ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1991, p. 135–140
0066-4804/91/010135-06$02.00/0

Vol. 35, No. 1

Killing of Intracellular *Mycobacterium tuberculosis* by Receptor-Mediated Drug Delivery†

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Received 3 August 1990/Accepted 1 November 1990

***p*-Aminosalicylic acid (PAS) conjugated to maleylated bovine serum albumin (MBSA) was taken up efficiently through high-affinity MBSA-binding sites on macrophages. Binding of the radiolabeled conjugate to cultured mouse peritoneal macrophages at 4°C was competed for by MBSA but not by PAS. At 37°C, the radiolabeled conjugate was rapidly degraded by the macrophages, leading to release of acid-soluble degradation products in the medium. The drug conjugate was nearly 100 times as effective as free PAS in killing the intracellular mycobacteria in mouse peritoneal macrophages infected in culture with *Mycobacterium tuberculosis*. The killing of intracellular mycobacteria mediated by the drug conjugate was effectively prevented by simultaneous addition of excess MBSA (100 μg/ml) or chloroquine (3 μM) to the medium, whereas these agents did not affect the microbicidal action of free PAS. These results suggest that (i) uptake of the PAS-MBSA conjugate was mediated by cell surface receptors on macrophages which recognize MBSA and (ii) lysosomal hydrolysis of the internalized conjugate resulted in intracellular release of a pharmacologically active form of the drug, which led to selective killing of the *M. tuberculosis* harbored by mouse macrophages infected in culture. This receptor-mediated modality of delivering drugs to macrophages could contribute to greater therapeutic efficacy and minimization of toxic side effects in the management of tuberculosis and other intracellular mycobacterial infections.**

Mycobacterium tuberculosis owes its pathogenicity to its ability to survive and proliferate within mononuclear phagocytes (5). It is estimated that every year about 10 million persons develop tuberculosis and at least 3 million die from the disease (16). The most important factor in the treatment of tuberculosis is prolonged chemotherapy, which is often associated with serious unwanted side effects (7, 24). In the chemotherapy of such intracellular pathogens, it is necessary to achieve relatively high levels of the drug in blood to attain therapeutically effective concentrations in infected cells, which presumably leads to adverse effects on healthy tissues. The present challenge in the therapy of intracellular infections, therefore, is to develop systems capable of delivering the drugs to the intracellular sites of action at relatively low levels in blood (reviewed in reference 6). Multilamellar liposomes are being explored for intracellular delivery of antimicrobial drugs (1, 4, 20) because of their preferential uptake by reticuloendothelial cells with various degrees of success. Recently, we have developed an alternative approach for selective intracellular delivery of drugs to macrophages (3, 15) by using endocytosis of macromolecular drug conjugates mediated presumably by scavenger receptors (2, 10, 17) present only on macrophages (and, to a lesser extent, on some endothelial cells). We have shown that effective chemotherapy of experimental leishmaniasis in hamsters can be achieved by homing an antileishmanial drug, methotrexate, to macrophages by using maleylated bovine serum albumin (MBSA) as the carrier to which the drug was chemically conjugated (3, 15). In this study, we investigated the possibility of using MBSA as a vehicle for intracellular delivery of an antimycobacterial drug, *p*-aminosalicylic acid (PAS), to cultured mouse peritoneal macrophages infected with *M. tuberculosis*. We chose PAS as the targeted drug to determine whether the receptor-mediated modality of uptake of the drug reported here is likely to be effective at lower dosages so that the toxic side reactions of this potent antimycobacterial agent, and the drug of choice until the 1970s, could be reduced to currently acceptable levels (14).

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† Communication IMT-003/90 from the Institute of Microbial Technology.

MATERIALS AND METHODS

Organism. *M. tuberculosis* H37Ra obtained from N. K. Ganguly, Postgraduate Institute of Medical Education and Research, Chandigarh, India, was cultivated at 37°C for 7 days in Middlebrook 7H9 medium (Difco) containing Tween 80 and ADC enrichment. Bacteria were harvested in the exponential growth phase. CFU were counted by serial dilution on 7H10 agar plates (18). Cells were preserved in 1-ml aliquots at −70°C, thawed just before use, and subjected to mild sonication to get single-cell suspensions.

