Establishment of Mouse Cell Lines Expressing *Mycobacterium leprae* 65KDa Heat Shock Protein Gene

Kazue Nakanaga* Hiroko Nomaguchi and Masanori Matsuoka

National Institute for Leprosy Research, Tokyo, Japan.

[Received 2 April 1996/Accepted 7 May 1996]

Key words: *Mycobacterium leprae* gene, hsp65, gene transfection, gene expression, messenger RNA.

Neurotropism is one of the most important characteristics of *Mycobacterium leprae* (*M. leprae*). It is believed that nerve damage of leprosy is caused by various immune mechanisms, and so called "autoimmune reaction" is suspected to have one of the role in nerve damage.

*M. leprae* 65 kDa heat-shock protein (hsp65) has noticeable characteristics. Hsp65 is a highly conserved protein. It belongs to the family of GroEL proteins. The amino acid sequence of *M. leprae* hsp65 shows homology of more than 90% with other mycobacterial hsp65s and shares around 48% amino acid identity with the mammalian mitochondrial P1 or hsp60. Hsp65 was also reported to be a highly immunogenic protein of *M. leprae* in both B-cell and T-cell responses. Recently, many reports have described the relationships between hsp65 and some autoimmune diseases such as Kawasaki disease, rheumatoid arthritis and so on; the role of hsp65 in immunological disorders may be one of molecular mimicry. Freedman et al described the expression of hsp60 and hsp70 by human glial cells. Such hsps were supposed to be the recognition molecule(s) for γδT cells which mediated glial cell lysis *in vitro*. There are no providing mechanisms for nerve damage in leprosy just now, but the molecule(s) of hsp65 may at least be the possible candidate as a recognition molecule for γδT cells generated during *M. leprae* infection.

In this paper we reported establishment of mouse cell lines showing stable expression of *M. leprae* hsp65, such cell lines would work as a target cell in cytolysis assay *in vitro* for cytotoxic T cells and γδT cells generating during *M. leprae* infections.

**MATERIALS AND METHODS**

Cell lines and culture. The mouse cell line BALB/3T3 clone A31-1-1 which was provided by JCRB cell bank (Japan), was cultured in Ham's F12 medium supplemented...
with 10% fetal bovine serum (FBS) as growth medium. The 300 μg/ml G418 was added to growth medium, which was used as selection medium, or maintenance medium for the transfectants. For induction of the gene expression, 10 μM dexamethasone was added to selection medium.

**Plasmids and the host E. coli strains.** The plasmid pUC8/N5 carrying the gene of *M. leprae* hsp65 was propagated in JM109. The mammalian expression vector pMAMneo (Clontech Lab. Inc.) which has the mouse mammary tumor virus's long terminal repeat (LTR) as the expression promoter for foreign gene, and also has G418 resistant gene as a selection marker in mammalian cells, was propagated in DH5α.

**Construction of plasmids:** The hsp65 gene fragment which was digested with restriction enzymes Xmn I and BssH II was treated to be blunt end and ligated to Sal I linker. The resulting fragment was ligated to Sal I linker. The fragment containing hsp65 gene and Sal I digested pMAMneo were ligated to construct the plasmid pMAMneo65k-B (Fig. 1). The pMAMneo65k-B was digested with EcoR I to cut off ori region of pBR322 (EDpMAMneo65k-B) before transfection.

**Gene transfection to A31 cells.** Samples of 2x10⁵ A31 cells were seeded onto 60 mm dishes and 24h later, each 5 μg of vector DNA (pMAMneo or pMAMneo65k-B) or the EcoR I fragment of it (EDpMAMneo65k-B) was introduced into the cells by the calcium phosphate precipitation method. After the cells were cultured for 24h, they were washed and added with fresh growth medium. After 24h cultivation, the cells were spread onto 100 mm dishes at the concentration of 2~6x10⁴ cells/10 ml, and 24h later the culture medium was replaced with selection medium. Approximate 10 days after G418 selection, G418 resistant cell colonies were isolated for further propagation.

**Western blotting (WB).** The gene product for the hsp65 gene of *M. leprae* was tried to detect in the cell lysate of transfectants after 48h of cultivation in expression induce medium by WB. The mixture of anti-hsp65 monoclonal antibodies (MAbs) B2C, A5B and 3A were used as the first reaction antibody. The second reaction antibody was the anti-mouse IgG conjugated with alkaline phosphatase (AP), and BCIP/NBT was used as the substrate reagent. Recombinant hsp65 (rHsp65) was extracted from IPTG stimulated *E. coli* carrying pUC8/N5,
and affinity purified using MAb 3A. The rhsp 65 was used as a positive control for WB.

**Isolation and cloning of the hsp65 producing cells.** To prevent the heterogeneity of cell populations, limiting dilution was done twice per one WB positive cell colony. Every clone was immunologically stained to check the hsp65 expression. Each 0.3-0.5 cells/100μl/well was distributed into flat-bottomed 96 well microplate. The cells of more than 50% confluent wells were passaged into 24 well microplates.

