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In Vitro and In Vivo Intraleukocytic Accumulation of Azithromycin (CP-62, 993) and Its Influence on Ex Vivo Leukocyte Chemiluminescence

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The accumulation of azithromycin in phagocytic cells was studied both in vitro by using a radiolabelled drug and a bioassay and in vivo for 12 volunteers receiving 1.5 g (total dose) orally within 3 days. In vitro, neutrophils and unfractionated blood leukocytes accumulated azithromycin up to 160-fold the extracellular concentration within 1 h at 37°C but less than 3-fold at 4°C. Dead cells accumulated up to 30-fold azithromycin, whereas NaF-treated cells accumulated up to 60-fold arithromycin. The mean efflux from preloaded cells was at most 31.0% ± 10.6% (standard error of the mean) of the cell-associated concentration within 4 h of incubation at 37°C in drug-free buffer. In vivo, the azithromycin concentration was 45.2 ± 6.1 mg/liter of intracellular fluid at 2 h after the third dose and 36.6 ± 8.3 mg/liter at 1 week thereafter. The corresponding concentrations in serum were 0.2 ± 0.1 (2 h) and <0.05 (1 week). The luminol-enhanced chemiluminescence response induced by phorbol myristate acetate, opsonized zymosan, and two opsonized strains of *Haemophilus influenzae* (a type b capsulated strain and a noncapsulated strain) was also studied ex vivo by using the blood leukocytes from the 12 test volunteers and 4 control volunteers at 2 and 6 h after the third oral dose of azithromycin and at 2, 4, and 7 days thereafter. Azithromycin did not influence this response despite high levels of cellular accumulation.

Azithromycin (CP-62, 993; XZ-450) is a new 15-membered macrolide with high tissue penetration (9) and in vitro activity against intracellular pathogens such as *Legionella pneumophila* and *Chlamydia trachomatis* (8, 32). Macrolides are highly accumulated by phagocytic cells through at least two mechanisms: an energy-independent mechanism, which occurs even in dead cells or at 4°C and probably depends on pH and liposolubility (23), and an energy-dependent mechanism associated with the nucleoside transport system (22). Most of the intracellular drug is located in the cytoplasm (6). In vitro, the elution of the macrolides out of the phagocytes is very rapid (within minutes) once the extracellular drug is removed (13). Several investigators have shown that 14-membered macrolides can significantly impair oxidative metabolism of the neutrophils in vitro, as assessed by luminol-enhanced chemiluminescence, superoxide generation, H_2O_2 and OH· production, and myeloperoxidase-mediated iodination of proteins (1, 3, 11, 18, 21). Inhibition occurred at fairly high concentrations (50 mg/liter for roxithromycin and erythromycin) and was reversible by washing the cells (18). Other antimicrobial agents have been shown to impair the oxidative metabolism of human polymorphonuclear leukocytes (PMN) investigated in vitro (11, 21, 31). Very few studies have questioned the in vivo relevance of these findings. Labro et al. (17) showed the opposite results when testing roxithromycin ex vivo (enhancing of oxidative mechanisms) by comparison with their in vitro studies (16, 18). The mechanism of ex vivo enhancement of oxidative functions might be due to an indirect effect on macrophages, recruitment of neutrophils, or priming of the neutrophils (5a).

We report here the in vivo accumulation of azithromycin by blood leukocytes obtained from volunteers receiving azithromycin. Luminol-enhanced chemiluminescence (induced by phorbol myristate acetate [PMA], opsonized zymosan, and two strains of *Haemophilus influenzae*) was studied ex vivo to assess the oxidative metabolism and phagocytosis of human blood leukocytes exposed in vivo to azithromycin.

MATERIALS AND METHODS

Protocol. The protocol was reviewed and approved by the Ethics Committee of the Institut Jules Bordet. A written informed consent was obtained from each volunteer. Before inclusion and 1 week after the last dose of azithromycin was given, each volunteer was tested for blood hemoglobin, hematocrit, leukocytes and differential formula, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, total bilirubin, electrolytes, glucose, alkaline phosphatase, albumin, total protein, urea, and creatinine, and a urinalysis was done.

Volunteers. Included in the study (March to June 1990) were 16 volunteers who were determined to be healthy by their medical histories, physical examinations, and biology workups (values within the range of the means ± 2 standard deviations of normal values for each test). Four volunteers were used as internal controls for ex vivo chemiluminescence analysis. No medication or drug was allowed for at least 10 days before the study. Exclusion criteria were pregnancy, lactation, previous history of intolerance to macrolides, concomitant therapy, and participation in other studies of investigational drugs within 1 month prior to entering the present study. The 12 test volunteers, six males and six females, had a median weight of 66 kg (43 to 95 kg) and a median age of 32 years (23 to 41 years). The four