Isolation of mouse peritoneal macrophages. Five-milliliter volumes of heparinized phosphate-buffered saline (PBS) were injected into the peritoneal cavities of BALB/c mice weighing 30 to 40 g. The peritoneal fluids from 15 mice were pooled, and the cells were collected by centrifugation (250 × *g*, 10 min, room temperature). Cells were suspended in medium A (RPMI 1640 medium [GIBCO] containing 10% heat-inactivated fetal calf serum, 2 g of sodium bicarbonate per liter, 2 mM glutamine, and 100 U of penicillin G per ml). One-milliliter aliquots of the cell suspension (2×10^6 cells per ml) were dispensed into six-well tissue culture clusters (Costar, Cambridge, Mass.) containing 1 ml of medium A and incubated at 37°C for 1 h in a humidified incubator containing 5% CO_2. Nonadherent cells were removed by washing three times with sterile PBS. The monolayers were incubated for 24 h in 2 ml of fresh medium A at 37°C. These cells were used for further experiments.

Preparation of drug conjugate. Bovine serum albumin

(Sigma Chemical Co., St. Louis, Mo.) was maleylated at pH 8.5 by using maleic anhydride (Sigma) as previously described (11). The reaction mixture was dialyzed for 24 h against 0.1 M ammonium bicarbonate solution and for 48 h against glass-distilled water at 4°C, lyophilized, and stored at −20°C.

To prepare the drug conjugate, MBSA was allowed to react with the sodium salt of PAS (Sigma) at pH 7.4 in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) as previously described (8). Briefly, three reactants, MBSA, PAS, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were mixed at a molar ratio of 1:60:60, respectively, in 3 ml of PBS and allowed to react for 5 h at room temperature with constant shaking. The conjugate was separated from other low-molecular-weight components by Sephadex G-50 gel filtration, dialyzed against PBS, and stored frozen at −20°C in small aliquots.

The amount of PAS bound to MBSA was determined by fluorescence measurement in a KONTRON SFM-25 spectrofluorometer with 310 nm as the excitation wavelength and 405 nm as the emission wavelength. PAS showed a characteristic fluorescence spectrum at this range (22), but MBSA did not (data not shown). Protein concentration was determined by the biuret method. The molar ratio of PAS to MBSA was 33:1.

Radioiodination of PAS-MBSA conjugate. The PAS-MBSA conjugate was radioiodinated with $Na^{125}I$ (Bhabha Atomic Research Centre, Bombay, India) by the iodine monochloride method as previously described (9). The specific activity of the labeled product was about 600 cpm/ng of protein.

Binding of [^{125}I]PAS-MBSA to peritoneal macrophages at 4°C. Macrophage monolayers prepared in six-well tissue culture clusters as described earlier were washed twice with ice-cold PBS. Each well received 1 ml of medium B (RPMI 1640 medium without bicarbonate and fetal calf serum but containing 1 mg of bovine serum albumin per ml) and the concentrations of [^{125}I]PAS-MBSA indicated (see Fig. 1). The clusters were incubated at 4°C with gentle shaking. After 2 h, the monolayers were washed three times with ice-cold PBS containing bovine serum albumin (1 mg/ml) and once with PBS. The cells were dissolved in 1 ml of 0.1 N NaOH, and radioactivity was measured by placing an aliquot in a gamma counter. Cellular protein content was measured by using alkaline copper reagent as previously described (9).

For competition experiments, each monolayer received 10 μg of [^{125}I]PAS-MBSA per ml with or without the concentrations of free PAS or free MBSA indicated (see Fig. 2) and was processed as above.

Assay of uptake and degradation of [^{125}I]PAS-MBSA conjugate by peritoneal macrophages. Macrophage monolayers prepared in six-well tissue culture clusters as described earlier were washed twice with warm (37°C) PBS. Each well received 1 ml of medium C (medium B with 2 g of sodium bicarbonate per liter) containing the concentrations of [^{125}I]PAS-MBSA indicated (see Fig. 3 and 4), and the clusters were incubated at 37°C in a humidified incubator containing 5% CO_2 for 5 h or various other periods. The amounts of trichloroacetic acid-soluble degradation products released in the medium were estimated as previously described (9). The monolayers were washed, and cell-associated radioactivity and protein content were determined as described above.

