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Production of Fab Fragment Corresponding to Surface Protein Antigen of *Streptococcus mutans* Serotype c-Derived Peptide by *Escherichia coli* and Cultured Tobacco Cells

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The cDNA of a mouse Fab fragment was cloned from a hybridoma cell line that produces a mouse monoclonal antibody, KH5, that reacts with the peptide fragment of the surface protein antigen of *Streptococcus mutans* serotype c (PAc). After transfection with cDNA, recombinant Fab fragments were produced by *Escherichia coli* (T15 Fab) and cultured tobacco cells (X253 and X262 Fabs). The antipeptide activities of T15 and X253 were similar to that of KH5. X253 was secreted into the culture media, which had a specific affinity for the PAc peptide.

[**Key words:** recombinant Fab fragments, tobacco cell culture, *Streptococcus mutans*, surface protein antigen of *Streptococcus mutans* serotype c (PAc)]

Streptococcus mutans is thought to be a causative agent of dental caries and its cell surface protein antigen (PAc) has been implicated as a cariogenic factor (1). PAc is a major adhesion molecule of *S. mutans* responsible for colonization on the tooth surface and is an important target for the development of anticaries agents. An anti-PAc antibody has been reported to prevent recolonization by *S. mutans* (2). In addition, PAc peptides have been studied as caries vaccines and found to induce anti-PAc antibodies that inhibit the colonization on the tooth surface by *S. mutans* (3, 4). Several monoclonal antibodies (MAbs), designated as the KH and SH series, have been developed (4) that are candidates for anticaries reagents or caries risk diagnostic tools. KH5 is an IgG1, which is reactive to the PAc (361–377) peptide and one of the most reactive antibodies to recombinant PAc among the KH and SH MAb series.

Past studies of anti-*S. mutans* antibodies were focused on the prevention of recolonization (2). The removal of biofilms on the tooth surface by professional cleaning with chlorhexidine before treatment with a large amount of antibodies was necessary. An *S. mutans*-specific bacteriocide that could penetrate the biofilm on the tooth would prevent dental caries more effectively and be a simple medication. The use of an antibody-based drug delivery system (DDS) is one possible method for the development of *S. mutans*-specific anticaries reagents. An immunoliposome, which is a liposome fused with either single-chain antibodies (scFvs) or Fab fragments, is being studied for cancer therapy (5). Dental biofilms have a complex structure and are relatively impermeable to antimicrobial reagents (6). In addition, reagents are easily washed out from biofilms by saliva. However, small antimicrobial molecules with an affinity to *S. mutans* will work more effectively than reagents with no affinity. Antimicrobial peptides fused with small antibodies can be produced (7). PAc-specific recombinant antibodies can be good tools both for producing DDSs for caries prevention and for the diagnosis of caries risk.

Therapeutic antibodies are usually produced by mammalian cell cultures, and the costs are high. It would not be logical to spend as much on caries prevention as on cancer therapies. Moreover, prophylactic anticaries agents must be safer than therapeutic agents. Plants are inexpensive hosts for antibody production and their use reduces the risk of contamination by human pathogens (8). In this study, we investigated plant cells as practical candidate hosts for anticaries antibody production. We cloned Fab cDNA from a MAb KH5-secreting hybridoma cell line for expression in both *Escherichia coli* and cultured tobacco cells. The anti-PAc activities of Fabs were determined and conditions for expression in tobacco cultures were investigated.

MATERIALS AND METHODS

cDNA cloning of Fab fragments and construction of expression vectors Total RNA was isolated from KH5-secreting hybridoma cells (4) and cDNAs of the Fab heavy (H) and light (L) chains were amplified using an antibody cDNA cloning kit (Immunogene M; Nisshinbo Industries, Tokyo). The cDNAs were ligated into the pFab1-His2 vector (9). The selected cDNAs of the

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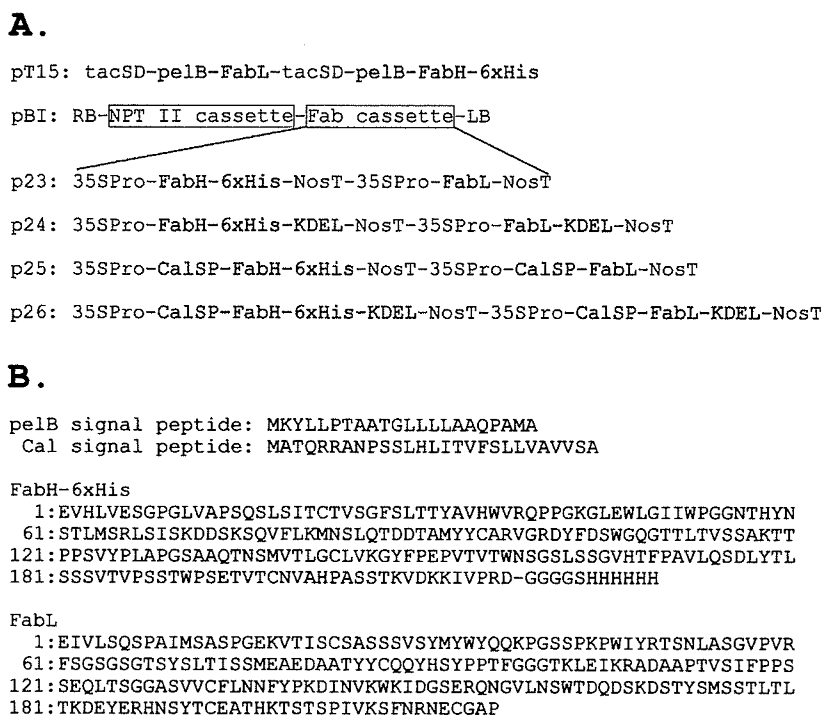

