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Inhibition of Granulopoiesis in Vivo and in Vitro by β-Lactam Antibiotics

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β-Lactam antibiotics can induce severe neutropenia by a hitherto unknown mechanism. Fifty cases of β-lactam antibiotic-induced neutropenia (<1,000 neutrophils/mm³) from 17 hospitals were analyzed and compared with 140 literature cases. The incidence of neutropenia was 5%->15% in patients treated for ≥10 days with large doses of any β-lactam antibiotic but <0.1% with shorter duration of therapy. In >95% of cases recovery occurred between one to seven days after withdrawal of β-lactam antibiotics. Bone marrow aspirates were characterized by a lack of well-differentiated myeloid elements in the presence of numerous immature granulocyte precursors. Nine penicillins and eight cephalosporins inhibited in vitro granulopoiesis in a dose-dependent manner. There was a good correlation between the inhibitory capacity of β-lactam antibiotics in vitro and the doses inducing neutropenia in vivo. These observations may be relevant for therapy in the granulocytopenic patient.

Penicillins and cephalosporins (β-lactam antibiotics) are remarkably nontoxic within a broad dose range. Nevertheless, they induce a variety of adverse reactions, most of which are believed to be of an allergic nature [1]. Severe neutropenia is a well-known complication of high-dose therapy with β-lactam antibiotics [2]. There are still many unanswered clinical and pathogenetic questions. It is not known whether β-lactam antibiotic-induced neutropenia is a result of direct toxicity to the bone marrow or of immune-mediated bone marrow injury. In an attempt to increase our understanding of β-lactam antibiotic-induced neutropenia, we analyzed 50 of our patients with this problem, found an additional 140 literature cases, and assessed the influence of 17 selected β-lactam antibiotics on in vitro granulopoiesis.

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Materials and Methods

Selection of cases. Neutropenia was defined as a neutrophil count of ≤1,000/mm³. Criteria for assessing the causal relation between neutropenia and β-lactam antibiotics were adapted from those of Karch and Lasagna [3]. Neutropenia was thus induced by β-lactam antibiotics only if it followed a reasonable time sequence from administration of the drug, disappeared on stopping administration of the drug, and was not explainable by the patient's clinical condition.

Cases were excluded in which other drugs had to be considered as a possible cause or in which the cause-and-effect relation remained otherwise questionable.

Cases of β-lactam antibiotic-induced neutropenia came to our attention in one of the three following ways: (1) they were encountered during a multicenter study concerning the side effects of penicillin G [3] and other β-lactam antibiotics (authors' unpublished observations); (2) they occurred from September 1980 to December 1983 in the Medical Clinic of the University Hospital, Zurich; or (3) specimens from such cases were sent to us for evaluation of suspected β-lactam antibiotic-induced reactions.

The literature cases were obtained through a computer search (MEDLINE; National Library of Medicine, Bethesda, Md) from 1945 to 1983.

Analysis of clinical data. The total dose of β-lactam antibiotic given within the course of therapy before neutropenia developed was determined for each patient, and the mean daily dose (MDD) was

then calculated. In children the doses per kilogram of body weight were translated to the same relative doses given to a patient weighing 65 kg. Therapy breaks of <48 hr were ignored. If more than one β-lactam antibiotic was involved, the drug given while neutropenia developed was considered as the main cause. If two β-lactam antibiotics were given simultaneously, the one given in the higher total dose was considered as the main cause.

Concomitant symptoms of neutropenia were defined as follows. Eosinophilia was ≥10% of the white blood cell count or an absolute count of ≥ 750/mm³. Thrombocytopenia was ≤100,000 platelets/mm³. Reticulocytopenia was ≤0.25% of the red blood cell count. Fever was a temperature ≥38.0 C after at least three afebrile days (≤37.2 C) with no other cause of fever evident.

Bone marrow cultures. Cancellous bone samples from hematologically normal donors undergoing orthopedic surgery were collected in Hanks' solution.

A modification of the method of Pike and Robinson [4] for culture of myeloid progenitor cells was used. Dextran-sedimented bone marrow cells were plated at a concentration of 10^5 mononuclear cells/ml of supplemented minimal essential medium containing 0.3% agar and 20% pretested fetal calf serum. Human placenta-conditioned medium [5] served as the source of colony-stimulating factor. Cultures were set up in triplicate and incubated at 37 C in a humidified atmosphere plus 5% CO_2. After eight days aggregates of ≥40 cells were scored as colonies and those of three to 39 cells as clusters. Because cluster formation always paralleled colony formation, only the latter was considered for final evaluation.

