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Intracellular Accumulation of Azithromycin by Cultured Human Fibroblasts

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Azithromycin was shown to achieve high concentrations in human skin fibroblasts. Intracellular penetration occurred rapidly (10 μg/mg of cellular protein after 3 h) and then increased progressively over a 3-day period; azithromycin accumulated up to 21 times more than erythromycin (61.1 versus 2.9 μg/mg of protein). Uptake was dependent on the extracellular concentration, was inhibited at 4°C, did not occur in nonviable cells, and was reduced by a low pH. Intracellular accumulation was not affected by the metabolic inhibitor 2,4-dinitrophenol or sodium fluoride or by the nucleoside transport inhibitor 2-chloroadenosine. Once concentrated in cells, azithromycin remained intracellular and was released slowly in the absence of extracellular drug, compared with erythromycin (17 versus 78% released after 1 h). After 48 h of incubation in drug-free medium, 27% of the initial amount of azithromycin remained cell associated. The release of azithromycin was not affected by various monokines reported to stimulate fibroblasts (interleukin-1 or tumor necrosis factor) or by exposure to bacteria. Incubation of azithromycin-loaded fibroblasts with human polymorphonuclear leukocytes resulted in a higher intracellular accumulation of azithromycin in polymorphonuclear leukocytes than in cells incubated with free nonintracellular azithromycin for the same time (8.3 versus 2.2 μg/ml after 2 h), suggesting a more efficient or rapid uptake through cell-to-cell interaction. The widespread distribution of fibroblasts in tissues suggests a potential for these cells, and possibly other lysosome-containing tissue cells, to serve as a reservoir for azithromycin, slowly releasing it for activity against extracellular organisms at sites of infection and passing it to phagocytes for activity against intracellular pathogens and potential transport to sites of infection.

Fibroblasts are found in virtually all tissues. They play a central role in wound healing by secreting collagen and facilitating wound repair (3, 14). They may also contribute to microbial virulence by secreting and binding fibronectin (17, 18), which can facilitate the adherence of infectious organisms to themselves and other tissue cells (1, 12, 23). Fibroblasts may also serve an immunoregulatory role by responding to endotoxin-induced monokines to produce factors which can increase the function (15, 23) and recruitment (21) of phagocytes.

The novel azalide antibiotic azithromycin (CP-62,993) (2, 19) has previously been reported to have improved efficacy against soft tissue infections, compared with erythromycin (10). However, its in vivo activity has been better correlated with extravascular concentrations, not with levels in serum (9). Previously, it has been reported to achieve and maintain high concentrations in tissues (7, 10, 20) and in phagocytic cells (11), achieving intracellular concentrations greater than 200 times that of the extracellular medium. Since fibroblasts are ubiquitous, being found in most tissues, the ability of azithromycin to concentrate intracellularly in this cell type was determined. The potential for fibroblasts to serve as a reservoir for azithromycin in tissues and at infection sites for passage to and intracellular accumulation by phagocytic cells was assessed.

MATERIALS AND METHODS

Antibiotics. Unlabeled and radiolabeled [^{14}C]azithromycin was synthesized by Pfizer Medicinal Research Laboratories, Groton, Conn. (2, 11). Labeled drug had a radiopurity of >97% and a specific activity of 15.4 mCi/mmol. [^{14}C]erythromycin was purchased from Dupont, NEN Research Products, Boston, Mass., and had a radiopurity of >95% and a specific activity of 54.3 mCi/mmol. Unlabeled erythromycin was purchased from Abbott Laboratories, North Chicago, Ill. The bioactivities of the labeled drugs were verified by comparing the MICs of the drugs with those obtained with the corresponding unlabeled antibiotics against a clinical isolate of *Staphylococcus aureus* (19).

Chemicals. 2,4-Dinitrophenol was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Sodium fluoride was obtained from Fisher Scientific Co., Fair Lawn, N.J. 2-Chloroadenosine was obtained from Sigma Chemical Co., St. Louis, Mo.

