MAJOR ARTICLE

Clinicopathologic Analysis of Coxsackievirus A6 New Variant Induced Widespread Mucocutaneous Bullous Reactions Mimicking Severe Cutaneous Adverse Reactions

Wen-Hung Chung,[1,a] Shin-Ru Shih,[5,a] Ching-Fen Chang,[1] Tzou-Yien Lin,[2] Yhu-Chering Huang,[2] Shih-Chen Chang,[5] Ming-Tsan Liu,[6] Yu-Shien Ko,[3,4] Ming-Chung Deng,[7] Yea-Ling Liau,[8] Lung-Huang Lin,[9] Tou-Hwei Chen,[9] Chih-Hsun Yang,[1] Hsin-Chun Ho,[1] Jheng-Wei Lin,[1] Chun-Wei Lu,[1] Chin-Fang Lu,[8] and Shuen-Iu Hung[10]

[1]Department of Dermatology, Drug hypersensitivity clinic and research center, [2]Department of Pediatrics, [3]First Cardiovascular Division, Chang Gung Memorial Hospital, College of Medicine, Chang Gung University, Keelung, Taipei and [4]Linko Medical Center, Taipei, Taiwan; [5]Research Center for Emerging Viral Infections, Chang Gung University; [6]Research and Diagnostic Center, Centers for Disease Control, Taipei, Taiwan; [7]Division of Hog Cholera Research, New Taipei City, Taiwan; [8]Department of Dermatology, Research Center of Cutaneous Disorders, Chung Shan Hospital, Taipei, Taiwan; [9]Department of Pediatrics, Cathay General Hospital, School of Medicine, Fu-Jen Catholic University, Taipei, Taiwan; and [10]Institute of Pharmacology, School of Medicine, Infection and Immunity Research Center, VYM Genome Research Center, National Yang-Ming University, Taipei, Taiwan

Background. The cutaneous manifestations of human enterovirus (HEV) infection are usually limited, such as hand-foot-mouth disease. By comparison, Stevens-Johnson syndrome (SJS) is a life-threatening severe cutaneous adverse reaction (SCAR), mainly caused by drugs. During the HEV outbreaks in 2010–2012 in Taiwan, we identified 21 patients who developed widespread blistering mucocutaneous reactions without any suspected drug causality.

Methods. We screened possible pathogen(s) for detecting human herpes virus (HHV1–HHV7), HEV, or *Mycoplasma pneumoniae* infections using throat swab virus cultures, real-time PCR, DNA sequencing, immunochemistry and electron microscopy analyses.

Results. Coxsackievirus A6 (CVA6) DNA was identified in the blistering skin lesions in 6 of 21 patients. Cytotoxic T lymphocytes and natural killer cells expressing granulysin predominantly infiltrated into the skin lesions, sharing the histopathological features with SJS. Intact CVA6 viral particles were identified in the blister fluids and skin lesions by electron microscopy. The phylogenetic analysis of the viral genome showed the CVA6 DNA sequence sharing higher similarity (97.6%–98.1%) to CVA6 strains reported from Finland at 2008.

Conclusions. This study identifies a new variant of CVA6 as the causative agent for severe mucocutaneous blistering reactions mimicking SCAR. An awareness of this unusual presentation of HEV infection is needed in the epidemic area.

Keywords. coxsackievirus A6; hand-foot-mouth disease; severe cutaneous adverse reactions; granulysin.

Human enteroviruses (HEV) are common pathogens that can cause a wide range of clinical manifestations, including nonspecific febrile illness, exanthema, respiratory infections, enteritis, aseptic meningitis, encephalitis, myocarditis, and even death [1]. Among HEV, coxsackieviruses, echoviruses, and enterovirus type 71 are frequent causes for cutaneous symptoms [2, 3]. A typical cutaneous manifestation for HEV infection is hand-foot-mouth disease (HFMD), characterized by multiple small papulovesicles on hands, feet, mouth, tongue, buttocks, and oral ulcers accompanied by fever. It is usually self-limiting, and one can recover within 4–6 days [4, 5]. HFMD and herpangina are common contagious diseases in children, and most of the patients were <4 years of age [6, 7]. Child patients infected with CVA16, CVA6, or enterovirus 71 mostly

Received 21 February 2013; accepted 23 May 2013; electronically published 31 July 2013.

[a]W.-H. C. and S.-R. S. contributed equally to this study.

Correspondence: Shuen-Iu Hung, PhD, Institute of Pharmacology, Infection and Immunity Research Center, National Yang-Ming University, 155, Linong St, Sec 2, Beitou, Taipei 11221, Taiwan (sihung@ym.edu.tw).

The Journal of Infectious Diseases 2013;208:1968–78

DOI: 10.1093/infdis/jit383

presented as HFMD [8, 9]. CVA6 was identified as a primary pathogen associated with HFMD during a nationwide outbreak in Finland in 2008 [9]. By comparison, the main clinical presentation for the CVA6 outbreaks in northern Taiwan between 2004 and 2009 was herpangina, and HFMD only accounted for 12.8% [10].

Comparing with HFMD, Stevens-Johnson syndrome (SJS) is a life-threatening severe cutaneous adverse reaction, mainly caused by drugs or medications [11, 12]. SJS and its related disorder, toxic epidermal necrolysis (TEN), principally involve the activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [13]. Our recent study shows that granulysin, a cytotoxic protein produced by CTLs or NK cells, is the key mediator for disseminated keratinocyte deaths resulting in blistering skin lesions or skin detachment in SJS/TEN [14]. The characteristic features of SJS include a rapid development of blistering exanthema and target-like lesions accompanied by mucosal involvement and skin detachment [15–18]. Though with a low incidence, SJS may progress to TEN and is potentially fatal. The survivors of SJS/TEN frequently suffer from permanent complications, such as eye sequelae [11]. Drugs are assumed or identified as the main cause of SJS/TEN in most cases [17, 18], but *Mycoplasma pneumoniae* and *Herpes simplex* virus infections are documented causes alongside rare cases in which the etiology remains unknown [19–21]. There are still about 20% cases without an identified causality [17, 18, 22].

During the HEV outbreaks from 2010 to 2012 in Taiwan, we identified 21 patients who presented unusual widespread mucocutaneous bullous reactions without the administration of any suspected offending drugs. We detected CVA6 virus DNA and the intact viral particles in the skin lesions of 6 patients with widespread blistering reactions. Further virus genome sequencing identified a novel variant of CVA6 that may explain the unusual cutaneous presentations and immune reactions.