β-Lactam antibiotics were dissolved in 0.9% NaCl at 100 mg/ml and further diluted with supplemented medium. In some experiments β-lactam antibiotics were added after the culture process was started by overlaying the drug in 0.1 ml of complete medium on top of the agar culture to the final concentration required.

Results

In vivo data. Table 1 summarizes data in 50 cases of β-lactam antibiotic-induced neutropenia from a total of 17 hospitals.

Ninety-six percent of these cases were detected only after therapy with β-lactam antibiotics of ≥10 days. In cases in which neutrophil counts were done daily, a decrease in count was seen over several days until total agranulocytosis was established or therapy with β-lactam antibiotics was discontinued.

In contrast to the duration of therapy, MDDs, and thus total doses given before neutropenia developed, varied markedly with different β-lactam antibiotics. In the cases of penicillin G, floxacillin, and ceftriaxone, with six or more cases each, these differences were significant (table 1).

Table 1. Data from 50 cases of β-lactam antibiotic-induced neutropenia from 17 hospitals.

Substance	No. of cases	Mean (range) duration of therapy (days)	Mean ± SD dose (g)		No. of cases with		
			Total	Daily	Rash	Eosinophilia	Fever
Penicillin G	26	23 (13–34)	268 ± 97*	11.6 ± 3.5†	11	10	11
Floxacillin	7	27 (21–40)	197 ± 67*‡	7.2 ± 1.1†§	4	2	3
Ceftriaxone	6	21 (8–25)	51 ± 29‡	2.3 ± 1.0§	0	3	2
Amoxicillin	2	13 (6–20)	216 ± 275	12.0 ± 12.0	0	0	0
Piperacillin	2	22 (17–27)	400 ± 232	17.4 ± 4.9	0	0	0
Cefotaxime	2	29 (27–31)	208 ± 101	7.0 ± 2.8	0	0	1
Apalcillin	1	16	162	9.0	0	1	0
Ticarcillin	1	15	516	34.4	0	1	0
Mezlocillin	1	36	594	16.5	1	1	1
Cephalothin	1	18	139	7.7	0	0	0
Cephacetrile	1	36	432	12.0	1	0	1
Total	50	23.2 (6–40)	243 ± 139	10.3 ± 5.9	17	18	19

* $P < .05$ by t test.
† $P < .01$ by t test.
‡ $P < .01$ by t test.
§ $P < .01$ by t test.

On discontinuation of therapy with β-lactam antibiotics, complete recovery took place within one to seven days.

Simultaneous eosinophilia, rash, and/or fever were observed in 35 (70%) of the cases (table 1). In 19 (38%) cases one symptom was seen, in 14 (28%) cases two symptoms were present, and in 2 (4%) cases all three occurred together. There did not appear to be any predictable combination of these symptoms, which disappeared within one to four days after cessation of therapy with β-lactam antibiotics.

Thrombocytopenia was noted in nine of 36 patients for whom platelet counts were done and reticulocytopenia in two of 19 cases.

Bone marrow findings. In 24 cases a bone marrow aspirate was available. Numerical values of differential counts are not presented because bone marrow aspiration was done at different intervals with regard to the nadir of neutropenia.

Lack of well-differentiated myeloid elements in the presence of still numerous granulocyte precursors was a typical finding. The mitotic pool of granulopoiesis was found to be normocellular or even hypercellular; however, occasionally it was hypocellular. Severe hypocellularity has only been found in cases of total agranulocytosis. Such a "maturation arrest" in aspirates taken immediately at the termination of therapy was usually at the promyelocyte stage, whereas ≥24 hr later it was at the myelocyte or metamyelocyte stage.

In four cases bone marrow aspirates were performed at the beginning of the decrease in number of peripheral granulocytes. In these cases myeloblasts and promyelocytes were still numerous; myelocytes were greatly reduced in number or lacking, whereas the numbers of metamyelocytes, band forms, and segmented granulocytes were still normal.