FIG. 1. Schematic representation of expression cassettes of Fab genes. (A) pT15 was the *E. coli* expression plasmid for the Fab genes. The cDNAs of the light (Fab L) and heavy (Fab H) chains were cloned under the control of the tac promoter and Shine–Dalgano sequence (tacSD). The periplasmic space secretion sequence of *Erwinia cartovora* (pelB) followed the tacSD sequences. A 6× His tag was attached to the C-terminal of the Fab H chain. Fab genes were cloned into the T-DNA region of the binary vector, which has right (RB) and left border (LB) sequences. The p23 and p24 plasmids have Fab H and L chain cDNAs under the control of the cauliflower mosaic virus 35S promoter and omega sequence (35SPro). The nopaline synthetase terminator (NosT) was located downstream. The p25 and p26 plasmids have the secretion sequence of calreticulin (CalSP) of *Nicotiana plumbaginifolia*. The p24 and p26 plasmids have the KDEL ER retention signal at the 3′ end of the Fab H and L chain cDNAs. (B) Deduced amino acid sequences of pelB and CalSP, Fab H-6x His and Fab L.

Fab H and L chains were cloned into the T-DNA region of the pBI101 binary vector with additional synthesized DNA coding for a signal peptide and an ER retention signal (10) (Fig. 1). Translational enhancers (omega sequence and initiation sequence) were introduced immediately downstream of the 35S promoter, as previously described (10), to enhance Fab production.

Expression in *E. coli* and extraction of soluble Fab fragments The ligated pFab1-His2 vector was transfected into *E. coli* JM109 cells (Toyobo, Osaka). Bacterial colonies were incubated at 28°C in 2 ml of Luria–Bertani (LB)/ampicillin (50 mg/*l*) then isopropyl-β-D-thiogalactopyranoside (IPTG: 0.5 mM) was added to induce the expression of the Fab fragments. *E. coli* pellets were suspended in B-PER PBS (Pierce Biotechnology, Rockford, IL, USA)/Complete (Roche Diagnostics, Tokyo) and the anti-PAc activity of the supernatants was determined by ELISA. Five hundred milliliters of the culture media from a selected bacterial clone centrifuged and the pellet obtained was resuspended in 25 ml of B-PER PBS/Complete/5 mM imidazole (Sigma-Aldrich Japan, Tokyo). The cell extract was centrifuged and the supernatant was filtered (0.45 μm) before liquid chromatography.

Transformation of tobacco cells and extraction of Fab fragments Suspension cultures of XD6S tobacco cells (11) were transformed using the recombinant binary vectors p23, p24, p25 and p26 by standard methods (10). Between 10 and 20 colonies from each of the four transgenic lines, X23–X26 series, were inoculated into the MS medium. The transgenic cells were propagated for 7 d under the same conditions used for XD6S cells (11). To measure both total soluble protein (TSP) concentration and the expression level of the antibody, 1 ml of suspension culture was transferred into a 2-ml microtube. One-tenth milliliters of 10× Complete (Roche) in 0.5 M Tris pH 7.5 and 0.15 g each of 0.1- and 1-mm diameter glass beads (Sigma-Aldrich) were added. The tubes were shaken at 30 Hz for 5 min using the Mixer Mill MM300 (Qiagen, Tokyo) and centrifuged at 15,000 rpm for 10 min to obtain the plant extract supernatants. TSP concentration was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

For antibody purification, tobacco culture suspensions in multiples of 100-ml volumes were sampled 10 d after the initiation of culture. The cells and media were separated by vacuum filtration through filter paper (Advantec, ToyoRoshi, Tokyo). The protein in the media was concentrated by ammonium sulfate precipitation and the resulting pellet was suspended in phosphate-buffered saline (PBS)/Complete/5 mM imidazole. The suspension was filtered (0.45 μm) before liquid chromatography. Cells were frozen overnight and thawed in 2 volumes (v/v) of extraction buffer (50 mM Tris pH 7.5/200 mM NaCl/Complete/5 mM dithiothreitol [DTT]). The cells were homogenized (Physcotron, Microtech, Tokyo) until over 80% of the cells were disrupted, as determined by microscopic observation. Cell debris was removed by centrifugation at 18,000 rpm for 30 min. The protein in the supernatant was concentrated by ammonium sulfate precipitation and the resulting pellet was suspended in PBS/Complete/5 mM imidazole. The suspension was filtered (0.45 μm) before liquid chromatography.

ELISA ELISA plates (SUMILON H type; Sumitomo Bakelite, Tokyo) were coated with 1 μg/well recombinant PAc isolated from *S. mutans* TK18 as described previously (4). Plates were blocked overnight with 3% skim milk (Wako, Tokyo) in PBS to inhibit nonspecific binding. After washing with PBS containing 0.05% Tween 20 (PBST), 100 μl of either 2-fold serially diluted extracts

or purified antibodies was added to the plates for 1-h incubation at 37°C. Plates were rewashed with PBST, 100 μl of alkaline phosphatase (AP)-conjugated goat anti-mouse Fab specific antibody (1 : 1000 dilution, Sigma-Aldrich) was added, and the plates were incubated for an additional 1 h at 37°C. Unbound AP conjugate was washed away with PBST, bound antibodies were detected by incubation for 1 h at 37°C with a *p*-nitrophenyl phosphate (pNPP) substrate (Bio-Rad), and $OD_{405-620}$ was read. ELISA antibody titer was expressed as the highest dilution giving an $OD_{405-620}$ of 0.1 unit higher than that of the control wells without antigen.

ELISA plates were coated with an anti-mouse Fab antibody (0.5 μg/well; Sigma-Aldrich). Then the test samples were added to the plates incubated for 1 h at 37°C, followed by blocking with 3% skim milk in PBS. Serially diluted mouse Fab (Jackson ImmunoReserch Laboratories, West Grove, PA, USA) was used as a standard in all assays. After incubation, AP-conjugated anti-mouse Fab antibody (1 : 1000 dilution, Sigma-Aldrich) was added and the plates were incubated for an additional 1 h at 37°C. The plates were washed and incubated with a pNPP substrate (Bio-Rad) for 1 h at 37°C and the $OD_{405-620}$ was read.

