Effects of Antimicrobial Agents on Survival of *Mycobacterium avium* Complex Inside Alveolar Macrophages Obtained from Patients with Human Immunodeficiency Virus Infection

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Measurements of the activities of antimicrobial agents against the *Mycobacterium avium* complex (MAC) usually do not take into consideration the intracellular location of the organism. A recent study using mouse macrophage continuous cell line J774 (D. M. Yajko, P. S. Nassos, C. A. Sanders, and W. K. Hadley, Am. Rev. Respir. Dis. 140:1198–1203, 1989) showed that certain combinations of antimicrobial agents are able to kill MAC inside macrophages and suggested that the J774 cell line could be used as a model for screening of drugs for intracellular activity against MAC. As a test of the validity of this model, alveolar macrophages were isolated from the bronchoalveolar lavages of 36 patients who had AIDS or an AIDS-related condition or were considered to be at risk for AIDS. The macrophages were infected with MAC and then treated with a drug or drug combination for 48 to 72 h. Survival of MAC was measured over time in drug-treated macrophages and untreated control macrophages. No single drug or two-drug combination that was tested was able to cause a decrease in the survival of every one of the MAC strains used in the study. However, several three-drug combinations that had been shown to cause a decrease in survival of all MAC strains isolated in the study (6) and untreated control macrophages. The good agreement between these results and those obtained previously with J774 cells gives further evidence of the usefulness of the simpler J774 model for screening of drugs for intracellular activity against MAC.

We previously studied the in vitro susceptibility of the *Mycobacterium avium* complex (MAC) to antimicrobial agents by determining MICs and MBCs for MAC in broth cultures (13, 14). Because MAC was found to be resistant to most of the antimicrobial agents tested, combinations of drugs were screened for synergistic activity against this organism (12, 14). The results of these and other studies indicated that certain combinations of drugs can act synergistically to kill MAC in broth (3, 7, 12, 16). However, the location of MAC inside macrophages of the host cellular defense system adds another level of complexity to antimicrobial susceptibility testing of this organism, since some drugs penetrate the eukaryotic cell membrane poorly while others are concentrated inside eukaryotic cells (2, 10). To address these considerations, we tested the abilities of antimicrobial agents, either singly or in combination, to kill MAC inside mouse macrophage continuous line J774 cells (15). The results of these experiments gave evidence of the activities of certain drug combinations against intracellular MAC and suggested a potential therapeutic value for these agents in treating MAC infections (15).

Disseminated infection due to MAC is seen most frequently in patients with AIDS. Monocytes-macrophages from these patients have been shown to harbor human immunodeficiency virus type 1 (HIV-1; 1). Although macrophages from patients with AIDS are able to phagocytize MAC efficiently (11), the effect of HIV infection on the uptake and activity of antimicrobial agents against MAC in macrophages is not known. The clearest in vitro evidence for the potential therapeutic value of antimicrobial agents against MAC infection in patients with HIV infection would come from experiments using macrophages obtained from HIV-infected patients. Such experiments are reported here. The results of these experiments are in agreement with earlier results obtained by using J774 cells (15) and give evidence that mouse macrophage continuous cell line J774 is a valid alternative to the use of alveolar macrophages from patients with AIDS to screen antimicrobial agents for intracellular activity against MAC.

MATERIALS AND METHODS

**Bacteria.** All 15 MAC strains used in these experiments were isolated from patients with AIDS. Growth of bacteria prior to infection of macrophages was as previously described (14).

**Antimicrobial agents.** The antimicrobial agents tested were chosen because they showed activity against MAC inside J774 cells (15) or because they have been suggested for use in treatment of MAC or *M. tuberculosis* infection (7, 9, 15, 16). They included clofazimine (1 μg/ml), ethambutol (EMB; 4 μg/ml), rifampin (RIF; 8 μg/ml), rifabutin (RTB [Ansamycin]; 0.5 μg/ml), ciprofloxacin (CIP; 2 μg/ml), amikacin (AMI; 32 μg/ml), and streptomycin (STR; 16 μg/ml). The concentration of each drug tested was based upon the achievable level of the drug in serum. Each drug or drug combination was tested in duplicate flasks.