In vitro data. Addition of various β-lactam antibiotics to normal bone marrow cultures resulted in a dose-dependent inhibition of myeloid colony formation, as illustrated for penicillin G, cloxacillin, and ceftizoxime in figure 1. There were impressive differences in absolute potency of the β-lactam antibiotics studied. However, the shape of the dose-effect curves was always similar. Concentration of β-lactam antibiotics leading to 50% inhibition of colony formation (IC_{50}) were interpolated from the dose-effect curves and served as a simple measure of in vitro toxicity (table 2). Cephalosporins were from three to 25 times more effective than semisynthetic penicillins. The sensitivity of cells from different bone marrow donors to a particular substance varied greatly. In some instances, especially in the low concentration range, deviations from median values exceeding 50% were seen. However, the relation of inhibition of a given bone marrow sample by different β-lactam antibiotics remained constant.

Overnight exposure (18 hr) of bone marrow cells to β-lactam antibiotics in concentrations otherwise resulting in 90%-100% inhibition, followed by washing for removal of drug, did not affect colony formation. The trypan blue-exclusion test showed full viability of cells during preincubation as compared with controls (viability, >95%).

However, when β-lactam antibiotics were added after the culture process had begun, the inhibitory effect was progressively diminished. In five culture

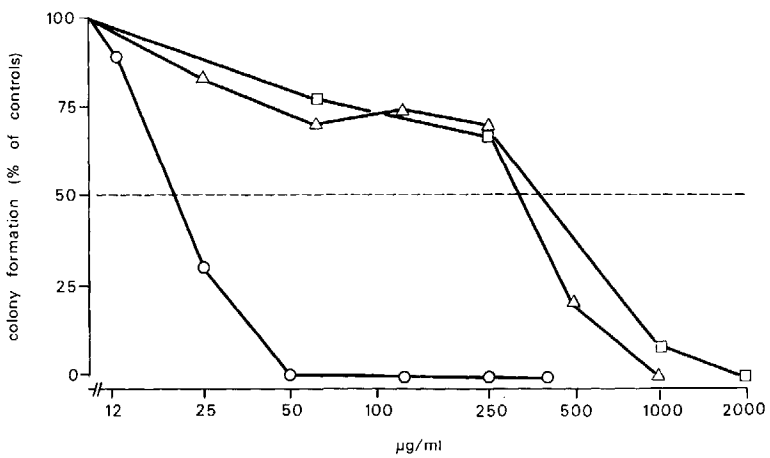

Figure 1. Inhibition of colony formation by ceftizoxime (○), cloxacillin (△), and penicillin G (□). Data are median values from differing numbers of cultures (table 2).

Table 2. IC_{50} values of penicillin G, eight semisynthetic penicillins, and eight cephalosporins in vitro.

Substance	Medium (range) IC_{50} (μg/ml)	No. of cultures
Penicillin		
Mezlocillin	550 (280->1,000)	4
Ticarcillin	520 (300->1,000)	9
Piperacillin	470 (153->1,000)	5
Methicillin	470 (155-670)	6
Penicillin G	380 (45-560)	15
Oxacillin	370 (62.5-560)	4
Cloxacillin	340 (251-380)	4
Azlocillin	320 (12-1,000)	6
Floxacillin	105 (<62.5-230)	6
Cephalosporin		
Moxalactam	35 (12-62)	4
Cefoxitin	32 (16-36)	3
Ceftriaxone	30 (2-43)	5
Ceftizoxime	20 (10-33)	4
Cephalothin	16 (16-17)	3
Cefazolin	15 (7-18)	3
Ceftazidime	12 (1-16)	3
Cefotaxime	7 (1-8)	4

experiments with ceftazidime we found inhibition to be reduced by ~50% when the drug was added after 24 hr and by ~75% after 48hr. Addition after ≥72 hr no longer induced significant inhibition.

Discussion

In vivo data. In the literature we found an additional 140 cases fulfilling the above criteria for β-lactam antibiotic-induced neutropenia (table 3). The literature data largely confirmed the observations with our 50 subjects and allowed estimation of the incidence of β-lactam antibiotic-induced neutropenia and the risk of a relapse when the patient is rechallenged.

Relation of neutropenia to duration and dosage of therapy with β-lactam antibiotics. In 91% of the literature cases neutropenia did not occur before day 10 after therapy was started. Indeed, duration of therapy with β-lactam antibiotics of at least 10 days appears to be a threshold for inducing neutropenia. In

Table 3. Data from 140 literature cases of β-lactam antibiotic-induced neutropenia.

