On The Beta-Glucuronidase Binding Protein (BGBP) Of Microorganisms.

Its Purification, The Antiserum Preparation Against That And Its Localization In Leproma And The Other Infectious Lesions Shown By Immunohistologic Method.

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SUMMARY

Our previous studies suggested that *M. leprae* (ML) grow in peripheral nerves and lepra cells because ML metabolize hyaluronic acid (HA), and use its component for their growth by the aid of host enzyme combined to the bacilli derived β -glucuronidase binding protein (BGBP). In this study, therefore, we examined the method to purify BGBP from a *mycobacterium* HI-75 originally separated from a leproma and cultured by modified Ogawa's medium containing split products of HA (glucuronic acid and N-acetylglucosamine). The distribution of BGBP in leproma and the other lesions consisting of hepatitis B virus infected liver and *M. avium-intracellulare* infected lung tissue were also immunohistologically examined.

As the result, the best method to get BGBP was preparatory electrophoresis in the final step of the purification and not the molecular sieving. The BGBP was actually proven in leproma and the other infected tissues as described, indicating the abilities of these microorganisms to utilize the metabolic machinery of the host with the similar ways to that of ML.

INTRODUCTION

The understanding of the metabolism of *M. leprae* (ML) to make the parasitism of ML in peripheral nerves and lepra cells of patients is one of the most interesting subject in leprology. We have assumed that ML have the affinity with and metabolize hyaluronic acid (HA), based on the results of histochemical studies on the above sites of parasitism showing the abundance of HA and its degrading enzyme encompassing ML¹. Therefore, we tried to extract β -glucuronidase (B-Gase), though unsuccessfully², from a *mycobacterium* HI-75 (HI-75) which was isolated from a leproma, claimed as ML by Skinsnes et al³. and has been kept on cultivating thereafter. The reason of the choice of HI-75 for that purpose is, the intraneural invasion and growth of the bacilli in the inoculated mice as one of its characteristics, though the reports by Stanford et al⁴ and Fujimiya et al⁵ concerning about the matter are quite controversial. The latter investigators reported that they observed the nerve invasion of HI-75 in the infected mice. However, besides Stanford et al., Tasaka identified HI-75 as *M. scrofulaceum* (MS) based on the observation of an immunoprecipitation line between his spe-

cific antiserum⁶ to alpha antigen of MS and the extract of HI-75 by the gel diffusion method. Genomic study consisting of ML-specific polymerase chain reaction amplification assay (PCR) and restriction fragment length polymorphism (RFLP) analysis by Williams, DL⁷ in 1991 revealed that HI-75 looks neither like ML nor MS. It rather resembled to either *M. fortuitum* or *M. smegmatis* according to her. Therefore, we have to evaluate highly the results on HI-75 by the current identification methods for ML especially of the genomic ones. We have to clarify the reason of these discrepancies in future by ourselves. In the mean time the ability of HI-75 to grow in nerves according to Fujimiya et al, provides us a basis to utilize the bacilli as the source to extract B-Gase², previously and the binding protein for the enzyme this time. The reason to take Fujimiya's report seriously is that the nerve involvement has been regarded as a characteristic of leprosy^{8,9}, and therefore, of ML. No other mycobacterium is known to show the intraneural growth of the bacilli so far. However, the HI-75 do not seem to have at least the extractable amount of B-Gase, in spite of our effort to get B-Gase in the past². Instead, we have observed human B-Gase in leproma which was immunohistologically stained utilizing anti-human B-Gase¹⁰.

In this study, we tried to get the evidence that ML in leprosy patient obtain B-Gase activity by fixing the host enzyme to the bacilli-derived B-Gase binding protein (BGBP), by immunohistologically confirming its presence in leproma. To do so, we also selected the best method to extract BGBP from HI-75.