Purification of Fab fragments The filtered protein solutions were loaded onto a Ni^{2+}-charged-5 ml HiTrap Chelating HP column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) at a flow rate of 5 ml/min using a chromatography system (BIO CAD sprint; Applied Biosystems Japan). After washing with 20 column volumes of PBS/5 mM imidazole, the bound protein was eluted with 5 column volumes of PBS/500 mM imidazole. The eluate was concentrated and the buffer was exchanged to PBS using a 30-kDa-molecular-size cut-off filter (CentriPlus; Millipore, Billerica, MA, USA), and filtered through 0.22-μm filters.

Kinetics studies Biacore 3000 (Biacore AB, Uppsala, Sweden) based on the principle of surface plasmon resonance was used to compare antibodies. The lysine-linked tandem repeat peptide antigen of KH5, TYEAALKQYEADLKKTYEAALKQYEADL (3), was immobilized on the sensor chip CM5. Bovine serum albumin (BSA; Sigma-Aldrich) was also immobilized as a negative control. Antibody samples were suspended in HBS-EP buffer (Biacore AB). Each sample was injected onto the sensor at 3.12, 6.25, 12.5, 25 and 50 nM at 30 μl/min for 120 s, followed by an injection of HBS-EP buffer. The kinetics was determined using BIAevaluation 3.2 (Biacore AB).

RESULTS

Cloning of Fab genes and generation of transgenic cells The cDNAs coding for the Fab H and L chains were cloned into the pFab-His2 vector (9). To identify bacterial clones expressing the Fab fragments of interest, their reactivity to PAc was tested by ELISA. The most reactive of the positive clones, T15, was used for subsequent studies. The data in Fig. 1 show that expression was controlled by the *E. coli* tac promoter and the Shine–Dalgarno sequence (tac-SD). The pelB signal peptides (pelB) directed the Fab fragments into the periplasmic space of the bacterial host (9). The 6x histidine tag sequence was fused to the C-terminal of the Fab H chain.

The cDNAs of the Fab H and L chains were transferred from pT15, the plasmid containing cDNA of the T15 Fab fragment, to the pBI 101 binary vector for the transformation of tobacco cells (Fig. 1). The ER retention signal sequence, KDEL, which is reported to enhance the production of recombinant proteins (12), was added at the C-terminal region of Fab in the p24 and p26 constructs to investigate its effects on increasing antibody recovery. The secretion signal of *Nicotiana plumbaginifolia* was also used to direct Fab fragments to the secretion pathway in the p25 and p26 constructs (10). Four series of transgenic tobacco XD6S suspension cells (X23, X24, X25 and X26) were obtained by *Agrobacterium*-mediated transformation with the corresponding binary plasmids.

Characterization of transgenic tobacco cell lines Between 10 and 20 kanamycin-resistant clones of each transgenic line were grown in a small liquid media and the total protein extracts from 1-ml cultures (including the proteins in the cells and media) were tested by anti-PAc ELISA. The data in Fig. 2 show the OD of each transgenic clone. The X23 and X24 clones had no anti-PAc activity. Many X25 clones showed higher ODs, which meant that they produced more anti-PAc Fabs per ml of culture, than the X26 clones. The X25-3 and X26-2 cell lines, with specific cell lines designated after the hyphen, were selected for further analysis because their ELISA ODs were higher than those of the other cell lines. Both cell lines grew as well as their parental XD6S cells.

The time courses of the anti-PAc activities and amounts of mouse Fab fragments were quantified from the TSP concentrations (Fig. 3). The Fab fragments were quantified by sandwich ELISA with anti-mouse Fab fragment antibodies. The anti-PAc titers, mouse Fab fragments and TSPs all showed similar time courses of production, with peak titers observed 10 to 14 d after inoculation. The X25-3 cell line had higher TSP concentrations, anti-PAc titers, and Fab amounts than the X26-2 cell line.

Recovery of Fab fragments from cultures The X25-3 and X26-2 cell lines were cultured for the recovery of the Fab fragments, designated X253 and X262. About 2 mg/*l* Fab fragments were detected from both cell extracts, but their amount in the culture media differed between X25-3 and X26-2 (Fig. 4A). The X253 Fab fragment was secreted into the media at levels greater than 10 mg/*l*, but X263 was secreted at levels less than 1 mg/*l*. The tobacco cell culture medium had only 0.1–0.4 g/*l* TSP in the 10-d culture of X25-3 and the ratio of the X253 Fab fragment to TSP was

FIG. 2. Anti-PAc ELISA of transgenic tobacco clones. Between 10 and 20 clones of each transgenic cell lines X23, X24, X25 and X26, were inoculated for 7 d. Protein extracts were prepared from 1 ml of culture of each clone and the anti-PAc activity of each clone was measured by ELISA. OD405-620 values were plotted.

