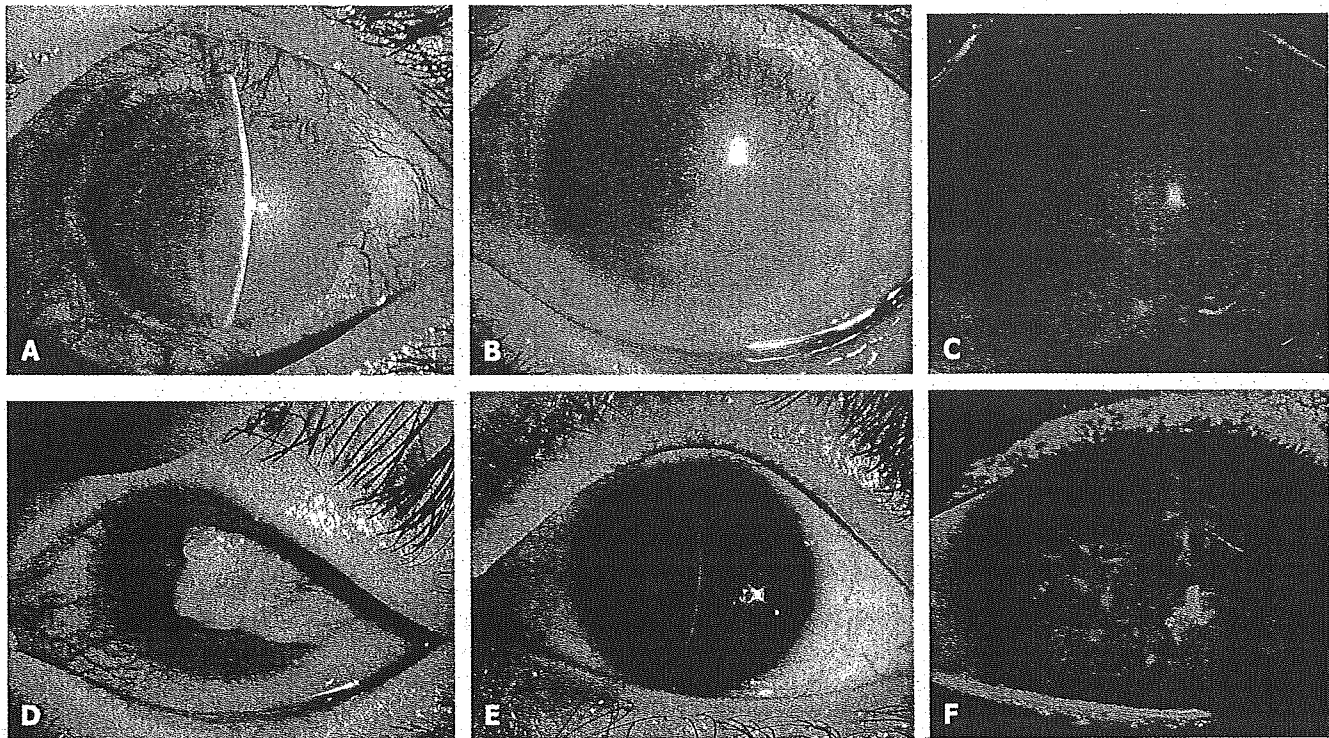

FIGURE 6. Cultivated autologous oral mucosal epithelial transplantation for acute phase chemical injury (A–C) and chronic-phase Stevens-Johnson syndrome (D–F). In acute-phase chemical injury, the corneal surface showed persistent corneal epithelial defect and intensive inflammation in adjacent tissue. In the chronic phase of Stevens-Johnson syndrome, total conjunctivalization with cicatrization was observed. Both cases were reconstructed by autologous cultivated oral mucosal epithelial sheet. It is noteworthy that peripheral neovascularization was observed to some extent in all cases (D, E). Fluorescein staining showed diffuse apical cell stains without any defects (C, F). Adapted from Nakamura et al.[16]

and decreased surgical wounds that retain healthy corneal tissue are ideal directions to reduce biologic responses that may interfere with the effect of treatment and prognosis. Immunologic response to an allogeneic graft is also a crucial aspect for transplantation. Thus, new approaches based on regenerative medicine are the closest strategy that allows surgeons to reconstruct the cornea without using allogeneic tissue components. As one of the leading clinical practices, cultivated mucosal epithelial sheet transplantations have already contributed not only to the improvement of clinical outcome but also to further knowledge for tissue engineering procedures as a whole.

In situations where original-tissue stem cells are lacking, eg, in corneal stem cell–deficiency diseases, alternative cell sources are the only remaining candidates for the generation of autologous grafts. In this regard, the success of cultivated oral mucosal epithelial sheet transplantation has provided much new information. Tissue-engineered oral mucosal epithelial sheet survives ectopically on the ocular surface, and its behavior and phenotype appear to be completely different from that of buccal mucosal graft transplantation. In addition to the biologic findings, visual recovery and alternative function of oral mucosal epithelium on the cornea are promising results. Our recent clinical experience with cultivated autologous oral mucosal epithelial sheet transplantation promises sufficient application of nonocular cells for future regenerative medicine.

Although clinical practice is ongoing, numerous questions regarding cultivated oral mucosal epithelial transplantation remain. An interesting aspect is the period for epithelial supply and biologic characteristics of ectopically surviving oral mucosal epithelium. Our recent study demonstrated that the stable survival of oral mucosal epithelium in the original area is achievable according to fluorescein staining, even in the longest observation period >2.5 years after surgery. No biologic evidence has demonstrated whether the cultivated sheet contains oral mucosal epithelial stem cells. Although the keratin expression pattern of surviving oral mucosal epithelium was similar to that of in vivo original oral mucosal epithelium, epithelial stratification and induction of neovascularization were distinguishable from the original characteristics. Compared with the success of clinical practice, not only biologic characterization but also genetic stability and differential gene expression remain to be investigated. Future studies may also provide new information regarding essential corneal epithelial characteristics including regarding corneal stem cells and avascularity of corneal tissue.

Recent improvements in surgical techniques have contributed much to the safety and quality of visual recovery. However, because the ocular surface itself organizes

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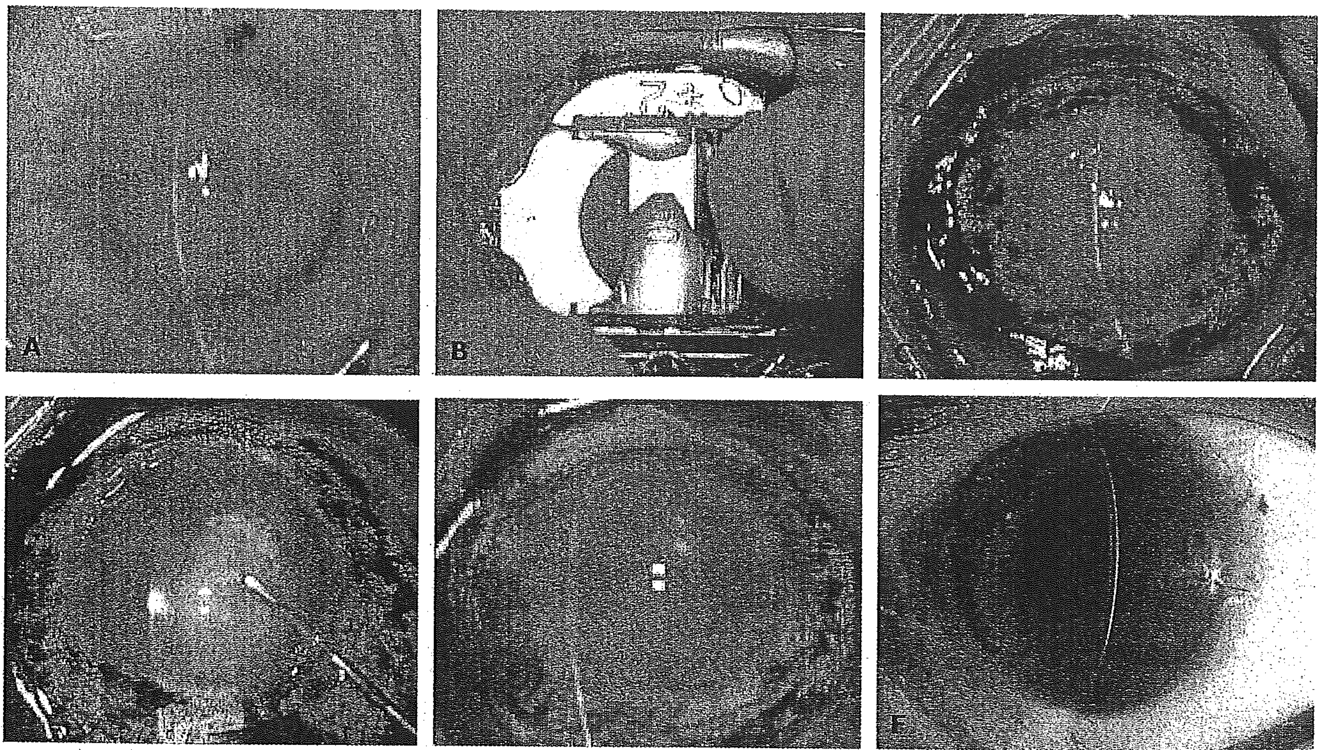

FIGURE 7. Surgical procedure in an eye with aniridia that has undergone ALTK and cultivated corneal epithelial transplantation. Before (A) and 4 months after (F) this surgical procedure. A microkeratome was used to remove scarred tissue (B). After removal of the opacified cornea (C), cataract surgery was safely performed through minimal residual opacity with indocyanine green staining (D). An identically sized corneal graft was sutured and covered with a cultivated oral mucosal epithelial sheet (E).

its complicated special environment including tear secretion, immunologic defense, and epithelial cell turnover, postoperative maintenance of constructed surface is crucial for a good prognosis. To treat severe ocular surface diseases, further studies are required for us better to understand the etiology and biologic characteristics of these entities.

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Dissemination of Methicillin-Resistant Staphylococci among Healthy Japanese Children

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Methicillin-resistant *Staphylococcus aureus* (MRSA), regarded as a tenacious pathogen in the hospital, has recently become increasingly prevalent as a community pathogen. We evaluated the prevalence and characteristics of methicillin-resistant staphylococci in the Japanese community by testing nasal samples of 818 children of five day care centers and two kindergartens in three districts. We found that methicillin-resistant staphylococci are already prevalent among healthy children. Among 818 children, 35 children (4.3%) carried MRSA and 231 children (28.2%) carried methicillin-resistant coagulase-negative staphylococci (MRC-NS). The types of staphylococcal cassette chromosome *mec* (SCC*mec*) found among 44 MRSA isolates were as follows: type IIa, 11 isolates; type IIb, 19 isolates; and type IV, 14 isolates. The type IIb SCC*mec* element was a new SCC*mec* element found in this study. Eleven (25%) strains which belonged to clonal complex 5 (CC5) carried type IIa SCC*mec*, and they produced type 2 coagulase and toxic shock syndrome toxin 1. They were indistinguishable from health care-associated MRSA (H-MRSA) strains in Japan, represented by strain N315. On the other hand, 33 (75%) strains, most of which belonged to CC78 or CC91, carried small SCC*mec* elements, such as type IIb or type IV, and they produced type 1 or type 3 coagulase and exfoliative toxin. The data indicated that MRSA clones distinct from H-MRSA have disseminated in healthy children. The fact that MRC-NS strains were prevalent in the community suggested that they might serve as a reservoir for the SCC*mec* element carried by MRSA strains disseminated in the community.