Substance	No. of cases	Mean (range) duration of therapy (days)	Mean ± SD dose (g)		Reference
			Total	Daily	
Oxacillin	22	24 (17-75)	249 ± 114	11.1 ± 4.8	6-16
Penicillin G	20	24 (10-36)	335 ± 301	14.1 ± 12	16-31
Cephalothin	18	20 (2-34)	141 ± 83	7.4 ± 2.9	11, 32-38
Nafcillin	18	18 (2-38)	169 ± 68	10.5 ± 2.8	39-48
Methicillin	9	17 (4-21)	171 ± 102	9.5 ± 3.4	49-54
Cloxacillin	8 (+ 1)*	25 (17-40)	216 ± 82	9.3 ± 3.5	24, 55-57
Ampicillin	8	20 (9-32)	198 ± 125	10.9 ± 7.7	16, 23, 58-62
Carbenicillin	6	20 (15-24)	536 ± 213	25.8 ± 9.3	23, 24, 63
Cefotaxime	5	22 (9-37)	171 ± 162	6.5 ± 3.7	16, 64, 65
Piperacillin	4	24 (22-26)	373 ± 142	15.4 ± 5.9	46, 66
Ticarcillin	4	15 (9-29)	246 ± 111	18.5 ± 5.5	67-69
Ceftazidime	3	14 (7-25)	66 ± 73	4.1 ± 2.3	16, 70
Amoxicillin	2	21 (20-22)	74 ± 2	3.5 ± 0.3	16
Cephaloridine	2	16 (5-27)	115 ± 70	9.6 ± 4.9	71, 72
Mezlocillin	2	17 (12-22)	134 ± 65	9.5 ± 7.8	73, 74
Ceftriaxone	2	6 (5-7)	30 ± 7	4.9	75
Azlocillin	1	19	223	11.7	76
Phenethicillin	1	23	138	5.0	29
Cefamandole	1	24	254	10.6	11
Cefuroxime	1	28	168	6.0	30
Cefoperazone	1	18	108	6.0	77
Cephapirin	1	6	24	4.0	78
Total	140	20.6 (2-75)	224 ± 178	10.95 ± 7.5	

* The additional case was seen after 263 days of peroral therapy (1.5 g per day).

Table 4. Incidence of neutropenia in four different series of patients treated with methicillin.

Reference	Mean duration of therapy (days)	Mean total dose (g)	Incidence (%)
Nahata et al. [44]	6.5	45	0
Kitzing et al. [51]	10	130	0
	21	273	12
Kancir et al. [85]	27	>325	12.2

patients treated with large doses of β-lactam antibiotics for ≥10 days, neutropenia developed with an incidence of from 5% to >15% [3, 44, 48, 51, 54, 64, 66, 75, 79–84]. With shorter therapy courses, however, the incidence can be estimated to be as low as 0.1%.

This point can be illustrated with three articles that studied a total of 144 patients receiving methicillin (table 4). Yow et al. [54], in a 10 year assessment of methicillin-associated side effects, found leukopenia to occur in 16 of 102 children who received 200 mg of methicillin/kg per day for ≥10 days. In four instances the absolute neutrophil count dropped to <500/mm³. In contrast, nearly 3,000 patients who received the drug for <10 days did not develop leukopenia.

Besides intrinsic myelosuppressive activity, pharmacokinetic properties might be important for the overall myelotoxicity of a given β-lactam antibiotic. Because ceftriaxone has an very long serum half-life, neutropenia already occurred in a dose range lower than with any other β-lactam antibiotic. However, this observation need not imply an increased risk because this substance reaches sufficient antibacterial activity with lower daily doses as well. Pharmacokinetic factors might also account for the fact that in the 15 literature cases in which treatment was oral [10, 16, 29, 56, 57, 61] or im [29, 33, 52, 53, 72, 78] total doses of β-lactam antibiotics given were lower than when treatment was iv, although duration of such treatment was the same.

Complete recovery within one to seven days after discontinuation of therapy with β-lactam antibiotics was observed in 94% of the well-documented literature cases. Duration of recovery of four or more days was only seen in cases of total agranulocytosis. Permanent damage of granulopoiesis was never seen. In one case a decreased neutrophil count remained constant [41], and in three cases the count even returned to normal levels during continuous therapy [32, 39, 42].