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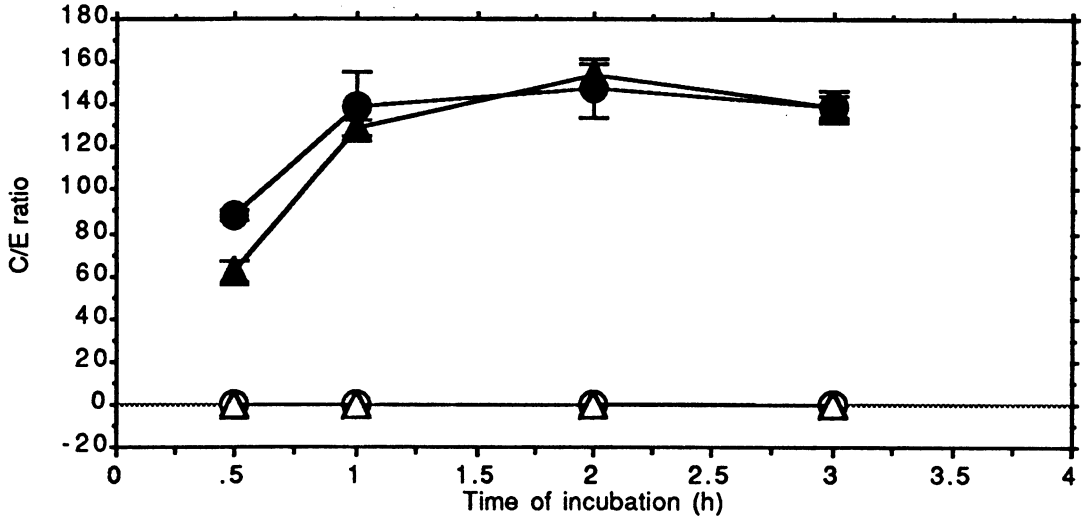

FIG. 1. Influx of azithromycin in vitro in two cell preparations (PBL [●, ○] and PMN [▲, △]) at 4°C (○, △) and 37°C (●, ▲). The mean ± standard error of the mean C/E ratio was determined by a bioassay; cells from three volunteers were tested in triplicate. The extracellular concentration of azithromycin was 10 mg/liter.

control volunteers, two males and two females, ranged in weight from 61 to 74 kg and in age from 23 to 37 years.

Drug administration. Azithromycin was provided by Pfizer as 250-mg capsules (lot no. ED-G-170-789). Six volunteers received 500 mg once a day for 3 days (at 7:00 a.m.), and blood samples were collected at 9:00 a.m. ("peak" concentration) on days 0, 2, 4, and 7 after the last dose. Six volunteers received 500 mg once a day for 3 days (at 3:00 a.m.), and blood samples were collected at 9:00 a.m. ("trough" concentration) on days 0, 2, 4, and 7 after the last dose. Alcoholic beverages were not allowed during drug administration and for 48 h after the last dose. A control volunteer was tested in parallel for each set of three test volunteers.

Sample collections and preparation of the cells. (i) Preparation of PMNs. PMNs were obtained from the peripheral blood, which was collected on citrate buffer (GIBCO Europe) (29–31) and sedimented in dextran (1 h). The supernatant was separated on Ficoll-Hypaque (Pharmacia). After hypotonic shock, the cells were resuspended in HBSS (Hanks balanced salt solution, pH 7.2; GIBCO Laboratories, Grand Island, N.Y.). Counting and viability tests (trypan blue exclusion and lactate dehydrogenase release) were done before and after each experiment.

(ii) Preparation of peripheral blood leukocytes (PBL). Dextran sedimentation was followed by a single wash in HBSS without calcium and magnesium. After centrifugation, hypotonic shock was performed to eliminate the contaminating erythrocytes. The number of cells resuspended in HBSS for the final preparation was counted, and a differential formula was calculated to determine the percentage of lymphocytes, monocytes, and neutrophils (Giemsa stain).