Since the discovery of the first clinical isolates in 1960, methicillin-resistant *Staphylococcus aureus* (MRSA) has remained a major hospital pathogen throughout the world (19). However, recent reports suggest that it became increasingly prevalent in the community as well since the 1990s (6, 8, 13, 31). Now, the MRSA strains designated community-acquired or community-associated MRSA (C-MRSA) are increasingly found in healthy individuals without conventional risk factors for MRSA colonization (2, 11, 14, 26, 33).

MRSA strains carry methicillin resistance gene *mecA*, encoded by a mobile genetic element designated staphylococcal cassette chromosome *mec* (SCC*mec*) (15, 22). We define the type of SCC*mec* by the combination of the type of *ccr* gene complex, composed of cassette chromosome recombinase genes and the surrounding open reading frames (ORFs), and the class of the *mec* gene complex, composed of the *mecA* gene and its surrounding ORFs. A total of five allelic types have been identified in SCC*mec* elements (16, 17, 21). Three types of SCC*mec* elements (type I, type II, and type III) are carried mostly by health care-associated MRSA (H-MRSA) strains throughout the world (9, 16). On the other hand, novel types of SCC*mec* elements (type IV and type V) have been widely disseminated among C-MRSA strains (7, 17, 25, 29). The type IV and type V SCC*mec* elements are characterized by their small sizes (21 to 28 kbp) and lack of resistance genes, other than *mecA* (17, 25, 29).

MRSA clones are defined by the type of SCC*mec* element and the genotype of the methicillin-susceptible *S. aureus* chromosome in which the SCC*mec* element is integrated (12). We have shown that the C-MRSA strains isolated in Australia and the United States were derived from more diverse *S. aureus* clones than H-MRSA strains by determination of the types of the SCC*mec* elements and the types of their chromosomes by multilocus sequence typing (MLST) (29). C-MRSA strains grew faster than H-MRSA strains and carried virulence genes, such as Panton-Valentine leucocidin (PVL) genes (1, 29, 37).

This study was undertaken to investigate the prevalence of MRSA strains and methicillin-resistant coagulase-negative staphylococci (MRC-NS) among healthy Japanese children. In addition, we describe the characteristic features of MRSA strains distributed in the Japanese community.

MATERIALS AND METHODS

Isolation of methicillin-resistant staphylococci from nasal swabs of healthy children. To establish the prevalence of methicillin-resistant staphylococci in the community, we have isolated staphylococci from nasal swabs of healthy children from five day care centers and two kindergartens in three different districts: Miyagi, Kyoto, and Saga. To understand the colonization of methicillin-resistant staphylococci, we obtained samples from children in Miyagi twice, with an 8-month interval. In the first sampling, in July 2001, 362 children were sampled;

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and in the second sampling, in March 2002, 292 children were sampled. Among the 292 children sampled in the second sampling, 236 children who had been sampled in the first test were resampled. In Kyoto and Saga, we sampled 150 and 250 children, respectively. The children who were absent on the day of investigation and the children who did not receive parental consent were not included in this study. A total of 818 children were tested.

Samples were obtained from both nares of the children by using a sterile dry-cotton swab (Medical Wire & Equipment Co., Ltd., Corsham, United Kingdom) and were inoculated directly onto mannitol-salt agar (Eiken Chemical Co., Ltd., Tokyo, Japan), with or without 10 mg/liter of ceftizoxime (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), and incubated at 37°C for 48 h. Yellow colonies that grew on the agar plates were tested for the production of clumping factor and protein A by using a Staphylo LA test kit (Denka Seiken Co., Ltd., Niigata, Japan) to distinguish *S. aureus* from other species. The species of the strains that showed negative reactions in the Staphylo LA test were determined by using an identification kit (StaphyoGram; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Identification of exotoxin genes and SCC*mec* elements. Chromosomal DNAs were extracted from cells that had been cultured overnight by the phenol-chloroform extraction methods described previously (29). Detection of the *mecA* gene and typing of the SCC*mec* elements were carried out by PCR, as described previously (16, 29). PCR amplification was performed in a 50-μl reaction mixture composed of 2 U of Ex *Taq* (Takara Shuzo Co., Ltd., Kyoto, Japan), 10 pmol of each primer, 0.2 mM deoxynucleoside triphosphate mixture, 10 ng of chromosomal DNA, 1× reaction buffer (Takara Shuzo Co., Ltd.), and H_2O. Thermal cycling was set at 30 cycles (30 s for denaturation at 94°C, 1 min for annealing at 50°C, and 2 min for elongation at 72°C) and was performed with a Gene Amp PCR system 9600 (Perkin-Elmer, Wellesley, Mass.). Localization of five exotoxin genes, the toxic shock syndrome toxin 1 gene (*tst-1*), the exfoliative toxin A gene (*eta*), the exfoliative toxin B gene (*etb*), the exfoliative toxin D gene (*etd*), and the Panton-Valentine leucocidin genes (*lukS* and *lukF*); and all MRSA isolates were examined by PCR with the sets of primers listed in Table 1. The *S. aureus* strains used as references for the five exotoxins were N315 for *tst-1*, ZM for *eta*, N20 for *etb*, TY114 for *etd*, and MW2 for *lukS* and *lukF*. *S. aureus* ZM and N20 were kindly provided by Y. Yoshizawa, Jikei University, Tokyo, Japan. *S. aureus* TY114 was kindly provided by M. Sugai, Department of Microbiology, Hiroshima University, Hiroshima, Japan (39–41).

Coagulase isotyping. The coagulase isotypes of MRSA strains were determined by the coagulation inhibition test for coagulation by using commercially available rabbit antisera specific to each of the eight isotypes of staphylocoagulases (Denka Seiken Co., Ltd., Tokyo, Japan), as described previously (29). Briefly, *S. aureus* cells were cultured overnight in brain heart infusion broth (Becton Dickinson Co., Ltd., Paramus, N.J.), and the supernatant was collected by centrifugation. The 0.1-ml aliquot of the supernatant, which was diluted appropriately with diluent (2.0% polypeptone, 1.0% sodium citrate, 0.1% sodium azide), was distributed into nine tubes, and 0.1 ml of anticoagulase type I to VIII sera was added to tubes 1 to 8. In the ninth tube, 0.1 ml of 5% normal rabbit serum in diluent was added as a control. Then, the tubes were incubated at 37°C for 1 h. After the incubation, 0.2 ml of diluted rabbit plasma was added to each tube, followed by incubation at 37°C for at least 1 h. The coagulation of the plasma was judged by visual inspection after 1, 2, 4, 6, and 24 h. The type of antiserum that inhibited the coagulation alone was regarded as the type of staphylocoagulase produced by the strain.

PFGE. Chromosomal DNAs of the MRSA strains were digested with SmaI and were separated by pulsed-field gel electrophoresis (PFGE) with a Gene Path system (CHEF-DR, PULS WAVE.760; Bio-Rad, Hercules, Calif.). The settings for PFGE were as follows: initial switch time, 5.0 s; final switch time, 40.0 s; included angle, 120°; current, 200 V; and run time, 22 h. The buffer temperature was maintained at 14°C. The correlations of the banding patterns were analyzed with BioNumerics software (version 2.5; Applied Maths, Kortrijk, Belgium). A similarity index was determined for each pair of strains by using the Dice coefficient with 0.5% band tolerance. Clustering correlation coefficients were calculated by using the unweighted pair group method of arithmetic averages. Isolates were considered "potentially genetically related" if their macrorestriction DNA patterns differed by less than seven bands (35).

MLST. The MLSTs of the MRSA strains representing each pulsotype were determined according to the method described previously (10). Sequencing reactions were performed with fluorescent dideoxy chain termination chemistry by using a Big Dye Terminator (version 1.1) cycle sequencing kit (Applied Biosystems). DNA sequencing was performed with an ABI Prism 3100 genetic analyzer (Applied Biosystems). The nucleotide sequences of each of the seven genes were assigned allele numbers by comparing them with those of the extant alleles listed on the MLST website (http://www.mlst.net). The sequence type (ST) was determined according to the pattern of the combination of the seven alleles, and the clonal complex (CC) was defined by the BURST (based upon related sequence types) program by accessing the MLST website.

Antimicrobial susceptibility testing. The minimal growth-inhibitory concentrations of isolates against nine antibiotics (MICs) were determined by the agar dilution method recommended by CLSI (formerly the National Committee for Clinical Laboratory Standards) (28). The antibiotics tested were as follows: oxacillin and gentamicin, Sigma Chemical Co., Ltd., St. Louis, Mo.; ceftizoxime, Fujisawa Pharmaceutical Co., Ltd.; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; erythromycin and tetracycline, Wako Pure Chemical Industries, Ltd.; tobramycin, Shionogi Co., Ltd., Osaka, Japan; kanamycin, Meiji Seika Kaisha, Ltd., Tokyo, Japan; and norfloxacin, Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan.

Nucleotide sequence accession number. The nucleotide sequence of the region containing the junkyard 1 (J1) region and the *ccr* gene complex of SCC*mec* type IIb of strain JCSC3063 has been deposited in the DDBJ/EMBL/GenBank database under accession no. AB127982.

RESULTS

Carriage of MRSA and MRC-NS in healthy children. We took nasal smear samples from healthy children in five day care centers and two kindergartens in three prefectures of Japan, Miyagi, Kyoto, and Saga, from north to south, respectively. To elucidate the stability of MRSA carriage by individuals, the Miyagi study was done twice with overlapping subjects: the first study was done in July 2001 and the second one was done in March 2002. The number of children who participated was 818 in total: 418 from the first and second studies in Miyagi, 150 from Kyoto, and 250 from Saga (Table 2). Table 2 summarizes the results of the surveillance for MRSA and MRC-NS among the children tested. Of the 818 children, 231 (28.2%) children carried *S. aureus*, and 35 (4.3%) children carried MRSA. The ratio of MRSA strains among the *S. aureus* isolates was 15.1%. The percentages of MRSA carriers among the children in the four studies were 5.2% (19 of 362) in the first Miyagi study, 3.7% (11 of 292) in the second Miyagi study, 4.0% (6 of 150) in Kyoto, and 3.2% (8 of 250) in Saga. Interestingly, 231 (28.2%) children carried MRC-NS. The rates of carriage of MRC-NS in each study were 39.7% (144 of 362) in the first Miyagi study, 24.0% (70 of 292) in the second Miyagi study, 19.3% (29 of 150) in Kyoto, and 15.2% (38 of 250) in Saga.

