は，しばしば下痢が長期化・重症化し，致死的となることもある[12]。いずれの場合も感染者の糞便には数週間にわたってオーシストの排出が続く。わが国においては全数把握が義務づけられた五類感染症である。

1907年，Tyzzerにより実験用マウスの胃より発見されたのがクリプトスポリジウムに関する最初の記録である[11]。1971年にヒトでの感染によりふたたび注目され，1976年には世界保健機関（WHO）により新興・再興感染症に指定された。クリプトスポリジウム症は全世界に分布してお

り，代表的な集団感染としては1994年，米国ミルウォーキーでの403,000人，1996年，埼玉県越生町での9,140人の集団感染があり，いずれも水系感染であった。人獣共通感染症でもあり，仔牛を中心とした体重減少による経済的な損失が問題視されている。

近年，米国ではバイオテロリズム関連の研究と基盤整備を強化しており，クリプトスポリジウムは米国国立衛生研究所（NIH）によりBiodefense Category Bの病原体にあげられている。また，わが国においてもクリプトスポリジウムは寄生原虫
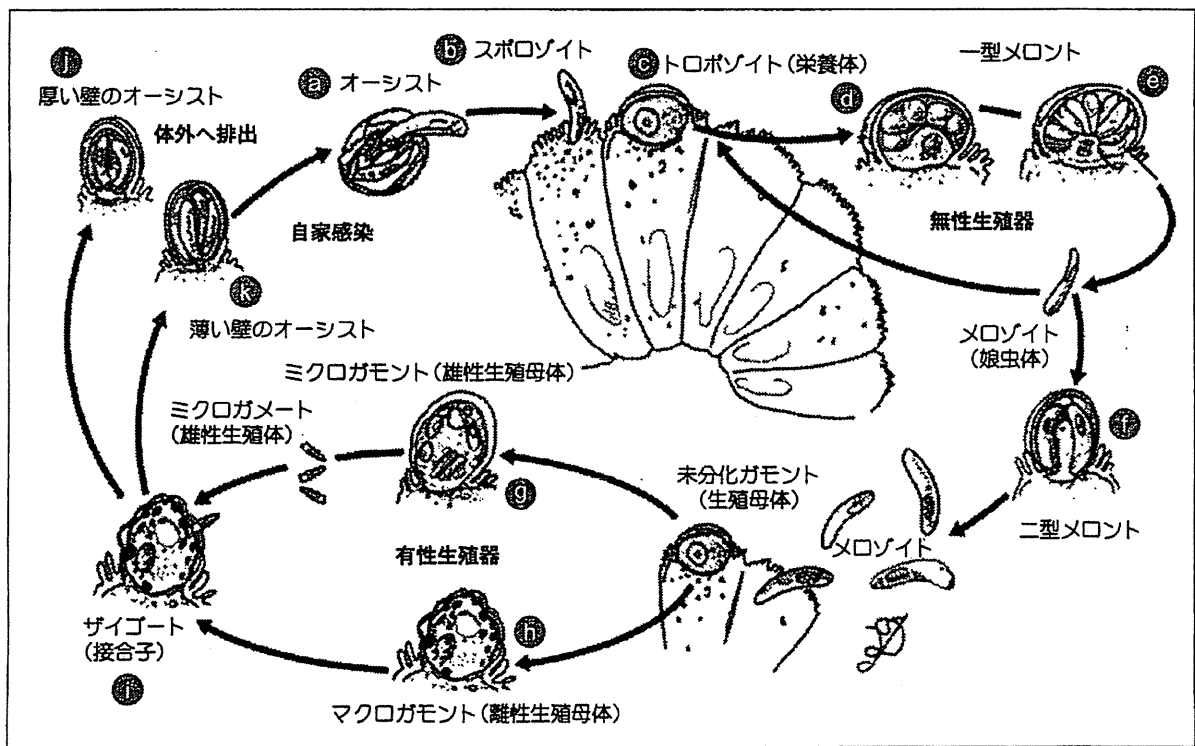

図3　クリプトスポリジウムの生活環

接合子嚢であるオーシストは経口感染後，温度・酸・胆汁酸によりオーシスト壁の縫合膜が刺激されて内部の4個のスポロゾイトを放出する（脱嚢）。スポロゾイトは胃腸上皮細胞に感染し無性生殖（メロゴニー）を開始する。トロホゾイト（栄養体），メロントを経て，I型メロントは8個のメロゾイト（娘虫体）を形成し，ふたたび他の上皮細胞に感染する。メロントの一部はII型メロントとなり4個のメロゾイトを形成して有性生殖（ガメトゴニー）を開始する。メロゾイトはミクロガモント（雄性生殖母体）またはマクロガモント（雌性生殖母体）へと性分化する。ミクロガモントは16個のミクロガメート（雄性生殖体）に分離し，細胞外へと放出された後にマクロガモントと受精しザイゴート（接合子）となる。ザイゴートは成長してオーシストとなり，その80%は宿主外に排出され，残りの20%は同一個体内での自家感染をくり返す。

（文献15より）

NIH（米国国立衛生研究所）

表　ヒトにおけるクリプトスポリジウムの感染症例数

国＼年	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
日本[16]	4	3	11	109	8	92	12	18	6	10
欧州[17]	6,456	7,833	6,389	4,940	8,413	6,164	7,960	6,802	6,253	7,032
米国[18]	2,769	3,128	3,787	3,016	3,505	3,911	8,269	6,479	11,657	10,500

（文献 16 〜 18 より）

で唯一,「生物テロに使用される恐れのある病原体等であり, 国民の生命および健康に影響を与える恐れがある感染症の病原体」として四種病原体に指定されている。迅速な検査法・ゲノム情報による病原因子と薬剤標的分子の同定・薬剤開発が急務となっている。

1．病原体

病原体であるクリプトスポリジウムはマラリア原虫やトキソプラズマの属するアピコンプレックス門に属し, 2010 年現在で 19 種が発見されている[14]。ヒトでの感染が問題となっているのは *Cryptosporidium parvum* および *C. hominis* である。クリプトスポリジウムは無性生殖期と有性生殖期をくり返しながら増殖し（図3）[15], 媒介昆虫（ベクター）は介さず, 中間宿主も報告されていない。

2．疫　学

ヒトでの感染について表[16]〜[18]にまとめた。わが国では 1999 〜 2008 年は年 3 〜 18 例の報告で推移しているが, 2002 年および 2004 年のみ 109 例, 92 例の報告があった。これはそれぞれ, 北海道における 100 例, 長野における 80 例の集団感染があったためである。国外に目を向けると 1999 〜 2008 年での症例数は, 欧州では年 4,940 〜 8,413 例, 米国では年 2,769 〜 11,657 例とより多くみられる。わが国の国土面積が狭いことに加えて, 1996 年の埼玉県越生町での集団感染を受けて, ろ過処理と紫外線処理が推奨されたことが要因であろう。

一方, 途上国での同症の罹患率は相変わらず高い。世界では下痢症により毎年 200 万人の子どもが命を落とすが, その 20％をクリプトスポリジウム症が占める地域もある[19]。このような背景から, わが国におけるクリプトスポリジウム症は輸入感染症の色合いが濃い。

3．診　断

クリプトスポリジウムの診断は検便によりオーシストを検出することによる。全国の衛生研究所および国立感染症研究所で作成したクリプトスポリジウム検出マニュアル(http://www.nih.go.jp/niid/para/atlas/japanese/manual/cryptosporidium.pdf)によると, 急性期の患者便には多数のオーシストが排出されるが, オーシストは 5 μm と小さいために通常の顕微鏡観察では見落とされる危険性が高い。したがって, 遠心沈殿法（MGL 変法）や浮遊法, 密度勾配遠心法（遠心浮遊法）によりオーシストの濃縮・精製を行い, さらに得られた試料を, 蛍光抗体染色, 抗酸染色, ネガティブ染色などで染色して観察に供することが推奨されている。もっとも高い検出感度が期待される方法は蛍光抗体染色であり, 簡便な染色用キットが市販されているものの, 検査試薬として未承認のため保険適用外である。集団感染などにおける感染経路や汚染源の特定には分子疫学的手法が活用されている。

4．治　療

軽度の下痢症に対しては対症療法として十分な水分摂取, 電解バランスの維持（補液管理）を行う。重度の下痢症に対しては食餌制限の上, 鎮痙

MGL（遠心沈殿法）

AF（アスコフラノン）

AOX（alternative oxidase；シアン耐性酸化酵素）

剤・止瀉薬を使用する。免疫機能不全患者の長期間継続する下痢症に対しては，アジスロマイシン，クラリスロマイシン，リファブチンの経口投与が推奨されている。パロモマイシンがこれまで第一選択薬として用いられてきたが，現在製造中止であり，一般には入手困難である。

近年，抗赤痢アメーバ症薬剤であるメトロニダゾールの類縁体であるニタゾキサニドが有効であることが示された。本薬剤は赤痢アメーバにおけるメトロニダゾールと同様の作用機序により原虫の増殖を抑制するが，AIDS にともなうクリプトスポリジウム症よりも小児の場合のほうが効果は高い。パロモマイシンおよびニタゾキサニドは熱帯病治療研究班から入手することができるが，治癒率はいずれも 6〜7 割である。AIDS の場合，慢性化・重症化する前にこれらの薬剤投与を行うことにより症状改善や完治が可能な例があるため，早期診断・早期治療が重要である [20]。免疫機能が回復すれば本症も寛解する場合があるため，AIDS 患者では抗 HIV 療法が欠かせない。

近年，Babel-24，パモ酸ピルビニウム，ネッカリッチ等有効な化合物が見出されているが [21)〜23)]，実用化までには至っていない。筆者らはアフリカ睡眠病の原因原虫：*Trypanosoma brucei* がもつミトコンドリアのシアン耐性酸化酵素（alternative oxidase：AOX），その阻害剤であるアスコフラノン（AF）に着目した研究を行っており，クリプトスポリジウムも AOX を有することを見出した [24]。AOX は植物・菌類・緑藻類・原虫・線虫の一部に存在し，ヒトには存在しないため，選択毒性をもつ格好の薬剤標的酵素である。アフリカトリパノソーマ原虫においては，組換え酵素・原虫の増殖に対する阻害効果に加えて，ヤギに対する治癒効果も認められており，実用化に向けた準備が進められている。

一方，クリプトスポリジウムに対しても，組換え酵素および原虫の増殖に対する阻害効果，マウスでの治癒効果を確認している。また，赤痢アメーバやクリプトスポリジウムは，マイトソームと呼ばれるミトコンドリアと共通の祖先に由来する DNA を失ったオルガネラをもつことが明らか

になっているが，標的酵素の解析のためにクリプトスポリジウムのマイトソームの単離法を確立し，AOX がマイトソームに局在することを見出した（投稿準備中）。クリプトスポリジウム症治療への AF の実用化とともに，特殊なオルガネラであるマイトソームを標的とした創薬が期待されている。

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Roles Played by Toll-like Receptor-9 in Corneal Endothelial Cells after Herpes Simplex Virus Type 1 Infection

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PURPOSE. To determine the roles played by toll-like receptor 9 (TLR9) in cultured human corneal endothelial (HCEn) cells after herpes simplex virus type 1 (HSV-1) infection and to characterize the TLR9-mediated antiviral responses.

METHOD. Immortalized HCEn cells were examined for TLR expression. The upregulation of inflammatory cytokines after HSV-1 infection was determined by real-time RT-PCR or protein array analyses. The TLR9-mediated HSV-1 replication was determined by real-time PCR and plaque assay. To determine whether there was an activation of the signal transduction pathway, HCEn cells that were transfected with pathway-focused transcription factor reporters were examined for promoter activity.

