altered biologic response by functional uterine-like tissue in the pelvis and their possible secretion of substance such as cytokines that can affect the inflammatory response by the host. It is possible that PGE_2 plays a role in tumor development by promoting angiogenesis through other factors such as vascular endothelial growth factor.[29,30] High PG levels are detected in various types of cancers, and associated with immunosuppression and tumorigenesis.[13] PGE_2 may inhibit the antigen processing and presentation by dendritic cells.[31] Moreover, PGE_2 acts to limit interferon-γ production directly and inhibits the effects interleukin (IL)-12 receptor expression and responsiveness,[32] and can inhibit the production of IL-2. Thus, PGE_2 can downregulate proliferation and activation of CTLs by altering the balance of these cytokines.

It has been shown that the intake of aspirin or non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs) is related to a reduced risk of ovarian cancer in epidemiological studies.[33,34] It is also reported that NSAIDs other than aspirin differentially suppress endometriosis in a murine model.[35]

In human, the treatment with a COX-2-specific inhibitor rofecoxib is effective in the management of pain related to endometriosis.[36] In animal models, COX-2 selective inhibitor prevents implantation of eutopic endometrium to ectopic sites in rats,[37] and suppresses the growth of endometriosis xenografts via antiangiogenic activity in severe-combined immunodeficiency mice.[38] Long-term NSAIDs use to treat pain associated with endometriosis may suppress progression of disease and decrease cancer incidence.

Taken together, our results suggest that the expression of mPGES-1 in addition to COX-2 contributes to an elevated level of PGE_2 production in cases of endometriosis. mPGES-1 may also play a role in the pathogenesis of endometriosis and its immunological aspects. Although the role of mPGES-1 in endometriosis development and its correlation with malignant transformation has yet to be established, the present findings provide the basis for future studies that will evaluate whether mPGES is a potential therapeutic target for the clinical treatment of endometriosis.

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Influenza A virus non-structural protein 1 (NS1) interacts with cellular multifunctional protein nucleolin during infection

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Abstract

Influenza A virus non-structural protein 1 (NS1) is the most important viral regulatory factor that controls cellular processes to facilitate viral replication. To gain further insight into the role of NS1, we tried to find novel cellular factors that interact with NS1. The complexes of NS1 and target proteins were pulled down from an infected cell lysate using anti-NS1 (A/Udorn/72) single-chain Fv and identified by peptide mass fingerprinting analysis. We identified nucleolin, a multifunctional major nucleolar protein, as a novel NS1-binding protein. The RNA-binding domain of NS1 was responsible for this binding, as judged by a GST (glutathione *S*-transferase) pull-down assay with the GST-fused functional domains of NS1. By laser confocal microscopy, we observed the co-localization of NS1 with nucleolin most clearly in the nucleoli, indicating that NS1 is interacting with nucleolin during infection. Our results suggest a novel function of NS1, namely, affecting cellular events via interaction with nucleolin.

Keywords: Influenza A virus; Non-structural protein; NS1; Nucleolin; Nucleolus; Single-chain Fv

Influenza A viruses cause outbreaks in humans and domestic animals every year and, occasionally, a catastrophic pandemic such as the "Spanish influenza" in 1918–1919. The influenza A viruses are members of the family *Orthomyxoviridae* with a genome consisting of eight separate negative-stranded RNA segments that encodes 11 viral proteins [1]. The non-structural protein 1 (NS1) is translated from a transcript of the segment eight and is the most important viral regulatory factor during infection, affecting cellular and viral gene expression and inhibiting interferon response [1–3]. NS1 consists of two functional domains, the C-terminal effector domain and the N-terminal RNA-binding domain. The effector domain interacts with many viral and cellular factors, for example, the virus RNA polymerase complex [4], cellular proteins involved in translation such as eIF4G, and cellular factors involved in the post-transcriptional processing of RNA, such as CPSF4 and PABPII [1–3]. Especially, by binding with CPSF4, NS1 inhibits the cleavage of mRNA at the polyA-binding site and thus shuts off the cellular protein synthesis [5,6]. The RNA-binding domain interacts with cellular mRNA and spliceosome component U6 snRNA [7], inhibiting the exportation of cellular RNAs from the nucleus. Similarly, NS1 inhibits the cellular anti-viral pro-

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teins, such as protein kinase R (PKR) [1], 2′5′ oligoadenylate synthetase (OAS)/RNase L pathway [8], and retinoic acid-induced gene I (RIG-I) [9].

To obtain further insight into the pathological function of NS1, we sought novel cellular proteins interacting with NS1 during infection. We identified nucleolin, a multifunctional protein abundant in nucleoli, as an NS1-binding protein. Laser confocal microscopy revealed that A/Udorn/72 NS1 co-localizes with nucleolin clearly in the nucleoli from 6 h post-infection, suggesting that NS1 affects cellular functions through interaction with nucleolin during the processes of influenza virus infection.

Materials and methods

Cells and virus infection. A549, a human lung carcinoma cell line, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For infection experiments, A549 cells were mock-infected (PBS-0.5% geratin alone) or infected with influenza A/Udorn/72 (H3N2) in PBS-0.5% geratin at a multiplicity of infection (MOI) of 5. After 1 h of incubation, the inoculum was removed, and culture was continued at 37 °C in modified Eagle's medium without serum.

Generation of an anti-NS1 single-chain Fv. A human naive single-chain Fv (scFv) phage-display library (2.6×10^9 clones) was constructed as described previously [10,11] using a phagemid vector TFV5 (DDBJ/EMBL/GenBank Accession No. AB158265) [10]. To make an expression construct for histidine-tagged influenza A/Udorn/72 NS1 (NS1His), the NS1 cDNA was amplified by PCR using primers 5′-TTTAGCTAGCATGGATTCCAACACTGTGTC-3′ (NheI site, underlined) and 5′-TTTAAGCTTATCAATCAGCCATCTTATCTCTT-3′ (HindIII site, underlined), digested with NheI and HindIII and ligated with the NheI–HindIII-digested pTrcHisB vector (Invitrogen, USA). The NS1His was induced with 1 mM IPTG for 3 h at 25 °C in *Escherichia coli* TG1 and purified with Ni–NTA agarose (QIAGEN) according to the manufacturer's protocol. Anti-NS1 scFvs were selected from the scFv phage-display library using NS1His immobilized on polystylene tubes [12]. A clone giving the most intense signal on phage immunoblotting [13], clone 4A, was chosen for subsequent experiments. To prepare the scFv with an epitope (myc)-tag, the cDNA of the scFv fragment (NheI–NotI digest) was subcloned into the NheI–NotI site of TFV6 vector (DDBJ/EMBL/GenBank Accession No. AB334775). To obtain free scFv of 4A, the protein was induced by 0.1 mM IPTG at 30 °C overnight in non-suppressor *E. coli* strain BL21. The free form scFv 4A was extracted from the periplasm fraction [14] and purified with Ni–NTA agarose.

Immunoblotting. When phage-form anti-NS1 scFv (clone 4A) was used as the primary antibody (working concentration, 1×10^9 pfu/ml), the following procedures were essentially as described previously [13]. When free form anti-NS1 scFv was used as the primary antibody (working concentration, 0.2 μg/ml), anti-myc mouse antibody (1:1000 dilution, Calbiochem) and HRP conjugated anti-mouse IgG (1:5000 dilution, Jackson ImmunoResearch) were used as the secondary and tertiary antibodies, respectively. When nucleolin was examined, the rabbit polyclonal anti-nucleolin antibody H-250 (1:1000 dilution, Santa Cruz Biotechnology) and HRP-conjugated anti-rabbit IgG (1:2500 dilution, Jackson ImmunoResearch) were used as the primary and secondary antibodies, respectively. In all experiments, the blot was visualized with an ECL Plus immunoblot detection system (GE Healthcare).

Immunofluorescence. A549 cells grown on glass coverslips in 24-well plates were infected with A/Udorn/72 at an MOI of 5. At the indicated time after infection, the cells were fixed with cold-methanol for 10 min, washed with PBS, and incubated with rabbit anti-nucleolin antibody H-250 (1:200 dilution) for 1 h at room temperature. After washing with PBS, the cells were incubated with Alexa 555-conjugated goat anti-rabbit polyclonal antibody (1:250 dilution, Molecular Probes) and Alexa 488-conjugated anti-NS1 scFv 4A (2.5 μg/ml), prepared using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes), for 1 h at room temperature. After washing, the cells were mounted with the SlowFade Gold antifade reagent (Molecular Probes), and the fluorescent images were collected by confocal laser scanning microscope FV1000 (Olympus) in a sequential scanning mode.