A new SCC*mec* subtype (type IIb) identified in MRSA strains from healthy children. To determine the clonal compositions of the MRSA strains carried by healthy children, we analyzed 44 MRSA strains isolated in this study. Among the 44 MRSA strains, 30 strains carried the type II SCC*mec* element and 14 strains carried the type IV SCC*mec* element. Among the 30 type II SCC*mec* strains, 11 strains carried the *kdp* operon and were judged to carry the same type IIa SCC*mec* element as strain N315. Since the subtype of the type II SCC*mec* elements of the other 19 strains seemed to be new, we determined the nucleotide sequence from the J1 region to the *mec* gene complex of a type II SCC*mec* element from strain JCSC3063 (SD036-1). Figure 1 illustrates the comparison of structures between a new subtype of SCC*mec* designated type IIb SCC*mec* and the type IIa SCC*mec* element that we reported on previously (18, 23). The J1 region of the type IIb SCC*mec* element in JCSC3063 (SD036-1) was shorter than that in the type IIa SCC*mec* element and contained two open reading frames which had no similarity to those found in the latter element. Since the element did not carry plasmid pUB110, which encodes bleomycin and tobramycin resistance,

TABLE 1. Primers used for SCC*mec* typing and toxin gene detection

Primer group and gene(s) or gene allele detected	Primer name	Nucleotide sequence (5'→3')	Reference or source
mecA	mA1	TGCTATCCACCCTCAAACAGG	29
	mA2	AACGTTGTAACCACCCCAAGA	29
ccr gene complex type			
ccrB	βc	ATTGCCTTGATAATAGCCITCT[a]	16
ccrA1	α1	AACCTATATCATCAATCAGTACGT	16
ccrA2	α2	TAAAGGCATCAATGCACAAACACT	16
ccrA3	α3	AGCTCAAAAGCAAGCAATAGAAT	16
ccrC	γ1	AGCCCAATTTTGATGGTTATTGA	This study
	γ2	TGGAGAAGTACTCGTTACAATGT	This study
mec gene complex class			
mecI-mecRI (class A)	mI4	CAAGTGAATTGAAACCGCCT	29
	mcR3	GTCTCCACGTTAATTCCATT	21
IS*1272-mecA* (class B)	IS5	AACGCCACTCATAACATATGGAA	29
	mA6	TATACCAAACCCGACAAC	21
IS*431mecL-mecA* (class C)	mA2	AACGTTGTAACCACCCCAAGA	21
	IS2	TGAGGTTATTCAGATATTTCGATGT	21
Primers for subtyping			
IIa	2a1	ATGTCAGAGCTTTCTAACTTAGTCA	This study
	2a2	TGAAAATGAAAGCCGTGCCG	This study
IIb	2b1	AGCAATTTTTTCTCCTTCTGCTA	This study
	2b2	TTATTAGATCAAGAGCCAAGTG	This study
IVa	4a1	TTTGAATGCCCTCCATGAATAAAAT	29
	4a2	AGAAAAGATAGAAGTTCGAAAGA	29
IVb	4b1	AGTACATTTTATCTTTGCGTA	29
	4b2	AGTCATCTTCAATATCGAGAAAGTA	29
IVc	4c1	TCTATTCAATCGTTCTCGTATTT	This study
	4c2	TCGTTGTCATTTAATTCTGAACT	This study
IVd	4d1	TTTGAGAGTCCGTCATTATTTCTT	This study
	4d2	AGAATGTGGTTATAAGATAGCTA	This study
Exotoxic genes			
tst-1	TSST-1A	TGATATGTGGATCCGTCAT	This study
	TSST-1B	AAACACAGATGGCAGCAT	This study
eta	ET-1	CTATTTACTGTAGGAGCTAG	39
	ET-2	ATTTATTTGATGCTCTCTAT	39
etb	ET-3	ATACACACATTACGGATAAT	40
	ET-4	CAAAGTGTCTCCAAAAGTAT	40
etd	ET-14	AACTATCATGTATCAAGG	41
	ET-15	CAGAATTTCCCGACTCAG	41
lukS and *lukF*	PVLup	AAGACTATTAGCTGCAACATTGTC	29
	PVLdn	AATCTATCTGTTTAGCTCATAGGA	29

[a] I, inosine.

its size was estimated to be approximately 29 kb, which was confirmed by the long-range PCR analysis of the entire element (Fig. 1). The remaining 19 strains were judged to carry the type IIb SCC*mec* element by PCR with a set of primers constructed to be specific for J1-region DNAs of type IIb, as shown in Fig. 1.

Among the 14 strains that carried the type IV SCC*mec* element, 5 of them carried the type IVa SCC*mec* element and the remaining 9 strains carried an unknown subtype of the type IV SCC*mec* element so far tested with sets of primers used to identify four subtypes. There was a distinct geographical distribution of the SCC*mec* types of the MRSA strains carried by healthy children. In the Kyoto and Saga surveys, 12 of 14 MRSA isolates carried the type IV SCC*mec* element, whereas in the Miyagi study 28 of 30 MRSA strains carried the type II SCC*mec* element (Table 3).

TABLE 2. Carriage of methicillin-resistant staphylococci by healthy Japanese children

District or group	No. (%) of children			
	Total tested	With *S. aureus*	With MRSA	With MRC-NS
Miyagi, first study	362	133 (36.7)	19 (5.2)	144 (39.7)
Miyagi,[a] second study	56	12 (21.4)	2 (3.6)	20 (35.7)
Children sampled a second time in Miyagi	236	56 (23.7)	9 (3.8)	50 (21.1)
Kyoto	150	28 (18.7)	6 (4.0)	29 (19.3)
Saga	250	58 (23.2)	8 (3.2)	38 (15.2)
Total[b]	818	231 (28.2)	35 (4.3)	231 (28.2)

[a] In the second test, a total of 292 children were tested. Among them, 56 children were sampled only in the second test, whereas 236 children were sampled twice, in both the first and the second tests. The numbers of children carrying *S. aureus*, MRSA, or MRC-NS among the 236 resampled children are also shown.

[b] To eliminate overlapping of the data, we excluded the children from the second sampling when calculating the total number of tested children. Therefore, the total numbers of children are the cumulative sums of the numbers of children participating in the first and second tests in Miyagi and the tests in Kyoto and Saga.

MRSA clones distinct from H-MRSA were carried by healthy children. We determined the pulsotypes of all MRSA isolates. A total of 20 pulsotypes were identified among the 44 strains, and they were classified into eight major groups, groups A to H, by the mutual correlations of their banding patterns (Fig. 2). All of the 19 MRSA strains that were isolated in Miyagi and that carried the type IIb SCC*mec* element belonged to pulsotype A. Strains that carried the type IIa SCC*mec* element belonged to pulsotypes E, F, G, and H, whereas type IV SCC*mec* strains belonged to four pulsotypes, B, C, D, and G, indicating that their genetic backgrounds are very divergent. Furthermore, the multilocus STs of 20 MRSA strains representing each pulsotype were determined. Six STs (ST5, ST8, ST78, ST89, ST90, and ST91) were identified, and they were classified into four clonal complexes (CC5, CC8, CC78, and CC91).

Table 4 shows the characteristic features of the 44 MRSA strains and the susceptibilities of the strains to various antibiotics.

All 11 strains that carried the type IIa SCC*mec* element produced type 2 coagulase and carried the toxic shock syndrome toxin 1 (*tst-1*) gene. They belonged to CC5, although their pulsotypes were not identical and they showed a multidrug resistance phenotype. The characteristic features of the strains were indistinguishable from those of the H-MRSA strains in Japan represented by strain N315 (23). On the other hand, 19 strains carrying the type IIb SCC*mec* element and 14 strains carrying the type IV SCC*mec* element differed greatly

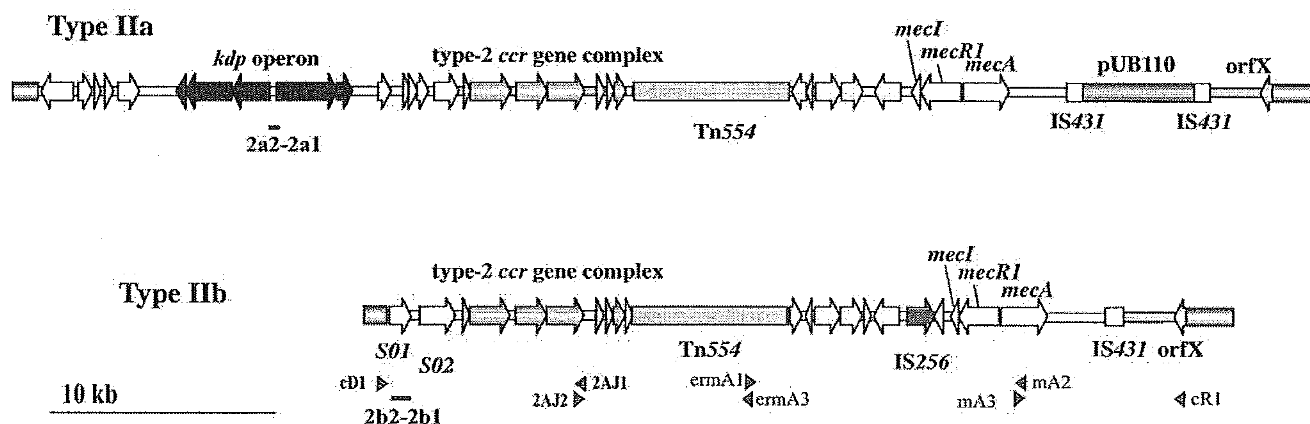

FIG. 1. Structural comparison of type IIa and type IIb SCC*mec* elements. The structures of the type IIa and type IIb SCC*mec* elements are illustrated based on the nucleotide sequences deposited in the DDBJ/EMBL/GenBank databases under accession nos. D86934 and AB127982, respectively. The entire SCC*mec* regions of the type IIb SCC*mec* elements were amplified by PCR with four sets of primers, indicated with arrowheads. The four sets of primers are as follows: cR1 and mA3, mA2 and ermA1 (5′-TGAAACAATTTGTAACTATTGA-3′), ermA3 (5′-TGGGTAAACCGTGAATATCGTGT-3′) and 2AJ1 (5′-ATTAGCCGATTTGGTAATTGAA-3′), and 2AJ2 (5′-TCGTACTTTGACGTAA ATAGCCT-3′) and cD1 (5′-TAGTAAAGACTGTGAAATCTCATAT-3′). The nucleotide sequences of primers cR1, mA2, and mA3 were reported previously (25, 29). Type II SCC*mec* is defined as an SCC*mec* element which typically possesses a class A *mec* gene complex combined with a type 2 *ccr* gene complex. The element was further subtyped according to the differences in the nucleotide sequences of the junkyard 1 regions. The novel subtype of type II SCC*mec* element, type IIb, carried Tn*554*, which encodes macrolide-lincomycin-spectinomycin resistance, but did not carry pUB110, which encodes aminoglycoside resistance. Although IS*256* was inserted upstream of the *mecI* gene, we are not sure whether or not it is happenstance that it was inserted in the element. The locations of the two sets of primers used for the identification of the type IIa SCC*mec*-specific J1 region and the type IIb SCC*mec*-specific J1 region are shown in bars. Two primers, primers 2a1 and 2a2, were constructed on the basis of the sequence of the *kdp* operon; and two primers, primers 2b1and 2b2, were constructed on the basis of the sequence of open reading frame S02.

TABLE 3. Types of SCC*mec* elements carried by 44 MRSA strains isolated from 42 healthy children[a]

District or group	No. of MRSA strains tested	No. of strains with the following type of SCC*mec* element:			
		IIa	IIb	IVa	IVn
Miyagi, first	19	7	11	0	1
Miyagi,[a] second	2	1	1	0	0
Children sampled a second time in Miyagi	9	1	7	1	0
Kyoto	6	1	0	2	3
Saga	8	1	0	2	5
Total	44	11	19	5	9

[a] Among the 236 children sampled twice, 2 children continuously carried MRSA strains, and in the second test 7 children carried newly acquired MRSA strains. Therefore, 44 MRSA strains were isolated from 42 children.

from the H-MRSA strains. The strains carrying the type IIb SCC*mec* element produced type 1 coagulase, and 12 of 19 strains carried exfoliative toxin B genes. All of them belonged to pulsotype A, and all six strains whose MLSTs were tested belonged to CC91. The strains carrying the type IV SCC*mec* element produced type 1 coagulase, and three of the five strains carried exfoliative toxin B genes. Although all three strains tested belonged to the same clonal complex (CC91) as the strains carrying the type IIb SCC*mec* element, their pulsotypes were different. They belonged to pulsotype B. On the other hand, the strains carrying the type IV SCC*mec* element of unknown subtype produced type 2 or type 3 coagulase. Two of them carried the *tst-1* gene, and the other three carried the exfoliative toxin A gene. Their chromosomal genetic backgrounds were judged to be divergent; at the least they covered three pulsotypes, pulsotypes C, D, and G, and three clonal complexes, CC5, CC8, and CC78.