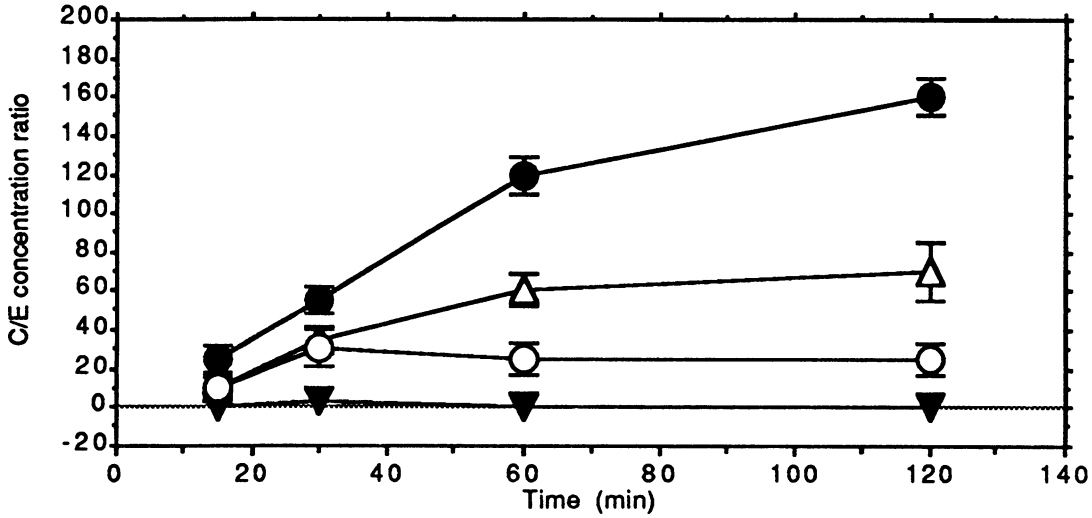

FIG. 2. Influx of ^{14}C-azithromycin in vitro in leukocyte preparations incubated at 4°C (▼) and 37°C (●), incubated with NaF (at 37°C) (△), or killed with formalin (○). The mean ± standard deviation C/E ratio was determined; [^{14}C]azithromycin was measured as counts per minute; cells from one volunteer were tested in triplicate.

TABLE 1. Efflux of azithromycin in vitro from blood leukocytes and neutrophils incubated at 4 and 37°C after being incubated for 60 min at 37°C in 10 mg of azithromycin per liter in PBS[a]

Temp (°C) of incubation	Duration of incubation (h)	Efflux (%) from[a]:	
		Leukocytes	Neutrophils
4	1	17.7 ± 8.9	13.3 ± 5.8
	2	24.3 ± 10.6	13.0 ± 7.0
	3	26.7 ± 9.7	17.7 ± 8.8
	4	27.7 ± 7.9	17.7 ± 5.6
37	1	22.3 ± 3.8	15.7 ± 2.3
	2	32.3 ± 4.6	17.0 ± 3.6
	3	29.7 ± 2.2	20.3 ± 4.7
	4	31.0 ± 10.6	26.0 ± 6.8

[a] Mean ± standard error of the mean efflux of arithromycin in the cells from three volunteers and tested in triplicate. Cell-associated azithromycin was measured by bioassay and expressed as miligrams per liter of intracellular fluid. Results are expressed as the percentage of initial cell-associated azithromycin lost during incubation in antibiotic-free medium for the indicated duration. By analysis of variance, $P = 0.016$, for type of cell preparation; $P = 0.2$, for temperature; and $P > 0.4$, for duration of incubation.

Bioassay of azithromycin. A modified agar diffusion micromethod was used for the bioassay (in triplicate) as described previously (30) by using *Micrococcus luteus* ATCC 9341 (0.5 ml of a suspension corresponding to a MacFarland standard of 0.5 to 1 in 100 ml of neomycin assay agar or antibiotic medium 11). Plates were incubated overnight at 37°C. The cell-associated concentration was determined by using a standard curve prepared from a pooled cell lysate (PMN and PBL gave the same results). The serum assay was done by using a standard curve prepared from pooled serum. The mean recovery for 32 tests (3 and 6 mg/liter for controls) was 106.3% ± 8.9% (standard deviation). The lower limit of detection was 0.04 mg/liter. The assay was linear from 0.08 to 1.6 mg/liter. In spiked neutrophil lysates, the recovery of azithromycin was 96.2% ± 10.3% (12 tests; 0.5 to 2 mg/liter; protein concentration, 0.3 to 1.2 mg/ml).

Accumulation of azithromycin in phagocytic cells in vitro. The azithromycin concentration was measured in neutrophils and leukocytes according to a previously described method (30) by using a bioassay and a radioassay ([^{14}C] azithromycin, 15.4 mCi/mmol; radiopurity, >97%) to assess cell-associated azithromycin. The intracellular volume was calculated considering 5 μl of intracellular fluid for 1 mg of protein (26, 30).

In a first assay, 10^7 cells per ml of HBSS were incubated with 10 mg of azithromycin per liter in HBSS (bioassay: 9.53 mg/liter) for 30 min and 1 h at both 4 and 37°C. The concentration of azithromycin in the supernatant after one wash of the cells (preincubated with the drug) was <0.05 mg/liter. During the incubation of cells (PMN and PBL) with azithromycin (8 mg/liter) for 3 h, the extracellular concentration of azithromycin was monitored by bioassay to exclude any metabolism. Three volunteers were tested. Extracellular concentrations of azithromycin (8.6 to 9.2 mg/liter) were not affected by the incubation with the two preparations of cells.

The influx of azithromycin was studied by using cells (three different volunteers) incubated for 30 min and 1, 2, and 3 h at both 37 and 4°C. In addition, dead cells (10% formaldehyde for 30 min; two washes with HBSS) and cells incubated in NaF (5 mM) were tested by bioassay and radioassay. Efflux was studied for cells preincubated with 10 mg/liter for 1 h and washed twice in HBSS, and the results were expressed as the decrease in the intracellular concentration of azithromycin for a given interval divided by the initial intracellular concentration (after the cells were exposed to azithromycin and washed) times 100.

