic foci of melanocarcinoma (for example), contrasting to the marked sensitivity of huge (1000 cu cm) masses of embryonal carcinoma.

Other lines of evidence which point against a purely kinetic or diffusion basis for tumor insensitivity can be developed from the large amount of data now available from *in vitro* clonogenic assays of clinical cancers (14, 59). In the performance of these assays, solid tumor masses are reduced to single-cell suspensions and are exposed for varying periods of times *in vitro* to cytotoxic drug concentrations. Under these conditions, the problems of drug access to the clonogenic cells do not arise, and likewise any *in vitro* response will be generated by individual cells and not by mass effects of the original tumor. Despite the enormous differences between the environment in which the assays are carried out and the *in vivo* clinical situation, there has been a degree of correlation between *in vitro* sensitivity and resistance and what is observed in the clinical treatment of the same neoplasm. The correlation is far from perfect, but it appears sufficiently good to argue for the hypothesis that a significant component of the drug responsiveness of the tumor can be traced to the properties of individual component cells.

Mathematical models based on either the kinetic or the diffusion hypothesis can be constructed (71, 74). A general feature of such models is that they consider drug resistance to be an aggregate property of tumor masses as opposed to a phenomenon residing at the individual cellular level. Such models tend to predict for the general lack of responsiveness of large tumor masses as opposed to small ones, although at least one such kinetic model has predicted greater resistance for small tumor populations as compared to larger ones (49). All of the models of these types have difficulty in accounting for the apparent unpredictable variability in response in individual tumors, for the superiority of combination chemotherapy over single agents, and for the effectiveness of certain complex treatment strategies such as the use of non-cross-resistant combinations. As will be discussed in this review, we feel that answers to these questions are more satisfactorily obtained if one postulates that a significant component of the drug resistance of tumors resides at the level of the individual tumor cell. The aggregate properties of the tumor may well contribute additional effects, but in general the pattern of treatment responsiveness will be dictated by the sensitivity and resistance of the individual cells.

Evidence for a Genetic Basis for Drug Resistance and Tumor Heterogeneity

There is an increasing amount of information that suggests that most malignant cell populations are characterized by what might be described as "genetic instability" (50, 51). By this is meant the spontaneous generation of variant forms which are characterized by different phenotypic and genotypic properties. The genetic changes involved include mutations, deletions, transposition of genetic elements, gene amplifications, chromosomal rearrangements, and translocations (57). These changes may be associated with altered, diminished, or increased gene products which in some cases can be shown to be directly involved with the generation of phenotypic drug resistance (11, 63, 81). Some workers suspect that this genetic instability underlies the phenomenon of tumor progression (51), with the associated progressive loss of normal function and acquisition of such properties as increased metastatic potential (28). This would imply an intimate association between the malignant state and the capacity to generate a great range of drug-resistant phenotypes.

Data suggesting a genetic basis for drug-resistant tumor cells go back at least to the early 1950s (44). Using the classic Luria-Delbruck fluctuation analysis, a number of workers were able to consistently demonstrate the spontaneous and stochastic origin of the drug-resistant phenotype in a variety of mammalian tumor cells and to a large number of antineoplastic agents (23, 52, 65). This evidence was admittedly somewhat indirect and did not exclude the possibility of a stochastic epigenetic process which

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was heritable. In more recent years, a large amount of evidence derived from studies in cell biology and molecular genetics has accumulated which is totally consistent with a genetic origin for drug resistance. This evidence has been reviewed recently by Ling (45), and the major points in favor of a genetic basis will be outlined below.

Random Variation in the Fraction of Resistant Cells Seen in a Series of Subclonal Populations. If a large number of parallel subclonal cell cultures are treated with a cytotoxic agent, there will be a great variation from culture to culture in the number of resistant colonies that are observed (47). This is the basis of the fluctuation test of Luria and Delbruck, and the phenomenon has been observed repeatedly with mammalian tumor cells *in vitro* and *in vivo* (44, 65). From such tests, the mutation rate can be calculated, as well as information about the expected frequency distribution of such mutants. While a positive result in a fluctuation test is not definitive proof of a genetic origin for the resistant phenotype, it is totally consistent with such an origin. The implication of such processes is that the occurrence of the resistant cell is random and not directed by the selecting agent itself. A number of implications for the expected behavior of a neoplastic cell population undergoing treatment by drugs will be discussed in greater detail below.

Characteristic Genetic and Karyotypic Changes Associated with Specific Drug Resistance. There are substantial data available indicating that phenotypic resistance to a number of antineoplastic agents is associated with amplified genetic sequences that contain extra gene copies coding for a specific protein (5, 10, 36, 43). This has been best studied in the case of methotrexate resistance where amplified genes coding for extra copies of the *dhfr* gene have been identified (63). These extra gene copies will result in markedly elevated intracellular levels of the enzyme dihydrofolate reductase which will in turn confer methotrexate resistance on the cell. Moreover, there is evidence that altered species of dihydrofolate reductase may be produced in association with the amplification of the normal *dhfr* gene (22, 36). Such altered forms may display high orders of insensitivity to methotrexate inhibition and thus act as an additional mechanism of resistance (35). The amplified genes have in some instances been specifically located in chromosomal regions. Stably resistant phenotypes have been shown to have amplified genes for *dhfr* in an expanded region of the chromosome known as a homogeneously staining region (37, 64). Unstably methotrexate-resistant cells have been shown to have amplified *dhfr* sequences associated with extrachromosomal fragments known as double minute chromosomes (3). These elements are known to segregate unequally at mitosis and hence tend to be quickly lost in the absence of continuous selection. Both homogeneously staining regions and double minute chromosomes have been seen in clinical tumor specimens resistant to methotrexate (3, 76, 82). In addition to methotrexate resistance, gene amplification has been demonstrated with resistance to phosphonacetyl-L-aspartate, ellipticine, and vincristine (6, 8, 43, 81).