Binding analyses. A549 cells (5×10^6), 7 h post-infection or uninfected (mock), were lysed with 1.5 ml of a TNE buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) with a protease inhibitor cocktail, Complete EDTA-free (Roche), for 30 min on ice and then centrifuged at 17,400g for 10 min at 4 °C. The supernatant was used as the cell lysate for subsequent binding analyses. For screening of NS1-binding host factors, the anti-NS1 scFv 4A was immobilized on NHS-activated Sepharose 4B beads (GE Healthcare). The anti-NS1-scFv beads were incubated with the cell lysate overnight at 4 °C, and, after washing extensively, the bound materials were eluted with stepwise (0.3, 0.5, 0.8 or 1 M) NaCl concentrations. After the elution, the beads were boiled in an SDS–PAGE sample buffer to collect the uneluted fraction. Then, the fractions were resolved on SDS–PAGE (10–20% linear gradient gel). For immunoprecipitation of the NS1-nucleolin complex, the cell lysate (1.5 ml) was incubated with 5 μg anti-nucleolin antibody H-250 or normal rabbit IgG (for the negative control) overnight at 4 °C, followed by the incubation with 30 μl of Protein G–Sepharose beads (GE Healthcare) for 1 h at 4 °C. Then, the beads were washed five times with 1 ml of a TNE buffer and resuspended with a 60 μl SDS–PAGE sample buffer. After boiling for 5 min and brief centrifugation, the sample (20 μl of the supernatant) was resolved on SDS–PAGE (15% gel) and the target antigens were detected by immunoblotting. To determine which domain, the RNA-binding domain (RBD) or the effector domain (ED), of NS1 binds with nucleolin, we prepared GST-fused NS1 constructs. NS1 fragments were amplified using the following primer sets: for full-length NS1 (NS1full, amino acids 1–237), 5′-TTAGGATCCATGGATTCCAACACTGTGTC-3′ and 5′-AAAGAATTCTTAATCAGCCATCTTATCTCT-3′ (BamHI and EcoRI sites, underlined, respectively); for the RBD of NS1 (NS1RBD, amino acids 1–81), 5′-TTAGGATCCATGGATTCCAACCACTGTGT-3′ and 5′-CATGGAATTCTTACATGGTCATTTTAAGTGCCT (BamHI and EcoRI sites, underlined, respectively); and for the ED of NS1 (NS1ED, amino acids 81–273), 5′-AGGCGGATCCGCCTCCACACCTGCTTCGCG-3′ and 5′-AAAGAATTCTTAATCAGCCATCTTATCTCTTC-3′ (BamHI and EcoRI sites, underlined, respectively). Each NS1 fragment was digested with BamHI and EcoRI and cloned into a BamHI–EcoRI-digested pGEX-2 T vector. The proteins were induced by 0.1 mM IPTG for 3 h at 30 °C in TG1 and purified with glutathione Sepharose beads (GE Healthcare). After free glutathione was removed by dialysis, the each GST-fused NS1 (200 μg) was added into the cell lysate (1.5 ml) and incubated overnight at 4 °C. The mixture was then incubated with glutathione Sepharose beads (25 μl) for 2 h at 4 °C, and, after extensive washing, the bound materials were eluted with 20 mM glutathione in 50 mM Tris–Cl pH 8.0. The eluate was resolved on SDS–PAGE (13% gel) and analyzed by immunoblotting using anti-nucleolin antibody H-250.

Peptide mass fingerprinting analysis. The protein bands were excised from the silver-stained SDS–PAGE gel, in-gel digested by trypsin, and analyzed by MALDI-TOF mass spectrometer AXIMA-QIT (Shimadzu-Biotech) [15]. Proteins were identified from peptide fragments by comparison to theoretical digests of the human proteome in silico using MASCOT search tools.

Results

A scFv clone 4A specific to A/Udorn/72 NS1

We performed four rounds of conventional panning [12] for the selection of anti-NS1 scFv from a human scFv phage-display library (2.6×10^9 clones) using purified Udorn NS1 (NS1His) as an antigen (Fig. 1A). Among the positive clones, the most reactive anti-NS1 scFv phage, clone 4A, was selected on the basis of the signal intensity

on immunoblotting with the recombinant antigen. The clone 4A was also effective to detect Udorn NS1 present in infected A549 cell lysate (Fig. 1B, left). The antigen epitope recognized by clone 4A was found to be in the C-terminal effector domain of NS1 (data not shown). The free form of the clone 4A also detected NS1 in the infected A549 cell lysate (Fig. 1B, right).

Nucleolin was pulled down together with NS1 using the anti-NS1 scFv 4A and identified as a novel NS1-binding protein

We tried to detect novel proteins that were interacting with NS1 during infection. For this purpose, the free form of the clone 4A scFv was immobilized on Sepharose beads to pull down the NS1-associated proteins from the infected A549 cell lysate. The anti-NS1 scFv beads were reacted with uninfected or infected cell lysate, and the bound proteins were eluted by the NaCl step gradient and resolved on SDS–PAGE. The representative result is shown in Fig. 2A. When the pattern of protein bands was compared between infected and uninfected lysates, we found two major bands specific to the infected cell lysate on silver-stained gels; one, about 105 kDa (asterisk), eluted with both 0.5 and 0.8 M NaCl, and the other, about 29 kDa (closed circle), detected in the uneluted fraction (boiled beads). Although there are many other minor bands, we concentrated on the 105 and 29 kDa bands. To identify these proteins, the observed 105 and 29 kDa bands were excised from the gel, subjected to trypsin digestion, and analyzed using a MALDI-TOF mass spectrometer. The peptide mass fingerprinting pattern was obtained successfully for both bands and applied to query a data base search engine, MASCOT. The best match of the 105 kDa protein was with nucleolin, a nuclear multifunctional protein that was first designated C23 (Fig. 2B). Overall, 13 peptides, ranging in size from 7 to 14 amino acids, were matched, representing a 17% sequence coverage of nucleolin (119 of a total of 710 amino acids). By immunoblotting using an anti-nucleolin monoclonal antibody, we confirmed that the detected 105 kDa band was nucleolin (Fig. 2C, left). It should be noted that even by immunoblotting, nucleolin was not detected in the eluates from uninfected (Mock) cell lysate (Fig. 2C, left). We also confirmed that no nucleolin band was detected when the same pull-down experiment was done with NHS Sepharose alone without scFv (data not shown). The mass fingerprinting analysis of the 29 kDa band revealed that it was A/Udorn/72 NS1. This was confirmed by immunoblotting (Fig. 2C, right). These results suggested that nucleolin and NS1 are associated in infected cells and pulled down together.

We also confirmed the association of nucleolin and NS1 in infected A549 cell lysate by conventional immunoprecipitation using an anti-nucleolin rabbit polyclonal antibody. As expected, NS1 was detected in the complexes immunoprecipitated by the anti-nucleolin antibody (Fig. 2D). No NS1 bands was detected when control normal rabbit IgG was used for the immunoprecipitation. Thus, we concluded that nucleolin is a novel NS1-binding protein.

The RNA-binding domain of NS1 is responsible for binding with nucleolin

Next, we determined which functional domain of NS1, the RNA-binding domain or the effector domain, binds to nucleolin. For this purpose, a GST (glutathione *S*-transferase) pull-down assay was performed (Fig. 2E). Each fragment of Udorn NS1, full-length (amino acids 1-273, GST-NS1full), the RNA-binding domain (amino acids 1–81, GST-NS1RBD), and the effector domain (amino acids 82–273, GST-NS1ED), was fused with GST and incubated with uninfected A549 cell lysate, and complexes were then pulled down using glutathione Sepharose. As seen, nucleolin was pulled down with GST-NS1full (Fig. 2E), indicating that *E. coli*-expressed, GST-fused NS1 can bind with nucleolin and, therefore, is suitable for the pull-down assay. Nucleolin was pulled down with GST-NS1RBD but not with GST-NS1ED, indicating that the RNA-binding domain of NS1 is responsible for binding with nucleolin.

