Suppressive Effect of Antibiotics on Colony Formation from Human Megakaryocyte Progenitors (CFU-M) and Granulocyte-Macrophage Progenitors (CFU-GM)

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Abstract—β-Lactam antibiotics, moxalactam (LMOX), cefotaxime (CTX), flomoxef (FMOX), cefamandole (CMD), carbenicillin (CBPC) and sulbenicillin (SBPC), suppressed colony formation from human megakaryocyte progenitors (CFU-M) and granulocyte-macrophage progenitors (CFU-GM) dose-dependently. The suppressive potencies for both progenitors were weakest for CBPC and SBPC, moderate for LMOX and FMOX, and strongest for CMD and CTX. The stainability of glycoprotein IIb/IIIa complex in the megakaryocyte colonies by the monoclonal antibody was decreased by LMOX and CTX. These data suggest that β-lactam antibiotics directly suppress proliferation of CFU-M and CFU-GM.

Prolonged administration of high doses of β-lactam antibiotics is reported to induce neutropenia (1, 2), and most of the cases are believed to be caused by an immunological reaction characterized by rapid destruction of peripheral neutrophils (3, 4). β-Lactam antibiotics also suppress colony formation from granulocyte-macrophage progenitors (CFU-GM) in semisolid culture (5), indicating that they exert a direct toxic (non-immunogenic) effect on the CFU-GM. Although there are many reports showing the occurrence of thrombocytopenia after prolonged treatment with β-lactam antibiotics (6–8), the effect on megakaryocytes or their progenitors is not yet known.

In the present experiment, we examined the effect of β-lactam antibiotics on megakaryocyte formation from the progenitors (CFU-M) in vitro. At the same time, we examined glycoprotein IIb/IIIa in the megakaryocytes formed, since they are common platelet membrane glycoproteins and are recognized even in the megakaryocytes from the early stages of their differentiation (9).

Materials and Methods

Cells: Normal human bone marrow cells were obtained by aspiration from the sternum and put into a plastic tube containing preservative-free heparin (100 U/tube). The marrow mononuclear cells were separated by a Ficoll-Conrey gradient and washed three times with antibiotics-free Iscove's modified Dulbecco's medium (IMDM).

Cell culture: The mononuclear cells were re-suspended in a medium containing the test antibiotics (0–2 mM), 0.9% methylcellulose, 30% plasma of a patient with paroxysmal nocturnal hemoglobinuria (PNH) of AB blood type, 5% phytohemagglutinin-stimulated-leucocyte condition medium (PHA-LCM) prepared as previously described (10), 0.5% bovine serum albumin (BSA), and 5 × 10^{-5} M 2-mercaptoethanol. One ml of the suspension containing 2 × 10^5 cells was cultured in a 35 mm Petri-dish. On day 14, the aggregates consisting of more than 4 megakaryocytes were scored as colonies from CFU-M. Morphological features of the megakaryocytes in the colonies were essentially identical to those reported by Messner et al. (11). Megakaryocytes distinguished from the other cells in situ had a smooth surface, translucent cytoplasm and distinct cell borders, and were similar to macrophages in size. In order to confirm our
observation, some colonies were individually picked up, made into samples using a centrifuge for cell collection (Cytospin 2®, Shandon Co.) and stained with May-Giemsa or monoclonal antibody to platelet glycoprotein IIb/IIIa complex using the avidin-biotin peroxidase complex (ABC) method.

For the CFU-GM assay, cells obtained from bone marrow were cultured in the IMDM containing the test antibiotics, 0.9% methylcellulose, 20% fetal calf serum (FCS), and 200 U/ml GM-colony stimulating factor (CSF-Chugai®, Chugai Co.). One ml of the suspension containing $2 \times 10^5$ cells was cultured in a 35 mm Petri-dish at 37°C under a 5% CO$_2$ humidified atmosphere. On day 10, the aggregates consisting of more than 50 cells were scored as colonies from CFU-GM.