RESULTS. TLR9 was abundantly expressed intracellularly in HCEn cells. The CpG oligonucleotide, a TLR9 ligand, stimulated the NF-κB activity in HCEn cells. HSV-1 infection also stimulated NF-κB and induced NF-κB -related inflammatory cytokines, including RANTES, IP-10, MCP-2, MIF, MCP-4, MDC, MIP-3α, IL-5, TARC, MCP-1, and IL-6. The induction of these cytokines was significantly reduced by blocking the activity of TLR9. In addition, viral replication in HCEn cells was significantly reduced by the inhibition of TLR9, but was preserved by a concomitant activation of the NF-κB cascade. Of the different HSV-1–induced inflammatory cascade–related transcription factors, TLR9 was found to activate NF-κB, cyclic AMP response element (CRE), and the CCAAT-enhancer-binding proteins (C/EBP) the most.

CONCLUSIONS. Corneal endothelial cells transcriptionally initiate inflammatory programs in response to HSV-1 infection related to NF-κB, CRE, and C/EBP and express arrays of inflammatory cytokine induction by TLR9. On the other hand, HSV-1 exploits TLR9-mediated NF-κB activation for its own replication. (*Invest Ophthalmol Vis Sci.* 2011;52:6729–6736) DOI:10.1167/iovs.11-7805

The tissues of the ocular surface help maintain the clarity of the cornea and protect the eye from numerous environmental pathogens or dead cell constituents. The endothelial cells lining the inner surface of the cornea are also responsible for maintaining the optical clarity of the cornea. Because endothelial cells do not replicate in vivo, a decrease in their density can lead to blinding bullous keratopathy.[1,2]

Generally, mucosal surfaces are armed with pattern-recognition receptors (PRRs) for sensing foreign materials. The PRRs recognize various types of ligands, such as bacterial and fungal cell wall components, bacterial lipoproteins, and nucleic acids derived from bacteria, virus, and self.[3] An invasion by viruses is recognized by the toll-like receptor (TLR) family, and the recently recognized categories of intracellular PRRs that detect nucleic acids in the cytoplasm.[4] The retinoic acid-inducible gene I (*RIG-I*) and melanoma differentiation-associated gene 5 (*MDA5*) detect the RNA of pathogens. DNA-dependent activator of interferon-regulatory factors (*DAI*), absent in melanoma 2 (*AIM2*), RNA polymerase III, leucine-rich repeat flightless-interacting protein 1 (*LRRFIP1*), and interferon γ–inducible protein 16 (*IFI16*) detect intracellular DNA.

The TLRs were the first discovered and major category of PRRs. The recognition of pathogens by TLRs leads to the induction of innate immunity, inflammation, and adaptive immunity. The TLRs on the mucosal body surface function not only to keep bacteria from invading the body but also to form mutually beneficial relationships.[5] TLRs recognize commensal or pathogen-associated molecular patterns to control the function of the mucosal surface cells. For example, the TLRs regulate the proliferation of epithelial cells after intestinal injury. An absence of TLRs significantly impairs the repair of the epithelial barrier.[5] Signaling by the TLRs leads to increased inflammation and promotes the development of inflammation-associated neoplasia.[6] Thus, intricate interactions operate for the host and microbes by the many functions of the TLRs.

Corneal endothelial cells have been recently found to act as immune modulators that suppress T cell receptor-mediated CD4^{+} T cell proliferation. They also stimulate the conversion of CD8^{+} T cells into regulatory T cells.[7,8] These functions may contribute to the immune privilege of the eye. TLRs are especially recognized as important modulators of innate and acquired immunity. Thus, understanding how the endothelial cells behave after TLR stimulation may provide important clues on how to control immune-mediated diseases.

TLR9 is a well-known sensor of the nucleic acids of viruses and microbes. HSV-1 is the most common viral pathogen permissive to the corneal endothelial cells, and an infection by HSV-1 is manifested as herpetic keratitis. To recognize herpesvirus, the host uses a distinct repertoire of TLRs. First, the surface glycoproteins ligate to TLR2.[9,10] Second, the DNAs of herpesvirus which are rich in CpG sequences, stimulate TLR9.[11,12] And third, double-strand RNAs, generated through

self-hybridization of viral genes, activate TLR3.[13,14] TLR9 has been reported to be a crucial component in corneal epithelial cells that recognize the HSV-1 infection.[15,16] However, the roles played by TLRs in corneal endothelial cells have not been determined.

The activation of TLR9 can also cause collateral damage or exacerbation of immune-mediated diseases. For example, when self nucleic acids activate TLR9 chaperoned by anti-DNA autoantibody,[5] the TLR9 activation initiates or exacerbates autoimmune diseases.[17-19] Thus, understanding the roles played by TLR9 may help develop effective strategies to prevent unwanted inflammatory responses in the anterior chamber or corneal endothelium.

The purpose of this study was to determine the response of the TLR9 in cultured human corneal endothelial (HCEn) cells after herpes simplex virus type 1 (HSV-1) infection and to characterize the TLR9-mediated anti-viral responses. We shall show that HCEn cells express TLR9 intracellularly, and HSV-1 infection leads to the upregulation of arrays of inflammatory cytokines mediated by TLR9. Especially important was that the NF-κB cascade downstream of TLR9 can be hijacked by HSV-1 and diverted for its own replication.

MATERIALS AND METHODS

Cells

An HCEn cell line was established as described in detail.[7] The HCEn cells were propagated to confluence on 6- or 96-well plates in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. Primary corneal endothelial cells were obtained from the corneoscleral rims of donor eyes after the central cornea was used for keratoplasty.

The procedures used conformed to the tenets of the Declaration of Helsinki.

Viruses

Confluent monolayers of Vero cells were infected with the KOS strain of HSV-1 (generous gift from Kozaburo Hayashi, National Institutes of Health [NIH], Bethesda, MD) After 1 hour of adsorption, the medium containing the virus was aspirated, and the monolayers of cells were refed with fresh HSV-1-free medium. After attaining the maximum cytopathic effect (48–72 hours after infection), the medium was discarded, and cells with the small amount of remaining medium were frozen, thawed, and sonicated. The supernatant was collected after centrifugation at 3000 rpm for 10 minutes and overlaid onto a sucrose density gradient (10–60% wt/vol). The solution was centrifuged with a swing rotor (SW28; Beckman Instruments, Fullerton, CA) for 1 hour at 11,500 rpm. The resultant visible band at the lower part of the gradient containing the HSV-1 was washed using centrifugation at 14,000 rpm for 90 minutes and resuspended in a small volume of serum-free DMEM. The virus was then divided into aliquots and stored at −80°C until use. To infect the HCEn cells with HSV-1, the HCEn cells were adsorbed with the sucrose-density gradient purified virus stock for 1 hour at a multiplicity of infection (MOI) of 0.01 to 1, and refed with fresh medium.

Flow Cytometry

Flow cytometry was used to determine the degree of TLR expression using the following monoclonal antibodies (mAbs): TLR2 (Alexis, Plymouth Meeting, PA), TLR4 (Monosan, Uden, Netherlands), TLR3 (abCam, Cambridge, UK), and TLR9 (Oncogene, San Diego, CA). Mouse isotype IgG was used as the control. FITC-conjugated anti-mouse IgG_1 or IgG_2 (BD Pharmingen, Franklin Lakes, NJ) was used as the secondary antibody.

For flow cytometric analysis of the surface expression of TLRs on the HCEn cells, a suspension of subconfluent cells was obtained by adding 0.5% trypsin/EDTA to the HCEn cells and incubated with anti-TLR antibodies. This was followed by incubation with FITC-conjugated anti-mouse IgG (BD PharMingen). For intracellular staining of the TLRs, HCEn cell suspensions were permeabilized (Cytofix/Cytoperm; BD Biosciences) before staining. After they were washed twice in PBS, the stained cells (live-gated on the basis of the forward and side scatter profile and propidium iodide exclusion) were analyzed by flow cytometry.

Luciferase Reporter Assays

HCEn cells were transfected with luciferase reporter plasmids for AP-1, C/EBP, CRE, Elk-1, ISRE, NFAT, or NF-κB (Agilent, Santa Clara, CA). For the internal control, HCEn cells were co-transfected with pRL-CMV (Promega, Madison, WI; using Geneporter 3000 transfection reagent; Genlantis, San Diego, CA).

For inhibition of TLR-9, TLR-9 inhibitory oligonucleotide (forward 5'-TCCTGGCGGGGAAGT-3') (Alexis, San Diego, CA) or TLR-9 siRNA (Qiagen, Hilden, Germany) was used. For activation of the NF-κB cascade, the IκBα on the HCEn cells was inhibited by IκBα siRNA (Invitrogen, Carlsbad, CA). For transfection of siRNA, HCEn cells were transfected (RNAifect; Qiagen) 2 days after transfection of the reporter plasmids, according to the manufacturer's protocol. HCEn cells were infected with HSV-1 48 hours after siRNA transfection. The luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

The target sequences of the siRNA were TLR-9 siRNA: forward 5'-CGGCAACTGTTATTACAAGAA-3', and IκBα siRNA: forward 5'-GAGCTCCGAGACTTTCGAGGAAATA-3'.

Pharmacologic Inhibition of NF-κB Cascade

An IKK inhibitor peptide or control peptide (Merck, Darmstadt, Germany) was used to block the IκB kinase activity. The IKK inhibitor peptide contained a sequence corresponding to the active IκB phosphorylation recognition sequence. For inhibition of NF-κB p65, NF-κB p65 (Ser276) inhibitor peptide or control peptide (Imgenex, San Diego, CA) was used.

Real-Time RT-PCR

Total RNA was isolated from HSV-1-infected HCEn cells and reverse transcribed (QuantiTect Reverse Transcription Kit; Qiagen). The cDNAs were amplified and quantified on a thermal cycler (LightCycler; Roche, Mannheim, Germany) using a PCR kit (QuantiTect SYBR Green; Qiagen). The sequences of the real-time PCR primer pairs were VEGF: forward 5'-GCAGCTTGAGTTAAACGAACG-3', reverse 5'-GGTTCC-CGAAACCCTGAG-3'; IL-6: forward 5'-GATGAGTACAAAAGTCCT-GATCCA-3', reverse 5'-CTGCAGCCACTGGTTCTGT-3'; HSV-1 DNA polymerase: forward 5'-CATCACCGACCCGGAGAGGGAC-3', reverse 5'-GGGCCAGGCGCTTGTTGGTGTA-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-AGCCACATCGCTCAGA-CAC-3', reverse 5'-GCCCAATACGACCAAATCC-3'.

To ensure equivalent loading and amplification, all products were normalized to GAPDH transcript as an internal control.

Enzyme-Linked Immunosorbent Assay

To determine the levels of secreted IL-6, supernatants collected from HSV-1-infected HCEn cells were assayed with a commercial ELISA kit (Peprotech, Rocky Hill, NJ).

For inflammatory cytokine and chemokine profiling after HSV-1 infection, supernatants were collected from HCEn cells 12 hours post infection (pi) and assayed with human cytokine antibody arrays (RayBiotech, Norcross, GA). This analysis determined the level of expression of 80 cytokines and chemokines. The intensity of the chemiluminescence signals was digitized (LAS-1000plus; Fujifilm, Tokyo, Japan, and MultiGauge software ver.2.0; Fujifilm) and normalized by using the positive control signals in each membrane.

Pathways Analysis

The set of extracted genes was analyzed for transcriptional networks of molecular events using computerized pathway analysis (Pathways Analysis 7.0; Ingenuity Systems, Redwood, CA; based on the Ingenuity Pathways Knowledge Base). The resulting networks were evaluated by the significance scores, which were expressed as the negative logarithm of the *P* value. The obtained score indicate the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone.

Statistical Analyses

Data are presented as the mean ± SEM. Statistical analyses were performed using *t*-tests or ANOVA, as appropriate.

RESULTS

TLR9 Expression in HCEn Cells

We used flow cytometry to determine whether TLRs are expressed on HCEn cells grown in culture or primary HCEn cells, because TLRs can be expressed on the cell surface in nonhematopoietic lineage cells. No significant cell surface expression was observed (data not shown).

