RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection

細菌感染分野

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Research in this division is directed toward understanding the complex interactions that occur between pathogenic bacteria and their human hosts at very early stage of bacterial infectious processes. Our special interest is focused upon the molecular pathogenicity of enteropathogenic bacteria, such as Shigella, enteropathogenic E. coli, enterohemorrhagic E.coli and Helicobacter pylori. We are also searching for effective methods to protect or regulate bacterial infection by using knowledge accumulated.

1. Hyper Adherence to Caco-2 Cells Caused by Disruption of *yhiE* and *yhiF* Genes in Enterohemorrhagic *Escherichia coli* O157:H7

Ichiro Tatsuno, Keiji Nagano[1], Kazuki Taguchi[1], Li Rong, Hiroshi Mori[1] and Chihiro Sasakawa: [1]Laboratory of Microbiology, Department of Public Health Pharmacy, Gifu Pharmaceutical University

Adherence of enterohemorrhagic *Escherichia coli* (EHEC) to intestinal epithelium is essential for initiation of the infection including diarrhea, and for the adherence, expression of the genes of the locus for enterocyte effacement (LEE) is thought to be crucial. To identify genes involved in modulating the adherent capacity, a collection of an EHEC O157:H7 strain (O157Sakai) mutagenized by mini-Tn*5*Km2 were screened for their ability to increase the number of microcolonies (MC) on Caco-2 cells, and eight hyper adherent mutants were isolated. Analysis of the mini-Tn*5*Km2-flanked DNA sequences indicated that one possessed the insertion within an O157 antigen gene cluster, the other within the *yhiF* gene, and the remaining 6 mutants had their insertions in the *yhiE* gene. *yhiE* and *yhiF* products share amino acid homology (23% identity) to each other and with the LuxR family known as transcriptional regulatory proteins. The mutant having the insertion within the O157 antigen gene cluster, but not the other seven mutants, did not express the O157 side chain as determined by agglutination test and immunoblotting with polyclonal O157-specific antiserum. Importantly, the other mutants showed enhanced type III secretion. Their related mRNAs of LEE, but not *ler* mRNA, were also increased as compared with those in the wild-type. Indeed, when we introduced an in-frame deletion into the *yhiE* or *yhiF* gene in O157Sakai, the capacity of the resultant mutants to adhere to Caco-2 cells was greatly increased. When one of the *yhiE* insertion mutants was orally inoculated into ICR mice, the number of bacteria shed into feces by day 14 was greater than that for wild-type. These results suggest that *yhiE* and *yhiF* are involved in the adherence of O157Sakai to epithelial cells as negative regulators for the expression of the genes required for the type III secretion system.

2. Grb2 is a Key Mediator of *Helicobacter pylori* CagA Protein Activities

Hitomi Mimuro, Toshihiko Suzuki, Jiro Tanaka, Momoyo Asahi[2], Rainer Haas[3], Chihiro Sasakawa: [2]Faculty of Nursing and Welfare, Fukui Prefectural University, and [3]Max von Pettenkofer Institut für

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CagA delivered from *Helicobacter pylori* into gastric epithelial cells undergoes tyrosine phosphorylation and induces host cell morphological changes. Here we show that CagA can interact with Grb2 both *in vitro* and *in vivo*, which results in the activation of the Ras/MEK/ERK pathway and leads to cell scattering as well as proliferation. Importantly, this ability of CagA is independent from the tyrosine phosphorylation, which occurs within the five repeated EPIYA sequences (PY-region) of CagA. However, the PY-region appears to be indispensable for the Grb2 binding and induction of the cellular responses. Thus, intracellular CagA via its binding to Grb2 may act as a transducer for stimulating growth factor-like downstream signals which lead to cell morphological changes and proliferation, the causes of *H. pylori*-induced gastric hyperplasia.

