MODE OF ACTION OF DEOXYPHEGANOMYCIN D ON *MYCOBACTERIUM SMEGMATIS* ATCC 607

KAYOKO SUZUKAKE-TSUCHIYA and MAKOTO HORI

Showa College of Pharmaceutical Sciences,
5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan

NOBUYOSHI SHIMADA† and MASA HAMADA

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication September 22, 1987)

Deoxypheganomycin D, a specific inhibitor of mycobacteria, inhibits the growth *in vitro* of *Mycobacterium smegmatis* ATCC 607 (M. 607) bacteriostatically at concentrations as high as 7×10^{-5} M. It shows no cross-resistance to paromomycin, capreomycin, viomycin, streptothricin, kanamycin and streptomycin. Deoxypheganomycin D at 2.8×10^{-7} M where the cell growth of M. 607 is only partially inhibited does not significantly inhibit DNA, RNA or protein synthesis but leads to marked decrease (13% of control) in [^{14}C]glycerol-derived radioactivity in cell-walls. In the presence of 7×10^{-6} M deoxypheganomycin D, the influx of leucine but not thymidine is affected while the reverse is true with efflux. The data suggest that the effect of deoxypheganomycin D on M. 607 may be related to both the cell membrane and specific mycobacterial lipid like components of the cell-wall.

Pheganomycins isolated from a fermentation broth of *Streptomyces cirratus* are specific inhibitors of mycobacteria. Their structures represent a new family of peptides sharing a chromophore[1,2]. The antibiotics are of interest because they are as effective against some drug-resistant strains of *Mycobacterium smegmatis* ATCC 607 (M. 607) as against the sensitive parent strain. The instabilities of the original compounds were improved in deoxypheganomycins which were derived from pheganomycins by chemical reduction of the chromophore moiety[1]. Deoxypheganomycins are more potent against M. 607 while maintaining the same level of toxicity as the original compounds. In the present paper we describe the results of the biochemical studies on how deoxypheganomycin D acts on M. 607.

Materials and Methods

Culture Media

A modified nutrient broth medium, referred to as MNB medium, consisted of nutrient broth (Difco) 0.8%, NaCl 0.1%, glycerol 1% (w/v) and Tween 80 1% (w/v). A semisynthetic medium, referred to as CGG medium, consisted of Casamino acids (Difco) 0.1%, glucose 0.2%, glycerol 1% (w/v), $MgSO_4\cdot7H_2O$ 0.025%, $CaCl_2$ 0.001%, KH_2PO_4 0.2%, Tris 0.73% and Tween 80 1% (w/v).

Cell-growth and Viability of M. 607

M. 607 was grown in a set of two L-shaped tubes, each containing 5.6 ml of MNB medium, at 37°C with shaking. When the cultures reached a cell density of 0.1 $A_{600\,nm}$, one tube received 0.4 ml of aqueous solution of deoxypheganomycin D to give a final concentration of 7×10^{-5} M. The other tube (control) received 0.4 ml of water. Incubation was resumed and the turbidity of the cultures was monitored. At the times indicated, 100 μl samples were withdrawn from the cultures to determine

† Present address: Research Laboratories, Pharmaceutical Group, Nippon Kayaku Co., Ltd., 3-31-12 Shimo, Kita-ku, Tokyo 115, Japan.

the number of viable cells by the layered plate method[3] using MNB medium. Colonies were counted after incubation at 37°C for 2 days.

Cell-free Protein Synthesis

Preparation of S-30 extract of M. 607 was performed according to the methods of YAMADA *et al.*[4] with a few modifications. M. 607 cells collected at a cell density of 1.2 $A_{600\,nm}$ were sonicated with a Branson sonifier at 80% power for the total period of 35 seconds with two intermissions for chilling. The sonicate was centrifuged at 20,000×*g* for 30 minutes. The supernatant was incubated with *Escherichia coli* deoxyribonuclease I (8 μg/ml) at 0°C for 20 minutes and at 35°C for 20 minutes, and centrifuged at 30,000×*g* for 30 minutes. The supernatant was dialyzed against 2 liters of buffer A for 2.5 hours at 10°C twice and stored at −70°C until use (S-30).