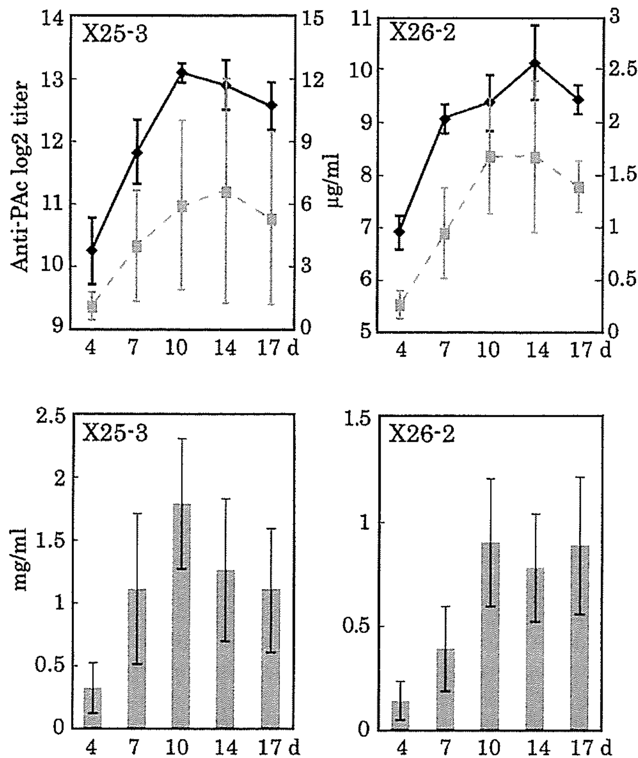

FIG. 3. Time course of antibody production in transgenic cell lines. X25-3 and X26-2 cultures were sampled from 4 to 17 d after inoculation. Both anti-PAc titer (black lines) and the amount of mouse Fab fragment (gray dashed lines) in samples were measured by ELISA. Total soluble protein concentration (gray bars) was measured by the Bradford assay. The results are presented as mean±s.d. (n=3).

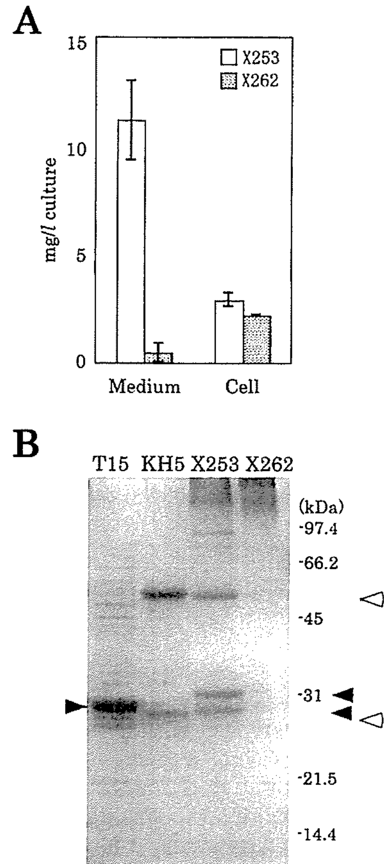

FIG. 4. Recovery of Fab fragments from transgenic cell lines. (A) X253 and X262 Fab fragments in either media or cell extracts. The media and cell extracts of 10-d cultures were prepared. The X253 and X262 mouse Fab fragments were quantified by ELISA. The results are presented as mean±s.d. (n=3). (B) SDS–PAGE analysis of antibodies. Purified KH5 (from hybridoma: open triangles), T15 (from *E. coli* extracts: closed triangles at the left side of the gel), X253 (from culture media: closed triangles at the right side of the gel) and the Ni^{2+} column effluent of X26-2 media were analyzed using 12.5% SDS–PAGE.

about 1:10 (w/w). In both the X25-3 and X26-2 cell extracts and in the media of X26-2, the amounts of Fab fragments were only 0.1–0.5% of the TSP concentrations.

Ten-day cultures of 100 ml of tobacco suspension cells were processed, and Fab fragments were recovered on a Ni^{2+} ion column via their affinity to the 6× His tag. Fab fragments from X26-2 cultures or X25-3 cell extracts were difficult to recover in sufficient amounts for further analysis. However, we did recover the X253 Fab fragment from the culture medium of X25-3. The T15 Fab fragment was also recovered from a Ni^{2+} column and the original mouse KH5 MAb was recovered from a protein A column using standard methods (10). Five micrograms of protein from the T15 and X253 Ni^{2+} column effluents, the KH5 effluents of the protein A column and 5 μg of protein from the Ni^{2+} column effluent from the X26-2 media as a negative control were subjected to 12.5% SDS–PAGE for analysis (Fig. 4B) (13). The heavy- and light-chain bands, indicated by open arrowheads, were mainly in the KH5 sample. Many extra bands were detected in the T15 and X253 samples. Proteins over 100 kDa in size in the media, particularly for X253 and X262, bound to the Ni^{2+} ion or resin of the column. The quantity of the Fab fragments increased from about 10% of TSP in the culture medium to about 30% of TSP in the column effluent. The molecular sizes, as deduced from the amino acid sequence, were calculated to be 25 kDa for the heavy chain and 24 kDa for the light chain of both T15 and X253. However, the putative bands were larger on the gel; specifically, 28 kDa doublet bands of T15 and 27 and 31 kDa bands of X253 were detected. It has been reported that Fab H and L chains expressed by a pFab1-His2 vector tend to be somewhat larger than expected on the basis of the deduced sequence (9). An approximately 50-kDa band of X253 might have been the H and L chain assembled into the Fab form. Two other bands were also observed at about 80 to 90 kDa that seemed to be three times the size of the X253 Fab H and/or L bands.

Kinetic studies Antigen binding activity was compared among the antibodies by ELISA (Fig. 5). KH5, T15 and X253 were the same samples used for SDS–PAGE shown in Fig. 4. The X25-3 medium was from a 10-d post inoculation culture. Antibodies were quantified by sandwich ELISA and then serially diluted over the same range used for the quantitative comparison of the ELISA PAc binding activities. The molecular mass of KH5 is 3 times larger than that of T15 or the X253 Fab and there are two antigen binding sites on KH5 compared with one on the Fab fragments. All antibodies showed similar curves on the graph, despite the

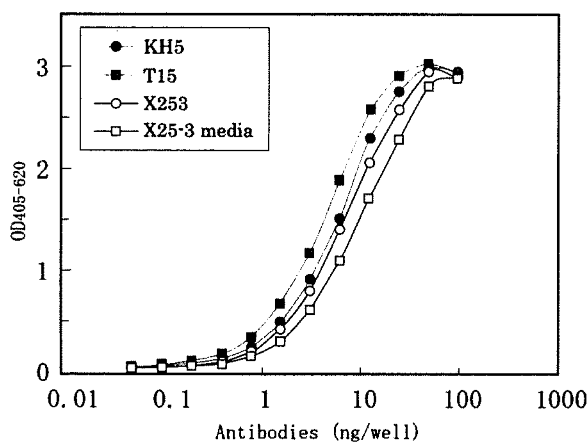

FIG. 5. Quantitative analysis of purified antibodies. Serially diluted solutions of KH5 (closed circles), T15 (closed squares), X253 (open circles) and media of X25-3 (open squares) cells were analyzed using anti-PAc ELISA. The means of triplicate experiments are shown.

difficulties in making a direct comparison of binding activities.