A recent report by Johnston *et al.* (42) examined the rate of spontaneous amplification of *dhfr* genes under nonselective conditions. Their data showed that amplification of the *dhrf* gene occurred at high rates in the absence of toxic selection pressure from the drug itself, confirming the spontaneous origin of the drug-resistant phenotype. Of special interest in this study was the very high rate of spontaneous amplification that was observed, up to 3×10^{-2} amplification events per cell division.

The data regarding gene amplification provide powerful support for the concept of a spontaneous genetic origin for the drug-resistant phenotype. Moreover, as this phenomenon is studied more extensively, it is apparent that gene amplification is a common process in tumor cells and may be fundamentally involved in neoplastic transformation itself (78).

Cell-Cell Hybridization Studies. Utilizing the technique of cell-cell hybrid formation, it has been possible to analyze the pattern of inheritance of the drug-resistant phenotype (*i.e.*, either dominant or recessive) (17, 31). Information has been obtained on the inheritance characteristics for a large number of antineoplastic agents, *i.e.*, 1-β-D-arabinofuranosylcytosine, methotrexate, hydroxyurea, melphalan, etc. (45). Such studies constitute strong evidence for the genetic origin of many types of drug-resistant phenotypes.

Drug Resistance Mediated by DNA Transfer. It has been possible using DNA transfection techniques to transfer cloned genes coding for a drug-resistant marker to drug-sensitive cells (2, 18, 19). The transfected cells can then be shown to display stable and hereditable specific drug resistance. These remarkable studies in many ways constitute the most powerful evidence obtainable to date for the genetic basis of many types of drug resistance. Indeed, it is difficult to imagine how more conclusive evidence could ever be obtained. This promises to be a very powerful tool for studying how genetic changes produce the specific alterations in protein structure that may then go on to confer phenotypic resistance on the transfected cell. Experiments utilizing the technique of directed mutagenesis whereby specific codons are changed in the transfected DNA allow a direct evaluation of some of the mechanisms involved in resistance production (79).

Pleiotropic Drug Resistance. Ling *et al.* (45, 46, 54) have demonstrated that tumor cells of both animal and human origin may as a consequence of a single mutation display significant orders of resistance to a variety of antineoplastic agents. This phenomenon, known as pleiotropic drug resistance, manifests itself principally as resistance to a variety of large-molecular-size antineoplastic agents of both microbial and plant origin (*i.e.*, anthracycline antibiotics, a variety of plant alkaloids, actinomycin D, etc.). The resistant phenotype in these cases is associated with the production of increased amounts of a plasma membrane glycoprotein which is thought to act by altering the permeability of the cell membrane to a variety of a large-molecule compounds. A number of other workers have shown similar results in other types of tumor cells (4, 6–8). Recently, data have been presented indicating that the pleiotropic drug-resistant phenotype can be identified in human neoplastic cell lines (4).

The pleiotropic phenotype appears to show all the classical manifestations of a phenomenon arising from a genetic mutation. That is, it can be identified in the absence of specific drug selection, its frequency in unselected subpopulations can be increased by mutagens, there is preliminary evidence that the phenotype can be transfected to other cell types using cloned DNA segments, and a specific protein product in a number of instances has been identified (46).

Although cells exhibiting pleiotropic drug resistance show significant levels of resistance to a wide variety of antineoplastic agents, they are not universally resistant to all such drugs (61). They may indeed even show increased sensitivity to unrelated classes of compounds such as alkylating agents and antimetabolites. The capacity of a cell to generate a broad range of

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resistance as a consequence of a single mutational event clearly has some serious implications for the utility of certain types of combination chemotherapy. However, this process need not be viewed in a totally pessimistic light as will be indicated subsequently.

Taken altogether, we believe that the amount of evidence implicating genetic instability and the generation of phenotypic variation with resultant degrees of biochemical sensitivity to antineoplastic drugs is very compelling. It is useful, therefore, to examine in a more quantitative fashion some of the implications of a spontaneous genetic origin for drug resistance with special references as to how this might influence the design and structure of chemotherapeutic protocols.

Mathematical and Computer Models of Drug Resistance

Relationship between Tumor Burden and Curability. If we apply the principles of the Luria-Delbruck fluctuation phenomenon to the behavior of growing neoplastic cell populations, it is possible to derive an average relationship between the size of the tumor in cell numbers and the likelihood of cure by any hypothetical chemotherapeutic regimen (32). If we consider the mutation rates as being a measure of the probability of occurrence of a new phenotype per cell division, then it is obvious that the probability of at least one such mutant cell type arising will be directly related to the size of the tumor population being considered. The larger the tumor cell population, the greater is the probability that at least one resistant phenotype will have appeared. Conversely, if we look at the tumor population when it is very small, there will be a significant probability that no resistant phenotypes will have as yet occurred. If we assume that our chemotherapy regimen will destroy all of the sensitive cells within the tumor and we further assume that we give enough courses of treatment to accomplish this, then the probability of there being no resistant cells present will be approximately equivalent to the potential probability of cure. The appearance of one resistant cell would signal a condition of incurability, although if the resistant fraction were very small compared to total tumor size it would not preclude significant regression of the tumor in the face of treatment. This relationship between tumor size and probability of cure can be expressed by the function p (cure) $= e^{-\alpha N}$ where α is the mutation rate per cell generation and N is the size of the tumor. This function is shown in Chart 1 and can be seen to have a sigmoid form when probability of cure is plotted against log tumor size. The point of downward inflection of the curve is dictated by the value of the mutation rate, higher values resulting in an earlier deflection. The shape of the curve, however, will be unaffected by the value of the mutation rate, and so will the steepness with which this probability-of-cure curve falls to an extremely low level of likelihood. The implications of this are that, except in those circumstances where the tumor burdens are on a flat portion of the curve (either in the highly curable or completely incurable range), relatively short delays on the average in the institution of chemotherapy might have an unexpectedly marked deleterious effect on end results. This appears to be definitely the case in experimental chemotherapy trials where analysis has shown that the distribution of observed cures for a particular drug falls very close to the theoretical curves calculated by the above formula (67). Whether this relationship holds true to the same extent in clinical cancer chemotherapy has yet to be established, although

