approximately the same as the binding constant [Burton et al., 1991].

### Flow Cytometric Analysis

The hepatocellular carcinoma cell line PLC/PRF/5 (Alexander cell line), which expresses HBs antigens (adw) on the cell surface, was cultured in 3 cm plastic dishes (Asahi Technoglass Co., Chiba, Japan). When cells reached confluence, medium was discarded, 1 ml of each antibody was added to $2–5 \times 10^5$ cells/dish, and cultures were held on ice for 1.5 hr. After washing twice with PBS, a 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG specific antibody (ICN Pharmaceuticals, Inc., Aurora, OH) was added and plates were incubated for 30 min at 37°C. At the end of the incubation, cells were trypsinized, filtered through nylon mesh, and sorted by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ).

### Microcytotoxicity Assay

Complement-dependent cytotoxicity activity was assayed to test the reactivity of the MAb Fc region with complement. The Alexander cells were cultured to confluency in a 96-well plate, incubated on ice for 1.5 hr with 50 μl of purified test antibody, followed by incubation with rabbit complement (Veritas, Tokyo, Japan). Ten microliters of Cell Counting Kit8 (DOJIN, Tokyo, Japan) was added and cell viability was estimated by measurement of $OD_{450}$, as specified by the manufacturer's protocol.

## RESULTS

### Generation of Transgenic Tobacco Cells

The constructs, designated p29, p30, and p31, are shown in Figure 1. To obtain the active full length IgG antibodies, protein fragments of heavy and light chain must be delivered to the secretary pathway through the ER. Three series of transgenic tobacco BY-2 suspension cells (B29, B30, and B31) were obtained by *Agrobacterium*-mediated transformation with the numerically corresponding binary plasmids. Small-scale protein extracts of 7 day cultures of several kanamycin resistant clones from liquid medium culture were used for ELISA. The B29-4, B30-3, and B31-7 cell lines, with the specific cell lines designated after the hyphen, were selected for further analysis because they gave OD values in ELISA and cell growth in the liquid medium that were higher than the other cell lines that were tested (data not shown).

The time course of the anti-HBs synthesis was determined by quantifying amounts of human IgG proteins in the total extracts of the culture that consisted of both the medium and cell extracts, and TSPs were quantified (Fig. 2). The anti-HBs titers and human IgG proteins showed the almost same time course of synthesis, with peak titers observed at 10 days after inoculation, but the time courses were different from that of TSP. The amounts of both IgG and TSP in the