A reaction mixture for *in vitro* protein synthesis (100 μl) consisted of Tris·HCl 50 mM (pH 7.8), ATP 2 mM, GTP 0.2 mM, NH_4Cl 160 mM, phosphoenol pyruvate 5 mM, pyruvate kinase 1.1 μg, dithiothreitol 2 mM, [^{14}C]Phe (5 μCi/ml, 504 mCi/ml) 10 μM, each of 19 amino acids 10 μM, $MgCl_2$ 15 mM, *E. coli* MRE 600 tRNA 30 μg, S-30 80 μg and poly U 20 μg. After incubation at 37°C for 20 minutes, a 90-μl sample was withdrawn and transferred to a paper disc (Whatman 3 MM, 2.4 cm diameter) to determine the radioactivity incorporated into TCA insoluble materials.

DNA Synthesis in Toluene-treated Cells

Preparation of toluene-treated M. 607 cells was performed as follows. Cells were grown in five L-shaped culture tubes each containing 10 ml of MNB medium at 37°C with shaking. When the cultures reached a cell density of 0.3 $A_{600\,nm}$ (about 2.5×10^7 cells/ml), they were centrifuged at 3,000×*g* for 15 minutes. Cells were collected, suspended in 10 ml of 0.1 M potassium phosphate buffer containing 1% toluene, pH 7.4, shaken slowly for 20 minutes at 25°C in a culture tube. The cells were washed in 5 ml of phosphate buffer twice, resuspended in 1 ml of phosphate buffer supplemented with 1% (w/v) Triton X-100 and kept at −70°C until use.

DNA synthesis in toluene-treated cells was performed according to the method of MATSUSHITA *et al.*[5] The suspension of toluene-treated cells (125 μl) and 0.17 μM [^{3}H]TTP (5 μCi/ml, 155 mCi/mmol) were added to a reaction mixture (500 μl). Otherwise the same as reported. The mixtures were incubated at 37°C with or without antibiotic. At the times indicated, 0.1 ml samples were transferred from the tubes to paper discs (Whatman 3 MM, 2.4 cm diameter) to determine the radioactivity incorporated into TCA insoluble materials.

Influx of Leucine, Thymidine, Uridine and Adenosine into Cells of M. 607

For determination of leucine influx, shake-cultures of M. 607 (0.1 $A_{600\,nm}$) were divided into two 14 ml portions in culture tubes. One tube received 70 μl of deoxypheganomycin D solution to give a final concentration of 1.8×10^{-6} M while the other received 70 μl of water. Each tube then received 40 μl of [^{3}H]leucine (0.5 mCi/ml, 5 Ci/mmol) and was incubated further at 37°C with shaking. At 0, 30, 60 and 90 minutes, 1 ml samples were withdrawn and centrifuged at 16,000×*g* for 1 minute. Cells (precipitate) were washed three times with 1 ml each of CGG medium, mixed with 0.5 ml of 2% perchloric acid and left standing for 30 minutes. After neutralization with 0.625 ml of 0.5 M alamine 336 (tricaprylyl tertiary amine) in 1,1,2-trichlorotrifluoroethane, the mixture was centrifuged at 650×*g* for 1 minute. Radioactivity in the aqueous phase was determined. Analyses were in triplicate. [^{3}H]Thymidine (250 μCi/ml, 22 Ci/mmol), [^{3}H]uridine (250 μCi/ml, 24.3 Ci/mmol) and [^{3}H]adenosine (250 μCi/ml, 22 Ci/mmol) were used for determination of influx of thymidine, uridine and adenosine, respectively. The procedures were the same as those for the determination of leucine influx.

Efflux of Macromolecular Precursors from Cells of M. 607

M. 607 was grown in four culture tubes each containing 10 ml of CGG medium at 37°C with shaking. When a cell density of 0.1 $A_{600\,nm}$ was reached, a culture received either 50 μl of [^{3}H]-adenosine (22 Ci/mmol, 1 mCi/ml), 15 μl of [^{3}H]thymidine, (22 Ci/mmol, 1 mCi/ml), 50 μl of [^{3}H]-uridine (24.3 Ci/mmol, 1 mCi/ml) or 50 μl of [^{3}H]leucine (5 Ci/mmol, 1 mCi/ml). After incubation for 1 hour, each culture was centrifuged at 3,000×*g* for 10 minutes to obtain isotope-labeled cells as

precipitate. Another six cultures were proceeded without isotope-labeling under similar conditions, combined, centrifuged, and the supernatant was used as 'conditioned medium' as follows. The isotope-labeled cells were washed with 2 ml of the 'conditioned medium' and resuspended in 10 ml of the 'conditioned medium' and the suspension was divided into a pair of 5 ml portions in two culture tubes. One tube of the pair received 25 μl of deoxypheganomycin D solution to give a final concentration of 7×10^{-6} M, while the other received water. Each cell suspension was incubated at 37°C with shaking. At 0, 30 and 60 minutes, 0.7 ml samples were withdrawn and centrifuged at $16,000\times g$ for 1 minute. Radioactivity released into the medium was determined. Analyses were in duplicate.