**Alveolar macrophages.** Alveolar macrophages were obtained from bronchoalveolar lavages (BALs) of patients with AIDS or an AIDS-related condition (ARC) or considered to be at risk for AIDS. Specimens were obtained from 74 consecutive patients as part of a diagnostic procedure for detection of *Pneumocystis carinii*. Macrophages from 38 (51%) of the 74 patients were subsequently determined to be unusable because of fungal contamination (27 patients

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(36%) or bacterial contamination (5 patients [7%]) or because an insufficient number of macrophages remained adherent after the washing procedure (6 patients [8%]). The initial intent of the experiments was to test every strain against every drug and drug combination. However, since many of the macrophage preparations became unusable during the course of the experiments, a full complement of data is not available for every strain. The data presented in this report are thus randomly distributed with regard to the strains and drugs tested and do not reflect a decision to test certain strains against certain drugs or drug combinations.

Among the 36 patients whose macrophages were used in this study, 25 had AIDS (by Centers for Disease Control definition) at the time of the BAL or developed AIDS within 6 months of the BAL. 2 had ARC, 3 were HIV seropositive but did not have AIDS or ARC, 4 had HIV risk factors but their HIV status was unknown, and 2 had no risk factors for HIV infection listed in their records. The BAL specimens from 12 (33%) of the 36 patients contained P. carinii. All of the BAL specimens used in this report were also cultured for acid-fast bacteria and found to be negative.

**Macrophage treatment.**

(i) **Time** \( T = \) 1 day. Cells in the lavage fluids were sedimented and then suspended in 4 ml of medium M199 (containing Earle’s balanced salt solution, 20% newborn calf serum, and penicillin and gentamicin, each at 1 μg/ml). The concentration of macrophages was estimated by counting cells in a hemacytometer. The volume of the macrophage cell suspension was adjusted with M199 so that 5 ml of the suspension contained ca. \( 5 \times 10^{4} \) to \( 1 \times 10^{6} \) macrophages. MAC cells were then added at a ratio of approximately five MAC cells per macrophage. The actual number of CFU of MAC cells added was determined by plating dilutions of the MAC culture suspension onto 7H10 agar plates. Five-milliliter aliquots of the suspension containing macrophages and MAC cells were transferred to polystyrene tissue culture flasks (25 cm²), and the flasks were incubated overnight at 35°C in 5% CO₂ to allow macrophages to adhere to the flask and phagocytize MAC cells.

(ii) **T = 0.** Following overnight incubation, any nonadherent cells and unphagocytized bacteria were removed from the flasks by decanting the supernatant fluid. The adherent macrophages were washed with 5 ml of Hank's balanced salt solution (HBSS), and the HBSS was replaced with 5 ml of medium M199. Antimicrobial agents were then added, either singly or in combination, to all but four (control) flasks.

(iii) **T = 0 controls.** The numbers of adherent macrophages in two of the control flasks at \( T = 0 \) were determined by washing the adherent macrophages with 5 ml of Ca²⁺ and Mg²⁺-free HBSS, adding 3 ml of prewarmed (35°C) STV (0.05% trypsin, 0.02% EDTA in saline), immediately decanting the STV, and the incubating the flasks for 15 min at 35°C until the cells became unattached. Medium M199 (0.5 ml) was then added, and the number of macrophages in the suspension was calculated by counting the cells in a hemacytometer. After counting, the macrophages were lysed by addition of 5 ml of 0.25% sodium dodecyl sulfate and the MAC cells in the lysate were dispersed by vigorously pipetting the entire volume 10 times. The number of CFU of MAC in this \( T = 0 \) lysate was determined by plating duplicate 0.1-ml portions of dilutions of the lysate onto 7H10 agar plates. After 7 days of incubation at 35°C colonies were counted with a dissecting microscope (13). The number of CFU of MAC per macrophage (CFU MAC/MP) was then calculated.

