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^{14}C-Labelling of hematopoietic immunomodulator (Z-100) consisting of polysaccharides obtained from cultivation of Mycobacterium tuberculosis

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SUMMARY

Mycobacterium tuberculosis was cultivated in sauton meium containing 31.4 GBq of ^{14}C-glycerol as a source of carbon to obtain ^{14}C-labelled form of hematopoietic immunomodulator (Z-100). ^{14}C-Z-100 was produced from the organisms with 0.11-0.15 % of radiochemical yield. The specific radioactivity of ^{14}C-Z-100 ranged from 3.3 to 7.5 kBq/eq.μ mole D-arabinose. ^{14}C-Z-100 was analyzed by gel filtration high performance liquid chromatography using a refractive index (RI) detector. The elution pattern of the polysaccharides of ^{14}C-Z-100 agreed closely with thay of Z-100, indicating that ^{14}C-Z-100 was equivalent to Z-100 in chemical properties. In addition, the elution pattern obtained from the radioactivity of ^{14}C-Z-100 was approximated to that obtained by using a RI detector, indicating that Z-100 was generally labelled with ^{14}C. Furthermore, ^{14}C-Z-100 was confirmed to have almost the same interleukin-3 inducing ability and antitumor activity as those of Z-100.

Key-word : ^{14}C-Labelling, Mycobacterium tuberculosis, ^{14}C-Glycerol Cultivation, Gel filtration high performance liquid chromatography

INTRODUCTION

Z-100 extracted from Mycobacterium tuberculosis with a hot water has been prepared as a formulation for injection contaning polysaccharides consisting of arabinomannan and mannan[1]. Z-100 has been reported to have

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not only antitumor activity[2] but also hematopoietic effect[3,4]. This drug has been studied more precisely to confirm an effectiveness on leukopenia associated with radiotherapy of patients with cancer. It is well known that metabolic disposition of a new drug in experimental animals is important to evaluate effectiveness and toxicity for extrapolation of these actions to human. In general, metabolic disposition has been studied by the tracer technique using radioisotope labelled compounds. However, in the case of Z-100, chemical synthesis of a test drug is impossible because of its complex composition. Considering the characteristics that the Mycobacterium tuberculosis consumes a large amount of glycerol as a source of carbon during growth in the medium[5], the present study was undertaken to biosynthesize ^{14}C-labelled Z-100 (^{14}C-Z-100) by cultivating this organism in the medium containing ^{14}C-glycerol.

MATERIALS

Z-100

Z-100 (Lot.No.8D0801) was manufactured by Zeria Pharmaceutical Co.,Ltd., which contains about 13.3 eq. μ mole D-arabinose per 1 ml.

^{14}C-glycerol

[2-^{14}C]glycerol (specific activity 1.0 MBq/μ mole, radiochemical purity 99.0 %) was supplied from Amersham Co., Ltd.(Buckingham, England), and [1,3-^{14}C]glycerol (0.40 MBq/μ mole, >98.0 % and 0.37 MBq/μ mole, >97.0 %) from Chemsyn Science Laboratories (Kansas, U.S.A.).

Sauton medium

The sauton medium [6] contaning ^{14}C-glycerol was used.

L-asparagine 4.0 g, dipotassium hydrogenphosphate 0.5 g, citric acid 2.0 g, magnesium sulfate 0.5 g, ammonium ferric citrate 0.05 g, and ^{14}C-glycerol 60 ml were dissolved in distilled water to make 1000 ml. A 200 ml of the sauton medium was dispensed into a 500 ml culture bottle.

Strain

Strain Aoyama B of Mycobacterium tuberculosis[5] from the stock preserved by Zeria pharmaceutical Co., Ltd. was used for cultivation of organisms.