The oxacillin MICs of the 33 strains carrying either the type IIb or the type IV SCC*mec* element were rather low compared with those of type IIa strains. Furthermore, they were susceptible to imipenem, tetracycline, and norfloxacin. Those features of the MRSA strains isolated from healthy children differed greatly from those of the highly methicillin-resistant and multiple-drug-resistant MRSA strains disseminated in Japanese hospitals.

Mode of dissemination and colonization of MRSA strains in children. We noticed that some children attending the same institutions or different institutions carried the same MRSA clone (Fig. 2). In Miyagi, SCC*mec* type IIb and pulsotype A1 strains were isolated from seven children in three different facilities, SCC*mec* type IIb and pulsotype A2 strains were isolated from seven children in three different facilities, and SCC*mec* type IIb and pulsotype A3 strains were isolated from two children who attended different classes (Fig. 2). Furthermore, SCC*mec* type IIa and pulsotype H3 strains were isolated from six children. Interestingly, four of the six children belonged to the same class in the same facility. In Saga, SCC*mec* type IVn and pulsotype G strains were isolated from three children in the same class; and also in Kyoto, SCC*mec* type IVn and pulsotype C2 strains and SCC*mec* type IVn and pulsotype B1 strains were isolated from three and two children in the same facility, respectively. In these cases, the data clearly showed that the dissemination of MRSA strains occurred in children attending a kindergarten or a day care center.

In the two studies conducted in Miyagi, we successfully col-

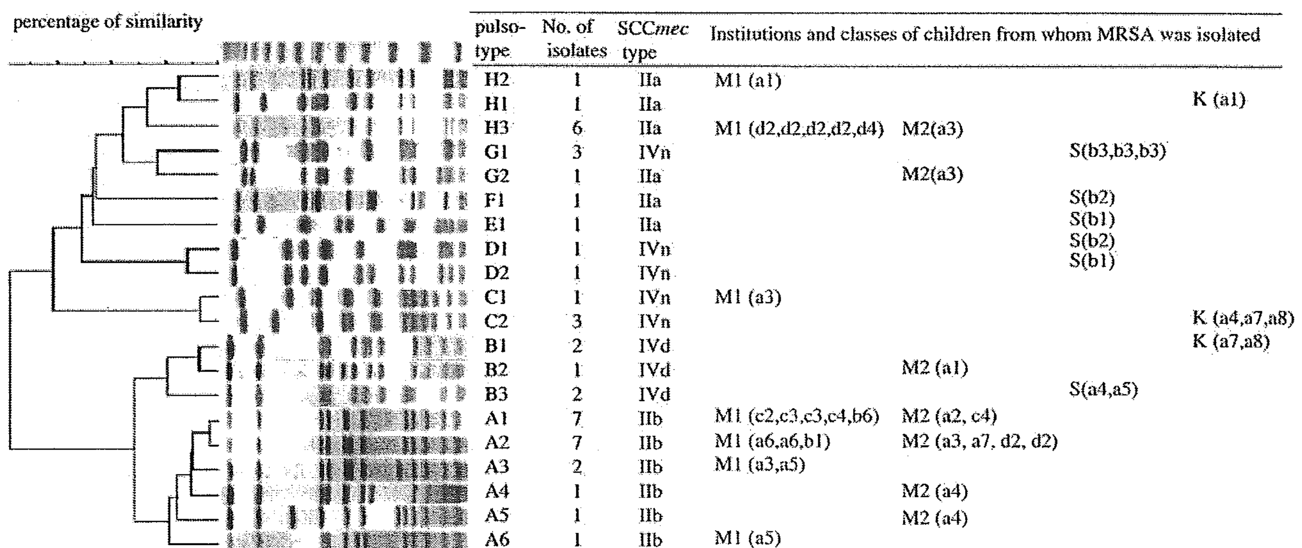

FIG. 2. Dendrogram of PFGE banding patterns of 44 MRSA isolates. A total of 44 MRSA strains were classified into eight pulsotypes. Pulsotypes of a representative strain of each type were compared by using the BioNumerics software program. The institutions and classes of children from whom MRSA strains were isolated are also indicated. The institution (kindergarten or day care center) to which a child belonged is indicated by the following abbreviations: M1, a child in Miyagi tested the first time; M2, a child in Miyagi tested the second time; S, a child in Saga; K, a child in Kyoto. Seven institutions have been subdivided according to the classes that they have. Their classes are indicated in parentheses by abbreviations. The names of the institutions are shown with letters, and the classes are indicated with numerals. Four institutions in Miyagi had multiple classes. They are indicated as a (a1 to a7), b (b1 to b7), c (c1 to c6), and d (d1 to d4). Two institutions in Saga and an institution in Kyoto are also indicated as a (a1 to a7) and b (b1 to b6) and a (a1 to a8), respectively.

TABLE 4. Characterization of MRSA strains isolated from healthy children

Pulsotype	No. of isolates	District[a]	coa[b]	SCCmec type	Presence of exotoxin gene(s)[c]			Strain chosen	MLST			MIC (mg/liter)[d]								
					et allele	tst-1	lukS and lukF		ST	Allelic profile	CC	CZX	OXA	IMP	TET	NOR	ERY	GEN	TOB	KAN
A1	7	M	1	2A2(IIb)	b	–	–	SD205-1	89	1-26-28-18-18-33-50	91	>128	4	0.13	0.5	2	>128	>128	>128	>128
A2	7	M	1	2A2(IIb)	b	–	–	SD036-1	89	1-26-28-18-18-33-50	91	>128	32	0.5	0.5	1	>128	64	32	>128
A3	2	M	1	2A2(IIb)	b	–	–	SD084-1	89	1-26-28-18-18-33-50	91	64	1	<0.06	0.5	2	>128	>128	32	>128
A4	1	M	1	2A2(IIb)	b	–	–	SD2:063-3	89	1-26-28-18-18-33-50	91	>128	64	1	0.5	4	>128	>128	>128	>128
A5	1	M	1	2A2(IIb)	b	–	–	SD2:050-2	89	1-26-28-18-18-33-50	91	>128	8	0.25	0.5	4	>128	>128	32	>128
A6	1	M	1	2A2(IIb)	–	–	–	SD007-1	89	1-26-28-18-18-33-50	91	>128	64	0.5	0.5	2	>128	32	>128	>128
B3	2	S	1	2B1(IVa)	–	–	–	SG25	89	1-26-28-18-18-33-50	91	64	4	0.25	0.2	2	0.5	64	64	>128
B2	1	M	1	2B1(IVa)	b	–	–	SD2:176-3	91	1-26-28-18-18-54-50	91	>128	16	0.13	0.5	4	0.5	64	>128	>128
B1	2	K	1	2B1(IVa)	b	–	–	W12	91	1-26-28-18-18-54-50	91	>128	16	0.13	0.25	1	>128	32	128	>128
E1	1	S	2	2A1(IIa)	–	+	–	SG144-1	5	1-4-1-4-12-1-10	5	>128	>128	64	64	>128	>128	64	128	>128
F1	1	M	2	2A1(IIa)	–	+	–	SD183-1	5	1-4-1-4-12-1-10	5	>128	>128	32	64	64	>128	32	>128	>128
G2	1	M	2	2A1(IIa)	–	+	–	SD2:083-2	5	1-4-1-4-12-1-10	5	>128	>128	32	0.5	>128	>128	2	>128	>128
H1	1	K	2	2A1(IIa)	–	+	–	W143	5	1-4-1-4-12-1-10	5	>128	32	0.5	0.25	1	>128	32	32	>128
H3	6	M	2	2A1(IIa)	–	+	–	SD370-1	5	1-4-1-4-12-1-10	5	>128	>128	32	8	64	>128	32	>128	>128
H2	1	M	2	2A1(IIa)	–	+	–	SD172-1	90	1-4-1-4-42-1-10	5	>128	>128	32	64	32	>128	0.5	>128	>128
G1	3	S	2	2Bn(IVnt)	–	–	–	SG167-2	5	1-4-1-4-12-1-10	5	128	2	0.13	0.5	2	128	0.5	0.25	4
C1	1	M	3	2Bn(IVnt)	–	–	–	SD179-1	88	22-1-14-23-12-4-31	78	>128	64	0.5	0.5	2	>128	32	16	>128
C2	3	K	3	2Bn(IVnt)	a	–	–	W17	88	22-1-14-23-12-4-31	78	>128	4	0.25	0.25	0.5	>128	32	8	>128
D1	1	S	3	2Bn(IVnt)	–	+	–	SG208	8	3-3-1-1-4-4-3	8	>128	8	0.25	0.5	1	0.25	0.25	128	128
D2	1	S	3	2Bn(IVnt)	–	+	–	SG143-1	8	3-3-1-1-4-4-3	8	>128	32	0.5	1	0.5	0.5	0.5	>128	>128

[a] The districts where the strains were isolated are designated as follows; M, Miyagi Prefecture; K, Kyoto Prefecture; S, Saga Prefecture.
[b] coa, coagulase type.
[c] The strains were tested for the carriage of exotoxin genes, *eta*, *etb*, *etd*, *tst-1*, and *lukS* and *lukF*.
[d] CZX, ceftizoxime; OXA, oxacillin; IMP, imipenem; TET, tetracycline; NOR, norfloxacin; ERY, erythromycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin. Boldface indicates resistance.
[e] nt, nontypeable.

lected consecutive nasal swab samples from 236 children. Among them were 13 children who were MRSA positive in the first survey. Of these 13 children, only 2 were positive for MRSA in the second test, and 7 MRSA-positive children were newly identified in the second survey. When we compared the pulsotypes of four MRSA strains isolated from the two children in the first and the second studies, only two MRSA strains isolated from a child (SD084-1 in the first study and SD084-15 in the second study) belonged to the same pulsotype (pulsotype A), with one band difference. It was also noted that the MRSA strain isolated from one of the two consistent carriers changed to a coagulase isotype 1-producing MRSA isolate with a type IIb SCC*mec* element from a coagulase isotype 2-producing MRSA isolate with a type IIa SCC*mec* element.

Our data suggested that the carriage of MRSA was a temporary phenomenon in most of the cases, with only a small number of persistent carrier cases.

Questionnaire survey. Prior to the collection of nasal swab samples, the parents were asked whether their children had taken antibiotics on the test day or in the past 1 month (in Kyoto and Saga) or in the past 3 months (in Miyagi) and whether their children had seen a clinician(s). The rates of response to the questionnaires were 91.4% in Miyagi (first), 93.5% in Miyagi (second), 100% in Kyoto, and 100% in Saga. A total of 19.3% of children had seen a clinician, 9.6% of the children had taken antibiotics on the test day, 34.3% of the children (in Kyoto and Saga) had taken antibiotics in the past 1 month, and 56.1% of the children (in Miyagi) had taken antibiotics in the past 3 months. However, no significant differences in the answers to the questionnaires were observed between the carriers and the noncarriers of MRSA.

DISCUSSION

Dissemination of methicillin-resistant staphylococci (MRS) in the community. Recent reports have indicated an increasing incidence of community-acquired MRSA infection, including increasing numbers of isolates from patients without the usual risk factors associated with MRSA infection or colonization (2, 8, 14, 26, 27, 31, 32).

