

# Glucocorticoid effects on the uptake of antibiotics by human polymorphonuclear leukocytes

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The effects of glucocorticoids on the uptake of 8 antibiotics by human polymorphonuclear leukocytes (PMNs), and the subsequent bactericidal activity of PMNs with 5 of the antibiotics, were examined. The experiments demonstrated that a synthetic glucocorticoid, methylprednisolone at a concentration of more than 50 mg/l, inhibited the uptake of clindamycin, erythromycin and josamycin, but didn't inhibit the uptake of rifampicin, ofloxacin, chloramphenicol and rokitamycin. The bactericidal activity of PMNs with or without antibiotics was also affected by the presence of methylprednisolone.

**Key words:** antibiotic, uptake, PMNs, methylprednisolone

## Introduction

In treating the infections caused by facultative intracellular bacteria which can multiply in phagocytes, such as *Mycobacterium tuberculosis*, *Salmonella typhi*, *Legionella pneumophila* and *Staphylococcus aureus*, it is important that the antibiotics enter phagocytes effectively. Johnson *et al.*<sup>1)</sup> described the uptake of several antibiotics by rabbit alveolar macrophages. Clindamycin and erythromycin became highly concentrated in phagocytes. Rifampicin, ethambutol and chloramphenicol entry phagocytes was moderately good, but beta-lactam penetration was poor. Almost identical results are obtained with human polymorphonuclear leukocytes<sup>2-5)</sup>, human alveolar macrophages<sup>6)</sup> and human monocytes<sup>7)</sup>. These results were fairly compatible with the observations in experimental therapeutics with some antibiotics against *L. pneumophila* pneumonia in guinea pigs<sup>8)</sup>.

High doses of methylprednisolone (MP) are sometimes used both for adult respiratory distress syndrome (ARDS) and for a status similar to ARDS caused by bacterial infections. Some researchers have reported that high doses are effective<sup>9,10)</sup> but others have reported the ineffectiveness of such regimens<sup>11,12)</sup>. Recently, Jerome *et al.*<sup>13)</sup> reported

the experimental efficacy of high doses of MP when the drug is administered at an early stage. This matter is still controversial and the effect of glucocorticoid on the treatment of infections with antibiotics has not yet been clarified. In this study, we have focused on the effect of glucocorticoid on the uptake of antibiotics by phagocytes.

## Materials and methods

### Isolation of human polymorphonuclear leukocytes (PMNs)

Heparinized venous blood was collected from healthy adults. PMNs were isolated from whole blood, as described by Koga<sup>3)</sup>. Four milliliters of Mono-Poly-Resolving-Medium (Flow Laboratories Inc. Mclean, USA) were placed in a sterile plastic tube (17 by 100 mm, Corning, Iwaki glass, Japan), and a 5 ml portion of fresh blood was layered on top of the Mono-Poly-Resolving-Medium and was then centrifuged at 300 × g for 30 min at room temperature. The PMN fraction was collected and washed with medium. The medium consisted of RPMI 1640 (containing 0.5% phenol red; Gibco Laboratories, Grand Island, N.Y., USA) supplemented with 2 g/l of sodium bicarbonate and 20 mM N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES; Dojin Chemical Laborato-

ries, Kumamoto, Japan) and its pH was adjusted to 7.2 by adding 5 N HCl. To lyse contaminating red blood cells, 0.2% NaCl was added for 20 sec, and then an equal volume of 1.6% NaCl was added to make the suspension isotonic. The PMNs were washed twice and resuspended in the medium containing 5% autologous serum to make an approximately  $5.0 \times 10^6$  cells/ml suspension. The collected cells were more than 95% PMNs. PMNs viability was more than 95%, as determined by the trypanblue exclusion method.

#### Preparation of Antibiotic solutions.

The antibiotics investigated in this study were clindamycin hydrochloride (CLDM; Japan Upjohn Ltd., Tokyo, Japan), rifampicin (RFP) and ofloxacin (OFLX; Daiichi Seiyaku Co. Ltd., Tokyo, Japan) and ceftizoxime (CZX; fujisawa Pharmaceutical Co., Osaka, Japan).  $^{14}\text{C}$ -labelled antibiotics were also used: [ $^{14}\text{C}$ ] erythromycin (EM; 4.56 mCi/mmol), [ $^{14}\text{C}$ ] josamycin (JM; 3.70 mCi/mmol) (both from Daiichi Pure Chemical Co. Ltd., Japan), [ $^{14}\text{C}$ ] rokitamycin (RKM; 4.34 mCi/mmol; Toyo Jozo Co. Ltd., Japan) and [ $^{14}\text{C}$ ] chloramphenicol (CP; 50 mCi/mmol; New England Nuclear, MA, USA). All but the chloramphenicol were donated from commercial sources. RFP and macrolides were dissolved in methanol, OFLX in 1 N NaOH, and then diluted with the medium for use.