Infection of macrophage monolayers with *M. tuberculosis*. Monolayers prepared as described above were washed twice with PBS and incubated in a humidified incubator containing 5% CO_2 with 2 ml of RPMI containing 1% fetal calf serum. After 1 h, 1 ml of medium was withdrawn from each well and replaced with 1 ml of a suspension of *M. tuberculosis* H37Ra (10^7 cells per ml) grown as described earlier. After incubation at 37°C for 4 h, the monolayers were washed twice with PBS to remove extracellular bacteria. Ziehl-Neelsen staining of the monolayers showed the presence of 9 to 12 bacteria per macrophage. Each monolayer then received 2 ml of medium A and was incubated further at 37°C for 24 h. Infected monolayers thus prepared were used to determine the effects of drugs.

Drug action against intracellular growth of phagocytosed mycobacteria. The concentrations of free PAS or PAS-MBSA indicated (see Fig. 5) were added to each infected monolayer prepared as described above, and incubation was continued for 4 h at 37°C. Monolayers were washed three times with PBS and incubated for 48 h in drug-free medium A. The macrophages were then lysed by adding 2.5% (wt/vol) sodium dodecyl sulfate (SDS), and appropriate dilutions were plated on Middlebrook 7H10 agar as previously described (18). CFU were counted after 3 weeks of incubation at 37°C. SDS did not affect the viability or growth of bacteria, as shown by control experiments (data not shown).

For determination of the time course of killing of *M. tuberculosis* H37Ra by PAS-MBSA conjugate or free PAS, infected monolayers (prepared as described earlier) were incubated with 2 μg of PAS per ml in free or conjugated form in medium A for various periods, washed, and incubated for up to 48 h at 37°C in drug-free medium A. The cells were lysed with SDS, and CFU were counted as described earlier.

RESULTS

Binding of [^{125}I]PAS-MBSA by peritoneal macrophages. To determine the nature of the interaction of PAS-MBSA with peritoneal macrophages, macrophage monolayers were incubated at 4°C with increasing concentrations of the labeled conjugate for 2 h, and after thorough washing, the radioactivity associated with the cells was measured. The experiments were done at 4°C to minimize the endocytic activities of the macrophages so that the cell-associated radioactivity largely reflects the binding of the ligand to discrete sites on the cell surface. Binding of [^{125}I]PAS-MBSA to the cells followed saturation kinetics, indicating a limited number of binding sites for the ligand (Fig. 1). Half-maximal binding occurred at a concentration of about 1 μg of the labeled conjugate per ml. Binding of radiolabeled PAS-MBSA was inhibited by free MBSA (half-maximal inhibition occurred at about 10 to 12 μg of MBSA per ml), but free PAS did not affect binding of the drug conjugate (Fig. 2). Addition of unlabeled PAS-MBSA to the medium eliminated binding of [^{125}I]PAS-MBSA to the macrophages as effectively as did MBSA (data not shown).

Uptake and degradation of [^{125}I]PAS-MBSA. When macrophages were incubated at 37°C with increasing concentrations of [^{125}I]PAS-MBSA, the cellular content of [^{125}I]PAS-MBSA, as well as trichloroacetic acid-soluble radioactivity released in the medium, increased in a saturable fashion (Fig. 3). Half-maximal saturation for uptake, as well as degradation, of radiolabeled PAS-MBSA was achieved at a conjugate concentration of about 6 μg of protein per ml.

The data in Fig. 4 show the time course of uptake and degradation of [^{125}I]PAS-MBSA by the macrophages. When macrophages were incubated with [^{125}I]PAS-MBSA at 37°C for various times, cellular radioactivity reached a steady-state plateau after about 2 h but acid-soluble radioactivity continued to appear in the medium linearly, reflecting con-