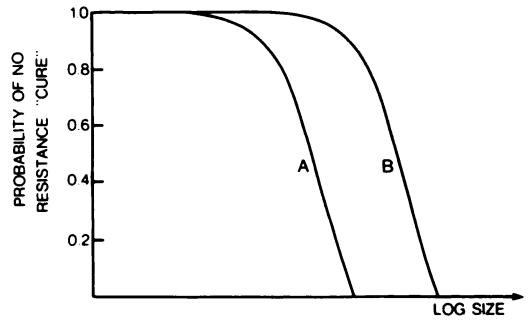

Chart 1. Plot of the function $p = e^{-\alpha N}$ for 2 different mutation rates, *A* and *B*, where $A > B$. The function defines the probability of their being zero resistant cells present for any given value of the tumor size and the mutation rate to resistance. Where treatment is capable of eradicating all nonresistant cells, then this probability approaches the value of potential cure. For tumor burdens distributed over the steep portion of the curve, relatively small increases in tumor mass will have a disproportionate effect on reducing curability. Combination chemotherapy might be envisaged as acting to lower the net mutation rate of the system from *A* to *B*. The impact of this on potential cure over certain critical tumor size ranges is apparent. Significant cross-resistance among the combined agents (*i.e.*, due to pleiotropic effects) may result in negligible shifts in the position of the curve away from *A* with the expected outcome of little or no improvement in treatment results.

retrospective analyses of some trials in adjuvant breast chemotherapy do suggest that, whatever the mechanism, relatively short delays in the institution of adjuvant treatment significantly reduce the subsequent disease-free survival (13, 48).

The spontaneous and random nature of the appearance of resistant mutants also provides a theoretical basis for the variation in response to cancer chemotherapy that is seen in both clinical and experimental trials. There will be substantial variation in the fraction of resistant cells from one tumor to another, even if the tumor mass and mutation rates to resistance are identical.

Treatment Strategies Aimed at Circumventing Drug Resistance. Two implications for improving chemotherapy results emerge directly from the above considerations. The first has to do with the increasing likelihood of cure as one deals with smaller tumor burdens. That this is a valid relationship is apparent from a large body of data from experimental chemotherapy and is totally consistent with much clinical observation as well (26, 66, 69). Even when dealing with categories of advanced disease, it is in those patients in whom a lower tumor burden can be inferred that the most favorable results are achieved (1, 27, 75, 83). Beyond that, one would anticipate that chemotherapeutic cure might be possible when neoplasms are treated in an adjuvant situation even when the same tumor is not curable at an advanced stage. Although controversies continue, there would seem to be no doubt that a number of types of human solid tumors can be cured when treatment is initiated before the tumor burden becomes clinically overt (9, 12, 26, 29, 30, 38, 41, 48, 55, 56). The actual degree of effectiveness of adjuvant chemotherapy intervention will depend on a number of factors including the effectiveness of the chemotherapy protocols themselves. However, the basic principle seems well established, and the use of treatment directed against minimal tumor burdens appears to be our best immediate hope for improving cure rates in a variety of human solid tumors.

The other therapeutic principle that clearly emerges is the enhanced curative potential of combination chemotherapy as opposed to single-agent treatment even given in maximal dose. Most of the treatment programs that are capable of producing significant cure rates in human cancers are characterized by the use of a large number of active antineoplastic agents (26). Only

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a few rare human tumors appear to be curable at the advanced stage by single-agent treatment, and even in these cases cure rates are increased by the application of combination chemotherapy (1, 83). The older theories behind combination chemotherapy implicating a type of sequential biochemical blockade induced by the drugs do not appear valid. If we imagine the tumor to be composed of a series of subsets of drug-resistant clones, then the role of combination treatment in overlapping the resistant populations becomes apparent. From the perspective of mathematical modeling, the use of a number of active non-cross-resistant drugs will be equivalent to having the tumor display a lower mutation rate to resistance (32, 34). The situation has many similarities to that seen in the treatment of tuberculosis by antibiotics. *Mycobacterium tuberculosis* displays relatively high mutation rates to resistance to most of the standard anti-tuberculosis drugs (21). The combination of 2 or 3 such agents greatly diminishes the probability that there will be any bacteria present that are not sensitive to at least one of the agents in the combination.

The effect of pleiotropic drug resistance in diminishing the effect of combination chemotherapy can be visualized by inspecting Chart 1. The distance between the 2 probability-of-cure curves can be considered to be a measure of the extent of hypothetical improvement that would occur with the application of non-cross-resistant agents. If the target tumor cells display a significant degree of cross-resistance to the additional agents being used due to a large pleiotropic dosage effect, then the actual shift to the right in the probability-of-cure curve may be minimal. Thus, highly heterogeneous systems which also display marked pleiotropic effects may show considerably less improvement in therapeutic response to what would otherwise be theoretically predicted. The disappointing results of combination chemotherapy in the treatment of a number of traditionally highly refractory tumors such as melanoma may possibly be traced in part to this phenomenon.