MATERIALS AND METHODS

Culture of a mycobacterium HI-75: Modified Ogawa egg media were prepared by either of the following two methods. In the first one¹¹, we added 1ml of autoclaved physiological saline

containing 1% each of both glucuronic acid (GA) and N-acetylglucosamine (NAG) to each prepared Ogawa egg medium (Eiken Chemical Company), containing 7ml of 3% medium in each tube, immediately after the bacilli were inoculated to the media. In the second method^{12,13}, for the recovery of the large amount of bacillary pellet, we put 100ml each of basal solution of egg medium containing 1% each of GA and NAG into Wheaton's liquid medicine bottles with the capacity of 400ml and inspissated at 95°C for 60 minutes or autoclaved at 121°C for 15 minutes to make slopes in the bottles. The bacilli showed full growth after the cultivation for three to four months at 37°C. The tubes and bottles containing medium and bacilli were autoclaved before the separation of the bacilli. The colonies of the HI-75 were washed off by vigorous pipetting with phosphate buffered saline, pH 7.4 (PBS) and the suspension of the bacilli were mixed to make the final ethanol concentration of 68% (v/v) and all precipitates including the bacilli, were recovered by centrifugation at 7,000G for 30 minutes and dehydrated with acetone (Fr. 1).

B-Gase activity of HI-75: The B-Gase activity of the viable bacilli, directly scraped off the culture bottles was tested in comparison with that of *E. coli* (rehydrated lyophilized product by Sigma) utilizing Swabzyme™ -Beta-glucuronidase, (E·Y laboratories, INC.).

Purification of BGBP from HI-75^{14,15}: Fr. 1, was suspended five times with PBS, containing 1mM of EDTA, and mixed by the ratio with 1mg of Difco's trypsin/250mg of Fr. 1 (dry weight). The suspension was kept at 37°C for 3 hours adjusting its pH. around 7.4 to 8.0. After the digestion the suspension was vigorously mixed with equal volume of chloroform up to 15 minutes using blender and centrifuged at 7000G. Ethanol was mixed to the recovered supernatant to make the final concentration of 68% (v/v) and the mixture was kept overnight

in a refrigerator. The resulted precipitate was collected by centrifugations and dehydrated with acetone (Fr. 2). Preparatory electrophoresis (with the Furicoverer, Fuji Riken) of the Fr. 2 were performed monitoring the absorbance (O.D.) of eluted fractions at 280nm. In the procedure, carrier was Gel Mix 6 (Life Technologies, Inc.), containing 6% (w/v) acrylamide/bis-acrylamide monomers, 100mM Tris-borate (pH 8.3), 1mM Na₂ EDTA, and 7M urea, which was solidified with 10% (w/v) ammonium persulfate in two glass columns, 2cm in diameters and 8cm in lengths. The lengths of two gels, separation and stopper ones, were 4cm, each. Charged amount of Fr.2 was 140mg/ml, each time. Electrophoresis was performed at 27mA. Total void volume was 162ml in 21 hours at the flow rate of 0.13ml/min.

High performance liquid chromatography¹⁶ (HPLC) of BGBP¹⁷: It was performed utilizing a column of octadecylsilica, 46mm × 150mm (Inertsil ODS 5, GL Sciences Inc. Tokyo) for the elution by a 0 to 100%, linear isopropanol concentration gradient in H₂O containing 0.1% trifluoroacetate. Sample applied was 50μl of 0.1% BGBP and its flow rate was 1ml/min. The absorbance of eluted fraction was monitored at 230nm. Apparatus utilized was Beckman System Gold consisted of the programmable solvent module 126, programmable detector module 166 and the personal chromatograph.

Molecular sievings of the BGBP of HI-75¹⁷: The first one of the two procedures was performed to get an elution curve of 10mg of BGBP with 5mg each of albumin (M.W.: 67,000) and chymotrypsinogen A (M.W.: 25,000) as markers (Pharmacia Fine Chemicals) which was performed using a column, sized 1cm in diameter and 55cms in the height of gel, packed with Sephacryl S-200, high resolution (Pharmacia). Elution was performed using 0.05M phosphate buffer, pH 8.0, containing 0.1M NaCl (cf. Fig. 3). The similar sieving of 10mg of BGBP was also

performed using 5mg each of ovalbumin (M.W.: 43,000) and ribonuclease A (M.W.: 13,700) as markers (cf. Fig. 4). Flow rates were 14.5ml/hour in both procedures.