NS1 and nucleolin co-localize in the nucleoli in infected cells

To clarify the distribution and movement of NS1 and nucleolin and their relationship during infection, we carried out double-immunofluorescence staining of infected A549 cells fixed with methanol (Fig. 3). To visualize nucleolin, the usual indirect immunofluorescence method was selected; on the other hand, for NS1, the free form of anti-NS1 scFv 4A was conjugated with fluorescent dye and used for staining. NS1 was apparent from 4 h post-infection (pi), mainly in nuclei as small dots. NS1 became

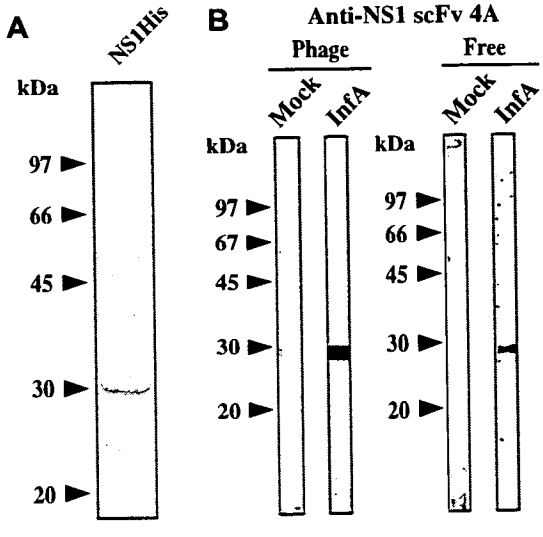

Fig. 1. Generation of an anti-NS1 scFv. (A) Purified NS1His stained by Coomassie brilliant blue. (B) Specificity of anti-NS1 scFv clone 4A. Infected (InfA) or uninfected (Mock) A549 cell lysate was immunoblotted using a phage form (Phage) or a free form (Free) of the anti-NS1 scFv 4A.

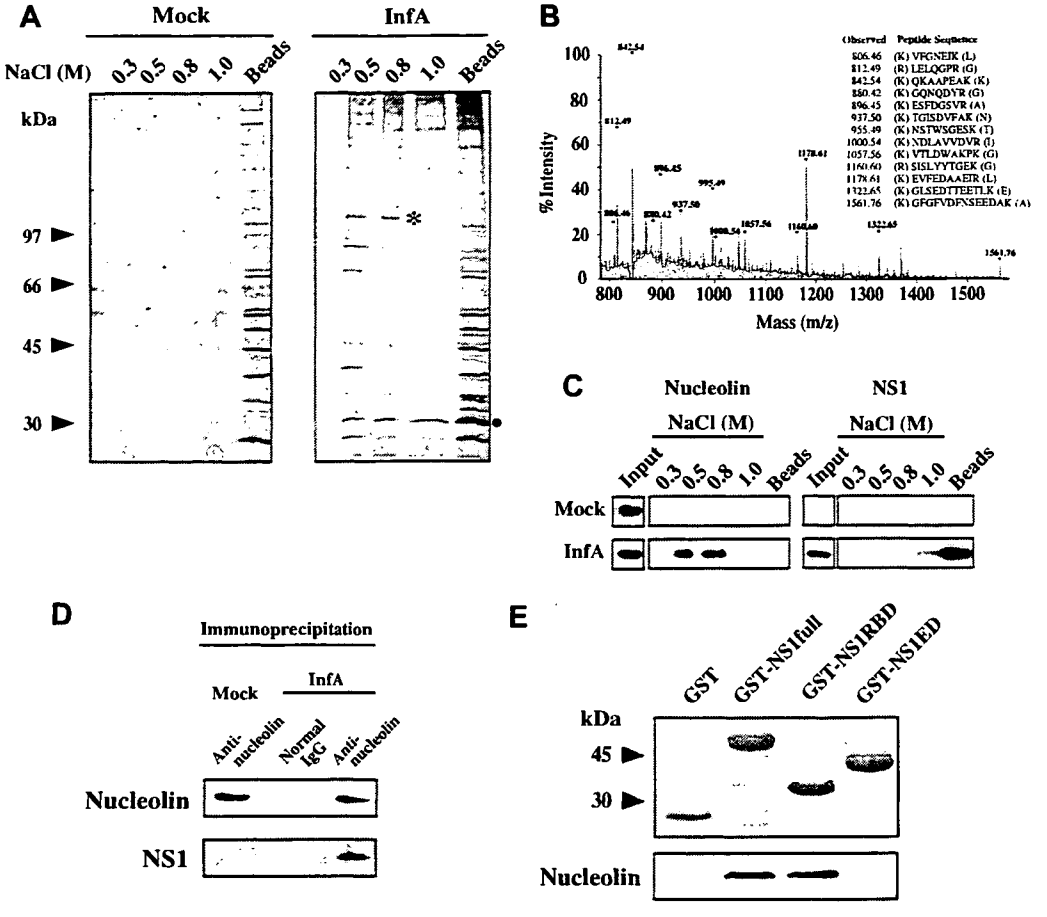

Fig. 2. Identification of nucleolin as a novel NS1-binding protein. (A) Pull-down of NS1-binding proteins with anti-NS1 scFv 4A-immobilized beads from the infected (InfA) or uninfected (Mock) A549 cell lysate. The elutions (0.3–1.0 M NaCl) and an uneluted fraction (Beads) were resolved on SDS–PAGE, and proteins were visualized by silver staining. The 105 and 29 kDa bands are indicated by an asterisk and a closed circle, respectively. (B) Peptide mass fingerprinting of the 105 kDa protein. The protein was identified as nucleolin using a program, MASCOT, and the peptides assigned to those of nucleolin are shown. (C) Confirmation of the 105 and 29 kDa bands as nucleolin and NS1, respectively. Each of the InfA or Mock fractions in (A) was immunoblotted with the anti-nucleolin antibody (left, at 105 kDa) or anti-NS1 scFv (right, at 29 kDa). Input, cell lysate applied to the beads in (A). (D) Complex of NS1 and nucleolin detected in infected cell lysate. The infected (InfA) or uninfected (Mock) A549 cell lysate was immunoprecipitated with an anti-nucleolin rabbit antibody (anti-nucleolin) or with normal rabbit IgG (Normal IgG). Then, the precipitant was immunoblotted by using the anti-nucleolin antibody or the anti-NS1 scFv. (E) The RNA-binding domain of NS1 binds with nucleolin. Full-length or each functional domain of NS1 was fused with GST (glutathione *S*-Sepharose), and the uninfected A549 cell lysate was examined by a pull-down assay: full, full-length; RBD, the RNA-binding domain; and ED, the effector domain. The top panel shows the purified GST-fused NS1 proteins stained by Coomassie brilliant blue. The bottom panel shows nucleolin detected by immunoblotting in the pull-down proteins.

detectable in nucleoli at 6 h pi, and, after that, NS1 was clearly seen in most of the nucleoli (8–24 h pi). In the cytoplasm, NS1 exhibited a granular pattern from 8 h pi. At 24 h pi, the nucleolar localization of NS1 was observed in almost all cells. On the other hand, nucleolin was stained in nucleoli and as particles in nuclei; similar patterns were observed within the observation period (0–24 h). The signals of NS1 and nucleolin clearly overlapped in nucleoli at 8–24 h pi, indicating that NS1 and nucleolin co-localize in the nucleoli during the processes of infection. Small parts of extranucleolar NS1 and nucleolin in nuclei seemed to co-localize. In Nomarski observation, as NS1 accumulated in nucleoli, the images of the nucleoli became clearer (Fig. 3 IV, 8–24 h). We also attempted paraformaldehyde (PFA) fixation (4%) for this examination (data not shown). Nucleolin was seen in nuclei diffusely and in the periphery of nucleoli. NS1 was observed in nuclei from 4 h pi and in nucleoli from 6 h pi as seen under methanol fixation. The signals of NS1 and nucleolin overlapped in the periphery of nucleoli under PFA fixation.