#### Incubation of PMNs with glucocorticoid.

MP was donated by Japan Upjohn Ltd., Tokyo, Japan. It was dissolved in 250  $\mu\text{l}$  of methanol, and was then diluted with the medium to make a final concentration of 5, 10, 20, 50, or 100 mg/l. The MP solution was pre-incubated in 5%  $\text{CO}_2$  in air at 37°C, which resulted in a medium pH of 7.2–7.4, as checked by the color of phenol-red. Then the solution was added to the purified PMN suspensions, mixed, and incubated at 37°C for 60 min prior to addition of the antibiotic.

#### Determination of antibiotic uptake with high-performance liquid chromatography (HPLC).

Antibiotic uptake was estimated with HPLC using the technique described by Koga<sup>3)</sup> Antibiotic solutions were added to PMNs and glucocorticoid combinations to make a final concentration of 50

mg/l. They were incubated at 37°C for 30 min, and the cell count and cell viability were again verified in a hemocytometer. They were centrifuged at  $250 \times g$ . After all of except 1 ml of the supernatant had been removed, the cells were re-suspended. To separate the extracellular antibiotics from the PMNs, the velocity gradient technique was used<sup>1,14)</sup>. Silicon oil (0.5 ml) (Toray Silicone Co. Ltd., Japan; a 6:5 mixture of SH 550 and SH 556) was placed in a microcentrifuge tube, and 0.5 ml of the PMN suspension was added. This was then centrifuged at  $12,000 \times g$  for 3 min at 4°C. The preparation was frozen at  $-70^\circ\text{C}$ . The extracellular solution layer and the PMN layer were obtained by cutting the frozen tube. The PMN layer was suspended in 1.0 ml of distilled water and then disrupted by two cycles of freezing and thawing. The supernatant was treated as the PMN layer. These samples were passed through a membrane filter (pore size, 0.22 micrometer; Millex-GV, Millipore Corp., Bedford, MA.) to remove cell debris, and the antibiotic concentration in these samples was measured.

HPLC was performed by using TRI ROTAR-V, UVIDEC-100-V, MODEL VL-614, MODEL DV-312 (Japan spectroscopic Co., Ltd.). The column used in these experiments was Nucleosil 5 C 18 (Sensyu Scientific Inc. Japan), and the conditions established for the drugs are shown in Table 1. Quantitative standards were run for each drug, and a standard curve was determined by using the total area under the peak of interest, as determined by electronic integration. The presumed cell volume of  $1 \times 10^7$  human PMNs was 2.7  $\mu\text{l}$ , assuming them to be spherical with a radius of 7–9  $\mu\text{m}$ .

#### Determination of antibiotic uptake using $^{14}\text{C}$ labelled antibiotics.

The methods used were described by Ishiguro *et al.*<sup>5)</sup> Antibiotic solutions (1.0 ml) were mixed with purified PMN suspensions (1.0 ml) to give a final concentration of 5 mg/l, and incubated at 37°C. Aliquots of the reaction mixture were sampled at an indicated interval. PMNs were separated from extracellular fluid by velocity gradient centrifugation as above with the following modification: 0.5 ml of silicon oil was placed on 0.02 ml of 88%

Table 1. HPLC apparatus and conditions<sup>a)</sup>

Antibiotic	UV (nm)	Mobile phase <sup>b)</sup>	RT (min)	Flow rate (ml/min)
Clindamycin	214	CH <sub>3</sub> CN (35%)	5.4	1.0
Rifampin	254	CH <sub>3</sub> CN (40%)	5.3	1.0
Ofloxacin	296	CH <sub>3</sub> CN (20%)	3.7	1.0
Ceftizoxime	254	CH <sub>3</sub> CN (10%)	5.0	1.0

<sup>a)</sup>HPLC was performed by using a TRIOTAR-V, Model VL-614, UVIDEC-100-V (Japan Spectroscopic Co., Ltd.). Column was Nucleosil 5 C18 (Sensyu Chemical Co., Ltd.).