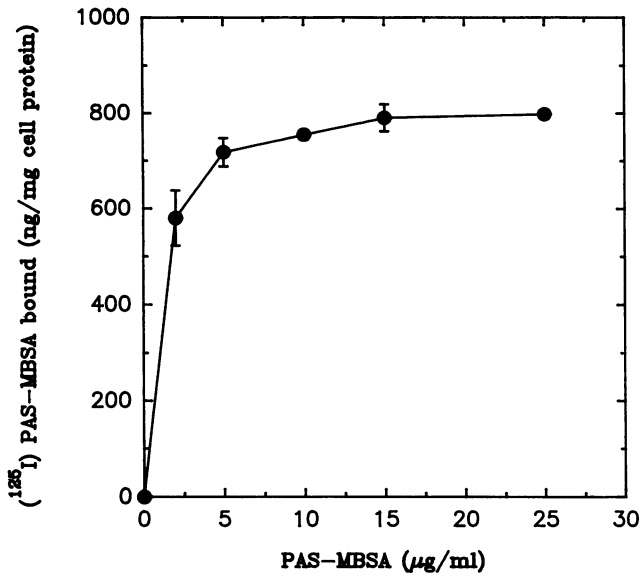

FIG. 1. Binding of [^{125}I]PAS-MBSA to mouse peritoneal macrophages at 4°C. Each monolayer received 1 ml of ice-cold medium B containing the indicated concentrations of [^{125}I]PAS-MBSA. After 2 h at 4°C, the monolayers were washed and the amount of [^{125}I]PAS-MBSA bound to the cells was determined as described in Materials and Methods. The results shown are means ± standard deviations for three independent experiments.

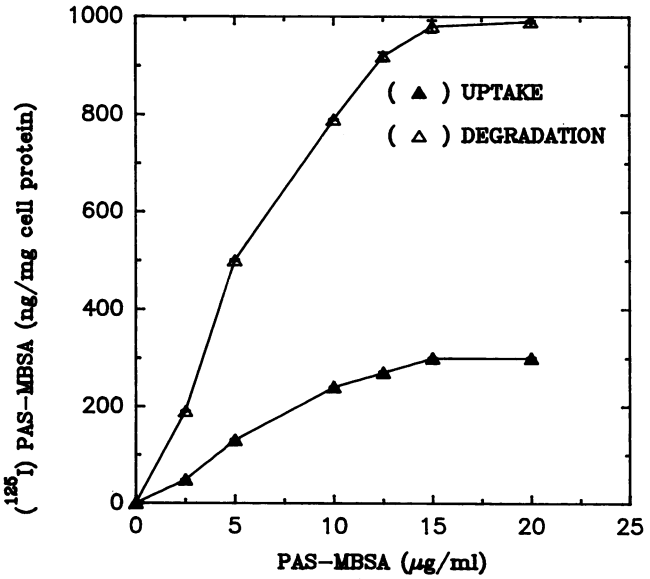

FIG. 3. Concentration dependence of uptake and degradation of [^{125}I]PAS-MBSA by mouse peritoneal macrophages at 37°C. Each monolayer received 1 ml of medium C containing the indicated concentrations of [^{125}I]PAS-MBSA. After incubation at 37°C for 5 h, the amount of [^{125}I]PAS-MBSA accumulated in the cells and the ^{125}I-labeled trichloroacetic acid-soluble degradation products released in the medium were determined as described in Materials and Methods. The results shown are means ± standard deviations for three independent experiments.

tinuing uptake and degradation of the conjugate. After about 4 h, the degradation process approached a steady state. At this time, about six times as much [^{125}I]PAS-MBSA was degraded as was contained within the cells.

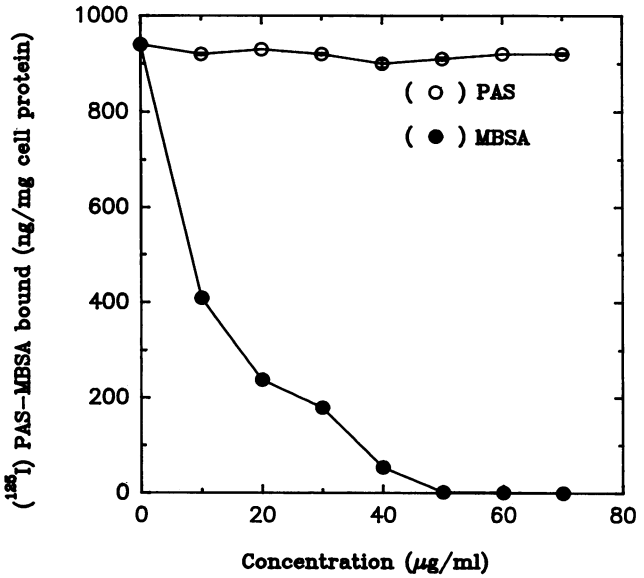

FIG. 2. Effects of MBSA and PAS on [^{125}I]PAS-MBSA binding by mouse peritoneal macrophages at 4°C. Each monolayer received 1 ml of ice-cold medium B containing 10 μg of [^{125}I]PAS-MBSA per ml and the indicated concentrations of either MBSA or PAS. After incubation for 2 h at 4°C, the monolayers were washed and the amount of [^{125}I]PAS-MBSA bound to the cells was determined as described in Materials and Methods. The results shown are means ± standard deviations for three independent experiments.