EXPERIMENTAL

Cultivation of ^{14}C-Z-100

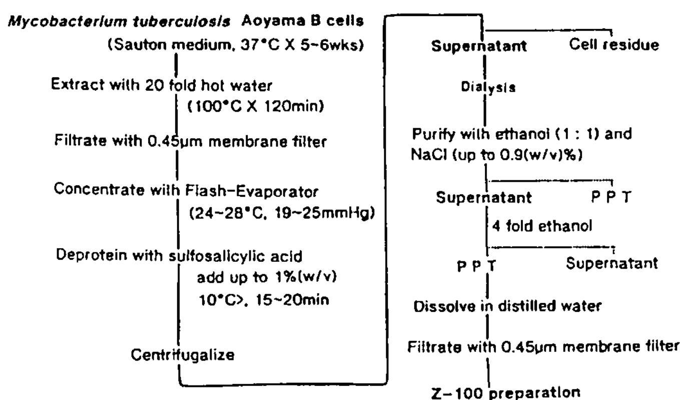
Mycobacterium tuberculosis was seeded onto the surface of the sauton medium of 200 ml in each bottle and incubated at 37°C for 5-6 weeks. After cultivation, the pellicles growing on the surface of the culture medium were collected by filtration and washed with distilled water for using as an extraction material.

Determination of Saccharides in Z-100

The amount of saccharides in Z-100 was determined as a D-arabinose by the method of Phenol-sulfuric acid[7].

Extraction procedure

The procedures for extraction and purification of ^{14}C-Z-100 was shown in the Scheme. Organisms obtained from the culture were suspended by 20 ml of distilled water per 1 mg of the organisms and heated at 100°C for 120



Scheme Purification procedure for Z-100 preparation

min, and then after allowed to cool, they were passed throgh a filter. The filtrate thus obtained was concentrated under reduced pressure to a sugar concentration of 33.3 eq. μ mole D-arabinose/ml. Sulfosalicylic acid was added to the concentrate to make 1 % (w/v). The mixture was stirred and allowed to stand. The precipitate formed was removed by centrifugation, and the supernatant was dialyzed by running water. The dialysate was concentrated under reduced pressure to a sugar concentration of 10.0 eq. μ mole D-arabinose/ml. To the concentrate, potassium chloride was added to make 0.9 % (w/v). Then an equal volume of ethanol was added, and the mixture was allowed to stand. The precipitate was removed by centrifugation, and to the supernatant, 4 volumes of ethanol was added, and after allowed to stand, centrifuged. The precipitated polysaccharides was dissolved in distilled water to make a sugar concentration of about 13.3 eq. μ mole D-arabinose/ml. This preparation was used as stock solution of ^{14}C-Z-100.

Incorporation of each carbon of ^{14}C-glycerol into Z-100 and radiochemical yield of ^{14}C-Z-100

[1,3-^{14}C]glycerol and [2-^{14}C]glycerol in culture media were diluted to 2.1 kBq/μ mole and 0.12 kBq/μ mole, respectively. Each group of ^{14}C-glycerol consisted of five culture bottles. After cultivation, ^{14}C-Z-100 was extracted and purified according to extraction procedure. The radiochemical yield of ^{14}C-Z-100 to each group of ^{14}C-glycerol was calculated.

Chemical equivalence between ^{14}C-Z-100 and Z-100, and ^{14}C-general label of Z-100

The ^{14}C-Z-100 (4.9 kBq) obtained from culture media with [1,3-^{14}C]glycerol was analyzed by gel filtration high performance liquid chromatography (gel filtration HPLC). The eluate was measured by a refractive index (RI) detector and its radioactivity was counted.

The gel filtration HPLC equipment consisted of a Model L-6000 pump

(Hitachi, Tokyo, Japan), and a Model ERC-7512 RI detector (8×10^{-5} RIU/FS; Eruma, Tokyo, Japan). The gel filtration HPLC separations were carried out with two TSK-G3000 SW columns (600 x 7.5 mm I.D.; Tosoh, Tokyo, Japan) and a TSK-G2000 SW column (600 x 7.5 mm I.D.; Tosoh, Tokyo, Japan). The mobile phase consisted of 0.05 M NaCl solution. The mobile phase was degassed by a Model ERC-3322 degassor (Erma, Tokyo, Japan). The assay was performed at ambient temperature with a flow-rate of 1 ml/min. Pullulan (Showa Denko, Japan) and raffinose were used as molecular weight reference standards.

