Influence of Antibiotics on Formylmethionyl-Leucyl-Phenylalanine-Induced Leukocyte Chemiluminescence

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Received 1 December 1986/Accepted 17 February 1987

The effect of three antimicrobial agents, penicillin G, ampicillin, and chloramphenicol, on luminol-enhanced chemiluminescence of polymorphonuclear leukocytes stimulated by the chemoattractant formylmethionyl-leucyl-phenylalanine was studied. An inhibitory effect of penicillin G and of ampicillin was demonstrated, whereas chloramphenicol gave rise to an enhancement of the chemiluminescence response from polymorphonuclear leukocytes. These effects could be due to interaction between the drugs and the polymorphonuclear leukocytes, but they could also be the result of interference with the generation of light without any effect on the cells. Therefore, the effects of the same antimicrobial agents on the chemiluminescence generated from a cell-free system consisting of myeloperoxidase and hydrogen peroxide were investigated in parallel. The results obtained in the cell-free system were almost identical to those obtained in the cell system; i.e., penicillin G and ampicillin caused an inhibition and chloramphenicol a caused an enhancement of the light emission. These results indicate that observed effects induced by drugs in a chemiluminescence assay are not necessarily due to interaction between the drug and polymorphonuclear leukocytes but may be caused by interference with other components of the assay. In view of these findings, the conflicting data reported in the literature on the effects of antimicrobial agents on phagocyte function are discussed.

Antimicrobial agents have biological effects other than simple antibacterial activity. For instance, they may influence the function of polymorphonuclear leukocytes (PMNL) (21, 28). When choosing antimicrobial agents one should therefore consider not only the susceptibility of the bacteria but also any possible negative or positive effects of the antimicrobial agent on the host defense system.

When PMNL interact with soluble or particulate matter, the cells respond and produce chemiluminescence (CL) (2), which is linked to the antimicrobial oxidative metabolism of the PMNL (24). The addition of luminol to a CL system has been shown to amplify the response and to simplify the measuring procedure (1). The luminol-enhanced system permits the use of very few cells (34), obviates the need for working under dark-adapted conditions (14), and can in fact be used to measure CL in samples of whole blood (25). Luminol-enhanced CL is used to investigate cellular functions associated with the oxidative metabolism, e.g., bactericidal capacity (24, 35), phagocytosis (16, 34, 35), and interaction with chemotactic factors (4, 7). Since luminol passes into the cell, measurements of CL reflects both extracellular and intracellular events during the respiratory burst (4, 7).

For these reasons luminol-enhanced CL has been regarded as a suitable method for investigating effects of pharmacological agents on PMNL. However, conflicting results have been reported with respect to the effects of antimicrobial agents on PMNL function (5, 15, 17-19, 22, 29, 31-33, 35). In this report we have investigated the effects of some commonly used antibiotics on the production of CL from PMNL stimulated by the chemotactic peptide formylmethionyl-leucyl-phenylalanine (FMLP). The drug-induced effects observed in this assay could be due to the direct effect on the cellular production of oxidative metabolites, but they could also be the result of some interference with the generation of light other than cellular interaction.

Since the light-generating mechanism in luminol-enhanced CL has been shown to be dependent on the reaction between myeloperoxidase (MPO) and hydrogen peroxide (H2O2) (9, 12, 13), the effects of the same drugs in a cellfree system consisting of MPO and H2O2 were studied in an attempt to assess whether the observed effects on the CL from PMNL were due to cellular interaction.

MATERIALS AND METHODS

PMNL. Blood from healthy laboratory personnel was collected in EDTA-containing tubes, and the PMNL were separated as described by Boyum (6). After removal of the remaining erythrocytes by hypotonic lysis, the PMNL were washed twice in Krebs-Ringer phosphate buffer (KRG; pH 7.3) and suspended in the same buffer. The number of cells was determined with a Coulter Counter (Coulter Electronics Ltd, Dunstable Bedfordshire, England), and the PMNL suspension was adjusted to a concentration of 2 x 10^6 cells per ml in KRG. The PMNL suspensions were stored on ice until use.

Antimicrobial agents. Ampicillin (Doktacin; Astra, Sweden), penicillin G (Bensylpenicillin; Astra), and chloramphenicol (Dumex Ltd, Denmark) were diluted in KRG to the proper concentrations. The pH of the highest concentration used for each agent was tested and found to be 7.2 to 7.3.