Exposure to the same or a different β-lactam antibiotic induced a relapse in only 12 (31%) of the 39 patients rechallenged (including our cases) [6, 7, 11, 13, 26, 27, 29, 30, 32, 36, 40–42, 46, 49, 52, 59, 63, 67, 69, 85]. The induction of a relapse depended clearly on the duration and dosage of therapy with β-lactam antibiotic, as did the first induction of neutropenia. This dependence was true irrespective of the time between complete recovery from neutropenia and rechallenge. There have been relapses reported months and even years after the first episode of neutropenia [27, 29].

Relation of neutropenia to other symptoms. Symptoms accompanying neutropenia in the literature cases were similar to those seen in our cases (table 5).

Eosinophilia, rash, and fever combined were reported in 10 cases, two of the symptoms appeared in 17 cases, and an isolated symptom was seen in 37 cases. The three symptoms appear to represent a syndrome that is pathogenetically related to neutropenia.

Thrombocytopenia and reticulocytopenia occurring along with neutropenia suggest that β-lactam antibiotics can inhibit all three hematopoietic cell lines. However, when bone marrow aspirates were analyzed no clear substrate for this inhibition could be identified.

In vitro data. All of the β-lactam antibiotics studied inhibited in vitro granulopoiesis dose dependently. However, progenitor cells were not directly killed by β-lactam antibiotics. If the bone marrow cells were incubated overnight with β-lactam antibiotics followed by washing and plating, no inhibition of colony formation was observed, and the developmental potential of the cells appeared to be maintained. However, β-lactam antibiotics were only fully effective during the first 24 hr of the culture pro-

Table 5. Concomitant symptoms in 140 literature cases of β-lactam antibiotic-induced neutropenia.

Symptom	No. positive/no. of patients studied (%)
Eosinophilia*	25/79 (32)
Rash	26/64 (41)
Fever*	52/76 (68)
Thrombocytopenia	9/69 (13)
Reticulocytopenia	12/27 (44)

* If exact values were lacking, these symptoms were considered present when they were at least stated qualitatively.

cess. An inhibitory effect of β-lactam antibiotics on in vitro granulopoiesis is thus seen only when these drugs are present during the early proliferative phase. Furthermore, we have noted that addition of acute-phase serum and/or lymphocytes from two patients with penicillin G–induced neutropenia to autologous bone marrow cultures did not alter the effects of β-lactam antibiotics [86]. These in vitro findings taken together are consistent only with a nonimmunologic pathogenesis of β-lactam antibiotic–induced neutropenia, assuming that similar mechanisms are operative in vivo.

Several articles have reported drug-dependent antibodies to neutrophils, which could explain β-lactam antibiotic–induced neutropenia by a mechanism similar to that seen in penicillin-induced immunohemolytic anemia [16, 25, 87, 88]. With a solid-phase RIA we found circulating IgG antibodies to penicillins in a series of patients after treatment with large doses of penicillin G [2, 89] as well as ampicillin, floxacillin, or piperacillin [90], whether the subjects had neutropenia or not. Circulating antibodies to penicilloylated cells may only reflect an increase in level of circulating antibodies to drug, which appears to be common after very high-dose therapy with β-lactam antibiotics.

Inhibitory capacity (IC_{50}) of different β-lactam antibiotics in bone marrow cultures correlates well with the MDDs inducing neutropenia in patients (figure 2). Bone marrow cultures seem to allow for an approximate estimation of the myelotoxicity that would be expected in vivo. However, there are important discrepancies between semisolid agar cultures and the situation in vivo, mainly concerning the kinetics and protein binding of β-lactam antibiotics. On the basis of preliminary experience, we believe that with a liquid culture system allowing for multiple addition and removal of the drug during the culture process, an improved test model could be built up.