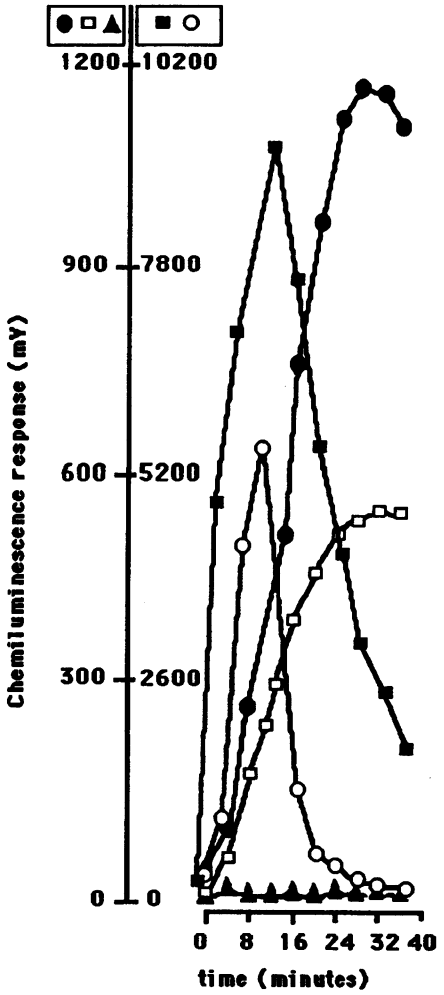

FIG. 3. Luminol-enhanced chemiluminescence response of human PBL after stimulation with PMA (○), opsonized zymosan (■), and two opsonized strains of *H. influenzae* (□ [strain 2] and ● [strain 3]). ▲, control.

Accumulation of azithromycin in leukocytes ex vivo. Blood leukocytes (PBL without erythrocytes) were tested by bioassay after lysis as described above (standard curves for pooled leucocyte lysates). Using PBL has the advantage of reducing the preparation time, which minimizes drug efflux.

Serum concentration. The concentration of azithromycin in serum was measured by bioassay according to the method of Bennet et al. (4) and with the same microorganism as described above, with standard curves for pooled serum (variability, 9.2%; recovery, 102.5%).

Luminol-enhanced chemiluminescence. Luminol-enhanced chemiluminescence was performed in vitro with the cells (PBLs) obtained from the 12 test volunteers and the 4 control volunteers. Four different stimuli were tested: opsonized zymosan (a C3b-dependent activation mechanism; 1.5 mg/ml, final concentration), PMA (Sigma) (a protein-kinase C agonist; 3×10^{-6} M), two opsonized clinical strains of *H.*

TABLE 2. Concentration of azithromycin in serum and cell-associated azithromycin for 12 volunteers receiving azithromycin orally[a]

Group no. (no. of volunteers)	Day	Concn of azithromycin (mg/liter)[b]			
		In serum		Cell associated	
		Mean ± SEM	Range	Mean ± SEM	Range
1 (6)	0	0.22 ± 0.09	0.06–0.58	45.22 ± 6.11	25.58–57.73
	2		ND[c]–0.04	74.51 ± 15.71	32.84–124.36
	4		ND–0.05	56.79 ± 12.78	25.54–98.70
	7		ND	36.56 ± 8.26	17.31–62.15
2 (5)	0	0.11 ± 0.02	0.08–0.15	41.51 ± 6.90	32.27–63.30
	2		ND–0.04	28.58 ± 6.39	20.86–48.68
	4		ND	25.01 ± 4.59	20.00–39.27
	7		ND	16.36 ± 2.47	11.32–24.21

[a] Sampling was done at 2 h (for group 1) and at 6 h (for group 2) after dosing. One volunteer from group 2 was excluded because no azithromycin was detected in any sample.
[b] The concentration of azithromycin is given in milligrams per liter of intracellular fluid.
[c] ND, not detected.

influenzae (strain 2, or EQ52, is a type b capsulated strain producing a β-lactamase and strain 3, or ET27, is a noncapsulated strain) (immunoglobulin- and C3b-dependent mechanisms). The two strains were prepared as follows. Overnight colonies grown on chocolate agar were diluted in 20 ml of HTM (*Haemophilus* test medium [14]) and incubated at 37°C for 2 h to reach logarithmic growth. Washed bacteria were incubated in pooled fresh serum (10%) and incubated at 37°C for 25 min on a rotator. The opsonized inoculum was diluted in phosphate buffer with calcium and magnesium (phosphate-buffered saline [PBS], pH 7.2) at a concentration of 6×10^6 CFU/ml, aliquoted, and then stored at −80°C until used.

The number of test leukocytes was adjusted to 10^7 neutrophils per ml of PBS. An automatic computerized luminometer (LKB) was used for measuring the luminol (5×10^{-5} M, final concentration)-enhanced chemiluminescence. For PMA and opsonized zymosan, the peak chemiluminescence response (in millivolts) was used for comparisons. For the two opsonized strains of *H. influenzae*, the maximal rate of the chemiluminescence response (millivolts per minute) was measured graphically and used for comparisons.