Reagents. FMLP (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide to a concentration of 10^-2 M and further diluted in KRG to 10^-6 M. Hydrogen peroxide (Perhydrol, 30%; E. Merck AG, Darmstadt, Federal Republic of Germany) was diluted in KRG to a concentration of 1 mM; 10 mg of 5-amino-2,3-dihydro-1,4- phthalazinedione (luminol; Sigma) was dissolved in 1 ml of 0.1 M NaOH and further diluted in KRG to a concentration of 5 x 10^-5 or 2.5 x 10^-5 M. Purified myeloperoxidase was a generous gift from Inge Olsson (Lund, Sweden).

CL cell system. Measurements of luminol-enhanced CL were made in a biolumat LB 9505 (Berthold Co, Wildbad,
Federal Republic of Germany) at 37°C. Reaction mixtures in 4-ml polycarbonate tubes were obtained by adding 0.6 ml of KRG, 0.1 ml of luminol (5 × 10⁻⁵ M, final concentration), 0.1 ml of an antimicrobial agent or control buffer, and 0.1 ml of the PMNL suspension (2 × 10⁶ cells per ml, final concentration). After incubation for 45 min at 37°C the samples were placed in the biolumat, and 0.1 ml of FMLP (10⁻⁵ M, final concentration) was added as a stimulus. For each antimicrobial agent one analysis was also done in which the antimicrobial agent was added at the same time as FMLP. In these experiments the samples were incubated for 5 min at 37°C before the addition of the antimicrobial agent and FMLP.

CL cell-free system. The reaction mixtures were obtained by mixing 0.65 ml of KRG, 0.1 ml of luminol (2.5 × 10⁻⁵ M, final concentration), 0.1 ml of the antimicrobial agent or control buffer, and 0.05 ml of H₂O₂ (0.05 mM, final concentration) into 4-ml polycarbonate tubes. The samples were stirred and placed in the biolumat at 37°C, and the light emission was measured after the addition of 0.1 ml of MPO (0.125 µg/ml, final concentration).

MPO assay. The effect of the antibiotics on the MPO-H₂O₂ reaction was also determined by using guaiacol as the substrate for the reaction as previously described (30).

Viability test. The viability of PMNL after having been incubated for 45 min at 37°C was tested for the highest concentration of each antimicrobial agent by using the trypan blue exclusion method.

Statistical analysis. Statistical differences between the CL results from experiments with antimicrobial agents and experiments run in parallel without antimicrobial agents were determined by using the Student t test for paired observations.

**RESULTS**

Procedure for antimicrobial agent-PMNL interaction. The PMNL with luminol were incubated either with penicillin G, ampicillin, or chloramphenicol at different concentrations for 45 min at 37°C. The final concentrations for each agent were as follows: penicillin G and ampicillin, 300, 100, 50, 10, and 1 mg/liter; chloramphenicol, 100, 15, 10, 1, and 0.1 mg/liter. The samples were in duplicate or triplicate for each concentration, and every experiment was repeated 6 to 11 times. For every sample containing an antimicrobial agent there was one sample containing buffer only that was run in parallel. The viability of PMNL after having been incubated with the highest concentration of each antimicrobial agent was studied by using the trypan blue exclusion technique. No difference in the number of stained cells could be found between the samples containing antimicrobial agents and those with control buffer. Furthermore, to eliminate the possibility of interference with the CL by absorption of light, a spectrophotometrical analysis was made for each antimicrobial agent. No absorbance of light in the range of 300 to 700 nm (luminol emits light at 425 nm [8, 27]) could be seen with any of the antimicrobial agents tested.

**FIG. 1.** Time trace of the CL response from PMNL incubated with buffer for 45 min at 37°C and stimulated with FMLP.

**FIG. 2.** (A) Effect of penicillin G on the CL response from PMNL stimulated with FMLP. (a) Penicillin G added simultaneously with FMLP. (b) PMNL incubated with penicillin G at different concentrations for 45 min at 37°C. The figures represent the peak values of the CL of the samples containing penicillin G expressed as percentages of the peak values of the samples containing control buffer run in parallel. Each value represents the mean ± standard deviation of 7 to 11 analyses where every analysis was made in triplicate or duplicate. (B) Effect of penicillin G on the CL in a cell-free, MPO-H₂O₂ system. The figures represent the peak values of the samples containing penicillin G expressed as percentages of the peak values of samples containing control buffer. Each value represents the mean ± standard deviation of four or five analyses, where every analysis was made in duplicate. (*, P < 0.05; **, P < 0.01; ***, P < 0.001.)