The inhibitory effect of β-lactam antibiotics on cell proliferation appears not to be restricted to granulopoiesis. Some cephalosporins were found to dose dependently inhibit lymphocyte proliferation after stimulation with mitogens [91, 92]. Preincubation with moxalactam followed by washing did not affect viability and subsequent proliferation of cells [92]. Similar effects were shown with penicillin G in cultures of isolated rat hepatocytes [93, 94]. It remains unclear how this inhibition of cell proliferation is achieved. Because the effect can be abolished by thorough washing after preincubation, stable

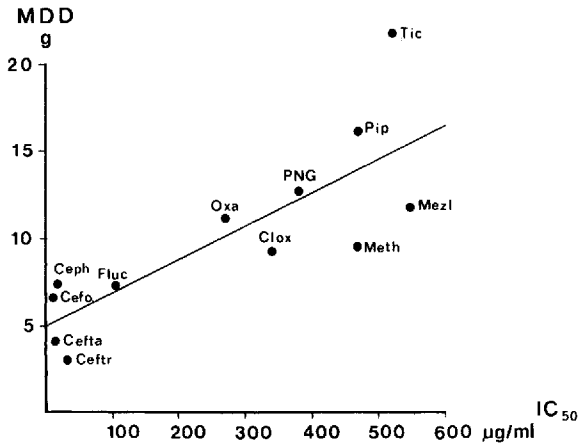

Figure 2. Correlation ($r = .804$; $P < .01$) of MDDs inducing neutropenia in vivo with IC_{50} values in vitro: PNG = penicillin G; Cefo = cefotaxime; Cefta = ceftazidime; Ceftr = ceftriaxone; Ceph = cephalothin; Clox = cloxacillin; Fluc = flucloxacillin (Floxacillin); Meth = methicillin; Mezl = mezlocillin; Oxa = oxacillin; Pip = piperacillin; and Tic = ticarcillin.

covalent binding of carbonyl groups to amino groups of membrane proteins or simple uptake and binding to intracellular structures are unlikely to be decisive pathogenic steps.

Although the in vitro observations as a whole cannot yet be interpreted comprehensively, they match well the typical findings in the bone marrow aspirate of patients with β-lactam antibiotic–induced neutropenia. As described above, immature myeloid precursor cells are usually numerous, whereas more mature forms are lacking. Furthermore, bone marrow aspirates taken early during development of neutropenia suggest that postmitotic maturation is not affected. β-lactam antibiotics appear not to affect the granulocytic stem cell itself but might exert their inhibitory effects on other precursor cells within the mitotic pool. Consequently, "proliferation arrest" would describe what seems to happen better than the generally used phrase "maturation arrest." This particular proliferation disturbance may explain the rapid recovery from neutropenia generally seen after withdrawal of therapy with β-lactam antibiotics.

Clinical consequences. In only eight (4.2%) of the 190 cases analyzed did a secondary infection ascribable to neutropenia develop, and it was never fatal. There might be some bias in that cases without documented recovery on discontinuation of therapy with β-lactam antibiotics were not considered. There are literature cases otherwise fullfulling our

criteria in which death occurred from various causes before the peripheral granulocyte count rose again [73]. Nevertheless, the risk for important sequelae of β-lactam antibiotic–induced neutropenia appears to be low.

The more important question is what consequences do the myelosuppressive effects of high-dose therapy with β-lactam antibiotics have in the treatment of the granulocytopenic patient, especially after cytostatic treatment? In such cases long-term therapy with cumulative doses, often of >200 g, of one or more β-lactam antibiotics are given. In hematologically normal individuals this treatment would lead to neutropenia in 5% to 15% of those treated. Currently several studies are being conducted to assess whether double β-lactam antibiotic regimens can reduce toxicity of β-lactam–aminoglycoside combinations without giving up double antibiotic coverage. Indeed, in a recent trial a double β-lactam antibiotic regimen resulted in lower nephrotoxicity but also in a significantly longer duration of granulocytopenia [95].

In an ongoing retrospective study we have analyzed 227 granulocytopenic episodes (granulocyte count, <500/mm³) in patients with acute myelogenous leukemia. We found that beginning therapy with β-lactam antibiotics simultaneously with or very early after cytostatic treatment is associated significantly ($P < .001$) with a longer duration of granulocytopenia. Consequently, in patients with granulocytopenia for >20 days, the mean total dose of β-lactam antibiotics and the mean dosage of β-lactam antibiotic per granulocytopenic day were disproportionately higher as compared with the remaining patients treated with β-lactam antibiotics (authors' unpublished observations).

Duration and severity of granulocytopenia remain the most important prognostic factors for the patient with neutropenia after cytostatic therapy for malignant disease. It is very important to clarify whether β-lactam antibiotics can delay regeneration of myelopoiesis after cytostatic therapy and how such an effect could be recognized.

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