In this study, we investigated the prevalence of MRS among healthy children. The prevalence of MRSA nasal carriage in children was 4.3%. This ratio was higher than those (0.2 to 2.2%) reported in previous surveys of healthy children and outpatients (14, 33, 36). Furthermore, we identified MRC-NS from 28.2% of the children in seven institutions across three districts. Although the prevalence of methicillin-resistant staphylococci among healthy children was high, no apparent correlation was found between the carriage of MRS and antibiotic use or medical examination at the individual level. However, the questionnaire survey clearly showed that the population itself was a high-risk one, in that more than 34.3% and 56.1% of the children had taken antibiotics in the last 1 month or the last 3 months before the tests, respectively. The medication seemed to have been prescribed by physicians on the occasion of the children's visits for common colds. In addition, determination of the genotypes of the MRSA strains showed that endemic strains were disseminating among children, presumably through close physical contact among the children in the same institution.

Forty-three percent of the MRSA strains carried the type IIb SCC*mec* element, which is rarely found among the strains in Japanese hospitals; and 32% of the MRSA strains carried the type IV SCC*mec* element, which is mostly found in community-acquired MRSA isolates. These data suggest that children are acquiring and disseminating MRSA clones which are not associated with hospital MRSA strains, although some minor populations do temporarily carry a typical H-MRSA strain (type IIa SCC*mec* and CC5). H-MRSA were seen in the first Miyagi study, and most of them disappeared in the second Miyagi study (Table 3). Colonization with MRSA did not continue for a long period; instead, it seemed to circulate among children. This is because 11 of 13 previously MRSA-positive children did not carry MRSA in the second test; instead, 7 previously MRSA-negative children newly acquired the same MRSA strains as those found among MRSA-positive children in the first test. Outbreaks of C-MRSA have been reported among some members of sport teams and in jails, where sweating and a greater chance of direct physical contact are expected (3–5, 24, 30, 38). Day care centers are the same in this regard. Children play in close physical contact, especially in the summertime, with tens of children sharing a small pool in the garden. The first Miyagi study was done during the hot season, which might have been the reason why the MRSA carriage rate was more pronounced than in the other investigations done in the colder seasons. There is a report that Japanese physicians prescribe antibiotics to children under 6 years old for as many as 90% of the cases of common colds (20). The high frequency of antibiotic use and the high chance of physical contact may be reasons why MRSA and MRC-NS prevail in Japanese day care centers and kindergartens.

Characteristic features of MRSA and MRC-NS isolates. Until now, five types of SCC*mec* elements have been reported. Four subtypes of the type IV SCC*mec* element are known. SCC*mec* typing has become an important tool in MRSA epidemiology. We have reported previously that well-defined C-MRSA and nonmultiresistant oxacillin-resistant *Staphylococcus aureus* strains carry the type IV or the type V SCC*mec* element, whereas H-MRSA strains carry the type I, II, and III SCC*mec* elements. Enright et al. (9) have reported that the H-MRSA strains in the world belong to five major clones. In this study, we found that a type IIb SCC*mec* element of a new subtype was carried by 63.3% of MRSA strains in Miyagi, and 83.3% and 87.5% of MRSA strains isolated in Saga and in Kyoto carried the type IV SCC*mec* element. Both elements were rather short and did not carry many antibiotic resistance-conferring genes on the element, although Tn*554*-encoding macrolide-lincomycin-spectinomycin resistance was carried by the type IIb element. Thus, the rule for community-acquired SCC*mec* of a small size and carriage of smaller numbers of resistance genes still applies to the new type IIb element. Evidently, the kinds of antibiotic selective pressure for day care centers and kindergartens are limited compared to those for hospitals. Oral cephalosporins and macrolides constitute more than 90% of the antibiotics used for the treatment of Japanese children (20). Thus, only the carriage of Tn*554* and the *mecA* gene is required for Japanese C-MRSA strains so that they can survive in the community.

Small SCC*mec* elements may be more prone to transfer from strain to strain, presumably via phage-associated trans-

duction. We proposed the hypothesis based on the extreme heterogeneity of the C-MRSA genotypes (29). Here, again, we observed diversity in the MRSA strains in the community. CC91 is a novel chromosome type, and two MRSA clones of SCC*mec* type IIb and CC91 and SCC*mec* type IVa and CC91 are novel MRSA clones transformed by the introduction of the community-type SCC*mec*. Further study to determine non-typeable subtypes of type IV SCC*mec* elements is ongoing and may add to the repertoire of the highly efficient transformer SCC*mec*.

Highly virulent C-MRSA strains carrying PVL genes are known to prevail in the world. No PVL-positive strains were identified in the present study. However, the possibility that some C-MRSA strains carrying PVL are distributed in the Japanese community cannot be excluded, since in the early 1980s we found that many type IV SCC*mec* strains carry PVL genes. In fact, in 2002 we found a type IV SCC*mec* strain carrying PVL from an outpatient who visited our facility with a deep skin abscess (X. X. Ma, unpublished data). However, the MRSA strains found in the 1980s declined, and a new MRSA represented by strain N315 appeared in the 1990s (34). Thus, we believe that PVL-positive strains are not widely disseminated in Japan at this moment.

MRC-NS in the community. We found that MRC-NS strains are widely disseminated in the Japanese community and investigated the characteristics of those MRC-NS strains. Most MRC-NS strains carried the type IV SCC*mec* element (K. Kuwahara-Arai, unpublished data). The data might suggest that MRC-NS in the community may serve as a reservoir for type IV SCC*mec* elements, which are widely disseminated among the C-MRSA strains in the world. A larger number of surveys in the community is warranted to explore from where and how the SCC*mec* element enters the *S. aureus* chromosome.

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Midterm Results on Ocular Surface Reconstruction Using Cultivated Autologous Oral Mucosal Epithelial Transplantation

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• PURPOSE: To perform a midterm assessment of the integrity and reproducibility of cultivated autologous oral mucosal epithelial sheets, and to evaluate the clinical efficacy of their transplantation in ocular surface.
• DESIGN: Observational case series.
• METHODS: Cultivated autologous oral mucosal epithelial sheets were created using amniotic membrane and buccal mucosal epithelium from 12 patients with Stevens-Johnson syndrome, chemical and thermal injury, pseudo-ocular cicatricial pemphigoid, and idiopathic ocular surface disorder. They were transplanted onto 15 eyes from these patients who were then followed up for a mean of 20 months; with the longest follow-up being 34 months. We assessed their clinical outcomes with special reference to neovascularization.
• RESULTS: Cultivated autologous oral mucosal epithelial sheets could be generated from all patients. On the second postoperative day, 14 of 15 sheets transplanted demonstrated total re-epithelialization on the cornea. During the follow-up, the ocular surface was stable and transparent without any major complications in 10 of 15 eyes (67%), and the transplanted epithelium survived for at least 34 months. There were five eyes (33%) with small but long-standing epithelial defects, three of these healed spontaneously, and two (13%) required reoperation. In 10 eyes, postoperative visual acuity was improved by more than 2 lines. All eyes manifested some peripheral corneal vascularization.
• CONCLUSIONS: We established a successful tissue-engineering technique to generate cultivated autologous oral mucosal epithelial sheets and succeeded in reconstructing the ocular surface. We suggest that this surgical modality may be both safe and useful, especially in younger patients with the most severe ocular surface disorders. (Am J Ophthalmol 2006;141:267–275. © 2006 by Elsevier Inc. All rights reserved.)

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THE COMPLETE LOSS OF CORNEAL EPITHELIAL STEM cells attributable to acute or chronic ocular surface disorders leads to limbal deficiency that results in the conjunctivalization of the corneal surface, that is, conjunctival epithelial invasion with superficial vascularization and subepithelial scarring. Various degrees of pathologic keratinization, symblepharon, and entropion also occur, resulting in serious visual loss. Surgical approaches to ocular surface diseases such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical injury include limbal transplantation[1] and amniotic membrane (AM) transplantation.[2] These approaches were both developed in the 1990s and have produced some positive therapeutic results.

The more recently developed and improved surgical modality that uses cultivated corneal epithelial stem cell sheets has already been implemented widely.[3–7] The primary concept and cultivation technique for epithelium is an extension of the method first introduced in the 1970s by Rheinwald and Green[8] that employed tissue-engineered epidermal sheets to treat thermal skin injuries.

Despite a number of failures, in part attributable to a lack of knowledge regarding stem cells, in 1997 Pellegrini and associates[9] successfully restored damaged human corneal surfaces by transplanting autologous cultivated corneal epithelium. Subsequently, patients with unilateral damage received transplants of cultivated corneal epithelial stem cells obtained from the healthy contralateral eye. This has become an established, successful approach.[3,10,11] Patients with bilateral eye damage required the transplantation of cultivated corneal epithelial stem cells from

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TABLE 1. Baseline Data of Patients Receiving an Oral Mucosal Epithelial Culture Reconstruction

Case	Age/Gender	Disease	Condition of Oral Cavity	Feeder Cell Condition	Culture Serum	Density of Cell Seeding (Cell/Well)	Days Reach Confluence	Integrity of Culture Sheet
1	33/M	Chemical	Good	Good	FBS	1.0×10^5	5	Excellent
2	33/M	Chemical	Good	Good	FBS	1.0×10^5	5	Excellent
3	27/M	Chemical	Good	Good	FBS	1.0×10^5	6	Excellent
4	24/M	SJS	Moderate	Good	FBS	0.9×10^5	6	Excellent
5	14/F	SJS	Moderate	Good	FBS	0.7×10^5	6	Excellent
6	24/M	SJS	Moderate	Good	FBS	1.1×10^5	8	Excellent
7	65/F	SJS	Moderate	Good	FBS	0.7×10^5	6	Fair
8	61/F	OSD	Good	Moderate	FBS	1.0×10^5	7	Excellent
9	69/M	Chemical	Good	Good	FBS	1.0×10^5	6	Excellent
10	65/F	SJS	Moderate	Good	AS	1.5×10^5	7	Excellent
11	70/M	SJS	Moderate	Good	AS	1.3×10^5	6	Excellent
12	67/F	SJS	Moderate	Good	AS	1.5×10^5	6	Excellent
13	29/M	Thermal	Moderate	Good	AS	1.0×10^5	5	Excellent
14	81/F	pOCP	Good	Good	AS	1.5×10^5	6	Excellent
15	64/M	Chemical	Moderate	Good	AS	1.5×10^5	7	Excellent

AS = autologous serum; Chemical = chemical injury; FBS = fetal bovine serum; OSD = idiopathic ocular surface disorder; pOCP = pseudo-ocular cicatricial pemphigoid; SJS = Stevens-Johnson syndrome; Thermal = thermal injury.

cadaver donors or a living-related eye. While this method also yielded some success,[4,12] immunologic rejection and microbial infection as a result of immunosuppressive therapy after allogeneic transplantation continue to present challenges.

In the context of regenerative medicine, the transplantation of cultivated mucosal epithelial stem cell sheets created from autologous cell sources presents a viable alternative in cases with bilateral eye damage that vitiates the use of autologous corneal epithelial stem cells. Oral mucosal epithelium has attracted attention as a cell source, and favorable results have been obtained in animal- and preliminary human pilot studies.[13–16]

Here we present midterm clinical data on 15 eyes grafted with cultivated autologous oral mucosal epithelial transplants. The corneal surface in 13 of our 15 eyes was stable and remained fairly transparent despite some peripheral corneal neovascularization.

METHODS

THIS STUDY WAS APPROVED BY THE INSTITUTIONAL REview Board for Human Studies of Kyoto Prefectural University of Medicine; prior informed consent was obtained from all patients. We report on 15 eyes from 12 patients with bilateral total limbal deficiency; their ages ranged from 14 to 81 years. The preoperative diagnosis was SJS in five patients, chemical injury in four, and thermal injury, pseudo-ocular cicatricial pemphigoid, and idiopathic ocular surface disorder of unknown etiology in one patient each. Preoperatively, all 15 eyes manifested severe destruction of the ocular surface with limbal deficiency, but also reasonable reflex tearing with some meniscus height.