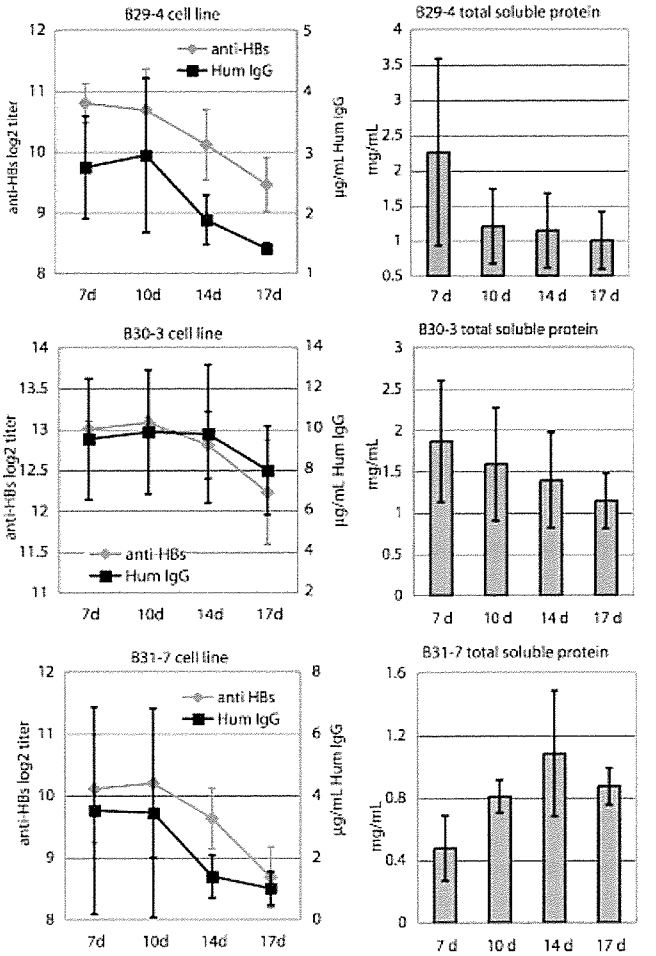


Fig. 2. Time course of antibody production in transgenic cell lines. B29-4, B30-3, and B31-7 cultures were sampled from 7 to 17 days after inoculation. Both anti-HBs titers and human IgG in samples were measured by ELISA. The amounts of total soluble proteins (TSP) were measured by Bradford assay. The results are presented as the mean ± SD (n = 3).

extracts of B30-3 cells were relatively stable from 7 to 17 days. In contrast, the B29-4 cells had rapid reductions in the amount of TSP after 10 days. The B31-7 cells also had reduced levels of IgG after 10 days, despite the peak of TSP detected at 10 days. The percentages of human IgG in the TSP were approximately 0.2% for B29-4, 0.6% for B30-3, and 0.25% for B31-7 cells. Despite the differences in the SSs for the three cell lines, the ratios of antibodies in the TSP were of same order, but the time points at which saturation density was reached were different. B30-3 cells grew well and rapidly reached saturation density. However, B31-7 cells grew slowly and did not reach saturation density until 10–14 days.

### Purification of the Antibodies From Transgenic Tobacco Cells

Every 10 days the cells and medium from the cultured suspension cells were separated by paper filtration for purification of the recombinant MAb. Figure 3 shows the maximum yields of the preparative scale IgG purifica-

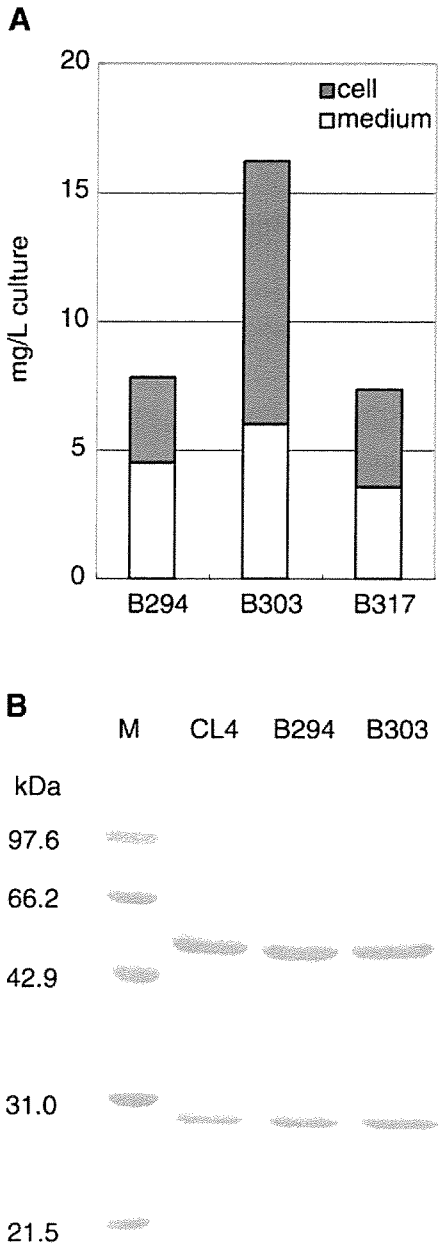


Fig. 3. Antibody purification from transgenic cell lines. **A**: IgG protein yields from B29-4 and B30-3 cultures. IgGs were purified from both cells and media of 10 day cultures with protein A columns. The B294 and B303 MAbs were quantified by ELISA. The maximum yield of the six times preparative scale purification procedures were indicated. **B**: SDS–PAGE analysis of purified antibodies. Purified CL4 (from culture medium), B294, and B303 MAbs (from medium and cell extracts) were analyzed using 12.5% SDS–PAGE. M, molecular weight marker.

