Rapid Prenatal Diagnosis and Exclusion of Epidermolysis Bullosa Using Novel Antibody Probes*

Adrian H. M. Heagerty, B.Sc., M.B., M.R.C.P., Andrew R. Kennedy, B.A., David B. Gunner, A.I.M.L.S., and Robin A. J. Eady, M.B., F.R.C.P.

Department of Electron Microscopy and Cell Pathology, Institute of Dermatology, London, U.K.

Prenatal diagnosis of recessive dystrophic epidermolysis bullosa was successfully achieved at 19 weeks' gestation by indirect immunofluorescence examination of a fetal skin biopsy sample using the monoclonal antibody LH 7:2. The abortus displayed marked blistering and the diagnosis was confirmed by transmission electron microscopy (TEM). In 3 further pregnancies at risk for lethal junctional epidermolysis bullosa the diagnosis was excluded using the polyclonal antibody AA3. In all these studies the results were available within 4 h of receiving the samples. These new techniques offer a quick and simple alternative to TEM for midtrimester prenatal diagnosis of 2 severe recessive forms of epidermolysis bullosa. *J Invest Dermatol 86:603–605, 1986*

Since lethal junctional epidermolysis bullosa (JEB) and recessive dystrophic epidermolysis bullosa (RDEB) were first diagnosed in utero about 6 years ago [1,2], they have continued as the main genetic skin diseases to be investigated prenatally using fetoscopy and fetal skin biopsy [3]. The procedure is usually performed at 18 weeks' gestation. The fetoscopic skin samples are examined by light and electron microscopy (TEM) to determine whether the fetus is affected. Even with rapid techniques [4] the results of the investigation are not usually available until a minimum of 36–48 h after biopsy. The interval will be longer when the samples have to be sent considerable distances between fetoscopist and electron microscopist. There is a need, therefore, for diagnostic techniques that are simpler and faster than TEM, without the loss of precision, which is a great strength of this method.

It has recently been shown that the monoclonal antibody LH 7:2 binds to the epidermal basement membrane (EBM) in normal adult skin [5] and in fetal skin at 10 weeks' gestation (A. H. M. Heagerty, A. R. Kennedy and R. A. J. Eady, unpublished findings), but binding is absent or markedly reduced in the skin of patients with severe generalized RDEB and patchily reduced in the localized form of RDEB [6].

Similarly, the polyclonal antibody AA3 has been reported to bind to the EBM of normal human skin [7,8] but displays markedly reduced binding in the lethal form of JEB both in neonatal and fetal skin, as seen in a total of 6 subjects [8]. We concluded that the abnormal binding of both antibodies was sufficiently specific and unambiguous in the 2 forms of epidermolysis bullosa (EB), that they could be used as precise diagnostic probes for these conditions.

We now report the successful use of these novel probes in the rapid prenatal diagnosis of RDEB, and in the prenatal exclusion of JEB.

MATERIALS AND METHODS

Case Histories *Case 1* was a 27-year-old woman in her second pregnancy. Her first child, aged 9 months, was affected since birth by widespread trauma-induced blisters resulting in scarring and milia. There was no family history of a similar disorder. The clinical diagnosis of RDEB was confirmed by TEM using established criteria [9]. Indirect immunofluorescence (IIF) using the monoclonal antibody LH 7:2 showed no staining, consistent with a diagnosis of RDEB [6] (see below). The possibility of prenatal diagnosis was offered to the patient and her husband, who accepted, knowing there was a 1 in 4 chance that the pregnancy would be affected.

Case 2 was a 32-year-old woman in her fourth pregnancy. Her first child died at 14 weeks of age with JEB. The diagnosis was confirmed by TEM. In 1981, the second pregnancy was found to be affected by JEB, using TEM to examine fetoscopic skin samples according to the method of Rodeck et al [1]. The third pregnancy in 1984 resulted in a spontaneous abortion at 8 weeks. During the present pregnancy the patient again asked for prenatal testing for JEB.