<sup>b)</sup>Acetonitril mixed with 0.05 M KH<sub>2</sub>PO<sub>4</sub> (pH 6.0) for all but ofloxacin, and mixed with 0.2 M tetraethylammonium phosphate buffer (pH 1.85).

formic acid in a micro-centrifugation tube, and 0.5 ml of the PMN suspension was layered on top. This was then centrifuged at  $12,000 \times g$  for 3 min at 4°C. The PMN layer and the extracellular fluid fraction were separated by cutting the microtube and placing each of them in the tubes containing 10 ml of scintillation liquid (ACS-II, Amersham Ltd., USA). Their radioactivities were determined with a liquid scintillation counter (Aloka LSC 900, Japan).

#### MICs and MBCs against *S. aureus*

*Staphylococcus aureus* (ATCC 25923) was stocked in sterile skimmed milk at -70°C until use. It was subcultured on blood tryptic soy agar for 24 hours. The MICs were determined by the macrodilution method<sup>15)</sup> in cation-supplemented Mueller-Hinton broth. The MBCs were evaluated by the method described below. One hundred microliter of the aliquot in the tube without bacterial growth was inoculated on antibiotic-free tryptic soy agar plates. After 24 hours incubation at 37°C, the growth of bacteria on the new agar plates was evaluated. The MBCs were defined as the lowest drug concentration indicating 99.9% or greater killing of the initial inoculum.

#### Assays of intraphagocytic bactericidal activity

*Staphylococcus aureus* (ATCC 25923) was prepared as described above, harvested and suspended in pyrogen-free normal saline at a concentration of  $2 \times 10^8$  cfu/ml. The suspension was mixed with  $2 \times 10^7$ /ml of PMNs in medium containing 5% fresh autologous serum and incubated in 5% CO<sub>2</sub> in air for 45 min, and then lysostaphin (5 u/ml; Sigma Chemical Co., St. Louis, USA) was added; after 15

min, it was washed three times by centrifugation at 250 g for 5 min, and MP was then added as necessary. One hour later, the cell suspensions were mixed with antibiotics and incubated for 3 hours. The suspensions were diluted ten times with sterile distilled water and glass beads (Iuchi Seieido Co, Japan), and then vortexed for 20 sec. to disrupt the PMNs. The resulting solutions were appropriately diluted with sterile normal saline and inoculated onto tryptic soy agar plate (Difco Laboratories, Detroit, USA). Colonies were counted after two days incubation.

#### Statistical analysis

The statistical significance of differences between results was calculated by Student's *t*-test.

#### Results

##### Uptake of antibiotics by human PMNs

Penetrations into PMNs of the antibiotics investigated are shown in Table 2. CLDM and all macrolides showed high penetrations into PMNs, with intracellular concentrations being more than 10 times those of the extracellular compartment. The ratios of intracellular/extracellular concentration of OFLX, RFP and CP was 4 to 5, but that of CZX was extremely low ( $0.4 \pm 0.1$ ).

##### Effect of methylprednisolone on antibiotic uptake

The effect of MP on antibiotic uptake is shown in Fig. 1. MP, at a concentration of more than 50 mg/l, inhibited the uptake of CLDM, EM and JM significantly. But no effects were observed on RKM, OFLX and RFP. Penetration of CZX into PMNs was too small for the effect of MP to be evaluated (data not shown). Little change in the

Table 2. Uptake of antibiotics by polymorphonuclear leukocytes

Drug	Cin/Cex <sup>b)</sup>	Drug	Cin/Cex
Clindamycin	18.8±4.6	Erythromycin	15.5±0.2
Rifampicin	5.2±0.5	Josamycin	21.3±1.7
Ofloxacin	5.2±1.1	Rokitamycin	35.8±2.9
Ceftizoxime	0.4±0.1	Chloramphenicol	4.2±0.1

<sup>a)</sup>These are expressed as the ratio of intracellular/extracellular concentrations.

<sup>b)</sup>All data are the mean ± SD of three-five experiments.