tion from each of the tobacco cell lines cultured in n × 100 ml scale cultures for 10 days. The quantities of purified antibodies from medium were similar among the B29-4, B30-3, and B31-7 cell lines. However, the quantities of the antibodies recovered from the cells were varied. The maximum value after purification was 16.2 mg of human IgG from 1 L of a 10 day culture of B30-3 cells. The Coomassie-stained SDS–PAGE of purified MAbs (10 μg each), parental CL4MAb, MAb B294, and MAb B303, from both medium and cell extracts is shown in Figure 3B. The total amount of antibody purified from B31-7 culture was insufficient for analysis. The major bands of heavy (H) and light (L) chains of the MAbs were almost all the same size. A difference among the three MAbs was the presence of weakly staining bands between the positions of the H and L chains. The positions of the extra bands were different for the original CL4 and the MAbs produced in plants. MAbs B294 and B303 showed similar patterns of extra bands. It was not clear whether the extra bands were the result of nonspecific binding of the plant proteins to Protein A column or degradation products of the IgGs.

### Quantitative Analysis of Plant Produced Antibodies

The antigen binding activities were compared among the purified MAbs, parental CL4, B294, and B303 MAb. The MAbs were quantified by ELISA and then serially diluted over the same range as for quantification for comparison by ELISA of the HBs binding activities by ELISA (Fig. 4.). No obvious differences in antigen-binding were observed among the antibodies when tested from 0.3 μg/ml (=2.0 nM) to 73 pg/ml (=49 pM).

For a more detailed comparison among these MAbs, inhibition ELISA was done to determine the relative affinity constants (Fig. 5). In five independent assays, (data not shown) that used the HBsAg concentrations giving 50% inhibition, the relative affinity constants, which ranged from 1 to 1.26 × $10^{-8}$ M, were almost the same among MAbs B294, B303, and CL4.

### Antibody Reaction With Cell Surface HBs

The Alexander cells were incubated with HBIg or HBs MAbs and the binding of antibodies was confirmed by flow cytometric analysis (FACS). Human cytomegalovirus (CMV) neutralizing MAb, TI23 (IgG1/kappa), was

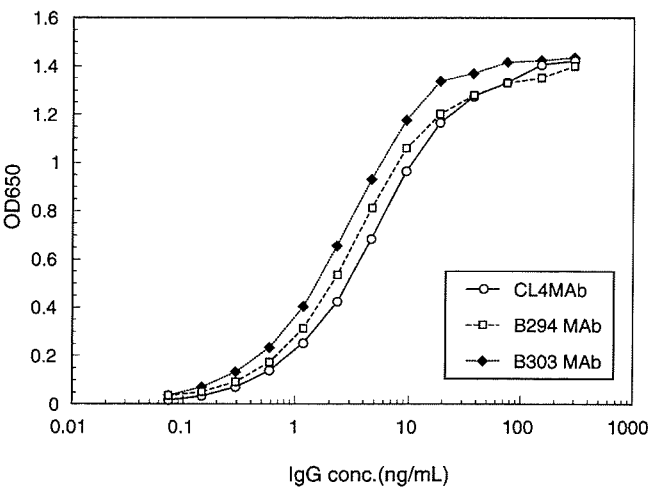


Fig. 4. Quantitative analysis of purified antibodies. Serially diluted solutions of the CL4, B294, and B303 MAbs were analyzed using the anti-HBsAg ELISA. The typical result of five experiments are shown.

