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SPECIFICITY OF TRANSPORT OF BLEOMYCIN AND COBALT-BLEOMYCIN IN L5178Y CELLS

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SUMMARY: The mechanism of transport of [^{3}H]peplomycin (PEP), a new member of bleomycin group antibiotics, was studied in cultured L5178Y mouse leukemic cells. Cobalt ions enhanced the uptake of PEP, but Cu, Zn, Fe(II) and Fe(III) had no effect. The initial rate of uptake of cobalt chelated PEP [PEP(Co)] was several times higher than that of free or Cu-chelated PEP and was temperature independent. A double reciprocal plot of the data demonstrated both saturable (K_m = 4.5 μM, V_{max} = 1.3 x 10^{-18} mole/min/cell) and non-saturable components of the uptake of PEP(Co). The saturable component was inhibited specifically by cobalt chelated bleomycin analogs. PEP-chelates with metals other than cobalt, such as PEP(Cu) were metabolically unstable. These results suggest that bleomycin enters into cells as a metal chelate through a specific transport site.

Bleomycin, a glycopeptide antibiotic, is clinically effective against squamous cell carcinoma and malignant lymphoma (1,2). Since its discovery, many studies have been published concerning its effect on cells both *in vivo* and *in vitro* and on the mechanism of its cytotoxic action (2,3). However, the mechanism of its transport through the cell membrane has not been established. Only recently, the involvement of trypsin-sensitive components of the cell membrane in the toxicity of bleomycin has been reported by Barranco *et al.* (4), suggesting the possible presence of a transport carrier. In the present paper, we report the first study on the transport mechanism of bleomycin by using tritium labeled peplomycin, a new member of bleomycin group antibiotics (5).

METHODS

L5178Y cells were grown in RPMI1640 medium supplemented with 10% heat-inactivated horse serum. They were harvested in the logarithmic phase of

Abbreviations: PEP, peplomycin; BLM, bleomycin.

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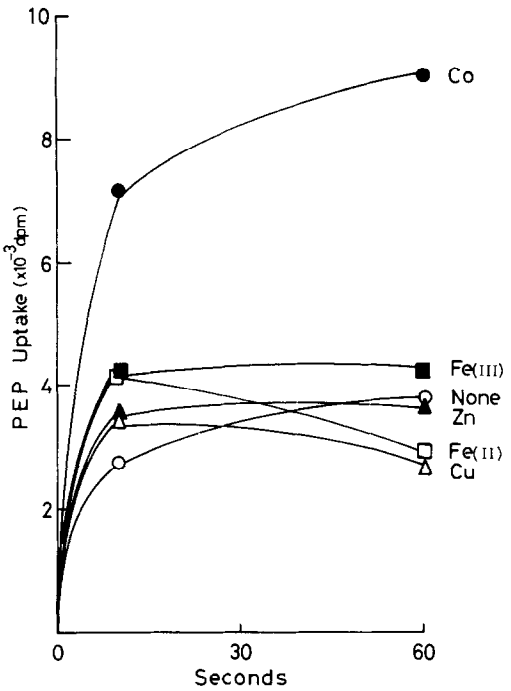

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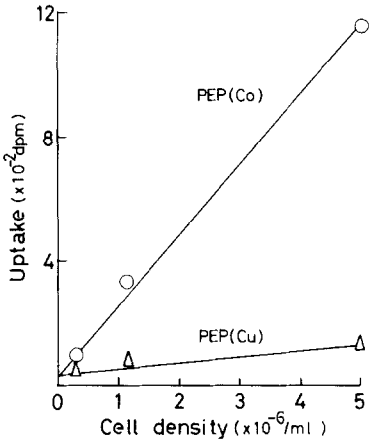

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Fig. 1. The effect of various metal ions on the uptake of PEP. L5178Y cells, at a concentration of 5 x 10^6 cells/ml, were incubated with [^{3}H]PEP (0.6 μM) in the absence or presence of various metal ions (2 μg/ml) at 25°. One ml of the incubation mixture was layered over the oil and uptake was terminated by centrifugation of the cells through the oil at indicated times. Radioactivity associated with the cell fraction was estimated as described in METHODS.