**FIG. 3.** (A) Effect of ampicillin on the CL response from PMNL stimulated with FMLP. (a) Ampicillin added simultaneously with FMLP. (b) PMNL incubated with ampicillin at different concentrations for 45 min at 37°C. The figures represent the peak value of the CL of the samples containing ampicillin expressed as percentages of the peak values of the samples containing control buffer run in parallel. Each value represents the mean ± standard deviation of 8 to 10 analyses where every analysis was made in duplicate or triplicate. (B) Effect of ampicillin on the CL in a cell-free, MPO-H₂O₂ system. The figures represent the peak values of the samples containing ampicillin expressed as percentages of the peak values of the samples containing control buffer. Each value represents the mean ± standard deviation of four or five analyses, where every analysis was made in duplicate. (*, P < 0.05; **, P < 0.01; ***, P < 0.001.)
due to cellular interaction, the effects of the drugs on the CL generated in a cell-free system consisting of MPO and H₂O₂ were studied. Figure 5 shows the kinetics of the CL generated by the MPO-H₂O₂ reaction. The addition of the antibiotics did not affect the kinetics, but the maximal value of the CL was altered.

The effects induced by all three antibiotics in the cell-free system were almost identical to the effects seen in the cell system. Penicillin G and ampicillin at a concentration of 300 mg/liter caused an almost total inhibition of the CL in the MPO-H₂O₂ system (Fig. 2B and 3B). Just as in the cell system, this inhibition was found to be dose dependent with decreased inhibition when the concentration of the antibiotics was reduced. When chloramphenicol was added to the MPO-H₂O₂ system there was no effect on the CL at high concentrations of the antibiotic, but when the concentration was reduced to 0.1 to 1 mg/liter there was an enhancement of the CL as compared with MPO-H₂O₂ without any chloramphenicol (Fig. 4B).

**Effect of the antibiotics on the MPO-H₂O₂ reaction in the guaiacol assay.** The effects of the antibiotics in the CL assay could be due to direct interaction with the MPO activity. Therefore, the effects of the antibiotics on the MPO-H₂O₂ reaction were determined by using another substrate. Each of the three antibiotics was tested in a concentration that caused a pronounced effect on the CL generation in the cell-free system (penicillin G [100 mg/liter], dactacin [100 mg/liter], and chloramphenicol [1 mg/liter]). However, none of the antibiotics had any detectable effect on the MPO-H₂O₂ reaction in the guaiacol assay (data not shown).

**DISCUSSION**

The luminol-enhanced CL assay has been used to study the metabolic activity of PMNL associated with microbicidal activity (24). The technique is rapid and easy to perform, making it possible to run several samples in parallel in a short time. For these reasons the technique has been found suitable for studying drug-induced effects on PMNL function (15, 17, 24). Both penicillin G and ampicillin inhibited the CL from PMNL induced by FMLP in a dose-dependent manner. Concentrations that are achieved during therapy in vivo significantly inhibited the CL response. This inhibition was apparently not due to an increased number of dead cells in the samples containing antimicrobial agents, since there was no difference in the number of stained cells when the trypan blue exclusion method was used, regardless of whether the cells had been incubated with an antimicrobial agent. Furthermore, spectrophotometrical analysis of the drug suspensions did not demonstrate any absorbance of light in the range of 300 to 700 nm. The effect of the drugs was not

**FIG. 5.** Time trace of the CL generation in the cell-free system consisting of MPO and H₂O₂.
dependent on any preincubation with the PMNL, and the CL from a cell-free system consisting of MPO and H2O2 was even more inhibited than the CL from the cell system. No inhibition of the CL was obtained with chloramphenicol. Instead, the presence of chloramphenicol caused an increased CL. Furthermore, the enhancement of the CL response from PMNL stimulated with FMLP could be reproduced in the cell-free system.

These results indicate that the observed effect induced by penicillin G, ampicillin, and chloramphenicol on the CL response from PMNL stimulated by FMLP is not a direct effect on the cellular production of oxidative metabolites but rather an interference with other components involved in the production of light. Since no effect was seen with either of the antibiotics when added to the MPO-H2O2 reaction in the guaiacol assay, it is unlikely that the effect on the CL was due to inhibition of the peroxidase activity or interference with H2O2. However, penicillin G and ampicillin may act as scavengers for substances generated by the MPO-H2O2 reaction, e.g., hypochlorous acid, which is capable of oxidizing luminol and thereby generating CL (13), or singlet oxygen, which is also proposed to be involved in the generation of CL (26). The observed effects on the CL may have biological relevance, despite the lack of cellular interaction, if the effects of the antibiotics are due to interference with these species, since both hypochlorous acid and singlet oxygen participate in the bacterial activity of the cell (26).