Case 3 was a 26-year-old woman in her fifth pregnancy. Her fourth child had been affected with JEB, confirmed by TEM, and died aged 9 weeks. Fetoscopic skin biopsy to exclude the diagnosis of JEB in the present pregnancy was performed at 19 weeks' gestation.

Case 4 was a woman aged 33 years in her third pregnancy. Her first child had been affected by JEB and died at 1 year of age. Fetoscopy and fetal skin biopsy demonstrated an affected fetus in her second pregnancy. During the 19th week of the present pregnancy, she presented for further prenatal diagnosis.

Fetoscopy and Fetal Skin Biopsy Ultrasound examination, fetoscopy, and fetal skin biopsy [1] were performed in each case at an estimated gestational age of 18–20 weeks. Intrauterine blistering was not evident in any fetus. Five biopsies were taken from each fetus under direct vision from the buttock and outer thigh.

Antibodies LH 7:2 is a supernatant murine monoclonal antibody produced by immunization with a 1% Nonidet P 40 extract of trypsin-separated epidermal cells. Preliminary results of char-

acterization of the antibody have shown no reaction to laminin, entactin, fibronectin, or types I–VI collagen [5]. It recognizes an epitope in the lamina densa of the basement membrane of stratified squamous epithelia [5,6]. Its use in the diagnosis of RDEB has been described elsewhere [6].

AA3 is a rabbit polyclonal antibody raised against deoxycholate-solubilized human amnion which binds to the EBM (chiefly lamina lucida) [7]. We have previously described its use as a diagnostic probe in JEB [8].

Processing for Immunofluorescence   One specimen from fetus 1, 2 from fetus 2, 1 from fetus 3, and 2 from fetus 4 were immediately embedded in OCT compound (Lab-Tek Products, Naperville, Illinois), oriented on cork, and snap-frozen in isopentane over solid carbon dioxide.

Five-micrometer cryostat sections of unfixed tissue, cut perpendicular to the skin surface, were air-dried on glass slides and washed for 15 min at 4°C in phosphate-buffered saline (PBS). Nonspecific secondary antibody staining was blocked by preincubation for 10 min at 20°C with nonimmune serum of the species in which the secondary antiserum was derived. The primary antibody LH 7:2 (undiluted) or AA3 (at a titer of 1 in 40) was incubated on sections for 30 min at 37°C. After washing in excess PBS for 15 min at 4°C, the sections were incubated with either rabbit antimouse or goat antirabbit fluorescein isothiocyanate (FITC)-conjugated IgG antibodies for 30 min at 37°C. The secondary antibodies were first preabsorbed against human skin acetone powder. After a final PBS rinse (15 min at 4°C) the sections were mounted in glycerol:PBS (9:1, v/v) pH 8.4, containing 0.1% paraphenylenediamine [10].

Parallel sections were incubated with a rabbit antihuman type IV collagen polyclonal antiserum, at a titer of 1 in 600, or with a rabbit antimouse laminin polyclonal antiserum at a titer of 1 in 800, localization being visualized using goat antirabbit IgG-FITC conjugates as described above.

Dilutions of nonimmune sera, antisera, and antibody preparations were made in PBS/3% bovine serum albumin solution. Sections were examined using a Zeiss standard 14 microscope, equipped for narrow band excitation/transmission epifluorescence, and photographed using Kodak Ektachrome 200 ASA film. Further parallel sections were air-dried, lightly fixed with phosphate-buffered 4% formalin for 5 min at room temperature, and stained with methylene blue and azure II for standard light microscopy.

Processing for Light and Electron Microscopy   The remaining biopsy specimens were immersed in half-strength Karnovsky fixative in cacodylate buffer (pH 7.4) containing 5% sucrose and 0.05% $CaCl_2$ at 20°C for 30 min. After one rinse in buffer, the tissues were postfixed in 2% osmium tetroxide containing 0.01 M potassium ferrocyanide at 20°C for 30 min. After dehydration in graded ethanols, the specimens were embedded in Epon and semithin sections were stained with methylene blue and azure II for light microscopy. Ultrathin sections were cut with a diamond knife using a Reichert Ultracut microtome, and stained with uranyl acetate and lead citrate prior to ultrastructural examination using a JEOL 100 CX electron microscope.

