ance to other antibiotics such as aminoglycosides, minocyclin and fluoroquinolones has been on the rise.[11–15] *Staphylococcus epidermidis* has developed the same bacterial resistance as *S aureus* and has now been termed as methicillin-resistant *S epidermidis* (MRSE). Moreover, previous reports have shown that MRSE can cause ophthalmic infections and blindness.[4 16]

Vancomycin, a glycopeptide antibiotic, is known to be effective for treating MRSA infections. Since its injection formulation was first approved for the indication of infectious disease due to Gram-positive bacteria in 1958 in the USA, vancomycin has become an approved antibiotic throughout the world and is highly valued particularly for the treatment of MRSA infections.[9 15] In the therapy of ocular infections, a topical application of vancomycin solutions prepared by in-house prescription is often used.[16 17] However, vancomycin is unstable in an aqueous solution. In addition, vancomycin solutions prepared by in-house prescription using saline are acidic, and the irritation of the solutions to tissues causes patient compliance problems.[17]

We first prepared vancomycin ophthalmic ointments for the treatment of destructive MRSA keratitis postlamellar keratoplasty and found that the infectious keratitis healed dramatically.[4 18] Considering the fact that vancomycin is a drug that exerts its actions time dependently,[19] an ophthalmic ointment with high tissue retentivity, is well suited for clinical use. Indeed, vancomycin ophthalmic ointments remained at least 3 h after administration in a 5-year-old boy with severe MRSA keratitis. It has been suggested that vancomycin ophthalmic ointments remain longer on the ocular surface compared with vancomycin solutions.[18] However, those ointments have proved to be difficult to prepare, and a commercially made product with long-term stability that can be distributed at an effective concentration to the site of an infection has been in demand.

Vancomycin Ophthalmic Ointment 1% (Toa Pharmaceutical Co., Ltd, Toyama, Japan) was developed for the treatment of MRSA/MRSE ocular infections.[20] In 2001, it was designated as an orphan drug for the treatment of 'ocular infections, such as blepharitis, conjunctivitis and keratitis caused by MRSA and MRSE' (Grant No. 13–152, dated 23 April 2001). Thereafter, a phase I study confirmed the safety and tolerability of vancomycin ophthalmic ointment in healthy adult volunteers. In this study, we investigated the efficacy and safety of Vancomycin Ophthalmic Ointment 1% in patients with external ocular infections caused by MRSA or MRSE.

MATERIALS AND METHODS

Study design

This study was a multicentre, open-label, uncontrolled study approved as orphan drug status. The study protocol was designed to evaluate the efficacy and safety of Vancomycin Ophthalmic Ointment 1% in patients with MRSA or MRSE external ocular infections. The study included a 3-day (or more) screening period with the treatment of fluoroquinolone eye drops, and a 14-day treatment period during which patients received Vancomycin Ophthalmic Ointment 1% (four times daily; figure 1). It was approved by the institutional review board at each study site. The study was carried out in accordance with the tenets set forth in the Declaration of Helsinki and in compliance with the 'Good Clinical Practice (GCP)' stipulated by the Ministry of Health, Labour and Welfare of Japan. Written informed consent was obtained from each patient at the respective institution before the initiation of the study protocol.

Screening and eligibility

The subjects involved in this study were patients with external ocular infections caused by MRSA or MRSE who were diagnosed with conjunctivitis, blepharitis, hordeolum, meibomitis, dacryocystitis and keratitis after presentation at 1 of 20 medical institutions in Japan between February 2006 and February 2007. Patient inclusion and exclusion criteria are shown in box 1. Only the patients who met all of the inclusion criteria were enrolled in this study.

Dosage regimen of the study drug

Vancomycin Ophthalmic Ointment 1% (containing 10 mg (potency) of vancomycin hydrochloride per gram) was administered at a dose of around 1 cm (approximately 50 mg) four times (morning, noon, evening and before bedtime) daily. The study treatment was started in the morning. The maximum treatment period was 14 days, and the treatment was terminated before 14 days in cases with diminishing subjective and objective findings of ocular infection.

Evaluation methods

Efficacy

The results of the bacteriological evaluation and clinical symptom assessment at days 3, 7 and 14 after the study

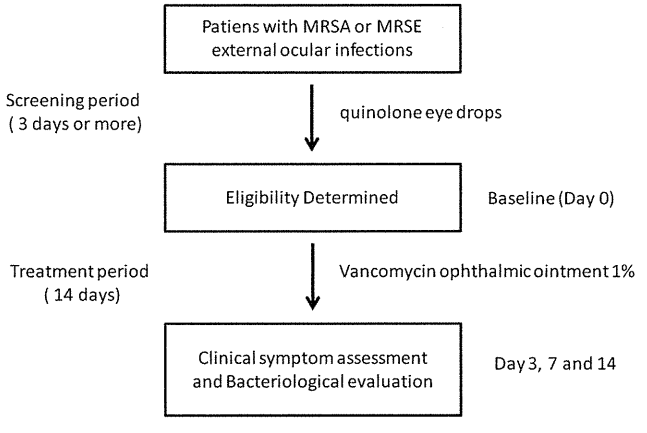

Figure 1 Study design. External ocular infections caused by methicillin-resistant *Staphylococcus aureus* or methicillin-resistant *Staphylococcus epidermidis* and cases in which fluoroquinolone eye drops showed no clinical effect were enrolled.

Box 1 Inclusion/exclusion criteria.