Overcoming a heterogeneous population of cells would dictate that ideally all of the active agents should be used concurrently. Because of their considerable toxicity to normal cells, there are definite limits to the number of antineoplastic agents that can be given simultaneously without major reductions in drug dosage. Because lowering the dose will not only reduce the fractional cell kill for that particular agent but will also in effect raise the mutation rate to resistance to it, it is clear that significant dose reductions to accommodate more drugs will at some point become a self-defeating strategy. We are then confronted with the problem as to how to use most effectively a larger number of cytotoxic agents than can be safely administered concurrently. Analysis of this problem leads to the conclusion that one solution is to alternate at each cycle between 2 roughly equivalent treatment programs (34). (These could represent single agents but in practice would more often constitute complex drug combinations themselves.) Alternating chemotherapy can therefore be seen as a mechanism to allow one to introduce therapeutic diversity early into the treatment program. In evaluating the effectiveness of such a strategy, the standard of comparison should be to what would be expected if the 2 treatment blocks were used in a sequential as opposed to an alternating fashion. That is, would 6 courses of Treatment A followed by 6 courses of Treatment B be equivalent to alternating A and B at each cycle? Testing this approach at the clinical level is not easy because of the requirement of (*a*) a sufficiently large number of patients to allow one to draw valid statistical inferences and (*b*) the prior necessity of having established the near quantitative equivalence of the treatment arms that are to be used.

The mechanism of how alternating chemotherapy might be expected to work can be illustrated by reference to the following data from the work of Skipper *et al*. (68) (see Chart 2). In these results, Skipper used a computer program to simulate the effect of chemotherapy on a transplantable mouse tumor, the Ridgway osteosarcoma. In this experimental system, reasonably accurate estimates of such critical parameters as tumor mass, tumor-doubling time, fractional cell kill per dose of drug, and approximate mutation rates to resistance can be made. Assigning appropriate values for these various parameters, one can then simulate the effects of a variety of treatment programs upon the tumor and compare these with the actual observed experimental results. In Chart 2*A*, we see the computer simulation of treatment of the neoplasm with cyclophosphamide alone. The program predicts that the treatment will fail because of the outgrowth of cyclophosphamide-resistant cells. This conforms to what is observed, and this is a noncurative strategy. In Chart 2*B*, the effect of repetitive daily courses of another active agent, 6-MP,[3] is simulated. As in the previous simulation, we see an initial tumor response but that ultimately there is overgrowth by 6-MP-resistant cells with consequent treatment failure. Again, this conforms to the actual observed experimental data where 6-MP produced temporary regression of tumor mass but was invariably followed by the outgrowth of 6-MP-resistant tumor cells.

Chart 2*C* shows the therapeutic effect of alternating cyclophosphamide with 6-MP. Note that the course of 6-MP, although given over several days, is quantitatively equivalent to the cyclophosphamide course in terms of net log kill on the neoplasm. The computer simulation now predicts cure (at least on average). When this treatment protocol was in fact used against the tumor, it resulted in 100% cures being observed. Again, it is instructive to examine the computer plots to see that the mechanism of cure has come about because the 2 non-cross-resistant agents were each effective in eradicating the tumor lines resistant to the other drug.

There is obviously a great gulf between the levels of complexity of a transplanted experimental tumor and of a clinical cancer. Nonetheless, it is of interest to note that, utilizing the assumptions of spontaneous mutations to resistance, it is possible to simulate and to predict the behavior of certain types of chemotherapy regimens in an experimental setting. It seems that this approach has considerable potential for further refinement and development. It may be possible to drastically reduce the amount of trial and error in chemotherapy experiments in attempting to establish effective treatment protocols. If one can predict with a significant degree of accuracy the outcome of experimental chemotherapy trials, then this would increase one's confidence that similar approaches may be attempted in clinical treatment.

Although the transplanted tumors of rodents have been criticized as being invalid models for the human disease, it appears to us that they have in fact accurately predicted for most of the major chemotherapeutic strategies that have proven to be beneficial at the clinical level. It is true that such matters as specific drug selection cannot be readily extrapolated from the animal

[3] The abbreviations used are: 6-MP, 6-mercaptopurine; MOPP, nitrogen mustard-vincristine-procarbazine-prednisone; ABVD, Adriamycin-bleomycin-vinblastine-imidazolecarboxamide.

