Effect of Chemotherapeutic Agents upon Microorganisms Isolated from Cancer Patients

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Attempting to explain the predominance of *Pseudomonas* in leukemic patients, five of the most common gram-negative organisms isolated from sites of infection in cancer patients were exposed to several of the chemotherapeutic agents used in the treatment of this disease (methotrexate, cytosine arabinoside, cyclophosphamide, and 6-mercaptopurine). At concentrations of 125 µg/ml or higher, methotrexate inhibited all organisms except *Pseudomonas*. Cytosine arabinoside inhibited *Escherichia* and *Klebsiella* but appeared to stimulate the growth of *Pseudomonas* slightly at the higher concentrations. Thus, significant differences existed in individual susceptibilities to these agents. Clinical isolates were more resistant than the corresponding laboratory strains not previously exposed to these compounds. The resistance of *Escherichia coli* to cyclophosphamide was decreased 26% when it was grown in mixed culture with *Pseudomonas*. Only *Pseudomonas* was resistant to all of these compounds whether in pure or mixed culture. These observations may help to explain, in part, the predominant role that *Pseudomonas* plays as an infectious agent in leukemic patients.

Increased susceptibility to microbial infection in patients on chemotherapeutic and immunosuppressive drug regimens is a well known and constant factor in cancer patient management as well as in the field of organ transplantation. The variations in resistance of microorganisms, particularly gram-negative bacilli, to some of these drugs has been documented for several organisms (7, 11, 16). A microorganism not inhibited by these compounds would have a definite advantage in establishing an infection in the cancer patient.

There appears to be a relationship between the type of cancer and the types of bacterial infections occurring in these patients. For example, *Pseudomonas* was isolated in over 60% of the leukemic patients who developed a bacterial infection (2, 3). It is possible that the chemotherapeutic drugs used in these patients may be partially responsible for the selection of infecting organisms as is the type of neoplasm per se (9).

In this paper, we investigate this relationship and report on the effect of various chemotherapeutic agents in the in vitro survival of several of

the most common organisms usually isolated from cancer patients.

MATERIALS AND METHODS

The resistance to several chemotherapeutic agents was determined quantitatively with both stock cultures and clinical isolates. The strains designated as "stock cultures" were obtained from the culture collection of M. Goldschmidt. These cultures have been carried at least 7 to 10 years and were never previously exposed to the chemotherapeutic drugs used in this study. The organisms were: *Escherichia coli* (B/r), *Proteus morganii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (Nima). The corresponding clinical isolates were obtained from leukemic patients at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston. The bacteria used as an inoculum for the shake flasks were grown for 24 to 48 hr on slants of antibiotic medium no. 3 (Difco 0234). The shake flasks, 250-ml Erlenmeyer flasks containing 30 ml of antibiotic medium no. 3, were incubated for 18 hr at 37 C on a New Brunswick rotary shaker at a speed of 200 rev/min. These were used to inoculate tubes of sterile antibiotic medium no. 3 (Difco 0234) containing various concentrations of the chemotherapeutic agents. After incubation at 37 C for 24 hr, the organisms were diluted into saline to stop

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the action of the drugs. The standard plate count method published by the Food and Drug Administration (8) was used to determine the number of viable organisms.

The following chemotherapeutic agents were used: methotrexate (4-amino- N^{10} -methyl pteroylglutamic acid, Lederle Laboratories); cyclophosphamide [N,N -bis(β -chloroethyl)- N' - α -propylene phosphoric acid, Mead Johnson Laboratories]; cytosine arabinoside (1- β -D-arabinofuranosyl cytosine·HCl, Ben Venue Laboratories); and 6-mercaptopurine (purine-6-thiol hydrate, Burroughs Wellcome and Co.). These were dissolved in distilled water and sterilized by membrane filtration (Millipore Corp.).

RESULTS

Figures 1 through 4 illustrate the effect of several of these chemotherapeutic agents on clinical isolates and the corresponding stock cultures. Each curve represents the average of three to five experiments. The variability between experiments with each organism was <5%. Several different clinical isolates of each representative genus were investigated and showed similar results (within a 10% variation). These strains were obtained at

FIG. 1. Effect of cytosine arabinoside on the survival of several bacteria. The organisms were grown for 24 hr at 37 C in various concentrations of the drug and then assayed for viability.

FIG. 2. Effect of methotrexate on the survival of several bacteria. The organisms were grown for 24 hr at 37 C in various concentrations of the drug and then assayed for viability.

3- to 4-month intervals from various patients and were assumed to be different from those obtained originally.

The effect of methotrexate on the clinical strains was observed by sampling at various intervals over a 24-hr period. Figure 5 shows these results for *E. coli* and *P. aeruginosa*. Under the same conditions, the data for *Klebsiella*, *Proteus*, and *Serratia* were similar to those obtained with *E. coli*, in that the curves leveled off at 4 to 6 hr.