*Inclusion criteria**
- Age: 20 years or older and 90 years or younger (at the time of informed consent)
- Ocular infections due to MRSA or MRSE including the following target diseases: conjunctivitis, blepharitis, hordeolum, meibomianitis, dacryocystitis, keratitis and corneal ulcer
- Patients whose symptoms did not improve after local treatment with a fluoroquinolone antibacterial agent for the eyes for 3 days or more

*Exclusion criteria**
- Prior episode of hypersensitivity to vancomycin hydrochloride
- Prior episode of hypersensitivity to teicoplanin, peptide antibiotics or aminoglycoside antibiotics
- Patients who were on vancomycin hydrochloride or drugs of the same class and with the same effect (arbekacin sulfate and teicoplanin)
- Patients with a clinically significant disease of the autoimmune, cardiovascular, haematological, nervous, endocrine, hepatic, renal or digestive system
- Pregnant women, women of childbearing potential and lactating women

MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*.
* Pertains to study eyes, except where otherwise noted.

treatment initiation, and at study treatment completion or discontinuation, were evaluated and judged in comparison with those at the study treatment initiation (day 0; baseline). In cases with bilateral infection, either the severely affected eye or the right eye was evaluated.

In the clinical symptom assessment, symptoms and findings were scored according to the evaluation criteria, and the course of clinical symptoms at screening, at day 0 (baseline) and at days 3, 7 and 14 after the study treatment initiation, and study treatment completion or discontinuation were evaluated. Eye discharge, eye pain, foreign body sensation, photophobia and lacrimation as symptoms and redness (hyperaemia) and oedema (swelling), swelling of the eyelid, lacrimal-sac fluid reflux and keratitis as objective findings were classified into four levels and recorded as follows: notably severe (+++), 3 points; marked (++), 2 points; obvious (+),1 point; and none (−), 0 points.

In the bacteriological evaluation, samples for bacterial culture and identification were collected with sterile swabs from the eyes of patients at screening, at day 0 (baseline) and at days 3, 7 and 14 after the study treatment initiation, and at study treatment completion or discontinuation. These samples were inoculated into aerobic media, and the antibiotic sensitivity of the isolated bacterial strains was tested at the central laboratory for microbial testing (Research Foundation for Microbial Diseases of Osaka University). The effect towards negative conversion at days 3, 7 and 14 was calculated and evaluated.

The efficacy was determined as *complete response* (eradication of detected bacteria (estimated causative bacteria, hereinafter referred to as 'the bacteria') within 4 days and the disappearance of main symptoms within 1 week), *partial response* (1) eradication of the bacteria within 1 week and the disappearance of main symptoms within 2 weeks, (2) eradication of the bacteria within 4 days and a symptom score changed to ≥1/4 to ≤1/2 within 1 week or (3) no eradication of the bacteria but a symptom score changed to ≤1/3 within 1 week), *no response* (efficacy not corresponding to partial response or better) and *worsening* (deterioration of the main symptoms or symptom score compared with those at baseline).

Safety evaluation

All adverse drug reactions (ADRs) were recorded, and the frequency and incidence of the ADRs were then evaluated.

Analysis methods

Efficacy

The main efficacy analysis population was defined as a 'full analysis set (FAS)' not including patients with major GCP violations. Analyses in a 'per protocol set (PPS)', the population meeting the protocol criteria, were also performed. The results of the bacteriological evaluation and clinical symptom assessment were classified into five levels (complete response, partial response, no response, worsening and indeterminate) and a frequency table was then prepared. In addition, the percentage of patients with complete response and a partial response was calculated as an 'efficacy rate' that was evaluated by a one-sample exact test (two-sided significance level of 0.05: null hypothesis, efficacy rate: 10%) based on a binomial distribution. The 95% CIs for the efficacy rates were also calculated. In the bacteriological evaluation, the percentages of patients with eradication of MRSA or MRSE at the treatment completion or discontinuation were calculated as eradication rates.

Safety

In a 'safety population (SP)', patients who received at least one dose of the study drug and excluding those with major GCP violations, the frequency (number of patients with ADRs, number of ADRs and incidence) was tabulated by a system organ. Causal relationship, severity and outcomes in each ADR were judged by the attending physician.

RESULTS

Disposition of patients

In regard to the analysis populations, 25 patients, not including a patient with a major GCP violation, were adopted to the SP. Of the 25 patients in the SP, 4 patients

Table 1 Frequency tabulation of patient background characteristics: FAS, PPS and SP

Analysis population Item	FAS Number of patients (%)	PPS Number of patients (%)	SP Number of patients (%)
Number of patients	21 (100.0)	18 (100.0)	25 (100.0)
Sex			
Male	8 (38.1)	8 (44.4)	9 (36.0)
Female	13 (61.9)	10 (55.6)	16 (64.0)
Age (years)			
20≤ to <40	1 (4.8)	1 (5.6)	1 (4.0)
40≤ to <60	1 (4.8)	1 (5.6)	2 (8.0)
60≤ to <75	7 (33.3)	6 (33.3)	8 (32.0)
75≤ to ≤90	12 (57.1)	10 (55.6)	14 (56.0)
Bacterial strain			
MRSA	19 (90.5)	16 (88.9)	20 (80.0)
MRSE	2 (9.5)	2 (11.1)	5 (20.0)
Diagnosis (target disease)			
Blepharitis	3 (14.3)	3 (16.7)	3 (12.0)
Hordeolum	0 (0.0)	0 (0.0)	0 (0.0)
Meibomianitis	1 (4.8)	1 (5.6)	1 (4.0)
Conjunctivitis	14 (66.7)	11 (61.1)	16 (64.0)
Dacryocystitis	2 (9.5)	2 (11.1)	2 (8.0)
Keratitis	1 (4.8)	1 (5.6)	3 (12.0)
Severity			
Mild	19 (90.5)	16 (88.9)	21 (84.0)
Moderate	2 (9.5)	2 (11.1)	4 (16.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)

FAS, full analysis set; PPS, per protocol set; SP, safety population.

with treatment discontinuation due to negative results for bacterial culture during screening or at baseline were excluded, and 21 patients were included in the FAS. Of the 21 patients adopted into the FAS, 3 patients with protocol deviations were excluded and 18 patients were included in the PPS (table 1). As to the demographic characteristics of the patients, the mean age was 72.1±14.0 years (hereinafter: mean±SD).