After the RI detection , the eluate from the gel filtration HPLC was fractionated every 1 ml by a Model FRAC-100 fraction-collecter (Pharmacia, Upsala, Sweden). The fractionated solution (1 ml) was added to 3 ml of liquid scintillator (Pico-aqua, Packard, ILL, USA), and counted by a Model 2000 CA scintillation counter (Packard, ILL, USA).

The effect of radioactivity on growth of organisms

For culture of organisms, [1,3-^{14}C]glycerol in a culture medium was respectively diluted to 6.39 kBq/μ mole (group I), 10.5 kBq/μ mole (group II) and 20.9 kBq/μ mole (group III). Each group consisted of three to five culture bottles. Growth of organism was observed weekly during cultivation. After completion of cultivation, ^{14}C-Z-100 was prepared according to the procedures of extraction and purifications described above. The chemical equivalence between ^{14}C-Z-100 and Z-100 was examined.

Large scale of biosynthesis of ^{14}C-Z-100

[1,3-^{14}C]glycerol in culture medium was diluted to make 2.2-4.5 kBq/μ mole. The organism was cultivated in 55 culture bottles with a medium contaning 31.4 GBq of [1,3-^{14}C]glycerol. ^{14}C-Z-100 was obtained through the procedures of extraction and purifications. The chemical equivalence between ^{14}C-Z-100 and Z-100 was examined.

Biological equivalence between ^{14}C-Z-100 and Z-100

Using aliquots of ^{14}C-Z-100 obtained in large scale of biosynthesis, the biological activity of ^{14}C-Z-100 was compared with that of Z-100 by the method described bellow.

Interleukin-3 (IL-3) inducing ability

Lymph node cells were collected from the inguinal region in C57BL/6 mice (Japan SLC) inoculated with 125 μg of Freunds incomplete adjubant (Difco)-emulsified live BCG organisms (Japan BCG Laboratory). These lymphocytes were suspended in an RPMI-1640 medium (Nissui Pharmaceutical,Co.,Ltd) containing 10 % fetal calf serum (FCS) and prepared to produce a concentration of 5 x 10^6 cells/ml. To this preparation, ^{14}C-Z-100 was added in a reasonable amount to give a concentration of 33.3 n mole D-arabinose/ml. After the mixture was incubated in a 5 % CO_2 atmosphere at 37°C for 24 hours, the supernatant was removed. To the 50 μl of supernatants in a 96-well microtiter plate, FDC-P2 cells (5 x 10^6 cells/50 μl, Tohoku University)[8] of the IL-3 dependent cell clones were added, and the mixture was incubated in a 5 % CO_2 atmosphere at 37°C for 48 hours. Then, 10 μl of 0.5 % MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) was added to each well and the plates were incubated at 37° C for 6 hours. Moreover, acid isopropanol (150 μl of conc. HCl-isopropanol (1:249)) was added and mixed thoroughly. The plates were read on a micro Erisa Auto Reader at the wavelengths of 550 and 630 nm. The ability of IL-3 was estimated by taking advantages of the difference in the OD at the two wavelengths[9].

Antitumor activity[10]

IMC tumor cells (1x10^6/mouse; Institute of Micobial Chemistry, Tokyo, Japan) were implanted intradermally into the flank of CDF1-mice (7 week, SLC, Japan), and ^{14}C-Z-100 was administered once a day at a dose of 1.33 eq. μ mole D-arabinose/mouse into developing tumor nodules on days 3, 7, 10, 14, 17, 21. Physiological saline (0.1 ml/mouse) was administered

into non-^{14}C-Z-100-treated mice as a control. At 24 days following tumor implantation, the tumor nodules were excised and weighed to calculate the percentage inhibition of tumor growth by the following equation:
Inhibition of tumor growth (%) = { 1 - (tumor weight in ^{14}C-Z-100 treated mouse/tumor weight in control mouse) } x 100