3. Neural Wiskott-Aldrich syndrome protein (N-WASP) is the specific ligand for *Shigella* VirG among the WASP family and determines the host cell type allowing actin-based spreading

Toshihiko Suzuki, Hitomi Mimuro, Shiro Suetsugu[4], Hiroaki Miki[4], Tadaomi Takenawa[4], and Chihiro Sasakawa: [4]Department of Biochemistry, Institute of Medical Science, University of Tokyo

Shigella cause bacillary dysentery , a disease provoking a severe inflammatory diarrhea in humans and primates. *Shigella* is capable of directing its movement within the cytoplasm in infected host cells by forming an actin comet tail. The VirG (IcsA) protein, encoded on a 230kb large plasmid, expressed at one pole of the bacterium recruits neural Wiskott-Aldrich syndrome protein (N-WASP), a member of the WASP family, which in turn stimulates actin-related protein (Arp) 2/3 complex-mediated actin polymerization. In mammalian cells, five WASP family members, N-WASP, WASP and WAVE1-3 have been identified. Since all of the WASP family proteins induce actin polymerization by recruiting Arp2/3 complex, we investigated their involvement in Shigella motility. We showed that VirG binds to N-WASP but not the other WASP family proteins. Using a series of chimeras obtained by swapping N-WASP and WASP domains, we demonstrated that the specificity of VirG to interact with N-WASP lies in the N-terminal region containing the pleckstrin homology (PH) domain and calmodulin-binding IQ motif of N-WASP. A conformational change in N-WASP was important for the VirG-N-WASP interaction, since elimination of the C-terminal acidic region, which is responsible for the intramolecular interaction with the central basic region of N-WASP, affected the specific binding to VirG. The expression of WASP is limited to cells of hematopoietic origin such as lymphocytes or platelets. In contrast, N-WASP is thought to be ubiquitously expressed. However, whether the level of N-WASP expression in hematopoietic cells would be the same or not as that in other epithelial or fibroblastic cells has never seriously investigated yet. Since macrophages and polymorphonuclear leukocytes (PMNs) have been indicated to play significant roles of mucosal innate immunity in the infection of *Shigella* including the inflammatory responses, we investigated macrophages and PMNs for the expression of N-WASP and WASP. We observed that in hematopoietic cells such as macrophages, polymorphonuclear leukocytes (PMNs) and platelets, WASP was predominantly expressed, while the expression of N-WASP was greatly suppressed. Indeed, unlike *Listeria*, *Shigella* was unable to move in macrophages at all, though the movement was restored as N-WASP was ectopically expressed. Thus, our findings demonstrate that N-WASP is a specific ligand of VirG, which determines the host cell type allowing actin-based spreading of *Shigella*.

4. IcsB, secreted via the type III secretion system, is chaperoned by IpgA and required at post invasion stage of *Shigella* pathogenicity

Michinaga Ogawa, Toshihiko Suzuki , Ichiro Tatsuno , Hiroyuki Abe and Chihiro Sasakawa

Shigella deliver a subset of effector proteins such as IpaA, IpaB and IpaC via the type III secretion system (TTSS) into host cells during the infection of colonic epithelial cells. Many bacterial effectors including some from *Shigella* require specific chaperones for protection from degradation and targeting to the TTSS. In this study, we have investigated the role of the *icsB* gene located upstream of the *ipaBCDA* operon in *Shigella* infection, since the role of IcsB as a virulence factor remains unknown. Here, we found that the IcsB protein is secreted via the TTSS of *Shigella in vitro* and *in vivo*. We show that IpgA protein encoded by *ipgA*, the gene immediately downstream of *icsB*, serves as the chaperone required for the stabilization and secretion of IcsB. We showed that IcsB was bound to IpgA in bacterial cytosol, and the binding site was in the middle of the IcsB protein. Intriguingly, although its significance in *Shigella* pathogenicity is as yet unclear, the *icsB* gene can be read-through into the *ipgA* gene to create a translational fusion protein. Furthermore, the contribution of IcsB to the pathogenicity of *Shigella* was demonstrated by plaque-forming assay and the Sereny test. The ability of the *icsB* mutant to form plaques was greatly reduced as compared with that of the wild type in MDCK cell monolayers. Furthermore, when guinea pig eyes were infected with a non-polar *icsB* mutant, the bacteria failed to provoke keratoconjunctivitis. These results suggest that IcsB is secreted via the TTSS, chaperoned by IpgA, and required at the

post-invasion stage of *Shigella* pathogenicity.