Efficacy

In the clinical response evaluation (efficacy rate) defined as the primary endpoint, the efficacy rate was 66.7% in both the FAS and PPS. It was significantly higher in both populations as compared with the efficacy rate of 10% specified in the null hypothesis ($p<0.001$). The 95% CIs for the efficacy rate were 43–85.4% in the FAS and 41–86.7% in the PPS. In the evaluation by bacterial strain, the efficacy rates for MRSA were 63.2% in the FAS and 62.5% in the PPS. The efficacy rates for MRSE were 100% in both the FAS and PPS. In the evaluation by disease, conjunctivitis was most frequent, and the efficacy rates were 71.4% in the FAS and 72.7% in the PPS (table 2). In the bacteriological evaluation, the eradication rates were 68.4% (13 of 19 cases) for MRSA and 100% (2 of 2 cases) for MRSE in the FAS (figure 2).

Safety

Ten ADRs occurred in seven (28%) patients, and all the ADRs occurred at the local administration site. The main ADRs were eyelid oedema in three (12%) patients and conjunctival hyperaemia in three (12%) patients. Eyelid oedema, increased eye discharge and swelling of the face were moderate, and the other events such as conjunctival hyperaemia, abnormal sensation in the eye

Table 2 Clinical response evaluation by disease (full analysis set, FAS)

Target disease	Number of patients	Clinical response					Efficacy rate (%)
		Complete response	Partial response	No response	Worsening	Indeterminate	
Conjunctivitis	14	2 (14.3%)	8 (57.1%)	3 (21.4%)	0	1 (7.1%)	71.4
Blepharitis	3	0	2 (66.7%)	1 (33.3%)	0	0	66.7
Meibomianitis	1	0	1 100.0%)	0	0	0	100.0
Dacryocystitis	2	0	1 (50.0%)	1 (50.0%)	0	0	50.0
Keratitis	1	0	0	1 (100.0%)	0	0	0.0

Efficacy rate: (number of patients with 'complete response' or 'partial response'/number of patients studied)×100.

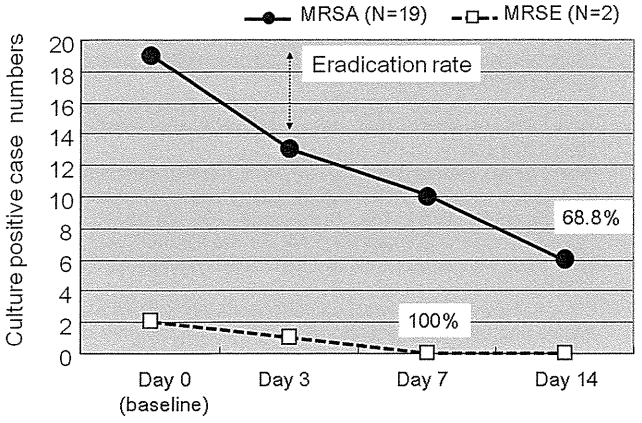

Figure 2 Bacteriological evaluation at 3, 7 and 14 days after initiation of treatment.

and pruritus at the application site were mild. Treatment was discontinued only in one patient with atopic dermatitis who developed swelling of the face and bilateral swelling of the eyelid. All the ADRs were confirmed to have resolved after the study completion.

DISCUSSION

In recent reports on drug-susceptibility of detected bacteria in the field of ophthalmology, the resistance rates of MRSA to ophthalmic antibiotics such as levofloxacin, cefmenoxime and erythromycin have risen. In contrast, the susceptibility rate of MRSA to vancomycin is reportedly still 100%.[2 11 13–15] Physicians in the clinical setting use ophthalmic solutions prepared by in-house prescription from bulk powder for injection, and their efficacy for MRSA or MRSE ocular infections has been previously reported.[4 16 17] Nonetheless, vancomycin for local ophthalmic use has yet to become available on the open market.

Since vancomycin exerts its actions time dependently,[19] an ophthalmic ointment with high tissue retentivity is well suited for clinical use. Vancomycin Ophthalmic Ointment 1% is a product with good stability achieved by creating an ophthalmic ointment in which vancomycin is dispersed in an oily base.[20 21] This case series showed that Vancomycin Ophthalmic Ointment 1% is useful for the treatment of external ocular MRSA or MRSE infections.

In this study, the subjects were defined as patients in whom MRSA or MRSE was detected in a bacterial test, and moreover, whose symptoms did not improve after local treatment with fluoroquinolone eye drops. Due to such strict inclusion criteria, the number of patients enrolled is small. It was difficult to obtain participants in whom acutely severe infections occurred. In most of the hospitals involved in this study, vancomycin solutions prepared by in-house prescription had already been used for sight-threatening severe infections such as severe MRSA keratitis. Most of the cases in this study were chronic and/or prolonged mild infections in elderly patients.

Of the total 25 patients, 10 ADRs occurred in 7 (28%) patients, and all the ADRs occurred at the local administration site. The main ADRs were eyelid oedema in three (12%) patients and conjunctival hyperaemia in three (12%) patients. All of the ADRs were confirmed to have resolved after the study completion. In terms of the systemic distribution following the administration of vancomycin ophthalmic ointment, plasma concentrations after administration were below the detection limit in all subjects in the phase I study. Vancomycin ophthalmic ointment was presumably a product that would be quite unlikely to cause systemic ADRs based on its pharmacokinetics.