Killing of intracellular *M. tuberculosis* by free PAS and PAS-MBSA conjugate. The data in Fig. 5 show the comparative efficacies of free PAS and PAS-MBSA conjugate in killing *M. tuberculosis* H37Ra harbored by cultured peritoneal macrophages. During a 4-h exposure period, PAS (5 μg/ml) in the conjugated form killed 80% of the intracellular mycobacteria. In contrast, an equivalent amount of free PAS under the same conditions eliminated only 12.5% of the mycobacteria. The superior antimycobacterial activity of PAS-MBSA was more apparent at lower concentrations. Thus, 50% of the intracellular bacteria were eliminated at a concentration of 0.2 μg of PAS per ml presented as the conjugate, whereas the free form killed only 0.5% of the bacteria.

When infected macrophages were incubated for various periods with PAS (2 μg/ml) in the conjugated form, about 55% of bacteria were eliminated within 3 h of exposure. In contrast, exposure to the same concentration of the free drug for the same duration killed only 10% of the bacilli (Fig. 6). Exposure to PAS or PAS-MBSA did not affect the viability of macrophages, as determined by the criterion of trypan blue exclusion (data not shown).

The antimycobacterial activity of the drug conjugate was suppressed by an inhibitor of lysosomal functions, chloroquine, or an excess of MBSA, but neither chloroquine nor MBSA had any effect on the antibacterial activity of free PAS (Table 1). These data show that uptake by macrophages, followed by lysosomal degradation of the drug conjugate, was necessary to elicit the antimycobacterial activity of the drug conjugate. Addition of PAS-MBSA to an axenic culture of *M. tuberculosis* did not affect the growth of the mycobacteria (data not shown).

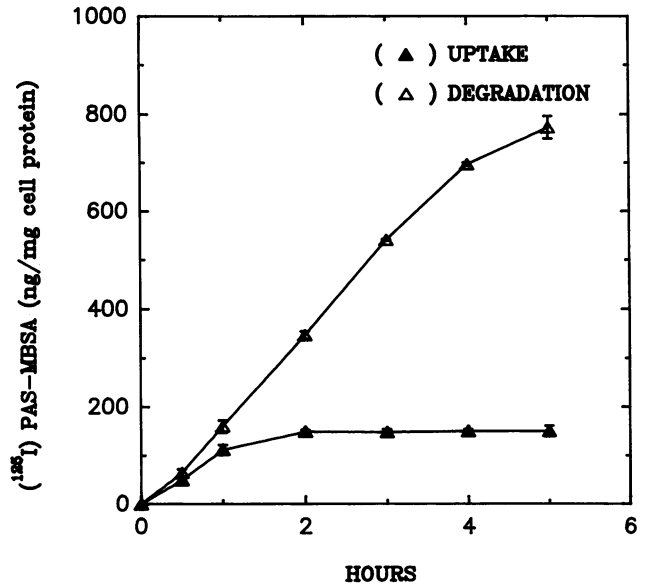

FIG. 4. Time course of uptake and degradation of [^{125}I]PAS-MBSA by macrophages at 37°C. Each monolayer received 1 ml of medium containing 4 μg of [^{125}I]PAS-MBSA per ml and was incubated at 37°C. At the indicated times, the amount of ^{125}I-labeled trichloroacetic acid-soluble material in the medium and the amount of [^{125}I]PAS-MBSA in the cells were determined as described in Materials and Methods. The results shown are means ± standard deviations for three independent experiments.