Analysis of Intracellular Nucleic Acid Precursors by HPLC

M. 607 was grown in 26 culture tubes each containing 15 ml of CGG medium at 37°C with shaking. At a cell density of 0.1 $A_{600\,nm}$, 13 cultures received 100 μl each of deoxypheganomycin D solution to give a final concentration of 7×10^{-6} M (group a) while the other 13 cultures received water (group b). After incubation for 1 hour under the same conditions, the cultures of (a) and (b) were combined separately, and centrifuged at $3,000\times g$ for 20 minutes. The cells were washed with 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, and centrifuged at $16,000\times g$ for 1 minute. To the cells 0.5 ml of 2% (w/v) perchloric acid was added and the suspension was mixed vigorously for 3 minutes. After standing at 0°C for 30 minutes, the suspension received 0.625 ml of 0.5 M alamine 336 in trichlorotrifluoroethane and was mixed. The mixture was centrifuged at $650\times g$ for 1 minute and the supernatant was filtered through a filter disc (Millipore, 0.45 μm pore). A 20-μl portion of the filtrate was applied to a Senshu Pak Hitachi-Gel 3013-N column (4.6×250 mm) equilibrated with buffer A (4% CH_3CN, 0.06 M NH_4Cl, 0.01 M KH_2PO_4, 0.01 M K_2HPO_4). The column was eluted with buffer A for 30 minutes, with a linear gradient between buffer A and buffer B (4% CH_3CN, 0.3 M NH_4Cl, 0.05 M KH_2PO_4, 0.05 M K_2HPO_4) for 90 minutes and with buffer B for 20 minutes, in this order. Flow rate was 0.5 ml/minute. Nucleic acid precursors were detected by absorbance at 254 nm.

Incorporation of [^{14}C]Glycerol into Whole-cells and Subcellular Fractions of M. 607

Preparation of cell-walls was performed according to the method of WINDER and COLLINS[6] with minor modifications. M. 607 was grown in 18 flasks each containing 100 ml of the medium which consisted of Casamino acids 1%, glycerol 0.6% (w/v), $MgSO_4\cdot7H_2O$ 0.025%, $CaCl_2$ 0.001%, KH_2PO_4 0.2%, Tris 0.73%, [^{14}C]glycerol (15 mCi/mmol) 0.7 μM and Tween 80 1% (w/v), pH adjusted to 7.4. Nine flasks received 200 μl of deoxypheganomycin D solution to give a final concentration of 2.8×10^{-7} M, while the other flasks received 200 μl each of water, and all were incubated at 37°C for 38 hours with shaking. The treated and control flasks were combined separately and centrifuged at $6,000\times g$ for 30 minutes and the volume of each cell pellet was determined. The cells were washed three times with 0.9% (w/v) NaCl solution, centrifuged at $10,000\times g$ for 20 minutes, suspended in 0.1 M phosphate buffer, pH 7.8 (1 g wet weight of cells in about 4.5 ml of buffer), and sonicated with a Branson Sonifier at 80% power by 20 cycles of 1-minute-sonification and 1-minute-chilling. Each sonicate was heated at 90°C for 15 minutes, mixed with DNase I (to 10 μg/ml), RNase (to 10 μg/ml) and $MgCl_2$ (to 5 mM), incubated at 37°C for 2 hours, and centrifuged at $330\times g$ for 20 minutes to remove unbroken cells. The supernatant was centrifuged at $4,200\times g$ for 1 hour and the precipitate (including cell-walls) was taken and washed four times with 0.05 M phosphate buffer by dispersing and centrifuging at $8,000\times g$ for 30 minutes (optical densities at 260 nm and 280 nm of the supernatant became low and constant). The precipitate was dispersed in 0.05 M phosphate buffer and treated with 100 μg/ml trypsin at 37°C for 2 hours. After centrifugation at $8,000\times g$ for 45 minutes the precipitate was washed six times with 0.05 M phosphate buffer by dispersing and centrifuging at $8,000\times g$ for 30 minutes and was freeze-dried (crude cell-wall fraction). The crude cell-wall fraction was suspended in a 2 : 1-mixture of $CHCl_3$ - MeOH (25 ml of the mixed solvent per 100 mg cell-walls) and kept standing at room temp for 2 days with occasional shaking. The suspension received extra MeOH to make its final concentration 50% and was centrifuged at $8,000\times g$ for 30 minutes. The precipitate was suspended in 50% EtOH (100 mg of crude cell-walls in 20 ml of 50% EtOH) and heated at 40°C for 1 hour and centrifuged at $8,000\times g$ for 30 minutes. The precipitate was freeze-dried (purified cell-wall fraction).