(iv) **T = 48 to 72 h.** All remaining flasks were reinfused at 35°C in CO₂ for an additional 48 to 72 h. Trypan blue staining of macrophages showed that viability was >90% among macrophages that remained attached after 72 h. Incubation for longer than 72 h resulted in detachment and loss of significant numbers of macrophages. Macrophages in drug-treated flasks were washed with 5 ml of HBSS, the HBSS was decanted, and the macrophages were lysed with 5 ml of 0.25% sodium dodecyl sulfate. Portions of the lysate were plated, and the numbers of surviving CFU were determined as described above.

(v) **T = 48 to 72 h controls.** Macrophages in the two remaining untreated control flasks were detached and counted as described above for the \( T = 0 \) control flasks. Macrophages were washed, and the lysate was plated to determine the number of surviving CFU of MAC and the number of CFU MAC/MP in the 48- to 72-h untreated macrophages.

**Analysis of data.** The data were analyzed and plotted first by determining the total number of CFU of MAC in the macrophage lysates without consideration of the MAC-per-macrophage burden. Next, the numbers of CFU MAC/MP at the time of drug addition \( T = 0 \) and after incubation for 48 to 72 h were calculated. This was done to determine whether MAC counts in macrophages increased or decreased over time in the absence of antimicrobial agents. Survival of MAC in macrophages of drug-treated samples was then plotted as a percentage of the \( T = 0 \) control number of CFU MAC/MP. Data plotted in this manner appeared similar to a plot of the total number of CFU of MAC in macrophage lysates; this is the method of analysis presented here. A two-way analysis of variance was used to compare the activities of single drugs and two-drug combinations with those of three- and four-drug combinations. A paired \( t \) test was used to compare the activities of RIF versus RBT and AMI versus STR.

**RESULTS**

At \( T = 0 \), each macrophage contained an average of five MAC cells, a number that is in good agreement with the target number that was added to the flasks, indicating that virtually all of the MAC cells were phagocytized during the overnight incubation. All 15 of the MAC strains used in this study were able to grow inside alveolar macrophages, as evidenced by an increase in the number of CFU MAC/MP over time. MAC strains were unable to grow, or grew only poorly, in the cell culture medium without macrophages. In control flasks incubated without drugs for 48 to 72 h, MAC grew inside the alveolar macrophages from 30 (83%) of the 36 patients. The mean increase for all MAC strains in these patients’ macrophages was 280%. From this value, the mean generation time for MAC inside alveolar macrophages was estimated to be 29 h. Macrophages from 6 (17%) of the 36 patients appeared not to support the growth of MAC, as demonstrated by a decrease in the number of CFU MAC/MP over time. The mean survival of MAC in these macrophages after incubation was 68% of the \( T = 0 \) control value. This decrease could not be attributed solely to an effect of prior antimicrobial treatment, since only three of these six patients had received documented antimicrobial therapy at the time of the BAL (one patient received trimethoprim-sulfamethoxazole, one received sulfamethoxazole plus erythromycin, and the third received sulfamethoxazole, nafcillin, clindamycin, and tobramycin). When the BALs were obtained, the clinical status of the six patients whose macrophages did not support the growth of MAC was as follows: one patient had AIDS (diagnosed by the presence of \( P. \)
carinii in this BAL), one had ARC, and four did not have AIDS or ARC. Two of the latter four patients remained status quo during the next 6 months (one had no known risk factor for HIV infection), and two patients were diagnosed with AIDS within 6 months after the BAL was obtained.

For data analysis, patients were divided into two groups. Group 1 included the 30 patients whose macrophages permitted growth of MAC; group 2 included the 6 patients whose macrophages did not permit growth of MAC. For group 1 patients, survival of MAC after treatment with drugs was expressed as a percentage of the number of CFU MAC/MP in the $T = 0$ control. Since MAC counts in untreated macrophages decreased over time in macrophages from group 2 patients, survival of MAC in drug-treated macrophages from these patients was compared with the number of CFU MAC/MP present in the untreated controls at the end of the incubation period instead of the number present at $T = 0$. This was done to ensure that any decrease observed in the MAC-macrophage ratio was due to the drugs rather than killing by macrophages.