Elution from the cells during preparation and chemiluminescence. Purified neutrophils were exposed to azithromycin (10 mg/liter) during 1 h at 37°C, and then the cells were exposed either to hypotonic shock, vortexing, and centrifugation or to 30 min of incubation in HBSS at 37°C, vortexing, and centrifugation. An aliquot of the cells was used to measure cell-associated azithromycin (bioassay). The remaining cells were used for chemiluminescence, an aliquot was tested for cell-associated azithromycin just after and 30 min after the addition of the various stimuli (PMA and opsonized strains 2 and 3).

Statistics. Descriptive and comparative statistics were computed with MacIntosh SE/30 hardware and Statview 512+ software (version 1.1; Abacus Concepts, 1986).

The influence of azithromycin given in vivo on the chemiluminescence response ex vivo was studied by using

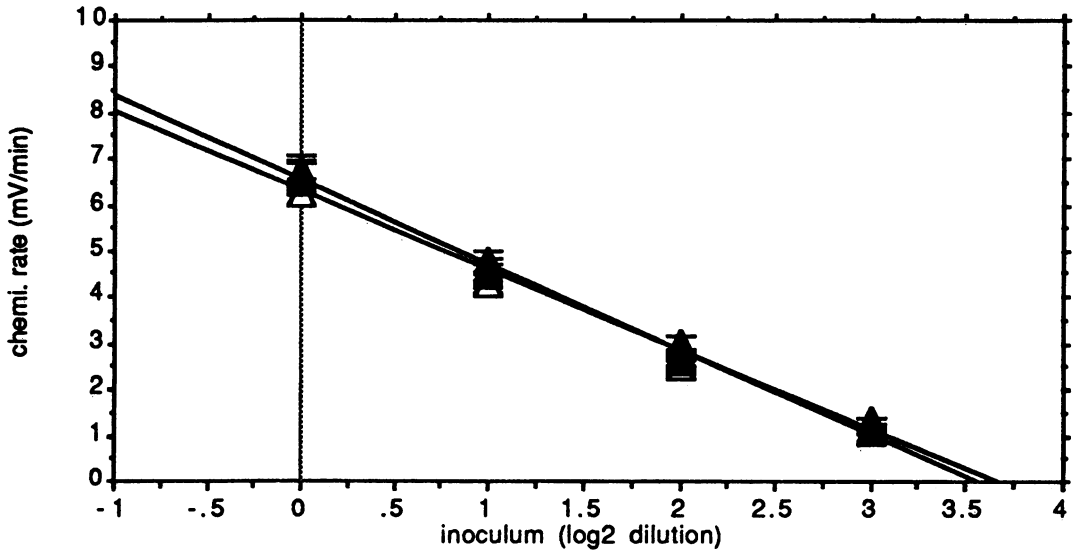

FIG. 4. Relationship between inoculum size and chemiluminescence (chemi.) response of PBL induced by opsonized *H. influenzae* (strain 3). Cells from one volunteer were tested in duplicate in two different buffered saline solutions: calcium- and magnesium-supplemented HBSS (△) and PBS (▲).