Activity of BGBP¹⁴: The BGBP solution was 0.164% by protein concentration which was estimated by micro TP-Test Wako kit (Wako Pure Chemicals). The solvent was PBS containing 1mM each of CaCl₂ and MgCl₂ (CMPBS). Each of these specimens, 0.025ml per a well, was placed for 30 minutes in the apparatuses for enzyme-linked immunosorbent assays (ELISA). Then the wells were washed several times with CMPBS. Specimens treated in the boiling water up to 5 minutes after dissolving with either CMPBS containing 6M urea or Laemmli's solution which contains sodium dodecyl sulfate (SDS) were also tested for the combination. After the blocking with 0.025ml of 3% bovine serum albumin (BSA), 0.025ml of CMPBS containing 16.7mg (833.3 units) of B-Gase (β-D-glucanohydrolase; EC 3.2.1.31) was placed in each well. The B-Gase utilized was purified one from bovine liver, type B-1 (Sigma). After the washing of nitrocellulose sheet with CMPBS the activity of bound B-Gase was examined utilizing Naphthol AS-BI glucuronide as substrate prepared according to the method of Hayashi et al¹⁸.

Anti-BGBP-antiserum: Three male rabbits weighing about 2kg, each at the start of the immunization were injected with the BGBP, suspended with physiological saline and homogenized with incomplete adjuvant, initially into foot pads then into muscles of buttocks, 1.2, 2.2mg, respectively at the beginning and 9 weeks thereafter. Then each rabbit was intravenously injected with 1ml of saline each time containing 8.5mg once and 10mg twice of BGBP at 15, 28 and 29 weeks after the start of immunization. The rabbits were exsanguinated a week after the last immunization. Two rabbits out of three survived until the

exsanguination. The sera were inactivated at 56°C for 30 minutes, pooled together and 50ml of them were fractionated by mixing with 7g of sodium sulfate. The precipitate was centrifuged down at 10,000G. and was reconstituted with 10ml of PBS and diluted 500X with PBS when used for immunohistologic stain.

Immunoassays of the anti-BGBP antiserum: Those were performed using ELISA¹⁹ utilizing multi-wells plastic plates and the apparatus for dot ELISA and passive hemagglutination²⁰. In the ELISA assays, BGBP was combined with BSA at the ratio of one to one by weight, according to the method of Avrameas²⁰, and suspended to make the 0.05% solution. The antigen solution was placed in the wells for 30 to 40 minutes at room temperature in both procedures. Blockings were made with 10% normal goat serum in ELISA and with 1% BSA in dot ELISA. Washing and dilution throughout the study was made using 10mM phosphate buffered saline, pH 7.2 in ELISA and 0.1% Tween 20 containing PBS in dot ELISA. For antigen antiserum reaction, the plates were left in a refrigerator, overnight or 20minutes at 37° C. The combined antiserum was developed using biotinilated anti-rabbit-IgG goat antibody, streptoavidine peroxidase, followed by the solution containing 0.05% each of o-phenylenediamine and H₂O₂, 5 minutes in case of ELISA and 0.025% 4-chloro-1-naphthol and 0.05% H₂O₂ for up to 15 minutes in case of dot ELISA. The reaction was, then, suppressed by 1N-HCl and was estimated by the absorbances at 405 and 450nm in ELISA. In passive hemagglutination test (PHT)²¹, 0.5% BGBP was combined to glutaraldehyde fixed and tanned sheep red blood cells (TSRBC). TSRBC suspension (2%) was introduced into wells already containing antiserum serially diluted with PBS and left for 2 hours before the assay.