RESULTS AND DISCUSSION

Metabolic disposition of a new drug, generally, provides an important information to evaluate efficacy and toxicity of the drug. Usually, drugs synthesized chemically by using ^{3}H- or ^{14}C-labelled compound have been widely used for a study of drug metabolism. Z-100, however, is unable to be radioisotopically labelled by means of chemical reaction because of its complex chemical compositions. Some organic compounds of the complex chemical composition have been tritiated by the method of Wilzbach. This method, however, has a problem of causing radiation decomposition of the compound. For instance, Lentinan similar in polysaccharide to Z-100 was tritiated by the method of Wilzbach. However, ^{3}H-labelled Lentinan was reported to be remarkably decomposed, resulting in decrease in biological activities[11,12]. Considering the fact, in the present study, that Mycobacterium tuberculosis consumes a large amount of glycerol as a souce of carbon during its growth, ^{14}C-Z-100 could be obtained biosynthetically by cultivation of Mycobacterium tuberculosis in the medium containing ^{14}C-glycerol.

Incorporation of each carbon of ^{14}C-glycerol into Z-100 and radiochemical yield of ^{14}C-Z-100

Whether incorporation of glycerol carbons into Z-100 would be affected by the labelled site of ^{14}C-glycerol was investigated. 2.1 kBq/μ mole of [1,3-^{14}C]glycerol and 0.12 kBq/μ mole of [2-^{14}C]glycerol in culture medium were prepared. Both groups of organisms were normally grown, and no hazardous effect of radiation on growth of organism was observed. The radiochemical yield of ^{14}C-Z-100 was 0.13 % for [1,3-^{14}C]glycerol and 0.16

% for [2-^{14}C]glycerol. This result showed that organisms utilized carbons in glycerol molecule irrespective of chemical disposition of the carbon. Therefore, less expensive [1,3-^{14}C]glycerol was used in subsequent experiments.

Chemical equivalence between ^{14}C-Z-100 and Z-100, and ^{14}C-general label of Z-100

When Z-100 is analyzed with gel filtration HPLC, Z-100 can be separated into three fractions, i.e., Fr-A (molecular range : 24000-8000), Fr-B (8000-3000) and Fr-C (3000-500) in the increasing order of elution time according to gel filtration HPLC (Fig. 1).

The chemical equivalence, in the present experiment, was determined by comparing the elution pattern of ^{14}C-Z-100 obtained from a RI detecter with that of Z-100. On the other hand, ^{14}C-general label of Z-100 was studied by comparing the elution pattern obtained from radioactivity with that from the RI detector.

4.9 kBq of ^{14}C-Z-100 obtained from the group of [1,3-^{14}C]glycerol described above was analyzed by gel filtration HPLC. The eluate was measured by using a RI detector and was followed by counting its radioactivity. Fig. 2 shows the elution pattern. The elution pattern of ^{14}C-Z-100 obtained by the RI detector closely agreed with that of Z-100, indicating ^{14}C-Z-100 was chemically equivalent to Z-100 (Fig. 1, 2).

Percentage of total radioactivity obtained from three fractions to the radioactivity injected into gel filtration HPLC was over 96 %. Furthermore, the elution pattern of radioactivity for ^{14}C-Z-100 closely agreed with the pattern obtained by the RI detector, indicating that Z-100 was generally labelled with ^{14}C.

The effect of radioactivity on growth of organisms

[1,3-^{14}C]glycerol which has relatively high specific radioactivity was mixed with a culture medium to investigate an effect of radiation on growth of organisms. [1,3-^{14}C]glycerol in the culture medium was diluted