Cells. Primary human fibroblasts were derived from minced collagenase-treated foreskin tissue. The cultures were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum (DMEM; Hazelton Biologicals, Lenexa, Kans.). Cells were split once or twice weekly and utilized between passages 4 and 20. The cells were dislodged by using trypsin-EDTA, placed in 24-well plates at approximately 10^4 cells per well, and incubated for 3 days before use or until confluent. Human polymorphonuclear leukocytes (PMNs) were obtained from heparinized blood collected by venipuncture. The PMNs were isolated by using Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, Va.) and washed four times prior to use. The preparations were found to contain >98% neutrophils.

Cell volume was determined by fluid displacement (11) and related to the number of cells. Fibroblast volume was also related to the amount of cellular protein (Coomassie blue; Bio-Rad Laboratories, Richmond, Calif.). Fibroblasts were found to have a volume of 2.55 μl/mg of cellular protein or /6.4 × 10^6 cells, which is slightly lower than that reported

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for cultured rat fibroblasts (5 μl/mg of protein or /2.7 × 10^6 cells) (22). Human PMNs had a volume of 2 μl/10^7 cells, as previously reported (11).

Procedure for determining the uptake of radiolabeled antibiotic. The uptake and release of azithromycin were compared with those of erythromycin, since erythromycin is readily available commercially and its uptake has been studied extensively in other cell types (4, 11, 13, 16). The methods utilized for uptake in this study were essentially the same as those previously described (11). Confluent cells were washed with DMEM, and various concentrations of the radiolabeled drugs were added. After various incubation periods, the cells were washed four times by using warm Hanks balanced salt solution (a procedure requiring less than 1 min) and lysed by using 0.05% Triton X-100 (Sigma), and the amount of radioactivity was determined in a liquid scintillation counter. The concentration of antibiotic was then determined from a standard curve prepared with various dilutions of the labeled drug. In addition, the concentrations of azithromycin in selected samples of cell lysates were determined by comparing the inhibitory dilution of the lysate determined against a recent clinical isolate of *S. aureus* with the MIC obtained with unlabeled drug by using standard techniques (19). The amount of cellular protein in a sample of each cell lysate was also determined. The amount of antibiotic associated with 1 mg of cellular protein was then determined. The intracellular/extracellular ratio (I/E) was calculated on the basis of the amounts of antibiotics in the supernatants at the end of the incubation period, and the cellular volume was determined as described above. Studies examining the effects of various inhibitors on uptake utilized a 1-h preincubation period prior to adding the radiolabeled antibiotic. By using these techniques, the intracellular accumulation of erythromycin was 2.5 times higher than that previously reported (16); however, in that study, uptake was determined for cells maintained in suspension rather than for adherent cells.

Potential for fibroblasts to serve as a reservoir for azithromycin. The amount of time azithromycin or erythromycin remained in fibroblasts after removal of the extracellular drug was determined by incubating the cells with 10 μg of the appropriate antibiotic per ml for 24 h, washing them, and then adding fresh medium to the culture. After various times, the amounts of antibiotics in the supernatants and in the washed-cell lysates were determined. The release of azithromycin was also determined in the presence of various cytokines and bacteria.

In order to determine the potential for azithromycin contained within fibroblasts to be passed to other cell types, 10^7 PMNs were incubated with azithromycin-loaded and washed fibroblasts. After 2 or 3 h, the nonadherent PMNs were removed by washing the culture with warm Hanks balanced salt solution. The intracellular concentration of azithromycin in the fibroblasts was determined as described above. The PMN suspension was washed by centrifugation, and the amount of azithromycin in the supernatant, as well as the amount of intracellular azithromycin, was determined as previously described (11). For comparison, the intracellular accumulation of azithromycin in PMNs (incubated with fibroblasts) was compared with that which occurs in the absence of fibroblasts by using a previously described procedure (11). Briefly, PMNs were incubated with azithromycin added in increasing amounts every 30 min at levels which approximated those found in fibroblast supernatants at the same times. At 2 or 3 h, the PMNs were washed and the concentration of intracellular drug was determined.

TABLE 1. Uptake of azithromycin and erythromycin by human fibroblasts[a]

Time of exposure (h)	Azithromycin		Erythromycin	
	μg/mg of protein (mean ± SD)	I/E	μg/mg of protein (mean ± SD)	I/E
1	4.52 ± 0.24	174	0.63 ± 0.04	25
3	10.04 ± 0.98	379	0.96 ± 0.43	41
5	15.50 ± 0.38	620	0.86 ± 0.12	38
24	25.88 ± 1.74	1,316	0.96 ± 0.06	35
48	48.89 ± 5.49	2,472	1.97 ± 0.43	71
72	61.19 ± 8.80	3,738	2.89 ± 0.22	97

[a] Cells were exposed to 10 μg of azithromycin or erythromycin per ml.