Controls   Normal age-matched fetal skin (3 samples) and normal adult skin (9 subjects) were examined by IIF using LH 7:2 and AA3 antibodies, in addition to anti-type IV collagen and antilaminin sera. Skin from 3 fetuses with JEB previously diagnosed prenatally by TEM between 18–20 weeks was also examined with AA3. A skin biopsy from the proband of case 1, and skin from the second affected fetus of case 2 were similarly examined. As negative controls, for LH 7:2, nonfused Sp2 OAg14 myeloma cell line supernatant was regularly tested, and for AA3, nonimmunized rabbit serum was used.

RESULTS

Immunofluorescence Microscopy   *Case 1:*   Examination of the entire biopsy sample under a dissecting microscope and serial frozen sections showed gross dermal-epidermal separation with a small residual piece of adherent epidermis. Staining for LH 7:2 was totally absent (Fig 1a). Staining for type IV collagen showed linear fluorescence at the base of the epidermal portion (Fig 1b). These results were available within 4 h of receiving the specimen.

Cases 2–4:   No dermal-epidermal separation was evident in any section. AA3 antibody staining at the EBM was intense in all 3 fetuses as was the labeling with antilaminin antiserum (Fig 2a,b).

Controls   LH 7:2 (Fig 1c), AA3 (Fig 2c), and antisera to type IV collagen and laminin produced strong linear staining of the EBM in all samples of age-matched normal fetal skin. LH 7:2 binding was absent in the proband for case 1 (Fig 1d), while anti-type IV collagen antiserum stained the skin normally. AA3 staining was reduced in intensity in skin of the third fetus of case 2 (Fig 2d) and in the skin of the 3 previously positively diagnosed fetuses in the presence of normal laminin labeling.

Light and Electron Microscopy   Examination of semithin sections confirmed gross subepidermal separation in all fetal samples from case 1 only. TEM demonstrated the level of cleavage to be below the lamina densa (Fig 3) with an absence of normal anchoring fibrils in adjoining unseparated skin. The samples from cases 2–4 showed no separation, with the presence of normal-appearing hemidesmosomes.

Termination of Pregnancy and Postmortem Examination   Elective termination of pregnancy was performed in case 1 at 20 weeks' gestation, using prostaglandin $F_{2\alpha}$ infusion. The fetus was delivered with the intact fetal membranes after 10 h. Unbroken blisters were noted on the palms and soles and frontal ridges. The nails were present and normal. The biopsy sites could not be found. Skin samples were removed from the back, limbs, scalp, and feet. The diagnosis of RDEB was confirmed by demonstrating the absence of normal anchoring fibrils by TEM and absent IIF staining with LH 7:2.

DISCUSSION

We have shown that 2 novel immunohistochemical probes can be used in the prenatal diagnosis or exclusion of 2 serious forms of recessive EB. We encountered no major difficulty with the handling of small fetoscopic skin samples either during sectioning in a cryostat or in subsequent processing for immunofluorescence. The binding characteristics of both antibodies (AA3 and LH 7:2) had previously been extensively assessed in studies on normal skin from fetuses of different gestational ages and from neonates and adults. Skin samples from a large pool of patients and fetuses affected by different types of EB have also been examined. We are satisfied with the value of these antibodies as precise diagnostic markers in JEB and RDEB.

Before studies are contemplated using antibodies for prenatal diagnosis, we feel it important that certain criteria should be met. First, the immunofluorescence staining should be reproducible and unambiguous; second, the antigens being studied must be known to be expressed by the time of fetoscopy, that is, at 18–20 weeks' gestation; and third, extensive baseline studies should be carried out in a sufficiently large number of patients with the disease being investigated and especially, wherever possible, in the index cases.

Ideally, sufficient quantities of antibodies should be available to ensure reproducible results and for use, if required, by other laboratories engaged in similar work. With regard to the present study, the antibody LH 7:2 is a murine monoclonal and can be produced in large amounts; AA3 is a rabbit polyclonal antiserum which is in limited supply. However, we have been able to substitute the murine monoclonal antibody GB3, which displays similar binding characteristics to AA3 in both normal skin and in JEB-affected skin.