MATERIALS AND METHODS

Patient Recruitment

From June 2010 to July 2012, we enrolled 21 young patients with mucocutaneous bullous reactions diagnosed as SJS without the administration of suspected offending drugs from Chang Gung Memorial Hospital, Cathay General Hospital, and Chung Shan Hospital in Taiwan. The study was approved by the institutional review board (IRB) of each studying site, and informed consents were obtained from all of the participants or their parents or guardians. We complied with the human experimentation guidelines of Taiwan Department of Health with regard to patient consent for the conduct of clinical research.

Diagnosis of SJS was made by clinical presentation and/or histopathology findings as the criteria previously established by international experts in the field of drug-induced skin reactions, the Registry of Severe Cutaneous Adverse Reactions (RegiSCAR) consortium [15]. SJS is characterized by a rapidly developing blistering exanthema of purpuric macules and target-like lesions accompanied by mucosal involvement with skin detachment of <10% of the body surface area (BSA). All of the 21 patients have epidermal detachment involving <10% of total body surface area (TBSA). We collected clinical specimens, including throat swabs, skin biopsies, blister fluids/blister cells, peripheral blood mononuclear cells (PBMCs) and serum, for laboratory examinations. The blister fluids from burn patients were used as the controls.

Viral Culture and Virus DNA Detection in Clinical Samples

Clinical specimens from throat swabs were inoculated with 4 cell lines, including the Rhesus monkey kidney (RhMK) cells, human rhabdomyosarcoma (RD) cells, human embryonic lung fibroblasts (MRC-5), and rhesus monkey kidney epithelial cells (LLC-MK-2). The infected cells were maintained in minimal essential media (MEM) containing 1% penicillin-streptomycin (Gibco, Grand Island, NY) with 2% fetal calf serum (FCS, Gibco, Grand Island, NY) at 35°C. All cultures were observed daily for examination of cytopathic effect (CPE).

We extracted the total virus nucleic acids from clinical samples by the viral RNA extraction miniprep system kit (Viogene, Sunnyvale, CA). For HEV detection, complementary DNA (cDNA) was generated through reverse transcription reaction by using M-MLV reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan) with random primers. We performed real-time PCR for HEV detection by amplifying the 5′ untranslated region (UTR) (forward primer: 5′-CAAGCACTTCTGTNWCCCGG-3′; reverse primer: 5′-GAAACACTGGACACCCAAAGTAGT-3′) [23]. For HHV1-7 virus and *Mycoplasma pneumoniae* detection, we adapted real-time PCR protocols of the previous studies [23–25]. We processed appropriate negative and positive controls in each PCR to exclude any contamination and to establish the specificity of primer-directed amplification.

Histopathology, Immunohistochemistry, and Immunofluorescence Staining of Skin Lesions and Blister Cells

The skin specimens were alcohol-formalin-acetic acid fixed and paraffin-embedded and subjected to the hematoxylin and eosin (H&E) staining. For histopathology, the paraffin in the sections was dissolved, and dewaxed in xylene solution and a gradient of ethanol. The specimen was hematoxylin staining for 3 minutes and rinsed with running water, 1% hydrochloric acid alcohol, and then water rinsing. The counter stain was done by eosin staining for 30 seconds, followed by water rinsing, then dehydration in ethanol solutions. The staining was then made transparent with 2 changes of xylene, then mounted with neutral gum and dried.

For immunohistochemistry analyses on the skin biopsies, we incubated the sections with the monoclonal antibodies against CD3, CD4, CD8, CD20, CD56 (BD Biosciences), granulysin (RB-1; MBL international corporation), CD1a (marker of

dendritic cells), and S100 (Dako, Glostrup, Denmark). Sections of skin lesions were subjected to the immunoperoxidase technique (DAB Detection Kit, Invitrogen).

For immunofluoscence analyses on the blister cells, the blister cells were incubated with GolgiStop (BD Biosciences), fixed with Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4°C. Then, the cells on slides were incubated with distinct fluorochromes labeled monoclonal antibodies against human CD8, CD56, or granulysin. The antibodies were labeled with fluorescein isothiocyanate (FITC), or R-phycoerythrin (PE). The cells were examined by the confocal microscopy (Olympus FV1000).

Enzyme-linked Immunosorbent Assay (ELISA) for Granulysin Level Measurement

The levels of granulysin in the blister fluids of patients and the burn control subjects were measured as described elsewhere [14]. Briefly, the plates (Nunc) were coated with 2 μg/mL RB-1 monoclonal antibody (MBL International Corporation) in sterile phosphate-buffered saline (PBS) overnight at room temperature. The nonspecific sites on the plates were blocked with 1% bovine serum albumin in washing buffer (PBS containing 0.1% Tween-20) and serially reacted at room temperature with the following materials, with washing steps between each reaction: samples or standards in blocking buffer for 2 hours, 1 μg/mL of biotinylated RC-8 MAb (recognizing granulysin epitopes) in blocking buffer for 1 hour, and 2 μg/mL of horseradish peroxidase-conjugated streptavidin (R&D system) in washing buffer. The plates were finally incubated with substrate solution containing H_2O_2 and tetramethylbenzidine (R&D system) for 5–10 minutes. Then, the optical density of each well was determined by using a microplate reader set to 620 nm. Samples were performed in triplicate. The assay sensitivity for granulysin was 2.5 ng/mL.

Electron Microscopy

Visualization of virus particles from skin biopsies and blister fluids was acquired by transmission electron microscopy (TEM) on high magnification (X4K, X30K, and X50K). Skin samples from the skin lesions were cut into small pieces and ground in PBS in a microcentrifugal tube, then prepared for negative staining (2% phosphotungstic acid) and observed in a JEM 1010 electron microscope (JEOL USA Inc., Peabody, MA).

Virus Nucleotide Acids Sequencing and Phylogenetic Tree Analysis

The virus RNA was extracted from the blister fluids and the cDNA was synthesized by M-MLV reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan) using pan-enterovirus primer -(5′-AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$VN-3′, where V is G, A, or C and N is any nucleotide). We used a consensus primer (5′-GTTTTCCCAGTCACGACTGGTATCARACDAA-3′) of human enterovirus A (HEV-A) VP1 gene, which encoded the viral capsid and KOD-PLUS DNA polymerase (Toyobo, Osaka, Japan) for PCR and cDNA sequencing. The virus nucleotide sequences were subjected to Blast analysis (NCBI), showing high similarity to the human CVA6. We further performed sequencing experiment for determining CVA6 5′-untranslated genomic region [26]. We constructed phylogenetic tree based on 377 nucleotide long partial VP1 sequence (nucleotide [nt] 2930 to nt 3306) of our viral sequence data using a neighbor-joining method and the Kimura 2-parameter distance model by a *MEGA* version 4 software with 1000 replications of bootstrap analyses [27].