Monoclonal antibody to glycoprotein IIb/IIIa complex: The monoclonal antibody to platelet glycoprotein IIb/IIIa complex (HPL-1) was a gift from Dr. H. Shiku, the Faculty of Medicine of Nagasaki University.

Liquid culture: The mononuclear cells were suspended in the IMDM containing 30% PNH patient plasma, 5% PHA-LCM, 0.5% BSA and $5 \times 10^{-6}$ M 2-mercaptoethanol. Two ml of the suspension containing $10^5$ cells/ml were cultured using a tissue culture chamber slide (Lab. Tek® No. 4801). On day 14, the chamber was rinsed gently with PBS, dried by blowing and fixed with 10% formalin-ethanol mixture (1:9) containing 1 mM NaN$_3$. Next, the chambers were washed with PBS over night. The anti-glycoprotein IIb/IIIa complex monoclonal antibody was mounted on the chamber slide and incubated for 1 hr and stained with a kit for ABC staining (Vectastain® ABC kit, Vector Lab.). All the positive colonies were graded into one of three groups, marked (+ +), moderate (+), and weak (+), according to the intensity of the staining (Fig. 1).

Antibiotics: Six $\beta$-lactam antibiotics, moxalactam (LMOX), cefotaxime (CTX), flomoxef (FMOX), cefamandole (CMD), carbenicillin (CBPC) and sulbenicillin (SBPC), were freshly dissolved in the IMDM, filtered through a 0.22 $\mu$m milipore filter® and added to the culture medium to give final concentrations of 0.125, 0.25 and 0.5 mM. To the liquid culture medium, LMOX or CTX was added to give a final concentration of 0.125, 0.25 and 0.5 mM.

Results

All the six $\beta$-lactam antibiotics examined suppressed the megakaryocyte colony formation dose-dependently (Fig. 2). CBPC and SBPC were the weakest, LMOX and FMOX were moderate, and CMD and CTX were the strongest. The concentration for 50% inhibition of colony formation (IC50) of each antibiotic was calculated from the
Fig. 2. Effect of antibiotics on megakaryocyte colony formation. Data are expressed as percentages of the control values obtained in the culture mixture containing no antibiotics. • moxalactam, ○ FMOX, ■ cefotaxime, □ cefamandole, ▼ carbenicillin, ▽ sulbenicillin. The t-test was used for statistical analysis (*P<0.05, **P<0.01).

Table 1. IC50 of β-lactam antibiotics to colony formation from CFU-M

| Drugs          | IC50 (μg/ml) |
|---------------|-------------|
| Cephalosporins |             |
| moxalactam    | 268.7       |
| FMOX          | 148.5       |
| cefotaxime    | 87.5        |
| cefamandole   | 53.3        |
| Penicillins   |             |
| carbenicillin | 591.7       |
| sulbenicillin | 489.8       |

IC50: Concentration of drugs inducing 50% inhibition of colony formation.

average of suppressive ratio obtained from experiments using cells from different bone marrow donors (Table 1). Figure 3 shows the effects of the antibiotics on CFU-GM. Although CMD and CTX showed somewhat stronger suppression on CFU-GM than CFU-M, the order of the suppressive potency was the same as that for CFU-M.

The glycoprotein IIb/IIIa complex on megakaryocytes which was stained with the anti-glycoprotein IIb/IIIa complex monoclonal antibody was decreased by both LMOX and CTX, as shown in Fig. 4.