Next, we assessed the intracellular expression of TLRs by staining permeabilized HCEn cells and primary corneal endothelial cells. TLR9 was found to be significantly expressed intracellularly, whereas expression of TLR2, TLR3, and TLR4 was barely detectable (Fig. 1).

TLR9-Mediated NF-κB Promoter Activation in HCEn Cells after HSV-1 Infection

To determine whether the input from TLR9 is functional, we examined whether TLR9 ligand activates the NF-κB cascade, since NF-κB is the representative signaling cascade of TLR-mediated signaling. When TLR9 was stimulated with B class CpG oligonucleotide, a TLR9 ligand, there was a significant upregulation of NF-κB promoter activity, indicating that TLR9 is functional in HCEn cells (Fig. 2).

We next evaluated whether HSV-1 infection would activate the NF-κB cascade in HCEn cells and whether TLR9 plays a role in this activation. We found that HSV-1 infection significantly stimulated the promoter of NF-κB as early as 6 hours pi, and the level of expression continued to increase up to 12 hours pi (Fig. 3) The elevated NF-κB promoter activity was significantly

reduced by an inhibition of TLR9. Thus, the TLR9 cascade that stimulates NF-κB is activated after HSV-1 infection.
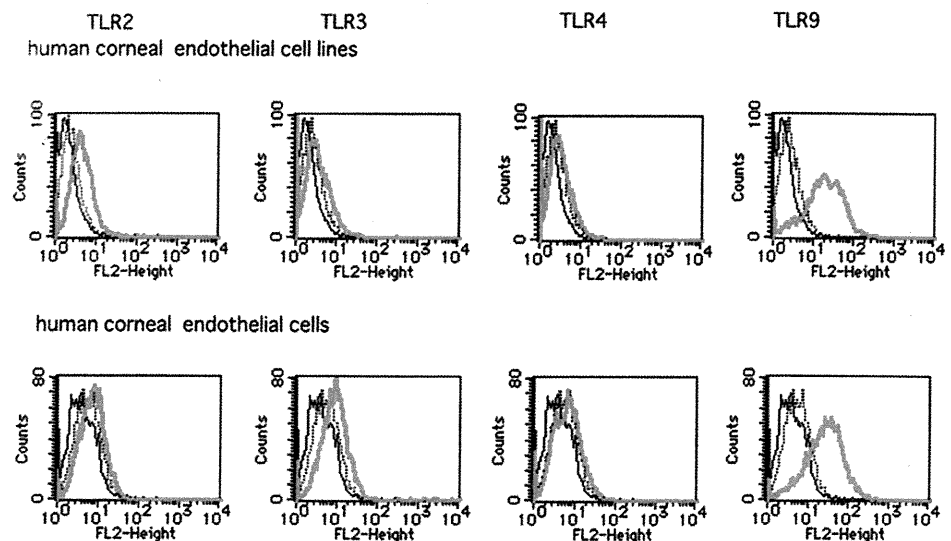
TLR9-Mediated Inflammatory Cytokine and Chemokine Induction in HCEn Cells after HSV-1 Infection

Next, we examined whether TLR9 is involved in the induction of cytokines and chemokines in HCEn cells after HSV-1 infection. After HCEn cells were infected with HSV-1, the level of IL-6 transcript was significantly increased at 12 hours pi (i.e., the IL-6 expression relative to GAPDH was $1.4 \times 10^{-4} \pm 1.0 \times 10^{-5}$ at an HSV-1 MOI of 0.1 and $2.0 \times 10^{-6} \pm 3.1 \times 10^{-7}$ for mock infection ($P < 0.01$). The level was lower at 24 hours pi: $1.1 \times 10^{-4} \pm 4.0 \times 10^{-6}$ IL-6/GAPDH at an HSV-1 MOI of 0.1 and $2.3 \times 10^{-6} \pm 3.3 \times 10^{-7}$ for mock infection.

To examine the contribution of TLR9 to the IL-6 induction, HCEn cells treated with TLR9 inhibitory oligonucleotide were infected with HSV-1 and assessed for IL-6 induction by real-time PCR. The HSV-1 infection significantly elevated IL-6 induction at 12 hours pi (Figs. 4A, 4B). The level of IL-6 after HSV-1 infection was significantly reduced by blocking TLR9 with a TLR9 inhibitory oligonucleotide (Fig. 4A). Inhibition of TLR9 by siRNA transfection also had a similar inhibitory effect on the IL-6 induction (Fig. 4B).

We then determined whether HSV-1 infection can stimulate IL-6 secretion through TLR9. When supernatants of HSV-1–infected HCEn cells were assessed for IL-6 by ELISA, we found that HSV-1 infection significantly stimulated IL-6 secretion (Figs. 4C, 4D). This HSV-1 infection–induced IL-6 secretion was significantly suppressed by a TLR9 inhibitory oligonucleotide in a dose-dependent manner (Figs. 4C, 4D).

Next, we assessed how HSV-1 infection modulated the cytokine and chemokine milieu of HCEn cells through TLR9. Supernatants from HSV-1–infected HCEn cells were assayed for 80 cytokines and chemokines using protein array analysis and were tested for their sensitivity to TLR9 inhibition. Twenty cytokines and chemokines were significantly upregulated after HSV-1 infection, and of them,TLR9 inhibition significantly reduced the upregulation of RANTES (CCL5), IP-10 (CXCL10), MCP-2 (CCL8), MIF, MCP-4 (CCL13), MDC (CCL22), MIP-3α (CCL20), IL-5, TARC (CCL17), and MCP-1 (CCL2) (Fig. 5).

TLR2   TLR3   TLR4   TLR9
human corneal endothelial cell lines

human corneal endothelial cells

FIGURE 1. Intracellular expression of TLRs in HCEn cells. TLR 9 was significantly expressed in HCEn cells and primary human corneal endothelial cells. Expression of TLR2, -3, and -4 was barely detectable. *Solid line*: unstained; *dotted line*: control IgG stained, *gray line*: anti-TLR antibody stained.
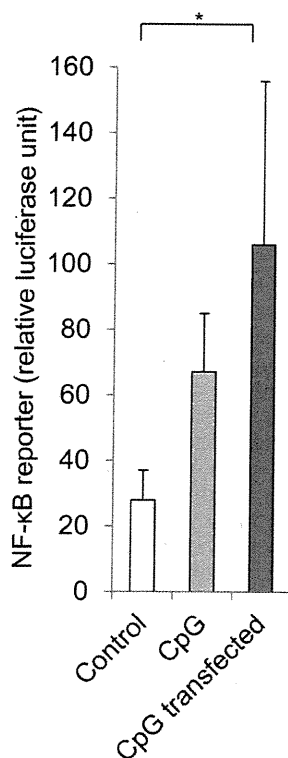
Figure 2. NF-κB promoter activation in HCEn cells by TLR9. HCEn cells transfected with NF-κB reporter plasmids were stimulated with CpG oligonucleotide for 24 hours and measured for luciferase activity. CpG transfection significantly elevates the NF-κB promoter activity ($n = 6$; $^*P < 0.05$).

Role of TLR9-Mediated NF-κB Activation and HSV-1 Replication

TLR9 participates in the primary defense systems against viral infection and functions to induce inflammatory cytokines after infection of HCEn cells by HSV-1. We examined whether TLR9 affects the entry and replication of HSV-1 into HCEn cells. To accomplish this, HSV-1 was adsorbed on HCEn cells, and the number of HSV-1 copies was determined by real-time PCR of HSV-1 DNA polymerase. After TLR9 was inhibited by pretreatment with TLR9 inhibitory oligonucleotide, a significant reduction in the copy number was not observed (Fig. 6A).

The contribution of TLR9 to viral replication was determined by real-time RT-PCR, and the results showed a significant reduction in the expression of the mRNA of HSV-1 DNA polymerase in HCEn cells after exposure to TLR9 inhibitory oligonucleotide (Fig. 6B). This reduction was also confirmed by titration (control oligo-treated at an MOI of 0.1: $9.3 \times 10^8 \pm 0.3 \times 10^8$; TLR9 inhibitory oligonucleotide-treated at an MOI of 0.1: $9.8 \times 10^7 \pm 0.9 \times 10^7$, $P < 0.01$).

We then assessed whether the TLR9-mediated viral replication in HCEn cells was related to NF-κB activation. The classic NF-κB cascade is regulated by IκB kinase (IKK), leading to the nuclear translocation of p65, a component of the NF-κB pathway. When HCEn cells were treated with IKK inhibitory peptide, which contained sequences corresponding to the active IκBα phosphorylation recognition sequence, the induction of HSV-1 DNA polymerase was significantly inhibited in HSV-1–infected HCEn cells (Fig. 6C). Treatment with a p65 inhibitor also significantly reduced the number HSV-1 copies (data not shown). These findings indicate that the classic NF-κB cascade is involved in HSV-1 replication in HCEn cells.

We next examined whether TLR9-inhibition–mediated suppression of HSV-1 replication can be restored by NF-κB activation. Because the activation of the classic NF-κB cascade is regulated by the degradation of IκBα, the NF-κB cascade is activated by siRNA-mediated inhibition of IκBα. Exposure to TLR9 inhibitory oligonucleotide reduced the copy number of HSV-1 DNA polymerase mRNA, and the transfection of IκBα siRNA reduced the effect of TLR9 inhibition (Fig. 6D). Collectively, these findings indicate that HSV-1 used the TLR9-mediated NF-κB activation for its own replication in HCEn cells.

Alternative Transcription Factor Activation by TLR9 in HSV-1–Infected HCEn Cells

HSV-1 infection induces an array of inflammatory cascades. This can be summarized by the transcriptional induction profiles of representative transcriptional factors. To characterize the profiles of the signaling cascades activated by HSV-1 infection and show the possible involvement of TLR9, we determined whether HSV-1 infection can activate representative transcriptional factors related to the TLR9 cascades by using transfection of reporter plasmids. The activities of transcriptional factors of cascades of NF-κB, MAPK/ERK, cAMP/PKA, MAPK/JNK, C/EBP, interferon response, and PKC/calcium were measured using reporter plasmids for NF-κB, Elk-1, cyclic AMP response element (CRE), AP-1, C/EBP, ISRE, and NFAT, respectively. HSV-1 infection significantly stimulated the transcription factors of NF-κB, Elk-1, CRE, AP-1, C/EBP, and NFAT at 24 hours pi (Fig. 7).

We then tested whether TLR9 contributes to the induction of transcription factor activities of the inflammatory cascades. When TLR9 was inhibited by siRNA transfection, the HSV-1–induced activation of CRE and C/EBP reporters was significantly reduced (Fig. 8). The other transcription factor activities, including Elk-1, AP-1, and NFAT, were not appreciably affected (data not shown). Thus, HCEn cells used TLR9 leading to various types of promoter activation, including NF-κB, after HSV-1 infection.