Statistical analysis. Statistical analysis was done by using the Student *t* test. A *P* value of less than 0.05 was considered significant. All data are expressed as means ± 1 standard deviation determined from three separate experiments.

RESULTS

Intracellular accumulation of azithromycin. The uptake of azithromycin and erythromycin by human fibroblasts is shown in Table 1. Azithromycin concentrated in human fibroblasts, increasing progressively over a 3-day period in an almost linear fashion. In contrast, the uptake of erythromycin occurred mostly during the first few hours of incubation. After 72 h, the intracellular accumulation of azithromycin was almost 21 times higher than that of erythromycin (61.2 versus 2.9 μg/mg of protein). Incubation of fibroblasts with various concentrations of azithromycin indicated a direct relationship between the extracellular concentration of azithromycin and the degree of intracellular accumulation (Table 2). However, the concentration gradient (I/E) achieved was similar regardless of the extracellular concentration.

Effects of various factors and conditions on azithromycin uptake. In an attempt to characterize the uptake of azithromycin into fibroblasts, the effects of various conditions and inhibitors were determined (Table 3). No uptake was observed when cells were incubated with azithromycin at 4°C, compared with the controls incubated at 37°C. Azithromycin was also not concentrated in nonviable intact cells (exposed for 2 h to 10% phosphate-buffered Formalin and then washed). When the pH of the extracellular medium was reduced from 7.2 to 6.0, uptake was reduced 69% relative to the controls. Increasing the pH to 8.6 had no effect on uptake. Preincubation of fibroblasts for 1 h with 100 μM 2,4-dinitrophenol, 200 μM sodium fluoride, or 100 μM 2-chloroadenosine had no effect on uptake.

Potential for fibroblasts to serve as a reservoir for azithro-

TABLE 2. Effect of extracellular concentration on the uptake of azithromycin by human fibroblasts[a]

Extracellular concn (μg/ml)	Azithromycin concn (μg/ml of protein; means ± SD)	I/E[b]
1.0	1.13 ± 0.1	443
5.0	6.48 ± 0.4	508
10.0	11.82 ± 0.2	463
20.0	23.11 ± 0.2	453

[a] Cells were exposed to various concentrations of azithromycin for 3 h.
[b] No significant differences were observed between the I/E values for any of the groups relative to one another.

TABLE 3. Effects of various factors and conditions on the uptake of azithromycin by human fibroblasts

Factor or condition[a]	I/E[b]
Control[c]	437 ± 27
4°C	0[d]
Formalin treatment	0[d]
pH 6	137 ± 1[d]
Sodium fluoride (200 μM)	506 ± 75
2,4-Dinitrophenol (100 μM)	429 ± 51
Chloroadenosine (100 μM)	497 ± 51

[a] Cells were exposed to the factors listed for 1 h prior to the addition of azithromycin.
[b] The uptake of azithromycin was determined after a 2-h incubation period. Values are means ± standard deviations.
[c] Control cells were incubated at 37°C (pH 7.2).
[d] Significantly different from the controls.

mycin. Incubation of azithromycin- or erythromycin-loaded fibroblasts in drug-free medium indicated that azithromycin had a longer half-life in cells than erythromycin (Fig. 1). Whereas over 75% of the intracellular erythromycin was released during the first 5 h, only 38% of the azithromycin was released. Even after 48 h of incubation, fibroblasts still retained 27% of the initial amount of azithromycin and 10% of the initial amount of erythromycin, which may reflect an equilibrium between the extracellular and intracellular concentrations at this time.

In view of the long half-life of azithromycin in cells, the effects of various factors reported to stimulate fibroblasts (15, 25) on release were examined (Table 4). No effect was observed when fibroblasts were incubated with 100 U of recombinant interleukin-1 (Genzyme Corp., Boston, Mass.) per ml, 100 U of recombinant tumor necrosis factor (Amgen Biologicals, Thousand Oaks, Calif.) per ml, or a dilution (5×10^{-2}) of a 24-h lipopolysaccharide (1 μg/ml)-stimulated monocyte soup. Since previous studies have indicated that bacteria can enhance the release of azithromycin from macrophages (11), the effect of opsonized *S. aureus* on the release of azithromycin from fibroblasts was examined. No enhanced release of azithromycin from fibroblasts was observed when they were incubated in the presence of opsonized *S. aureus* (40 bacteria to 1 fibroblast).