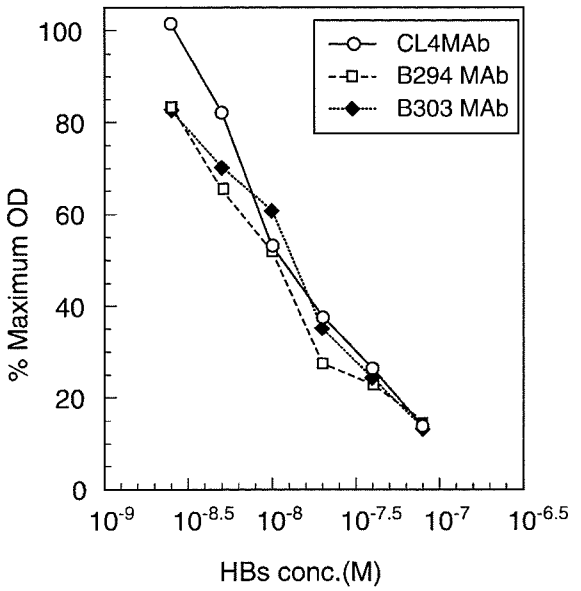


Fig. 5. Inhibition ELISA assays of purified antibodies. Affinities of the CL4, B294, and B303 MAbs were estimated by inhibition ELISA. The HBsAg was used as solid-phase antigen and serially diluted soluble HBsAg was used as inhibitor. The concentration of HBsAg, which gave 50% inhibition of maximum binding reflected MAb affinity, which is approximately the same as the binding constant. The typical result of five experiments is shown.

used as negative control [Masuho et al., 1987]. These cells were clearly labeled by HBIg, CL4, and B303 MAbs (Fig. 6). When the concentration of antibodies was increased to 40 μg/ml for CL4Mab or 20 μg/ml for MAb B303, the FACS profile was altered to a greater extent than when the HBIg concentration was increased to 20 IU/ml.

### Complement-Dependent Cytotoxicity

It has been reported that anti-HBs antibodies have complement-dependent cytotoxicity in cells expressing HBs antigens on their surface [Shouval et al., 1982]. To confirm that the Fc domains of plant produced MAbs are functionally equivalent to those of HBIg, complement-dependent cytotoxicity test were done. When Alexander cells were reacted with the parental CL4Mab, MAb B303, positive control HBIg, or negative control TI23 MAb in the presence of rabbit complement, a dose-dependent increase in cytolysis was observed with all anti-HBs antibodies tested, but not with the T123 negative control (Fig. 7). There were no significant differences between the human antibodies and the plant-produced antibody for the complement binding function of the Fc region.

## DISCUSSION

This is the report of the production in plant cells of a full-length human IgG that was synthesized using the gene encoding an HBV neutralizing MAb. As such, the plant derived MAb would be expected to be fully active in neutralizing HBV in vivo. The quantities of antibody