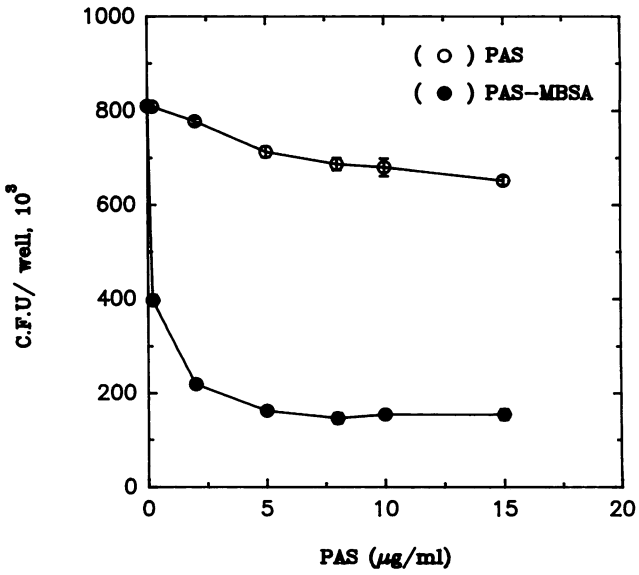

FIG. 5. Comparison of the antimycobacterial actions of PAS and PAS-MBSA in cultured mouse peritoneal macrophages. Macrophage monolayers were infected with *M. tuberculosis* as described in Materials and Methods. Each infected monolayer containing 2 ml of medium A received the indicated concentrations of PAS in the free or conjugated form and were incubated at 37°C for 4 h. The monolayers were then washed and incubated in 2 ml of drug-free medium A. After 48 h, the macrophages were lysed and the number of CFU of *M. tuberculosis* released in the lysate was determined as described in Materials and Methods. The results shown are means ± standard deviations for five independent experiments.

DISCUSSION

Macrophages from different organs of a variety of animals express high-affinity binding sites which recognize a number of structurally disparate polyanionic macromolecules like acetylated low-density lipoprotein, fucoidin, polyinosinic acid, and MBSA. Binding is followed by internalization and lysosomal degradation of the protein ligands (2, 10). The determinants for recognition and uptake of such polyanionic macromolecules, including MBSA, are present primarily on macrophages (2, 3, 10, 17) and are referred to generically as scavenger receptors which may comprise several distinct molecular species (13, 19, 23).

This receptor system is beginning to find applications in drug delivery research. Thus, previous work from our laboratory has shown the utility of methotrexate coupled to MBSA for elimination of intracellular amastigotes of *Leishmania donovani* and *L. mexicana amazonensis* in macrophages (3, 15). Similar results have been obtained by using acetylated low-density lipoprotein as the vehicle for delivering antileishmanial agents (12). Also, acetylated low-density lipoprotein-containing lipophilic muramyl tripeptide has been shown to be delivered to macrophages, resulting in enhancement of their tumoricidal activity (21). In this study, we used MBSA as a homing device to deliver an antimycobacterial agent, PAS, selectively to macrophages with the expectation that uptake, followed by lysosomal degradation of the PAS-MBSA conjugate, would lead to intracellular release of a pharmacologically active form of the drug, as in our previous studies with conjugates of methotrexate with MBSA.

We conjugated PAS with MBSA by using a water-soluble carbodiimide and determined whether the drug conjugate binds to macrophages through the receptor which recognizes MBSA. [^{125}I]PAS-MBSA conjugate bound to macrophages in a saturable fashion at 4°C (Fig. 1), indicating the presence of a limited number of binding sites on the cell surface. Binding of [^{125}I]PAS-MBSA was inhibited by free MBSA but not by free PAS (Fig. 2), indicating that the drug conjugate was recognized by MBSA receptors. At 37°C, the radiolabeled drug conjugate was efficiently taken up and degraded by mouse peritoneal macrophages with saturation kinetics (Fig. 3 and 4). Both MBSA and unlabeled PAS-MBSA effectively competed for uptake and degradation of [^{125}I]PAS-MBSA (data not shown). Conjugation of PAS with MBSA significantly increased the antimycobacterial efficacy of the drug. The conjugated drug at low concentrations was nearly 100-fold as effective as the free drug (Fig. 5). Over a 5-h period, an average of about 17% of the bacteria were eliminated per h by the PAS-MBSA conjugate (2 μg of PAS per ml) but only 2.5% of the bacilli were killed per h by the same concentration of free PAS (Fig. 6). Active lysosomal functions are necessary to elicit the antibacterial activity of the drug conjugate. The presence in the medium of 3 μM chloroquine, an inhibitor of lysosomal functions, largely abolished the microbicidal activity of the drug conjugate, indicating that lysosomal hydrolysis of the drug conjugate is necessary for intracellular release of the drug (Table 1). In separate experiments, we found that chloroquine (3 μM) effectively suppressed [^{125}I]PAS-MBSA degradation (data not shown). Chloroquine did not significantly affect the antimycobacterial action of free PAS (Table 1). An excess of MBSA in the medium effectively abolished the antimycobacterial action of PAS-MBSA but not that of free PAS. These results, taken together, indicate that uptake of PAS-MBSA through the MBSA receptors on macrophages, followed by lysosomal degradation of the conjugate, led to intracellular

