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MINIREVIEW

Effects of β-Lactam Antibiotics on Proliferating Eucaryotic Cells

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INTRODUCTION

Most fungal toxins interact specifically with well-defined target structures, thereby reaching very high selectivity for the cell type attacked (17). The susceptibility of bacteria to beta-lactam antibiotics, as an example, is due to a number of penicillin-binding proteins on the bacterial plasma membrane. The eucaryotic cell lacks such target structures. Its insusceptibility to beta-lactams seems to be a logical consequence. Accordingly, beta-lactams are generally considered to be remarkably nontoxic in humans, with most of their side effects believed to be immune mediated (6, 10).

Clinical and experimental observations shed some doubt on this belief. In this minireview, recent evidence that beta-lactam antibiotics can act on cultured eucaryotic cells is reviewed and an evaluation is made concerning potential clinical impact.

EARLY EVIDENCE FOR BETA-LACTAM EFFECTS ON PROLIFERATING EUCARYOTIC CELLS

From 1973 to 1982, several reports presented evidence of antiproliferative effects of beta-lactam antibiotics on cultured eucaryotic cells. The cell types included rat liver cells (46, 50), human fibroblasts (16), and lymphoid cells (1, 3, 25, 26, 45). These studies examined parameters such as cell proliferation, incorporation of amino acids into cell protein, and incorporation of thymidine into DNA. Regardless of the parameters examined, the effects of beta-lactams were clearly dose dependent. However, viability of cells was not directly affected, at least with short-term exposure, even to high concentrations of beta-lactams.

In part, these studies were done to evaluate methodological implications of in vitro procedures. The underlying mechanisms and possible consequences for the clinical use of beta-lactam antibiotics remained unclear. Although beta-lactam concentrations leading to 50% inhibition of the various biological activities assessed were generally rather high, in some experiments they were in the range reached in the sera of patients or when beta-lactams were added routinely to cell culture media.

BONE MARROW TOXICITY OF BETA-LACTAMS

Recently, data from 190 cases of beta-lactam-induced neutropenia (neutrophil count, ≤1,000/μl) were analyzed (32). The incidence of neutropenia was estimated to range from 5% to more than 15% in patients treated for more than 10 days with large doses of any beta-lactam but to be less than 0.1% with shorter-term therapy. On beta-lactam withdrawal, rapid recovery within 1 to 7 days occurred. Reexposure to the same or a different beta-lactam induced relapses in only one-third of the patients. Similar to the first induction of neutropenia, relapses again were dependent on duration and dosage of beta-lactam therapy. Bone marrow aspirates typically showed a lack of well-differentiated myeloid elements in the presence of numerous immature granulocyte precursors. Thrombocytopenia and reticulocytopenia frequently occurred along with neutropenia, indicating that beta-lactams can simultaneously affect all three hematopoietic cell lines. On the whole, these findings suggested a toxic rather than an immune mechanism underlying beta-lactam-induced neutropenia and the other accompanying cytopenias.

Indeed, it was found that a large variety of beta-lactam antibiotics dose dependently inhibit human in vitro granulopoiesis in a semisolid agar system (32, 33). There were, however, marked differences in absolute inhibitory potency of beta-lactams. Beta-lactam concentrations leading to 50% inhibition of myeloid colony formation (aggregates of 40 or more cells after a 6-day culture period) varied within a range as broad as 6 μM (cefotiam) to >2 mM (aminopenicillins and monobactams). Cephalosporins as a group were clearly 3 to 25 times more potent than penicillins, and imipenem behaved like a cephalosporin, whereas monobactams were practically ineffective.

Interestingly, some degradation products of beta-lactam antibiotics (as spontaneously formed in aqueous solution) appeared to underly proliferation inhibition rather than the native molecules themselves (33). In the case of penicillin G, the degree of inhibition was closely correlated with the extend of breakdown, as assessed by thin-layer chromatography. However, with some compounds longer degradation periods of 10 or more days eventually led to a gradual loss of inhibitory capacity. Cell viability was not significantly affected by 24-h exposure to even very high beta-lactam concentrations; on the other hand, beta-lactams were fully effective only when added during the first 12 to 24 h of the culture period and hence appeared to affect particularly the early proliferative states of the granulocyte precursor cells but not any maturation step. Addition of lymphocytes or acute-phase sera from patients with beta-lactam-induced neutropenia to autologous bone marrow cultures did not alter the effects of beta-lactams (31).