Immunohistologic staining for BGBP²⁸: 10% formalin fixed and paraffin embedded tissue

sections were studied. Those were skin with lepromatous leprosy, liver with hepatitis B, and lung with *M. avium-intracellulare* infection. The causative organisms of those diseases were confirmed by Nyka and O'Neill's modification of acid-fast stain²⁴ for the mycobacteriosis and modified Orcein method for hepatitis B surface antigen²³ prior to the immunohistologic staining. After thin sectioning, the tissue sections were deparaffinized with chloroform and then ethanol before the rehydration. Then, the sections were digested with small amount of hyaluronidase solution from Streptomyces hyalurolyticus containing 100 TRU per an ampoule (Seikagaku Kogyo), for two hours mounted to cover the sections at room temperature. After washing with PBS and blocking with PBS containing 1% BSA, the sections were served for immunohistologic stain with avidine biotin complex method²⁵. Controls consisted of the sites of same tissues without the bacilli apart from the site with the bacilli. In case of hepatitis B virus infected liver, the sites of coexisted hepatocellular carcinoma which was not stained with Orcein method served as the control.

RESULTS

Cultivation of HI-75: The bacilli grew well forming many confluent orange-yellow colonies in the surface of media, which could be easily washed off by splashing PBS by Komagome pipette. The recovery of the Fr. 1 from 353 tubes by the first method was 62.8g. The recovery by the second method was not much different from that by the first one. The yield of Fr. 2 from 62.8g of Fr. 1 was 18.6g by dry weight (29.6%).

Activity of B-Gase of HI-75: The pellets of the viable bacilli did not show the activity of B-Gase with the Swabzyme™ β -glucuronidase unlike *E. coli* which showed deep red color of cotton swabs.

Separation of BGBP from HI-75 and its activity: As shown in the Fig. 1, BGBP was separated very well by means of preparatory electrophoresis. The BGBP appeared in the last fraction eluted, which was separated very discretely from the other fraction. The recovery of BGBP from 140mg of Fr. 2 was 34mg (24.3%). The fraction seemed to be very well purified according to the elution curve by HPLC as shown in the Fig. 2. Molecular sievings of BGBP of HI-75 with marker proteins did not separate the BGBP well as shown in the Figs. 3 and 4. There were atypical absorbances in the foot of the peaks of markers and absence of the solitary peaks of BGBP in both experiments.

Activity of BGBP: The activity of BGBP could be visualized by the method as written in the "materials and methods". The previous treatment of BGBP by SDS nullified its activity as shown by the Fig. 5.

Anti-BGBP-antiserum: The titers of anti-BGBP was 179200X by passive haemagglutination, 32000X by dot ELISA and 12800X with ordinal ELISA using multi-wells plastic plates.

Immunohistologic staining for B-Gase and BGBP: Anti-BGBP antiserum stained not only leproma (cf. Fig. 6) but *M. avium-intracellulare* (MAI) (cf. Fig. 7). In leproma, globi were also stained well like the other lepra cells containing ML. However, some of the immunoreactive sites seemed to be easily dispersed without the careful handling of the stained sections. In the cytoplasm of macrophages which were full with MAI the immunoreactive sites for BGBP were arranged discretely as though stained for the MAI themselves. In the hepatitis B virus infected liver, immunohistologically stained sections showed the similar localizations of BGBP in the liver cell cytoplasm (cf. Fig. 8) to those of hepatitis B surface antigen with modified Orcein method (cf. Fig. 9). The tissue section of hepatoma were stained neither by the immunohistologic stain for BGBP (Fig. 10) nor

by the modified Orcein method (Fig. 11).