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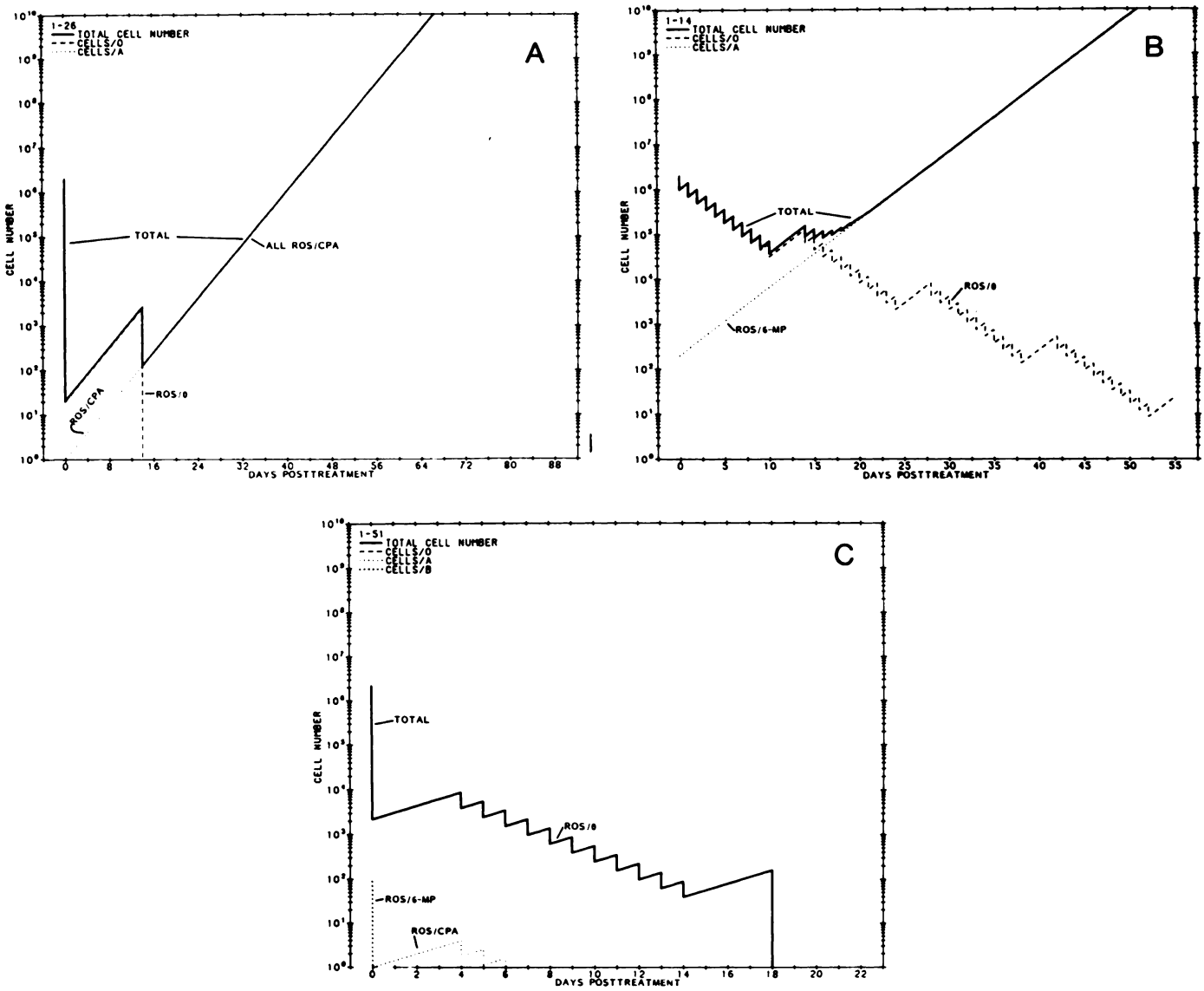

Chart 2. Computer simulations of the treatment of the Ridgway osteosarcoma (*Ros*) with cyclophosphamide (*CPA*) and 6-MP. Initial inputs include tumor size, growth rates of sensitive and resistant cells, log kills per dose of each drug, treatment intervals, and average burden of drug-resistant cells based on estimates of mutation rates to resistance for each agent. *A*, treatment with cyclophosphamide alone, which produces significant but transient regression, but failure occurs due to existence and outgrowth of cyclophosphamide-resistant cells. *B*, treatment with 6-MP alone given as a series of repetitive daily doses. As in *A*, response is followed by failure due to outgrowth of 6-MP-resistant cells. *C*, alternating cyclophosphamide and 6-MP. Both resistant subpopulations are extinguished, and the last course of cyclophosphamide eliminates the residual sensitive tumor cells. Chart reproduced with the kind permission of Dr. H. E. Skipper.

systems, but we would suggest that the general principles can.

Chemotherapeutic Scheduling: Drug Combination, Dose Rate and Intensity. A more rigorous analysis of the phenomenon of alternating chemotherapy would suggest that the theoretical enhanced therapeutic potential of such a maneuver has a more fundamental basis than the question of simple alternation *per se*. In the experimental example given above, we could consider the protocol of cyclophosphamide alternating with 6-MP not as an alternating schedule but simply as a 2-drug block in which cyclophosphamide is followed by 6-MP. Likewise, at the clinical level, we could envisage the effective alternating protocol for Hodgkin's disease of MOPP-ABVD (60) as not "alternating" chemotherapy but rather as a very complex regimen designated MOPP-ABVD which is given over a 48-day cycle and which is in effect not alternated but simply repeated at 2-month intervals. We have recently published a mathematical analysis as to how complex treatment schedules of this type might be expected to exert their benefit (20). It can be shown, in purely theoretical terms, that they appear to act by maximizing the amount of growth inhibition of all of the subsets of cells (both sensitive and resistant) that are present within the tumor. The most effective strategies will be those in which there is the least amount of regrowth of the various subsets between treatment cycles. This not only argues for drug diversity in terms of numbers of non-cross-resistant agents but also suggests that they should be given at minimum intervals and in maximum tolerated dose. The delayed introduction of active agents in the protocol will be inferior to those strategies that use all active agents as early as possible. The idealized optimal approach would of course be to apply all of the active agents in a therapeutic dosage simultaneously. Where large numbers of drugs are available and required, this is not feasible, and effective strategies will be ones in which

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this optimum is most closely approached.

We might illustrate this effect by considering the specific example of the chemotherapy of Hodgkin's disease. The standard regimen for the chemotherapy of disseminated Hodgkin's disease is the multidrug protocol designated MOPP (25). The structure of the MOPP protocol at first sight seems to violate some of the principles that have been developed with respect to minimizing host toxicity with chemotherapy. Two courses of myelosuppressive therapy are given at 7-day intervals in the first part of the treatment block. MOPP is in fact quite myelosuppressive, and it is common for patients to require some dosage attenuation before completion of 6 courses of therapy. It would be reasonable to question in fact how much of the therapeutic activity of MOPP is retained in the last few courses and how much is crucially dependent on the major therapeutic impact given at the beginning.