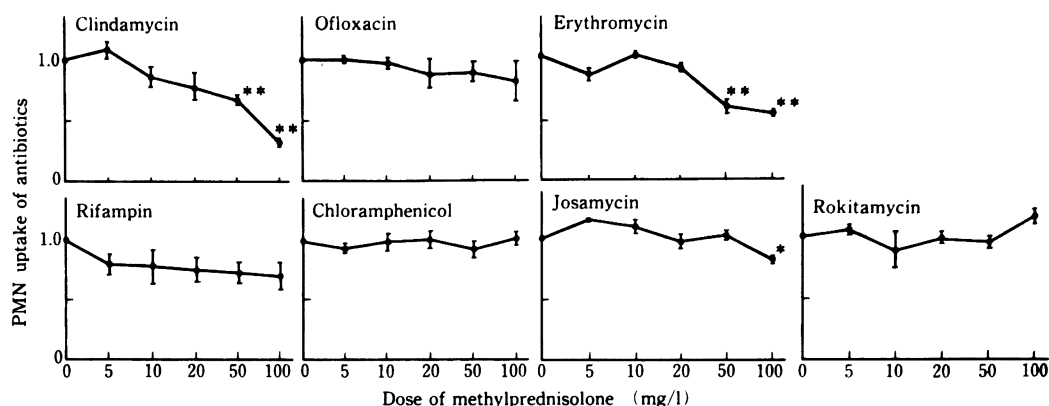


Fig. 1. The effect of methylprednisolone on antibiotic uptake. The ratios of the uptake of each antibiotic to uptake of that without methylprednisolone (as a control) are expressed. \*represents a significant difference from the control value ( $p < 0.05$ ). \*\*represents  $p < 0.01$ . These results are the mean ± SE of three-five experiments.

cell count or its viability was observed during these procedures.

#### MICs and MBCs

The MICs and MBCs of each drug against *S. aureus* were as follows: CLDM, 0.0313 mg/l and 16 mg/l respectively; RFP, 0.0078 mg/l and 0.0313 mg/l; EM, 0.25 mg/l and 32 mg/l; OFLX, 0.25 mg/l and 1.0 mg/l; CZX, 4 mg/l and 16 mg/l.

#### Effect of MP on bactericidal activity of PMNs with or without antibiotics

During phagocytosis of *S. aureus* by PMNs, viable bacteria decreased to one-tenth the number of the initial inoculum, but after another 3 hours the numbers of viable bacteria remained unchanged. At the time of antibiotic challenge, the numbers of viable bacteria were  $1.5\text{--}1.9 \times 10^5$  cfu/ml. After incubation for three hours, the number of surviving bacteria in control was  $1.53 \times 10^5$  cfu/ml. The

ratios of surviving bacteria to control are shown in Fig. 2. MP, at a concentration of 60 mg/l, affected bactericidal activity of PMNs alone, and it also affected the bactericidal activity of PMNs with all antibiotics except RFP

#### Discussion

Our results on uptake of the antibiotics investigated are fairly compatible with those reported elsewhere<sup>2-4)</sup> CLDM has been reported to be actively transported into phagocytes via a nucleoside transport system<sup>3,16)</sup>. The same mechanism was reported as being compatible for EM<sup>17)</sup> and other macrolides<sup>5)</sup>, but other investigators have reported that the uptake of EM into phagocytes was due to passive phenomenon and the pH gradient between the extracellular and intracellular environments<sup>4,18)</sup>. These questions are still controversial. RFP and CP also entered phagocytes effectively, but their entra-

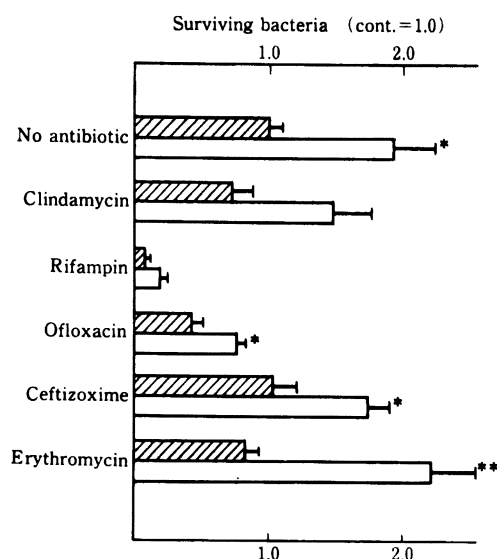


Fig. 2. Effect of methylprednisolone on the intracellular bactericidal activity of human polymorphonuclear leukocytes and antibiotics. These data are expressed as the ratio of the numbers of surviving bacteria to those of PMNs without antibiotic and methylprednisolone. Shaded columns express PMNs and 20 mg/l of each antibiotic. Open columns express those with methylprednisolone at a concentration of 60 mg/l. \*represents a significant difference between those with and without methylprednisolone ( $p < 0.05$ ). \*\*represents  $p < 0.01$ . These data are the mean  $\pm$  SE of triplicate tubes using PMNs from one healthy volunteer.

nce was independent of cell viability, and different from that of CLDM<sup>21</sup>. This may not be an active transport system but, rather, a simple diffusion. These drugs have an advantage in lipid-solubility. On the other hand, poor entrance of beta lactams into the cells may be related to their low lipid-solubility.