Results and Discussion

Antimicrobial Spectrum of Pheganomycins

As shown in Table 1, the antibacterial spectra of the pheganomycin group antibiotics are very narrow, primarily limited to mycobacteria and are as effective against various drug-resistant strains of M. 607 as against the original parent strain.

Effect of Deoxypheganomycin D on the Cell-growth and Viability

Deoxypheganomycin D was bacteriostatic for M. 607. Although the growth of M. 607 in liquid culture medium was inhibited almost completely within an hour by 7×10^{-5} M deoxypheganomycin D

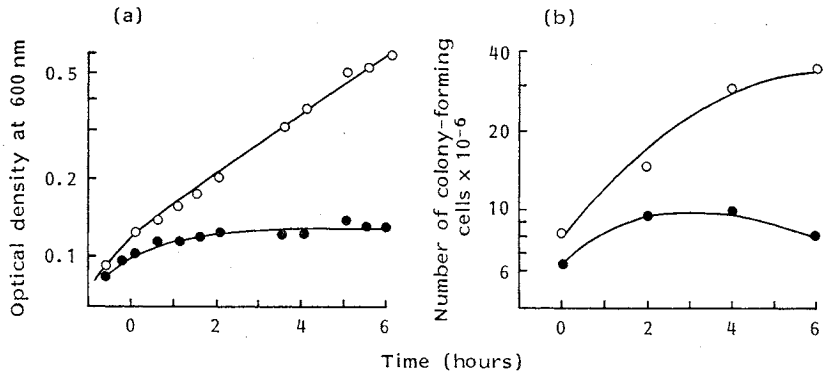
Table 1. Antimicrobial spectrum of pheganomycins.

Organisms	MIC (μg/ml)				
	PHG	PHG-D	PHG-DR	PHG-DGPT	Deoxy PHG-D
Staphylococcus aureus FDA 209P	>100	>100	>100	>100	
S. aureus Smith	>100	100	>100	>100	
Micrococcus flavus FDA 16	>100	12.5	>100	100	
M. luteus PCI 1001	>100	>100	>100	>100	
Bacillus subtilis NRRL B-558	>100	>100	>100	>100	
B. subtilis PCI 219	>100	>100	>100	>100	
Corynebacterium bovis 1810	>100	>100	>100	>100	
Escherichia coli NIHJ	>100	>100	>100	100	
E. coli K-12	>100	>100	>100	100	
E. coli ML1629	>100	>100	>100	>100	
Shigella dysenteriae JS11910	>100	>100	>100	25	
S. sonnei JS11746	>100	>100	>100	>100	
Salmonella typhi T-63	>100	>100	>100	25	
S. enteritidis 1891	>100	100	>100	25	
Klebsiella pneumoniae PCI 602	>100	50	>100	50	
Proteus vulgaris OX19	>100	>100	>100	100	
Serratia marcescens	>100	>100	>100	>100	
Pseudomonas aeruginosa A3	>100	>100	>100	>100	
Xanthomonas oryzae	>100	100	100	100	
Candida albicans 3147	>100	>100	>100	>100	
Saccharomyces cerevisiae	>100	>100	>100	>100	
Pyricularia oryzae	>100	>100	>100	>100	
Aspergillus niger F-16	>100	>100	>100	>100	
Nocardia asteroides IFO 3423					>100
N. asteroides IFO 3424					>100
N. asteroides IFO 3384					>100
Mycobacterium smegmatis ATCC 607	100	12.5	6.25	12.5	6.25
Mycobacterium 607 PM^R	>100	25	12.5	25	25
Mycobacterium CPM^R	100	12.5	3.12	6.25	6.25
Mycobacterium VM^R	>100	12.5	6.25	6.25	6.25
Mycobacterium ST^R	>100	12.5	6.25	6.25	6.25
Mycobacterium KM^R	100	12.5	6.25	12.5	12.5
Mycobacterium SM^R	100	25	6.25	6.25	25
M. phlei	100	12.5	6.25	12.5	6.25

Abbreviations: PHG; Pheganomycin, PM; paromomycin, CPM; capreomycin, VM; viomycin, ST; streptothricin, KM; kanamycin, SM; streptomycin, ^{R}: resistance.