The proportion of MRSA in conjunctival bacterial flora is reportedly high in elderly individuals and in patients with atopic dermatitis or neonates.[22–24] Postoperative endophthalmitis or keratitis can occur in these MRSA carriers, and the application of vancomycin in conjunctival MRSA carriers might be effective in preventing MRSA infections.

There has been concern about the growing resistance of *S aureus* to vancomycin.[25] Particular attention should be paid to not facilitate the growth of bacterial resistance to vancomycin. MRSA isolated from ocular infections is often susceptible to chloramphenicol, fourth-generation fluoroquinolones and other antibiotics.[15 26] Thus, it is preferable to use Vancomycin Ophthalmic Ointment 1% only for a short period of time and only for patients who specifically require this new drug.

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Competing interests YS is an employee of Toa Pharmaceuticals Co Ltd.

Patient consent Obtained.

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REFERENCES

1. Jevons MP, Coe AW, Parker MT. Methicillin resistance in staphylococci. *Lancet* 1963;1:904–7.
2. Maple PA, Hamilton-Miller JM, Brumfitt W. World-wide antibiotic resistance in methicillin-resistant Staphylococcus aureus. *Lancet* 1989;1:537–40.
3. Brennen C, Muder RR. Conjunctivitis associated with methicillin-resistant Staphylococcus aureus in a long-term-care facility. *Am J Med* 1990;88:14N–7N.
4. Sotozono C, Inagaki K, Fujita A, *et al.* Methicillin-resistant Staphylococcus aureus and methicillin-resistant Staphylococcus epidermidis infections in the cornea. *Cornea* 2002;21:S94–101.
5. Forster W, Becker K, Hungermann D, *et al.* Methicillin-resistant Staphylococcus aureus keratitis after excimer laser photorefractive keratectomy1. *J Cataract Refract Surg* 2002;28:722–4.
6. Shanmuganathan VA, Armstrong M, Buller A, *et al.* External ocular infections due to methicillin-resistant Staphylococcus aureus (MRSA). *Eye* 2005;19:284–91.
7. Sahu DN, Thomson S, Salam A, *et al.* Neonatal methicillin-resistant Staphylococcus aureus conjunctivitis. *Br J Ophthalmol* 2006;90:794–5.
8. Wroblewski KJ, Pasternak JF, Bower KS, *et al.* Infectious keratitis after photorefractive keratectomy in the United States army and navy. *Ophthalmology* 2006;113:520–5.
9. Solomon R, Donnenfeld ED, Perry HD, *et al.* Methicillin-resistant Staphylococcus aureus infectious keratitis following refractive surgery. *Am J Ophthalmol* 2007;143:629–34.
10. Shimada H, Nakashizuka H, Hattori T, *et al.* Incidence of endophthalmitis after 20- and 25-gauge vitrectomy causes and prevention. *Ophthalmology* 2008;115:2215–20.
11. Maffett M, O'Day DM. Ciprofloxacin-resistant bacterial keratitis. *Am J Ophthalmol* 1993;115:545–6.
12. Cimolai N. Ciprofloxacin and multiresistant staphylococci. *Lancet* 1997;349:1030.
13. Marangon FB, Miller D, Muallem MS, *et al.* Ciprofloxacin and levofloxacin resistance among methicillin-sensitive Staphylococcus aureus isolates from keratitis and conjunctivitis. *Am J Ophthalmol* 2004;137:453–8.
14. Cavuoto K, Zutshi D, Karp CL, *et al.* Update on bacterial conjunctivitis in South Florida. *Ophthalmology* 2008; 115:51–6.
15. Elsahn AF, Yildiz EH, Jungkind DL, *et al.* In vitro susceptibility patterns of methicillin-resistant Staphylococcus aureus and coagulase-negative staphylococcus corneal isolates to antibiotics. *Cornea* 2010;29:1131–5.
16. Goodman DF, Gottsch JD. Methicillin-resistant staphylococcus epidermidis keratitis treated with vancomycin. *Arch Ophthalmol* 1988;106:1570–1.
17. Fleischer AB, Hoover DL, Khan JA, *et al.* Topical vancomycin formulation for methicillin-resistant Staphylococcus epidermidis blepharoconjunctivitis. *Am J Ophthalmol* 1986;101:283–7.
18. Fujita A, Sotozono C, Inatomi T, *et al.* Severe MRSA infection of the eye treated with vamcomycin ointment. *Atarashii Ganka* 2000; 17:93–5.
19. Chua K, Howden BP. Treating Gram-positive infections: vancomycin update and the whys, wherefores and evidence base for continuous infusion of anti-Gram-positive antibiotics. *Curr Opin Infect Dis* 2009;22:525–34.
20. Fukuda M, Hanazome I, Sasaki K. The intraocular dynamics of vancomycin hydrochloride ophthalmic ointment (TN-011) in rabbits. *J Infect Chemother* 2003;9:93–6.
21. Eguchi H, Shiota H, Oguro S, *et al.* The inhibitory effect of vancomycin ointment on the manifestation of MRSA keratitis in rabbits. *J Infect Chemother* 2009;15:279–83.
22. Kato T, Hayasaka S. Methicillin-resistant Staphylococcus aureus and methicillin-resistant coagulase-negative staphylococci from conjunctivas of preoperative patients. *Jpn J Ophthalmol* 1998;42:461–5.
23. Nakata K, Inoue Y, Harada J, *et al.* A high incidence of Staphylococcus aureus colonization in the external eyes of patients with atopic dermatitis. *Ophthalmology* 2000;107:2167–71.
24. Hori Y, Nakazawa T, Maeda N, *et al.* Susceptibility comparisons of normal preoperative conjunctival bacteria to fluoroquinolones. *J Cataract Refract Surg* 2009;35:475–9.
25. Hiramatsu K, Aritaka N, Hanaki H, *et al.* Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. *Lancet* 1997;350:1670–3.
26. Fukuda M, Ohashi H, Matsumoto C, *et al.* Methicillin-resistant Staphylococcus aureus and methicillin-resistant coagulase-negative Staphylococcus ocular surface infection efficacy of chloramphenicol eye drops. *Cornea* 2002;21:S86–9.