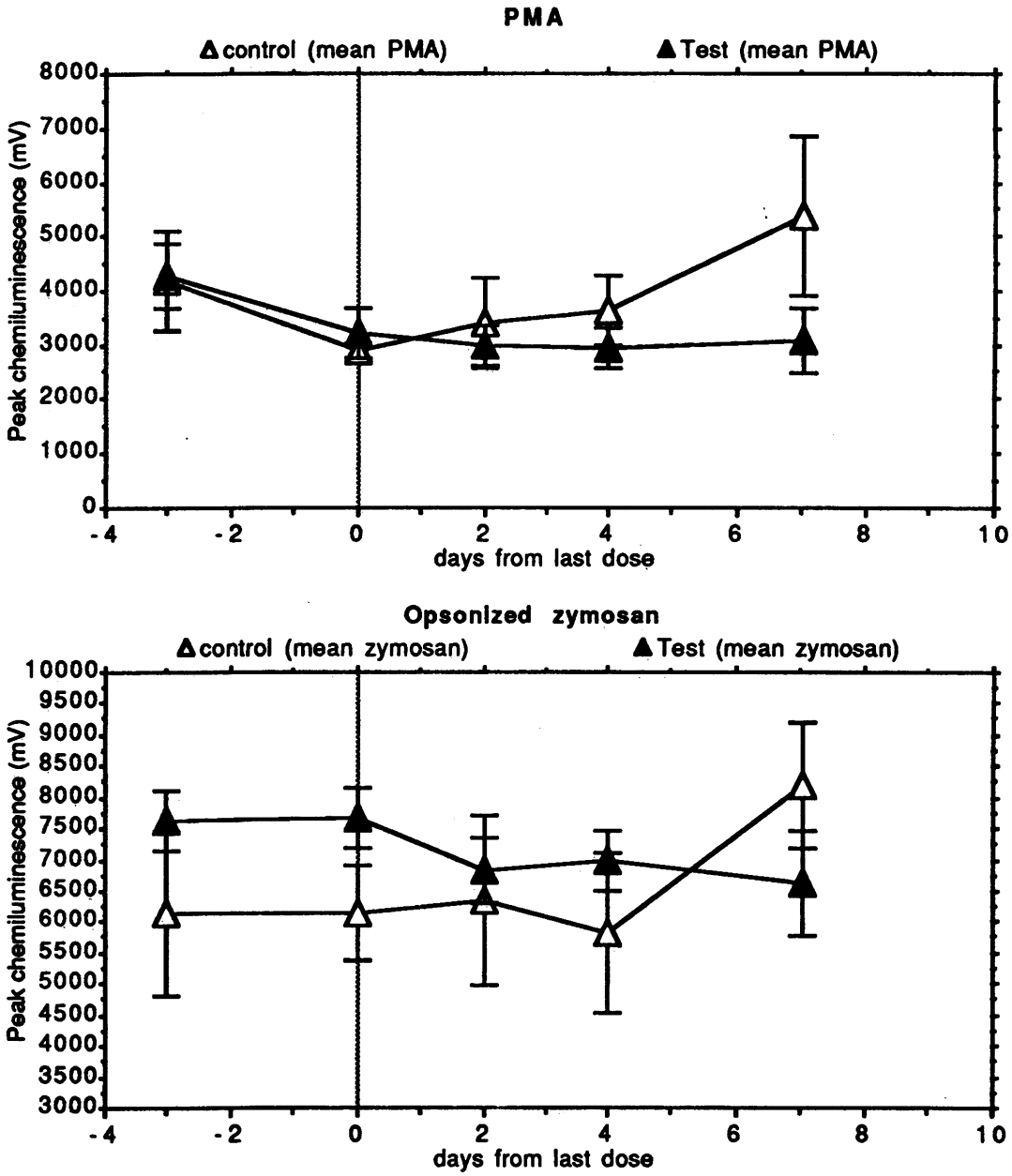

FIG. 5. Luminol-enhanced chemiluminescence response of blood leukocytes obtained from 4 control volunteers and 12 test volunteers. Test volunteers received three doses of 500 mg of azithromycin orally for 3 days. On the same day of the third dose (day 0), leukocytes were purified from peripheral blood collected 2 (for two control and six test volunteers) and 6 h (for two control and five test volunteers) after dosing. Results shown are the means ± standard errors of the means.

an analysis of variance test (factors: control and test volunteers and time from last dose).

RESULTS

The tolerance for azithromycin was excellent. One volunteer (weight, 95 kg) from group 2 had undetectable levels of azithromycin in all samples. Therefore, he was excluded from the analysis.

Azithromycin accumulation in cells in vitro. The purification of the neutrophils had a marginal effect on the cell-associated azithromycin content. The hypotonic shock induced a 6% decrease in the azithromycin content, whereas a 30-min incubation in HBSS at 37°C induced a further decrease of 8%.

At 4°C, none of the two cell preparations accumulated azithromycin intracellularly as measured by bioassay (Fig. 1). When the concentration of azithromycin was measured by radioassay, little accumulation (ratio of the concentration of cell-associated azithromycin to the extracellular azithromycin concentration [C/E ratio], ≤3) occurred with PBLs (Fig. 2). On the other hand, accumulation was very large (C/E ratio, >100) at 37°C for both cell types (Fig. 1), which were saturated after 1 to 2 h. Leukocytes accumulated more