Discussion

The nucleolus is a dynamic subnuclear structure in which ribosomal RNAs are synthesized and ribosomes are assembled. In recent years, many investigators reported that viruses interact with the nucleolus and its components, where viruses can utilize the components or affect nucleolar functions to facilitate viral replication [16,17]. Our results strongly suggest that influenza A viruses also target and

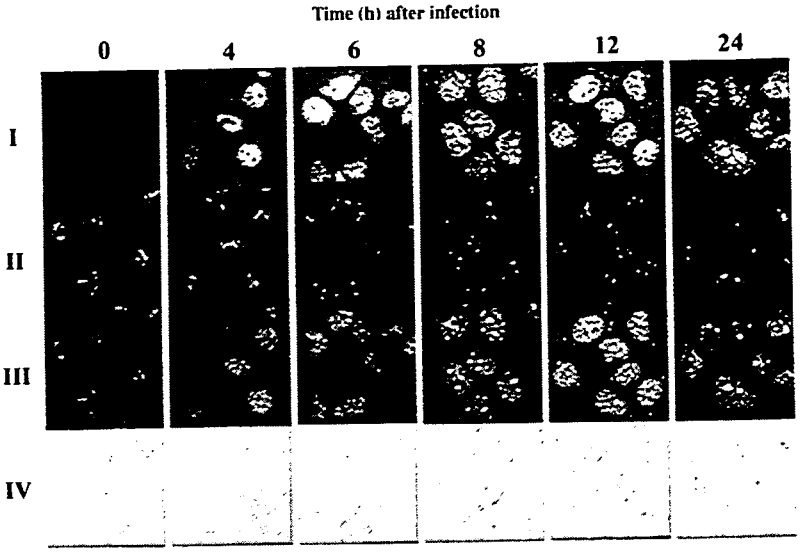

Fig. 3. Subcellular localization of NS1 and nucleolin during infection. A549 cells were infected with A/Udorn/72 at an MOI of 5, and, at each indicated time after infection, NS1 and nucleolin were visualized using the immunofluorescent technique (see text): I, NS1; II, nucleolin; III, merged images; and IV, Nomarski.

utilize the nucleolus. Consistently with our results of laser confocal microscopic study, the nucleolar localization of NS1 was reported in infected cells [18,19]; however, the target protein in nucleoli was undetermined. Nucleolin is a major nucleolar phosphoprotein implicated in rDNA transcription, rRNA maturation, ribosome assembly, nucleolus formation, and chromosome congression [16,20]. In addition, the involvement is suggested in cell signal transduction, apoptosis, regulation of cell proliferation and growth, cytokinesis, embryogenesis, and nucleogenesis [21]. Nucleolin localized not only to the nucleolus but also to the nucleoplasm, the cytoplasm, and even the outer surface of the cell membrane [16]. When the influenza A virus NS1 interacts with nucleolin, NS1 may inhibit some of the above functions of nucleolin by blocking its active site(s). Interestingly, we found that the binding site on NS1 locates in the RNA-binding domain of NS1. Nucleolin has also the binding activity to multiple RNA targets. Whether NS1 binds to nucleolin via RNA or not is under investigation.

NS1 inhibits cellular antiviral responses based on complex mechanisms, including the shut-off of cellular protein synthesis and inhibition of cellular antiviral proteins. Which activity appears dominantly in the infected cells depends on the sequence of NS1, in other words, on the virus strain (presumably, also on the cell-line employed); for example, A/Udorn/72 NS1 is closely associated with the shut-off of the host translation [5], while A/PR/8/34 NS1 is not [22]. Melén et al. reported that whether NS1 targets the nucleolus depends on the C-terminal sequence of NS1, especially regarding the combination of basic amino acid residues in the region [18]. The efficiency of the interaction between NS1 and nucleolin may also depend on the virus strain, although it occurs via the N-terminal RNA-binding domain of NS1. Differences in the efficiencies of nucleolar targeting and binding with nucleolin may also be an explanation of the difference in the virulence of NS1 seen among various strains.

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1 女性生殖器における免疫機構

早川 智，真島洋子，相澤志保子

女性生殖器粘膜における免疫応答は，消化器や呼吸器とは異なった特徴を有する．すなわち，腟や子宮内膜（脱落膜）には月経周期や妊娠，閉経など刻々と変化する内分泌環境の影響を強く受けて，ダイナミックな機能的変化をとげる局所免疫系が存在する．未熟なNK細胞やNKT細胞，マクロファージ，樹状細胞などからなる女性生殖器粘膜免疫系は宿主を感染症から防御すると同時に，非自己である精子，胎児胎盤を許容して妊娠を成立させる．妊娠現象とその破綻，また，HIV感染を含む性行為感染症（sexually transmitted disease：STD）や垂直感染の理解と制御には女性生殖器粘膜における免疫応答の理解が必須である．

はじめに

個体の寿命が有限であることの必然として，遺伝子はその乗り物を次々に変えてゆく必要がある．バクテリアのように無性生殖で同一のコピーを増やすことがもっとも手っ取り早く，コストがかからないが，プラスミドなどによる遺伝子の交換には自ら限界がある．雌雄両性による有性生殖の本質は世代ごとの遺伝子のシャッフリングにより，環境の変化，特にあらたな病原微生物の出現や変異に対する宿主の多様性を確保することにあると考えられている．それには異なった遺伝的背景を有する個体との間で情報の交換が必須であり，さらに子宮内で胎児胎盤を育てる真胎生動物では胎児胎盤を許容するため特異免疫系と折り合いをつけねばならない．

1953年，Sir Peter Medawarが免疫学的異物である胎児胎盤がなぜ，拒絶されないのかという生殖免疫学の中心的命題を提起して半世紀が過ぎた現在でも，妊娠現象は謎に満ちている．彼が想定した四つの仮説，① 母体の免疫応答は妊娠中低下する．② 胎児胎盤は免疫学的に未熟である．③ 子宮腔内は免疫学的に特異な場所であり，免疫応答が生じない．④ 胎児循環と母体循環は胎盤によって完全に隔離されている．①は，現在ではいずれも否定され，1）胎盤におけるextravillous trophoblastは単型のHLA-Gを発現し細胞傷害性T細胞（cytotoxic T lymphocye：CTLを誘導できないと同時にNK細胞のネガティブシグナルとなる．2）脱落膜局所のリンパ球は活性化し種々のサイトカインを分泌するがこれは胎児胎盤の成長を促進する（immunotrophism）[2]．3）脱落膜局所に制御

[キーワード&略語]
脱落膜，生殖内分泌，有性生殖，γδT細胞，胸腺外T細胞，Th1/Th2パラダイム

dNK細胞：decidual Natural Killer細胞（脱落膜NK細胞）
STD(STI)：sexually transmitted disease (sexually transmutted infection)（性行為感染症）

Mucosal immune system of female reproductive tract
Satoshi Hayakawa/Hiroko Majima/Shihoko Komine-Aizawa：Division of Microbiology and Infectious Diseases, Department of Pathology and Microbiology, School of Medicine, Nihon University（日本大学医学部病態病理学系微生物学分野）

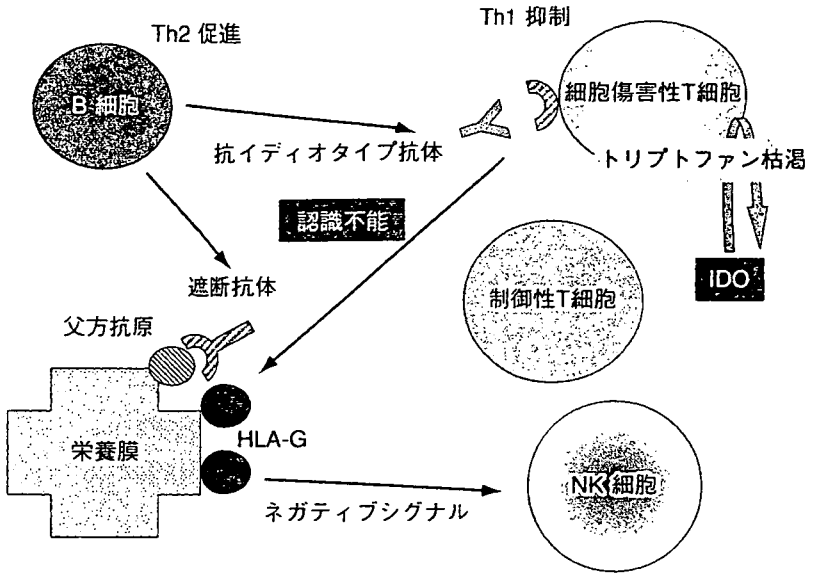

図1　脱落膜における免疫応答
母体との接点にある栄養膜細胞は単型のHLA-Gを発現し，細胞傷害性T細胞の認識を免れると同時にNK細胞に負のシグナルを送る．妊娠中はTh2優位の免疫環境となり，細胞傷害性T細胞の機能は抑制される．胎盤が産生するIDOは局所のトリプトファンを代謝して細胞傷害性T細胞の機能は抑制する一方，制御性T細胞の機能を活性化する