Although the cycles of MOPP chemotherapy are given at 28-day intervals, this time frame is essentially dictated by the 7-day week. If we examine a sequence of MOPP chemotherapy and draw a line arbitrarily at the end of the fifth week of treatment, we can see that during this time interval the patient has received a total of 4 cycles of vincristine and nitrogen mustard, 21 days of procarbazine p.o., and 14 days of prednisone. Thus, a very considerable amount of chemotherapy has been administered during an initial short time period. All of the active agents have been given at least once and some of them several times.

If we study the situation with MOPP-ABVD, we can see that the major difference now is that, at the end of the fifth week of chemotherapy, 4 additional agents have been added to the protocol structure, replacing the repetitive application of some of the MOPP agents that were used at the beginning of the treatment program. Because all of these additional agents are highly active against the disease, especially when used in the schedule that they are, one would expect that their integration into the protocol should significantly enhance the therapeutic effect. In theory, an even earlier integration of the agents from the ABVD program might be expected to further improve therapeutic results.

Many multiple-agent protocols used in cancer chemotherapy, particularly in a number of pediatric cancers such as acute lymphoblastic leukemia, tend to use active agents in protracted blocks before switching to the next set of therapeutically active drugs. This is visualized in phenomenological terms as a sequence of stages "remission induction → remission consolidation → remission maintenance or intensification, etc." The theoretical considerations outlined above would suggest that this approach may be inferior to ones where there is earlier integration of active drugs into the protocol structure.

This theory is open to testing at the clinical level, although the number of factors that have to be considered in terms of drug pharmacology, toxicity patterns, etc., clearly make it a complex problem.

Relationship between Tumor Growth Rate, Biological Age, and Drug Resistance. The model of drug resistance described above, based essentially on the Luria-Delbruck experiments, provides a useful conceptual framework to examine the relationship between tumor mass and curability. It helps to explain the phenomenon of variability of response in individual tumors, and it directly predicts the superiority of certain chemotherapeutic strategies as opposed to others. It does not, as it stands, effectively answer the question as to why certain aspects of tumor kinetics, particularly overall growth rate and biological age, should influence drug sensitivity and curability. As was described earlier, the purely kinetic explanations for this relationship break down at certain levels and do not appear to provide a comprehensive explanation for the phenomenon.

We have attempted to examine this question from the point of view of integrating the somatic mutation theory with certain time-dependent processes that are believed to occur within neoplastic populations. A detailed analysis of this approach has been published elsewhere (33), but the broad outlines can be indicated as follows. Studies on the behavior of cell renewal systems, of which neoplasms can be considered to be one type, point towards the extent of self-renewal of the stem cell compartment as being the major parameter in dictating overall growth rate (15, 16, 82). The growth rate of a system will be maximized under conditions in which no cell loss (*i.e.*, differentiation) occurs from the stem cell compartment. Such a system will grow in a pure birth rate mode very much like a population of bacteria. Such growth rates are approximated by certain experimental neoplasms that have been maintained for prolonged periods *in vitro* or by propagation in suitable animal hosts (69). There is, however, now substantial evidence that this is not an accurate description of the growth behavior of most types of clinical neoplasms. Many lines of evidence suggest that only a small proportion of the cells within a tumor are truly stem cells or clonogenic in type (14, 16, 59, 71). Most of the morphologically malignant cells in the neoplasm have only limited proliferative potential, and many have ceased cell division permanently. There is evidence to suggest that the probability of self-renewal in the clonogenic subpopulation of clinical tumors is substantially less than 1 and may well lie within the range of 0.5 to 0.6 (14, 15). If we assume a generation time of the clonogenic cells varying from 24 to 48 hr, then such a range of renewal probabilities could encompass the observed values of clinical doubling times of human cancer (15).

Although not intuitively obvious, our analysis of this phenomenon indicates that the closer the renewal probability lies to 0.5 (*i.e.*, the slower the growth rate of the system), the greater will be the extent of phenotypic heterogeneity for any given value of the mutation rate. This comes about essentially because it now requires many more division cycles of the clonogenic cells for the population as a whole to reach any given size. When the renewal probability is 1, then it takes 36 generation cycles (or doublings) for a single cell to expand to size 10^{11}. If the renewal probability is 0.51, then the stem cell compartment will grow far more slowly, and it will require on average about 1500 cell generations to reach size 10^{11}. During this long growth period, many cell lineages will become extinct, but the ones that survive to the 10^{11} level will have an enormously elongated genetic "history." Their genetic apparatus will have "fired" an average of 1500 times or more, providing a greatly enhanced opportunity for mutations to occur and to accumulate. Moreover, any process which is conditional, in the sense that the state of the genome at time t_1 will increase the likelihood of transition to a subsequent state at time t_2, will be enormously enhanced by this process. Such phenomena could include gene amplification, chromosomal instability with translocation and deletion, etc. Either the cell loss processes generating this effect could arise as a consequence of stochastic stem cell differentiation (with loss of proliferative

