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Full Length Research Paper

Expression, purification and characterization of recombinant exfoliative toxin A from *Staphylococcus aureus*

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For the expression of *Staphylococcus aureus* exfoliative toxin A in *Escherichia coli* regulated by a T5 promoter, the gene was amplified by polymerase chain reaction and cloned into expression vector pQE-30 to generate pQE-ETA. The maximum production of His6-tagged protein by *E. coli* M15 (PQE-ETA) was obtained with 0.1 mM IPTG induction for 4 h at 25°C. The expressed protein was purified by Ni^{2+}-nitrilotriacetate resin to a specific activity of 0.25 mg/ml recombinant protein. The molecular mass of the purified protein was estimated to be 27 kDa by SDS-PAGE. Western blot showed that, the recombinant protein was recognized by sheep anti-ETA antibody and the recombinant protein could split the stratum granulosum of the mouse skin. In conclusion, it was found that the recombinant ETA exhibited no important differences from those properties described for the native protein.

Key words: *Escherichia coli*, exfoliative toxin A, *Staphylococcus aureus.*

INTRODUCTION

The exfoliative toxins (ET) produced by *Staphylococcus aureus* (*S. aureus*) are the causative agents of the bullous impetigo and its generalize form, staphylococcal scalded-skin syndrome (SSSS) (Ladhani, 2001).SSSS is a disease of infants and young children characterized by extensive erythema and exfoliation of outer epidermal layers of skin, but adults with immunocompromised states and chronic renal failure can also be affected. The mortality of SSSS is still 3% in children and over 50% in adults even with appropriate antibiotic treatment (Iwatsuki et al., 2006; Nishifuji et al., 2008).

There are two serological forms of staphylococcal ETs (ETA and ETB) which are responsible for human SSSS. The gene encoding ETA is situated in the bacterial chromosome, but the gene encoding ETB is located on the plasmid. ETA and ETB are composed of 242 and 246 amino acids, respectively and they have approximately 40% amino acid similarity (Ladhani et al., 1999). Recent researches revealed a possible correlation between the clinical manifestation of SSSS and the serotype of ETs. Yamasaki et al. report that ETA is related to bullous impetigo and ETB is related to the generalize SSSS (Yamasaki et al., 2005).

In the early 1970s, Melish et al.(1970) found that ET produced by *S.aureus* causes the blistering in neonatal mice like that in patient with bullous impetigo and SSSS, but the mechanisms by which ETs cause intraepidermal separation was still unknown for over three decades. In

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Abbreviation: ET, exfoliative toxin; **SSSS,** staphylococcal scalded-skin syndrome; **Dsg,** desmoglein; **LB,** Luria-Bertani; **PCR,** polymerase chain reaction; **IPTG,** isopropyl-D-thiogalactopyranoside; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **TEMED,** N,N,N'N'-tetra methyl ethylene diami ne; **PVDF,** Polyvinylidene difluoride; **ECL,** enhanced chemiluminescence.

recent decade, some studies about x-ray crystal structures and amino acid sequence (Vath et al., 1999; Yamaguchi et al., 2001) of ETA and ETB indicate that, ETs are the members of the trypsin-like serine protease family and they specifically cleave desmoglein (Dsg) 1 which is a desmosomal cadherin-type cell-cell adhesion molecule of keratinocytes in the granular layer (Amagi et al., 2000; Hanakawa et al., 2002; Hanakawa et al., 2004). Currently, therapy for SSSS in humans mainly consists of antibiotic therapy. Because antibiotics are targeted to the offending organism, exfoliation will continue for 24 to 36 h after antibiotic administration. The development of novel therapies may be invaluable. There have been studies showing that antitoxin antibodies will protect neonatal mice from lethal doses of ET (Plano, 2004). To carry on future studies for the precise mechanisms by which ETs split the epidermis and manufacture antitoxin antibodies, large quantities of purified ETs are needed. This work described efforts to synthesis recombinant ETA in a biologically active form in the *Escherichia coli* expression system and large amount of active protein can be purified by the process of metal-chelate chromatography.