RESULTS

Clinical Features and Identification of CVA6 DNA From the Blistering Skin Lesions

We obtained the throat swab samples, skin biopsies, or skin blister fluids from 21 patients with widespread mucocutaneous blistering reactions. We screened possible pathogen for detecting of the infections of human herpes virus (HHV1–HHV7), *Mycoplasma pneumoniae*, or HEV using throat swab virus cultures and real-time PCR. CVA6 DNA sequence was identified in the blistering skin lesions of 6 patients by real-time PCR and sequencing experiments (Table 1). No *Mycoplasma pneumoniae* or HHV1–HHV7 DNA amplicons was detected in the samples of the 6 patients (Table 1). CVA6 was isolated by a direct throat swab viral culture of 2 patients (Table 1). The clinical cutaneous manifestations of the 6 patients were multiple to widespread central purpuric atypical target-like lesions on limbs, faces, and trunks in the early stage and then progressed to large blisters with purpuric bases at the maximal stage with mild to moderate lip erosions (Figure 1, and Supplementary Figure 1).

Four patients received only supportive treatments, and 2 patients received systemic corticosteroids (Table 1). All 6 patients recovered within 1–3 weeks without complications. The mean hospitalization duration of the patients was 14 ± 4.43 days, which is significantly longer than that of the outbreaks of HFMD caused by CVA6 in Taiwan in 2004–2007 (mean duration, 4.21 ± 0.11 days) [10].

Predominant CTLs and NK Cells Expressing Granulysin in Blistering Skin Lesions

The histopathological findings showed an extensive epidermal necrosis and keratinocyte dyskeratosis resulting in separation of the epidermis from the underlying dermis with blister formation and inflammatory cells infiltrations (Figure 2, Supplementary Figure 2). Immunohistochemistry staining of skin lesions showed the predominate infiltration of $CD8^+$ T cells, $CD56^+$ NK/natural killer T (NKT) cells, and granulysin expression in the epidermis (Figure 2, Supplementary Figure 2). The immunofluorescent microscopy study further confirmed that the majority of blister cells were composed of $CD8^+$ CTL or $CD56^+$ NK/NKT cells

Table 1. Clinical Manifestations and Laboratory Findings in Patients With CVA6 Infection-induced Severe Cutaneous Reactions

Case no.	Age/ Sex	Distribution of Blistering Lesions	Systemic symptoms	Internal Organ Involve-ment	Drug Exposure Before the Cutaneous Reactions	HE Stain on Skin Biopsies	IHC Stain on Skin Biopsies	IF Stain on Blister Cells	Throat Swab Virus Culture	Skin Samples for Viral Genome Detection	PCR and Sequen-cing for HEV	PCR for HHV 1–7	Treatment and outcome	Disease duration (days)
1	3/F	Face, lips, 4 limbs, trunk, palms/soles, buttock	Fever, diarrhea	No	Antihistamines[a]	Full thickness of epidermal necrosis	CD8, CD56 dominant, GNLY (+)	CD8, CD56, GNLY (+++)	CVA6	Blister fluids	CVA6	(–)	Systemic corticosteroid	21
2	2/F	Lips, palms/soles, buttock	Fever, sore throat	No	No	Many dyskeratic cells in epidermis	CD8 (+), CD56 (+) GNLY (+)	ND	(–)	Blister fluids	CVA6	(–)	Supportive care	14
3	5/M	Lips, lower limbs, palms/soles	Fever, sore throat	No	No	ND	ND	ND	(–)	Blister fluids	CVA6	(–)	Supportive care	14
4	6/M	Palms/soles, lips	Fever, sore throat	No	No	ND	ND	ND	(–)	Blister fluids	CVA6	(–)	Supportive care	7
5	4/M	Face, lips, four limbs, trunk, palms/soles, buttocks	No fever, sore throat abdominal pain, vomiting	No	No	Partial epidermal necrosis	CD8, CD56 dominant, GNLY (+)	ND	(–)	Blister fluids	CVA6	(–)	Supportive care	14
6	10/M	Face, lips, four limbs, trunk, palms/soles, buttock	Fever, sore throat	No	Acetaminophen, antihistamines[a]	Partial epidermal necrosis[b]	CD8 dominant, CD56 (+), GNLY (+)	CD8, CD56, GNLY (+++)	CVA6	Skin biopsy	CVA6	(–)	Systemic corticosteroid	14

Abbreviations: (+), positive; (–): negative; CVA6, Coxsackievirus A6; F, female; GNLY, granulysin; HE, hematoxylin and eosin stain; HHV, human herpes virus; IF, immunofluorescence stain; IHC, immunohistochemistry stain; M, male; ND, not determined.

[a] Patients had exposed to the same medications before and after the CVA6 infection episode.

[b] The biopsies were obtained from a regeneration stage.