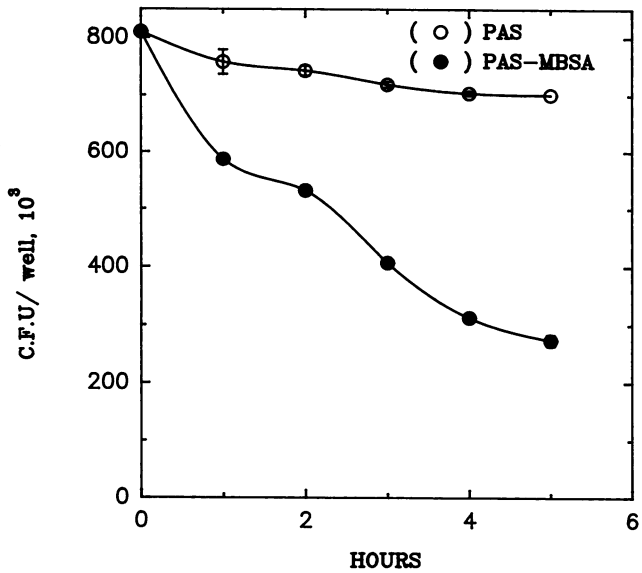

FIG. 6. Time course of killing of *M. tuberculosis* in cultured mouse peritoneal macrophages by PAS or PAS-MBSA. Each infected monolayer containing 2 ml of medium A received 2 μg of PAS per ml in the free or conjugated form. After incubation at 37°C for the indicated interval, the monolayers were washed and the media were replaced with 2 ml of drug-free medium A. After 48 h, the macrophages were lysed and the number of CFU of *M. tuberculosis* released in the lysate was determined as described in Materials and Methods. The number of CFU in the untreated control group of infected macrophages did not change significantly over the 5-h period of the drug pulse. The results shown are means ± standard deviations of three independent experiments.

release of a pharmacologically active form of the drug, which effectively eliminated the mycobacteria residing in the infected macrophages.

Thus, we have shown that it is possible to increase the antimycobacterial efficacy of PAS through conjugation of the drug to MBSA. We have also shown that the polyanion receptor system known to be present primarily on cells of macrophage lineage (2, 10, 17) mediates delivery of the conjugate to the macrophages. The efficiency of this receptor system appears to be primarily responsible for an increase in the effective concentration of the antitubercular agent within macrophages. Inasmuch as macrophages are the primary cell type in which various pathogenic mycobacteria reside and proliferate, the receptor-mediated mode of drug delivery to macrophages described here merits serious consideration. Further studies with different antimycobacterial drugs and animal models, currently under way, will establish the value of our approach for selective drug delivery in the chemotherapy of tuberculosis and other diseases caused by mycobacteria.

TABLE 1. Inhibition of antimycobacterial activity of PAS-MBSA by MBSA and chloroquine[a]

Addition (concn)	No. of CFU/well (10^3)		
	Untreated control	PAS	PAS-MBSA
None	921	507	276
MBSA (100 μg/ml)	923	479	820
Chloroquine (3 μM)	875	415	847

[a] Macrophage monolayers infected with *M. tuberculosis* were incubated at 37°C with medium alone, MBSA, or chloroquine. After 2 h, PAS (5 μg/ml) was added in free or conjugated form and incubation was continued for 48 h. Monolayers were then washed twice with PBS, and the macrophages were lysed with SDS for determination of the number of CFU of *M. tuberculosis* per well as described in Materials and Methods. The results shown are means of three independent experiments.

ACKNOWLEDGMENTS

This work was supported by grants from the Council of Scientific & Industrial Research, India. S.M. was supported by a research fellowship from C.S.I.R.

We thank R. K. Srivastava for skilled technical assistance and A. Mukhopadhyay and S. Srimal for helpful discussions.

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