The 12 patients presented displayed total limbal deficiency in either the acute or chronic phase. This was diagnosed by the complete absence of the palisades of Vogt. The four eyes in the acute phase had sustained chemical (n = 3) or thermal injury (n = 1) and manifested persistent epithelial defects involving the entire cornea, complete limbal deficiency, and sustained conjunctival inflammation. The injury to these four eyes was of grade IIIb or IV according to the grading system we proposed elsewhere.[17] The 11 eyes in the chronic phase included seven with SJS, two with chemical injuries, and one each with pseudo-ocular cicatricial pemphigoid and idiopathic ocular surface disorder. All 11 eyes manifested total conjunctivalization on the cornea with conjunctival cicatrization. Of the 15 eyes, seven had received previous treatment consisting of AM transplantation alone (n = 2), limbal transplantation with AM transplantation (n = 1), keratoepithelioplasty with AM transplantation (n = 1), and penetrating keratoplasty (n = 1); both eyes in one patient had been grafted with cultivated allogeneic corneal epithelial sheets in the acute phase. The mean follow-up period in our midterm study was 20 months; the longest follow-up was 34 months.

• PROCEDURE FOR THE TISSUE-ENGINEERING OF AUTOLOGOUS ORAL MUCOSAL EPITHELIAL SHEETS: After obtaining informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects, we harvested human AM at the time of elective Cesarean section. Under sterile conditions, the membranes were deprived of their amniotic epithelium by

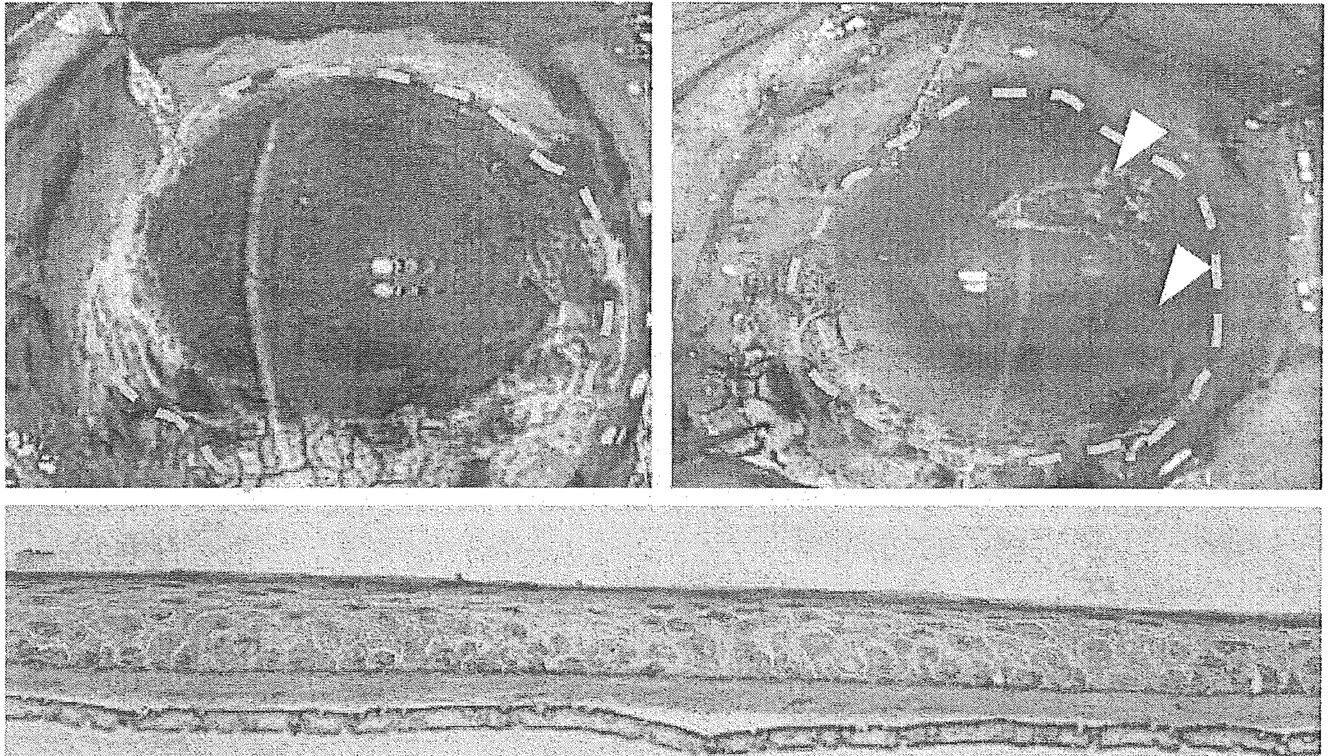

FIGURE 1. The integrity of all transplanted cultivated oral mucosal epithelial sheets confirmed by fluorescein staining at the end of ocular surface reconstruction. The yellow broken line encompasses negatively-stained cultivated stratified oral mucosal epithelium. Arrows indicate the region harboring the epithelial defect in the sheet that was considered to be of only fair quality before grafting. Of the 15 sheets, 14 (93.3%) were of excellent quality and without epithelial defects (Top left); one case was scored as fair with some epithelial defects (Top right). Histologic examination using hematoxylin and eosin staining revealed good stratification throughout the entire sheet (Bottom). EP: cultivated oral mucosal epithelium; AM: amniotic membrane; CI: culture insert.

using a 2-hour incubation at 37°C with ethylene diamine tetraacetic acid (EDTA) 0.02% to loosen cell adhesion. This was followed by gentle scraping with a cell scraper.

The presence of healthy oral mucosa in our patients was confirmed by a dentist before biopsy. All patients were monitored to confirm their adherence to required tooth-decay treatment, their abstinence from alcohol or tobacco use, and their regular performance of tooth brushing and iodine gargling. Under local anesthesia, oral mucosal biopsy specimens, each measuring approximately 2 to 3 mm^2, were obtained 2 to 3 weeks before the planned transplantation procedure. Submucosal connective tissues were removed with scissors to the extent possible, with the resulting samples being cut into small explants that were then immersed three times (10 minutes, room temperature) in phosphate-buffered saline solution containing antibiotics (50 IU/ml penicillin-streptomycin and 5 μg/ml amphotericin B). The explants were then incubated at 37°C for 1 hour with 1.2 IU dispase as previously described[13] and treated with trypsin-EDTA 0.05% solution for 10 minutes at room temperature to separate the cells. Enzyme activity was stopped by washing with culture medium comprised of DMEM and Ham's F12 medium (1:1) containing insulin (5 μg/ml), cholera toxin (0.1 nmol/l), human recombinant epidermal growth factor (10 ng/ml), and penicillin-streptomycin (50 IU/ml). In cultures for eyes no. 1 to 9, the medium also contained 10% fetal calf serum. In cultures for eyes no. 10 to 15, we included 10% autologous serum. The oral mucosal epithelium was then seeded onto denuded AM spread on the bottom of culture inserts, and cocultured with mitomycin-C (MMC)-inactivated 3T3 fibroblasts. The culture was submerged in medium for 2 weeks and then exposed to air by lowering the level of the medium (air lifting) for 1 to 2 days. Cultures were incubated at 37°C in a 5% CO_2-95% air incubator; the medium being changed daily. Baseline data on the oral mucosal epithelial cultures are summarized in Table 1.

• SURGICAL PROCEDURE FOR OCULAR SURFACE RECONSTRUCTION USING CULTIVATED AUTOLOGOUS ORAL MUCOSAL EPITHELIAL TRANSPLANTATION: The surgical procedure was as described in our previous report.[4] Stated briefly, after a 360-degree conjunctival peritomy, we either scraped the area with the epithelial defect, or completely removed the conjunctivalized tissue by thin superficial keratectomy on the corneal surface. Subconjunctival spaces were treated with MMC 0.04% for 5 minutes, followed by a vigorous washing with saline. Then,

TABLE 2. Characteristics of Cases and Clinical Outcome of Patients With Oral Mucosal Epithelial Culture Reconstruction

Case	Age/Gender	Eye	Disease	Prior Op	Combined Op	Visual Acuity			Complication	Follow-up (mos)
						Pre Op	Post Op	Last VA		
1	33/M	OS	Chemical (acute)	AMT		HM	20/200	20/40		34
2	33/M	OD	Chemical (acute)	AMT		HM	HM	HM		34
3	27/M	OS	Chemical (chronic)	None	AMT	HM	CF	HM		32
4	24/M	OS	SJS	CCET		HM	20/2000	CF	ED	29
5	14/F	OS	SJS	None		CF	20/1000	20/1000		28
6	24/M	OD	SJS	CCET		HM	20/2000	CF	ED	28
7	65/F	OD	SJS	AMT + KEP	PEA + IOL	CF	20/400	20/500	ED	26
8	61/F	OD	OSD	AMT + LT	PEA + IOL	HM	20/500	20/800		23
9	69/M	OD	Chemical (chronic)	PK	PK*	HM	HM	20/50		18
10	65/F	OS	SJS	None	PEA + IOL	HM	20/320	20/320		12
11	70/M	OS	SJS	None	PK*	HM	HM	20/1000		11
12	67/F	OD	SJS	None	PEA + IOL	HM	20/2000	20/2000	ED	8
13	29/M	OD	Thermal (acute)	None	Lid	20/500	20/1000	20/32	ED	8
14	81/F	OS	pOCP	None	PEA + IOL + PPV	20/400	20/63	20/63		6
15	64/M	OD	Chemical (acute)	None	PEA + IOL + Lid	20/500	20/250	20/500		3

AMT = amniotic membrane transplantation; CCET = cultivated corneal epithelial transplantation; CF = count finger; Chemical = chemical injury; ED = epithelial defect; HM = hand motion; IOL = intraocular lens; KEP = keratoepithelioplasty; Lid = lid plastic surgery; LT = limbal transplantation; OSD = idiopathic ocular surface disorder; PEA = phacoemulsification; PK = penetrating keratoplasty; pOCP = pseudo-ocular cicatricial pemphigoid; PPV = pars plana vitrectomy; SJS = Stevens-Johnson syndrome; Thermal = thermal injury.
*Two cases received PK after primary surgery.

the cultivated autologous oral mucosal epithelial sheet in a culture dish was cut with a 19-mm diameter trephine, transferred onto the corneal surface, and sutured with 10-0 nylon. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of surgery (Figure 1), and the ocular surface was protected with a medical-use contact lens.

• CLINICAL EVALUATION: Preoperative and postoperative best-corrected visual acuity was measured, and ocular surface manifestations were inspected with a slit-lamp microscope and fluorescein staining. Corneal superficial vascularization was monitored photographically and graded according to extent and intensity, where grade 1 indicates peripheral vascularization, grade 2 peripheral and midperipheral vascularization, grade 3 modest vascularization involving the entire cornea, and grade 4 massive vascularization of the entire cornea.

RESULTS

• CULTIVATED AUTOLOGOUS ORAL MUCOSAL EPITHELIAL SHEETS: There were no complications during or after the excision of oral mucosa. Cell suspensions of approximately 1×10^5 seeded oral mucosal epithelial cells began to form colonies on the denuded AM within 3 days. After 5 to 8 days in culture, a confluent primary culture of oral mucosal epithelial cells was established on the whole AM. After 2 weeks, the cultivated oral mucosal epithelium consisted of five to six cell layers and was similar to the cultivated corneal epithelial sheets we reported previously.[4,13] The oral mucosal epithelial sheet was composed of a well-conserved basal layer formed by cuboidal cells, several suprabasal cell layers, and flat apical cell layers (Figure 1). In 14 of 15 instances, the quality of the cultivated epithelial sheets was excellent. In one instance (Case 6), it was merely fair because only 70% of the entire cultivated epithelial sheet showed mature stratification as determined by fluorescein staining under a phase-contrast microscope and an operating microscope at the end of surgery (Table 1, Figure 1).