性T細胞[3]やTr1細胞など抑制性の細胞が存在する．4）妊娠中はTh2優位の免疫学的環境にある．5）IDO（indoleamine-2, 3-dioxygenase）による局所のトリプトファン欠乏がCTLを抑制する[4]．などの機構が関与すると考えられている（図1）[5]．

1 子宮内膜・脱落膜の粘膜免疫

特異免疫系の起源は有顎脊椎動物の消化器粘膜にある．[6] 消化器粘膜における免疫応答系は進化のうえで，その祖形に最も近いものであり，呼吸器や生殖器における粘膜免疫や皮膚の免疫はこれから派生したものと考えられる．粘膜免疫の研究は消化管を中心に進められてきたが，女性生殖器における粘膜免疫応答は① 月経周期など内分泌因子による調節を受ける．② 異物である精子・胎児抗原の認識と受容を行う．という2点で他の局所免疫系とは異なった側面がある．臨床的には① STDの侵入門戸となる．② 妊娠の生理と病理に直接関与する．③ 婦人科腫瘍の発生と進展に関与する．といった点が重要である．

腟内には細菌叢が存在するが，子宮腔内や卵管内は無菌である．女性生殖器は種（厳密には自己の遺伝子）を保存するために，胎児の生存には危険を及ぼすことなく，病原体から自己を防御する免疫機構を進化させた．胎児に対し，拒絶的に働く個体は子孫を残せない一方，無制限に異物を受け入れる個体は感染症に対する抵抗性を維持できないからである．ここに，胎児の生存を守り，かつ潜在する病原体から保護していくという女性生殖器独自の免疫機構の存在理由がある（図2）．

2 上皮細胞

内性器粘膜の構成細胞は上皮細胞，マクロファージ，樹状細胞（DC），好中球，NK細胞，T細胞，B細胞，NKT細胞などである．上皮細胞はその最前線に位置し，連続的なバリアーとして微生物の侵入を防止する．腟粘膜，子宮内膜とも性ホルモンの直接的な影響を強く受ける．腟から子宮腟部は扁平上皮に覆われ，エストロゲンによってグリコーゲンを産生する．角化して剥離した上皮細胞中のグリコーゲンは腟内のデーデルライン桿菌によって分解されて乳酸となり，腟内を酸性環境に保つことで，雑菌に増殖を抑制する．（腟の自浄作用）

先に述べたように子宮腔内は無菌であるが，性周期を有する女性では月経により約28日毎に剥離脱落と再生を繰り返す．子宮腔と腟内は頸管粘液で遮断され，細菌の侵入を防ぐが原則的に精子の侵入は許す．

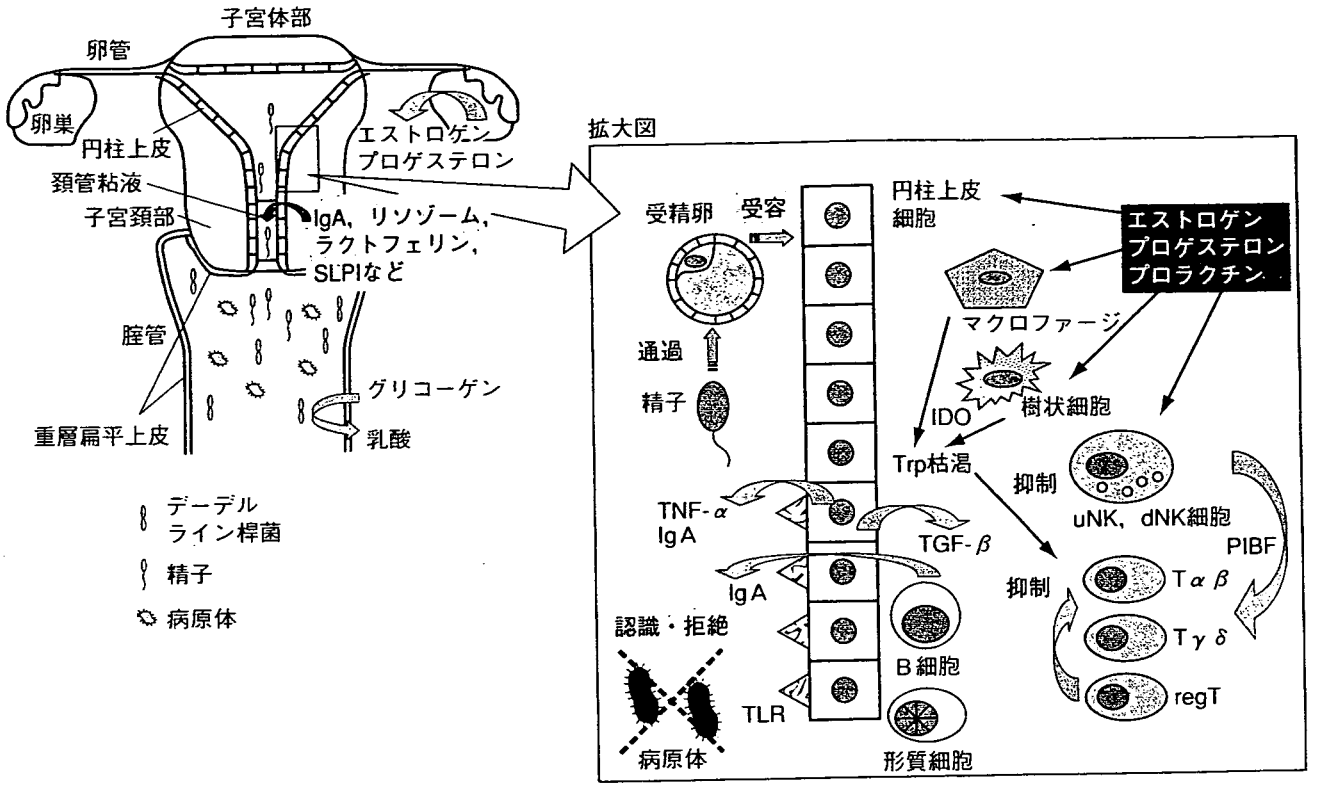

図2　女性生殖器の粘膜免疫

膣上皮の産生するグリコーゲンはデーデルライン桿菌によって乳酸に代謝され，膣内を強酸性に保つ．子宮頸管は粘液栓により，膣腔と隔てられ，頸管内に分泌されるIgA,ラクトフェリンなどが細菌や真菌，原虫，ウイルスの進入を抑制する一方，精子の進入を許す．半非自己（semi allograft）である胎児胎盤は子宮内膜が性ステロイドによって変化した脱落膜に着床し，拒絶されることなく妊娠期間（ヒトでは280日間）を過ごす

頸管腺によって産生される粘液の量や性情はエストロゲンによって調節される．常在菌が存在する膣や子宮膣部は重層扁平上皮細胞に覆われ，機械的損傷に抵抗性があるが，子宮頸管や内膜，卵管の円柱上皮は一層であるため，物理的損傷や感染には脆弱である．しかし，妊卵を許容するためには欠くことのできない構造である．実際，子宮腔内の慢性炎症では扁平上皮化成を見ることがあるが，このような場合には着床不全を伴う．近年，上皮細胞が単なる物理的バリアーとしての役割だけでなく，感染制御に積極的にかかわること，これに性ホルモンが密接に関与することが明らかになった．獲得免疫系は，特異的に活性化して病原体に対する反応性を発揮するまでに一定の時間を要するため，病原体の侵入初期には非特異的な防御機構が作用する．その1つは，上皮細胞による殺菌性の可溶性物質産生である．重要なものにデフェンシンやSLPI（secretory leukocyte protease inhibitor），LF (lysozyme and lactoferrin)，tracheal anti-microbial peptideなどがある．実際臨床的に，膣部頸管粘液中のSLPIの濃度は閉経前の女性の方において，閉経後よりも有意に高いこと，月経周期によって変動することが報告されている．妊娠中や羊水中にはさらに増加しており，その分泌はプロゲステロンに依存する．子宮内腔の上皮細胞は構造的，機能的に極性をもった配置をとっており，内腔にTNF-αやIgAを分泌する一方，基底膜下にTGF-βを分泌する．その分子的基盤として，Wiraらは培養上皮細胞を用いてTER (transepithelial resistance) という概念を提唱し，これがエストロゲンによって濃度依存性に調節されていることを明らかにした．上皮細胞は間質細胞とも，直接接触あるいはケモカインやEGF (epidermal growth factor)，VEGF (vascular endothelial growth factor) など増殖因子を介した密接な機能的関連を有してしており，これもエストロゲンの支配を受ける．