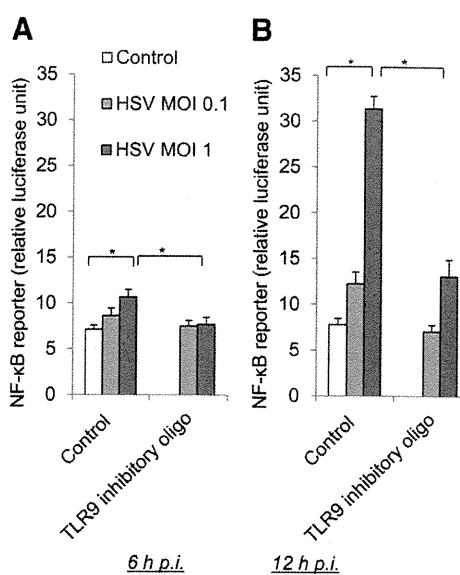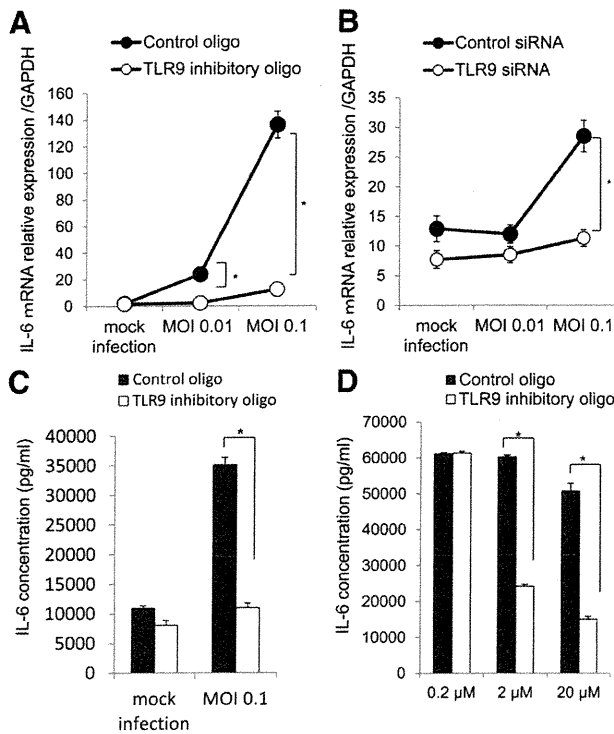**Figure 3.** Inhibition of HSV-1 infection–induced NF-κB promoter by TLR9 inhibition. HCEn cells transfected with reporter plasmids were stimulated with HSV-1 infection for 6 (**A**) and 12 (**B**) hours, and measured for luciferase activity. Treatment by TLR9 inhibitory oligonucleotide significantly inhibited NF-κB promoter activation ($n = 6$; $^*P < 0.05$).

FIGURE 4. Inhibition of HSV-1 infection–induced IL-6 activation. Effect of inhibiting HSV-1 infection–induced IL-6 mRNA induction by TLR9 inhibitory oligonucleotide treatment (A) and by transfection of siRNA of TLR9 (B) at 12 hours. TLR9 blockade by inhibitory oligonucleotide or siRNA significantly reduced the HSV-1 infection–induced IL-6 mRNA activation. $n = 4$; $^{*}P < 0.01$. (C, D) Reduction of HSV-1 infection–induced IL-6 secretion by TLR9 blockade. TLR9 inhibitory oligonucleotide significantly reduced IL-6 secretion at 12 (C) and 24 (D) hours pi in a dose-dependent manner ($n = 6$; $^{*}P < 0.01$).

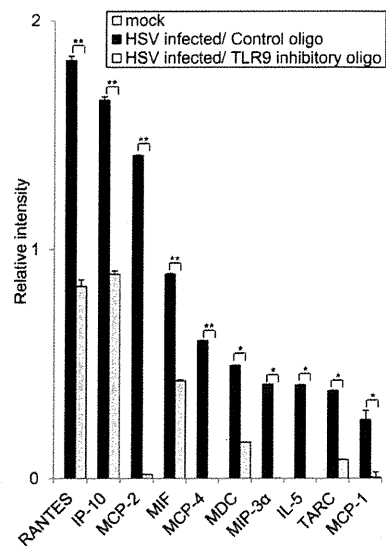## TLR9-Mediated Inflammatory Network after HSV-1 Infection

To summarize how HCEn cells used TLR9-mediated signals after HSV-1 infection, the TLR9-dependent cytokines induced after HSV-1 infection were analyzed for signaling interactions using a systems biological approach. Using a database of known signaling networks (Ingenuity Pathways Knowledge Base; Ingenuity Systems), we successfully generated two major biological networks with high significance scores (network 1, $P < 10^{-31}$; network 2, $P < 10^{-15}$). The most significant network was network 1, which was annotated as cell-to-cell signaling and interaction, hematologic system development and function, and immune cell trafficking, where NF-κB was centrally positioned (data not shown).

DISCUSSION

Our results showed that TLR9 was abundantly expressed in HCEn cells and was used to initiate inflammatory responses after HSV-1 infection. HSV-1 exploited the TLR9-mediated NF-κB activation for its own replication. To resist the assault, HCEn cells transcriptionally initiate an array of inflammatory programs related to the cascades of NF-κB, ERK, MAPK (P38), JNK, cAMP/PKA, PKC, and interferon responses. Of these, TLR9 activation was especially used for the signal transduction cascades of NF-κB, CRE, C/EBP, and arrays of inflammatory cytokines, including IL-6.

In sensing microbial pathogens, conserved structural moieties are recognized by germline encoded PRRs, including the TLRs, NOD-like receptors, and C-type lectin receptors.[20] Apoptotic or necrotic cells or degradation products of the extracellular matrix, damage-associated molecules or cytokines, such as dsDNA, RNA, high-mobility group box 1 (HMGB1), ATP, hyaluronan, versican, heparin sulfate, and heat shock proteins, are abundantly present. These damage-associated molecular patterns (DAMPs) are also recognized by PRRs. Of the different PRRs, the TLRs are the most important class of receptors that are able to sense pathogen-associated molecular patterns (PAMPs). Nucleic acids, especially DNAs, are a major class of molecules that stimulate TLRs. Previously, the DNAs derived from bacteria had been considered the exclusive ligand of TLR9. However, viral genomes and self DNAs derived from necrotic or apoptotic cells have also been shown to activate TLR9. Physiologically, ligands of TLRs, including TLR9, are ubiquitous, and the corneal endothelium is continuously exposed to various components of PRR ligands. Thus, the cornea and the host are exposed to and sense the environment using combinations of PRRs. In this setting, cascades initiated from such PRRs generally converge to NF-κB or inflammasomes, where the converged signal inputs can elicit robust immune responses in synergy.[21]

For entry of HSV-1 into the host, glycoproteins, gB, gD, gH, and gL are required. For example, gB binds to paired immunoglobulin-like type 2 receptor α (PILRα) on the host.[22] gD binds to herpesvirus entry mediator (HVEM), nectin-1, or 3-O sulfated heparan sulfate, after which the host recognizes the viral invasion by the PRRs. In the TLR-mediated recognition cascade, three major molecular components—TLR2, TLR9, and TLR3—are engaged to activate innate immune responses.[23] However, the TLR-mediated interaction does not appear to affect viral entry (Fig. 6A). The sequential recognition of TLR2 and -9 that occurs after HSV-1 infection leads to a robust NF-κB activation which then induces a wide array of cytokines, chemokines, and interferons, where NF-κB plays a central role in regulating numerous cellular metabolic events. Concomitantly, HSV-1 redirects the host transcriptional machinery to

FIGURE 5. TLR9-mediated inflammatory cytokine and chemokine induction by HSV-1–infected HCEn cells. HCEn cells were adsorbed with HSV-1 at an MOI of 0.1 for 1 hour and refed with the DMEM. After 12 hours of incubation, the supernatant of HSV-1–infected HCEn cells was assayed for cytokines. TLR9-induced inflammatory cytokines and chemokines were significantly reduced by exposure to TLR9 inhibitory oligonucleotide ($n = 4$/group; $^{*}P < 0.05$, $^{**}P < 0.01$).
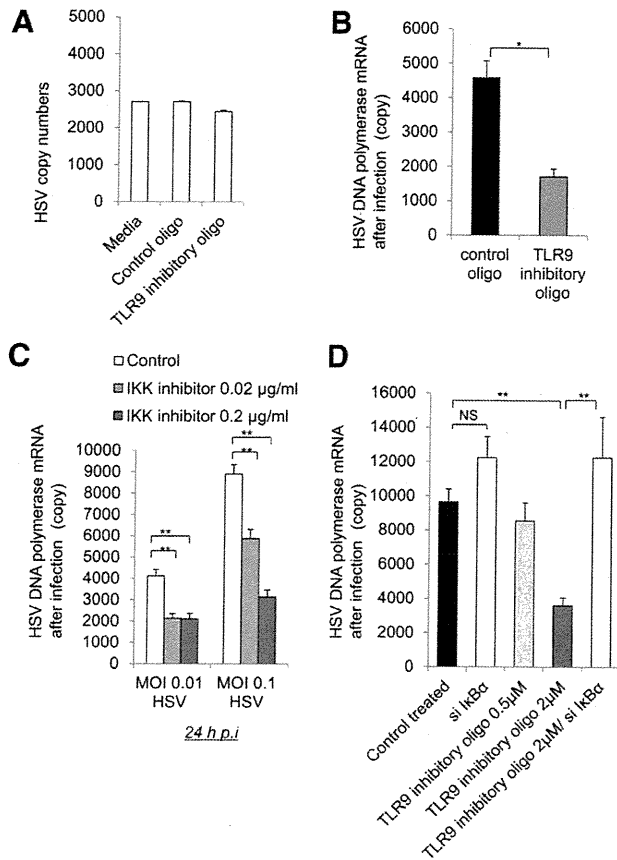
FIGURE 6. Inhibition of HSV-1 proliferation by blockade of TLR9 and NF-κB signaling cascade. (A) Unperturbed entry of HSV-1 into the HCEn cells by TLR9 inhibition. HSV-1 was adsorbed on HCEn cells for 1 hour. HCEn cells were washed and assessed for HSV-1 DNA polymerase copy number by using real-time PCR (n = 8). TLR9 inhibitory oligonucleotide did not appreciably affect HSV-1 absorption. (B) TLR9 inhibitory oligonucleotide impaired HSV-1 replication, shown by the reduction in copy number of HSV-1 DNA polymerase mRNA (n = 4, 24 hours pi). (C) Reduced proliferation of HSV-1 by IKK inhibitor. HCEn cells were infected with HSV-1 at the indicated MOI and assessed at 24 hours pi for copy number of HSV-1 DNA polymerase mRNA, with reverse transcription real-time PCR (n = 4; $P < 0.01$). (D) Restoration of TLR9 inhibition-mediated reduction of HSV-1 proliferation by IκBα inhibition. Treatment of TLR9 inhibitory oligonucleotide significantly reduced the copy number of HSV-1 DNA polymerase mRNA at 24 hours pi. This reduction was restored by NF-κB activation using transfection of siRNA of IκBα (n = 4; *$P < 0.05$, **$P < 0.01$).

nome of different members of the herpes virus family.[28,29] Moreover, HSV-1 is equipped with the ability to effectively block multiple innate signaling for its survival. For example, virion host shutoff protein (VHS) degrades the host mRNA by its RNase function, US11 or γ34.5 inhibits PKR (RNA-activated protein kinase), and ICP47 inhibits MHC class I loading.[30-37] After the viral replication is completed, ICP-0 directs the inhibition of inflammatory responses by ubiquitin-specific peptidase 7 (USP7) translocation, which leads to the inhibition of NF-κB and JNK.[30] Thus, HSV-1 hijacks and exploits the crucial components of the host immune system, TLR9 and NF-κB, for its own use.