Although not formal proof, these in vitro findings are consistent with a nonimmunologic pathogenesis of beta-lactam-induced neutropenia, provided that similar mechanisms take place in vivo. In addition, the inhibitory capacity of different beta-lactam antibiotics in bone marrow cultures has been found to correlate well with the mean daily beta-lactam doses having induced neutropenia in patients (32).

The suppressive effects of beta-lactam antibiotics on colony formation from bone marrow-derived progenitor cells have been confirmed. Marie et al. showed similar toxicities with mezlocillin, piperacillin, ceftriaxone, and ceftazidime for human in vitro granulopoiesis (27), and Maruyama et al.

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reported analogous suppression of megakaryocyte colony formation by six more beta-lactams (four cephalosporins and two penicillins) (28). The detailed results in these studies were in basic agreement with the findings noted above.

BETA-LACTAM TOXICITY FOR OTHER CELLS

Evidence for analogous antiproliferative effects was obtained with normal and malignant cells from various sources other than normal bone marrow. In chick embryo liver cells, beta-lactam antibiotics similarly inhibited [^{14}C]valine incorporation into trichloroacetic acid-insoluble material as previously found by Vonen and Morland (50) and Schwarze and Seglen (46) in rat liver cells (34). Beta-lactams concentration dependently inhibited two continuously growing T-cell lymphomas, namely EL-4 and YAC-1, as well as generation of cytotoxic T cells, in a secondary in vitro antiviral immune response (21). Further corroboration of the antiproliferative beta-lactam effects was obtained in cultures of the human erythroleukemic cell K-562 (4), the promyelocytic cell HL-60 (51), and the mouse B-cell lymphoma BCL-1 and the mouse myeloma cell NS-1 (I. Guldenschuh, P. Cottagnoud, and K. A. Neftel, Abstr. Annu. Meet. Swiss Soc. Microbiol. 1987).

Again, in all studies in which beta-lactam degradation products have been considered, their suppressive potency was strongly increased compared with that of native compounds. Studies in which freshly dissolved and degraded beta-lactam antibiotics were added at various times to the culture media were compatible with the assumption that beta-lactam degradation products are the only active substances (4, 21).

MECHANISM OF BETA-LACTAM EFFECTS

Some attempts to elucidate the mechanisms underlying beta-lactam effects on eucaryotic cells were made by Cottagnoud and Neftel in liquid cultures of K-562 cells (4). First, although this cell type proved to be three to five times less susceptible to beta-lactam antibiotics than normal human myeloid precursor cells in the same culture system, an identical order of efficacy among 14 different beta-lactams emerged, as previously found in human bone marrow cultures. Second, in all experiments that were done in this system, positive effects of beta-lactams were again clearly and invariably enhanced upon previous degradation of beta-lactams. Sequential monitoring of K-562 cultures over 4 to 5 days revealed a particular divergence of beta-lactam effects on different parameters: with beta-lactam concentrations already inducing a significant decline of cell proliferation and protein synthesis, a transient but marked increase of [^{3}H]thymidine incorporation was seen before complete proliferation inhibition occurred. Flow cytometrically, this thymidine peak corresponded to stainable cellular DNA. However, the phenomenon was observed only with very high beta-lactam doses and within a narrow dose range, e.g., 500 to 800 μg of freshly dissolved ceftazidime per ml. When aged beta-lactam solutions were added, extra thymidine uptake not only increased but occurred much earlier during the culture process, again hinting that beta-lactams are possibly not effective until ad hoc formation of degradation products in cell cultures occurs.

The effects of beta-lactam antibiotics in K-562 cells proved to be cell cycle dependent. Cells that were arrested at the G_1-S boundary were almost completely protected from beta-lactam effects, and cells in the S phase were much more susceptible to beta-lactams than were unsynchronized ones. Hence, the evidence suggests that beta-lactams or their degradation products act on eucaryotic DNA synthesis and that their effects are restricted to the S phase of the cell cycle.