5. *Shigella* deliver an effector protein to trigger host microtubule destabilization, which promotes Rac1 activity and efficient bacterial internalization

Sei Yoshida, Eisaku Katayama[5], Asaomi Kuwae, Hitomi Mimuro, Toshihiko Suzuki, and Chihiro Sasakawa: [5]Department of Basic Medical Sciences, Institute of Medical Science, University of Tokyo

Many bacterial pathogens enter non-professional phagocytic cells by remodeling the host surface including cytoskeletal networks in various ways. *Shigella* deliver a subset of effectors into the host cell cytosol via the type III secretion system, that stimulate host cell signal pathways to modulate the actin dynamics required for directing its own internalization into the cell. Here we show that one of the effectors delivered by *Shigella*, called VirA, can interact with tubulin to promote microtubule (MT) destabilization, and elicit protrusions of membrane ruffling. Under in vitro conditions, VirA inhibited polymerization of tubulin and stimulated MT destabilization. Upon microinjection of VirA into HeLa cells, a localized membrane ruffling was rapidly induced. Overexpression of VirA in COS-7 or HeLa cells caused MT destruction and protruding membrane ruffles which were absent when VirA was coexpressed with a dominant negative Rac1mutant. Consistent with this the wild type *Shigella* but not the *virA* mutant stimulated Rac1 activity including the formation of large-scale membrane ruffles in infected HeLa cells. Importantly, the MT structure beneath the protruding ruffling was destroyed. Furthermore, the MT growth induced in HeLa cells by washing out nocodazole greatly enhanced the Shigella entry. These results indicate that VirA appears to be a novel type of bacterial effector capable of inducing lamellipodial membrane ruffling through the stimulation of MT destabilization.

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Department of Microbiology and Immunology

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免疫調節分野 (1)

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Self-defense against invaded pathogenic microorganisms and foreign antigenic molecules is strictly controlled by the immune system. Our major research interests are to elucidate cells and effector molecules in innate and acquired immunity and inflammation. In particular, we are focused on cellular and molecular mechanisms of development and activation of B cells and IgH class switch recombination under the influence of T cells, cytokines, and adaptor proteins. Another interest is to elucidate cellular mechanisms of preferential induction of Th1 cells upon immunization with Mycobacteria peptide.

1. Role of interleukin-5 (IL-5) in the B cell differentiation

a. Molecular mechanisms of IL-5-induced B cell maturation

Keisuke Horikawa, Hiroaki Kaku, Kiyoshi Takatsu

Interleukin-5 (IL-5) is a cytokine with pleiotropic activities on the B-1 and B-2 cell differentiation. We reported that IL-5, but not IL-4, directly induces μ-γ1 class switch recombination (CSR) and Ig secretion on CD38-activated murine splenic B-2 cells. We also showed that Stat5, one of transcription factors involved in the IL-5R signaling pathway, is essential for IL-5-dependent μ-γ1 CSR and Ig production. Accumulating data indicate that protein tyrosine kinases, such as JAK, Src and Btk are involved in IL-5-dependent μ-γ1 CSR and Ig production. However, downstream signaling molecules of these kinases and their target genes are still remained unclear.

To elucidate mechanisms underlying IL-5-dependent μ-γ1 CSR and Ig production, we used DNA micro-arrays to analyze genes whose expression are increased or decreased upon IL-5 stimulation. Of 36,000 genes surveyed, expressions of about 300 genes were enhanced by IL-5 stimulation. We isolated some of interesting genes and determined their nucleotide sequences. Results revealed that the enhanced expression of genes encoding immunoglobulin family, cytoskeletal components, and molecules involved in DNA replication and cell cycles, metabolism, signal transduction, transcription, translation and transport was observed. Among them, we focused on the enhanced gene expression of *Blimp-1*, *XBP-1*, and *AID*, which are thought to be involved in differentiation of activated B cells to Ig producing cells and Ig CSR. Effect of enforced expression of *Blimp-1*, *XBP-1*, and *AID* genes in CD38-activated B cells on IL-5-dependent B cell differentiation was examined. The retroviral induction of *Blimp-1* gene to CD38-stimulated B cells enhanced the frequency of IL-5-induced differentiation to plasma cell. Interestingly, a significant and a lesser extent of differentiation to plasma cell were observed upon IL-4 stimulation by the enforced expression of Blimp-1 in CD38-stimulated B cells. Intriguingly, the retroviral induction of *AID* gene enhanced the differentiation of CD38-stimulated B cells to sIgG1 cells in response to IL-4. Results suggest that the enhanced gene expression of Blimp-1 and AID is involved in maturation of activated B-2 cells to μ-γ1 CSR and Ig production.