Incubation of PMNs with either washed azithromycin-loaded fibroblasts or equivalent escalating levels of free (nonintracellular) drug resulted in higher intracellular levels of azithromycin when cells were incubated with fibroblast-associated azithromycin. After a 2-h incubation period, the intracellular concentration of azithromycin in PMNs incubated with fibroblasts was 3.7 times higher than it was when they were incubated with free azithromycin (8.3 ± 0.4 versus 2.2 ± 0.1 μg/ml; $P < 0.05$). After 3 h, the intracellular concentration of azithromycin within those PMNs incubated with fibroblasts was 26.8 ± 3.0 mg/ml, compared with 16.7 ± 1.0 μg/ml in those cells incubated in the absence of fibroblasts (with free azithromycin, $P < 0.05$). Since PMNs in culture rapidly settle, placing them in immediate contact with the adherent fibroblasts, this higher intracellular accumulation may be a reflection of the higher level of released drug in the immediate area of the fibroblasts and PMNs than that apparent in the total supernatant at any given time. In fact, periodic swirling of the PMN-fibroblast culture resulted in intracellular concentrations of azithromycin similar to that obtained with cells incubated with free drug (data not shown).

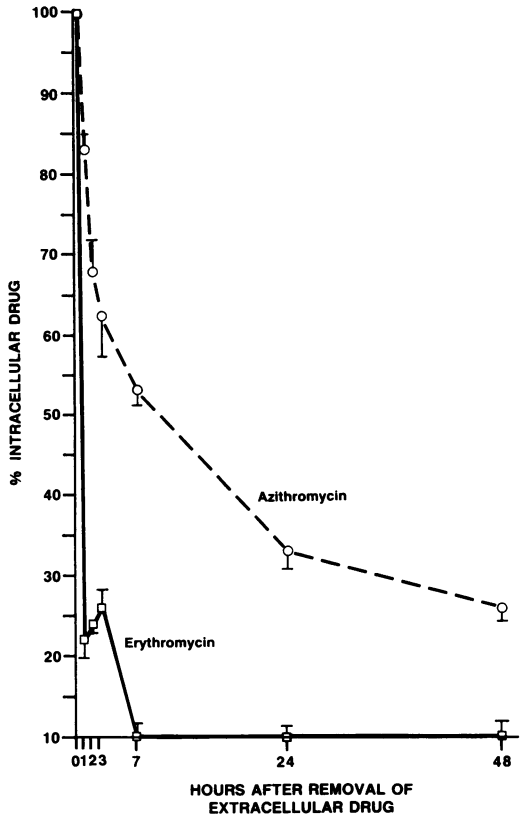

FIG. 1. Persistence of azithromycin and erythromycin in human fibroblasts following removal of extracellular drug. Cells were exposed to azithromycin or erythromycin (10 μg/ml) for 24 h, washed, and then incubated in antibiotic-free medium for the designated times.

DISCUSSION

The results demonstrate that azithromycin can concentrate in human fibroblasts. As previously observed with phagocytic cells, the intracellular accumulation of azithromycin exceeded that of erythromycin (up to 27 times) at all times examined. The degree of intracellular accumulation of erythromycin was higher than that previously reported by Martin et al. (16), who utilized different incubation conditions (i.e., the use of suspended rather than adherent cells). Nevertheless, the differential uptake between azithromycin and erythromycin should remain the same and may be a reflection of the higher tissue levels of azithromycin reported (7, 10, 20). The ability of fibroblasts to accumulate large amounts of some antibiotics was also previously shown for streptomycin, for which an intracellular accumulation of 20 μg of cell protein per ml was observed in vitro (22). However, unlike streptomycin, azithromycin achieved high intracellular concentrations (up to 61 μg/mg of cell protein) by using 100-fold-lower extracellular concentrations.