Recent experimental data suggest that an intracellular target of beta-lactam antibiotics is the cellular replicative enzyme DNA polymerase α (20; U. D. Huynh, K. A. Neftel, S. Spadari, and U. Hübscher, submitted for publication). Beta-lactams inhibit both crude and highly purified DNA polymerase α from a variety of eucaryotic sources, including human sources. On the other hand, DNA polymerases β and γ involved in DNA repair and mitochondrial DNA replication, respectively, were insensitive to beta-lactams or to any beta-lactam derivative tested. In addition, beta-lactams inhibited the two DNA polymerases from herpes simplex and vaccinia viruses.

In sum, the effect of beta-lactam antibiotics is evident in DNA polymerases that are sensitive to aphidicolin (47), a reversible blocker of various replicative DNA polymerases, which interestingly is itself produced by a *Cephalosporium* species, namely *Cephalosporium aphidicolae*.

The inhibitory effect is enhanced by up to several hundredfold if degraded beta-lactam antibiotics are tested, thus confirming the observations made with cell cultures (Huynh et al., submitted). Furthermore, treatment of beta-lactams with beta-lactamase similarly decreases the K_i, suggesting that the effect of degradation might be linked to opening of the beta-lactam ring.

STRUCTURE-EFFECT RELATIONSHIP

By analyzing general effects of beta-lactam antibiotics on various parameters as observed in very different cell types, two features concerning the structure-effect relationship clearly emerge. First, 3-aminomonobactamic acid and its derivatives, i.e., monobactams, were invariably inactive. Hence, a second ring fused to the beta-lactam ring appears to be indispensable. Second, cephalosporins as a group, as well as some of their degradation products, were from 3 to over 25 times more active than freshly dissolved or "aged" penicillins. This was true regardless of the side chains present on C-6 in penicillins or on C-3 and C-7 in cephalosporins. The relationship of efficacies between penicillins and cephalosporins was reproduced when their basic structures, namely 6-aminopenicillanic acid and 7-aminocephalosporanic acid, were compared. Minor differences within the individual beta-lactam subclasses appear to be due to variability in side-chain configuration.

In cell-free DNA replication systems, this hierarchy of beta-lactam toxicity was broadly reproduced. However, there were some discrepancies, e.g., penicilloic acid, which has practically no effect on living cells (31), significantly inhibited in vitro DNA replication (Huynh et al., submitted). The most likely explanation for this is differences in penetration behavior of various beta-lactams into the cell. However, the observation that degraded beta-lactams are more active when tested in living cells was invariably confirmed. The difference in activities between fresh and aged compounds was even more pronounced, and in some instances the increase of activity due to degradation was more than 200-fold (Huynh et al., submitted).

An open beta-lactam ring appears to be required for the ultimate interaction of beta-lactam derivatives with the relevant receptor structures. Opening of the beta-lactam ring with beta-lactamase immediately before testing led to in-

creases in activity similar to those found with the most active degradation product (Huynh et al., submitted). On the other hand, further spontaneous degradation of some beta-lactamase-treated beta-lactams eventually led to inactive products. Because the activity of some derivatives is increased by more than 200-fold compared with that of the native molecules, it is conceivable that the active products are formed from only a very small fraction of the whole beta-lactam amount available, e.g., in culture media.

Nevertheless, most in vitro effects both in cultured cells and in cell-free DNA replication systems are seen only at moderate-to-very-high beta-lactam concentrations. When it is assumed that the effects observed in cell-free DNA replication operate also in living cells, significant accumulation of beta-lactams or their degradation products and, possibly, intracellular formation of the latter have to be postulated. Little is known about cellular uptake of beta-lactam antibiotics, and no information is available about uptake, distribution, or intracellular formation of beta-lactam derivatives. Except in erythrocytes (23), beta-lactams do not accumulate intracellularly. In different cell types, the ratio of cellular-to-extracellular concentration has been found to be less than 1.0 (2, 8, 9, 14, 18, 22, 40), although there is recent evidence for a common transport system of beta-lactams in isolated rat hepatocytes (48). Very recently, however, conversion of penicillin G into a basic derivative led to accumulation within macrophages by one order of magnitude (41). Identification of the structures that are most active in cell cultures on the one hand and in cell-free DNA replication systems on the other hand is necessary to decide whether intracellular conversion of beta-lactams plays any role at all.