MATERIALS AND METHODS

Materials, bacterial strains, vector and growth conditions

Luria-bertani (LB) media for bacterial culture were purchased from MD Bio, Inc. (Taipei, Taiwan). *Taq* DNA polymerase enzyme, dNTP, restriction enzyme, T4 DNA ligase enzyme and protein size markers were acquired from Takara Biotechnology Co. (Dalian, LL, China). The oligonucleotide primers were synthesized by invitrogen (Shanghai, China). Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resin was obtained from Qiagen Inc. (Valencia, CA, USA). Reagents for polyacrylamide electrophoresis such as acrylamide, bis-acrylamide, ammonium persulfate and TEMED were obtained from Beyotime Institute of Biotechnology (Haimen, JS, China). Sheep polyclonal to *Staphylococcus aureus* ETA was obtained from Abcam (Hong Kong) Ltd (Hongkong, China). Rabbit anti-sheep IgG-HRP was obtained from Bioworld technology Co., Ltd (Minneapolis, MN, USA). Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence (ECL) were purchased from Keygen biotech. Co., Ltd (Nanjing, JS, China). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or other comer-cial sources.

ETA-producing *S. aureus* was isolated from the patient with generalized SSSS. *E. coli* DH5α was used for the preservation and amplification of expression plasmid. The overproduction of ETA protein was performed in *E. coli* M15 (Qiagen). The plasmid used was pQE-30 (Qiagen). ETA-producing S. aureus was grown in 100 ml of LB medium at 37℃ for 12 h and was then, harvested for chromosomal DNA isolation. Host cells harboring pQE-ETA were cultured aerobically in LB medium supplemented with 100 µg ampicillin/ml for DH5α strain or with 100 µg ampicillin/ml and 25 µg kanamycin/ml for M15 strain.

Construction of expression plasmid

The DNA fragment of the *eta* gene encoding the open reading frame corresponding to the mature form of ETA was obtained by utilizing PCR and DNA from ETA-producing *S. aureus*. A primer set, 5'-CCG GGA TCC TTA GGA TGA TTA ATA ATG AAT-3' and 5'- CGT AAG CTT GCA GTC AGC TTC TTA CTG-3', was designed to amplify a 927 bp DNA fragment encoding mature ETA and contained restriction endonuclease sites (*BamHI* and *HindIII*) that facilitated insertion of the fragment into the expression vector PQE30. The DNA sequence of ETA-encoding fragment was from the GenBank database sequence (accession number L25372). The PCR reaction mixture, in 50 µl contained 5 ng template DNA, 0.05 mM each dNTP, 10 pmole each primer, 10×PCR buffer and 2.5 U Taq DNA polymerase. After an initial denaturation at 94℃ for 4 min, the PCR reaction was performed for 32 cycles under the following conditions; denaturation at 94℃ for 30 s, annealing of primers at 55℃ for 30 s and primer extension at 72℃ for 1 min, followed by a final extension at 72℃ for 5 min. Then, 50 µl PCR products were analyzed on 1% agarose gel and purified by the BioSpin gel extraction kit (BioFlux Tokyo, Japan).The recovered products were cloned as a *BamHI-HindIII* fragment into the corresponding sites of pQE-30 vector and transformed into *E. coli* DH5α. Transformants containing the recombinant vector were selected on LB medium, supplemented with 100 µg /ml ampicillin. After the selected clones were verified by DNA sequencing, one recombinant plasmid was transformed into *E. coli* M15 again. The recombinant plasmid was constructed as described in Figure 1.

Expression and purification of ETA

E. coli M15 containing pQE-ETA recombinant plasmid was grown at 37℃ in 10 ml LB medium supplemented with ampicillin (100 µg/ ml) plus kanamycin (25 µg/ml) until the optical density (OD) at 600 nm of the culture was 0.9. Then, IPTG were added to a final concentration of 0.01, 0.05, 0.1, 0.5, 1 and 2 mM, respectively. The induced cells were incubated at 20, 25 and 37℃, respectively, for 24 h and cells were harvested by centrifugation (9,000 × g for 10 min at 4℃) at 0, 1, 2, 3 and 4 h after induction (Lin et al., 2006).

The protocol of purifying the recombinant ETA was followed. 100 ml of the induced cells was incubated at 25℃ for 4 h and harvested by centrifugation. The pellets were resuspended in lysis buffer (10 mM imidazole, 300 mM NaCl and 50 mM NaH_2PO_4, PH 8.0) at 2 to 5 ml per gram wet weight and were sonicated on ice after adding lysozyme. The supernatant was mixed with Ni^{2+}-NTA resin, washed with wash buffer (pH 8.0) containing 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole to remove impurities. Finally, the recombinant ETA with His_6-tagged was eluted with elution buffer (250 mM imidazole, 300 mM NaCl and 50 mM NaH_2PO_4, pH 8.0).