The IIF techniques described in the present study should provide a useful alternative to TEM for the prenatal diagnosis or exclusion of RDEB and JEB and would be especially valuable where TEM facilities are not available. A further advantage is
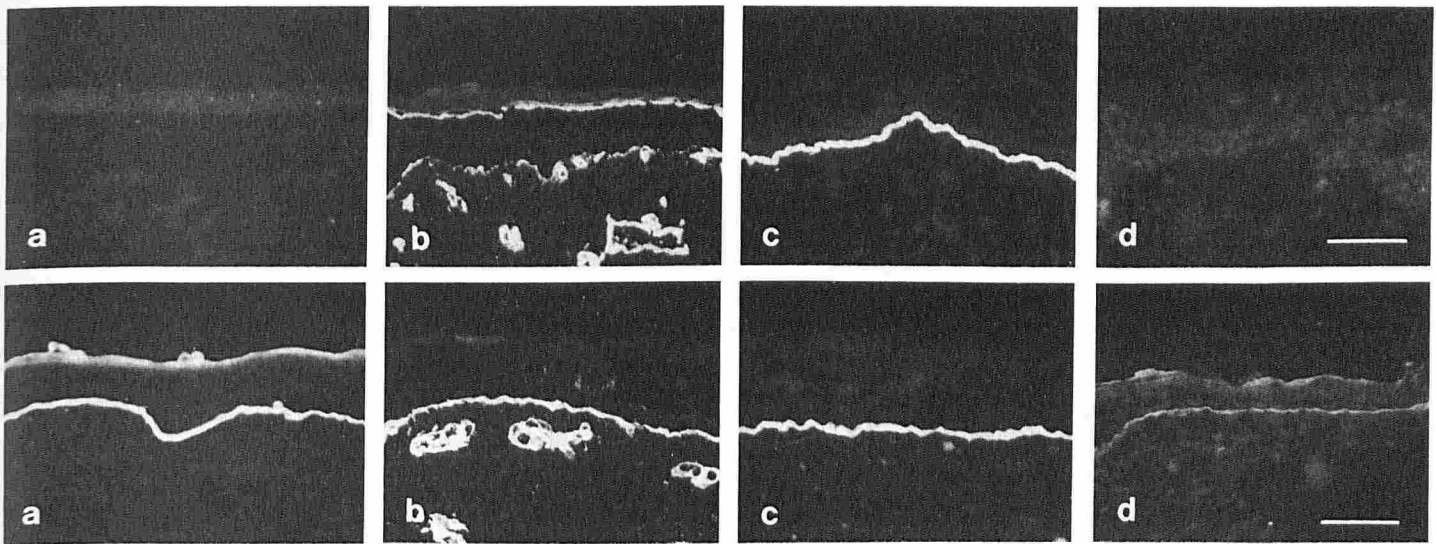
Figure 1. Immunofluorescence photomicrographs. *a*, Absent labeling of EBM with LH 7:2 in fetus affected with RDEB. *b*, Normal type IV collagen labeling of EBM with sublamina densa split in fetus affected with RDEB. *c*, Age-matched (19 week) normal fetal skin labeled with LH 7:2. *d*, Absent labeling with LH 7:2 in skin of proband for case 1. *Bar* = 50 μm.

Figure 2. Immunofluorescence photomicrographs. *a*, Intense labeling with AA3 of EBM in fetus from case 2. *b*, Normal laminin labeling in same fetus. *c*, Age-matched (19 week) normal fetal skin labeled with AA3. *d*, Reduced labeling with AA3 in skin from patient with JEB (index for case 2). *Bar* = 50 μm.

that very rapid results can be obtained, which is an important consideration in late midtrimester prenatal diagnostic studies.

In conclusion, we feel that the methods described here will have an important place in future prenatal diagnostic studies of EB. However, we still recommend careful monitoring by TEM until more experience has been gained with these new techniques. Further work will determine whether the antibodies examined in this study can be used at an earlier stage in pregnancy, using chorionic villus samples.