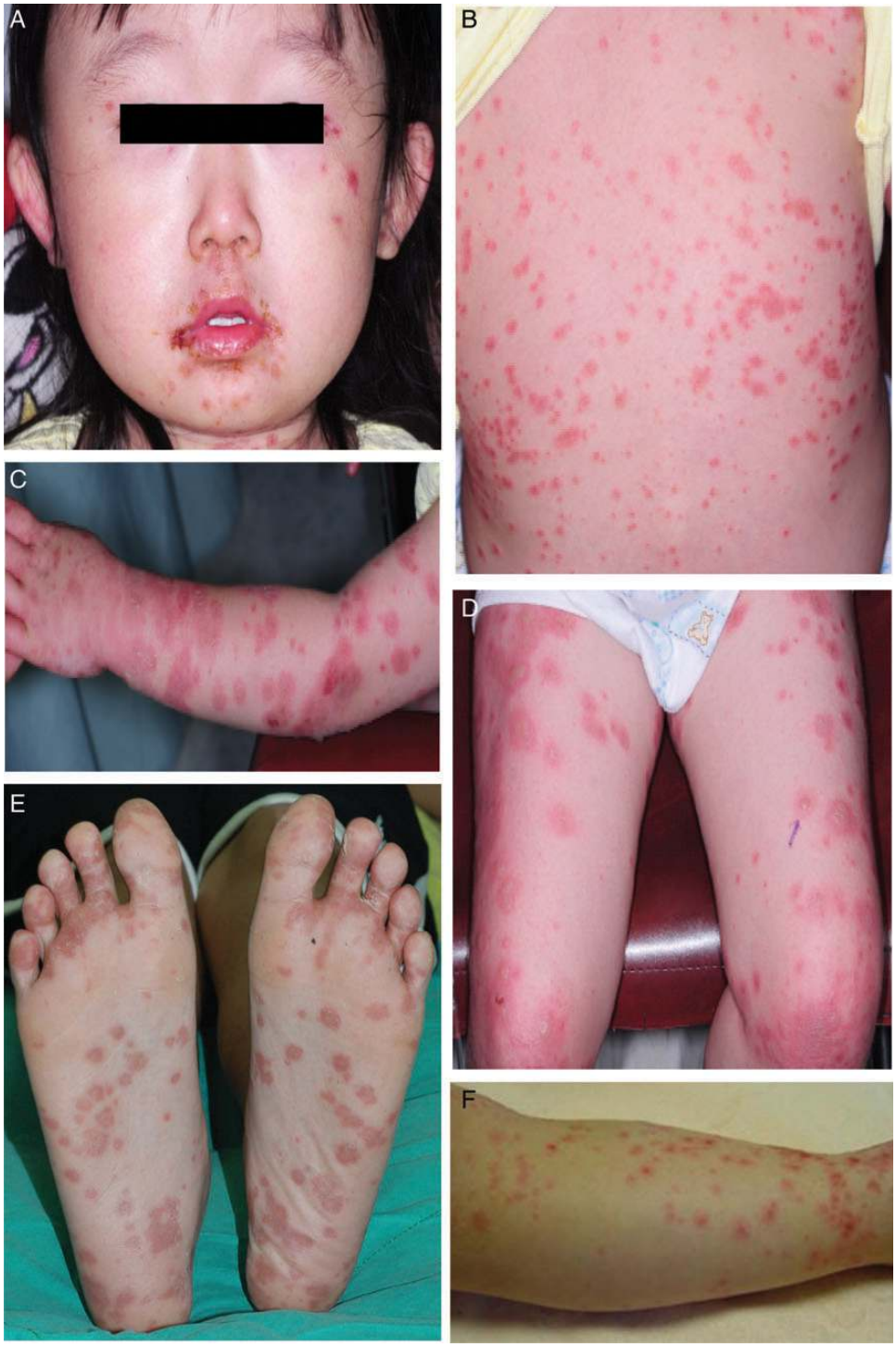


Figure 1. Clinical presentations of patients. *A–D*, Representative pictures showing mild lip erosions, generalized painful SJS-like blisters, and atypical target lesions on 4 limbs and trunk (case 1). *E*, A representative picture showing SJS-like painful blisters on palms and soles (case 6). *F*, A picture showing EM-like target lesions on lower limbs (case 5). Abbreviations: EM, erythema multiforme; SJS, Stevens-Johnson syndrome.

expressing granulysin (Figure 3, Supplementary Figure 3). We found about 45%–78% of the blister cells expressed CD8, CD56, or granulysin protein (Figure 3*J*). Moreover, the levels of granulysin highly increased (573 ± 41 ng/mL, n = 3) in the blister fluid of our patients, when compared to that of burn lesions (26 ± 3 ng/mL, n = 10).

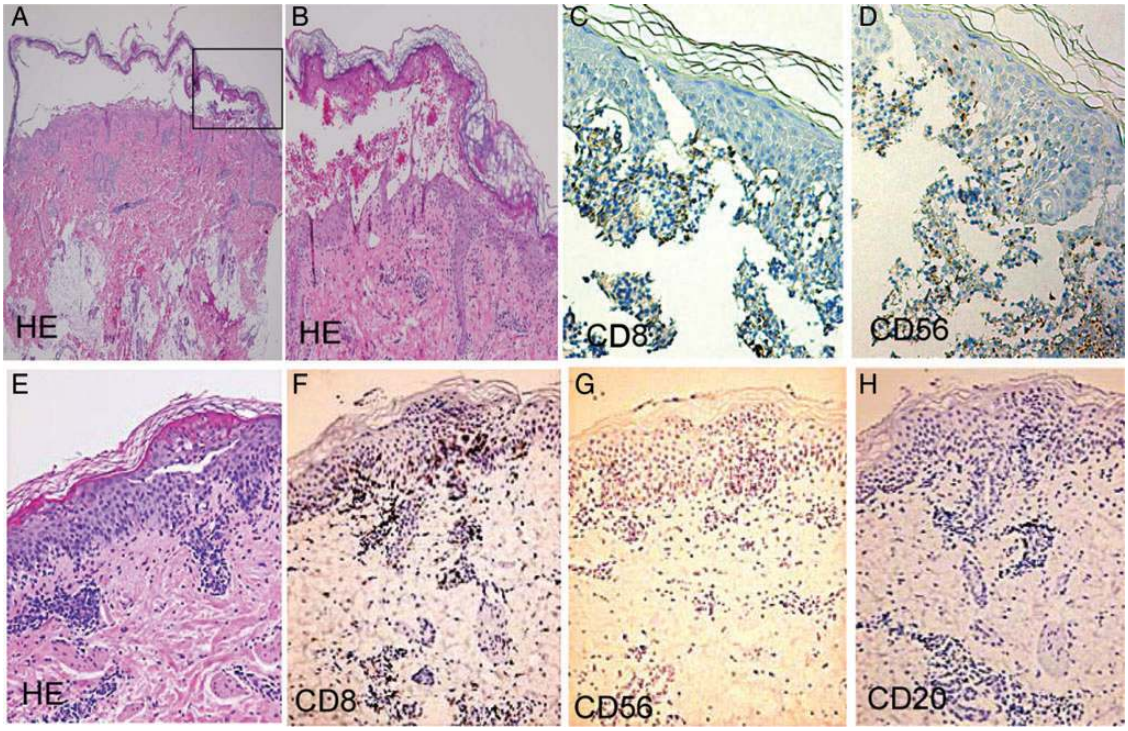


Figure 2. Histophathological characterization of the infiltrated immune cells in the skin lesions of the patients. *A–D, I–P*, The representative images showed separated dermo-epidermal junction and blister formation from the skin biopsies of case 1 (*A–D*) and case 5 (*I–P*). *E–H*, The histopathological images of a nonblistering skin lesion from case 1. Hematoxylin-eosin (HE) staining (*A, I*, magnification: 40×; *B, E, J*: 100×). Immunohistochemical staining showed many infiltrated cells expressed CD8 (a marker for cytotoxic T lymphocytes, *C, F, K*: 100×; *L*: 400×), and CD56 (a marker for NK cells or NKT cells, *D, G, M*: 100×; *N*: 400×), but only a few of cells expressed CD20 (a marker for B cells, *H*: 100×). Granulysin protein was detected in the epidermis (granulysin, *O*, magnification: 100×, *P*: 400×). Abbreviations: NK, natural killer; NKT, natural killer T cells.