Protein methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% acrylamide slabs using a Protean III mini gel system (Bio-Rad Laboratories, Beverly, MA, USA). Before electrophoresis, the samples were heated to 100℃ for 5 min in SDS-PAGE loading buffer containing, 2% SDS and 5% 2-mercaptoethanol, and centrifuged at 12,000 g for 5 min. Protein size markers were phosphorylase b (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). SDS-PAGE was carried out at 100 V for 2 h at room temperature. The gel was stained with coomassie brilliant blue R-250. ETA protein concentration was determined according to the Bradford method, using bovine serum albumin (BSA) as the standard.

Western blotting

After SDS-PAGE, the ETA on the SDS-PAGE gel was electrophoretically transferred onto a PVDF membrane for 35 min at 10 mA.

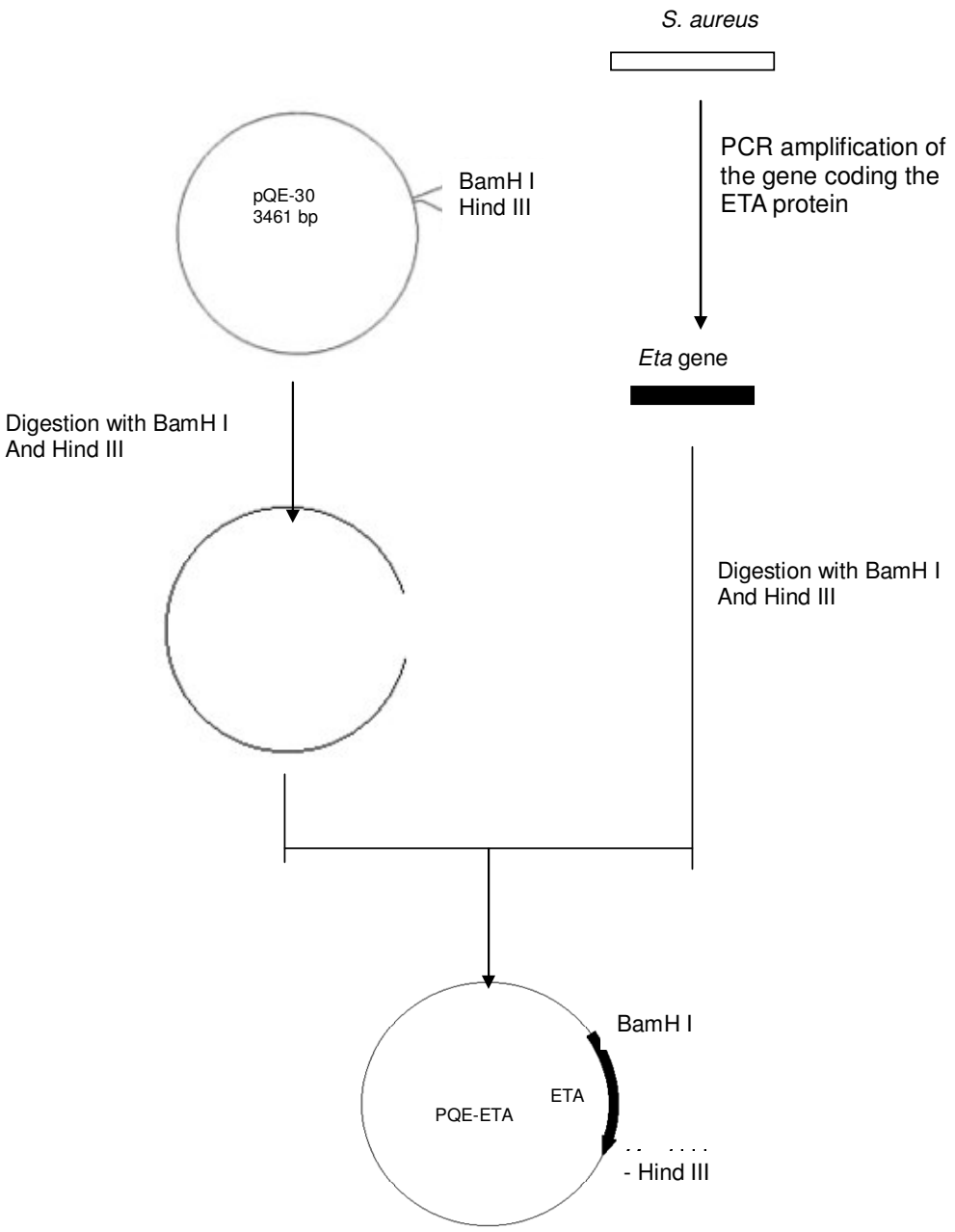

Figure 1. Strategy for cloning and construction of pQE-ETA, which expresses the recombinant ETA protein of *S.aureus.*