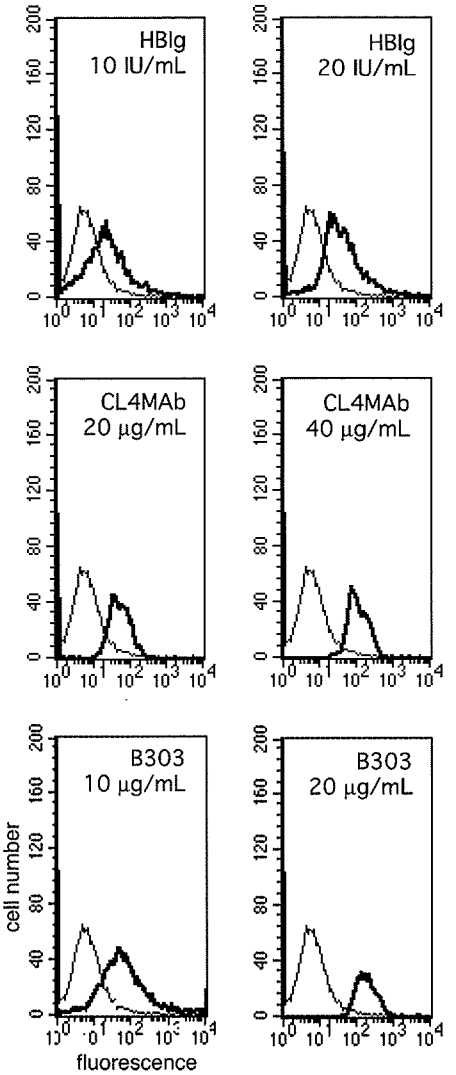


Fig. 6. Binding assay of the antibodies to cell surface HBsAg. The Alexander cells with surface HBsAgs were treated with HBIg, CL4, B303, and TI23 MAbs. Cell-antibody complexes were detected with FITC-conjugated goat anti-human IgG specific antibody and analyzed by flow cytometry. The number of cells are shown on the vertical and immunofluorescence intensity is shown on the horizontal axis. The result of the TI23 MAb (20 μg/ml) treatment is overlaid on each graph as negative control and is shown with a thin line. The typical result of five experiments are shown.

produced were consistent with levels that would be expected from a plant production system [Conrad and Fiedler, 1998]. The average yield of the recombinant protein in our system using plant SS (B303) was usually 0.1–2% of TSP. The MAbs were expressed using three types of signals; specifically, human derived LS, dicotyledonous calreticulin derived SS, and monocotyledonous hordothionin derived SS. The use of hordothionin derived SS transgenic tobacco cells did not consistently result in expression levels of antibody that were sufficient for preparative scale purification. Overexpression of some proteins, when the monocotyledonous signal peptide was used or the processing site of the SS was not correct, were burden for the protein secretion pathway and

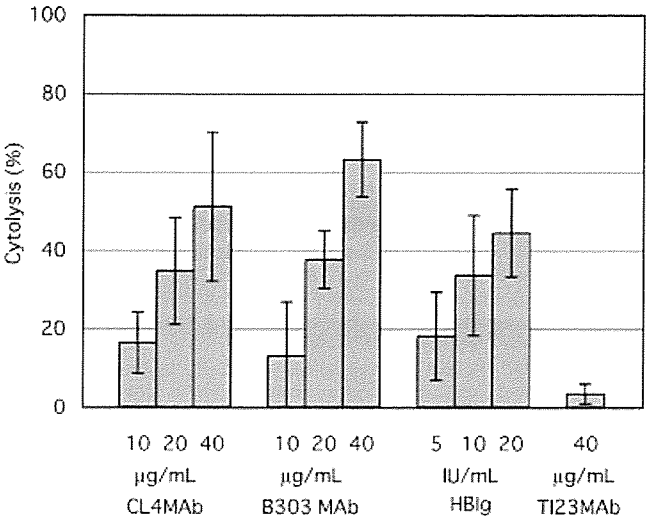


Fig. 7. Antibody induced complement-dependent cytotoxicity. The Alexander cells were treated with CL4 and B303 MAb. Hepatitis B immunoglobulin (HBIg) was used as positive control, and TI23 MAb was used as negative control. Columns and vertical bars represent the mean cytotoxicity values ± SD (n = 3).

resulted in less-than-optimum cell culture conditions. The transgenic tobacco cells containing dicotyledonous SS grew more vigorously and expressed the antibodies more consistently than cells made transgenic with human LS. The human derived signals that originate from the immunoglobulin leader sequence have usually been used for production of antibodies in transgenic plants because it is thought that the correct antibody processing is dependent upon the signal peptide. However, plant derived signal peptide was more suitable for both overexpression of the protein and stable growth of the host culture. Similar results have been reported for the stable expression of the HBs antigen in tobacco cells by plant signal peptide [Sojikul et al., 2003]. When culture conditions and the purification protocols are optimized, yields of full-length recombinant antibodies in tobacco suspension culture were 25 mg/L culture [Fischer et al., 2003]. In addition, when transgenic plants are used as bioreactors as an alternative to suspension cultures, it is easy to scale-up production simply by enlargement of the field area for plant growth.