DISCUSSION

Culture of HI-75 and its activity of B-Gase: Our basis of the choice of HI-75 to extract BGBP was the ability of the bacilli to grow in the peripheral nerve of nude mice as was briefly reported by Fujimiya et al.⁵. Therefore, we have taken the nerve invasion of HI-75 most seriously over the other characteristics. However, it does not mean that we have neglected the other reports. In fact, we admit that it would be fascinating research subjects in future to study whether other microorganisms besides ML grow in nerves like HI-75 and its difference from ML by PCR and RFLP after the recovery from host animals besides that harvested directly from culture bottles. Meanwhile, though there might be some risk of misinterpretation of our past results without all of the answers to the above questions, the nerve invasion of HI-75, absence of B-Gase in HI-75 as shown previously² and this time again and the presence of BGBP, cross-immunoreactive with that of ML¹⁵ as described below, facilitated us to draw the temporal conclusion that ML combine host B-Gase to its BGBP to use metabolic machinery of patients. The accelerated growth of HI-75 on culture, by GA and NAG¹¹, split product of HA, than by HA itself, also support the speculation. It might be also interesting to cultivate and examine the nature of microorganisms directly derived from lepromata by either of two methods of proposed media preparation.

Purification of BGBP from HI-75: Concerning about the nature of this BGBP from HI-75, the present study, as was the previous one¹⁵, clearly indicated the weak negative charge at pH 8.3 and a good separability by that condition by preparatory electrophoresis probably due to the presence of urea and EDTA. The fraction seemed to be very well purified according to the

Fig. 1: An elution pattern of BGBP (arrow) by preparatory electrophoresis monitored by the absorbance at 280nm showing a distinct peak, well separated from the others.

Fig. 3: An elution pattern on molecular sieving of BGBP with albumin and chymotrypsinogen A. Note the presence of atypical absorbance (arrows) in the foot of the peaks of markers and the absence of the peak of BGBP itself.

Fig. 4: An elution pattern on molecular sieving of BGBP with ovalbumin and ribonuclease A as markers. Note the presence of atypical absorbance (arrows) in the foot of the peaks of markers and the absence of the peak of BGBP itself.

Fig. 5: The sites of the activity of B-Gase, combined to BGBP which were bound to nitrocellulose sheet were stained with the method according to Hayashi et al. The dots 1 and 2 which combined BGBP, treated with SDS instead of original BGBP did not show the enzyme activity. The dots 3 and 4 which combined BGBP showed the enzyme activity. The dots 5 and 6 which were placed with CMPBS alone without BGBP did not show the enzyme activity.

Fig. 6: A leproma, immunohistologically stained for BGBP, counterstained with hematoxylin. Gray tone of lepra cells and globi (arrows) indicates the sites of the immunoreactivity, and therefore, the localization of the BGBP.

Fig. 7: An inflammatory lesion, immunohistologically stained for BGBP and counterstained for nuclei with hematoxylin, showing the histiocytic infiltration associated with the infection of *M. avium-intracellulare*. The gray tone of the cytoplasm of histiocytes indicate the immunoreactivity for BGBP and the presence of the bacilli (arrows).

Fig. 2: An elution curve of BGBP (arrow) by HPLC showing some tailing but the good purity.

Fig. 8: The photomicrograph of liver in the same case as that of Fig. 9 immunohistologically stained for BGBP. The immunoreactive sites (arrows) are located in the cytoplasm of some liver cells and therefore showing the similar staining pattern as that of Orcein stain except the connective tissue (arrow C) of portal triad was not stained by this method.

Fig. 10: The photomicrograph of a hepatoma immunohistologically stained for BGBP. No positively stained cells were present.

Fig. 9: The photomicrograph of liver section stained with modified Orcein method for hepatitis B surface antigen. The positive stain (arrows) located in the cytoplasm of some liver cells. The connective tissue (arrow C) of portal triad was also stained by this method.