The membrane was washed in T-PBS and blocked with 2% BSA in T-PBS for 1 h. The membrane was reacted with sheep anti-staphylococcal ETA IgG diluted to 1:500 in 1% BSA in T-PBS for 14 h at 4℃. The membrane was washed thrice in T-PBS for 10 min per time. Peroxidase-rabbit anti-sheep IgG antibody diluted to 1:10000 was then added and incubated for 2 h, wash as described earlier and stained with ECL.

Exfoliative activity assay

The exfoliative activity of the recombinant ETA was tested in neonatal mice. One-day-old BALB/c mice were injected subcutaneously at the nape of the neck with 10 μg/g of body weight of rETA or PBS (negative control). All mice were returned to lactating mothers and were observed at various times post injection for symptoms of SSSS (Plano et al., 2001).

RESULTS

Expression and purification of the recombinant ETA

The PCR-amplified DNA fragment encoding mature ETA of *S. aureus* was digested with *BamHI* and *HindIII*

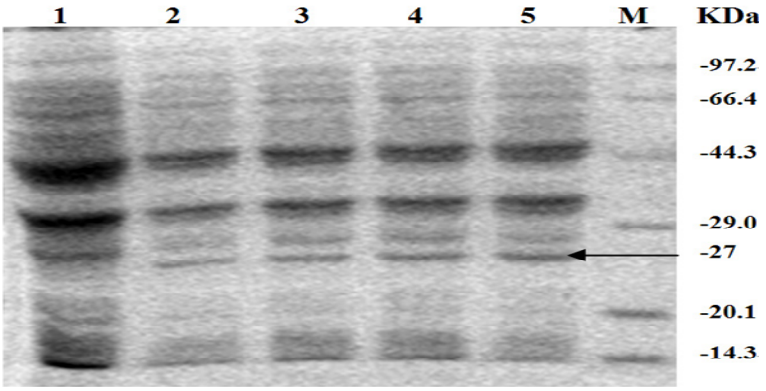

Figure 2. Analysis of the total cell proteins from *E. coli* M15 (pQE-ETA).The expressed protein was separated on 12% SDS-PAGE gel stained with coomassie brilliant blue R-250. Lanes: M, molecular weight marker; 1, cultivation without IPTG induction; 2, 1 h cultivation with IPTG induction; 3, 2 h cultivation with IPTG induction; 4, 3 h cultivation with IPTG induction; 5, 4 h cultivation with IPTG induction.

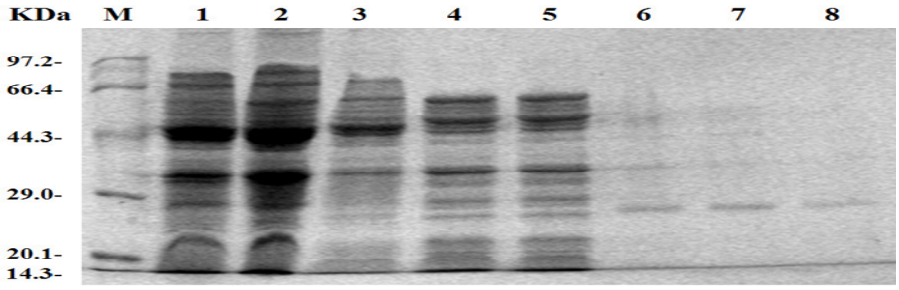

Figure 3. SDS-PAGE analysis of the eluted fractions from nickel-chelate chromatography. Lanes: M, molecular weight marker; 1, *E. coli* M15 including pQE30-ETA induced without IPTG; 2, *E. coli* M15 including pQE30-ETA induced with IPTG; 3, Supernatant of *E. coli* M15 including pQE30-ETA after sonicating; 4 Flow-through ; 5, Wash fraction; 6 to 8, purified protein fraction .

and inserted into the expression vector PQE30 under the control of T5 promoter. *E. coli* M15 competent cells were transformed with the recombinant plasmid, pQE-ETA and the cloned gene was confirmed by restriction analysis and DNA sequencing. The sequencing data corresponded with the *eta* gene sequence on GenBank database.