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Vancomycin Ophthalmic Ointment 1% for methicillin-resistant *Staphylococcus aureus* or methicillin-resistant *Staphylococcus epidermidis* infections: a case series

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Immunohistochemical analysis of inflammatory limbal conjunctiva adjacent to Mooren's ulcer

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ABSTRACT

Background/aims To examine the characteristics of infiltrating cells in conjunctival tissues adjacent to the peripheral corneal ulcers of Mooren's ulcer.

Methods This study involved four eyes of four patients with Mooren's ulcer and who were considered to be in need of surgical treatment. The patients' resected conjunctival tissues were embedded and frozen. The tissue sections were then subjected to H&E and immunohistochemical staining. The stained sections were observed and the characteristics of the infiltrating cells in the conjunctival tissues were pathologically examined.

Results In all patients, infiltration of inflammatory cells was observed in the submucosal connective tissue of the conjunctiva. Immunohistochemical analysis revealed inflammatory cell infiltration into the submucosal layer of the conjunctiva that was mainly composed of CD3-positive and CD45RO-positive cells. Some of these cells also showed positive reactivity with CD4, yet very few cells showed positive reactivity with CD8. In addition, infiltration of the cells indicating CD68 positivity was frequent in a few cases.

Conclusions In the four Mooren's ulcer cases, infiltrating cells in the submucosa of the conjunctival tissues adjacent to the ulcerative cornea were found to be mainly composed of helper T lymphocytes and macrophages. Our results show that helper T cells and macrophages contribute to the pathogenesis of Mooren's ulcer.

INTRODUCTION

Mooren's ulcer (rodent corneal ulcer) is a rare disorder first described by Mooren in 1867[1] involving chronic and painful ulceration of the cornea.[2] It occurs in the absence of any systemic disorder such as collagen diseases. The ulcerative lesion with overhanging edges typically starts on the periphery of the cornea and tends to spread progressively to the entire circumference or towards the centre of the cornea.[2–5] In such cases, severe inflammation sometimes occurs on the ocular surface, progresses rapidly and may cause corneal perforation. Since Mooren's ulcer is a rare disorder, the aetiology or mechanisms of pathogenesis remain uncertain. Topical administration of corticosteroids[6] and systemic[7 8] or topical[6 9] administration of immunosuppressive agents such as cyclosporin A are commonly used as a conservative treatment for the disorder. However, for cases that are resistant to such medical treatments, various surgical treatments such as peritectomy or keratoepithelioplasty[10] are indicated.

A few studies have reported that autoimmunity[11 12] is involved in Mooren's ulcer. Histopathologically, inflammatory cell infiltration is observed in the cornea and conjunctiva adjacent to Mooren's ulcer.[2 13] Moreover, steroid and/or immunosuppressive therapies have been shown to be effective. Therefore, it is considered that the primary pathogenesis of Mooren's ulcer is an immunological reaction. However, few reports have focused on examining the pathological findings of this disease in detail.

To elucidate the pathology of Mooren's ulcer, in this study we examined the characteristics of the infiltrating cells in the conjunctival tissues adjacent to the peripheral corneal ulcers using an immunohistochemical technique.

MATERIALS AND METHODS

Patients

This study involved four eyes of four patients (two men and two women; age range: 56–82 years) who were diagnosed with Mooren's ulcer at the Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan. These patients were considered to be in need of surgical treatment because they were resistant to the systemic administration of betamethasone and cyclosporin A, and the topical administration of betamethasone. Macroscopic images of the ocular surface in these four patients are shown in figure 1.

Limbal conjunctival tissues adjacent to the ulcerative lesions resected during each patient's surgery were used for this study. As a control, the conjunctival tissues resected at the time of surgery for one woman (79 years of age) with conjunctivochalasis (CCh) were also used.[14]

This research was approved by the Committee for Ethical Issues on Human Research, Kyoto Prefectural University of Medicine and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients after they had received a detailed explanation of the procedures.

Histological and immunohistochemical analysis

Preparation of the sections and H&E staining

The resected conjunctival tissues were embedded in Tissue Tek OCT compound (Sakura Finetek Japan Co., Tokyo, Japan) and snap frozen in liquid nitrogen. Next, serial sections approximately 5 μm in thickness were cut using a cryostat (CM3050S; Leica Biosystems Nussloch GmbH., Nussloch, Germany). The sections were then placed on aminosilane-coated glass slides (MAS slide glass; Matsunami Glass Ind., Osaka, Japan), air dried, fixed with Zamboni's fixative (phosphate buffer containing 2% paraformaldehyde and 0.19% picric acid) for 1 min, and subjected to standard H&E staining.