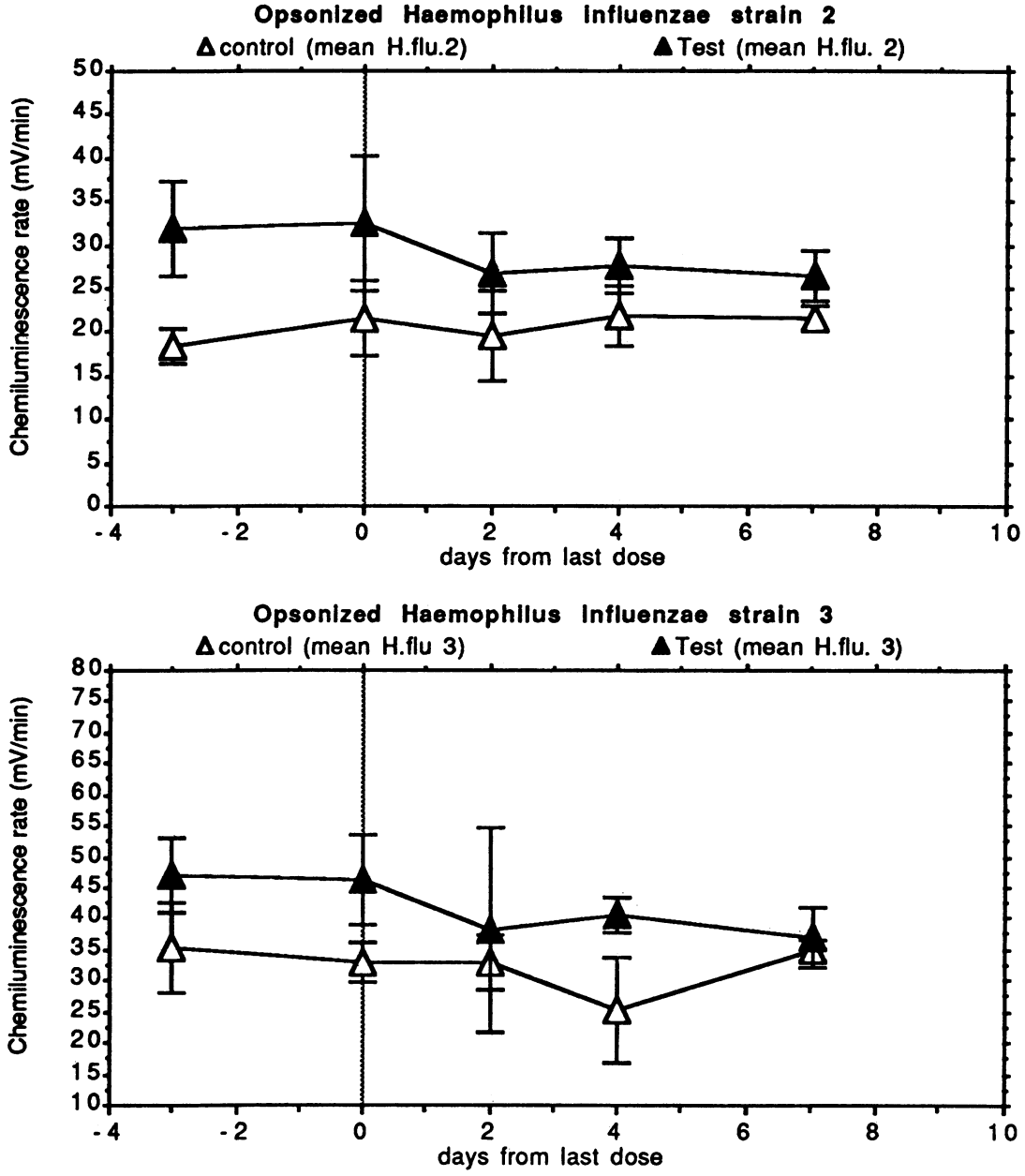

FIG. 5—*Continued.*

azithromycin than did neutrophils for two volunteers, whereas neutrophils accumulated more azithromycin for one volunteer (data not shown). Dead cells accumulated azithromycin to a C/E ratio of 30 (Fig. 2), whereas NaF-treated neutrophils accumulated azithromycin to half the extent as did viable cells at 37°C. Table 1 shows that the efflux stabilized at 20 to 46% after 1 h in drug-free medium. Efflux was similar at 4°C and 37°C (Table 1). The two cell preparations showed a similar pattern of efflux, although the efflux was significantly higher from PBLs than from PMNs (analysis of variance test; $P < 0.02$). The results were identical for the two assay methods (bioassay and radioassay).

Effect of stimulation. The stimulation of preloaded purified neutrophils with PMA for 30 min had no influence on the azithromycin content, whereas stimulation with opsonized *H. influenzae* strains 2 and 3 induced a release of 51.8 and 46.4% of the cell-associated drug, respectively.

Azithromycin accumulation in vivo. The intraleukocytic concentration of azithromycin was very high from 2 h after the third dose of 500 mg of azithromycin, corresponding to a 200-fold increase in the azithromycin concentration in serum at the same time and a 400-fold increase after 6 h (Table 2). The azithromycin concentration in serum was less than the level of sensitivity for the bioassay for the samples on days 2, 4, and 7, whereas the azithromycin concentration in the leukocytes remained very high even 1 week after the last oral dose (Table 2).

Luminol-enhanced chemiluminescence ex vivo. A characteristic chemiluminescence curve is shown in Fig. 3 for the four stimuli used. Both PMA and opsonized zymosan pro-

vided a very strong response of short duration (peak within 15 min), whereas the two strains of *H. influenzae* provided a more progressive response. The chemiluminescence response, as measured by the rate in millivolts per minute, induced by the two bacterial strains was linearly correlated with the inoculum (Fig. 4 for the uncapsulated strain). The administration of azithromycin had no statistically significant (analysis of variance test) influence on the chemiluminescence response of blood leukocytes (Fig. 5) despite a very high intracellular concentration of azithromycin.