We also demonstrated that a high dose of MP affected the uptake of CLDM, EM and JM, but not that of RFP, OFLX, CP and RKM. Because CLDM has been reported to be transported into phagocytes via the nucleoside active transport system, high dose of steroid may block this active transport system. The mechanism(s) of transport of ma-

crolides into phagocytes remains controversial, as stated above, so how the steroid affects uptake is unclear. In these experiments, because the pH of the extracellular compartment was stable, decreases in the uptakes of macrolides cannot be explained by pH changes during the procedures.

Furthermore, some questions occur relative to the discrepancies between EM and RKM. RKM were more highly concentrated in PMNs than EM and JM, as we demonstrated. Dette & Knothe<sup>17)</sup> described how adenosine inhibited the uptake of EM by both human lymphocytes and PMNs. Ishiguro *et al.*<sup>5)</sup> reported that adenosine inhibited the uptake of RKM and JM, but not of EM. Kohno *et al.*<sup>19)</sup> demonstrated that the uptake of clarithromycin, a new macrolide, displayed saturation kinetics typical of a carrier-mediated membrane transport system, but it was different from that for EM. It can be speculated that the transport system(s) differs among the macrolides. OFLX has also been reported to be actively transported into phagocytes and this could be related to an amino acid transport system<sup>20)</sup>, but our experiments demonstrated that MP did not affect the transport of OFLX into PMNs. The mechanisms of the inhibitory effect of MP on uptake have not been clarified, but we speculate that the steroid might affect certain specific active transport systems.

Our data on the subsequent intracellular bactericidal activity of each antibiotic are consistent with the report of Hand & King-Thompson<sup>21)</sup>. But some discrepancies exist between the results of the killing assay for intracellular *S. aureus* and the uptake and MIC of the drugs. For example, EM and CLDM had strong anti-*S. aureus* activity and good penetration into PMNs, but little effect on intracellular *S. aureus*. These discrepancies might have resulted from differences in the incubation times of each experiment with or without PMNs. Another possibility is that the antibiotic alters PMNs activities. Hand & King-Thompson<sup>22)</sup> pointed out CLDM affected superoxide production by PMNs. Whether or not the intracellular environments are suitable for the bactericidal activity of the drugs should also be considered.

Glucocorticoids affect phagocytic functions, as

well as cell-mediated and humoral immunity<sup>23)</sup>. Indeed, superoxide production<sup>24)</sup> and the antimicrobial activity<sup>25)</sup> of phagocytes were affected by glucocorticoids. We demonstrated that MP affected the killing of intracellular *S. aureus* by human PMNs. Furthermore, the killing of intracellular bacteria by PMNs with antibiotics, especially with EM, was also affected. These results were related mainly to inhibition of the PMNs' bactericidal activity by MP, but might be slightly related to the inhibition of uptake of the drug by the steroid. However, we can not determine the clinical significance of these results with MP at a concentration of 60 mg/l, because this dose exceeds the clinical therapeutic drug level.

In summary, a high concentration of MP affected the uptake of CLDM, EM and JM by PMNs. It could be speculated that the steroid affected some specific active transport systems utilized for antibiotic uptake. This may be one of the important mechanisms by which glucocorticoids affect the treatment of infections caused by bacteria which can survive in host phagocytes. Further investigations of these phenomena should be carried out.

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### 抗生物質のヒト多核白血球内移行におよぼす糖質コルチコイドの影響

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8種の抗生物質のヒト多核白血球内への移行および細胞内抗菌活性におよぼす糖質コルチコイドの影響を検討した。実験では、50  $\mu\text{g/ml}$  以上の合成糖質コルチコイドである methylprednisolone (MP) を加えた場合に、clindamycin, erythromycin および josamycin の細胞内移行を抑制した。しかし、rifampicin, ofloxacin, chloramphenicol, rokitamycin の細胞内移行に対する影響はなかった。多核白血球単独の抗菌活性および抗菌剤との協調抗菌活性も MP の存在下に、抑制された。

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