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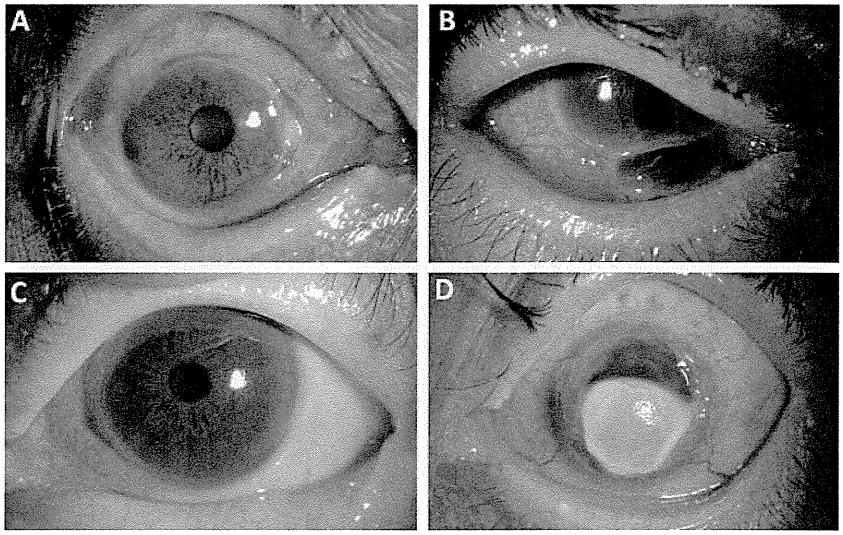

Figure 1 Macroscopic images of the patients' ocular surface. (A) Case 1, the right eye of a 60-year-old man. (B) Case 2, the right eye of a 56-year-old man. (C) Case 3, the left eye of an 82-year-old woman. (D) Case 4, the left eye of a 64-year-old woman. This figure is only reproduced in colour in the online version.

Immunohistochemical staining

The prepared tissue sections were subjected to an indirect immunohistochemical staining. In brief, the sections were fixed with Zamboni's fixative for 10 min at 4°C. They were then washed in 0.01 mol/litre phosphate buffered saline (PBS), preincubated with PBS containing 2% bovine serum albumin (Nacalai Tesque Inc., Kyoto, Japan) at room temperature (RT) to eliminate any non-specific reaction, and continuously diluted primary antibody solutions (table 1) were applied to the sections for approximately 16 h at 4°C. To confirm specificity of the immunohistochemical staining, diluted solutions of normal mouse IgG_1 or mouse IgG_{2a} (Dako Japan, Tokyo, Japan) were applied instead of the primary antibody solutions. The sections were then washed with PBS and immersed in methanol containing 0.3% H_2O_2 for 30 min at RT to eliminate endogenous peroxidase activity. The sections were washed again with PBS and peroxidase conjugated secondary antibody (Histofine Simple Stain MAX-PO MULTI; Nichirei BioSciences Inc., Tokyo, Japan) was applied for 45 min at RT. The sections were then washed with PBS and purified water, and incubated with 3, 3′-diaminobenzidin solution (Peroxidase Substrate Kit DAB; Vector Laboratories, Inc., Burlingame, California, USA) for 1–2 min to visualise the immunoreaction. After counterstaining was performed with haematoxylin, the sections were dehydrated and mounted.

Analysis

The H&E and immunohistochemical stained sections were observed by light microscopy (AX-70; Olympus Corporation, Tokyo, Japan) and pathologically examined. Images of the sections were obtained using a CCD camera (DP50; Olympus).

RESULTS

H&E staining

In all four cases of Mooren's ulcer in this study, infiltration of small to slightly large-sized round-shaped cells was observed in the submucosal connective tissue of the conjunctiva (figure 2A–D). In case 1 (figure 2A) and case 2 (figure 2B), round-shaped cells and a number of slightly large-sized cells with many vacuoles were found to have been infiltrated. In case 3 (figure 2C), severe fibrosis and necrotic change was observed in the submucosa of the lesion site of the conjunctiva. In the tissue obtained from the female patient with CCh (figure 2E), no remarkable changes were observed in the mucosa or submucosa of the conjunctiva.

Immunohistchemical staining

The results of the immunohistochemical staining are shown in table 2. In all four cases, inflammatory cell infiltration mainly composed of CD3-positive cells (figure 3A,E,I,M) and

Table 1 List of primary antibodies

Antibody	Maker	Immunised animal/clonality	Subtype of immunoglobulin	Clone name	Cat. No.	Specificity
CD3	Dako Japan	Mouse Monoclonal	IgG1-k	T3-4B5	M0756	Pan T cell (mature)
CD4	Dako Japan	Mouse Monoclonal	IgG1-k	MT310	M0716	Helper/inducer T cell, monocyte
CD8	Dako Japan	Mouse Monoclonal	IgG1-k	C8/144B	M7103	Suppressor/cytotoxic T cell
CD20cy	Dako Japan	Mouse Monoclonal	IgG2a-k	L26	M0755	Pan B cell (except plasma cell)
CD45RO	Dako Japan	Mouse Monoclonal	IgG2a-k	UCHL1	M0742	Pan T cell
Mast cell tryptase	Dako Japan	Mouse Monoclonal	IgG1-k	AA1	M7052	Mast cell
Neutrophil elastase	Dako Japan	Mouse Monoclonal	IgG1-k	NP57	M0752	Neutrophil, monocyte
CD68	Dako Japan	Mouse Monoclonal	IgG1-k	KP1	M0814	Macrophage, histiocyte