Fig. 2. Uptake of PEP(Co) and PEP(Cu) by L5178Y cells at various cell densities. L5178Y cells, at indicated cell concentrations, were incubated with 0.5 μM of [^{3}H]PEP(Co) or [^{3}H]PEP(Cu) at 25°. Duplicate 0.4 ml aliquots of the incubation mixture were layered over the oil in microcentrifuge tubes and uptake was terminated by centrifugation of the cells through the oil after 1 min incubation.

growth, washed and suspended in fresh medium at a concentration of up to 10^7 cells/ml. Then [^{3}H]PEP or its metal chelate was added and the incubation carried out as indicated in invidual experiments. Aliquots of the incubation mixture were layered on a mixture of silicone oil and liquid paraffin (84 : 16 by volume, specific gravity 1.04 at 25°) in a microcentrifuge tube and uptake was terminated by centrifugation of the cells through the oil at 12,000 g for 1 min in an Eppendorf microcentrifuge. After the removal of reaction mixture and oil, the cells were solubilized in 0.5N KOH, liquid scintillation fluor was added, and the sample counted on a liquid scintillation counter.

[^{3}H]Peplomycin (0.75 mCi/mg) was obtained from Nippon Kayaku Co., Ltd., Tokyo.

RESULTS

Enhancement of uptake of PEP by cobalt

The effect of various metal ions on the uptake of [^{3}H]PEP is shown in Fig. 1. Cobalt ion enhanced the initial rate of uptake of PEP, while Cu,

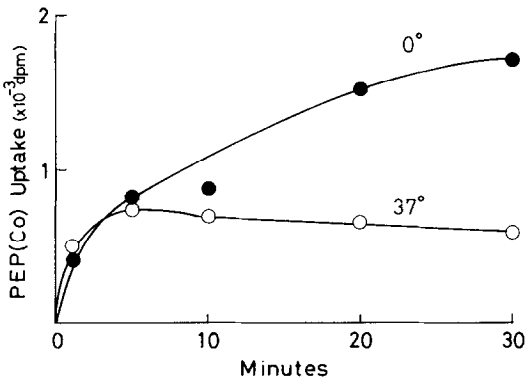

Fig. 3. The effect of temperature on the uptake of PEP(Co). L5178Y cells (4 x 10^6 cells/ml) were incubated at 37° and 0° with 0.3 μM of [^{3}H]PEP(Co), and at indicated times duplicate 0.2 ml aliquots of the incubation mixtures were removed and uptake terminated by centrifugation of the cells through the oil.

Zn, Fe(II) and Fe(III) had no effect. The enhancement of the Co on the uptake of PEP suggested that PEP may enter cells as the PEP(Co) chelate most efficiently. To determine this possibility, the [^{3}H]PEP(Co) complex was prepared by mixing solutions of PEP and $CoCl_2$ and purified by chromatography on a CM Sephadex C-25 column. The rate of the uptake of PEP(Co) increased linearly with the cell density and was several times higher than that of metal free (data not shown) or PEP(Cu) (Fig. 2). Due to the insufficient amount of uptake for the analysis of the latter two bleomycins we used [^{3}H]PEP(Co) for the detailed study.

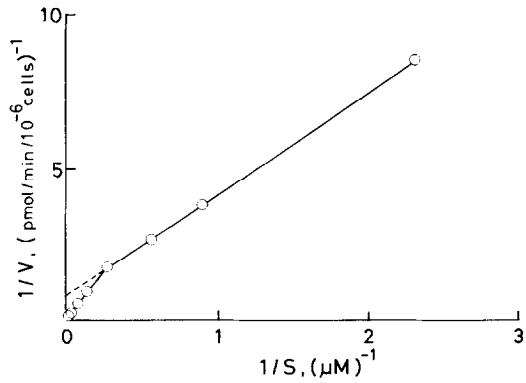

Fig. 4. Kinetic analysis of PEP(Co) uptake by L5178Y cells. Duplicate cell suspensions (10^7 cells/ml) were incubated with various concentrations (0.43 - 67 μM) of [^{3}H]PEP(Co) at 0° for 1 min. The reciprocal of drug uptake V, in pmole/min/10^{-6} cells is plotted against the reciprocal micromolar concentration of PEP(Co) as substrate.