PRACTICAL CONSEQUENCES

To what extent the in vitro studies summarized here have clinical consequences remains to be clarified. Meanwhile, some information is available pointing to possible directions of future investigations.

(i) Pathogenesis of adverse reactions. As outlined above, there are good reasons to assume that beta-lactam-induced neutropenia is the result of a direct toxic effect of beta-lactam antibiotics on proliferating myeloid precursor cells (32). Whether analogous mechanisms might underly other adverse reactions to high-dose, prolonged beta-lactam therapy has not been studied thoroughly. Several reactions are striking because they share some prominent clinical features with beta-lactam-induced neutropenia. The incidences of methicillin-induced nephritis and of oxacillin-induced hepatitis, for example, were found to be dependent on dosage and duration of beta-lactam therapy in a manner similar to that of beta-lactam-induced neutropenia. Both reactions occurred in about 15% of patients treated with high doses for 15 to 20 days (38, 44) but were very rare with short-term treatment. In parallel to beta-lactam-induced neutropenia, both reactions are accompanied in about 80% of cases by a syndrome consisting of eosinophilia, fever, or rash.

A dose- and time-dependent incidence of adverse reactions to cephalosporins as high as 100% was seen by Sanders et al. (43). These researchers gave cefapirin and cephalothin in increasing doses up to a final daily dose of 8.0 g (day 5) to 30 volunteers. All 30 treated volunteers and none of the 10 controls developed reactions between days 11 and 28, such as lymphadenopathy, rash, fever, eosinophilia, leukopenia, arthralgia, and others. Interestingly, cephalosporin solutions for injection were prepared in advance, allowing for some degradation of the drugs in vitro.

Degradation of penicillin G in vitro was also found to contribute to the generation of adverse reactions in that their overall incidence in prolonged intravenous treatment was much lower when the drug was given in freshly dissolved bolus doses instead of 24-h infusions (35).

Clinical data on patients with beta-lactam-induced neutropenia suggest that bone marrow toxicity of different beta-lactam antibiotics, when given simultaneously or sequentially, is additive (32). It is not clear whether this also applies to other untoward reactions to beta-lactams. This aspect will have to be considered when double-beta-lactam combinations are compared with single-compound regimens or other combinations.

The suspicion has been advanced that bone marrow toxicity of high-dose beta-lactam antibiotics might prolong neutropenia induced by cytostatic agents (32). There are preliminary observations both in favor of (32; B. Osterwalder, C. Haberthuer, A. Gratwohl, and B. Speck, Abstr. Annu. Meet. Swiss Soc. Int. Med., abstr. no. 80, 1986) and against (C. Kibbler, personal communication) such a hypothesis. In another study, a double-beta-lactam combination was associated with more patients having persistent granulocytopenia ($\leq$100 granulocytes per mm^3) than occurred with a beta-lactam–aminoglycoside combination (52). In mice, no evidence was found that beta-lactam antibiotics delayed recovery from cytosine arabinoside-induced bone marrow damage (51), one problem in this species being the very short serum half-lives of beta-lactams compared with that in humans. A final answer to the question awaits data.