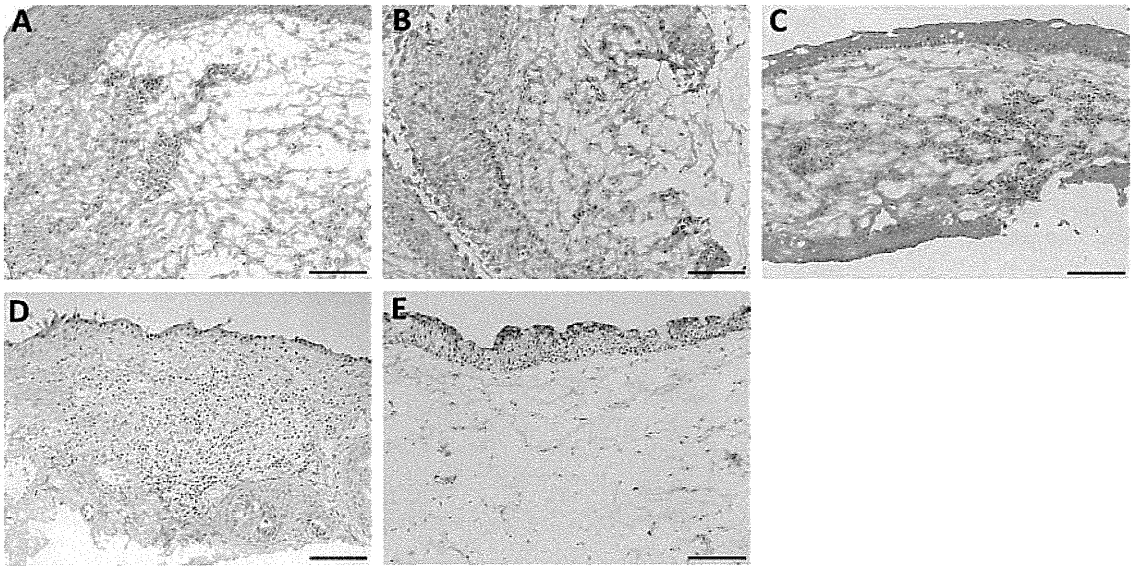

Figure 2 H&E staining images. (A) Case 1, (B) case 2, (C) case 3, (D) case 4 and (E) control with conjunctivochalasis (CCh). Some case-specific differences of grade can be seen, and small to slight large-sized round-shaped cells infiltrate into the submucosal connective tissue of the conjunctiva in all cases. (A) In case 1, infiltration of slight large-sized cells which have many vacuoles can be observed. (E) In CCh, no remarkable changes can be seen. Magnification ×200. Scale bar 100 μm. This figure is only reproduced in colour in the online version.

CD45RO-positive cells (figures not shown) was observed in the submucosal layer of the conjunctiva. These cells were characterised as T lymphocytes due to the pattern of their immunoreactivity. Some of these cells also showed positive reactivity with CD4 (figure 3B,F,J,N), although very few cells showed positive reactivity with CD8 (figure 3C,G,K,O). In addition, T lymphocytes and the infiltration of cells indicating CD3 negativity and CD4 positivity were frequent in case 1 (figure 3A,B). These cells were thought to be macrophages because, as with the large cells, they showed vacuolisation of their cytoplasm on H&E staining and positive reactivity with CD68. In addition, during immunohistochemical analysis, a large number of macrophages showing positive reactivity with CD68 were observed in case 1 (figure 3D) and also in case 2 (figure 3H), case 3 (figure 3L) and case 4 (figure 3P). In contrast, a small number of CD20cy-positive, mast cell tryptase-positive or neutrophil elastase-positive cells were observed in all four cases, and there were no specific localisation patterns (figures not shown).

In the CCh specimen, a small number of cells showed positive reactivity to antibodies (figure 3Q–T). In the negative control, sections stained using normal mouse IgG_1 or IgG_{2a} showed no positive reactivity (figure 3U,V).

In summary, in the four cases of Mooren's ulcer in this study, infiltrating cells in the submucosa of the conjunctival tissues adjacent to the ulcerative cornea were mainly composed of CD3-positive, CD45RO-positive and CD4-positive helper T lymphocytes and CD68-positive macrophages, whereas the infiltration of B lymphocytes, neutrophils and mast cells was minimal.

Table 2 Results of immunohistochemistry

Antibody/cases	1	2	3	4	CCh
CD3	+*	+	++	++	+/–
CD4	++	+	+/–	+	+/–
CD8	+/–	+/–	+/–	+/–	+/–
CD20cy	+/–	+/–	+/–	+/–	+/–
CD45RO	++	++	+	++	+/–
Mast cell tryptase	+/–	+/–	–	+/–	+/–
Neutrophil elastase	+/–	+	+/–	+/–	+/–
CD68	++	+	+	+	+/–

*Infiltration of inflammatory cells was scored as follows: –, no positive cells are observed; +/–, a small number of positive cells are observed; +, a large number of positive cells are observed; ++, any of numerous positive cells are observed, and/or aggregations of numerous positive cells are observed.
CCh, conjunctivochalasis.

DISCUSSION

In the four patients with Mooren's ulcer in this study, infiltrating cells in the submucosa of the conjunctival tissues adjacent to the ulcerative cornea were found to be mainly composed of CD3-positive, CD45RO-positive and CD4-positive helper T lymphocytes and CD68-positive macrophages. In addition, the CD4-positive ratio of infiltrating T lymphocytes was clearly higher than the CD8-positive ratio. However, a small number of CD20cy-positive, mast cell tryptase-positive and neutrophil elastase-positive cells were observed in the submucosa of the conjunctival tissues but there were no characteristic patterns.

Wang *et al*[15] previously reported that in the adjacent bulbar conjunctiva of Mooren's ulcer the CD4/CD8 ratio is significantly higher than in normal controls, which is consistent with our results.

It is known that immunosuppressive reagents such as cyclosporin A are effective for the treatment of Mooren's ulcer.[6–9] The primary effective treatment mechanism of cyclosporin A is the inhibition of the activation of helper T cells by suppressing the production of inflammatory cytokines such as interleukin-2 by binding to calcineurin.[16 17] In the inflammatory lesion of Mooren's ulcer, it is thought that cyclosporin A inhibits the function of helper T lymphocytes and stimulates suppressor/cytotoxic T lymphocytes.[7–9] Therefore, the helper T lymphocytes are more likely to participate in Mooren's ulcer.