b. Role of nuclear factor (NF)-κB in CD38-mediated induction of the germline γ1 transcripts

Hiroaki Kaku, Keisuke Horikawa, Yoichi Obata[1], and Kiyoshi Takatsu: [1]Department of Pathology, Aichi Cancer Center

CD38 is a 45kDa type II transmembrane glycoprotein with a short cytoplasmic part and a long extracellular domain. CD38 is an ectoenzyme with both ADP ribosyl cyclase and cADP ribosyl hydrolase activity. Ligation of CD38 on mouse B cells with CS/2, an agonistic anti-CD38 mAb, induces B cell proliferation, IL-5Rα chain expression, and the expression of germline γ1 transcripts. This leads to Ig class switch recombination from the μ to γ1 heavy chain gene and high levels of IgM and lgG1 production particularly in response to anti-CD38 and IL-5 costimulation in an IL-4 independent manner. Although some of the post-receptor signaling events initiated by CD38 ligation has been characterized, signaling pathways involved in CD38-mediated germline γ1 transcript expression in B cells are poorly understood.

We examined NF-κB activation in CD38-stimulated mouse B cells by electrophoretic mobility shift assay (EMSA). Results revealed that CD38 ligation of murine splenic B cells activates members of the nuclear factor (NF)-κB/Rel family of proteins including c-Rel, p65, and p50. The activation patterns and kinetics of NF-κB-like proteins in CD38-stimulated B cells differ somewhat from those seen in CD40-stimulated B cells. Activation of NF-κB-like proteins by CD38 ligation is not observed in splenic B cells from Btk-deficient ($Btk^{-/-}$) mice, with inhibitors of PKC and phosphatidylinositol (PI)-3 kinase also suppressing NF-κB activation in CD38-activated B cells. We infer from these results that activation of Btk, PI-3 kinase, and PKC play, at least in part, important roles in the induction of NF-κB in CD38-stimulated murine B cells. Consistent with a role for NF-κB/Rel signaling in CD38-mediated germline γ1 transcript expression, $p50^{-/-}$ B cells show significant impairment of germline γ1 transcript expression in response to CD38 ligation, whereas the CD40-induced response was not altered. In contrast, $c\text{-}Rel^{-/-}$ B cells show a severe impairment of germline γ1 transcript expression in response to CD38 or CD40 ligation. These results indicate an essential role for NF-κB proteins in the induction of germline γ1 transcripts by CD38-ligated murine B cells giving rise to IL-5-induced IgG1 production.

c. Molecular cloning of genes induced by IL-5 in murine B cells

Keisuke Horikawa and Kiyoshi Takatsu

IL-5 enhances proliferation and differentiation of activated B cells. Accumulating data suggest the involvement of protein tyrosine kinases, such as JAK, Src, and Btk in IL-5 signaling pathway. However, downstream signaling molecules and transcription factors of above kinases still remain unclear. To elucidate IL-5 signaling pathway in molecular levels, we compared the expression patterns of various genes and Est-related genes expressed in CD38- and IL-5-stimulated B cells with these of CD38-stimulated B cells using an Affymetrix GeneChip system. The genes inducible by IL-5 included immunoglobulin-related genes such as Cγ1, IgL, and J-chain, genes encoding cytoskeletal components and molecules involved in DNA replication and cell cycle, cell metabolism, signal transduction, transcription, translation, and transport.