Table 1. The effect of various compounds on uptake of [^{3}H]PEP(Co) by L5178Y cells

Compound	% of Control	
	25°	0°
PEP(Co)	41	47
BLM A2(Co, green)	33	66
BLM A2(Co, brown)	32	48
BLM Acid(Co)	-	54
PEP	91	102
PEP(Cu)	-	84
BLM Acid	103	98
BLM Acid(Cu)	86	97
Co	86	97

Cell suspensions (5 x 10^6 cells/ml) were incubated with 0.6 μg/ml of [^{3}H]PEP(Co), with or without the compound (45 μg/ml) at 25° or 0° for 1 min. The uptake was estimated as described in METHODS.

Temperature independency of the uptake

Initial velocities of uptake of PEP(Co) by L5178Y cells were similar at 0° and 37° (Fig. 3). A steady state level of accumulation reached after about 5 min incubation at 37° with a cell/medium distribution ratio of 0.9. At 0°, however, uptake continued after 5 minutes and net accumulation was about 2 to 3 times higher than at 37°.

Specificity of PEP(Co) transport

Kinetic analysis of initial uptake of PEP(Co) by L5178Y cells over a concentration range of 0.43 to 67 μM appeared biphasic (Fig. 4). The K_m for transport in the low concentration range of PEP(Co) was 4.5 μM and the V_{max} 1.3 x 10^{-18} mole/min/cell. A nonsaturable component of uptake is obtained with higher concentrations.

The effect of a 70-fold excess of several structural analogs of bleomycin, including bleomycinic acid and cobalt ion on transport of 0.4 μM [^{3}H]PEP(Co) was evaluated at different temperatures (Table 1). The uptake was inhibited by PEP(Co), two isomers of bleomycin A2(Co) and bleomycinic acid(Co), but not by PEP, PEP(Cu), bleomycinic acid or cobalt ion. This indicates the importance of Co-chelated form for the specificity of trans-

port. Terminal amine moieties are not involved in the specificity of the transport of cobalt chelated bleomycins.

DISCUSSION

Bleomycin-Fe(II)-O_2 complex has been shown to cause DNA strand scission (6,7). Cobalt complex of bleomycin has no biological activity, because the cobalt in the complex is not replaced by ferrous ion (8,9). The present paper suggests that bleomycin penetrates through the membrane as a metal chelate form because PEP(Co) is more efficiently incorporated into cells than metal free PEP (Fig. 1). Although Cu or Fe(II) complex of bleomycin may be a real form for the transport, we could not see a sufficient amount of uptake of these complexes in our assay system, probably in part due to the rapid disintegration in these cells (data not shown). Therefore, we believe that the transport system of PEP(Co) presented here could be a model system for the transport of bleomycin group antibiotics. Support for the transport mechanism of PEP by L5178Y cells as the metal chelate is indicated by the following observations: (a) Uptake at 0° and 37° (Fig. 3) correlates with the fact that bleomycin, at low concentrations, kills the cells equally (10) and induces DNA damage (11,12) in the cells at both 0° and 37°; (b) presence of a carrier mediated transport system for PEP(Co) at the low concentration range (Fig. 4) may be one of the physical bases for why very low bleomycin doses are effective *in vitro* (13) and *in vivo* (14); (c) bleomycin (^{57}Co) as a tumor scanning agent for the diagnosis of pulmonary and cerebral tumors has been demonstrated (9,15).

Although no inhibitory effect of metal free PEP and PEP(Cu) on the uptake of PEP(Co) (Table 1) was observed, the fact does not necessarily prove the presence of independent transport mechanisms, because rapid disintegration of PEP(Cu) in the cells was observed. On the other hand, PEP(Co) was stable in our system (data not shown) and also in tumors *in vivo* (16). This uptake of cobalt chelate may provide a clue to the

differences in uptake and their efficacy of bleomycin among different tumor types (1).

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