The possibility existed that a bacteriostatic effect could occur and not be observed. If methotrexate was merely bacteriostatic for *Pseudomonas* rather than bactericidal, one would expect to see a final count equivalent to the control cells when the drug was diluted beyond effectiveness during the routine plate count procedures. This would allow subsequent growth of the organisms on the assay plates. To investigate this possibility, samples of the inoculum were diluted and streaked directly on plates containing 1,000 μ g of metho-

FIG. 3. Effect of 6-mercaptopurine on the survival of several bacteria. The organisms were grown for 24 hr at 37 C in various concentrations of the drug and then assayed for viability.

trexate per ml. Control plates without the drug were also streaked in parallel. When the plates were observed after 24 hr, the control and experimental plates had approximately the same number of organisms (1.7×10^8 /ml for the former and 1.6×10^8 /ml for the latter), indicating that no bacteriostatic effect existed.

The effect of cytosine arabinoside, cyclophosphamide, and methotrexate on mixed cultures of *E. coli* and *P. aeruginosa* were investigated. These data are presented in Table 1. Cultures grown together in the absence of the drugs indicated that there was no inhibitory effect of one organism on the other because final cell counts were approximately the same for both.

Cyclophosphamide inhibited both organisms to the same extent when they were exposed to the drug in pure culture. However, when cyclophosphamide was added to the mixed cultures, there was a 26% decrease in the survival of *E. coli*, indicating a preferential inhibition under these conditions. This allowed *Pseudomonas* to dominate in the final mixed population (73%). With cytosine arabinoside and methotrexate, the inhibition of *E. coli* or the resistance of *Pseudomonas* was not significantly altered from their response

FIG. 4. Effect of cyclophosphamide on the survival of several bacteria. The organisms were grown for 24 hr at 37 C in various concentrations of the drug and then assayed for viability.

in pure culture. Again, this allowed *Pseudomonas* to dominate the final mixed population (96–98%).

S. marcescens was also very susceptible to methotrexate, showing a 1.7% survival. Mixed cultures of this organism and *P. aeruginosa* were also exposed to 1,000 µg of methotrexate per ml. The results were similar to the *E. coli*-*P. aeruginosa* experiments as there were no significant differences in the survival data for either organism regardless of the exposure conditions. *Pseudomonas* again dominated the final mixed population (98%).

DISCUSSION

Many factors account for the pathogenicity of the autogenous bacterial flora, especially the gram-negative enteric organisms, in the cancer patient. The increasing use of chemotherapeutic drugs and steroid hormones may alter the host resistance to its indigenous flora (4, 9, 10, 12, 13). Maiztegui et al. (14) reported that patients with gram-negative bacteremias were infected by or-

FIG. 5. Effect of exposure time on the survival of *Escherichia coli* and *Pseudomonas aeruginosa* in methotrexate. The organisms were grown in two concentrations of methotrexate and were sampled at various time intervals for viability.

TABLE 1. Effect of various chemotherapeutic agents on the survival of *Escherichia coli* and *Pseudomonas aeruginosa* in pure and mixed culture^a

Cultural conditions	Per cent survival		
	Cytosine arabinoside	Cyclophosphamide	Methotrexate
Pure			
<i>E. coli</i>	5.1	85.5	0.7
<i>P. aeruginosa</i>	107.1	87.6	88.4
Mixed			
<i>E. coli</i>	2.3	59.6	2.5
<i>P. aeruginosa</i>	83.6	82.7	77.6

^a Organisms were grown singly or mixed for 24 hr at 37°C in the presence of the drugs (1,000 µg/ml) and then assayed for viability. Results are averages of three to five experiments. The per cent survival was determined by comparing the survival of the organisms (singly or mixed) in the presence and absence of the drugs. There was no inhibitory effect in the mixed cultures by one organism on another in the absence of the drugs since final cell counts were approximately the same.

organisms from their own urinary and intestinal tracts or from the skin. Their results indicated that defects in host resistance were important in the establishment of gram-negative infections.

Microorganisms have also been used as assay systems for potential antitumor agents (15). The differential biochemical resistance mechanisms of microorganisms, particularly the gram-negative organisms, have been documented (3, 6, 11, 16). Various microbial systems have been used to study the effects of these drugs. Cardeilhac and Cohen (5) indicated that cytosine arabinoside was not incorporated into the deoxyribonucleic acid and ribonucleic acid of *E. coli*. However, other compounds, such as 6-mercaptopurine and several pyrimidine analogues, were effectively incorporated, thus interfering with the normal metabolic pattern of these organisms (1). Cyclophosphamide, a nitrogen mustard derivative, must be converted to its active form in the body, but these organisms all possessed active phosphatases which could convert it to its active form.