There are two major signaling pathways for TLR: NF-κB and MAPKs. In the MAPK cascade, the JNK, p38, and ERK pathways are conventionally activated, leading to the activation of AP-1, CRE, Elk-1, and C/EBP elements in the promoters. In addition, the C/EBP family of transcription factors is involved in many biological functions, including regulating cytokine expression, proliferation, and tumor progression.[38-40] We found that the reporter activity of NF-κB, CRE, and C/EBP are activated by TLR9 after HSV-1 infection. Analysis of the HSV-1 infection-induced transcriptome of HCEn cells showed strong inductions of CREBBP and C/EBPα, which are representative transcription factors related to CRE and C/EBP.
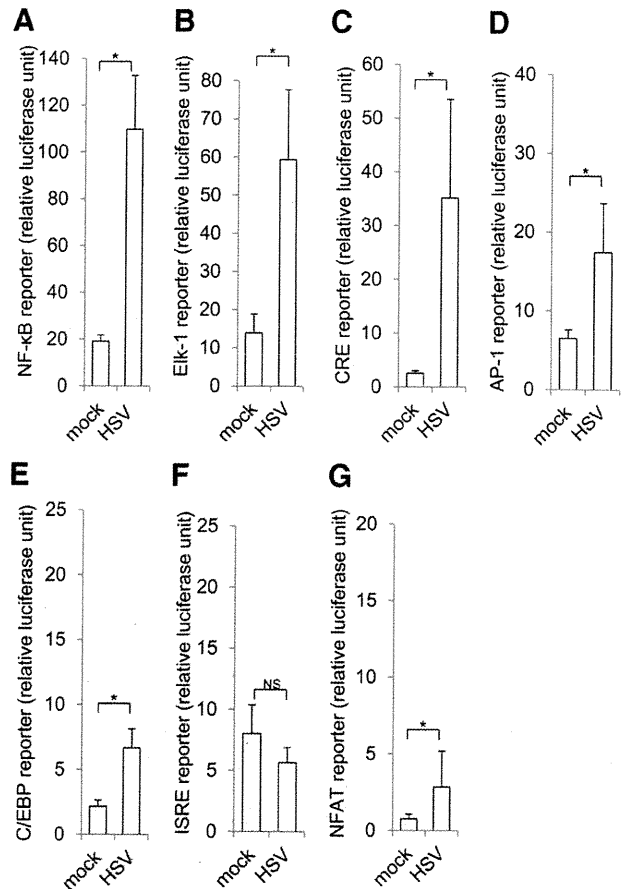
express its own genes in a tightly regulated temporal cascade.[24] The three classes of genes, the immediate-early (IE) genes, including ICP-0, -4, -22, -27, and -47, followed by the early and the late genes are sequentially expressed.

To initiate productive replication of HSV-1, ICP0 plays a crucial role as a strong activator of all classes of HSV-1 genes and a propagator of lytic infections. ICP0 possesses NF-κB-binding elements on its promoter. The transcription of ICP0 is dependent on activation of NF-κB of the host, which is triggered by the recruitment of p65/RelA.[24] Inhibition of the NF-κB cascade, including the inhibition of IKK or dominant negative IκBα, significantly suppresses viral replication (Fig. 6).[24-26] Very recently, the UL31 of HSV-1 was also shown to be necessary for optimal NF-κB activation and expression of ICP4, ICP8, and glycoprotein C.[27]

The use of host NF-κB for viral replication is not limited to HSV-1 because NF-κB-binding sites are also located in the ge-

FIGURE 7. Signaling cascade-focused promoter activation in HCEn cells by HSV-1 infection. HCEn cells transfected with reporter plasmids were stimulated with HSV-1 infection for 24 hours at an MOI of 0.1 and measured for luciferase activity for (A) NF-κB, (B) Elk-1, (C) CRE, (D) AP-1, (E) C/EBP, (F) ISRE, and (G) NFAT. HSV-1 infection significantly elevated promoter activities of NF-κB, ELK-1, CRE, AP-1, C/EBP, and NFAT (n = 6; *$P < 0.05$).
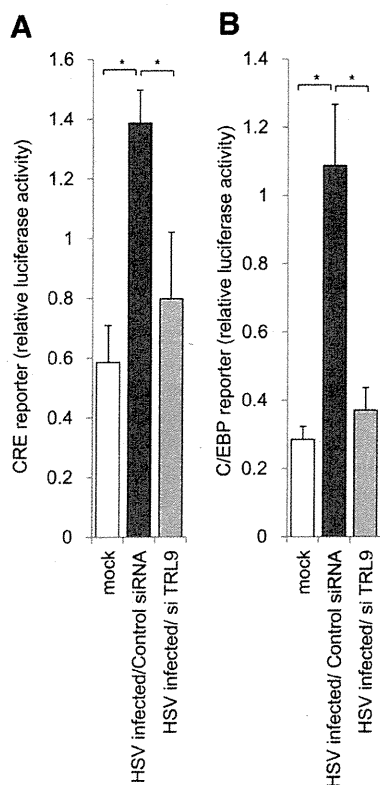
FIGURE 8. Inhibition of HSV-1 infection–induced CRE and C/EBP promoter activation by TLR9 inhibition. HCEn cells transfected with reporter plasmids were infected with HSV-1 at an MOI of 0.1 and measured for luciferase activity at 12 hours pi. Transfection of siRNA of TLR9 significantly inhibited the reporter activities of CRE (**A**) and C/EBP (**B**). ($n = 6$; $*P < 0.05$).

Generally, transcriptional activation is regulated by different levels of transcriptional factor activation and interactions. On infection by *Helicobacter pylori*, the AP-1 and CRE elements in the cyclooxygenase promoter are activated by TLR2 and -9.[41] In TLR-mediated activation of IL-6 and TNF-α, both NF-κB and C/EBP binding elements in the promoter are critical for their transcriptional activation.[42-44] In HSV-1–infected HCEn cells, TLR9 input activated the NF-κB signal transduction cascade (Fig. 3), and our bioinformatic analysis of the induced cytokines and chemokines which are sensitive to TLR9 inhibition, were summarized as NF-κB-dependent inflammatory cascade. However, the NF-κB cascade may not be sufficient to fully explain the transcriptional activation of inflammatory cytokines. In HCEn cells, the activations of CRE, C/EBP, and NF-κB were involved in the TLR9-mediated signaling cascade (Fig. 8) and presumably in the TLR9-mediated induction of inflammatory cytokines and chemokines. At least two of the recognition sequences of these transcription factors exist in the promoters of TLR responsive cytokines and chemokines (data not shown). This may explain the unexpectedly wide array of inflammatory cytokines that was inhibited by TLR9 suppression.

We used immortalized HCEn cells as models of corneal endothelial cells in situ. The HCEn cells have similar capabilities as primary corneal endothelial cells and organ cultured corneal endothelial cell in inducing representative cytokines including MCP-1, IL-6, IL-8, CXCL2, TGFβ2, and thrombospondin 1.[8,45] However, there is still some question of whether immortalized HCEn cells can truly reflect the in vivo properties of corneal endothelial cells such as HSV-1 infection–induced endotheliitis. For this, in vivo analysis may help in gaining a

better understanding of the physiological roles of the endothelial cells during a viral infection.

At present, a murine model of HSV-1–induced corneal endotheliitis is not available. We used the KOS strain for this study because our initial hypothesis was based on the findings of our earlier studies.[46,47] Very recently, the KOS strain has been reported to have a mutation of the *US8A* gene and defective *US9* gene.[48] *US9* is especially involved in neuronal virulence. However, a defective *US9* does not appear to affect the cell-to-cell spread in permissive epithelial cells.[49] In addition, no apparent dysfunction was reported for the elongated US8A by mutation.

To summarize, corneal endothelial cells express TLR9 intracellularly to recognize dsDNAs and HSV-1 infection. HSV-1 usurps this TLR-mediated NF-κB activation for its own replication.

Acknowledgments

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前眼部・外眼部感染症における起炎菌判定
—日本眼感染症学会による眼感染症起炎菌・薬剤感受性多施設調査(第一報)—

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要　　約

　目　的：前眼部・外眼部感染症の起炎菌の判定基準を考案し, それに基づいた評価を行った.

　対象と方法：眼感染症起炎菌・薬剤感受性動向調査参加施設(全国 18 施設)において 2007 年 9 月 22 日から 2008 年 8 月 25 日の間に 476 症例から分離された 909 株のうち真菌を除いた 890 株について, 直接分離培養・増菌培養・塗抹検鏡・菌量の測定を行い, 検鏡と培養結果の一致性, 多核白血球の有無, 菌量を勘案して起炎菌を判定した. 判定にあたっては, 起炎菌と推定起炎菌の 2 段階とし, 黄色ブドウ球菌, モラクセラ桿菌, インフルエンザ菌, 肺炎球菌, 緑膿菌, 淋菌の 6 菌種は特定菌として別に取り扱った.

　結　果：全分離菌 890 株のうち 18.8% が起炎菌, 15.1% が推定起炎菌と判定された. 常在菌の表皮ブドウ球菌, アクネ菌, コリネバクテリウムにおいても各々 2.0%, 2.6%, 38.3% が起炎菌と判定された.

　結　論：起炎菌判定には塗抹検鏡や菌量を勘案する必要がある. 常在菌であっても起炎菌となっている例があると考えられた.(日眼会誌 115：801—813, 2011)

キーワード：起炎菌, 塗抹検鏡, 多核白血球, コリネバクテリウム, 特定菌

Determination of Causative Agents in Ocular Infection of External Adnexa and Anterior Segments
—Multicenter Study of Causative Agents and Drug Sensitivity of Ocular Infection by the Japanese Association for Ocular Infection Part I

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Abstract

Purpose：To determine the causative agents of ocular infection of external adnexa and anterior segments due to selected criteria.

Subjects and Methods：Between September 22, 2007 and August 25, 2008, 890 bacterial strains were collected from 476 patients in 18 facilities nationwide participating in the Drug Sensitivity for Ocular Infection Study Group. Usual aerobic and anaerobic cultures, enrichment cultures, smears, and measurements of bacterial quantity were performed and the determination of causative agents was made from the results of smears and culture, the presence of polymorphonuclear cells and bacterial quantity. The selection was divided into two categories, causative agents and presumed causative agents. *Staphylococcus aureus*, Moraxella bacilli, *Haemophilus influenzae, Streptococcus pneumoniae, Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae* were distinctively considered as specified bacteria.

Results：Among 890 strains, 18.8% were deter-

mined to be causative agents, and 15.1% were determined to be presumed causative agents. Among the bacteria detected in normal flora, 2.0% of *Staphylococcus epidermidis*, 2.6% of *Propionibacterium acnes*, and 38.3% of Corynebacterium spp. were determined to be causative agents.

Conclusion：To determine the causative agents, the results of smears and bacterial quantity should be considered together with culture results. Bacteria constituting normal flora have the potential of becoming causative agents.

Nippon Ganka Gakkai Zasshi（J Jpn Ophthalmol Soc 115：801—813, 2011）

Key words：Causative agent, Smear, Polymorphonuclear cells, Corynebacterium, Specified bacteria

I　緒　言

眼感染症の診断と治療において，原因となっている起炎菌を検出し，その薬剤感受性を測定することは最も重要なステップであり，それが不明なままでは，十分な治療を行うことはできない．しかし，前眼部・外眼部においては非病原菌が結膜嚢や皮膚に常在菌として存在しているために，分離培養を行って検出された菌が必ずしも起炎菌であるとは限らない．今まで前眼部・外眼部感染症について，菌の薬剤感受性を調べた報告は大変多い[1]~[14]が，そのいずれもが，起炎菌ではなくあくまで分離された菌についての薬剤感受性を調べているのは，起炎菌かどうかを見極めることがきわめて困難であることに由来している．厳密な起炎性を確かめるのであれば，分離された菌を，分離されたときの菌量で，分離された本人の局所に戻し，再び炎症が惹起されるかどうかを確かめる必要があるが，これは倫理的に許されない以上，起炎菌であるかどうかをある基準を決めて推定する以外に方法はない．感染症の中で診断法が最も確立している肺炎では，検出された菌量について，病原菌と判断する基準が提唱されている．具体的には tracheal aspirate で 10^6cfu/ml，broncho-alveolar lavage（肺胞洗浄液）で 10^4~10^5cfu/ml，protected specimen brush で 10^3cfu/ml あれば起炎菌の可能性が高いと報告されている[15]．ただ，この値も絶対的なものと考えるべきではないと注釈がなされており，絶対的な基準を定めることの困難性が示されている．眼科領域では，過去に三井らにより，眼感染症研究会（日本眼感染症学会の前身）としての起炎菌の推定基準が報告されている[16]．その中では，前眼部・外眼部における病原菌としての特定菌が定められ，特定菌が分離された例ではそれのみを起炎菌とし，それ以外の菌が分離された例ではすべてを起炎菌またはその協力菌であると推定しているが，起炎性を検証するそれ以上の基準は設けられていない．肺炎で行われているような菌量を勘案するという提案については，眼科領域の感染症でもなされたことがあるが，実際に疫学調査の結果を踏まえ，個々の菌について菌量の基準を設定して起炎菌の判定が行われたことはない．