Identification of Viral Particles in the Skin Lesions and Blister Fluids

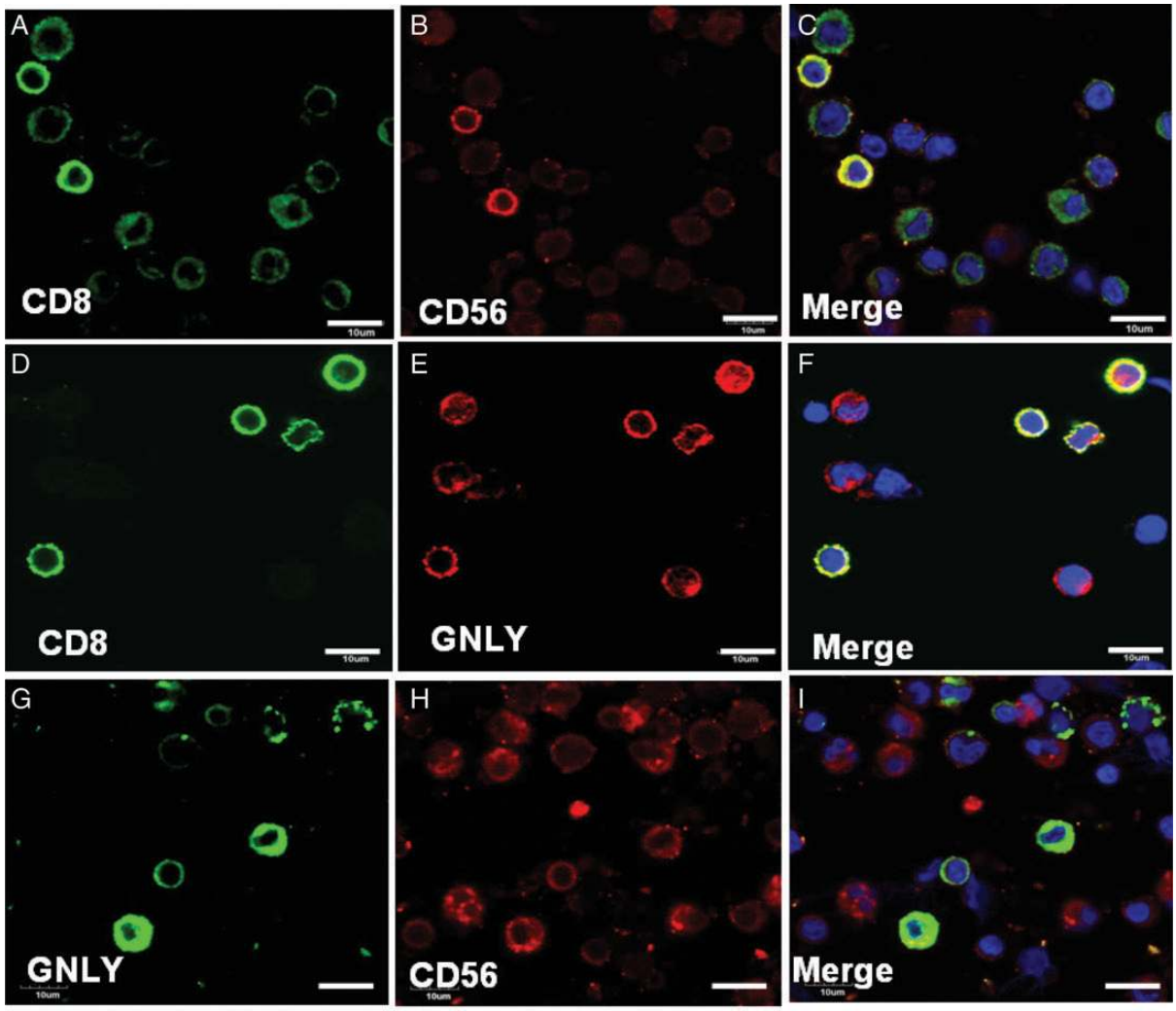
By transmission electron microscopy, we found numerous intact viral particles in both skin lesions and in the blister fluids (Figure 4). The virus particles can be seen around the cells (red blood cells [RBC], macrophages, and Langerhans cells) or in the blister fluids (Figure 4). Our data revealed that the skin lesions and blisters contained intact viral particles.

Viral Genome Sequencing and Phylogenetic Analysis Revealed a New CVA6 Variant

The NCBI BLAST analysis of amplified DNA sequence from blister fluids of skin lesions showed that the etiological agent was highly similar to human coxsackievirus A6 (CVA6) and was confirmed as CVA6 by 5′ untranslated region of viral genome sequencing. We constructed the phylogenetic tree based on 377 nucleotide long partial VP1 sequence (nt 2930–3306) using a neighbor-joining method and the Kimura 2-parameter distance model by *MEGA* version 4 software with 1000 replications of bootstrap analyses [27]. The results of the phylogenetic analysis (Figure 5) showed that the nucleotide sequence of our CVA6 isolates (TW/1537/2011, GenBank accession no. JN582001) shares higher similarity (97.6%–98.1%) to that of CVA6 strains found in Finland at 2008 than that of Taiwan isolates (Taiwan 2004–2007; 92%–93.6%), or the Gdula strain in the United States, 1949 (CA-V6/Gdula AF081297; 85.1%; (Figure 5). The nonstructural region sequence showed <80% homology to the Gdula strain and had 83% of the homology of the Coxsackievirus A16 (nt 3358–5091), 85% to enterovirus 71, Coxsackievirus A2 and A4 (nt 5092–7350), suggesting that there may be an enormous evolutionary change in the CVA6 genome in recent years.