To precisely compare the antibodies, Biacore 3000 was used to determine dissociation constant (KD: Table 1). The di-lysine-linked tandem repeat peptide of the antigen was immobilized on a sensor chip to investigate the interaction with antibodies. Concentration-dependent antibody-antigen interactions, which supported the specificities of the antibodies, were detected and no interactions were detected between BSA and antibodies (data not shown). The association rates (ka) of antibodies were approximately the same, but the dissociation rate (kd) varied from 2.2×10^{-5} to 1.3×10^{-3} s^{-1}. The variation in kd had an effect on the differences of KDs, which is defined as kd/ka. KD values indicated that the strengths of the affinities were in the order KH5 > T15 > X253 > media, with KH5 having the highest affinity. Considering the differences between IgG and Fab, the KD values are appropriate.

DISCUSSION

Many recombinant antibodies are being developed for use as therapeutic antibodies (14). Antibodies function as a component of the immune system. Specific targets, such as cancer tissues or viruses, are destroyed via complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) or neutralization (15). Small antibodies, Fab or scFv, are applicable as targeting tools for the DDS (5). To clone the cDNA of antibodies, *E. coli* is a useful host, but it cannot produce large proteins or antibacterial peptides. Plant cells are potentially good hosts for the production of antibodies if the antibodies are fused with antibacterial peptides (7, 10, 16, 17). The *E. coli* system described in this study has been well established and we have published our experiences with cloning and producing several antibodies using this system (9, 18, 19). Usually, 10 mg of soluble Fab fragments was recovered from 1 *l* of *E. coli* cultures. In this study, we obtained approximately the same amount of soluble Fab fragments in the culture media as shown in Fig. 4. This result indicates that plant culture sys-

TABLE 1. Kinetic interactions between antibodies and PAc peptide

Sample tested	ka (M^{-1} s^{-1})	kd (s^{-1})	KD (M)
KH5	2.4×10^{5}	2.2×10^{-5}	9.1×10^{-11}
T15	4.0×10^{5}	5.0×10^{-4}	1.3×10^{-9}
X253	3.2×10^{5}	9.1×10^{-4}	2.9×10^{-9}
X25-3 media	3.6×10^{5}	1.3×10^{-3}	3.7×10^{-9}

tems are candidates for the production of small antibodies and their derivatives.

The addition of the ER retention signal, the KDEL tetrapeptide, to the C-terminal of the protein is a standard method for the enhancement of protein production in plant cells (12). When KDEL was fused at the C terminus, the expression levels of scFv were one to two orders of magnitude higher than those of scFv without KDEL (12). The Fab X262 has a KDEL motif at the C terminus of both the H and L fragments. However, these signals did not enhance the accumulation of X262 in the cells, but prevented the secretion of X262 into the culture media because of ER retention. However, X253 was actively secreted into the media. The plant cell culture media mainly contained sucrose, minerals and plant hormones and Fab fragments constitute about 10% of the TSP levels. Defects in Fab production in the X23 and X24 series pointed to the importance of protein secretion signals. The Fab fragment has a disulfide bond between the H and L chains, which is formed in the secretion pathway (20). When we use XD6S cells for antibody production, protein recovery from the media might be more efficient than recovering proteins that have accumulated in the ER.

Ni^{2+} columns did not work well for trapping the Fab fragments containing a 6x His tag at the C-terminal of H chains. Particularly for plant cell cultures, many proteins and carbohydrates may bind to the Ni^{2+} column, including some substances that bind non specifically to the carrier of the ion. We were unable to improve either the purity or recovery rate of Fab fragments, despite the use of stringent wash conditions for the Ni^{2+} column. Other affinity purification procedures, possibly antigen or anti-Fab antibody columns, should be tried to achieve efficient recovery. In the culture media, there are some impurities that affect the dissociation of Fab from the antigen, because kd differs between the media and X253. However these impurities do not influence the association of the Fab with the antigen, because the ka of the media is almost the same as that of X253. This finding means that we can use media containing an antibody as antibody reagents without purification if the end use of the Fabs is in *in vitro* examination.

The binding specificities and affinities were similar among antibodies from mouse hybridomas, *E. coli* and cultured tobacco cells. However, the size of each antibody fragment was different from that deduced from the amino acid sequences. Some modifications, *e.g.*, *N*-glycosylation, should be considered; however, there are no experimental data to explain the differences between the band sizes of T15 and X253.

In conclusion, some aspects of the production of Fab fragments by plant cell culture need further improvement, such as the recovery of Fab fragments. However, plant sys-

tems have unique properties that allow the secretion of Fabs to their media, with only a limited secretion of proteins from cells. If we can utilize these characteristics effectively, plant cells will become a powerful tool for the production of small antibodies.