• CLINICAL OUTCOMES: All eyes, including the eye transplanted with the sheet whose quality we judged as only fair, demonstrated total re-epithelialization of the corneal surface 2 days after surgery. During the follow-up period, in 10 of 15 eyes the ocular surface grafted with cultivated autologous oral mucosal epithelial sheets remained silent and fairly transparent. However, five eyes, including four with severe SJS, developed small but long-standing epithelial defects; two eyes proceeded to be completely healed by adjacent oral mucosal epithelium, one eye demonstrated conjunctival replacement, and the other two eyes required reoperation. Except for the latter two eyes, all ocular surfaces became stable without any

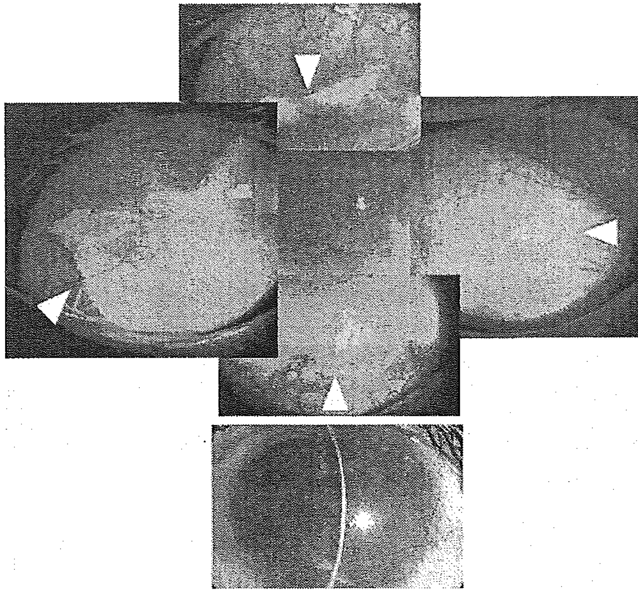

FIGURE 2. The clinical appearance of case 1 at 34 months after cultivated autologous oral mucosal epithelial transplantation. Fluorescein staining confirms the long-term survival of oral mucosal epithelium identified by the different levels of staining density. Arrows indicate the margin of the outgrowth of survived oral mucosal epithelium (Top). Slit-lamp photograph showing the appropriately resurfaced cornea. Note the modest vascularization involving the entire cornea beneath the amniotic membrane sheet and the preexisting corneal stromal opacity (Bottom).

major postoperative complications such as microbial infection or secondary glaucoma (Table 2).

Cultivated corneal epithelial stem cell sheets and ectopically surviving cultivated oral mucosal epithelial sheets are somewhat different in terms of their fluorescein staining patterns at the apical cell surface. In fact, regenerating epithelium that had originated from cultivated oral mucosal epithelium was clearly demarcated from adjacent conjunctival epithelium even as late as 34 months after surgery, the longest follow-up period in this series. This observation strongly suggests the long survival and epithelial supply of presumed oral mucosal epithelial stem cells (Figure 2).

Preoperative best-corrected visual acuity in our series was hand motion (HM) or counting fingers (12 eyes), 20/500 (two eyes), and 20/400 (one eye). Postoperative visual recovery ranged from HM to 20/32; best-corrected visual acuity was improved by more than 2 lines in 10 eyes (67%) at 3 months, and in 10 eyes (67%) at their latest follow-up examination. Three eyes with severe corneal opacity were scheduled for ocular surface reconstruction before penetrating keratoplasty. In cases 9 and 11, we performed a triple procedure with penetrating keratoplasty at 5 and 6 months after the ocular surface reconstruction procedure, respectively; visual acuity achieved in these two eyes was 20/50 and 20/1000. Of the 15 eyes, six were treated with cataract surgery immediately after the removal of ocular surface scarring using either a surgical slit-lamp or a special lighting device, and two eyes were treated with eyelid plastic surgery for entropion attributable to the primary injury (Table 2).

CASE REPORTS

FIGURES 3 AND 4, SHOW REPRESENTATIVE CASES OF CULTIvated autologous oral mucosal epithelial transplantation.

• CASE 1: A 33-year-old man in the acute phase of alkali injury graded IIIb with severe corneal stromal opacity in March 2002. AM transplantation was initially performed to cover the total damaged corneal surface, however, persistent corneal epithelial defect and severe inflammation prolonged for more than 1 month. Cultivated autologous oral mucosal epithelial transplantation was performed on June 24, 2002. Postoperatively, the ocular surface showed stabilized epithelialization with peripheral corneal vascularization (Figure 3). Even after 34 months of follow-up, surviving oral mucosal epithelium was distinguishable from conjunctival epithelium. The latest visual acuity was maintained at 10/20.

• CASE 5: A 14-year-old girl in the chronic phase of SJS with severe symblepharon over the cornea. The primary SJS occurred at the age of 5. The ocular surface was totally conjunctivalized with severe symblepharon without any surgeries. The ocular surface was reconstructed using cultivated oral mucosal epithelial transplantation, and the postoperative corneal surface was maintained fairly transparent. Best-corrected visual acuity improved from counting fingers to 20/1000 although the damaged corneal stroma was somewhat opaque (Figure 4).

• CASE 8: A 61-year-old woman with limbal deficiency of unknown etiology following AM transplantation and conventional allogeneic limbal transplantation. Primary surgery was performed in November 2000, but subsequent failure resulted in total conjunctivalization. After removal of scarred tissue and previously transplanted lenticles, the ocular surface was covered with a cultivated oral mucosal epithelial sheet. Postoperatively, the corneal surface showed complete epithelialization with minimal vascularization; some calcium deposits were observed (Figure 4).

• CASE 10: A 65-year-old woman with SJS. The primary SJS occurred at the age of 28. Visual acuity was reduced to CF, because of the conjunctivalization and the progression of cataract. Ocular surface was reconstructed in April 2004 using cultivated oral mucosal epithelial transplantation and cataract surgery. Postoperatively, the ocular surface was stable and transparent (Figure 4). Visual acuity improved to 20/320.

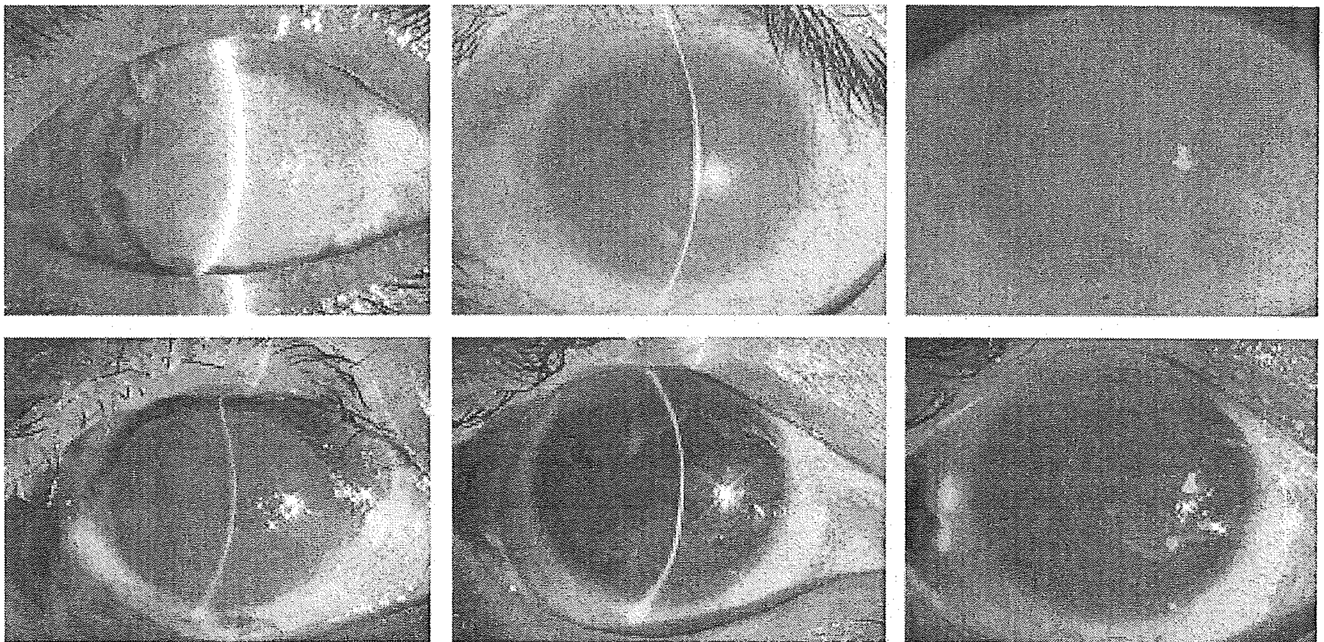

FIGURE 3. Slit-lamp photographs of two patients in the acute phase of chemical/thermal injury shown before and after ocular surface reconstruction using cultivated oral mucosal epithelial transplantation. Case 1 (33-year-old man): acute phase of alkali injury graded IIIb with severe corneal stromal opacity. (Top left) The ocular surface in preoperative condition. (Top center) Postoperative condition. (Top right) After fluorescein staining. Case 13 (29-year-old man): acute phase of thermal injury with total corneal stem-cell loss and a persistent epithelial defect. (Bottom left) The ocular surface in preoperative condition. (Bottom center) Postoperative condition. (Bottom right) After fluorescein staining.

• CASE 13: A 29-year-old man in the acute phase of thermal injury with total corneal stem cells loss and a persistent epithelial defect. He was injured in July 2004, and a persistent epithelial defect prolonged for more than 1 month. Simultaneously, progression of cicatrisation was observed. Therefore, we performed cultivated oral mucosal epithelial transplantation, and the ocular surface became stable after combined eyelid plastic surgery for cicatricial entropion (Figure 3).

• NEOVASCULARIZATION: All eyes grafted with cultivated oral mucosal epithelial sheets manifested various degrees of superficial corneal vascularization between the AM and corneal stroma. Preoperatively, most of the corneas had been covered with highly vascularized conjunctiva and had been given a grade of 4. Sparse or modest peripheral vascularization began after the first postoperative month (grade 1 to 2); in most cases, vascularization gradually progressed toward the center and peaked at 3 to 6 months. Although all grafted eyes manifested some degree of neovascularization, it gradually abated and over time it ceased to interfere markedly with visual function. At the 1-year follow-up, the neovascular formations were stable and none of the grafted eyes converted to their preoperative condition or exhibited oral mucosal tissue characteristics (Figure 5).

DISCUSSION

THIS MIDTERM STUDY DEMONSTRATES THE EFFECTIVENESS of cultivated autologous oral mucosal epithelial sheet transplantation and supports our earlier, preliminary report[14] by documenting multiple successful clinical results. According to their preliminary clinical study, Nishida and associates,[15] who grafted oral mucosal epithelial cell sheets cultured by methods different from ours,[13,14] also obtained positive results. This suggests that the transplantation of cultivated autologous oral mucosal epithelial sheets holds promise as a novel surgical treatment for severe ocular surface disorders such as SJS, ocular cicatricial pemphigoid, and chemical injury.