Among those genes we are currently focusing on acidic epidydimal protein 1 (AEG1), which is evolutionary conserved from plants to mammals. AEG1 is a secretory protein, originally identified from the *Caudal epidydimis* and shows sequences homologous to plant PR-1 (pathogenesis related) proteins, which are accumulated after pathogen infection and has been shown to have anti-fungal activities both *in vitro* and *in vivo*. However, the molecular mechanisms underlining expression of PR-1 activity and function of mammalian homologue of AEG1 still remain unclear. RT-PCR analysis revealed that AEG1 was induced upon IL-5 stimulation of CD38-activated splenic B-2 cells and B-1 cells in peritoneal washouts. Interestingly, LPS stimulation did not induce the AEG1 expression in both cell types. We also found the decreased expression of AEG1 in peritoneal exudate cells from IL-5Rα-deficient mice and anti-IL-5-treated mice as well. These data suggest that AEG1 is induced by IL-5 in B-2 cells and is constitutively expressed in B-1 cells in peritoneal cavity, provably upon endogenous IL-5 stimulation. AEG1 may play important roles in both innate and acquired immunity.

d. IL-5/IL-5R system in homeostatic regulation of B-1 cell compartment

Byoung-Gon Moon, Satoshi Takaki, and Kiyoshi Takatsu

IL-5R consists of two membrane proteins, IL-5Rα and βc. The IL-5Rα specifically binds IL-5 and forms a functional receptor complex together with βc, the signal transducing subunit shared with receptors for IL-3 or GM-CSF. We have demonstrated that the membrane-proximal proline-rich region (PPVP motif) of IL-5Rα is also critical to transduce signal for both cell growth and differentiation *in vitro* and *in vivo*. The C-terminal region of IL-5Rα is important for both IL-5-induced IgM production and μ to γ1 switch recombination in B cells.

B-1 cells are distinguished from conventional B-2

cells by their surface markers, anatomical localization and self-replenishing activity. Most of B-1 cells constitutively express IL-5R, and differentiate into IgM producing cells upon stimulation of IL-5. We reported that the number and cell size of B-1 cells was decreased in *IL-5Rα*$^{-/-}$ mice. We tried to distinguish whether observed abnormalities of *IL-5Rα*$^{-/-}$ B-1 cells are due to impaired development or to inefficient maintenance of mature B-1 cells. Injection of anti-IL-5 mAb into adult wild type mice resulted in decreased number and cell size of B-1 cells within 6 days after mAb injection. *IL-5Rα*$^{-/-}$ B-1 cells transferred into normal mice showed impaired survival activity compared to normal B-1 cells. When transferred into *RAG2*$^{-/-}$ host animals lacking mature lymphocytes, *IL-5Rα*$^{-/-}$ B-1 cells did not proliferate as well as normal B-1 cells. These indicate that IL-5/IL-5R system plays critical roles for survival and homeostatic proliferation of B-1 cells. Moreover, responsiveness to anti-CD40 cross-linking or to LPS was significantly impaired in *IL-5Rα*$^{-/-}$ B-1 cells, indicating importance of IL-5/IL5R system for full activation and function of B-1 cells.

e. Mutual regulation between BTK and BAM11, BTK-associated molecule

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Bruton's tyrosine kinase (Btk) is required for normal B cell development and signal transduction through cell surface molecules, and its defects lead to X-linked immune deficiency (Xid) in mice and X-linked agammaglobulinemia (XLA) in humans. We isolated a molecule that binds to PH-domain of BTK, BAM11 that is murine homologue of human LTG19/ENL, a fusion partners of *MLL/ALL-1/HRX*, in leukemia cells, and has been supposed to be a transcriptional factor. The region of BAM11 required for binding to Btk was localized between amino acid residues 240 and 256. Forced expression of a truncated form (BAM-B) of BAM11 (aa246-368) significantly inhibited IL-5-induced proliferation and the kinase activity of Btk.