Discussion

Neutropenia and thrombocytopenia are common blood disorders induced by β-lactam antibiotics (12). These complications are believed to be caused by an immunological reaction (13), but there remains a possibility that a direct toxic effect of β-lactam antibiotics on the bone marrow is involved. Murphy et al. (14) suggested that an immune mechanism similar to the well-recognized penicillin-induced immune haemolytic anemia was involved in both penicillin induced neutropenia and thrombocytopenia from the observation that a complement-
Fig. 3. Effect of antibiotics on granulocyte-macrophage colony formation. Data were expressed as percentage of control values obtained in the culture mixture containing no antibiotics. • moxalactam, ○ FMOX, □ cefotaxime, □ cefamandole, ▼ carbenicillin, ▽ sulbenicillin. The t-test was used for statistical analysis (*P<0.05, **P<0.01).

Fig. 4. Effect of antibiotics on the stainability of glycoprotein IIb/IIIa complex in the megakaryocytes formed in the liquid culture mixture.

Fixing IgG penicillin antibody reacted with granulocytes and platelets of a patient in the presence of the drugs. However, they did not negate the direct effect of these drugs on neutrophils and thrombocytes. Schiffer et al. (15) reported that drug-dependent immune destruction of platelets was involved in the methicillin induced thrombocytopenia, but in addition, they suggested that the mechanism of platelet destruction would be more complex than a common explanation for “innocent-bystander” destruction of platelets coated with drugs. On the other hand, Neftel et al. (5) reported a direct toxic effect of β-lactam antibiotics on CFU-GM in vitro, and they showed a positive correlation between the IC50 of drugs to suppress granulocyte-macrophage colony formation in vitro and mean daily dose of drugs to induce neutropenia in clinical use. The data of the present study suggested that β-lactam antibiotics directly suppressed proliferation of CFU-M as evidenced by the decrease in megakaryocyte colony formation and glycoprotein IIb/IIIa complex formation in vitro. The suppressive effect of β-lactam antibiotics on the
CFU-M may be one of the causes for thrombocytopenia in their clinical use. In our study, the antibiotics also suppressed the granulocyte and macrophage colony formation in addition to the megakaryocyte formation. Neftel et al. (16) reported that β-lactam antibiotics suppressed the proliferation of not only CFU-GM but also chicken embryo liver cells and human and murine lymphoid cell lines. It is conceivable that the potency of the effect is dependent on the chemical structure of drugs and in the range of the influence is widespread including all the eucaryotic cells.

CMD and CTX showed somewhat stronger suppression on CFU-GM than on CFU-M. In our assay system, a granulocyte-macrophage aggregate containing less than 49 cells was regarded as a cluster and excluded from the colony count, while a megakaryocyte aggregate containing more than 4 cells was distinguished as a colony. This manner of evaluation may cause the difference in the effects of CMD and CTX on CFU-GM and CFU-M. However, it is also possible to speculate that the sensitivity of CFU-GM to these drugs is higher than that of CFU-M, since the other antibiotics used in the present study, LMOX, FMOX, CBPC and SBPC, showed no stronger suppression on CFU-GM.

Neftel et al. (17) found that a stocked penicillin-G solution showed a stronger suppressive effect on CFU-GM than a freshly prepared solution, and the degree of inhibition was closely correlated with the extent of breakdown of penicillin-G as assessed by thin layer chromatography. Similar data were obtained with some of the other penicillins and cephalosporins (16). Therefore, it is possible to speculate that the degradation products have caused such a suppressive effect on CFU-M and CFU-GM. We used freshly prepared drug solutions in this study, but some decomposition could have occurred in culture medium during the experiments. Neftel et al. (18) reported some cases of severe neutropenia following intravenous therapy with "aged" penicillin-G. They have suggested that penicillin-G degradation products are responsible for neutropenia (1), and neutropenia and most of the other adverse reactions after intravenous treatment with high dosages of penicillin-G will be prevented if the penicillin-G is always given in the form of fresh bolus doses (17). Few data are available about degradation and transformation products of β-lactam antibiotics, but such products have a chance to be relevant to neutropenia, thrombocytopenia or the other side effects. Further studies will be necessary to elucidate the effect of degradation products of β-lactam antibiotics on hematopoietic progenitor cells.

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