E. coli M15 cells harboring pQE-ETA were induced with 0.1 mM IPTG. After 1, 2, 3, and 4 h inductions, 1 ml of the bacterial culture was centrifuged and resuspended in the loading buffer and the total cellular proteins were separated by 12% SDS-PAGE. The protein patterns of the total cell extracts with or without IPTG induction are shown in Figure 2. SDS-PAGE analysis of the total proteins from IPTG-induced *E. coli* M15 exhibited a predominant protein band at 27kDa (Figure 2). The result also indicated that, the synthesis of ETA reached a maximum after a 4 h induction. To obtain high expression of active rETA, several different growth temperatures and IPTG concentration were tried. The optimal temperature for the production of active rETA was 25℃. In fact, the results obtained at 20, 25 and 37℃ were very similar. The optimum IPTG concentrations for the expression of rETA were in the range of 0.05 to 1 Mm. There was a significant reduction in the level of the active enzyme when IPTG concentration exceeded 1 mM. The best conditions for the maximal production of rETA by *E. coli* M15 were; IPTG at a final concentration of 0.1 mM and induction temperature and time of 25℃ and 4 h, respectively. Under these conditions, the expressed protein comprise up to 12% of the total soluble proteins of IPTG-induced *E. coli* M15 (pQE-ETA). The rETA in the crude extract was further purified by nickel column chromatography. As shown in Figure 3, the purified protein had a molecular mass of 27 kDa (Figure 3) and a production of 0.25 mg/ml rETA was obtained.

Western blot showed that the recombinant protein could be recognized by sheep polyclonal anti-ETA IgG and was visualized as a single band. As a control, ETA-producing *S. aureus* was observed as two bands, one

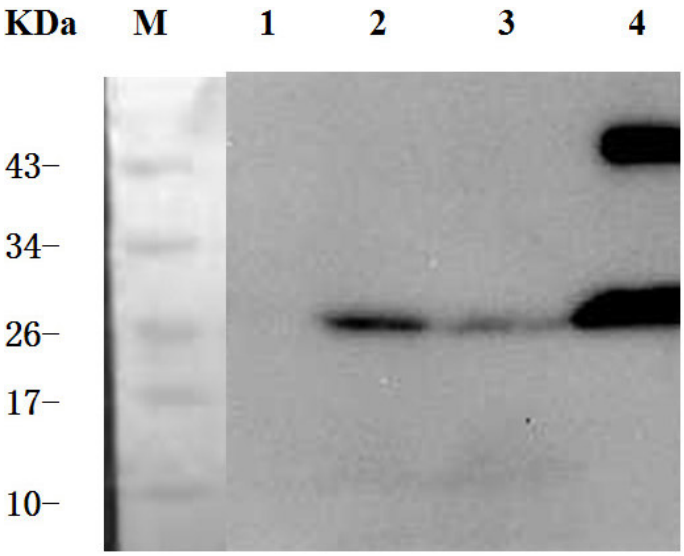

Figure 4. The identification of rETA by Western blot. Lanes: M, molecular weight marker; 1, *E. coli* M15 without vector PQE30-ETA; 2, induced protein of *E. coli* M15 with vector PQE30-ETA exhibited with sheep against ETA antibody and rabbit anti-sheep IgG-HRP; 3, purified rETA protein exhibited with sheep against ETA antibody and rabbit anti-sheep IgG-HRP; 4, ETA-producing *S. aureus.*

was the same location with recombinant ETA protein and the other showed a non-specific broad cross-reacting band of molecular masses at 45 kDa. This band is most probably staphylococcal protein A, which can bind the Fc portion of domestic animals IgG subclasses (Figure 4).

Exfoliative activity

6 h after the injection of rETA to a new born mouse, epidermal splitting was observed on the dorsal skin. The control mouse injected with PBS (Figure 5) showed the characteristic splitting at the stratum granulosum in the mouse with rETA and intact skin in the mouse with PBS (Figure 5).

DISCUSSION

In this study, the 927 bp gene encoding the mature form of ETA was successfully inserted into vector PQE-30 and expressed recombinant ETA in *E. coli* regulated by a T5 promoter. The expressed protein was most soluble and was present in its exfoliative active form. Additionally, the His6-tagged ETA was efficiently purified in simple steps by Ni^{2+}-NTA resin to produce a protein with a specific biological activity.