Promoter assay using firefly luciferase gene revealed that BAM11 acts as a transcriptional factor. Since BAM11 has nuclear localization signals, we speculated that BAM11-BTK complexes localize in the nucleus. Analysis using GFP-fused Btk protein demonstrated that a proportion of BTK, which has been reported to locate in the neighbor of surface B cell receptor complex (BCR), exist in the nucleus by making complex with BAM11. This finding is supported by biochemical analysis of fractionating cells into cytoplasmic fraction and nucleus fraction, and analyzing by immunoblotting. We previously reported that BAM11 suppresses BTK kinase activity and abrogate transmitting signals to downstream molecules when BAM11-BTK complex is localized in the neighbor of BCR. In addition to this, our finding strongly suggests that BTK up-regulate transcriptional activity of BAM11 when BAM11-BTK complex is localized in the nucleus. This "positive-negative mutual regulation system" between BAM11-BTK may provide attractive model to elucidate a novel mechanism to transmit signals in B-lymphocytes.

2. Regulatory functions of adapter proteins in the immune system

a. Impaired B-lymphopoiesis and altered B-subpopulations in transgenic mice overexpressing Lnk adaptor protein

Satoshi Takaki, Sang-Mo Kwon, Chiyomi Kubo and Kiyoshi Takatsu

Lnk is a 68-kD adaptor protein expressed mainly in lymphocytes and hematopoietic progenitor cells. Together with APS and SH2-B, Lnk forms part of an adaptor protein family, whose members share the presence of a homologous N-terminal domain with putative proline-rich protein interaction motifs, followed by PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site. Lnk regulates B cell production by negatively controlling pro-B cell expansion. In *lnk*$^{-/-}$ mice, pre-B and immature B cells accumulated in the spleens, and B precursor cells were proportionately increased from early pro-B cell stage in the bone marrow.

We used a transgenic approach to define critical aspects of Lnk function in more detail, and showed that Lnk overexpression resulted in impaired expansion of lymphoid precursor cells and altered mature B cell subpopulations. The representation of both B-lineage and T-lineage cells was reduced in transgenic mice overexpressing Lnk under the control of a lymphocyte-specific promoter. The C-terminal tyrosine residue, conserved among Lnk family adaptor proteins, was dispensable for the negative regulatory roles of Lnk in lymphocyte development. In addition to its importance in lymphopoiesis at the early developmental stages, Lnk also plays a role in peripheral maturing B cells. Whereas the overall number of B and T cells was correlated with Lnk protein expression levels, marginal zone B cells in spleen and B1 cells in the peritoneal cavity were relatively resistant to Lnk overexpression. In transgenic mouse spleens, abnormalities in B cell morphology and cell cycle status were also observed. Our results illuminate the novel negative regulatory mechanism mediated by the Lnk adaptor protein in controlling lymphocyte production and function.

b. Lnk regulates expansion and function of hematopoietic progenitor cells

Satoshi Takaki, Hitoshi Takizawa and Kiyoshi Takatsu

Mutant mice lacking the lnk gene show enhanced B cell production. This B cell overproduction is due to the hypersensitivity of B cell precursors to stem cell factor (SCF), a c-Kit ligand. C-Kit is well known as an important tyrosine kinase receptor in hematopoietic stem and progenitor cells and that give rise to variety of hematopoietic cells and are responsible for blood production throughout adult life. Hypersensitivity of $lnk^{-/-}$ B precursors to SCF prompted us to characterize hematopoietic progenitor cells in $lnk^{-/-}$ mice. We revealed that Lnk is also expressed in hematopoietic progenitors, and that the number of hematopoietic progenitors and the ability to generate various lineages of hematopoietic cells were greatly enhanced by the absence of Lnk

The $c\text{-}Kit^{+}Sca\text{-}1^{+}$ fraction containing hematopoietic stem cells in lineage-marker negative bone marrow cells was significantly expanded in lnk-deficient mice. The number of hematopoietic progenitors having ability to produce CFU-S d12 in irradiated hosts was also increased. Competitive repopulation assays in irradiated host animals demonstrated that the ability of progenitors to generate various lineages of hematopoietic cells was greatly enhanced by the absence of Lnk. Our observations provide useful clues to regulate expansion and hematopoietic ability of progenitor cells. Now we are trying to generate various Lnk mutants that effectively inhibit negative regulatory function of Lnk in cell growth. We found some of Lnk mutants, carrying a point mutation in the SH2 domain acted as dominant negative mutants in MC9 mast cell line overexpressing wild-type Lnk.