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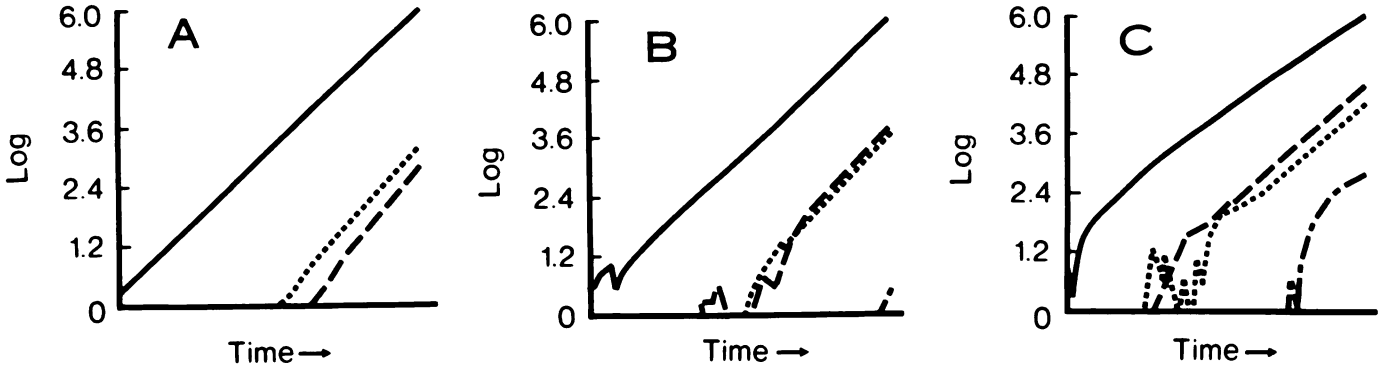

Chart 3. Each section plots the total number of stem cells (——) and the numbers of singly resistant (- - - - -, – – – –) and doubly resistant (- – - –) stem cells for 3 simulations using different values of the renewal probability p, and log population size *versus* time. All mutation rates are 10^{-4} for each simulation. The time scale is not uniform for each of the figures but is compressed in inverse proportion to the p value of the simulation; *i.e.*, the growth rate of the stem cells decreases as p decreases. *A*. This simulation has a renewal probability of 1, which implies no cellular differentiation and thus leads to overall exponential growth. Each of the singly resistant stem cell compartments shows very regular growth, although their time of emergence is different due to the random nature of the mutation process. No doubly resistant mutants emerge. *B*. In this simulation, $p = 0.6$. The overall growth of the stem cell compartment is quite regular, although initially it displays some variability. Similarly, the growth of the singly resistant compartments fluctuate initially (with the extinction of an early mutation) but settles down to faster than exponential growth. Comparisons between *A* and *B* show that the fraction of singly resistant cells is greater for the simulation where $p = 0.6$ than where $p = 1.0$. This is found to be the case in the majority of simulations. Also, a doubly resistant clone has emerged. Although random by nature, the probability of existence of a doubly resistant clone is greater in the case of $p = 0.6$ than $p = 1.0$ because of the greater number of singly resistant cells. *C*, simulation for $p = 0.51$. *C* shows a pattern similar to that in *B* with an increase in the variability when small numbers of cells are present and an increase in the number of singly and doubly resistant cells. The doubly resistant cells have increased so rapidly that there are nearly as many doubly resistant cells in *C* as singly resistant cells in *A* when there are 10^9 cells overall. It will be noticed that the resistant stem cell compartments seem to vary much more than the overall stem cell compartment at the same size. This phenomenon arises because we require that in the long run the stem cells as a whole must grow. We do not require this specifically of any one compartment of the stem cells. (For a detailed description of the mathematical assumptions in this model, see Ref. 33.)

capacity of the clonogenic cells), or alternatively it could be as a consequence of environmental conditions surrounding the tumor, resulting in enhanced rates of cell death. With a constant birth rate, any process that enhances the death rate will result in slower overall growth and a magnification of drug resistance. This can be illustrated by a series of computer simulations shown in Chart 3. In this sequence of simulations, we have allowed a clonogenic cell population to grow to size 10^6. The mutation rates to single levels of resistance are 10^{-4} in each case and in the 3 simulations displayed the only parameter that is changed is the renewal probability. In the first simulation (Chart 3*A*), the renewal probability is 1, and the system grows by a pure birth process with maximal growth rate. Under these conditions, the doubling time of the system is equal to the generation time of the component cells. Small singly resistant compartments are generated during the time elapsed to reach size 10^6, but no doubly resistant cells appear. If the renewal probability is now reduced to 0.6, the system now grows significantly more slowly, both singly resistant compartments are increased as compared with Chart 3*A*, and there is as well a small doubly resistant compartment that has appeared. In Chart 3*C*, the renewal probability has been further reduced to 0.51, producing a very slowly growing system, and the magnification in size of all of the resistant subcompartments is apparent.

If overall growth rates and rates of cell death do enhance the effect of drug resistance, then this does suggest that many types of slow-growing tumors will display extreme phenotypic heterogeneity unless they are characterized by very low mutation rates to resistance. Successful treatment of such neoplastic systems by essentially empirically derived treatment schedules would appear to be doomed to failure, and the lack of significant progress in treating many forms of advanced solid tumors would be the expected outcome.

Treatment Strategies Directed at Extremely Heterogeneous Neoplastic Systems. Many of the drug treatment strategies discussed above would appear to have the greatest potential utility when directed at neoplastic systems that are inherently curable to begin with. If the chance of curing an advanced solid tumor is in the order of 10^{-20}, then even a 1000-fold improvement in effect is not going to be perceived as a useful advance. Can one on the basis of existing knowledge define any directions where cytotoxic cancer chemotherapy can be made to have an impact on the refractory segment of human cancers?

One approach that can be and of course is being used extensively is to utilize the chemotherapy in an adjuvant setting wherever feasible. Even here there appear to be major limitations to chemotherapeutic effect, because the upper limit of curable size of many solid tumors appears to be only at the lower range of even microscopic tumor burden. Advances utilizing this approach may require more effective early diagnosis combined with better selective identification of the subgroups of patients who are likely to benefit from the adjuvant treatment. Minimizing time delays between diagnosis and drug intervention is an additional approach, and programs are under way to evaluate the utility of pre- and perioperative adjuvant chemotherapy (53).