In this study, infiltration of macrophages was also observed in the conjunctival submucosa. Since infiltration of T lymphocytes

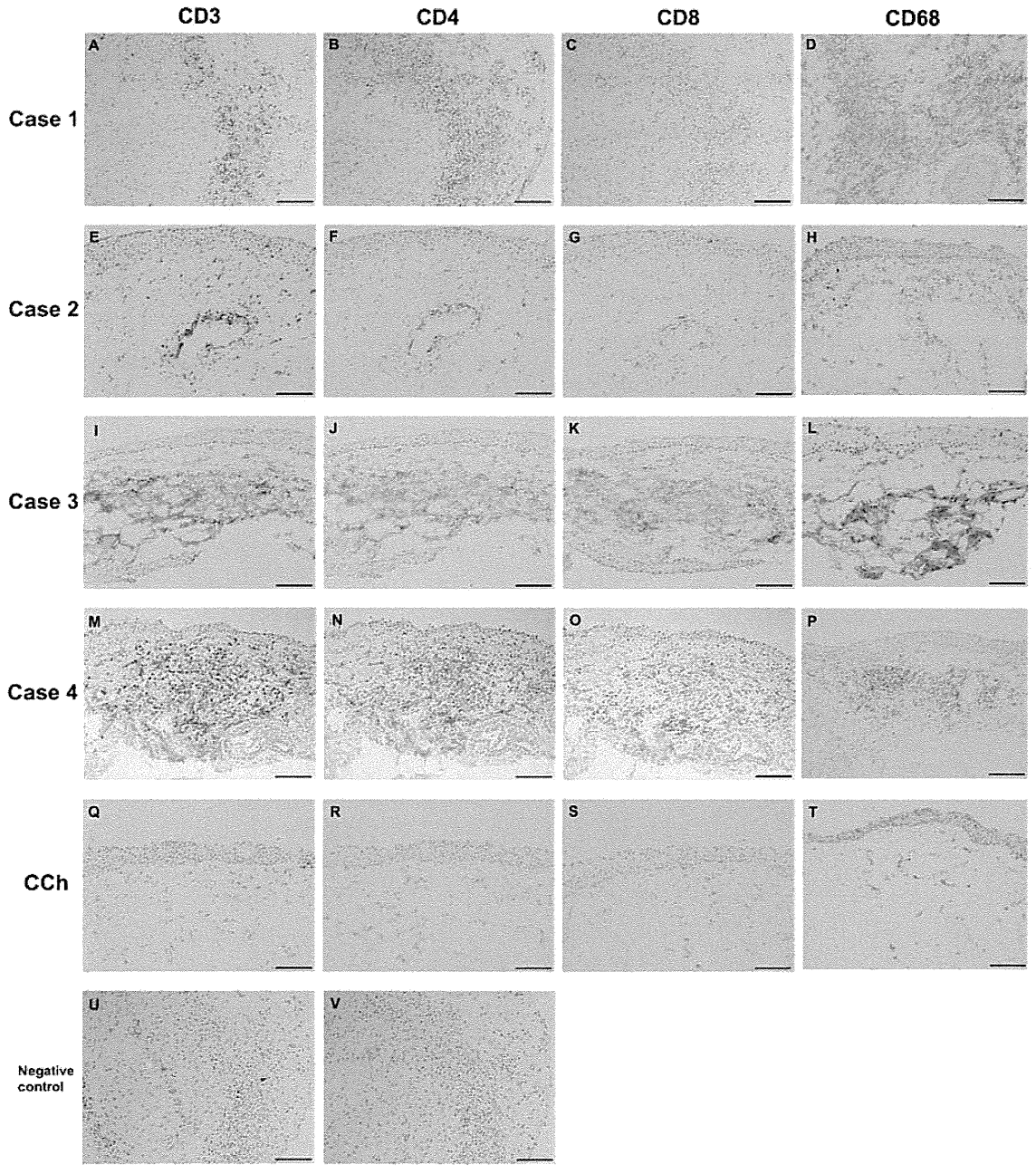

Figure 3 Immunohistochemical staining images (CD3, CD4, CD8 and CD68). (A–D and U, V) Case 1, (E–H) case 2, (I–L) case 3, (M–P) case 4, (Q–T) control with conjunctivochalasis (CCh). (A, E, I, M) Submucosal infiltrating cells show positive reactivity with CD3 in all cases. (B, F, J, N) Some CD3-positive cells also show positive reactivity with CD4. (C, G, K, O) A small number of cells show positive reactivity with CD8. (D, H, L, P) Many submucosal infiltrating cells show positive reactivity with CD68, indicating that they are macrophages. (D) In case 1, a large number of CD68-positive cells form a granulomatous lesion in the submucosa. (Q, R, S, T) In the control with CCh, only a small number of cells show positive reactivity with CD3, CD4, CD8 and CD68. (U,V) Negative controls, no positive reactivity is observed. Magnification ×200. Scale bar 100 μm. This figure is only reproduced in colour in the online version.

and macrophages was observed in the Mooren's ulcer lesion site, it seems that some abnormalities of the immune system are involved in the pathogenesis of the disorder. Previous reports have shown that an autoantibody against cornea-associated antigen was significantly increased in the serum of patients with Mooren's ulcer.[12]

As for the four cases involved in this study, systemic or topical treatments were applied consecutively. The histological or immunohistochemical findings may undergo various modifications with treatment. At a minimum, it can be posited that the infiltration of helper T lymphocytes and macrophages might be related to the pathogenesis of Mooren's ulcer.

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