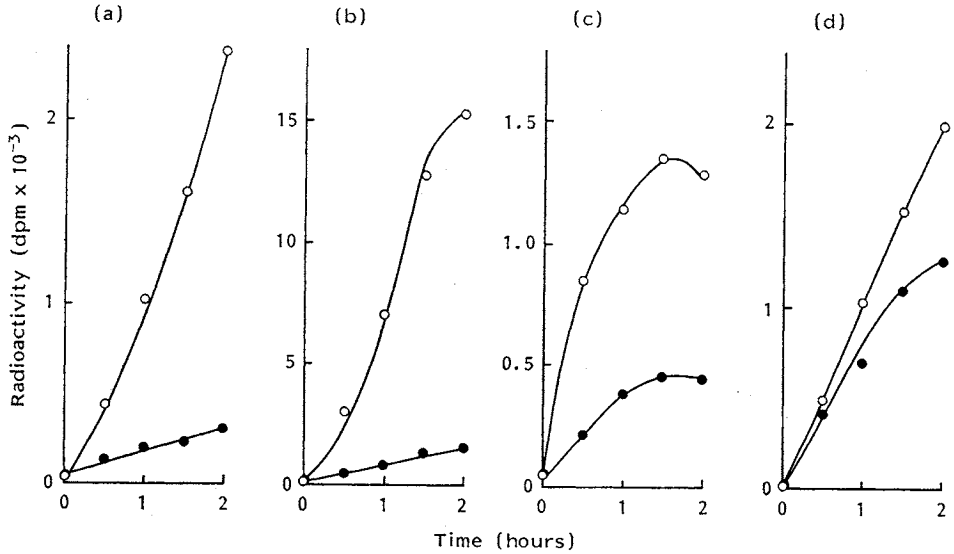
Resistant strains were established from *Mycobacterium smegmatis* ATCC 607 at the Institute of Microbial Chemistry. MICs were determined by the agar dilution method.

Fig. 1. Effect of deoxypheganomycin D on growth of *Mycobacterium smegmatis* ATCC 607.
○ Without antibiotic, ● with 7×10^{-5} M deoxypheganomycin D.



The experiment was conducted as described under "Materials and Methods". Deoxypheganomycin D was added at 0 time.

Fig. 2. Effect of deoxypheganomycin D on macromolecular synthesis in *Mycobacterium smegmatis* ATCC 607.
○ Without antibiotic, ● with 1.7×10^{-6} M deoxypheganomycin D.



M. 607 was grown in CGG medium at 37°C with shaking until the cultures reached a cell density of 0.1 $A_{600\,nm}$. The cultures were combined and divided into eight 2 ml portions in culture tubes which were grouped into 4 sets (a, b, c and d) of two tubes each.

To a tube of each set, 50 μl of deoxypheganomycin D solution was added to give a final concentration of 1.7×10^{-6} M while to the other, 50 μl of water (control). After 5 minutes, both tubes of set (a), (b), (c) and (d) received 10 μl of [^{3}H]leucine (1 mCi/ml, 5 Ci/mmol), 50 μl of [^{3}H]thymidine (1 mCi/ml, 26 Ci/mmol), 10 μl of [^{3}H]uridine (1 mCi/ml, 23.4 Ci/mmol) and 10 μl of *N*-[^{14}C]acetylglucosamine (0.1 mCi/ml, 58.18 mCi/mmol), respectively.

(Fig. 1a), the number of viable cells in the culture did not decrease significantly for 6 hours after addition of the antibiotic (Fig. 1b).

Effect of Deoxypheganomycin D on Synthesis of Cellular Macromolecules in Mycobacteria

The effects of deoxypheganomycin D on the syntheses of cell-walls, proteins and nucleic acids in exponentially growing cells of mycobacteria is shown in Fig. 2. Deoxypheganomycin D at 1.7×10^{-6} M strongly inhibited the incorporation of the radioactive leucine and nucleosides but not *N*-acetylglucosamine into the acid-insoluble cell material. Higher concentrations of the antibiotic (7×10^{-5} M) did not inhibit protein or DNA synthesis (Table 2 and Fig. 3).