Bodey (2) reported that a substantial number of

patients with leukemia became carriers of *Pseudomonas* after 2 to 4 weeks of hospitalization and chemotherapy. Although *Pseudomonas* was isolated from the skin, throat, and feces of only 25% of these patients initially, a carrier rate of 54% was found after 2 to 4 weeks. Thus, patients with leukemia have a particularly high risk of becoming *Pseudomonas* carriers while undergoing anti-leukemic therapy in the hospital environment (2). No one has isolated organisms from cancer patients undergoing various chemotherapeutic drug regimens and determined the changes in overall sensitivity to these drugs compared with the corresponding laboratory cultures. In an attempt to understand the dominance of *Pseudomonas* in many infections occurring in cancer (particularly leukemia) and transplant patients, in vitro drug sensitivity studies were conducted on four or five of the most commonly isolated gram-negative strains.

Several chemotherapeutic agents used in the treatment of leukemia, cytosine arabinoside, cyclophosphamide, methotrexate, and 6-mercaptopurine, were used in these studies. They are usually administered parenterally. However, the latter three are sometimes given orally. The dosages (per meter² of body surface) in the course of treatment would be as follows: cytosine arabinoside, 200 mg per day for 5 days; cyclophosphamide, 800 mg every 2 weeks; methotrexate, 30 mg two times weekly; 6-mercaptopurine, 90 mg per day. An adult has between 1 and 2 m² of body surface. The actual levels in the bowel lumen after the oral administration of drugs have not been determined. They would probably be high initially. Since an effect can be observed within 4 hr (Fig. 5), our highest experimental concentration of 1,000 µg/ml probably is representative of an initial concentration in the bowel lumen of a drug such as cyclophosphamide if 0.8 to 1.6 g was administered orally. It would be too high for tissue levels, except possibly for a brief initial period.

In a comparison of differences, methotrexate (Fig. 2) appeared to be the most toxic of the compounds. Cytosine arabinoside (Fig. 1) was slightly more toxic than 6-mercaptopurine (Fig. 3) and cyclophosphamide (Fig. 4), but not significantly so. The latter two drugs had the least effect on viability.

Many differences were noted in the response of the organisms to these chemotherapeutic agents. The minimal activity of cyclophosphamide probably was caused either by inability of the bacteria to convert it to the active metabolite or by the ability of the bacteria to detoxify this compound if it was formed. The clinical strains of *Proteus* were much more resistant to cyclophosphamide and 6-mercaptopurine than were the stock cul-

tures, whereas both types were susceptible to methotrexate and cytosine arabinoside. Again, the clinical isolates were more resistant than the stock cultures in each case. The differential resistance of these organisms suggests that the type of drug used in therapy might influence, in part, the microbial population which may infect the patient.

Pseudomonas strains were by far the most resistant of all the organisms studied. In fact, cytosine arabinoside and 6-mercaptopurine perhaps even slightly enhanced the growth of these organisms. This slight increase in numbers was observed in every experiment with these two drugs, but not with cyclophosphamide or methotrexate. This latter drug, the most toxic at the lower levels for the other organisms (including *Serratia*), had no effect at all on either the clinical isolates or the laboratory stocks of *Pseudomonas*.

Bacteria are usually more susceptible to physical and chemical agents during their early period of growth. Therefore, the clinical strains were grown in broth containing either 500 or 1,000 µg of methotrexate per ml, the most toxic of the compounds tested. Samples were removed at various intervals both to assess the effect of exposure time on survival and to observe possible periods of maximum susceptibility. As can be seen in Fig. 5, *P. aeruginosa* was not affected at any point during a 24-hr period whereas *E. coli* was particularly susceptible at 4 hr. The majority of the cells were killed at this point, and the curve remained essentially level thereafter. The other organisms were similar to *E. coli* in their reactions. The lower drug concentration, as expected, was less toxic. Both a greater survival and a slightly delayed period of maximum susceptibility were observed in all cases.

The data from the mixed-culture experiments (Table 1) deserve mention for several reasons. *Pseudomonas* did not exert a "sparing" or "synergistic" effect on *Escherichia* when these organisms were simultaneously exposed to methotrexate or cytosine arabinoside in mixed culture. Similar results were obtained with mixed cultures of *Pseudomonas* and *Serratia*. Thus, *Pseudomonas* easily dominated the final population in both cases. In addition, the toxicity of cyclophosphamide for *Escherichia* was enhanced under these conditions; survival decreased 26% from that observed in pure culture. This fact is as yet unexplained. It is also important to note that the mixed-culture environment did not particularly increase the susceptibility of *Pseudomonas* to any of the drugs so tested.

Therefore, *Pseudomonas* can not only survive but can also dominate a mixed population by virtue of a high degree of drug resistance both to

most antibiotics and now to several of these chemotherapeutic agents. In addition, it did not exert a "sparing" effect and even enhanced drug toxicity for other organisms under certain conditions. Thus, these studies may have helped to elucidate some of the reasons for the dominance of this opportunistic organism in the debilitated cancer or transplant patient on certain drug regimens.

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