最近我々は多施設研究として結膜炎の分離菌について薬剤感受性検査を行ったが[13]，その際も従来のように起炎菌でなく検出菌に対する薬剤感受性検査を実施してまとめた．しかし，それに対して実際の病原菌の薬剤感受性が検討されていないとの批判があった．そこで今回，日本眼感染症学会で，結膜炎のみならず前眼部・外眼部感染症からの分離菌について，その薬剤感受性を比較するスタディを計画するにあたって，臨床面へのフィードバックを考慮し，一定の基準のもとで起炎菌と判定された分離菌のみを対象に薬剤感受性検査を行うこととした．薬剤感受性検査結果の詳細については別報に譲り，ここでは起炎菌判定基準設定の経緯と判定結果について報告したい．

II　実　験　方　法

対象は，日本眼感染症学会による眼感染症起炎菌・薬剤感受性動向調査参加施設〔全国18施設（表1）〕にお

表1　参加施設一覧

医療機関名	所在地
医療法人社団　大橋眼科	北海道
江口眼科病院	北海道
阿部眼科	秋田県
庄司眼科医院	千葉県
医療法人社団馨風会　徳島診療所	東京都
ルミネはたの眼科	神奈川県
安間眼科	愛知県
いこま眼科医院	石川県
バプテスト眼科クリニック	京都府
大橋眼科	大阪府
魚谷眼科医院	鳥取県
医療法人眼科康誠会　井上眼科	岡山県
医療法人社団ひかり会　木村眼科内科病院	広島県
松本眼科	徳島県
医療法人幸友会　岡本眼科クリニック	愛媛県
出田眼科病院	熊本県
医療法人明和会　宮田眼科病院	宮崎県
医療法人水晶会　安里眼科	沖縄県

直接分離培養（定量培養）　　　　　　　　　　　　　　　増菌培養

検体　　　　　　　　　　　　原液

原液　　　10倍希釈液（滅菌生理食塩水900μl＋原液100μl）

臨床用TGC培地

36.5℃ 1日～2週間

＋（菌発育）

分離培養

サブロー寒天培地｜①5%羊血液加コロンビア寒天培地 ②チョコレート寒天培地各1枚｜5%羊血液加コロンビア寒天培地｜5%羊血液加コロンビア寒天培地｜5%羊血液加コロンビア寒天培地

30℃ 10日間｜36.5℃ 24～48時間 5%CO₂培養｜36.5℃ 1～5日 連続嫌気培養｜36.5℃ 24～48時間 5%CO₂培養｜36.5℃ 1～5日 連続嫌気培養

*①5%羊血液加Trypticase Soy Agar ②5%羊血液加コロンビアCNA寒天培地 ③マッコンキー寒天培地｜*5%羊血液加コロンビア寒天培地

36.5℃, 5%CO₂, 24～48時間｜36.5℃, 嫌気培養

発育集落数を菌種別にカウント　　　　発育菌

検体1mlあたりの菌量を算出

純培養 同定検査

薬剤感受性試験
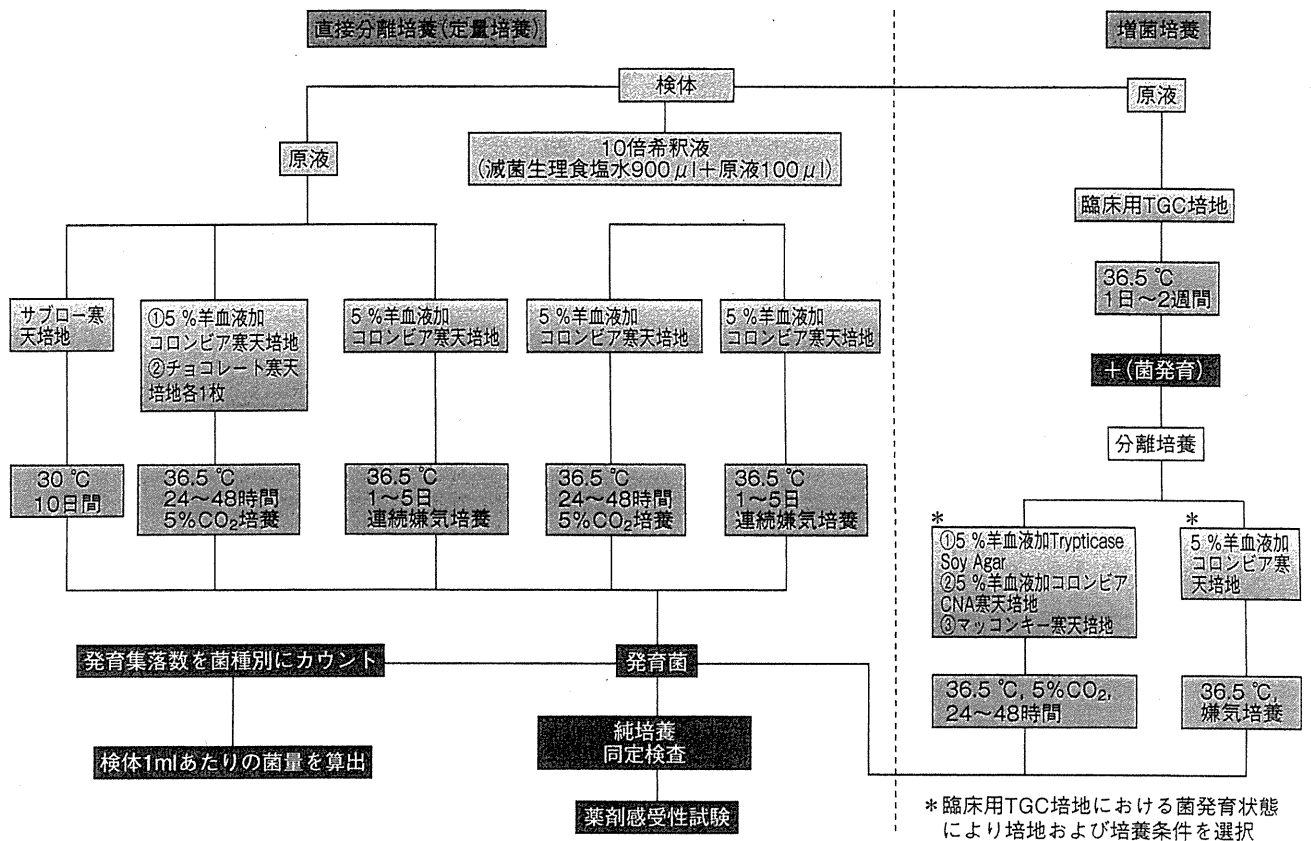
＊臨床用TGC培地における菌発育状態により培地および培養条件を選択

図 1　培養方法の流れ.
培養用に送られた検体は，直接分離培養と増菌培養に分けて各種培地に塗布した.

いて，2007年9月22日から2008年8月25日の間に前眼部・外眼部感染症疑いで受診した患者（抗菌薬の投与症例を含む）である．対象患者には本調査の説明を十分に行い，文書による同意を取得した．感染部位から，滅菌綿棒（日本綿棒2P753S）2本で擦過採取し，1本は塗抹検鏡用にリング付きスライドガラスに塗抹し，メチルアルコールで固定した．また，もう1本は分離培養のため，輸送用培地（ANAポート微研®）に採取し，輸送や保存期間の影響を受けないようにするために凍結保存した．なお，検体の採取にあたっては滅菌生理食塩水で滅菌綿棒を湿らせ，結膜炎の場合，滅菌綿棒を下眼瞼の円蓋部まで挿入し，1往復ぬぐい検体を採取した．また，塗抹にあたっては，4つのリング付きスライドガラスを用い，一番外側から滅菌綿棒をスライド上で転がして単層の塗抹となるようにした．また，検体の残りがあれば次のリングにも塗抹を行った．角膜炎の場合は浸潤部を綿棒でぬぐい，一番外側のスライド上で滅菌綿棒をスタンプした．その他の感染症については症例の状態に応じて採取を行い，採取部位を記録した．

2種類の検体を一般財団法人阪大微生物病研究会へ輸送し，検鏡ならびに好気性・嫌気性培養を実施し，菌の分離・同定を行った．なお，本研究については参加施設を一括して中央倫理審査を行った．

塗抹検鏡では，グラム染色を実施し，グラム陽性菌・陰性菌の有無，多核白血球とその菌貪食像の有無を確認した．細菌数は1,000倍の検鏡にて1視野あたりの数が20未満の場合には1＋，20以上で50以下の場合には2＋，50を超える場合には3＋とグレード分けを行った．多核白血球は1,000倍で1視野あたりの数が1未満の場合には1＋，1以上で2未満の場合には2＋，2以上で10未満は3＋，10以上は4＋とグレード分けを行った．

培養検査は，直接分離培養（好気および嫌気，定量培養）と増菌培養を並行して行った（図1）．定量培養については以下の手順で行った．

1）もとの検体（ANAポート微研®）をvoltexでよく混和する．

2）滅菌生理食塩水900μlに検体（ANAポート微研®）100μlを加え10倍希釈検体を調製する．

3）検体原液（ANAポート微研®）と10倍希釈検体各々50μlを5%羊血液加コロンビア寒天培地各々2枚に滴下し，コンラージ棒で塗抹する．原液については他にチョコレート寒天培地とサブロー寒天培地でも各々1枚培養する．

4）36.5℃で24～48時間，5%CO₂条件下での培養，および36.5℃で1～5日間連続嫌気培養を行う．

5）各培地の発育集落数を菌種別に数え，希釈倍率から検体1mlあたりの菌量を算出する．
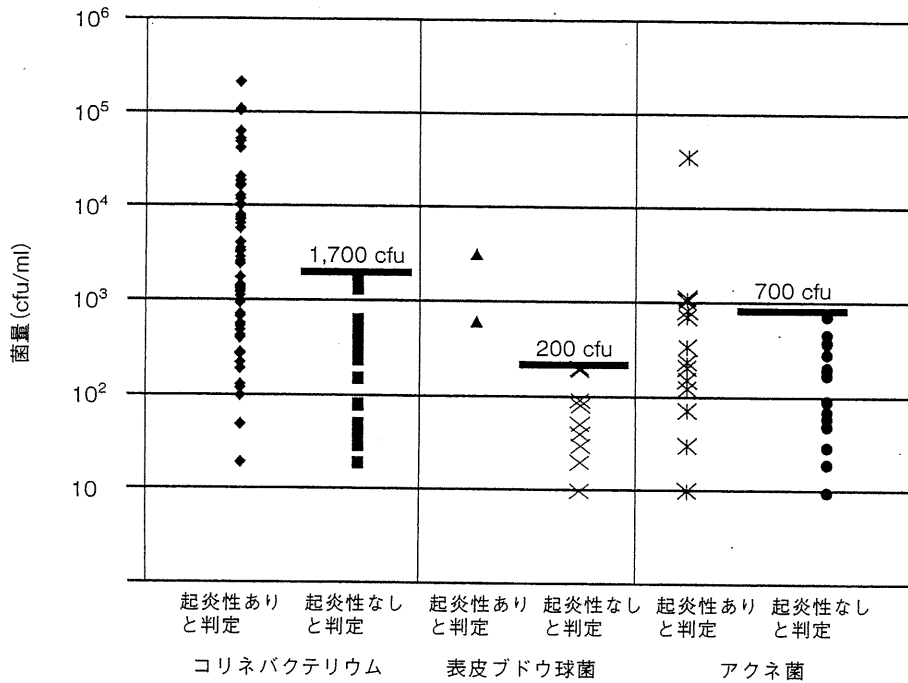
以上のようにして得られた分離菌の起炎菌判定につい

図 2　(1)〜(3)の判定基準による3大常在菌の菌量散布図.