Effect of Deoxypheganomycin D on the Cellular Permeability of Mycobacteria

The effect of deoxypheganomycin D on the membrane-transport (influx) of radioactive precursors into acid soluble cell fractions indicated that at 1.8×10^{-6} M the rate of influx of leucine was considerably reduced. The influx of thymidine leveled off too quickly (within 3 minutes of labeling) to determine an effect. Uridine influx was stimulated and adenosine was only slightly affected (Fig. 4).

Studies on the release (efflux) of the same compounds using 7×10^{-6} M deoxypheganomycin D showed that deoxypheganomycin D strongly stimulated the release of thymidine, uridine and adenosine while only weakly the release of leucine (Fig. 5).

Deoxypheganomycin D, which hardly inhibited the growth of *E. coli* K-12, did not interfere with the influx of leucine into- and the efflux of adenosine from cells of *E. coli* K-12 (data not shown).

Since it was already shown that deoxypheganomycin D did not affect protein or DNA synthesis (Table 2 and Fig. 3) the effect of the antibiotic on the influx and efflux of these precursors is probably related to an interaction with some component(s) of the cell-wall and/or cell membrane. Since most cells were viable under these condition, as already shown in Fig. 1, the alterations induced by deoxypheganomycin D should be limited and reversible after removal of the antibiotic.

As deoxypheganomycin D altered the rate of transport of macromolecular precursors into cells of mycobacteria, possible effects of deoxypheganomycin D on the amounts of nucleic acid precursors in cells were determined (Fig. 6). In cells treated with 7×10^{-6} M of deoxypheganomycin D, the amount of nucleosides and nucleotides generally decreased.

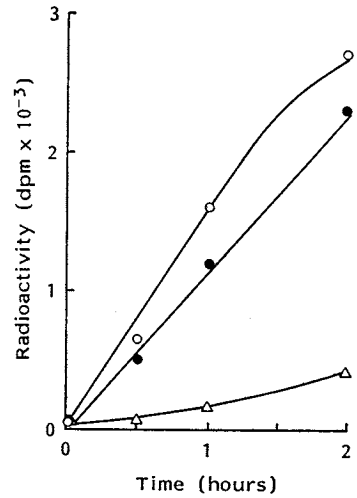
Table 2. Protein synthesis with S-30 from *Mycobacterium smegmatis* ATCC 607.

Antibiotic	Radioactivity (dpm)
None	19,920
Deoxypheganomycin D	
10^{-5} M	20,294
10^{-4} M	21,585
Neomycin	
10^{-6} M	948
10^{-5} M	976

The experiment was performed as described under "Materials and Methods".

Fig. 3. Effect of deoxypheganomycin D on DNA synthesis in toluene-treated cells of *Mycobacterium smegmatis* ATCC 607.

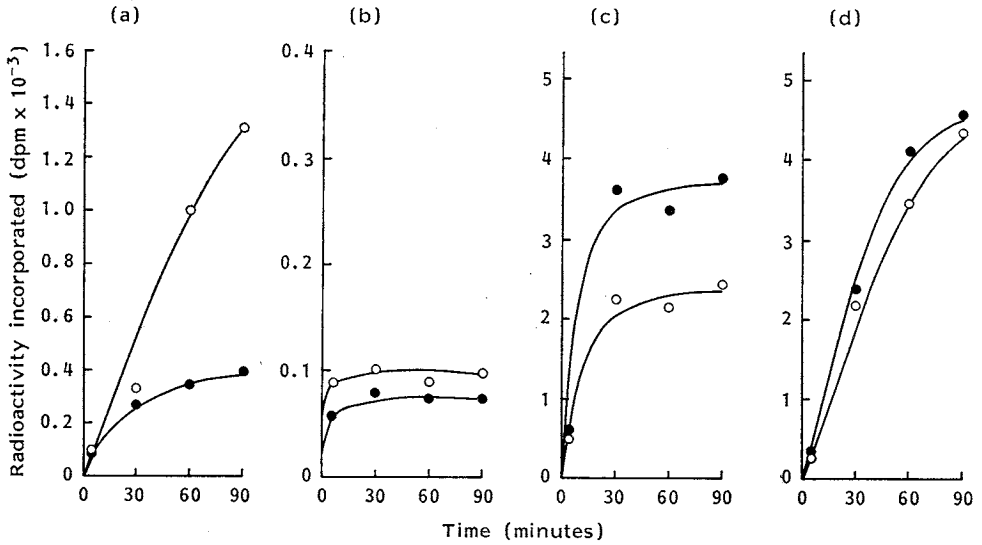
○ Without antibiotic, ● with 7×10^{-5} M deoxypheganomycin D, △ with 1.6×10^{-5} M novobiocin as a positive control.