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12 新生児聴覚スクリーニング後に当科を受診した症例の経過

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2）福島県立医科大学　医学部　耳鼻咽喉科

【はじめに】

福島県では平成13年頃から産科医療機関において私的に新生児聴覚スクリーニングが行われるようになり、また平成16年1月からは一部の地域で公的なスクリーニングも開始され、当科にも精査のためrefer児が紹介されるようになった。最近3年間に、新生児聴覚スクリーニング後の精査目的に紹介された症例16名につき受診後の経過について報告する。

【対象】

平成15年4月〜平成18年3月までに新生児聴覚検査でreferとなり当科に紹介された乳幼児16名。現在の年齢は6ヶ月から4歳1ヶ月、性別は男児10名、女児6名である。

【当科での検査、療育の流れ】

1．初診時は顕微鏡下での鼓膜、外耳道所見の観察、BOA、CORまたは遊戯聴力検査（受診時期に応じた聴力検査）、津守式発達検査。原則として難聴の診断が確定するまでは1ヶ月1回程度、受診し、聴力検査を行う。生後6ヶ月まではAABRの再検査も併用した。AABR両側passした場合はその時点で経過観察は終了とした。

2．生後3ヶ月以降にASSR、ABRを行い、聴力検査の結果と矛盾がなく、家族の同意もえられれば補聴器をフッティングし、経過観察する。保護者の訴え、聴力検査と脳波による検査に矛盾がある場合には引き続き検査のみで経過観察を行う。難聴が確定した時点で療育に移行する。

【検討項目】

1．初診時期、スクリーニング機器、refer耳

2．難聴確定時の年齢、初診時の聴力との比較

3．診断確定後の経過

【結果】

1．初診時期

1ヶ月…4人、2ヶ月…2人、3〜6ヶ月…4人、6〜12ヶ月…5人、1歳以降…1人他の耳鼻咽喉科を受診しないで産婦人科から直接を紹介されたものは3人で、1ヶ月で受診していた。他の13人は当科以外の耳鼻咽喉科でABRやASSRを受けてから紹介されていた。

スクリーニング機器　　AABR…7人、OAE…9人

Refer耳　　両側…11人、片側…5人

2.診断確定年齢

2ヶ月…1人、3ヶ月…1人、6ヶ月…2人、7ヶ月…1人、10ヶ月…1人、1歳0ヶ月…4人、1歳2ヶ月…1人、1歳4ヶ月…1人、1歳7ヶ月…1人、経過観察中…1人

初診から診断確定まで平均6ヶ月を要していた。

初診時の聴力と確定後の聴力

5症例において初診時の聴力評価と経過観察後の聴力評価に差が見られた。3症例は難聴

診断確定後の聴力より閾値が低く、2症例は閾値が高く出たが、5例とも経過観察後の聴力評価はASSRの結果に近いものになった。

3． 経過

症例5は2ヶ月でAABR両側passしたため、その時点で聴覚障害なしと診断した。1歳4ヶ月で診断確定した高度難聴児（症例2）は2歳4月で人工内耳埋め込みを行った。高度難聴と確定診断された2例（症例9、11）は人工内耳装用に向けて訓練中である。両側難聴が確定した症例は症例15を除き、補聴器を装用し訓練中であるが、症例15については中等度難聴であり、補聴器なしでもある程度音に対する反応があることから保護者の同意が得られないため引き続き経過観察中である。

【考察】

福島県では新生児聴覚スクリーニングを行っている産科医療機関に対し、出生後入院中にreferの場合は1ヶ月健康診査時に再検査することを推奨していることから生後1ヶ月以内に受診した症例はいなかった。産科医療機関からABRのある他の耳鼻咽喉科へ紹介された場合はABRを施行された後に当科へ紹介されたために初診が3ヶ月以降である症例が16例中10例と多かった。診断確定までには初診から平均して6ヶ月を要しており、初診時のBOAやCORのよる評価が難しいことがわかる。初診時のBOAまたはCORの結果とASSR、ABRの結果が不一致な場合、CORや遊戯聴力検査の結果が徐々にASSRの結果に近づく傾向があったことから他覚的な聴力検査であるASSRは乳幼児の難聴の評価に有用であった。自験例では高度難聴児は3例（症例2、9、11）であった。症例2は初診年齢が1歳1ヶ月であったため早期診断とはいえないが、症例9、11については3ヶ月、7ヶ月で診断確定できた。また、中等度難聴児、片側難聴児は1歳7ヶ月までに診断されており明らかにスクリーニングが導入される以前より早期に診断が確定し、療育に移行していると思われた。

表1. 症例のまとめ（聴力は4分法：dB）

症例	性	スクリーニング	refer耳	初診年齢	診断確定年齢	初診聴力評価	現在聴力評価	ASSR（推定聴力）	診断
1	男	AABR	両	9m	1y7m	COR40	右50左50	右55左60	両感難
2	男	OAE	右	1y1m	1y4m	COR40	右100左90	右100左97.5	両感難
3	女	OAE	両	1m	1y0m	BOA反応良	右50左50	右45左50	両感難
4	女	OAE	両	8m	10m	COR40	ピープショウ45	右110以上左40	両感難
5	男	AABR	両	1m	2m	BOA反応良	未検査	未検査	異常なし
6	男	OAE	左	11m	1y6m	COR20	COR30	右45左110	左感難
7	女	AABR	左	1m	1y2m	COR80	COR25	未検査	左感難
8	女	OAE	両	6m	1y0m	COR90	ピープショウ60	右55左47.5	両感難
9	女	AABR	両	1m	3m	BOA反応不良	COR100	右95左100	両感難
10	男	OAE	右	11m	1y0m	COR25	COR25	右95左1k15，4K0	右感難
11	男	AABR	両	3m	7m	COR60	COR100	右105左110以上	両感難
12	男	AABR	両	10m	1y0m	COR70	COR45	右20左25	両滲中
13	男	AABR	両	4m	7m	COR65	COR70	右75左80	両感難
14	男	OAE	両	2m	経過観察中	COR45	COR45	右15左30	両難聴疑
15	女	OAE	両	2m	6m	BOA反応不良	COR60	右50左80	両感難
16	男	OAE	左	5m	6m	COR40	COR40	右7.5左55	左感難