We are still faced, however, with the formidable problems of patients who present with advanced solid tumors of many types and in whom adjuvant chemotherapy was never available as a useful option. Does the extreme heterogeneity that such systems are almost certain to express render the clinical utilization of cytotoxic agents an exercise with low probability of success? Given the limitations of existing protocols, this certainly seems to be true in many cases. However, if extreme heterogeneity is postulated to be the major barrier, then this at least points in certain directions where therapy can be improved.

The phenomenon of pleiotropic drug resistance was described earlier. The capacity of the cell to express resistance to many drugs simultaneously would appear to make a bad situation immeasurably worse. However, the corollary to this phenomenon is that, if it can be circumvented or reversed, then sensitivity to

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a great range of drugs will be reestablished. Such approaches are presently being evaluated experimentally, and preliminary results appear encouraging (70, 77). A number of workers have reported that the use of calcium channel-blocking agents such as verapamil simultaneously with the administration of drugs such as Adriamycin appears to reverse the Adriamycin resistance associated with the pleiotropic state. The mechanism of this reversal is uncertain but may involve an alteration in the cell membrane impermeability induced by the presence of the phosphoglycoprotein.

Considerably more work along these lines will be required, particularly in delineating the mode of action of the calcium channel-blocking agents themselves so that drugs which maximize this effect can be identified. Moreover, resistance to drugs such as Adriamycin, many of the alkaloids, etc., is probably multifactorial (11, 40), and abrogating one resistance pathway may not necessarily ensure restoration of the tumor line to complete drug sensitivity. However, this does represent the application of knowledge about the drug-resistant state to the development of rational approaches to circumvent resistance.

Another direction which seems to warrant greater activity than has been carried out hitherto is to concentrate efforts on the development of at least a few types of second-generation antineoplastic agents that are designed to specifically retain their effect in the presence of a resistance mechanism. There have been efforts along these lines, but it has tended to be fragmented and is often done in association with other types of analogue development in which the main effort is towards the reduction of toxicity and other undesirable side effects.

The development of semisynthetic penicillins to specifically circumvent the problem of penicillinase-producing bacteria is an example of this type of rational approach. There has up till now not been a single case of a generally useful second-generation antineoplastic drug. Resistance is usually dealt with either by dose escalation or by the use of a non-cross-resistant agent with an entirely different mechanism of action. This method works when one is dealing with low heterogeneity systems, but it appears to be much less effective as a means of overcoming a great range of phenotypic diversity within a tumor. Second-generation agents, used in combination with their parent compounds, might in theory overcome the problem of marked heterogeneity. Some consideration might be given towards targeting programs to develop second-generation drugs based on, say, half a dozen broadly useful clinical antineoplastics. Although there may be several distinct mechanisms of resistance to an individual drug, it seems unlikely that there would be hundreds or thousands. A combination of 7 or 8 different types of antifolates might conceivably saturate all of the common resistant modes expressed by many types of tumor cells. This would be one way of providing the tumor with what DeVita *et al.* (26) have described as "an impossible choice."

Conclusion

There are still many unresolved questions in the area of drug resistance, especially as applied to the behavior of human cancer *in vivo*. Most of the details of biochemical mechanisms of resistance have been worked out in experimental systems, of animal or human origin. To what extent these specific molecular processes also occur in clinical cancer will require much further elaboration. Measuring such phenomena as *in vivo* mutation rates and drug-induced fractional cell kills will be extremely difficult and may have to be approached indirectly. The extent to which the patterns of sensitivity and resistance of a specific type of tumor are dictated by the properties of the normal tissue from which it is derived is unclear. Likewise, the mechanisms of acquired resistance to biological agents such as hormones and the interferons are poorly understood. A matter not specifically addressed in this review is the question as to what extent nonstem or differentiated cells of the neoplasm determine overall treatment effects. If tumors retain to some degree the feedback growth control processes that exist in normal cell renewal systems, then a complete theory of how cancers respond to chemotherapy will have to include some consideration of these phenomena (39).

Until such time as therapies based on deeper insights into the biology of cancer are developed, traditional cancer chemotherapy will remain the main treatment approach for disseminated cancer. We have presented evidence in this review that the major impediment to more successful cancer chemotherapy is the clonal heterogeneity of tumors arising as a consequence of genetic variability. This hypothesis is open to testing and if valid suggests certain directions in which research efforts in drug resistance might be directed.

More information about the molecular processes involved in drug resistance should lead to more effective means for preventing or circumventing it. The instructive examples derived from work in antimicrobial chemotherapy should provide useful insights in these areas.

The ability to simulate the effects of experimental chemotherapy trials by appropriate mathematical and computer-based models opens up the possibility for reanalyzing the vast amounts of such data available to gain better insights into the causes of treatment success and failure. Such techniques can also be used to prospectively plan new chemotherapeutic and combined modality experiments. The application of appropriate quantitative techniques to biological studies has always resulted in a greater understanding of the phenomena concerned. The first quantitative studies of experimental chemotherapy a generation ago provided the beginnings of a true insight into how anticancer drugs exerted their therapeutic effect (69).

Drug resistance appears as an inevitable consequence whenever one attempts to selectively destroy an undesirable biological population. Nevertheless, the successes that have been achieved in the clinical management of a number of types of human cancers indicate that the problem is not an insuperable one. The study of drug resistance should provide a basis for making the drug treatment of cancer more rational in concept and more effective in implementation.

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James H. Goldie and Andrew J. Coldman

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