結膜の3大常在菌，コリネバクテリウム，表皮ブドウ球菌，アクネ菌を(1)〜(3)の判定基準（次ページ本文）
によって起炎性の有無を判定した結果で分けて菌量の散布図を書き，ここで起炎性なしと判定した症例の上
限値（コリネバクテリウム 1,700 cfu，表皮ブドウ球菌 200 cfu，アクネ菌 700 cfu，それぞれバーで示してい
る）を，結膜炎において特定菌も塗抹培養一致菌もない症例で，この3大常在菌が分離された場合に推定起
炎菌と判定する菌量の基準値として定めた.

て，以下に示す判定基準の原則を定めた．なお三井らの
報告にある特定菌という考え方は採用したが，起炎性の
推定の度合いにはどうしても差があることから三井らの
報告のようにすべての判定を一律に白黒で判定すること
はせずに，重みづけを行って，より確からしいものを起
炎菌，起炎菌である可能性があるが根拠が弱いものを推
定起炎菌とした.

① 感染を示唆する臨床所見があること

② 起炎菌と推定起炎菌の2段階とする.

③ 黄色ブドウ球菌，モラクセラ桿菌，インフルエン
　ザ菌，肺炎球菌，緑膿菌，淋菌の6菌種を特定菌
　とする.

④ 特定菌が直接分離培養で分離された場合は起炎菌
　とする.

⑤ 特定菌が増菌培養で分離された場合は推定起炎菌
　とする.

⑥ 塗抹検鏡と直接分離培養もしくは増菌培養の結果
　が一致した菌は菌量も勘案して起炎菌とする.

⑦ 塗抹検鏡と直接分離培養もしくは増菌培養の結果
　が一致しなくても菌量が多い場合は推定起炎菌と
　する.

⑧ ③〜⑥以外で角膜炎の角膜病巣から直接分離培養
　で分離された菌は推定起炎菌とする.

⑨ ③〜⑥以外で結膜炎・麦粒腫・化膿性霰粒腫・眼

瞼炎・涙嚢炎・涙小管炎で病巣から直接分離培養
で分離された菌は，多核白血球を伴っていれば推
定起炎菌とする．ただし，表皮ブドウ球菌，アク
ネ菌，コリネバクテリウムでは一定の菌量を上回
る場合とする.

III　結　　果

今回検体を採取した 478 症例の内訳は，男性 191 例，
女性 287 例，平均年齢は 59 歳（0〜97 歳）であった．疾
患別では結膜炎が 306 例と最も多く，角膜炎が 86 例，
麦粒腫・化膿性霰粒腫（眼瞼炎を含む）が 41 例，涙嚢炎
（涙小管炎を含む）が 43 例という結果であった．2 例は
後眼部の感染であったため対象から除外した.

今回対象となった 476 例の前眼部・外眼部感染症症例
から 890 株の菌が分離（直接分離培養 598 株，増菌培養
292 株）された．最も多かったのは *Propionibacterium
acnes* 267 株，次いで Corynebacterium spp. 167 株，
Staphylococcus epidermidis 99 株，*Staphylococcus aur-
eus* 64 株，Bacillus spp. 19 株であった.

以上の分離菌について起炎菌の判定を判定基準の原則
によって行おうとしたが，実際の症例では複数の菌が分
離されるため，条件はより複雑となる．また，具体的な
菌量の基準の策定も必要となる．そこで，前述の原則を
もとに，菌の優先順位と菌量から策定した起炎菌の判定

表 2　具体的な起炎菌判定基準

特定菌/非特定菌	分離法	特定菌の同時分離	塗抹と培養の一致性	疾患	多核白血球	菌種	菌量	起炎菌(○)推定起炎菌(△)
特定菌[※1]	直接		NC	NC	NC	NC	NC	○
	増菌		NC	角膜炎[※2]	NC	NC	NC	○
				角膜炎以外	NC	NC	NC	△
非特定菌	直接	+（特定菌は直接分離）	+[※3]	NC	NC	NC	NC	○
			NC	NC	NC	NC	特定菌の 10 倍を超える量	○
	直接	+（特定菌は増菌分離）	+[※3]	NC	NC	NC	NC	△
			NC	NC	NC	NC	1,000 cfu 以上	△
	直接/増菌	−	+	NC	NC	NC	その症例の最大菌量	○
			NC	NC	NC	NC	その症例の最大菌量の分離菌の菌量の 1/100 を超える	△
			−	NC	NC	NC	塗抹培養一致菌が別に分離されており，その菌量の最大値の 10 倍を超える[※4]	△
	直接	−	−	結膜炎	+	コリネバクテリウム	1,700 cfu を超える	△
						表皮ブドウ球菌	200 cfu を超える	△
						アクネ菌	700 cfu を超える	△
						上記 3 大常在菌以外	NC	△
				角膜炎	NC	NC	NC	△
				麦粒腫・化膿性霰粒腫・眼瞼炎[※5]	+	NC	NC	△
				涙嚢炎・涙小管炎[※6]	+	NC	NC	△
	増菌	−	−	角膜炎	NC	*Serratia marcescens*	NC	△

[※1]：特定菌は黄色ブドウ球菌，モラクセラ桿菌，インフルエンザ菌，肺炎球菌，緑膿菌，淋菌の 6 菌種.
[※2]：他に起炎菌の候補となる菌が分離されない場合に限る.
[※3]：起炎菌・推定起炎菌とした特定菌と塗抹の上で区別できない菌は除く.
[※4]：塗抹培養一致菌が増菌培養の場合は最大値 10 cfu と解釈する（増菌培養では菌量測定をしていないため）.
[※5]：膿から分離された場合に限る.
[※6]：涙嚢・涙小管から分離された場合に限る.
NC：not concerned（如何を問わない）.

基準を以下のように定めた. ここで, 常在菌であるコリネバクテリウム, 表皮ブドウ球菌, アクネ菌の 3 菌種については図 2 に示すような菌量散布図を以下の(1)〜(3)の基準の結果に基づいて作成し, そこで「起炎性なし」と判定された症例の菌量の最大値をもとに(4)の基準を定めた.

(1) 特定菌が直接分離培養で分離された場合, その特定菌を「起炎菌」とする. ただし, その菌量の 10 倍を超える菌が他にある場合, あるいは塗抹と直接分離培養の結果が一致した菌が他にある場合は, それも「起炎菌」とする. ただし, 「起炎菌」とした特定菌と塗抹の上で区別ができない菌はこの限りではない.

※なお, 以下を特定菌とする.
黄色ブドウ球菌, モラクセラ桿菌, インフルエンザ菌, 肺炎球菌, 緑膿菌, 淋菌.

(2) 特定菌が増菌培養で分離された場合,「推定起炎菌」とする. ただし, 菌量が 1,000 cfu 以上の菌が他にある場合, あるいは塗抹と直接分離培養の結果が一致した菌が他にある場合は, それも「推定起炎菌」とする. ただし,「推定起炎菌」とした特定菌と塗抹の上で区別ができない菌はこの限りではない. また, 角膜炎において他に起炎菌の候補となる菌が分離されない場合には, 増菌培養で分離された特定菌を「起炎菌」とする.
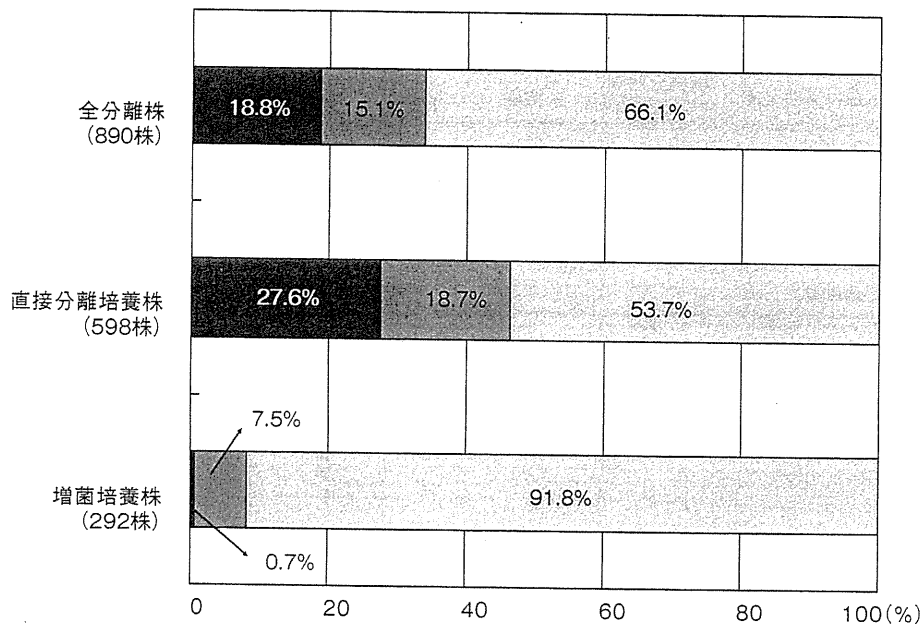
(3) 上記の(1), (2)以外で, 塗抹と直接分離培養ある

図 3　全分離株における起炎菌・推定起炎菌の割合.
■：起炎菌，▨：推定起炎菌，□：非起炎菌.
33.9％ が起炎菌あるいは推定起炎菌と判定された.

いは増菌培養結果が一致した場合，一致した菌の菌量が当該症例の分離菌において最大であれば「起炎菌」とする．一致した菌の菌量が最大でない場合には，当該症例の分離菌における最大菌量の 1/100 を超える量を認めるものに関しては「推定起炎菌」とする．また，塗抹と直接分離培養あるいは増菌培養結果の一致した菌の菌量の最大値（最大値が増菌の場合は 10 cfu と解釈）の 10 倍を超える菌量を認めた不一致菌も「推定起炎菌」とする．

(4) 上記の(1)〜(3)以外で，結膜炎において多核白血球が陽性であり，コリネバクテリウム，表皮ブドウ球菌，アクネ菌のいずれかが直接分離培養で分離された場合，(1)〜(3)の基準によってこの 3 菌種が分離されているが推定起炎菌と判定されなかった症例における最大菌量（コリネバクテリウムは 1,700 cfu，表皮ブドウ球菌は 200 cfu，アクネ菌は 700 cfu）を超える菌量を認めるものを「推定起炎菌」とする．それ以外の菌の場合には直接分離培養にて分離された細菌を「推定起炎菌」とする．

(5) 上記の(1)〜(3)以外で，角膜炎において病巣から採取された検体で，直接分離培養にて分離された菌は「推定起炎菌」とする（多核白血球の有無は問わない）．また，角膜炎において病巣から採取された検体で，増菌培養にて分離された *Serratia marcescens* を「推定起炎菌」とする．

(6) 上記の(1)〜(3)以外で，麦粒腫・化膿性霰粒腫・

眼瞼炎において膿から採取された検体であり，多核白血球が陽性で，直接分離培養により分離された菌を「推定起炎菌」とする．

(7) 上記の(1)〜(3)以外で，涙嚢炎・涙小管炎において涙嚢・涙小管から採取された検体であり，多核白血球が陽性で，直接分離培養により分離された菌は「推定起炎菌」とする．