The antibiotics may also directly interfere with luminol, making it less or more available for oxidation and thereby affecting the generation of light.

There are several reports on the effect of different antimicrobial agents on the CL from PMNL, but the results are difficult to interpret because of conflicting data. Trimethoprim and sulphamethoxazole have been shown to cause an inhibition (15, 33, 35) as well as an enhancement (31) of the CL from PMNL. Ampicillin B was found to inhibit the CL in one study (5), whereas another study failed to demonstrate any inhibitory effect (33). The CL from PMNL isolated from persons undergoing treatment with clindamycin was found to be inhibited (17), whereas treatment of PMNL by clindamycin in vitro resulted in an enhancement of the CL (31, 32), and the CL generated in whole blood was found to be unaffected by treatment with clindamycin (32). Tetraclines and doxycline were shown to inhibit the CL from PMNL (35), but in another study the inhibitory effect seen by doxycycline and aminocycline was abolished when an excess of Mg2+ was added to the samples (18), indicating that the inhibitory effect is not due to cellular interaction but rather to interaction with other components in the production of light. Treatment of PMNL with rifampin inhibits the CL response (22, 33), but rifampin added after the initiation of the CL resulted in an immediate drop in the CL, comparable to the level obtained when rifampin had been added before the stimulation of the PMNL (33). This indicates that the inhibitory effect was due to absorption of light, which could easily be explained since rifampin is orange. The inhibitory effect of several antimicrobial agents on the CL from PMNL has also been shown to be reversible. When the cells were washed, the inhibitory effect seen previously in the presence of the drugs was completely removed (33, 35).

Inhibition or lack of inhibition of the CL from a cell-free system consisting of xanthine and xanthine oxidase has been used to confirm a cell-directed effect (22, 33). However, it has been found that the light production in the xanthine-xanthine oxidase system differs from the light production in a cell system. The xanthine-xanthine oxidase system will produce superoxide anion (O2−), and this can dismutate to hydrogen peroxide (H2O2), but despite this there is no detectable light produced in the presence of luminol at a physiological pH (3, 4). Furthermore there is no CL detectable from cells deficient in MPO but still capable of producing O2− at a physiological pH (12). Therefore it is not possible to make any comparison between the CL generated by a xanthine-xanthine oxidase system and the CL from PMNL when luminol is included in the assay.

There are several possible explanations for the conflicting results obtained from different studies. The luminol-enhanced CL assay is a sensitive method that is influenced by several factors, such as the amount of glucose, calcium, and magnesium in the measuring medium (10, 20). Alterations in the pH influence the CL (10, 20). The presence of albumin, other protein solutions, and different amino acids in the medium has shown to inhibit the CL response induced by several different stimuli (7, 10, 20, 23). Changes in temperature also affect the CL response (16). The amount of light produced and the rate of light production increased when the temperature was raised from 20 to 37°C. The CL response is also dependent on the concentration of luminol (7, 16) and the amount of PMNL (16). Preincubation of the PMNL modifies the CL response in both magnitude and time course (11). The CL response is also dependent on the kind of stimulus used (10, 16, 23). In view of these possibilities of influencing the CL assay, it is obvious that there is a great need for standardization of the assay and that differences in results obtained in different laboratories can be due to methodological problems.

In summary, when choosing antimicrobial agents for therapy it is important to consider not only the sensitivity of the bacteria but also any possible effect on the host-defense system of which the PMNL is an important part (21, 28). Luminol-enhanced CL has been regarded as a suitable method for investigating drug-PMNL interactions, and it has become increasingly popular (15, 29, 32). Several investigators have stated the need for strict standardization of the assay since there are a number of factors that can influence the analysis (7, 10, 11, 16, 20, 23), and the conflicting data reported in the literature indicate that caution must be used when interpreting results on drug-induced effects on PMNL obtained with CL.

In this study we have demonstrated that alterations induced by antimicrobial agents on the CL response from PMNL stimulated with FMLP could be reproduced in a system lacking PMNL, indicating that drug-induced effects seen in a CL assay are not necessarily caused by interaction between PMNL and the drug but may be due to interaction with other components in the measuring system interfering with the production of light. However, whether the effects on light generation which have been described, i.e., increased CL induced by chloramphenicol and decreased CL induced by penicillin G and ampicillin, have any influence on the bacterial activity of PMNL requires further investigation.

ACKNOWLEDGMENT

This work was supported by grants from the County of Östergötland.

REFERENCES CITED