厚生労働科学研究・研究成果等啓発事業による成果発表会

一般公開講座「難聴とウイルス」

ウイルスできこえが悪くなる！なぜ、どうして？

日時：平成19年３月11日(日)　13:00～16:00

場所：エスパル福島　５階ネクストホール（JR 福島駅東口）

主催　公立大学法人 福島県立医科大学医学部耳鼻咽喉科
共催　財団法人 長寿科学振興財団
　　　日本耳鼻咽喉科学会福島県地方部会

〈プログラム〉

開会の挨拶　13：00－13：05　大森孝一

セクション1	13：05－13：55		
演題－1	きこえの仕組み	大森孝一	（福島県立医科大学耳鼻咽喉科教授）
演題－2	ウイルスと難聴	錫谷達夫	（福島県立医科大学微生物学教授）

休憩　13：55－14：05

セクション2	14：05－14：55		
演題－3	難聴児の手術と治療	小川洋	（福島県立医科大学耳鼻咽喉科助教授）
演題－4	難聴児とことばの訓練	馬場陽子	（福島県総合療育センター 福島県立医科大学耳鼻咽喉科講師）

休憩　14：55－15：05

医療相談　15：05－15：55

コメンテーター：髙取隆（大原綜合病院耳鼻咽喉科主任部長）
草野英昭（くさの耳鼻咽喉科クリニック院長）

閉会の挨拶　15：55－16：00　大森孝一

は　じ　め　に

福島県立医科大学医学部耳鼻咽喉科　教授
大　森　孝　一

「難聴とウイルス」とお聞きになって、ウイルスで難聴がおこるの？と不思議な感じがする方が多いかと思います。

難聴の子供さんは出生1,000人～2,000人に１人の割合と言われています。遺伝性の難聴はそのうち約５割と言われていますが、平成16年から平成18年の厚生労働省感覚器障害研究事業として福島県内での調査で、ウイルスによる難聴が約２割と遺伝性に次いで多いことが判明しました。予防に関する啓蒙活動や新しい治療技術の開発が必要となってきています。

難聴を早期に見つけることは大切で、正しく診断されて言語習得のための訓練を受けることで言葉の発達の遅れを防ぐことができます。福島県では難聴の早期発見のために平成16年１月より新生児聴覚検査事業が県中・県南・会津地域で行われています。１歳６ヵ月健診や３歳児健診で難聴をみつけることも重要ですし、みみ・はな・のどの病気の早期発見に必要な耳鼻咽喉科医による学校健診が望まれています。

現在、きこえを補う方法として、軽度から中等度の難聴の方には補聴器、高度の難聴の方には「人工内耳」があります。補聴器については、補聴器相談医がいますので耳鼻咽喉科にお気軽にご相談下さい。人工内耳などの外科治療については手術を行っている専門の病院にご相談下さい。

３月３日は「耳の日」です。「耳の日」は、難聴の方のために少しでもお役に立ちたいとの願いを込めて昭和31年から始まり、今年で52回目になります。この講演会も「耳の日」に関連する行事として企画しました。きこえの仕組みについては私、ウイルスと難聴については微生物学の錫谷教授、難聴児の手術と治療については小川助教授、難聴児とことばの訓練については福島県総合療育センターの馬場部長が解説します。さらに、きこえに関する質問を日本耳鼻咽喉科学会福島県地方部会から大原綜合病院の髙取主任部長、くさの耳鼻咽喉科クリニックの草野院長がお答えします。

皆様がより良い聴力を保って、明るい生活が送れることを心から願っております。

【演題－１】

きこえの仕組み

福島県立医科大学医学部耳鼻咽喉科　教授
大　森　孝　一

音がきこえるのは？

耳は外耳、中耳、内耳の３つの部分からできています。音は空気の振動です。耳介で集められ外耳道に入り、その奥にある鼓膜を振動させます。この振動は中耳にある３つの小さい骨の連鎖の働きで、内耳の蝸牛（かたつむりの形をした管があります）に伝えられ、この管の中の液体を振動させます。蝸牛の中にはこの液体の振動を電気信号に変換する器官があり、おもに有毛細胞で発生する電気信号がきこえの神経を経由して脳に伝えられ、音として感じます。

きこえの検査とは？

きこえの能力を聴力といい、聴力検査ではいろいろな高さの音を、強さを変えて聞いて、きこえるかどうかをご本人に尋ねます。かろうじてきこえる音の強さを聴力レベルとします。音の高さはヘルツ（Hz）で表し、聴力検査では125Hz、250Hz、500Hz、1000Hz、2000Hz、4000Hz、8000Hzの高さを検査します。生活には500Hz、1000Hz、2000Hzが重要です。聴力レベルをデシベル（dB）で表し、20dBまでを正常、40dBまでを軽度難聴、70dBまでを中等度難聴、100dBまでを高度難聴、101dB以上を重度難聴、全く聞こえない場合を聾とします。

乳幼児の場合はこれとは別の方法として、蝸牛からきこえの神経を通して脳に送られる電気信号を検出して聴力を検査する方法や有毛細胞の動きを検出して聴力を測る方法などがあります。

難聴になる病気

外耳道に耳あかがつまったり、急性中耳炎でうみがたまったり、滲出性中耳炎で液がたまったりすると聞こえにくくなります。外耳や中耳の病気は治療でよくなる可能性があります。

内耳については、蝸牛に生まれつき異常がある場合や、ウイルス、細菌、薬物が入ると難聴になることがあります。歳をとると有毛細胞が減少し聞こえにくくなります。内耳の病気の多くは治りませんので、補聴器や人工内耳などできこえを補う必要があります。

MEMO

【演題－2】

ウイルスと難聴

福島県立医科大学医学部微生物学　教授
錫　谷　達　夫

ウイルス感染が原因となって起こる難聴は、遺伝性の難聴に次いで多いことが知られています。難聴を起こすウイルス感染症には種々のものがありますが、頻度が高いものは流行性耳下腺炎（おたふく風邪）と胎児期の感染が原因となる風疹（三日ばしか）とサイトメガロウイルス感染です。