DISCUSSION

SJS is mainly caused by drugs and less common by infectious pathogens, such as *Mycoplasma pneumoniae* and *Herpes simplex* virus [11, 21, 28]. HEV had not been recognized to associate with SJS [19–21]. By comparison, erythema multiforme major (EMM) is mainly caused by viruses, which usually involves palms and soles and the patients' rapid healing without sequelae. In this study, we identified a new variant of CVA6 infection in the young patients with unusual manifestations of widespread blisters arising on erythematous to purpuric



Numbers of analyzed fields (N)	10	11	11	7	7	6
Percentages of positive cells (Mean ± S.E.M.)	57.3 ± 6.3	75.8 ± 3.7	77.9 ± 4.2	52.6 ± 7.5	44.9 ± 3.7	56.2 ± 10.3

Figure 3. Expression of CD8, CD56, and granulysin in the blister cells from the skin lesions of the patients. *A–I*, Immunofluorescence staining of CD8 (*A* and *D*), CD56 (*B* and *H*), granulysin protein (*E* and *G*), and merged images with DAPI staining (*C, F,* and *I*). The images were obtained from the sample of case 1. Bar = 10 μm. *J,* Percentages of cells expressing the cell markers (CD8, CD56) or granulysin protein detected by confocal microscopy fields analysis on the blister cells of case 1 and case 6. Abbreviation: DAPI, diamidino-2-phenylindole.

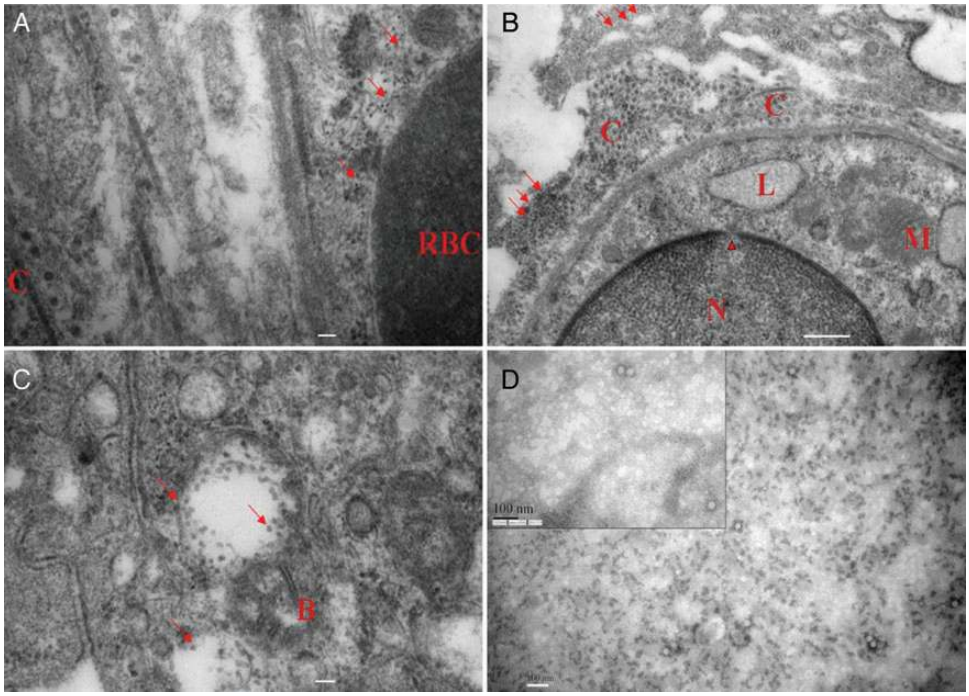


Figure 4. Transmission electron microscopy of the biopsies of skin lesions and the blister fluids. *A–C,* The red arrows indicate the virus particles in the blistering skin lesions obtained from case 1. The virus particles can be seen around a red blood cell (*A*) in a blood vessel, or around a macrophage (*B*) in the dermis. The virus particles aggregate to form a vesicle like structure in a Langerhans cell (*C*) in the epidermis. *D,* The viral particles can be detected in the blister fluid sample of case 1. The red letters in the panels represent the following: B, Birbeck granule in Langerhans' cell; C, collage fiber; L, lysosome; M, mitochondria; RBC, red blood cell. Bars: (*A, C,* and *D*) = 100 nm, (*B*) = 500 nm.

macules with irregular shape and size distributed over limbs and trunks and accompanied by mucous membrane erosions. This is the first report to our knowledge of HEV-induced widespread mucocutaneous blistering reactions. The clinical presentation, histopathology, and causative agent in this study are different from previous studies on CVA6 [10, 29].

Most cases with CVA6 infections in the previous outbreaks were presented as herpangina and only a few as HFMD [10, 30]. CVA6 was reported to be the pathogen associated with HFMD during a nationwide outbreak in Finland in 2008, and a recent epidemic in Boston, Massachusetts, in 2012 [9, 31, 32]. A typical HFMD is small vesicular lesions localized on palms and soles, which is distinct from the clinical presentation of SJS with characteristics of widespread blistering atypical target lesions and mucosal involvement [4, 5, 8, 15]. Herein we reported 6 young patients infected with CVA6 who developed severe skin reactions with blisters formation. Although the 6 patients showed blistering skin lesions as SJS or EMM, most of them had only mild erosions on the external part of their lips. These patients did not have severe bleeding erosions on inner lips or oral cavity mucosa involvement, which are usually seen in SJS or EMM. This can be one of the important clues for differentiating enterovirus-related severe blistering reactions from classic EMM or SJS. We found the young patients had prolonged hospitalization duration when compared with the data of the outbreaks of HFMD caused by CVA6 in Taiwan in 2004–2007 [10].

The evolution of nucleotide sequence of CVA6 may lead to the changes of virus characteristics and clinical features. In this study, our virus phylogenetic analysis identified a new CVA6 variant from the subjects' blistering skin samples; its nucleotide sequence showed high homology with that of Finland (2008) [31, 33] yet less similar to that of Taiwan (2004–2007) [10] or the Gdula strain of the United States (1949) [26,33]. In addition, our electron microscopy studies identified the intact viral particles in skin lesions and blister fluids. There were reports showing HSV DNA in the skin lesions of EMM [34, 35]. Whether the CVA6 viral particles in the blister fluids or skin lesions of the patients possess the infectious ability needs more studies.