The experiment was conducted as described under "Materials and Methods".

Fig. 4. Effect of deoxypheganomycin D on influx of amino acids and nucleosides into cells of *Mycobacterium smegmatis* ATCC 607.

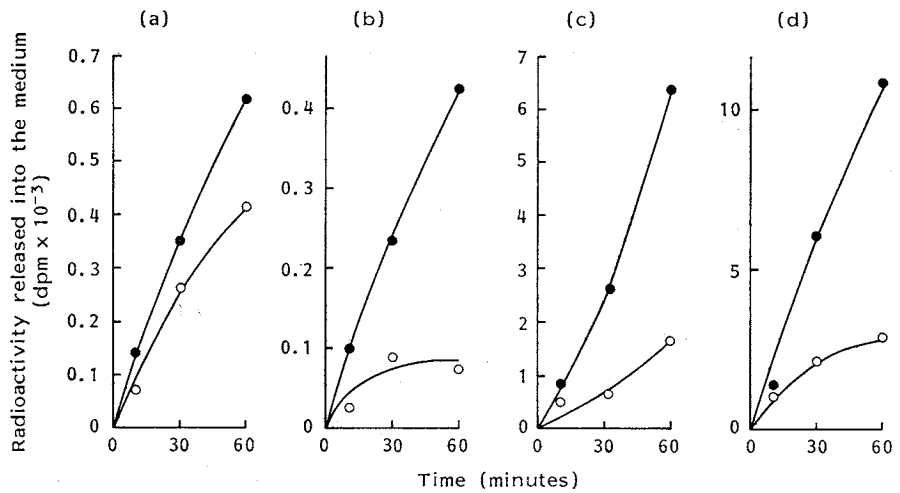
○ Without antibiotic, ● with 1.8×10^{-6} M deoxypheganomycin D.



The experiments were performed as described under "Materials and Methods". The amount of [^{3}H]leucine (a), [^{3}H]thymidine (b), [^{3}H]uridine (c) and [^{3}H]adenosine (d) incorporated into acid-insoluble fractions of cells of *Mycobacterium smegmatis* ATCC 607 are shown.

Fig. 5. Effect of deoxypheganomycin D on efflux of macromolecular precursors from cells of *Mycobacterium smegmatis* ATCC 607.

○ Without antibiotic, ● with 7×10^{-6} M deoxypheganomycin D.

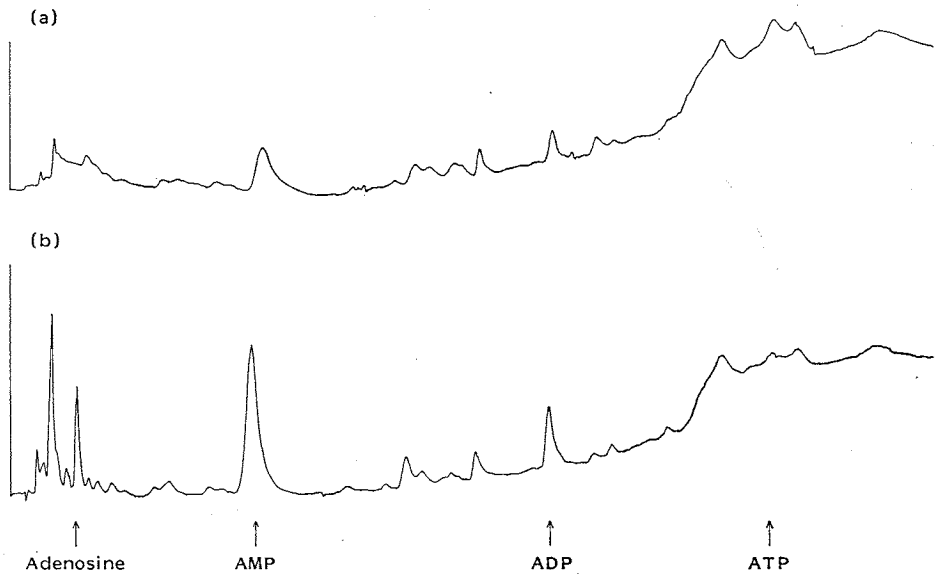


The experiments were performed as described under "Materials and Methods". Cells were labeled with [^{3}H]leucine (a), [^{3}H]thymidine (b), [^{3}H]uridine (c) or [^{3}H]adenosine (d).