Some factors detection have the choice of an expression system for the high-level production of recombinant proteins, such as cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications and biological activity of the protein (Hockney, 1994; Olins and Lee, 1993; Sørensen et al., 2005). PQE-30 vector was used for the expression of 6×His-tagged recombinant proteins in *E. coli* and the recombinant proteins was easily purified by Ni-NTA matrices. It was not necessary to remove the short 6×His affinity tag from the recombinant protein after purification and the function of the recombinant protein was affected hardly. If purification under native conditions is preferred, the 6×His-tagged protein must be soluble but be inclusion bodies (Carrio et al., 2001). Cultivation at reduced temperature could be used for the soluble expression of recombinant proteins in *E. coli* but in this study, there was no extra quantity of the expression of rETA and the inclusion body could not be formed. Thus, the soluble expression of rETA incubated at 20, 25 and 37℃ were very similar.

The particularity and the antigenicity of the rETA were confirmed by the reaction with anti-ETA antibody in western blot and the exfoliative activity was proved by Murine model. These indicated that the rETA was the same structure and functioned as the native ETA protein.

The frequency of *et*-positive strains of meticillin-resistant *S. aureus* (MRSA) in clinical is increasing and raising the possibility that those strains lead to SSSS (Ito et al., 2002; Yokota et al., 1996). The development of novel therapies may become urgent necessity. The manufacture of the anti-ETA antibody for the therapy and diagnosis of SSSS by utilizing rETA will be a goal for

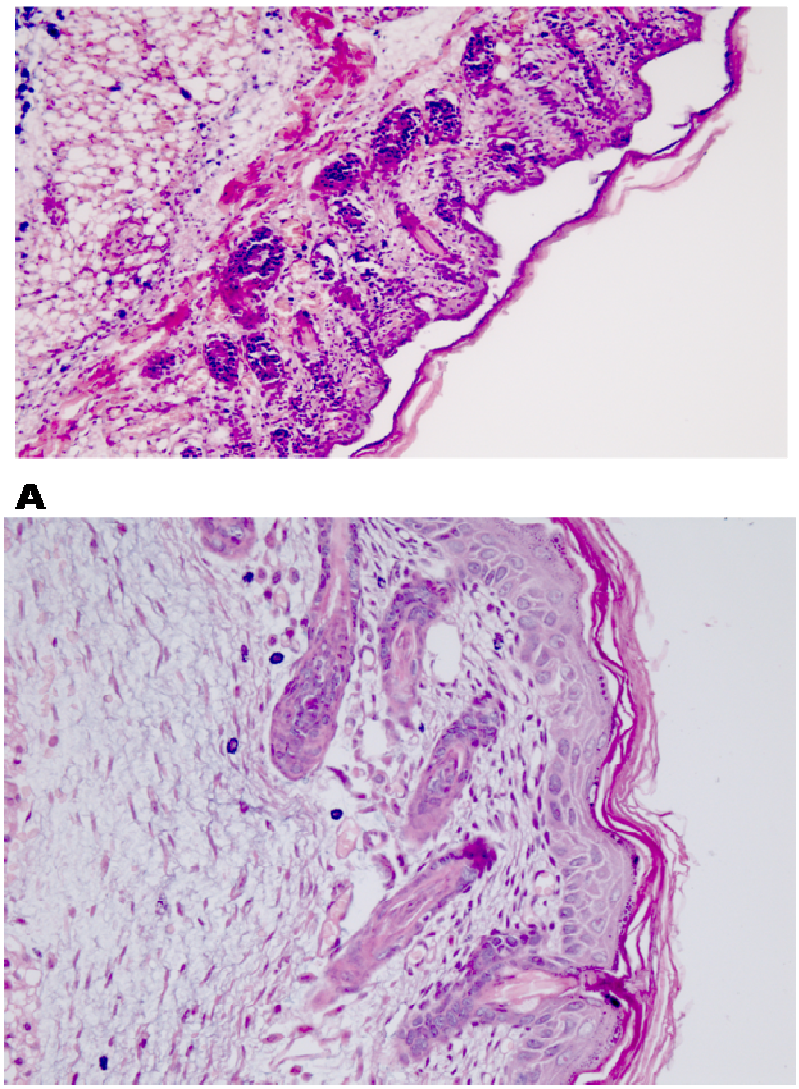

Figure 5. Histological examination of neonatal mouse skin exposed to rETA and PBS. Sections were from 1 day-old mice sacrificed at 24 h postinjection and were stained with hematoxylin and eosin. Magnification was ×40. A, Mouse injected with rETA showing the characteristic splitting at the stratum granulosum; B, control mouse injected with PBS showing intact skin.

future experiment.

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