We previously reported that granulysin, a cytotoxic protein produced by CTLs or NK/NKT cells, is the key mediator for disseminated keratinocyte deaths resulting in blistering skin lesions or skin detachment for SJS/TEN [14]. In this study, we found numerous intact CVA6 viral particles in the skin blistering tissues with predominant CTLs/NK/NKT cells expressing granulysin, mimicking the histopathological features of severe cutaneous adverse reactions (SCAR). These data indicate that these virus antigens induce a strong immune reaction in the epidermis, leading to the widespread mucocutaneous bullous

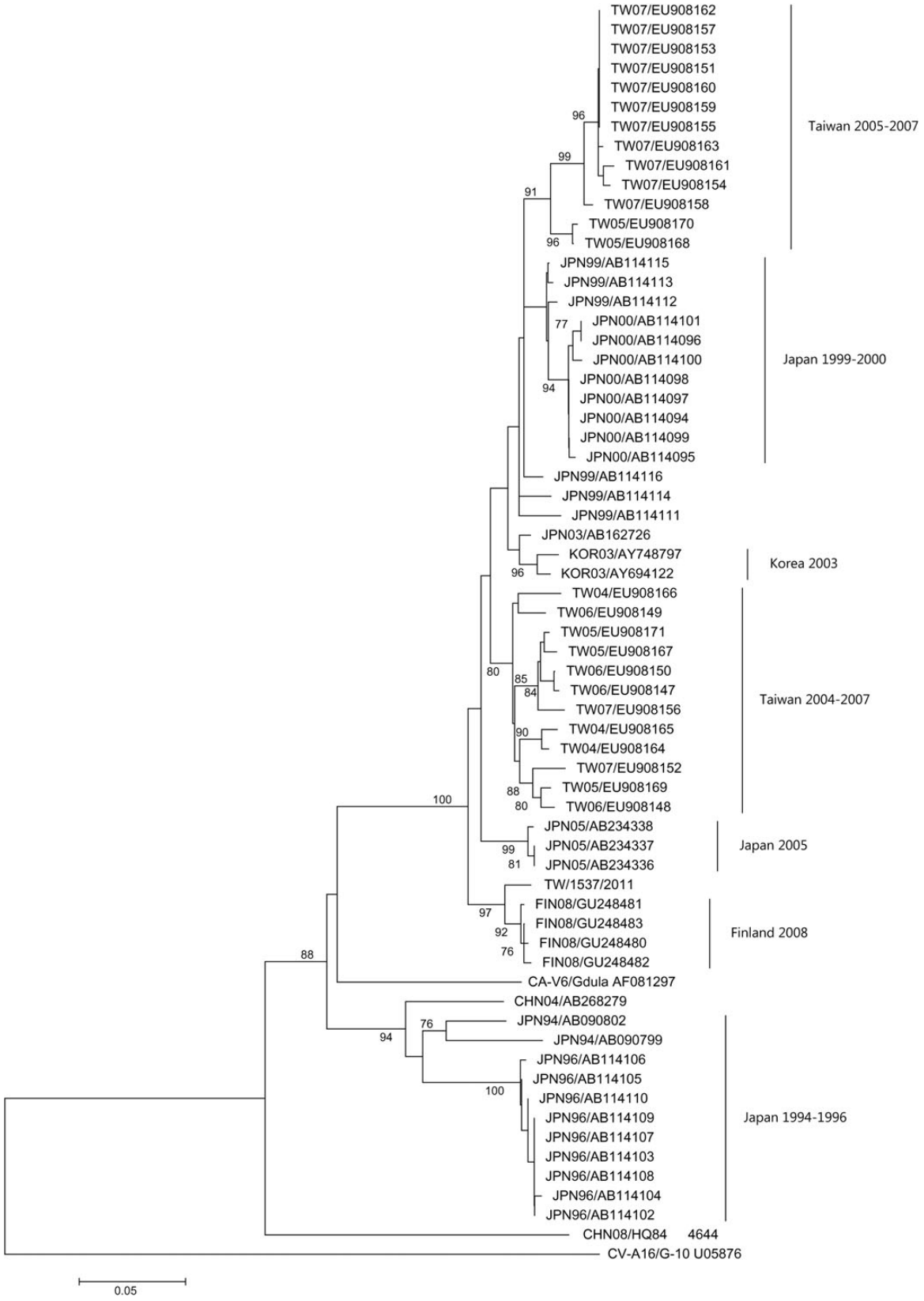


Figure 5. Phylogenetic analysis of coxsackievirus A6 DNA sequence. The 377 nucleotide long partial VP1 sequence (nt 2390–3306) of our CVA6 isolates (TW/1537/2011, GenBank accession no. JN582001) was used for the phylogenetic analysis. Phylogenetic tree was constructed using a neighbor-joining method and the Kimura 2-parameter distance model by MEGA version 4 software with 1000 replications of bootstrap analyses. CV-A16/G10 sequence (GenBank accession no. U05876.1) was used as the out-group.

reactions like SCAR. Similarly, rapid recruitment and activation of $CD8^+$ T cells and amplified inflammatory cascade were also observed after herpes simplex virus type 1 skin infection [36]. In addition, our data suggest the new CVA6 variant might induce a more intense immune reaction than the previous CVA6 isolates associated with HFMD. Further studies are needed to clarify the relationship between viral genome changes and clinical presentations.