(ii) Effects of beta-lactam antibiotics on immune cells. A broad array of effects on cells involved in functions of the immune system have been ascribed to antibiotics of all classes (5, 19, 29, 37, 39). Most studies in this field have been done in nonproliferating cells and need not be reviewed here. In addition to the well-documented growth inhibition of cultured lymphocytes in particular by cephalosporins, more recent studies hint that beta-lactams could also modify immune functions by their antiproliferative effects: generation of virus-specific cytotoxic T cells and proliferation of lymphocytes in a secondary in vitro antiviral immune response proved to be sensitive to beta-lactam concentrations ranging from 1/9 to 1 mM (~40 to 600 μg/ml) (21). With cephalosporins, these biological activities were both completely abolished by a 1 mM concentration of freshly dissolved compound and by much lower concentrations of degraded substances. In BALB/c mice, 7 days of chemotherapy with mezlocillin, piperacillin, or cefotaxime suppressed delayed-type hypersensitivity to oxazolone, as well as humoral responses against sheep erythrocytes (42). Furthermore, spleen cells from beta-lactam-treated animals exhibited markedly reduced proliferative capacity upon stimulation with concanavalin A or lipopolysaccharide. But there is no clear evidence for a direct antiproliferative effect of beta-lactam antibiotics in the living animal mediating these phenomena.

(iii) Amanita mushroom poisoning. It has long been recognized that very high doses of intravenous penicillin G have some antagonistic effect against amanita mushroom poisoning in humans and animals (11–13). The lethal principals of amanita mushrooms are the amatoxins, in particular α-amanitin, a selective blocker of DNA-dependent RNA polymerase II. It is therefore conceivable that beta-lactam antibiotics are protective against α-amanitin via their effects on eucaryotic DNA replication. In preliminary experiments in chicken embryo liver cells, various beta-lactams dose depen-

dently antagonized the effects of α-amanitin on cellular uptake of [^{3}H]uridine and ^{14}C-labeled amino acids. Again, cephalosporins were more effective than penicillins. Accordingly, α-amanitin–induced liver damage could be prevented by high-dose intraperitoneal or subcutaneous beta-lactam infusions in mice (K. A. Neftel, G. Keusch, P. Cottagnoud, U. Widmer, M. Hany, K. Gautschi, B. Joos, and H. Walt, Schweiz. Med. Wochenschr., in press). The clinical benefit of cephalosporins versus penicillins in human amanita mushroom intoxication remains to be evaluated. There is only anecdotal evidence of the beneficial effect of ceftazidime in one heavily intoxicated patient (Neftel et al., in press).

(iv) Interference with other agents. Knowledge of the mode of action suggests that beta-lactam antibiotics interfere with other agents acting on proliferating cells, particularly cytostatic agents. Indeed, preliminary experiments document that beta-lactams dose dependently reduce incorporation of cytosine arabinoside into the DNA of K-562 cells (I. Guldenschuh, P. Gottagnoud, and K. A. Neftel, Abstr. Bienn. Conf. Chemother. Infect. Dis., Munich, Federal Republic of Germany, 1987).

(v) Use of beta-lactam antibiotics in cell culture work. It is common practice to add beta-lactams, particularly penicillin G, to all sorts of cell culture media. Depending on concentration and extent of breakdown, beta-lactams may be a source of artifacts in cell culture work.

SUMMARY AND FINAL REMARKS

Selectivity of beta-lactam antibiotics for the bacterial cell is not absolute. Beta-lactams appear to have a target also within the eucaryotic cell, most likely the replicative DNA polymerase α. Interaction of beta-lactams with this target, however, requires previous modification to products that have not been identified. Another unexpected eucaryotic target for modified beta-lactams was recently recognized. Doherty et al. showed that modification of cephalosporins by various substitutions at position C-4 can lead to very potent inhibitors of human leukocyte elastase (7). Penicillin-binding proteins, on the other hand, appear not to be exclusively susceptible to beta-lactams in that lactivicin, a bicyclic dipeptide not bearing a beta-lactam ring, has been found to possess various biological activities otherwise exclusively ascribed to beta-lactams (36).

The clinical context in which the effects of beta-lactam antibiotics on proliferating cells and on in vitro DNA replication have to be considered remains largely undefined. Moreover, although observations first in patients and then in cultured cells finally led to studies on the effects of beta-lactams on in vitro DNA replication, the links between beta-lactam effects on living cells and cell-free DNA replication systems are not formally established. Further studies particularly require recognition of the relevant beta-lactam-derived structures and possibilities of detecting them in biological fluids by methods such as high-performance liquid chromatography.

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