Note: The antibody recognized by the antibody AA3 (Also called 6/2) has been found to be a protein with a molecular weight of 37 kilodaltons. (Venando P, Ortonne J-P, Hsi B-L, Yeh C-JG: Identification of a 37 kilodalton protein at the epidermal basement by an antiserum to human amnion. J Invest Dermatol, in press).
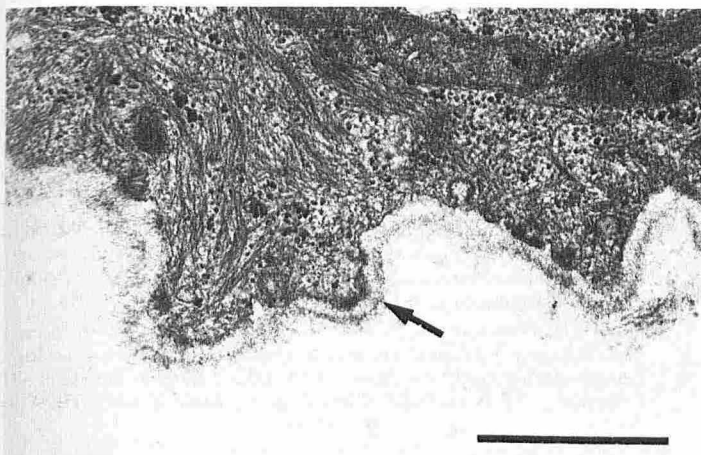
Figure 3. Electron micrograph of separated epidermis in fetoscopic skin biopsy from case 1. The split has occurred beneath the lamina densa (*arrow*) which remains adherent to the basal keratinocytes. *Bar* = 1 μm.

REFERENCES

1. Rodeck CH, Eady RAJ, Gosden CM: Prenatal diagnosis of epidermolysis bullosa letalis. Lancet 1:949–952, 1980

2. Anton-Lamprecht I, Jovanovic V, Arnold M-L, Rauskolb R, Kern B, Schenck W: Prenatal diagnosis of epidermolysis bullosa dystrophica Happopeau-Siemens with electron microscopy of fetal skin. Lancet 2:1077–1079, 1981

3. Eady RAJ, Gunner DB, Tidman MJ, Heagerty AHM, Nicolaides KH, Rodeck CH: Prenatal diagnosis of genetic skin disease by fetoscopy and electron microscopy: report on 5 years' experience. Br J Dermatol 113(suppl 29):45, 1985

4. Eady RAJ, Gunner DB, Tidman MJ, Nicolaides KH, Rodeck CH: Rapid processing of fetal skin for prenatal diagnosis by light and electron microscopy. J Clin Pathol 37:633–638, 1984

5. Leigh IM, Purkis PE: LH 7:2 a new monoclonal antibody to a lamina densa protein (abstr). J Invest Dermatol 84:448, 1985

6. Heagerty AHM, Kennedy AR, Leigh IM, Eady RAJ: LH 7:2 monoclonal antibody defines a common dermo-epidermal junction defect in recessive forms of dystrophic epidermolysis bullosa (abstr). J Invest Dermatol 84:448, 1985

7. Ortonne J-P, Hsi B-L, Schmitt D, Yeh C-JG, Faulk WP: Localization of an amnion antigen (AA3) at the dermo-epidermal junction (abstr). J Cutan Pathol 11:231–232, 1984

8. Kennedy AR, Heagerty AHM, Ortonne J-P, Hsi B-L, Yeh C-JG, Eady RAJ: Abnormal binding of an anti-amnion antibody to epidermal basement membrane provides a novel diagnostic probe for junctional epidermolysis bullosa. Br J Dermatol, 113:651–659, 1985

9. Tidman MJ, Eady RAJ: Evaluation of anchoring fibrils and other components of the dermal-epidermal junction in dystrophic epidermolysis bullosa by a quantitative ultrastructural technique. J Invest Dermatol 84:374–377, 1985

10. Huff JC, Weston WL, Wanda KD: Enhancement of specific immunofluorescent findings with use of a paraphenylenediamine mounting buffer. J Invest Dermatol 78:449–450, 1982