Fig. 11: The photomicrograph of a hepatoma stained with Orcein method for hepatitis B surface antigen. No positively stained cells were present.

elution curve by HPLC as shown in the Fig. 2. The molecular weight of BGBP was 11,990 according to the estimation using liquid-chromato-mass-spectrometry by the Department of instrumental analysis, Takara Shuzo Co²⁶. The molecular sieving, on the other hand, does not seem to be a good method for the separation, because of the absence of clear-cut peak of BGBP when the other fractions like marker proteins are present besides BGBP. It might also indicate that BGBP have the ability to combine the other enzymes besides B-Gase in the other organisms like hepatitis B virus which fit to use metabolic machinery of liver cells. The speculation should be justified since BGBP is not a specific product by ML^{17,27}, and it seems to combine other proteins beside B-Gase as suggested by the elution curves on column chromatography as written in results. The temporal conclusion, might also suggest the possibility of the growth disturbance of some microorganisms including ML in vivo by the elimination of BGBP function by immunological or biochemical means.

Activity of BGBP: Untreated, and treated BGBP with 6M urea but not SDS seemed to retain the activity, indicating again the best way to purify the BGBP is preparatory electrophoresis using the buffer containing urea without treating the Fr. 2 with SDS. It seemed that the present experiment to test the activity of BGBP was somehow successful but the results of repeated experiments indicated that the successful result seemed to depend on the proper choice of nitrocellulose sheets in which we need to work to improve more reproducibility.

Immunohistologic staining for B-Gase and BGBP: Our present and previous studies revealed that the rabbit antiserum against the BGBP of HI-75 react not only with ML but also with the other microorganisms, grown intracellularly, hepatitis B infected liver cell cytoplasm²⁸ and legume extracts¹⁷ indicating

vast distribution of BGBP among plants. This will assure us the equivalent immunogen to BGBP from HI-75, from the easier source to acquire. The knowledge on the nature of BGBP from HI-75 obtained by the present study will make the procedure easier. The immunoreactivity of BGBP in the tissue after hyaluronidase digestion of the tissue sections²⁸ might suggest that BGBP attract not only the enzyme but HA likewise. The coating of antigens by HA might mask the antigenicities of microorganisms and help microorganisms to be protected from immunosurveillance system of the host.

CONCLUSIONS

1. The present study showed that the best method to purify the β -glucuronidase binding protein (BGBP) from the *mycobacterium* HI-75 was preparatory electrophoresis in the final step of its purification.
2. Immunohistological study suggested that *M. leprae* in leprosy patient obtain the activity of β -glucuronidase by fixing the host enzyme to this BGBP.
3. This BGBP was shown to be present not only in *M. leprae* but apparently in the other microorganisms such as *M. avium-intracellulare* and hepatitis B virus.

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Beta-Glucuronidaseに対する微生物由来の結合蛋白質について。
その精製、抗体作成ならびに癰腫および他の感染症病巣における局在。

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キーワード：らい， β -グルクロニダーゼ， β -グルクロニダーゼ結合蛋白質。

らい菌は，末梢神経やらい細胞に多量に含まれるヒアルロン酸を自己に結合させた宿主由来の酵素を用いて分解し，その産物を代謝して増殖すると考えられる。よって本検索ではこの酵素結合蛋白質 (β -glucuronidase binding protein: BGBP) の精製方法，抗体作製並びに感染病巣における局在について検討した。

BGBP精製の原料としては，らい腫から分離された抗酸菌HI-75をヒアルロン酸の構成分子であるグルクロン酸とN-アセチグルコサミンを加えた小川培地で培養して用いた。なお，BGBPの分布はらい腫以外にB型肝炎やM. avium-

intracellulare(MAI)による感染病巣についても免疫組織学的に検討した。

以上の結果，BGBPの精製については，精製の最終段階で調整用電気泳動を用いる事が最良の方法であり，分子篩クロマトグラフィーは恐らくBGBPが共存する分子と結合する性質を有するため，適さない事が分かった。また，BGBPはらい腫ならびにB型肝炎ウイルスやMAIの感染病巣にも存在する事が分かった。以上は，これらの寄生体もまた，らい菌と類似した方法によって宿主の代謝系を利用する能力を有する事を示唆すると考えられた。