**Immuno-staining.** A part of each cell clone was cultured on a cover slip in expression induce medium for 48h. It was fixed in methanol at -20°C for 10 min, and stained with the MAb B2C as the first reaction antibody, the second reaction antibody and the substrate reagent were the same as those of WB. For counter staining, nuclear fast red was used, then the cover slip was mounted on slide glass with Canada balsam after ethanol dehydration and xylene treatment.

**Northern blot analysis.** Antisense hsp65 RNA probe labeled with 32P was generated as a transcript RNA\textsuperscript{17,18}. The template DNA of transcription contained hsp65 sequence downstream from a bacteriophage promoter T3. Total RNA was extracted from the cells\textsuperscript{19} after 12-24h cultivation in expression induce medium. Poly(A)+ mRNA was isolated from the total RNA using Oligotex-dT30 (TAKARA) and each 1 μg of poly (A)+ mRNA was separated by electrophoresis in 1% agarose gel supplemented with formaldehyde. The mRNA was transferred to a nylon membrane and hybridized with the probe. Positive signal was visualized by exposure of X-ray film at -80°C\textsuperscript{20}.

**RESULTS**

Fig. 2 WB analysis of the gene introduced cell extract: Hsp65 was detected with the mixture of anti-hsp65 MAbs as the first reaction antibody. The lysate of approximate 1.5 x 10^5 cells was loaded on each lane. Sizes are shown at the left. (A) Lanes: 1, affinity purified rhsp65 6 ng (extracted from E.coli carrying pUC8/N5); 2, No.1-3 (pMAM neo transfected A31); 3-5, No.141, No.102, No.121 (EDpMAM neo65k-B transfected A31). (B) lanes: 1, rhsp65; 2, No.6-1 (pMAMneo transfected A31); 3, No.141-3 (subclone derived from No.141); 4, mixture of the cell lysates (No.141-3) and rhsp65; 5, rhsp65.

**Detection and isolation of A31 cells expressing M. leprae hsp65.** Total 144 of the EDpMAM neo65k-B introduced and G418 resistant cell colonies were isolated and checked for hsp65 by WB. At first, positive bands of 65 kDa including faint ones were detected from 49 cell samples. However the expression of hsp65 was seemed to be transient in most of the cells, because the positive signal became faint or disappeared after 2 or 3 times passage. Among them colony No.141 showed stronger band than the band of 6 ng rhsp65 positive control (Fig.2A, lanes 1 and 3). Normal A31 cells and the pMAMneo introduced cells have been never stained with anti-hsp65 MAbs in this experiment. The expression of hsp65 in the cell No. 141 was stable after several passages. Several subclones expressing hsp65 were isolated from No.141 after twice limiting dilution.
Expression of protein. To compare the size of both hsp65 expressed in *E. coli* and A31 cell, rhsp65 was added to the cytosol of cell clone No.141-3. Rhsp65, the cytosol of cell clone No.141-3, and the mixture of them were analyzed by WB. Three lanes showed the same bands of approximate 65 kDa (Fig. 2 B lanes 3, 4 and 5).

Hsp65 was detected by immuno-staining at the cytoplasm of the cell colony No.141 and the subclones derived from No.141 (Fig. 3). By WB and immuno-staining, No.141 derived cell clones showed highest expression after 1-2 days cultivation in expression induce medium. Normal A31 cell and the pMAMneo introduced cells have been never stained with MAb B2C in the immuno-staining experiment.

Expression of mRNA. The mRNAs extracted from the gene introduced and G418 resistant cell colonies were hybridized with the hsp65 antisense RNA probe. Samples of cell clone No.141-3 and No.24-10 showed the bands of approximate 2.8 kb in size. The cell clone No.24-10 was the pMAMneo65k-B transfected and twice cloned A31 cell. However no apparent signal was detected from the cell clone No.6-1, which had been transfected with pMAMneo. The band of No.141-3 was stronger than that of No.24-10. For the control experiment, the same samples were hybridized with antisense β-actin RNA probe. The band of 2.2 kb β-actin mRNA was apparent on every three lanes showing the same strong signal.

**DISCUSSION**

Prolonged expression of *M. leprae* hsp65 gene was observed in A31 cell introduced with EDpMAMneo65k-B. That were demonstrated by both protein and mRNA analysis.

Our previous attempt was unsuccessful when using whole 3.6 kb *M. leprae* hsp65 gene as a insert into the same expression vector pMAMneo. That vector was stably integrated into A31 cell genome and G418 resistant cell colony was generated. However, hsp65 expression was undetectable even after dexamethasone induction. Hsp65 expression vector, pMAMneo65k-B, has a *Xmn* I-*BssH* II fragment of hsp65 gene without promoter and non-sense sequence of mycobacterial portion as an insert, it is likely that mycobacterial promoter and/or non-sense sequence region may prevent the expression in mammalian cells.