In the course of postoperative follow-up, their distinctive fluorescein staining pattern makes it easy to distinguish transplanted cultivated oral epithelial cell sheets from surrounding conjunctival epithelium. The staining pattern of epithelial cells of cultivated oral mucosal epithelial cell origin is more like that of superficial punctate keratopathy than conjunctival epithelium. Judging from their fluorescein staining at 2 days after surgery, with the exception of the sheet whose quality was considered only fair at the time of transplantation, almost all of the transplanted epithelial cells had attached on the cornea. In fact, histologically, the thriving oral mucosal epithelium at

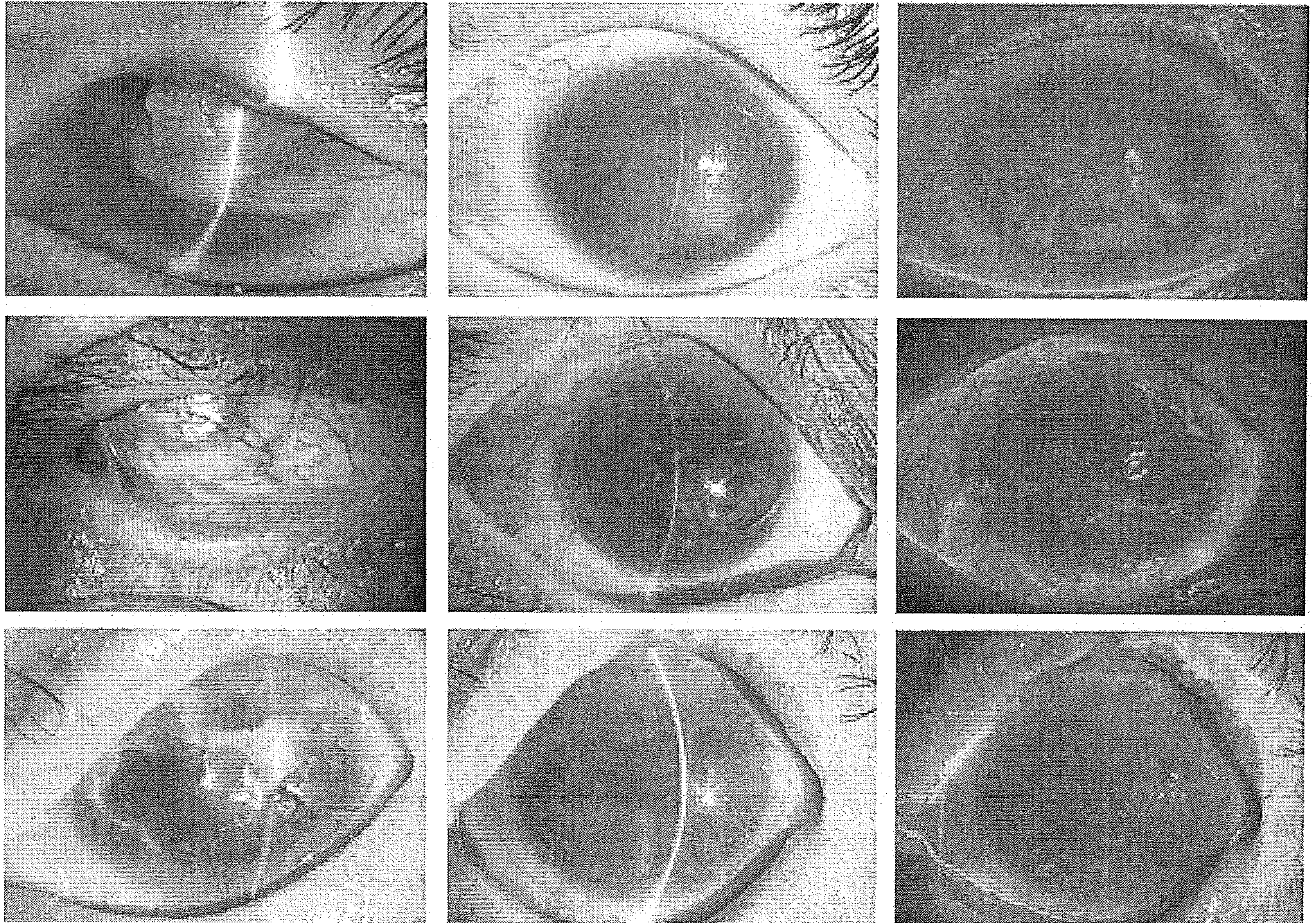

FIGURE 4. Slit-lamp photographs of three patients in the chronic phase of ocular surface disorders shown before and after ocular surface reconstruction using cultivated oral mucosal epithelial transplantation. Case 5 (14-year-old girl): chronic phase of SJS with severe symblepharon over the cornea. (Top row left) Preoperative condition. (Top row center) Postoperative condition. (Top row right) After fluorescein staining. Case 8 (61-year-old woman): limbal deficiency of unknown etiology. (Middle row left) Preoperative condition. (Middle row center) Postoperative corneal surface. (Middle row right) After fluorescein staining. Case 10 (65-year-old woman): chronic phase of SJS. (Bottom row left) Preoperative condition. (Bottom row center) Postoperative corneal surface. (Bottom row right) After fluorescein staining.

the central cornea that was removed at the time of penetrating keratoplasty (6 months after transplantation) was nonkeratinized stratified epithelium similar to corneal epithelium (data not shown). In the case followed for the longest period (34 months, Case 1), fluorescein staining results suggest that the cultivated oral mucosal epithelium cell sheet covered not only the entire cornea but also an area up to a few mm beyond the cornea. Although the transplanted epithelial sheets retained their transparency, there was a slight hazing, and the maximum corrected visual acuity we were able to obtain in our 15 eyes was 20/32. For most eyes, it was between 20/2000 and 20/32, suggesting the potential of visual recovery through the survived oral mucosal epithelium on the cornea may be around 20/200. This issue is currently under investigation at our laboratory.

The health of the oral mucosal epithelium in vivo depends on the existing disease. Patients with SJS always manifest mucosal epithelial damage in the acute phase. Ocular cicatricial pemphigoid, a type of mucous membranous pemphigoid, may also affect the oral mucosa. However, we were able to generate transplantable sheets from all 12 patients. In four instances, the transplantation of cultivated epithelium from patients with SJS resulted in small persistent epithelial defects, possibly because the oral mucosal epithelium was damaged. Alternatively, chronic ocular surface abnormalities may be different from other primary disorders. Although there is currently no solid evidence for the presence of stem cells in the human oral cavity, we posit that these cells are distributed as diffusely in the oral mucosal epithelium as in the human epidermis and conjunctival epithelium, and that oral mucosal epi-

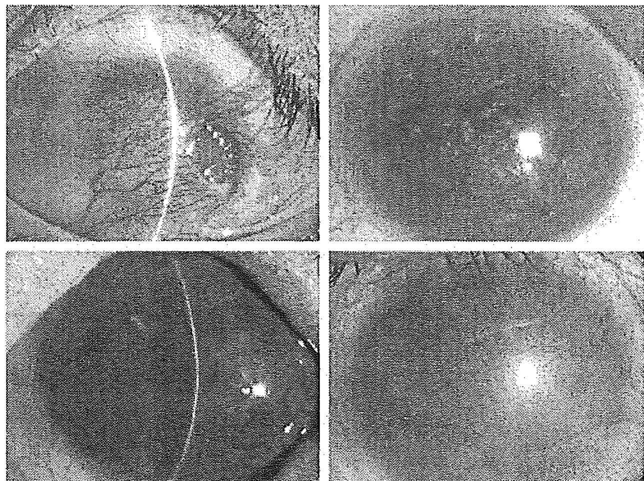

FIGURE 5. Slit-lamp photographs showing vascular formations after the transplantation of cultivated autologous oral mucosal epithelial sheets. (Top left) Preoperatively, most of the cornea manifested highly-vascularized conjunctivalization (case 3, grade 4). At the 1-year follow-up, vascular formations had abated and stabilized at grades 1 to 3. (Top right) Case 8 showed grade 1, (Bottom left) case 10 showed grade 2, and (Bottom right) case 1 showed grade 3.

thelial stem cells were present and impaired in these cases. Recently, Hayashida and associates[16] demonstrated p63 and β1-integrin positivity within the oral mucosa of rabbits, implying the presence of stem cells of oral mucosal epithelium in the oral cavity. In humans, we have a speculation that stem cells of oral mucosal epithelium may be diffusely located, similar to the rabbit study. This issue is also being investigated to rule out other factors in our laboratory.

In contrast to cultivated corneal epithelial stem cell transplants, the grafting of tissue-engineered oral mucosal epithelial cell sheets resulted in neovascularization in the superficial cornea. This suggests the presence of angiogenic activity whose level varies depending on the disorder and renders neovascularization inevitable. Transplanted buccal mucosa including subepithelial tissue survives by vessel recanalization. Gipson and associates,[18] who transplanted rabbit oral mucosal epithelium to the ocular surface, peeled the oral mucosal epithelial sheets by using dispase; their exfoliate transplantation results revealed vascularization. Tissue-engineered oral mucosal epithelial sheets may have weak, vascularization-inducing angiogenic activity. In fact, we found that some angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF) are present. Conversely, our preliminary data demonstrated that one antiangiogenic factor, thrombospondin 1, appeared to be expressed in a low level in cultivated oral mucosal epithelial cells, which may be a possible explanation for the induction of neovascularization. (data not shown) We are investigating the basis of our highly interesting observation that different eyes manifested different degrees of vascularization that tended to peak at 3 to 6 months post-transplantation and declined thereafter. Thus, from the point of long-term ocular surface rehabilitation in severe cases, modest corneal neovascularization can be expected not to interfere considerably with visual function.

As our procedure for tissue-engineered oral mucosal epithelial sheets for ocular transplantation is relatively new, it is too early for long-term results regarding the longevity of the improved corrected vision. We can, however, report that in our hands, cultivated autologous oral mucosal epithelial sheet transplantation is a safe procedure that led to no severe postoperative complications. Furthermore, our autologous transplantation provides rapid epithelial covering without the threat of an immunologic rejection. It also provides for a much-improved prognosis of ocular surface reconstruction compared with the conventional procedure. In fact, this study improved the surgical results of two cases failed by the conventional epithelial transplantation, indicating the superior advantages of our new procedure. Analysis of the biologic aspects of tissue-engineered oral mucosal epithelium sheets will lead to further improvements. Our autologous transplantation procedure may require short-term, postoperative immune suppression for the reduction of postoperative inflammation and control primary diseases, however, it can be safely performed even on very young patients. Cultivated autologous oral mucosal epithelial sheet transplantation constitutes a promising treatment in patients with severe ocular surface disorders.

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REPORTING VISUAL ACUITIES

The AJO encourages authors to report the visual acuity in the manuscript using the same nomenclature that was used in gathering the data provided they were recorded in one of the methods listed here. This table of equivalent visual acuities is provided to the readers as an aid to interpret visual acuity findings in familiar units.

Table of Equivalent Visual Acuity Measurements

Snellen Visual Acuities				
4 Meters	6 Meters	20 feet	Decimal Fraction	LogMar
4/40	6/60	20/200	0.10	+1.0
4/32	6/48	20/160	0.125	+0.9
4/25	6/38	20/125	0.16	+0.8
4/20	6/30	20/100	0.20	+0.7
4/16	6/24	20/80	0.25	+0.6
4/12.6	6/20	20/63	0.32	+0.5
4/10	6/15	20/50	0.40	+0.4
4/8	6/12	20/40	0.50	+0.3
4/6.3	6/10	20/32	0.63	+0.2
4/5	6/7.5	20/25	0.80	+0.1
4/4	6/6	20/20	1.00	0.0
4/3.2	6/5	20/16	1.25	−0.1
4/2.5	6/3.75	20/12.5	1.60	−0.3
4/2	6/3	20/10	2.00	−0.3

From Ferris FL III, Kassoff A, Bresnick GH, Bailey I. New visual acuity charts for clinical research. Am J Ophthalmol 1982;94:91–96.