c. Molecular mechanisms of cell growth inhibition by Lnk

Sang-Mo Kwon, Hitoshi Takizawa, Ikuo Nobuhisa[2], Tesuya Taga[2], Kiyoahi Takatsu and Satoshi Takaki: [2]Department of Cell Fate Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University

We have demonstrated that Lnk is phosphorylated by and associated with c-Kit, and selectively inhibited c-Kit-mediated proliferation by attenuating phosphorylation of Gab2 and activation of MAPK cascade using c-Kit-positive MC9 mast cell line. To further investigate how Lnk manifests its regulatory functions in cell growth, we generated various forms of Lnk mutants carrying substitutions or deletions. In transfected COS7 cells, Lnk formed multimeric complex via the N-terminal domain, and associated with phosphorylated c-Kit through the SH2 domain. The C-terminal tyrosine residue was a main target phosphorylated by c-Kit, while the N-terminal and PH domains somehow contributed to the efficient phosphorylation of Lnk.

It has been shown that Lnk associates with an actin binding protein, APB-280 (filamin), and that SH2-B, a member of the Lnk family adaptor proteins, plays a role in growth hormone-induced actin reorganization. We examined whether Lnk could control actin cytoskeleton and cytokinesis or not. Enforced expression of Lnk in NIH3T3 fibroblasts resulted in a drastic change in cell morphology and actin cytoskeleton. Lnk localized to cell membrane by virtue of the PH domain, which was indispensable for actin reorganization and association with ABP-280. Fibroblasts overexpressing Lnk showed impaired cell division and became multi-nucleated. In addition, immature transitional T1 B cells in spleen of the *lnk*-transgenic mice were larger in size. However, larger transgenic T1 cells were not actively proliferating as assessed via DNA content analysis, reflecting Lnk function in controlling the actin cytoskeleton and cytokinesis *in vivo*. Our results suggest that Lnk regulates cellular proliferation, migration or cytokinesis, in part, via effects on cytoskeletal remodeling by actin.

d. APS, a member of the Lnk adaptor protein family, regulates B cell and mast cell functions

Masanori Iseki, Chiyomi Kubo, Yuki Kataoka[3], Nobuaki Yoshida[3], Kiyoshi Takatsu and Satoshi Takaki: [3]Laboratory of Gene Expression and Regulation, Center for Experimental Medicine, IMSUT

To understand functions of Lnk family adaptor proteins, we attempted to identify other members of the Lnk family, and isolated the mouse APS (adaptor molecule containing PH and SH2 domains) using cDNA sequences conserved between Lnk and SH2-B as probes. We have shown that APS is expressed in various tissues including spleen, bone marrow, brain and muscle, and in mature B but not in T or immature B cell lines. APS is tyrosine phosphorylated at the C-terminal phosphorylation site upon stimulation with IL-5, IL-3 or anti-IgM.

We generated $APS^{-/-}$ mice to investigate the physiological roles of APS *in vivo*. $APS^{-/-}$ mice were viable, fertile and showed no anomalies or growth retardation. Lymphocyte or myeloid cell developments in bone marrow, thymus, spleen and lymph nodes were not perturbed in $APS^{-/-}$ mice. However, $APS^{-/-}$ mice had more B-1 cells in peritoneal cavity, and showed enhanced humoral immune responses against thymus-independent type-2 antigen. Bone marrow derived mast cells lacking APS manifested increased degranulation upon FceRI cross-linking. In transgenic mice overexpressing APS in lymphocytes, on the other hand, the numbers of peritoneal B-1 and

splenic B cells were reduced, and proliferation induced by anti-IgM stimulation were impaired. These results illuminate a novel negative regulatory role of APS in immune responses. APS controls B-1 cell compartment size by negatively modulating signals mediated through B cell receptor, and also negatively regulates mast cell functions induced by FceRI cross-linking.