Hsp65 has a high amino acid sequence
homology with the mitochondrial hsp P1 and with the hsp60 \(^{21}\). Moreover, anti hsp65 MAbs bound to the same unknown protein of neural cells \(^{22,23}\). However, these MAbs used in WB analysis were defined to have no cross-reactivity with mouse endogenous hsp60 (unpublished observation). So that the bands of near 60 kDa (Fig. 2) were thought to be identical to \(M. \text{leprae}\) hsp65 gene product. The same size bands were detected from the cytosol of hsp65 expressed cells by WB with individual MAbs (data not shown). In the pMAMneo65k - B, one of the initiation codons GUG, which was in the hsp65 gene at position 66 \(^{24}\) had been cut off by Xmn I digestion. The hsp65 protein expressed in A31 cells was thought to be translated from the codon AUG (position 207) to yield 56868 Da in size. Two kinds of hsp65 might be translated from the codon GUG (position 66) and AUG to yield proteins of 61856 Da and 56868 Da respectively in E. coli and host mycobacteria \(^{24}\), however the bands of No.141-3 and rhsp65 were shown to be equal in our experiment (Fig. 2B). At least the rhsp65 used in our experiment which was MAb 3A affinity-purified material, that was supposed to be the 56868 Da single protein.

The expression of hsp65 mRNA in A31 cell was confirmed by northern blot analysis. The band of 2.8 kb in northern blot was consistent with the size of mRNA estimated from the construction of pMAMneo65 k-B (Fig. 1 and 4). Shorter bands might be the processing form of the mRNA. Although there are approximate 50% homology between \(M. \text{leprae}\) hsp65 gene and the gene of mitochondrial P1 or hsp60, the sequence that was specific to \(M. \text{leprae}\) hsp65 gene was absent in the mRNA derived from host cell.

These established cell lines No.141-3 and No.24-10 would work as a target cell to analyze cytotoxic T cells and/or \(\gamma \delta\) T cells generating during \(M. \text{leprae}\) infections perhaps manifest nerve damage by autoimmune reactions.

Silva et al had described about the establishment of macrophage cell line J774 expressing \(M. \text{leprae}\) hsp65 gene using a retroviral vector, and the hsp65 antigen was presented by the transfected cells for recognition by CD8\(^{+}\) T lymphocytes, moreover by CD4\(^{+}\) T lymphocytes without MHC restriction \(^{25,26}\). Because of the fact that macrophages are able to present antigens in association with both MHC class I and class II molecules, choice of macrophage cell line has advantages when analyzing both antigen processing and recognition phase of cell-mediated immunity in \(M. \text{leprae}\) infection. On the other hand, the cytolysis mediated by \(\gamma \delta\) T cells may not require target cell expression of MHC antigen for interaction \(^{27}\). When recognition and cytolysis mediated by \(\gamma \delta\) T cells would be examined, not the macrophages but other simpler cell system was also needed. Our system of foreign gene expression in mammalian cells may apply \textit{in vivo} gene transfer such as genetic immunization \(^{28}\), which may not apply with retroviral vectors.

**REFERENCES**

4) Dudani AK, and Gupta RS. Immunological characterization of a human


21) Jindal S, Dudani AK, Singh B, Harley


らい菌熱ショック蛋白65kDa遺伝子発現マウス細胞株の樹立

中永和枝， 野間口博子， 松岡正典

［受付：1996年4月2日，受理：1996年5月7日］

国立多摩研究所

キーワード：らい菌遺伝子，hsp65，遺伝子導入，遺伝子発現，メッセンジャーRNA

ハンセン病における神経障害の機構を解析する手段の一つとして我々は，らい菌遺伝子を発現するマウス細胞株の樹立を試みた。モデルシステムとして，マウス細胞株BALB/c-3T3クローンA31-1-1（A31細胞）を，らい菌遺伝子は熱ショック蛋白65kDa（hsp65）遺伝子を選んだ。らい菌hsp65遺伝子のopen reading frameを含むXmn I-BssH II断片を哺乳動物高発現系ベクターpMAMneoに挿入してpMAMneo65k-Bを生成した。A31細胞への遺伝子導入は，リン酸カルシウム法にて行い，EcoR I消化によりpBR322のori領域を切り取ったpMAMneo65k-B（EDpMAMneo65k-B）を導入した。

らい菌hsp65の発現は，特異蛋白及びメッセンジャーRNAの解析により検出した。EDpMAMneo65k-Bを導入したA31細胞株No.141とそのサブクローンにおいて持続的ならびにらい菌hsp65遺伝子の発現が観察された。細胞クローンNo.24-10は，pMAMneo65k-Bを導入したA31細胞であるが，この細胞でもhsp65の発現が見いだされた。しかし，細胞クローンNo.24-10において発現しているhsp65遺伝子特異的メッセンジャーRNAの量は，細胞クローンNo.141-3のものに比べて少なかった。

これら樹立されたマウス細胞株は，らい菌の感染によって誘導される細胞傷害性細胞の標的細胞として解析に役立つものと思われる。

*Corresponding author:
〒189 東京都多摩市青葉町4-2-1
国立多摩研究所
Tel: 0423-91-8211

120