風疹や流行性耳下腺はワクチンによって予防が可能な疾患です。ところが日本でのワクチン接種率が低いため、未だ流行を防げずにいます。とくに流行性耳下腺炎ワクチンは定期接種（市町村で経費を支払う予防接種）とはなっていないこともあり、およそ50％程度のお子さんしか受けていないと推定されます。そのため３年に１度流行が起こり、流行のたびに500名程度のお子さんが難聴になっています。多くの先進国ではほとんど予防できている病気によって、多くのお子さんが難聴を患うのは大変残念なことと言わざるを得ません。このような悲劇をなくすためにも風疹や流行性耳下腺炎のワクチン接種率を何としても90％以上に高めたいものです。

サイトメガロウイルスという名前は聞き慣れない名前だと思いますが、日本人の80％程度の方にウイルスは感染し、生涯体内で生き続けます。このウイルスは普通病気を起こすことはないのですが、運悪く妊娠中に感染しますと胎児にも感染します。このようにして生まれながらにこのウイルスに感染してくるお子さんはおよそ400人に１人いるのです。最近、このような赤ちゃんの10％程度に難聴が発症することが解ってきました。現在、この難聴を防ぐ方法はなく、今後の研究が望まれるところです。

MEMO

【演題－３】

難聴児の手術的治療

福島県立医科大学医学部耳鼻咽喉科　助教授
小　川　　　洋

耳は大きく外耳、中耳、内耳の３つの部分に分けることができます。この３つの部分において何らかのトラブルが起きると聞こえが悪くなります。これらの中には、手術をすることによって聴力の改善が期待できるものがあります。手術によって聴力の改善が期待できるものには、外耳、中耳に原因がある場合、例えば、生まれつき外耳道ができていなかったり、音を伝える小さな骨（耳小骨）が無かったり、あるいは固まってしまったりしている場合や、特殊な中耳炎で小さな骨が溶けてしまったりしている場合、何度も何度も中耳炎を繰り返しているうちに鼓膜に穴があいてしまったりして聴力が低下している状態などが挙げられます。このような場合に外耳道を形成したり、小さな骨を形成したり、鼓膜の穴を閉じたりして聴力の改善を図ることができます。高度な病変の場合には聴力の改善が大変難しい場合もありますが、十分な検査を行うことである程度の聴力改善予想を立てることができます。内耳の病変で聴力が低下してしまった場合には、手術的な治療で聴力を改善させることはできませんが、唯一、高度の聴覚障害に対して人工内耳という手術的な治療方法があります。この治療は補聴器の効果がない両側高度感音難聴の患者さんに行うもので、耳の中の蝸牛という音を感じる器官に直接細い電極を挿入し、音を電気信号に変換して刺激を行い、聴覚を獲得させるものです。ウイルスにより高度の感音難聴を来した患者さんにもこの治療の有効性があり福島県立医科大学でもこの手術を行うことができます。今回はみなさま方に手術で聴力を改善できる病気、その手術の内容についてできるだけわかりやすくお話したいと思います。

MEMO

【演題－4】

難聴児とことばの訓練

福島県総合療育センター　部長
福島県立医科大学耳鼻咽喉科　講師
馬　場　陽　子

人間の耳は、内耳とよばれている音を聞く器官から常に音を放射しています。この内耳から放射される音は音が聴こえたときにさらに強くなるため、音を聞かせたときに内耳から放射される音を検出することでその人が聞こえているかを確認することができます。これを**耳音響放射検査**といいます。また、人間は音を聞いたときにはある決まったパターンの脳波を発生することがわかっており、この脳波を測定することで難聴の有無を確認できます。この検査を**聴性脳幹反応検査**といいます。これら２つの検査は、赤ちゃんでも眠っているときに簡単に行えるため、産科医療機関で生まれて２，３日目の赤ちゃんの聞こえの検査として行われ始めました。これが**新生児聴覚スクリーニング**といわれるものです。この検査が行われるようになり、生後３ヶ月でも、難聴の診断が可能となりました。

また、生まれたときには聞こえていても、その後徐々に難聴が進行する場合があります。乳幼児健診は、１ヶ月、４ヶ月、10ヶ月、１歳６ヶ月、３歳時に行われており、どの時期の健診でも言葉の遅れや音への反応について問診が行われています。特に３歳児健診では、ささやき声テスト、指こすりテストといわれる内緒話程度の小さい音が聞こえているかどうかを検査する方法が取り入れられています。この検査では、進行性の難聴や、軽度、中等度の難聴が発見できるよう工夫されています。

難聴児に、どのようにして言葉を覚えさせるかは、比較的古くから研究され、確立されており、**聴能訓練**と呼ばれています。具体的には補聴器や人工内耳を使用させ、音を聴くことに集中する態度を身につけること、耳で聞くことと目で見て理解したことを結びつけ、言葉を覚えることですが、この訓練を開始するのは脳が硬くならないなるべく早い時期がよいとされており、もし子供に難聴がある場合には健診等での早期発見が非常に重要といえます。

MEMO

一般公開講座

厚生労働科学研究・研究成果等普及啓発事業による成果発表会

難聴とウイルス

ウイルスできこえが悪くなる！ なぜ、どうして？

入場無料

日時

平成19年

3/11(日)

13:00～15:00
▶講　演

15:00～16:00
▶医療相談

会場

エスパル福島
5階ネクストホール
（福島駅東口）

主催

公立大学法人 福島県立医科大学医学部耳鼻咽喉科

共催

財団法人長寿科学振興財団
日本耳鼻咽喉科学会福島県地方部会

※医療相談：テーマである「難聴」に関する質問にお答えします。氏名・職業・電話・FAX番号・質問を明記の上、FAXでお申込み下さい。
締切：平成19年2月末日　　FAX番号 024-548-3011
※十分にお席はご用意しておりますが、席に限りがございますので、満席の場合には入場をお断りする場合もございますので、予めご了承下さい。