DISCUSSION

The mechanism of cellular accumulation of macrolides by phagocytes has been extensively studied. However, several points remain controversial (12, 13, 15, 22, 23, 25). At least two mechanisms seem to contribute to this accumulation: first, an energy-dependent pathway which corresponds to the nucleoside transport system as shown by competitive inhibition by various nucleosides (22) and, secondly, an energy-independent system as shown by the accumulation by dead cells or energy-deprived cells for which the driving forces are probably pH and hydrophobicity (13, 23). The respective contributions of the two mechanisms are variable from one macrolide to another (13). Furthermore, the source of energy seems to play a role in macrolide transport since NaCN is more inhibitory for erythromycin and josamycin than for other macrolides (13). Inhibition by 2,4-dinitrophenol affects clarithromycin transport, suggesting that for this antimicrobial agent, mitochondrial function is essential (13). This mitochondrial contribution was not due indirectly to the inhibition of proton pump activity since ouabain (an inhibitor of Na^+ K^+ membrane ATPase) resulted in only 2 to 13% inhibition of macrolide uptake (13). Particle phagocytosis (bacteria or zymosan) may increase macrolide uptake (13, 25). Unlike other macrolides which are 14 or 16 membered, azithromycin is the only representative of the 15-membered macrolides. The cellular uptake of azithromycin is very similar to that of the other macrolides; however, like clarithromycin, its transport is decreased by 2,4-dinitrophenol (10). Among all macrolides tested so far, azithromycin provides the largest C/E ratio (2, 6, 10, 12, 13, 15, 23, 30), as confirmed both in vitro and in vivo in our study, with values of approximately 160 obtained in vitro for azithromycin. The inhibition of in vitro cellular uptake of azithromycin by fluoride, as shown in the present study, is a characteristic of the 16-membered macrolides (such as josamycin and rokitamycin) (13). The fact that dead cells accumulated azithromycin at about one-fifth of the amount accumulated by viable cells is also a characteristic of josamycin and rokitamycin (13). The efflux of 14- and 16-membered macrolides is very rapid once the cells are incubated in a drug-free solution (13), whereas azithromycin efflux is very slow, with 50% of the intracellular concentration being released in about 4 h for mouse peritoneal macrophages (10). Ingestion of bacteria significantly increased azithromycin efflux from macrophages (10). Our results with human phagocytes showed that efflux was below 35% and remained so for the 4 h of the experiment. We have shown that stimulation with opsonized bacteria but not with PMA induced a marked efflux (almost 50% within 30 min) of neutrophil-associated azithromycin. Gladue et al. (10) have shown that for animals receiving azithromycin, the concentration in peritoneal lavage fluid was markedly increased proportionally to the number of neutrophils migrating into the peritoneal cavity. Interestingly, 75% of the azithromycin in the peritoneal fluid was neutrophil associated, and in other experiments in which animals were injected intraperitoneally, with caseinate, 92% of the azithromycin was associated with the peritoneal macrophages. It has been suggested that on-site delivery of intraphagocytic azithromycin may provide a significant amount of bioactive antimicrobial agent (10). As assessed by subcellular fractionation after incubation with radiolabelled molecules, intracellular 14-membered macrolides (erythromycin and roxithromycin) are mainly located in the cytoplasm of resting neutrophils and macrophages, with a third of the cell-associated drug being associated with the lysosomes (6). Very recently, it was shown that stimulation of *Mycobacterium avium*-infected macrophages with tumor necrosis factor alpha or gamma interferon significantly increased the uptake of azithromycin (5). As far as intracellular bioactivity is concerned, the macrolides are active against intracellular pathogens which prevent phagosome-lysosome fusion (*Legionella* spp. *Chlamydia* spp., and *Toxoplasma gondii*) (8, 20). On the other hand, their intracellular bioactivity against bacteria such as *Staphylococcus aureus* and *H. influenzae*, which are resistant to the normal phagolysosomal environment, remains very limited, with, at most, bacteriostasis of phagocytized *S. aureus* (1–3, 19, 28).

Azithromycin, like the 14-membered macrolides, was shown to be active against obligatory intracellular pathogens such as *T. gondii* and *Chlamydia trachomatis* but to have limited activity (bacteriostasis only) against intracellular *S. aureus* (19). When neutrophils are stimulated by soluble or particulate stimuli, they respond by emitting light (chemiluminescence). This response is due to the generation of oxygen metabolites and radicals (superoxide anion, singlet oxygen, hydroxyl anion, or hypochloride, etc.) (27).

When a particulate stimulus is used, chemiluminescence is concomitant to both degranulation and uptake (phagocytosis). Luminol-enhanced chemiluminescence seems to be associated more with the myeloperoxidase-H_2O_2-halide system and monitors both the production of H_2O_2 and the release of the granule enzyme myeloperoxidase (7). Therefore, the measure of chemiluminescence is an indirect measure of the respiratory burst reaction and of uptake. For the discussion of the clinical application of chemiluminescence, see the review by Steele (24).

We did not observe any influence of azithromycin on luminol-enhanced chemiluminescence response stimulated by a soluble stimulus (PMA) which acts on one of the final steps of the NADPH-oxidase activation pathway (protein kinase C) and three particulate stimuli (opsonized zymosan and two clinical strains of *H. influenzae*) which act on C3b and Fc receptors, resulting in phagocytosis. The delays between the collection of the PBL and the final preparation of sonicates for measuring cellular-associated azithromycin on the one hand and chemiluminescence response on the other hand were similar. Furthermore, the same procedure was used to prepare the cells for the determination of cell-associated azithromycin and chemiluminescence. Therefore, the cell-associated concentration of azithromycin measured was also present in the cells tested for chemiluminescence. It can be concluded that despite large cellular accumulation of azithromycin in phagocytic cells, which occurs both in vitro and in vivo, azithromycin did not impair the chemiluminescence response of these cells exposed to the drug in vivo and tested in vitro.

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