頸管上皮細胞，子宮内膜上皮細胞はTLRを発現し，微生物の侵入に対応するが，その発現もエストロゲン

の影響を受ける．すなわち，生殖年齢にある女性では微生物侵入の機会も高いためTLRの発現は高いレベルに維持されるが，閉経後はエストロゲンが低下すると同時にTLR発現も低下する．TLRについても原則的に無菌の上部生殖器とフロラのある下部生殖器を分けて考える必要があろう．

3 マクロファージと樹状細胞

単球・マクロファージは病原体や死細胞を認識して貪食する．さらに獲得免疫系に対して抗原提示を行うことにより，防御反応連鎖の第一線を担う．異物認識とその処理にかかわる機能は性ステロイドホルモンを含む可溶性分子の調節を受ける．解剖学的にはマクロファージはヒトの女性生殖器に広く分布し，組織白血球のおよそ10％を占めるが，特に内膜間質と筋層の結合組織の中に多く存在する．月経前の子宮間質に選択的に集まり，黄体消退に伴って他の内膜組織とともに剥離脱落する．Wiraらは子宮内膜マクロファージがER，PRを介して，性ステロイドホルモンの直接的調節を受ける可能性を報告したが，Ariciらは内膜間質細胞をエストラジオール処理することで，MCP-1発現が減少することから，これによるマクロファージの移動抑制が重要ではないかとしている．さらに，ヒトの内膜組織のマイクロアレイ解析により，他のケモカイン，MCP-3，FKN，MIP-1βなどは月経前に増加することが報告されている．

粘膜免疫からは若干外れるが，マクロファージは卵巣機能においても重要な因子となっている．子宮のマクロファージ同様，卵巣マクロファージの分布は月経周期による変化を示す．マクロファージは血管周囲の結合組織と，排卵前後の黄体の被膜に数多く存在する．排卵は生理的な炎症反応として説明できるが，その調節因子として卵巣のマクロファージはTNF-α，IFN-γ，IL-1，IL-6，IL-10，IL-12などのサイトカインを介し，また貪食作用による黄体融解や細胞外基質の処理を行う．その過程でマクロファージ由来のMMPによる，卵胞の発生と閉鎖の調節が重要な役割を果たす．

一般に自然免疫では，エストロゲンはERαを介してマクロファージの機能を抑制すると考えられている．LambertらによるERαのノックアウトマウス（KO）の実験では，LPSと*Mycobacterium avium*に感作させた腹腔マクロファージを*in vitro*で刺激したとき，ERα−/−動物では野生型の腹膜マクロファージよりも強くTNF-αを分泌するという．一方では，エストロゲンはB-1細胞や胸腺外T細胞など自然免疫系細胞の活性化を誘導することから，これのみで自己免疫性疾患発生率における性差や治療抵抗性を説明することは難しい．自己免疫疾患と性ホルモンについては興味ある問題であるが，本総説の趣旨から外れるので成書や総説を参考にしていただきたい[7) 8)]．

マクロファージと類縁の細胞に樹状細胞がある．樹状細胞とは樹枝状の突起を伸展させていることを形態的な特徴とし，全身の他の臓器同様，子宮内膜・脱落膜にも一定数が存在する．クラスⅡのMHCを恒常的に発現し，貪食した異物抗原をT細胞に提示することをその主要な機能とする．しかし，実際に抗原特異的な適応免疫応答が誘導されるためには，樹状細胞上にT細胞の活性化に必要とされる種々の補助刺激分子を発現し，サイトカインを産生する必要がある．脱落膜の樹状細胞はDC-SIGNを発現し，これが抑制性の免疫応答に必須であること[9)]，プロゲステロンが脱落膜におけるミエロイド型の樹状細胞の分化に必須であることが報告されている[10)]．宮崎らは脱落膜の樹状細胞がCD80とCD83，CD86を構成的に発現しさらにミエロイド型の表現型である$CD11c^+CD123^-$であること，この細胞がTh2型の免疫応答を誘導することを報告した[11)]．マクロファージ，樹状細胞はトリプトファン代謝の律速酵素で，T細胞の調節因子であるIDOを発現することにより，局所の免疫応答を負に調節する．われわれは，本来細胞性免疫応答の活性化因子であるIFN-γが高濃度のプロラクチン（PRL）存在下ではIDO産生発現を誘導することを明らかにした[12)]．PRLは下垂体前葉より分泌されるが，脱落膜間質細胞でも産生されることから局所の免疫調節機構に関与している可能性がある．

4 NK細胞

子宮内膜と腸管粘膜にはアズル顆粒陽性で$CD16^-CD56^+CD3^-$の未熟なNK細胞が多数存在する．1920年Weilは腸管粘膜と，脱落膜に存在する大顆粒リンパ球（*Les cellules granuleuses des musqueses in intestinale et uterine*）として報告した[13)]．NK細胞は全身に広く分布するが，末梢血のNK細胞の大部分

が$CD16^+CD56^{dim}$の表現系を示すのに対し，脱落膜NK細胞は，$CD16^-CD56^{bright}$である．$CD56^{bright}$細胞は胎生期初期に現れることや，骨髄移植後早期に出現することからきわめて未熟なNK細胞と考えられている．しかし，近年のマイクロアレイ解析から，子宮内膜NK細胞（uNK細胞）は血中の$CD16^-CD56^+$NK細胞とは異なった独自の細胞集団らしいことが明らかになってきた．この細胞群は妊娠により著しく増加し，脱落膜NK細胞（dNK細胞）として妊娠初期には脱落膜免疫細胞の80％を占めるに至る．現時点ではdNK細胞は血中のNK細胞に由来して，脱落膜という特殊な環境で分化したのか，脱落膜局所で複製しているかは不明であるが，その増殖や分化に性ステロイドが関与していることは間違いない．しかし，リアルタイムRT-PCRでER，PRを欠くとする成績もあり[14]，間質細胞や上皮細胞による間接的な支配を受けている可能性がある．一方，Szekeres-BarthoらはCD56uNK細胞（dNK細胞）がプロゲステロンによって誘導される免疫調節物質（PIBF）を産生し，これがB細胞の遮断抗体産生やTh2優位の免疫応答を誘導することから妊娠維持に必須であるという仮説を提唱している[15) 16)]．

5 脱落膜T細胞

免疫染色あるいはフローサイトメトリーによって検討すると脱落膜内には$\alpha\beta$もしくは$\gamma\delta$のT細胞受容体を有する成熟したT細胞が存在する．しかし，その割合は末梢血に比較して著しく少なく，その性状は長く明らかではなかったが，1994～1995年われわれとLundqvistらのグループが相次いで胸腺を経ないで分化する胸腺外T細胞がその多くを占めることを明らかにした[17) 18)]．T細胞を欠損したヌードマウスやRAG-1欠損マウスが妊娠可能であることから脱落膜のT細胞が妊娠維持に必須である可能性は低いがT細胞受容体を介した特異的認識が病的状態に関与する可能性がある．斎藤らは脱落膜T細胞ではT細胞受容体（TCR）／CD3複合体がほとんどすべての細胞で低下していること[19)]，しかしながらCD69，HLA-DR，IL-2Rβ鎖などの活性化抗原を発現していることを明らかにした[20)]．その意義として彼らは脱落膜において胎児胎盤抗原やサイトカインによって活性化されたT細胞に同時に寛容が誘導されたためと説明している．