以上，きわめて複雑な基準であるため，これを表の形にまとめたものを表 2 に示す．

本基準をもとに分離菌に対し，起炎菌ならびに推定起炎菌を判定した．全分離株 890 株中，起炎菌と判定された菌が 167 株（18.8％），推定起炎菌と判定された菌が 134 株（15.1％）であった．また，直接分離培養で検出された 598 株中，起炎菌と判定された菌は 165 株（27.6％），推定起炎菌と判定された菌は 112 株（18.7％）であり，増菌培養より検出された 292 株中，起炎菌と判定された菌は 2 株（0.7％），推定起炎菌と判定された菌は 22 株（7.5％）であった（図 3）．全分離菌の菌種ごと，疾患別の起炎菌判定一覧を表 3 に示す．また，主な菌種別の分離株数，起炎菌ならびに推定起炎菌と判定された株数の内訳を図 4 に示す．本研究では，起炎菌判定基準において特定菌とした黄色ブドウ球菌，モラクセラ桿菌，インフルエンザ菌，肺炎球菌，緑膿菌，淋菌の 6 菌種では，64 株が分離された黄色ブドウ球菌において，起炎菌と判定されたのは 45 株，推定起炎菌が 19 株，2 株が分離された緑膿菌では起炎菌が 1 株，推定起炎菌が 1 株であった他，2 株が分離されたモラクセラ桿菌，14 株が分離されたインフルエンザ菌，8 株が分離された肺炎球菌においては

表 3　全分離菌の疾患別起炎菌判定一覧

菌種		菌株数	起炎菌				推定起炎菌				非起炎菌			
			結膜炎	角膜炎	麦粒腫	涙嚢炎	結膜炎	角膜炎	麦粒腫	涙嚢炎	結膜炎	角膜炎	麦粒腫	涙嚢炎
グラム陽性球菌	*Staphylococcus aureus*	64	31	3	3	8	15	1	2	1	0	0	0	0
	Staphylococcus epidermidis	99	2	0	0	0	3	4	0	0	63	10	10	7
	Staphylococcus capitis	9	0	0	0	0	0	1	0	0	5	1	1	1
	Staphylococcus hominis	6	0	0	0	0	1	0	0	0	4	0	1	0
	Staphylococcus lugdunensis	6	0	0	0	0	1	0	0	0	4	0	0	1
	Staphylococcus warneri	5	0	0	0	0	0	1	0	0	4	0	0	0
	その他の Staphylococcus 属	16	0	0	0	0	1	0	0	0	12	2	1	0
	Streptococcus pneumoniae	8	6	0	0	2	0	0	0	0	0	0	0	0
	Streptococcus oralis	20	1	0	0	0	2	0	0	0	11	1	1	4
	Streptococcus mitis	14	0	0	0	0	5	1	0	1	6	1	0	0
	Streptococcus constellatus	6	0	0	0	0	1	0	0	1	2	0	0	2
	その他の Streptococcus 属	20	5	0	0	0	1	0	0	0	10	1	0	3
	Micrococcus 属	11	1	0	0	0	0	1	0	0	5	1	3	0
	Gemella 属	11	0	0	0	0	3	1	0	0	6	0	0	1
	Enterococcus 属	6	0	0	2	0	0	0	0	0	4	0	0	0
	その他のグラム陽性球菌	9	0	0	0	0	0	0	0	0	9	0	0	0
グラム陽性桿菌	Corynebacterium 属	167	47	1	9	7	15	6	1	1	64	2	12	2
	Bacillus 属	19	0	0	0	0	5	0	0	0	10	2	2	0
	その他のグラム陽性桿菌	13	0	0	0	0	1	0	0	2	4	3	2	1
グラム陰性球菌		5	0	0	0	0	0	0	0	0	4	0	1	0
グラム陰性桿菌	*Haemophilus influenzae*	14	14	0	0	0	0	0	0	0	0	0	0	0
	Moraxella 桿菌	2	1	1	0	0	0	0	0	0	0	0	0	0
	Pseudomonas aeruginosa	2	0	1	0	0	0	0	0	1	0	0	0	0
	Serratia marcescens	5	0	0	0	0	0	3	0	0	1	0	1	0
	Comamonas 属	9	0	0	0	0	1	0	0	0	5	2	0	1
	Acinetobacter 属	8	0	0	0	0	1	1	0	0	4	2	0	0
	Stenotrophomonas maltophilia	5	1	0	0	2	0	0	0	0	2	0	0	0
	その他のグラム陰性桿菌	36	5	1	0	1	3	2	1	1	10	8	1	3
嫌気性菌	*Propionibacterium acnes*	267	1	1	4	1	8	14	4	4	169	28	18	15
	その他の嫌気性菌	27	1	1	1	2	6	1	0	3	9	0	1	2
放線菌	放線菌	1	0	0	0	0	0	1	0	0	0	0	0	0
	計	890												

麦粒腫には，化膿性霰粒腫と眼瞼炎を含む．涙嚢炎には涙小管炎を含む．

いずれも起炎菌と判定された．また，今回，淋菌は分離されなかった．特定菌以外において，常在菌である表皮ブドウ球菌は 99 株が分離され，そのうち 2 株（2.0%）が起炎菌，7 株（7.1%）が推定起炎菌と判定され，アクネ菌は 267 株が分離され，そのうち 7 株（2.6%）が起炎菌，30 株（11.2%）が推定起炎菌と判定された．一方，167 株が分離されたコリネバクテリウムでは，64 株（38.3%）が起炎菌，23 株（13.8%）が推定起炎菌と判定され，他の常在菌に比し高い起炎性を示すと判定された．

結膜炎に関しては全分離株 616 株中，起炎菌と判定された菌が 116 株（18.8%），推定起炎菌と判定された菌が 73 株（11.9%）であった．また，直接分離培養された 425 株中，起炎菌と判定された菌は 116 株（27.3%），推定起炎菌と判定された菌は 58 株（13.6%）であり，増菌培養された 191 株中，起炎菌と判定された菌はなく，推定起炎菌と判定された菌は 15 株（7.9%）であった（図 5）．主

な菌種別の分離株数，起炎菌ならびに推定起炎菌と判定された株数の内訳を図 6 に示す．特定菌では黄色ブドウ球菌 46 株において，起炎菌と判定されたのは 31 株，推定起炎菌が 15 株であった．モラクセラ桿菌 1 株，インフルエンザ菌 14 株，肺炎球菌 6 株はすべて起炎菌と判定された．緑膿菌の分離はなかった．常在菌については，表皮ブドウ球菌は 68 株中 2 株（2.9%）が起炎菌，3 株（4.4%）が推定起炎菌と判定され，アクネ菌は 178 株中 1 株（0.6%）が起炎菌，8 株（4.5%）が推定起炎菌と判定された．コリネバクテリウムは 126 株中，47 株（37.3%）が起炎菌，15 株（11.9%）が推定起炎菌と判定された．

角膜炎に関しては全分離株 111 株中，起炎菌と判定された菌が 9 株（8.1%），推定起炎菌と判定された菌が 38 株（34.2%）であった．また，直接分離培養された 56 株中，起炎菌と判定された菌は 7 株（12.5%），推定起炎菌と判定された菌は 35 株（62.5%）であり，増菌培養され
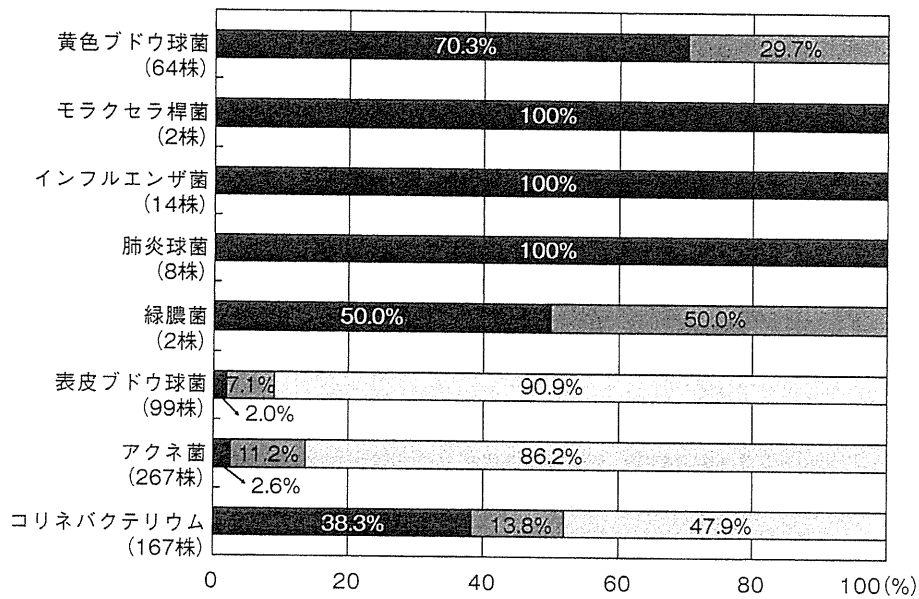
図 4　主要菌の菌別の起炎菌・推定起炎菌の割合.
■：起炎菌，■：推定起炎菌，□：非起炎菌.

特定菌と3大常在菌の起炎菌・推定起炎菌の判定結果である．淋菌は今回のスタディでは分離されていない．他の5つの特定菌は当然のことながら，起炎菌あるいは推定起炎菌として判定されている．3大常在菌についても，一部起炎菌と判定され，特にコリネバクテリウムが高い起炎菌，推定起炎菌率を示している．
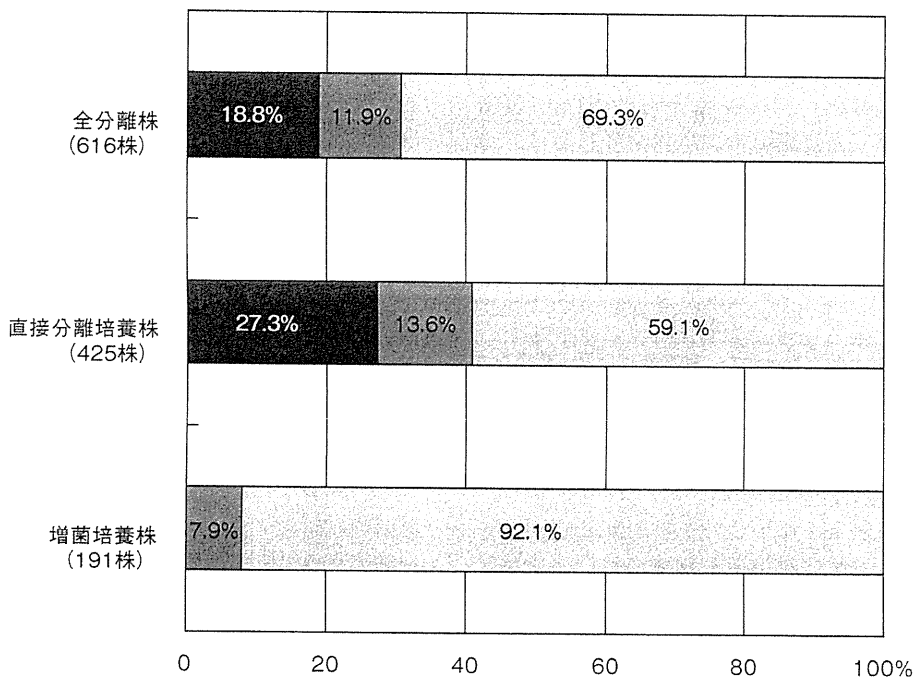

図 5　全結膜炎分離株での起炎菌・推定起炎菌の割合.
■：起炎菌，■：推定起炎菌，□：非起炎菌.
30.7% が起炎菌あるいは推定起炎菌と判定された.

た55株中，起炎菌と判定された菌は2株(3.6%)，推定起炎菌と判定された菌は3株(5.5%)であり，結膜炎と比較して起炎菌・推定起炎菌両者を合わせるとその割合は高く，起炎菌に限ると低い結果となった(図7)．主な菌種別の分離株数，起炎菌ならびに推定起炎菌と判定さ

れた株数の内訳を図8に示す．特定菌では黄色ブドウ球菌4株において，起炎菌と判定されたのは3株，推定起炎菌が1株であった．モラクセラ桿菌1株，緑膿菌1株は起炎菌と判定された．インフルエンザ菌，肺炎球菌の分離はなかった．常在菌については，表皮ブドウ球菌は