The characteristics of azithromycin uptake by fibroblasts were also similar to those previously observed with phagocytic cells (11). Like other weak bases, azithromycin (pK_a = 8.6) would be expected to concentrate in lysosomes. In lysosomes, the low pH would cause it to have a net charge of +2, which should inhibit its diffusion out of the cell (2, 5). In this way, diffusion of azithromycin into the cell with subsequent trapping may be one mechanism for intracellular accumulation. This is supported by the lack of inhibition by

TABLE 4. Effects of various factors on the release of azithromycin from human fibroblasts[a]

Factor[b]	Amt or concn	I/E (mean ± SD)	% Release
Control		602 ± 58	39
Monocyte supernatant	5%	565 ± 27	42
S. aureus	40:1 (bacterium:fibroblast)	543 ± 5	44
Recombinant tumor necrosis factor	100 U/ml	609 ± 62	48
Recombinant interleukin-1	100 U/ml	535 ± 36	46

[a] No significant differences were observed between any of the groups and the control ($P > 0.05$).
[b] Cells were exposed to 5 μg of azithromycin per ml for 24 h, washed, and then incubated with or without the factors listed for 2 h.

metabolic inhibitors, a reduction in uptake by a low extracellular pH (but not by a high pH), and a direct relationship between extracellular concentration and the extent of intracellular accumulation.The inhibition of uptake at low temperatures and by fixation might be explained by their effects on altering membrane fluidity and therefore diffusion (8).

Azithromycin has been reported to achieve and maintain high levels in all tissues examined in vivo in animals and humans (7, 20). The ability of azithromycin to concentrate in fibroblasts, together with their ubiquity, supports this finding and suggests that this cell type, together with other lysosome-containing cells, may be partly responsible for the high concentrations achieved in tissues. The ability to achieve high intracellular concentrations in fibroblasts may have several implications. First, fibroblasts may serve as a reservoir for azithromycin in tissue. Although azithromycin is released slowly from fibroblasts, the absolute amount released over time is high, because of the large amount that accumulates within the cell. The slow release of azithromycin from fibroblasts may allow activity against invading extracellular pathogens and potential accumulation by phagocytic cells as they pass through tissues. This was demonstrated in vitro, where PMNs incubated in contact with azithromycin-loaded fibroblasts not only accumulated azithromycin but did so to a greater extent than that achieved by incubating PMNs with azithromycin alone. This illustrates that phagocytic cells can pick up azithromycin from loaded tissue cells as it is released locally. Thus, the level of azithromycin in phagocytes may be regulated more by levels in local tissue than levels in serum. Once concentrated in phagocytes, these cells may transport azithromycin to the site of infection, producing locally high concentrations of active drug (11).

The second implication of azithromycin accumulation in fibroblasts may occur at the site of infection. Fibroblasts have been reported to produce neutrophil chemotactic factor in response to monokines (21) which serve to recruit PMNs to the site, potentially facilitating transfer of azithromycin from the intracellular stores in fibroblasts to the recruited PMNs. Fibroblasts may also contribute to walling off of infectious agents within abscesses (24), thus restricting phagocyte infiltration. Penetration of an antibiotic into such an infected area would be critical for eradication of the infection.Uptake of azithromycin by fibroblasts and subsequent release may allow efficacy against this type of localized infection. Indeed, in vivo activity has been reported for azithromycin in an abscess infection model for which high-dose levels of other antibiotics failed (10). Intracellular accumulation of azithromycin in fibroblasts may also help eradicate infectious agents penetrating into these cells (12). In this case, an intracellular antibiotic would be critical, since, unlike phagocytes, fibroblasts contain no autologous killing mechanisms. In addition, fibroblasts have been reported to secrete and bind fibronectin (17, 18), which may facilitate the adherence of microorganisms to themselves and other tissue cells, enhancing microbial virulence (1, 6, 12, 23). In this case, the release of azithromycin from fibroblasts may help inhibit these adherent organisms which may evade PMN and macrophage phagocytosis (12).

In summary, azithromycin was shown to achieve and maintain high intracellular concentrations in human fibroblasts. The ubiquity of fibroblasts in tissues and their ability to transfer released drug to PMNs suggest that fibroblasts may serve as a reservoir for the drug in tissue, allowing activity against invading organisms and possibly passing the drug to phagocytic cells for activity against intracellular organisms and delivery to infection sites (11).

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