MHC-TCRを介したactivationやanergyでは特定のエピトープを認識するクローンのみがTCRの発現低下や活性化マーカーの表出を行うのに対して脱落膜では全T細胞がこのような変化を示すことから抗原非特異的な刺激の存在や未熟な胸腺外T細胞としての性状である可能性がある．われわれはさらに脱落膜T細胞がCD161に加えて，NKT細胞特異的なTCR $V\alpha24$を使用することを明らかにした[21)]．胸腺外T細胞，NKT細胞はともにエストロゲン受容体を介してその機能を調節されるが[22) 23)]，抗原非特異的な感染防御や過剰な炎症反応の調節に関与するのみならず，胎児胎盤認識のうえで何らかの生理的な役割を果たしていると考えられる．正常妊娠脱落膜T細胞，NK細胞はともに通常の培養条件では自己絨毛細胞や絨毛癌細胞株に細胞傷害性を有さないがIL-2やIL-12の刺激によって傷害性を獲得する[24)]．興味深いことに妊娠中に胎盤が産生するG-CSFはIL-2やIL-12によって活性化した脱落膜リンパ球の絨毛細胞傷害性を強く抑制する[25) 26)]．妊娠時のG-CSFは一義的には局所の免疫抑制因子として作用し，母体の白血球増加は二次的な現象のようである．

6 $\gamma\delta$T細胞

子宮内膜には他の粘膜や表皮と同じく$\gamma\delta$T細胞が存在する．$\gamma\delta$T細胞の生理的機能は不明な点が多いが，多様性のない自己MHC類似抗原（CD1やTLaなど）や熱ショックタンパク質HSP60を認識し，初期T細胞防御反応として病原体や障害細胞の除去に働く一方，過剰な免疫反応を抑制し組織破壊を防御すると考えられている．Mincheva-Nilssonらは免疫電顕法によって脱落膜にはCD56陽性の$\gamma\delta$陽性T細胞が多数存在しT細胞の活性化マーカーであるCD45ROを発現していることを明らかにした．マウスでは脱落膜$\gamma\delta$細胞はリンパ球混合培養に対して抑制的に作用するが[27)]，われわれはこれら可移植腫瘍初期病変に浸潤する$\gamma\delta$T細胞と同一の細胞集団であることを明らかにした[28) 29)]．さらにわれわれは，脱落膜では腫瘍内リンパ球やアレルギー患者鼻粘膜[30)]と同一の抑制性$V\gamma I\delta1$細胞が多く存在しIL-10やIL-13などTh2サイトカインやTGF-βを産生することを報告した[31)]．先に述べたようにマウス脱落膜に存在する$\gamma\delta$T細胞はTGF-$\beta2$を産生しアロの免疫応答を抑制することが知られて

いる[32)～34)].

近年，マウス脱落膜γδT細胞は均一の細胞集団ではないことが明らかになってきた．Clarkらは習慣性流産モデルマウスとして有名なDBA/2VXBA/Jの系において妊娠6.5日asialo GM1陽性Vγ1.1δ6.3T細胞がIFN-γを産生して着床を阻害するのに対して，妊娠8.5日にみられる同一のT細胞受容体を使用する細胞はIL-10やTGF-β2を産生し妊娠維持に働くとしている[35)]．彼らによるとNKマーカーであるasialo GM1の有無がサイトカインの産生パターンと密接に関係しているという．すなわちasialo GM1陽性のγδNKT細胞は胎児胎盤に傷害性に作用しasialo GM1陰性の古典的γδT細胞はTh3やTr1の表現型を有し妊娠維持に働くらしい．また，抑制性のγδT細胞からのシグナル伝達経路には新たに発見された抑制性分子OX-2が関与するという．彼らは局所のTh1/Th2Th3比がそのまま妊娠予後に結びつくわけではないがLPSやストレスによる流産感受性に強く相関するとしている．γδT細胞の分化も，性ステロイドの支配を受けることが知られており，PIBFの関与が強く示唆されている．

7 Th1/Th2パラダイムとその限界

抗原特異的な免疫応答において中心的な役割を担うCD4陽性のヘルパーT（Th）細胞はB細胞による抗体産生や細胞傷害T細胞の機能を調節する．1990年代には，Th細胞が産生サイトカインによってTh1Th2の2種類に大別されること，そして2つのバランスの変化が自己免疫疾患やアレルギーなどの病態に密接に関係していることが明らかになった．Th1優位の免疫応答が生じるマラリアやリステリア感染では流早産がみられることから，妊娠の維持にはTh2優位が必須と考えられている．脱落膜局所におけるTh2の優位を示唆する所見として，Piccinniらはヒト正常妊娠脱落膜より樹立したT細胞クローンの多くがTh2サイトカインを産生するが流産患者では低下することを報告し[36)]，斎藤らは子宮内膜T細胞におけるTh1/Th2細胞比を解析し妊娠初期にTh2が優位であることを明らかにした[37)]．脱落膜局所にTh2優位の環境を誘導する因子としてマクロファージが産生するIL-10に加えてPGE2が重要と考えられている[38)]．また胎盤で産生されるプロゲステロンやhCGが直接的あるいは間接的にTh2型の免疫応答を誘導すると考えられるが，これに加えてわれわれはTh2特異的なケモカインであるTARCの関与を明らかにした[39)]．さらに長時間同一抗原に暴露するとTh1細胞が選択的にアポトーシスに陥ることが知られており[40)]，妊娠中は胎児胎盤抗原の存在自体がTh2を誘導している可能性がある．妊婦末梢血でもTh2優位となっている知見としてFCMにより細胞内サイトカインを定量すると妊娠中後期ではTh1/Th2比がTh2優位に傾くこと[41)]，夫リンパ球で妊婦末梢血を刺激すると選択的にTh2サイトカインが産生されること[42)]，などが報告されている．

習慣流産において，全身的にTh2が抑制されTh1優位となっていることを示唆する所見としては，末梢血T細胞をPHAなどの非特異的なmitogen[43)44)]や絨毛癌細胞株由来抗原[45)]，当該妊娠胎盤絨毛細胞[46)]で刺激したときに習慣流産患者では健常妊婦に比較してIL-2やIFN-γなどtype-1サイトカインの産生が増強しtype-2サイトカインであるIL-10の産生が抑制されること，FCMで細胞質内にTh1サイトカインが染色されるCD3細胞が有意に増加していること[47)]が報告されている．われわれは習慣流産患者に対し夫リンパ球による免疫療法を施行するとTh1/Th2比が低下し，これが妊娠予後とも相関することを報告した[48)]．しかしながら，Th1サイトカインであるインターフェロンγ受容体をノックアウトしたマウスは不妊であり[49)]，着床初期にはTh1型の炎症反応による血管構築の誘導が必須であると考えられる．また，われわれが行ったマウスにおけるサイトカイン活性化リンパ球移入実験では，Th1あるいはTh2いずれにも過剰活性化した動物では胎児胎盤の吸収と妊娠中毒症様の症状が認められた[50)]．妊娠はTh2善玉Th1悪玉という簡単なスキームでは必ずしも十分な説明はできない[51)]．

8 抑制性免疫応答の内分泌支配

真に免疫応答に対する負の制御を行うには，抗原特異的に活性化してTh1，Th2いずれもの応答も抑制する必要がある．この機能を担うのが坂口らにより発見された制御性T細胞である．$CD4^+CD25^+$の表現型を有するこの細胞は，免疫自己寛容を維持し，自己免疫疾患の発症阻止に関与するのみならず，感染症においては過剰な応答による組織破壊から宿主を防御し，また妊娠の維持に関与する．健常人では末梢血の5～

10％を占めるが，基底側脱落膜T細胞では70～80％に達する[52]．実際機能的にも，マウスならびにヒトの系で，着床と妊娠維持に制御性T細胞が必須であることがAluvihareらおよび[53]佐々木らにより報告された[54) 55]．制御性T細胞の誘導ならびに調節の分子機構は，癌の免疫療法や臓器移植の制御にもつながるため近年，注目を集めている領域であるが，近年，複数の施設よりエストロゲンがFOX-P3の発現をup-regulationすることにより，制御性T細胞の活性を調節することが明らかにされた[56) ～58)]．性ホルモン以外にも，胎盤，脱落膜で産生される複数のホルモンや神経伝達物質が局所の免疫応答を調節している可能性がある．われわれはβエンドルフィンがμ受容体非依存的にNK細胞，NKT細胞のサイトカイン産生を調節することを明らかにした．またβエンドルフィンはTLRを介したIFN-αの産生も負に制御することが明らかになった．胎盤は中枢神経，副腎髄質に次ぐエンドルフィンの産生臓器であるが，産生されるのはμ受容体に結合活性のないN-アセチルエンドルフィンであり，その生理的機能は謎であったが，われわれの研究より局所の免疫応答にかかわる可能性が示唆される．

おわりに

女性生殖器における粘膜免疫は，内分泌支配を受けていることが他の臓器と大きく異なる．その解析により，妊娠機構の解明や妊娠合併症の予防，性行為感染症を含む感染症の解析と制御に新たな道が開ける可能性がある．

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Rembrandt's Maria Bockenolle has a butterfly rash and digital deformities: Overlapping syndrome of rheumatoid arthritis and systemic lupus erythematosus

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Summary Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are the most common autoimmune disorders, although they each have very different pathophysiology. In general, RA is considered to be a Th1-mediated disease, while SLE is a Th2-mediated disease. Thus, their overlapping, in so called "rhupus", is a rare condition. In Rembrandt van Rijn's (1606–1669) portrait of the middle-aged Maria Bockenolle, we have what may be the earliest depiction of a case of rhupus syndrome: the coexistence of a butterfly rash and digital deformities. This suggests the possible historical importance of an RA epidemic which took place in the early 17th century.