Figure 1 shows the survival of MAC inside drug-treated macrophages as a percentage of the control number of MAC per macrophage. Each point in a column represents a different MAC strain. Some MAC strains were tested against a particular drug or drug combination by using macrophages from several different patients. In these cases, the value shown is the mean survival for all experiments. Panel A shows survival of MAC in macrophages after treatment with a single drug or after treatment with various two-drug combinations. No single drug or two-drug combination was able to decrease the survival of all of the MAC strains tested. The apparent activity of CIP alone (causing decreased survival of five of six strains tested) was determined to be caused by its interaction with the gentamicin that was used to suppress the growth of non-acid-fast bacteria which contaminated the BAL specimens (data not shown). Gentamicin had no apparent effect on the survival of intracellular MAC when CIP was used in combination with the other drugs tested. In contrast to the results obtained with single drugs and two-drug combinations, all three- and four-drug combinations, that were tested (panel B) resulted in decreased survival of every MAC strain included in the study. Statistical analysis of the data in Fig. 1 showed that survival after treatment with the three- and four-drug combinations was significantly lower than that after treatment with single drugs and two-drug combinations ($P < 0.001$). An analysis of antimicrobial susceptibility patterns of MAC in macrophages from group 1 and 2 patients indicated that MAC inside macrophages from group 1 patients was as susceptible to the action of antimicrobial agents as was the MAC in macrophages from group 2 patients.

The mean survival of MAC strains treated with various three- and four-drug combinations is shown in Table 1. A comparison of the relative activities of RIF and RBT (Ansamycin) showed that survival of MAC in alveolar macrophages after treatment with RIF was slightly but consistently lower than that after treatment with RBT ($P < 0.02$), regardless of which other drugs were present in the combination. The mean survival of MAC after treatment with AMI was slightly lower than that after treatment with STR when it was tested in three- and four-drug combinations. This difference approached, but did not achieve, statistical significance ($P = 0.09$). The greatest decrease in MAC survival inside macrophages was achieved with the combination of EMB-RIF-CIP-AMI. Only 15% of the number of MAC CFU present in the macrophages at $T = 0$ survived treatment with this combination for 48 to 72 h.
TABLE 1. Comparison of effectiveness of combinations of antimicrobial agents against MAC inside alveolar macrophages from patients with HIV infection.

| Drug combination       | No. of strains tested | No. of patients | Mean MAC survival* |
|------------------------|-----------------------|-----------------|--------------------|
| EMB-RBT-CIP            | 5                     | 7               | 34                 |
| EMB-RIF-CIP            | 10                    | 20              | 31                 |
| EMB-RBT-AMI            | 6                     | 8               | 31                 |
| EMB-RIF-AMI            | 6                     | 7               | 22                 |
| EMB-RBT-STR            | 6                     | 7               | 38                 |
| EMB-RIF-STR            | 7                     | 8               | 29                 |
| EMB-RBT-CIP-STR        | 6                     | 7               | 24                 |
| EMB-RIF-CIP-STR        | 6                     | 7               | 19                 |
| EMB-RBT-STR-AMI        | 6                     | 6               | 20                 |
| EMB-RIF-STR-AMI        | 6                     | 6               | 15                 |

* Survival was determined after treatment with a single dose of drugs for 48 to 72 h and is expressed as a percentage of the control.