Even though the transgenic expression systems provide an attractive vision for a source of plant derived MAbs for clinical use, there are problems that need to be solved before implementation of this strategy. Since HBIgs require intravenous injection to prevent the transmission of HBV from mothers to infants, recombinant antibodies used as substitutes for HBIg cannot be antigenic in humans. There are some differences in the protein glycosylation patterns between human and plants. However, studies of mice injected with a recombinant mouse IgG isolated from plants showed that, despite some differences in the glycan groups present on the recombinant antibody, neither the antibody nor the glycans were immunogenic [Chargelegue et al., 2000]. If necessary, the human type of glycosylated antibodies can be expressed in plants. The key technology for transgenic plant production of humanized glycosylated protein has been reported. The introduction of human galactosyltransferase (GalT) into plant cells results in the production of recombinant antibodies with human type β1,4-galactose residues [Palacpac et al., 1999; Bakker, 2001]. The next step for humanization of the glycosylation pattern will be to reduce the plant specific glycan residues, α1,3-fucose and β1,2-xylose. This may be possible by depression of glycosylation enzymes by inhibitory technologies such as RNA interference. Considering the progress in technologies for transgenic plants, many monoclonal antibodies would be produced by plant. Actually, several antibodies produced in plants are now undergoing phase II clinical trials [Fischer et al., 2003].

In this report, we showed that the B303 HBs antibody produced in plants had almost the same affinity and physiological activity as the virus neutralizing parental human monoclonal antibody CL4Mab. The MAb B303 has potential for both prevention and therapy of HBV infection. Characterization of the B294 and B303 MAbs supports the conclusion that these recombinant MAbs derived from the CL4MAb will be a clinical immunotherapeutic for preventing HBV transmission. However, data for CL4MAb or B303 have been obtained using sdr subtype HBV or HBs, and adw subtype HBs expressing cells. The problem of HBV escape mutants following HBV passive immunotherapy points to the need for further analysis of the MAbs against escape mutants. The most common mutations in the HBs protein were at amino acid positions 143–145. These positions are usually essential for recognition of antigen and neutralization by HBs antibodies [Mahoney, 1999]. In vitro studies of the HBV mutants indicated that some polyclonal antibodies more effectively inhibit the secretion of a mutant HBV from infected cells than MAbs [Schilling et al., 2003]. Preparations of polyclonal antibodies are composed of antibodies to single epitope determinants, much like MAbs. It is likely that the combination of some selected MAbs will be an effective means of neutralizing HBV in vivo. At this time, a combination of two MAbs to HBV is being developed as an anti-HBV drug [Galun et al., 2002]. It is necessary to obtain the cDNAs of other MAbs to HBs to produce the MAb cocktail, including CL4MAb, which will be a real substitute of HBIg. It would be desirable to produce the MAb cocktail in the GalT transformed plants. If this is possible, a safe alternative to HBIg will become available. We are confident that the transgenic plant derived biopharmaceuticals will become both safe and economical for promotion of global health.

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# 先天性聴覚障害 発見治療へ研究

## 福医大などのグループ

# へその緒で早期判定

## 言葉遅れ最小限に

県立福島医大などによる研究グループが新生児や幼児の聴覚障害の原因の三割を占める「サイトメガロウイルス（CMV）感染症」を早めに見つけ、子どもたちを難聴から救う取り組みをスタートした。CMV感染症は症状が重くなってから発見されるケースが多かったが、へその緒を使う新たな判定法で判別し、早めの治療や言語訓練などでハンディを最小限に食い止める。今年度の厚生労働省の感覚器障害研究事業に採択されており、治療法の確立などに大きな期待が寄せられている。

錫谷達夫教授

大森孝一教授

調査研究は県立福島医大の耳鼻咽喉科学講座と微生物学講座、県総合療育センター、国立感染症研究所などでつくる研究グループが一日から県内の医療機関に呼び掛け、実施している。乳幼児らのへその緒を親族の同意後に提出してもらい、グループの錫谷（すずたに）達夫県立福島医大微生物学講座教授らが開発した判定法を用いてCMV感染の有無をほぼ確実に診断する。