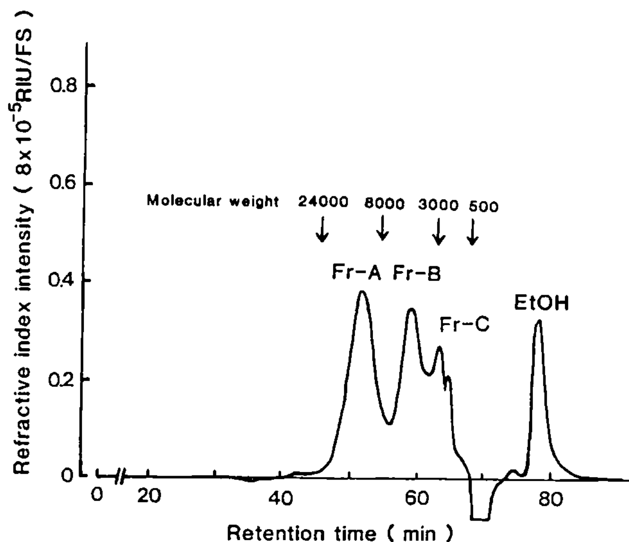


Fig.1 Gel filtration HPLC chromatogram of Z-100

Condition : Column, two TSK-G3000 SW columns (600x7.5mm I.D.) and a TSK-G2000SW column (600x7.5mm I.D.) : eluent, 0.05 M NaCl solution: flow rate , 1ml/min: Amount of sample injected , 1.3 eq. μ mole D-arabinose.

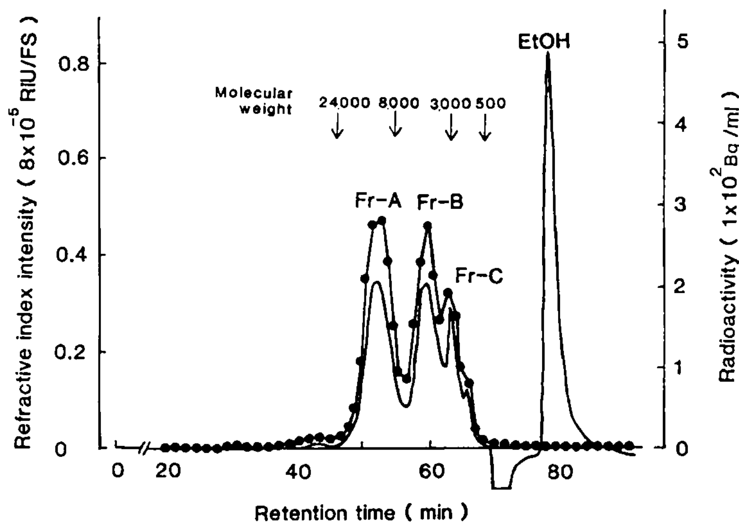


Fig.2 Gel filtration HPLC chromatogram of ^{14}C-Z-100

After the detection of refractive index detector, the radioactivity of the eluate from gel filtration HPLC was counted by a scintillation counter.
—— refractive index trace (left axis) —●— radioactive trace (right axis)
amount of sample injected : 4.9kBq, the other condition as in Fig.1.

to 6.39 kBq/μ mole (group I), 10.5 kBq/μ mole (group II) and 20.9 kBq/μ mole (group III), respectively. Organism of group I was found no inhibition in the growth by radiation, and a normal pellicle was formed on the surface of the culture medium. Growth of organisms of group II and III were inhibited around day 11 after cultivation , and at 3-4 weeks the culture growth of group III was extremely poor. Thus, extraction and purification from group III was discontinued thereafter because of poor formation of the pellicle. On the other hand, the poor formation of the pellicle was also observed in group II, and growth of the organism was delayed about a week at the third week compared to the normal growth. After cultivation for 3 to 4 weeks the pellicle of group II was still immature.

^{14}C-Z-100 extracted and purified from the organisms in group I and II respectively was tested for chemical equivalence to Z-100. As a result, ^{14}C-Z-100 from group I proved chemically equivalent to Z-100, but ^{14}C-Z-100 from group II showed chemical non-equivalence to Z-100 and an evident adverse effect of radioactivity on cell growth.

Thus, [1,3-^{14}C]glycerol was diluted to 6.39 kBq/μ mole or less for use in the cultivation of large scale biosynthesis of ^{14}C-Z-100.

Large scale biosynthesis of ^{14}C-Z-100

The organisms were cultivated in the medium contaning[1,3-^{14}C]glycerol at specific activity from 2.2 to 4.5 kBq/μ mole by using 55 culture bottles. The organisms in all bottles were normally grown during cultivation. The specific activity of ^{14}C-Z-100 was 3.3-7.5 kBq/eq. μ mole D-arabinose and the radiochemical yield was 0.11-0.15 %.