DISCUSSION

All 15 MAC strains that were tested in this study were able to grow inside alveolar macrophages. However, alveolar macrophages from only 83% of the patients allowed intracellular growth of MAC. This difference among macrophages from patients may be due to variability inherent in the experimental procedure, or it may reflect differences in macrophage activation. The number of patients whose alveolar macrophages caused a decrease in the MAC survival in the absence of antimicrobial agents (group 2) was too small to allow statistical comparison of their HIV condition with that of group 1 patients, but group 2 patients tended to be in the earlier stages of HIV disease. None of the macrophages used in our study were from patients who were known to be unaffected by HIV. Only 2 of the 36 patients had no risk factors for HIV infection listed in their records. Macrophages from one of these patients are among those of the six group 2 patients whose macrophages caused a decrease in MAC survival. Schnittman et al. (11) showed that during 14 days of incubation, MAC is able to grow inside monocyte-derived macrophages from control patients and in macrophages from patients with AIDS. Their study of the kinetics of MAC survival in normal macrophages showed an initial drop in MAC survival 1 to 2 days postinfection, followed by outgrowth of the organism. Although our results obtained with alveolar macrophages from group 1 patients did not demonstrate a decrease in MAC survival 48 to 72 h posttreatment, it is possible that the decrease observed in macrophages from group 2 patients is part of this phenomenon. Unfortunately, under the conditions of our experiments, most of the alveolar macrophages remained adherent for only 4 to 5 days, so it was not possible to determine whether a biphasic pattern of MAC survival occurred in macrophages from group 2 patients. Further studies are needed to determine the in vitro conditions under which MAC is killed inside alveolar macrophages from uninfected control patients.

The antimicrobial susceptibility data obtained here by using alveolar macrophages from patients are in good agreement with data obtained by using mouse macrophage continuous cell line J774 (15), although direct comparison of results is not possible because of differences in the experimental procedures. In our experiments, clofazimine, used alone, showed no appreciable killing activity against MAC inside either human alveolar macrophages or mouse J774 cells (15). Clofazimine also did not act synergistically in combination with EMB, CIP, and RBT in broth culture experiments (12). Its utility in treating disseminated MAC infection is therefore questionable. As previously found with J774 cells (15), two-drug combinations that included EMB were more effective in decreasing MAC survival than were single drugs, but a combination of at least three drugs was needed to achieve a decrease in survival of every MAC strain. The most potent drug combinations that were tested included EMB, RIF, and CIP, which caused a decrease in survival of 10 of 12 MAC strains inside J774 cells and all of 10 strains tested inside human alveolar macrophages, and EMB, AMI, and RIF (or RBT), which caused decreased survival of all 6 MAC strains tested in either J774 cells (15) or alveolar macrophages. Studies done in several different laboratories have shown that EMB can act synergistically with other antimicrobial agents against MAC (1, 3–7, 9, 11, 15, 16). EMB appears to be the critical component in antimicrobial combinations for achieving synergism against MAC (8). The data presented here suggest that a regimen that includes EMB, CIP, and RIF may have a therapeutic benefit in the treatment of MAC infection in patients with AIDS. It is worth noting, however, that none of the drug combinations tested caused killing of every MAC cell inside macrophages. Whether this is due, for example, to the relatively short period of treatment (48 to 72 h) or reflects a lack of therapeutic efficacy remains to be determined. We emphasize that in vitro data such as those presented here should not be interpreted to mean that a therapeutic benefit has been demonstrated. The data serve only as a basis for the possibility of conducting clinical trials to test the benefit of these drugs.

The good agreement between results obtained by using mouse macrophage continuous cell line J774 and alveolar macrophages from HIV-infected patients is evidence that J774 cells can be used to screen antimicrobial agents for intracellular activity against MAC. These results are significant because of the complexity of experiments which use human alveolar macrophages to measure antimicrobial susceptibility. Some of the problems associated with the use of human alveolar macrophages include (i) the relative scarcity of BAL specimens available for testing, (ii) the presence of other (non-acid-fast) bacteria in the BAL specimen with the concomitant need to incorporate other bacteria to inhibit their growth, (iii) the relatively short time (4 to 5 days) for which alveolar macrophages remain viable and adherent in vitro, and (iv) the apparent variability among alveolar macrophages from different patients in the ability to support MAC growth. Such problems can be overcome by use of the J774 continuous cell line. In addition, use of J774 cells gives more laboratories the ability to perform intracellular antimicrobial susceptibility tests of MAC isolates. This, in turn, should aid in the development of effective therapy for MAC infections.

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