CMVはヘルペスウイルスの一種。一般に乳幼児期に感染するが、発症は体力が低下した場合や、臓器移植手術などで免疫抑制剤の投与を受けた場合など限られている。胎児は母体からへその緒を通じ、CMVの働きを抑えるCMV抗体を受け継ぐが、抗体を持たない母親が妊娠中にCMVに感染すると母体には影響がなくとも、抵抗力の弱い胎児に発症する場合がある。重症だと脳障害による知的障害や身体障害などを引き起こす。

難聴は出生児の千人から二千人に一人発症するといわれており、できるだけ早く発見し、言語聴覚士らによる療育を実施することで言葉の遅れなどの二次的障害を最小限に抑えることができる。研究ではこの難聴の三割を占めるといわれるCMV感染症を早期に発見し、療育に結びつけると同時に、発症までのメカニズムを解明し、予防や治療などの対策確立を目指す。難聴の原因が遺伝ではないと特定できるため、次の妊娠に対する家族の不安も軽減できる。

研究は今年度から三年間の予定で、厚生労働省がすぐれた研究に対し費用を助成する「厚生労働科学研究費補助金」に採択されており、グループは今年度内に中間報告をまとめる。

グループの主任研究者で、県新生児聴覚検査事業推進会議会長を務める県立福島医大耳鼻咽喉科学講座の大森孝一教授は「将来はCMVによる障害発症を予防し、聴覚障害者を減らしたい」としている。

# 診療5分、難聴ハンデ最小限に

## 脳発達、言語習得…

## 新生児の聴覚検査、県中でスタート

### ろう学校▼言語聴覚士▼垣根越え連携▲医師▲保健技師

子どもたちの難聴を早期に発見して適切な訓練につなげようと、県が今年から、生まれたばかりの赤ちゃんの聴覚検査などを行う事業をスタートさせている。当面は県中地区だけで取り組む計画で、県内全域に広がるのは早くて二〇〇五年度になりそうだ。とはいえ、出産前後の親子のケアを担当する医師、ろう学校教諭、健診で乳幼児と接する機会が多い保健師などの自発的な連携が実現するなど、成果も表れている。

一月に始まったこの事業のうち、聴覚検査には、郡山、須賀川市と三春町の合わせて十の産婦人科病院が参加した。ここで生まれた赤ちゃんのうち、親が希望すれば、千八百円を自己負担することで米国製機器による聴覚検査を受けられる。機器は五分ほどで、「正常」か「精密検査が必要」かを判定する。その後の精密検査は郡山市内の総合病院が、必要な訓練は郡山市の県心身障害児総合療育センター（新年度から「県総合療育センター」と改称）などが、それぞれ引き受ける。

聴覚障害のある新生児の割合は、千人に五―六人といわれる。耳から得る情報は、脳の発達や言語の習得に大きな役割を果たす。ただ障害があっても、早めに見つけて適切な訓練を受ければ、ハンデは最小限にとどめることができるとされる。

県はこの事業を順次、他地域にも広げる方針。新年度は県北・県南地区が、二〇〇五年度には会津・浜通り地方が加わる予定となっている。

◇

事業化をきっかけに二月、「聴覚障害児療育支援連絡協議会」が結成された。これまで、生まれた直後に聴覚検査をする必要性を感じていた言語聴覚士、耳鼻科医師、教育関係者、保健技師などが自発的に集まり、手を携える場となった。初会合は二月五日に開かれた。委員十二人は新年度末までに五回ほど集まり、当面は現状を報告し、課題を話し合う。

委員長に就いた小柳津滋・県立ろう学校長によると、同校は以前から、聴覚障害のある子どもの言語訓練などの相談を引き受けている。だが、「どの親御さんも『ここへたどり着くまでの道のりは遠かった』とおっしゃる。必要な所に必要な情報が届いていないことを痛感してきた」（小柳津校長）という。

県中地区では既に、保健師の協力態勢も整いつつあり、事業開始にあわせた講習会も開かれた。土屋久・県子育て支援グループ参事は「難聴かどうか見つけたり、親の悩みに応じたりする役割が期待できる」と話している。