Effect of Deoxypheganomycin D on Incorporation of [^{14}C]Glycerol into Cells and the Subcellular Fractions of M. 607

To detect a possible alteration in the structure of the lipid-rich cell envelope, we labeled the cells with [^{14}C]glycerol in the presence of a low concentration of deoxypheganomycin D (2.8×10^{-7} M)

Fig. 6. Effect of deoxypheganomycin D on the amount of nucleic acid precursors in *Mycobacterium smegmatis* ATCC 607.



The experiment was performed as described under "Materials and Methods".

Table 3. Effect of deoxypheganomycin D on incorporation of [^{14}C]glycerol into cells and subcellular fractions of *Mycobacterium smegmatis* ATCC 607.

Fraction	Without deoxypheganomycin D			With 2.8×10^{-7} M deoxypheganomycin D		
	Weight	Radioactivity (dpm)	Specific activity (dpm/mg)	Weight	Radioactivity (dpm)	Specific activity (dpm/mg)
Cells	6.16 g	1,181,086		4.96 g	591,636	
Cell-walls						
Crude	139.7 mg	355,686	2,546 (100%)	30.6 mg	24,532	802 (32%)
Purified	39.4 mg	303,971	7,715 (100%)	7.9 mg	7,874	997 (13%)

The experiment was performed as described under "Materials and Methods".

where the cell growth was inhibited only slightly through 38 hours incubation (Table 3). When harvested, the wet weight of the deoxypheganomycin D-treated culture was more than 80% that of the control culture (4.96 g vs. 6.16 g). In contrast, wet weights of the crude cell-walls and of the purified cell-walls from the deoxypheganomycin D-treated cells were about 20% those from the control cells (30.6 mg vs. 139.7 mg, 7.9 mg vs. 39.4 mg). The specific radioactivity showed more pronounced differences; the crude cell-walls and the purified cell-walls from the deoxypheganomycin D-treated cells showed 32% and 13% of those from the control run, respectively (802 vs. 2,546, 997 vs. 7,715).

It is presumed therefore that deoxypheganomycin D acts at the level of the lipid-rich cell-walls, leading to altered permeability of the cells to various nutrients. Since deoxypheganomycin D is not bactericidal even at high concentrations, nonspecific degradation of cell-walls is unlikely. Mycolic acid, mycosides and arabinogalactan are characteristic components of mycobacterial cell-walls. Deoxypheganomycin D may act on some of these components.

Acknowledgment

The excellent technical assistance of Mr. T. TAKEDA, H. OHTAKI and M. TANAKA is greatfully acknowledged.

References

1) TAKITA, T.; N. SHIMADA, N. YAGISAWA, K. KATO, H. NAGANAWA, K. MAEDA & H. UMEZAWA: Chemistry of pheganomycins, new peptide antibiotics. Proc. of the 15th Symposium on Peptide Chemistry, pp. 121~126, Protein Research Foundation, Osaka, Nov. 4, 1977
2) Data on pheganomycins. Supplement to "Index of Antibiotics from Actinomycetes". J. Antibiotics 32: 79-64~79-68, 1979
3) GERHARDT, P. (*Ed.*): Manual of Methods for General Bacteriology. pp. 185~188, American Society for Microbiology, Washington D.C., 1981
4) YAMADA, T.; K. MASUDA, K. SHOJI & M. HORI: Analysis of ribosomes from viomycin-sensitive and -resistant strains of *Mycobacterium smegmatis*. J. Bacteriol. 112: 1~6, 1972
5) MATSUSHITA, T.; K. P. WHITE & N. SUEOKA: Chromosome replication in toluenized *Bacillus subtilis* cells. Nature [New Biol.] 232: 111~114, 1971
6) WINDER, F. G. & P. B. COLLINS: Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. J. Gen. Microbiol. 63: 41~48, 1970