Introduction

In 1936, Sigerist, the American medical historian wrote "Whenever a doctor goes on a vacation trip to Europe, accompanied by his wife, who insists

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on seeing the galleries, he spends his time hunting for pathological subjects, sure to make 'discovery' and to write paper about that" [1,2]. The same is true for certain Japanese doctors of today, during their travels to the United States and Europe.

Portrait of Maria Bockenolle

Recently, two of the authors (S.Hayakawa and Komine-Aizawa) visited the Boston Museum of Fine Arts and encountered Rembrandt's portrait of a middle-aged woman named Maria Bockenolle. The original oil-on-canvas from 1634 measures 174.9 cm × 124.1 cm (68 7/8 in. × 48 7/8 in.), and a digital-version can be seen on the homepage of the museum [3]. On closer inspection, the visiting authors noted a redness in the cheeks consistent with a butterfly rash and a remarkable deformity of the fifth finger of her left hand. Her right hand also showed evidence of swelling of the fingers and wrist joint (Fig. 1).

Figure 1 Portrait of Maria Bockenolle. By courtesy of the Museum of Fine Arts, Boston. The original from 1634 oil-on-canvas measures 174.9 cm × 124.1 cm (68 7/8 in. × 48 7/8 in.)

Rembrandt on the 400th anniversary of his birth

Rembrandt Harmenszoon van Rijn (July 15, 1606–October 4, 1669) is generally considered as one of the greatest painters of the West and may be the single greatest Dutch artist of all time. He produced about 600 paintings, 300 etchings, and 2000 drawings [4], and was a prolific portraitist during a period of three decades in the mid-17th century. He realistically depicted several pathogenic changes in portrait models as well as in himself. In fact, the tumorous changes in his female model for Bathsheva has been discussed by us [5] and others in the medical literature [6,7]. Marcus and Clarfield suggested that Rembrandt's self-portraits either faithfully represented observable details of illnesses from which he may have suffered, or may have simply reflected his failing eyesight. They also suggested probable psychological changes, including depression and mood disorders [8]. In his latter self-portraits, Espinel notes brow and eyelid posies, xanthelasma and arcus senilis, rosacea with complicating rhinophyma, and temporal vasculitis [9].

Possible diagnoses of Maria Bockenolle

Since there is no known biographical data on Ms Bockenolle, we based our diagnosis solely on our observations of the portrait, following in the best tradition of Sherlock Holmes, with careful attention to detail. The butterfly rash is the characteristic skin lesion of SLE. In this rash, erythema erupts in a butterfly distribution on the cheeks of the face and across the bridge of the nose. It is frequently precipitated by sun exposure, may precede SLE by weeks or months, and is often associated with other skin lesions. However, in the present portrait, no other signs of facial SLE, including cutaneous lupus erythematosus, vasculitis, and/or Raynaud's phenomenon, are visible. Further, because the model wore a black dress with long sleeves and a long skirt, other signs, such as lichen planus, leg ulcers, generalized vasculitis, thrombophlebitis etc., were not visible.

While it is possible that the reddened face was simply the result of over-zealous cosmetic applications on the part of Ms Bockenolle, the use of rouges being popular in her day – this is doubtful because the lines were clearly demarcated and involved her nose. Further, no such signs are visible in Rembrandt's many other portraits of young and middle-aged women, including: portrait of a Lady with an Ostrich-Feather Fan (c. 1660, National

Gallery of Art, Washington); portrait of a Young Woman with the Fan (1632, National Museum, Stockholm, Sweden); Cornelia Pronck, wife of Albert Cuyper (1633, Louvre, Paris, France); and portrait of Saskia (1633, Alte Meister Gallerie, Dresden, Germany). Thus, it is probable that Rembrandt accurately depicted the markings as they appeared on Ms Bockenolle [10].

In any case, the deformity of the fifth finger of her left hand is quite remarkable on its own. This kind of finger deformity can be variously attributed to congenital malformation, post-traumatic changes, and rheumatic disorders. While such congenital anomalies were probably as uncommon then as today, contracture deformity after a traumatic insult was probably more frequent during the 17th century, due to lack of appropriate medical care. However, even given these alternative causes, we concluded that she suffered from rhupus because of the coincidence of rheumatoid arthritis (RA) evident in her right hand as a slight digital deformity and swelling of the wrist. Based on these observations, we propose that Rembrandt's model suffered from an overlapping syndrome of RA and SLE.

Importance of the portrait

RA is traditionally considered a chronic, inflammatory autoimmune disorder. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction. The disease is also systemic in that it often also affects many extra-articular tissues throughout the body, including the skin, blood vessels, heart, lungs, and muscles.

Before its first description by Landre-Beauvais (1772–1840) in 1800, a house doctor at La Salpetriere hospital in Paris, RA was not considered to be an independent disease, and it was often confused with gout [11]. It is interesting, however, that there are no paleopathological samples, paintings, or descriptions suggesting RA before the 16th century in Europe [12], while ancient Native American skeletons, dated at between 4500 B.C. and 450 B.C., have shown traces of RA [13].

Appelboom et al. previously noted evidence of rheumatic changes in a painting by Peter Paul Rubens (1577–1640), a contemporary of Rembrandt, and suspected that Rubens himself suffered from RA in the last 30 years of his life [14,15]. Appelboom further suggested that unknown infectious pathogen(s) might have been imported from the New World to the Flemish port Antwerp, which was then a cultural center and the crossroads of Europe, under Spanish domination. Indeed, many other researchers have reported a possible epidemic of RA through the 17th–19th centuries in Western Europe.

By contrast, SLE has a relatively long history in Europe: cutaneous ulcers were first described by Hippocrates (460–375 BC), and dermatological descriptions of SLE were made by Herbernus of Tours in 916 AD and was termed herpes esthiomenos. The first clear description of lupus erythematosus was reported by Biett and Cazenave under the term erythema centrifugum in 1833. Later, Cazenave then renamed the disease lupus erythematosus [16].

So called "rhupus", which is an overlapping syndrome of RA and SLE, has only occasionally been recorded. Pannush et al. wrote that "rhupus indeed exists as a syndrome manifested by patients sharing features of probable coincidental concurrence of RA and SLE, but not as a unique clinical pathologic or immunologic syndrome". Although the incidence of rhupus may be as low as 0.1% based on their sample of 7000 patients with autoimmune disorders, they concluded that a recognition of this overlap among such patients is important since their optimum therapy and outcome differ from those having RA or SLE alone [17]. Rodriguez-Reyna and Alarcon-Segovia suggested that autoimmune rheumatic diseases may coexist due to an interplay of environmental factors with genes that control susceptibility of the diseases , which he termed "shared autoimmunity" [18].

In general, RA is considered to be a typical Th1 disease while SLE is a Th2 disease. Yet, even though both Th1 and Th2 cytokines play important roles in the pathophysiology of autoimmune disorders, this categorization is considered to be an over-simplification [19]. In this regard, the physical presentation of Maria Bockenolle, if accurately rendered, represents an important early example of a case of rhupus.

Conclusion

Without additional information on the clinical and physical signs and symptoms of the historical Maria Bockenolle, and in the certain absence of laboratory data on this "patient", no definitive diagnosis is possible. Nevertheless, a careful examination of representative paintings such as those of the 17th century Dutch masters, combined with recent clinical and immunological findings, suggests the presence of an overlapping syndrome and may be a