In conclusion, we identified a new CVA6 variant, which causes severe mucocutaneous blistering reactions mainly mediated by CTLs/NK/NKT cells expressing granulysin, mimicking the histopathological features of SJS or EMM. An awareness of the risk of this unusual presentation of HEV infections is needed in epidemic areas.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by grants from the National Science Council, Taiwan (98-2320-B-010-002-MY3, 98-2314-B-182A-027-MY3, NSC 101-2320-B-010-072-MY3, NSC 101-2321-B-010 -027, NSC101-2628-B-182-001-MY3; NSC101-2321-B-182-008), and Chang Gung Memorial Hospital.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Tseng FC, Huang HC, Chi CY, et al. Epidemiological survey of enterovirus infections occurring in Taiwan between 2000 and 2005: analysis of sentinel physician surveillance data. J Med Virol **2007**; 79:1850–60.
2. Pallansch MA, Roos RP. Enteroviruses: poliovirus, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe DM, Howley PM, eds. Field Virology. Philadelphia: Lippincott Williams & Wilkins, **2007**: 839–94.
3. Solomon T, Lewthwaite P, Perera D, et al. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis **2010**; 10: 778–90.
4. Richardson HB Jr, Leibovitz A. "Hand, foot, and mouth disease" in children; an epidemic associated with coxsakie virus a-16. J Pediatr **1965**; 67:6–12.
5. Frydenberg A, Starr M. Hand, foot and mouth disease. Aust Fam Physician **2003**; 32:594–5.
6. Chan KP, Goh KT, Chong CY, et al. Epidemic hand, foot and mouth disease caused by human enterovirus 71, Singapore. Emerg Infect Dis **2003**; 9:78–85.
7. Chen KT, Chang HL, Wang ST, et al. Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005. Pediatrics **2007**; 120:e244–52.
8. McMinn P, Lindsay K, Perera D, et al. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. J Virol **2001**; 75:7732–8.
9. Osterback R, Vuorinen T, Linna M, et al. Coxsackievirus A6 and hand, foot, and mouth disease, Finland. Emerg Infect Dis **2009**; 15: 1485–8.
10. Lo SH, Huang YC, Huang CG, et al. Clinical and epidemiologic features of Coxsackievirus A6 infection in children in northern Taiwan between 2004 and 2009. J Microbiol Immunol Infect **2011**; 44:252–7.
11. Mockenhaupt M. Epidemiology and causes of severe cutaneous adverse reactions to drugs. In: Pichler W, ed. Drug Hypersensitivity. 1st ed. Basel: Karger Verlag, **2007**:18–31.
12. Mockenhaupt M, Viboud C, Dunant A, et al. Stevens-Johnson syndrome and toxic epidermal necrolysis: assessment of medication risks with emphasis on recently marketed drugs.: The EuroSCARstudy. J Invest Dermatol **2008**; 128:35–44.
13. Nassif A, Bensussan A, Boumsell L, et al. Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. J Allergy Clin Immunol **2004**; 114:1209–15.
14. Chung WH, Hung SI, Yang JY, et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. Nat Med **2008**; 14:1343–50.
15. Roujeau JC. The spectrum of Stevens-Johnson syndrome and toxic epidermal necrolysis: a clinical classification. J Invest Dermatol **1994**; 102: 28S–30.
16. Roujeau JC, Stern RS. Severe adverse cutaneous reactions to drugs. N Engl J Med **1994**; 331:1272–85.
17. Rzany B, Mockenhaupt M, Baur S, et al. Epidemiology of erythema exsudativum multiforme majus, Stevens-Johnson syndrome, and toxic epidermal necrolysis in Germany (1990–1992): structure and results of a population-based registry. J Clin Epidemiol **1996**; 49: 769–73.
18. Auquier-Dunant A, Mockenhaupt M, et al. Correlations between clinical patterns and causes of erythema multiforme majus, Stevens-Johnson syndrome, and toxic epidermal necrolysis: results of an international prospective study. Arch Dermatol **2002**; 138:1019–24.
19. Fournier S, Bastuji-Garin S, Mentec H, et al. Toxic epidermal necrolysis associated with Mycoplasma pneumoniae infection. Eur J Clin Microbiol Infect Dis **1995**; 14:558–9.
20. Mulvey JM, Padowitz A, Lindley-Jones M, et al. Mycoplasma pneumoniae associated with Stevens Johnson syndrome. Anaesth Intensive Care **2007**; 35:414–7.
21. Harr T, French LE. Toxic epidermal necrolysis and Stevens-Johnson syndrome. Orphanet J Rare Dis **2010**; 5:39.
22. Mockenhaupt M. The current understanding of Stevens-Johnson syndrome and toxic epidermal necrolysis. Expert Rev Clin Immunol **2011**; 7:803–13.
23. Bonzel L, Tenenbaum T, Schroten H, et al. Frequent detection of viral coinfection in children hospitalized with acute respiratory tract infection using a real-time polymerase chain reaction. Pediatr Infect Dis J **2008**; 27:589–94.
24. Ito Y, Takakura S, Ichiyama S, et al. Multicenter evaluation of prototype real-time PCR assays for Epstein-Barr virus and cytomegalovirus DNA in whole blood samples from transplant recipients. Microbiol Immunol **2010**; 54:516–22.
25. Sugita S, Shimizu N, Watanabe K, et al. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. Br J Ophthalmol **2008**; 92:928–32.
26. Mohamed N, Elfaitouri A, Fohlman J, et al. A sensitive and quantitative single-tube real-time reverse transcriptase-PCR for detection of enteroviral RNA. J Clin Virol **2004**; 30:150–6.
27. Tamura K, Dudley J, Nei M, et al. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **2007**; 24:1596–9.
28. Chung WH, Hung SI. Recent advances in the genetics and immunology of Stevens-Johnson syndrome and toxic epidermal necrosis. J Dermatol Sci **2012**; 66:190–6.
29. Yamayoshi S, Iizuka S, Yamashita T, et al. Human SCARB2-dependent infection by coxsackievirus A7, A14, and A16 and enterovirus 71. Virology **2012**; 86:5686–96.

30. Yamashita T, Ito M, Taniguchi A, et al. Prevalence of coxsackievirus A5, A6, and A10 in patients with herpangina in Aichi Prefecture 2005. Jpn J Infect Dis **2005**; 58:390–1.
31. Blomqvist S, Klemola P, Kaijalainen S, et al. Co-circulation of Coxsackie viruses A6 and A10 in hand, foot and mouth disease outbreak in Finland. J Clin Virol **2010**; 48:49–54.
32. Flett K, Youngster I, Huang J, et al. Hand, foot, and mouth disease caused by coxsackievirus a6. Emerg Infect Dis **2012**; 18:1702–4.
33. Oberste MS, Maher K, Kilpatrick DR, et al. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol **1999**; 73:1941–8.
34. Ono F, Sharma BK, Smith CC, et al. CD34+ cells in the peripheral blood transport herpes simplex virus DNA fragments to the skin of patients with erythema multiforme (HAEM). J Invest Dermatol **2005**; 124:1215–24.
35. Kokuba H, Imafuku S, Huang S, et al. Erythema multiforme lesions are associated with expression of a herpes simplex virus (HSV) gene and qualitative alterations in the HSV-specific T-cell response. Br J Dermatol **1998**; 138:952–64.
36. Stock AT, Jones CM, Heath WR, et al. Rapid recruitment and activation of CD8+ T cells after herpes simplex virus type 1 skin infection. Immunol Cell Biol **2011**; 89:143–8.