From a number of pharmacological studies[3,4], the effective doses of Z-100 in animal experiment were determined to be from 16.7 to 66.6 eq. μ mole D-arabinose/kg (s.c.). The metabolic disposition in rat at 133.2 eq. μ mole D-arabinose/kg dose using ^{14}C-Z-100 obtained by the present method was clarified[13,14]. This dose was confirmed to be non-toxic in any toxicological parameters.

Biological equivalence between ^{14}C-Z-100 and Z-100

Results of the biological activity study of ^{14}C-Z-100, i.e., IL-3-inducing ability and antitumor activity were shown in Fig. 3 and 4, respectively. From these results ^{14}C-Z-100 had equivalent efficacies to Z-100, indicating biological equivalence of ^{14}C-Z-100 and Z-100.

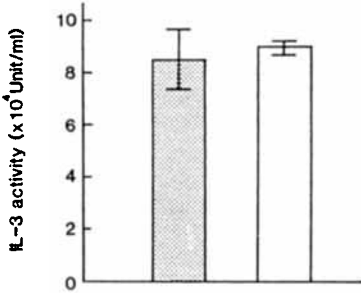


Fig .3 Effect of Z-100 on interleukin-3(IL-3) activity
Shaded column denotes Z-100 (n=3).
Open column denotes ^{14}C-Z-100 (n=3).

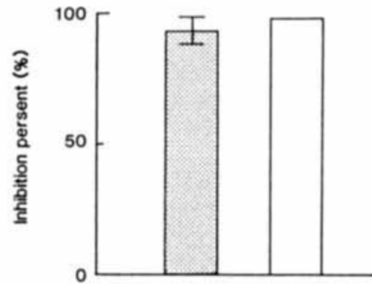


Fig.4 Effect of Z-100 on antitumor activity
Shaded column denotes Z-100 (n=3).
Open column denotes ^{14}C-Z-100 (n=1).

CONCLUSION

It became evident that ^{14}C-Z-100 obtained in this study was chemically and biologically equivalent to Z-100; Z-100 was generally ^{14}C-labelled; ^{14}C-Z-100 obtained has a specific activity sufficient for a study of metabolic disposition in animals.

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REFARENCES

1) Kobatake H., Suekane T., Murakami Y., Niwa S., Okahira A. and Kushida H. - Yakugaku Zasshi 101: 713 (1981)

2) Suzuki F, Brutkiewicz R.R. and Pollard R.B. - J.Natl.Cancer Inst. 77: 441 (1986)

3) Ohya O., Sasaki H., Nagatani Y., Hayashi Y. and Nomoto K. - Clin.Rep. 24 : 1965 (1990)

4) Ohya O., Sasaki H., Nagatani Y., Hayashi Y. and Nomoto K. - Clin.Rep. 24 : 1973 (1990)

5) Asami N., Hosoi M., Muramatsu K., Ikeda K. and Niijima Y. - Kyoubu Shikkan 3 : 912 (1959)

6) Sakazaki R. - "Bacterial Culture Media", Kindai Shuppan, 344 (1978)

7) Hodoger J.E. and Hofreiter B.T. - "Methods in Carbohydrate Chemistry Vol.1", Editors, Whistler R.L. and Wolform M.L., Academic Press, New York and London, 388 (1962)

8) Dexter T.M., Scott J.D., Scolnic E. and Metcalf D. - J.Exp.Med. 152 : 1036 (1980)

9) Mosmann T. - J.Immunol.Methods 65 : 55 (1983)

10) Shimizu T., Hayashi Y. and Kushida H. - Proceedings of the Japanese Cancer Association 44th annual meeting 1985

11) O'hara Y. - J.Toxical.Sci. 5 : 59 (1980)

12) O'hara Y. - J.Toxical.Sci. 5 : 73 (1980)

13) Sano H., Suzuki M., Ikeda Y., Sugimoto T., Sato H., Toyama S., Shinozaki U. and Tagashira E. - Jpn.pharmacol.Ther. 18 : 69 (1990)

14) Sano H., Suzuki M., Ikeda Y., Sugimoto T., Sato H., Furuta S., Miwa M. and Tagashira E. - Jpn.pharmacol.Ther. 18 : 85 (1990)