5. Mechanisms of preferential induction of Th1 response upon immunization with Mycobacteria peptide

a. Role of IFN-γ in the Peptide-25 dependent Th1 development

Ai Kariyone and Kiyoshi Takatsu

The α antigen is one of the major antigens secreted by *Mycobacterium (M.) tuberculosis* and *M. Bovis* BCG. We have shown that stimulation of lymph node cells from *M. tuberculosis*-primed C57BL/6 mice with α antigen (also known as Ag85B and MTP59) induces TCR Vβ11$^+$ CD4$^+$ Th1 cells in conjunction with antigen-presenting cells in an I-A^b-restricted manner. We identified the major antigenic epitope (Peptide-25) for a antigen-specific Vβ11$^+$ T cells as the 15-mer peptide, covering amino acid residues 240-254 of a antigen that contains the I-A^b binding motif. We also reported that the amino acid residues at positions 246, 248, 250, 251 and 252 of Peptide-25 would be important for recognition of TCRVβ11, and the residues at position 244, 247, 249 and 252 are possible I-A^b contact residues. Active immunization of C57BL/6 mice with Peptide-25 can induce the development of CD4$^+$ Th1 cells. In this study, we examined the roles of IFN-γ in the generation of Peptide-25-reactive CD4$^+$ T cells that produce of IFN-γ- and TNF-α. Peptide-25 was immunogenic in inducing the development of IFN-γ- and TNF-α-producing cells of CDT4$^+$ TCRVβ11$^+$ and CDT4$^+$ TCRVβ11- T cells. Treatment of C57BL/6 mice with anti-Vβ11 antibody before Peptide-25 immunization reduced the development of IFN-γ-producing CD4$^+$ T cells. Furthermore, B10.A(3R) mice, I-A^b-positive and TCRVβ11-negative strain showed remarkably lower Th1 development upon Peptide-25 immunization. In Peptide-25-immunized IFN-γ$^{-/-}$ mice expansion of TCRVβ11$^+$ CD4$^+$ T cell s in both TCRVβ11$^+$ and CDT4$^+$ TCRVβ11$^-$ T cell populations upon Peptide-25 stimulation *in vitro* decreased compared with WT mice. These findings indicate that IFN-γ plays an important role, at least in part, in the generation of Th1 cells including TCRVβ11$^+$ T cells in response to Peptide-25. Substituted mutant, Peptide-25(244D247V) capable of binding to I-A^k was immunogenic in C3H/HeN for Th1 development. These results provide useful information for delineating the regulation of Th1-cell development, for developing subunit vaccine peptides, and for inducing a Th1-dominant immune response.

b. Role of antigen-TCR interaction in the Th1 subset development

Toshiki Tamura, Haruyuki Ariga, Shu-I-chiro Uehara, Ai Kariyone, and Kiyoshi Takatsu

Activated CD4$^+$ Th cells can be classified into two subsets, Th1 and Th2, on the basis of cytokine production profiles. Development of each Th subset has been determined by cytokines, such as IL-4, IFN-γ and IL-12, in environment during the primary response of naive T cells to antigens. In addition to the cytokine environment, other mechanisms such as type of APC, co-stimulatory molecules and genetic background can also be involved in the development of naive CD4$^+$ T cells into Th1 and Th2 cells. Upon recognition of MHC/peptide complex, the T cell receptor (TCR) initiates a complex cascade of signaling events resulting in cytokine production, proliferation and differentiation. However, it is still unclear whether the TCR signaling events exert influence on Th1/Th2 differentiation.

As we demonstrated, CD4$^+$ T cells reactive to Peptide-25 express highly restricted TCR repertoire, Vα5-Vβ11. These results led us to re-examine whether Peptide-25 itself is able to directly contribute the determination of Th subset development. To address these issues, we generated transgenic (Tg) mice expressing TCRVα5-Vβ11 that recognizes Peptide-25 in the context with I-A^b molecule. The TCR-Tg mice did not show any abnormalities at glance. When naive CD4$^+$ T cells of TCR-Tg mice were stimulated *in vitro* with Peptide-25 for 5 days in the presence of I-A^b APCs, they produced large amounts of Th1 cytokines. This Th1 development was not abrogated by the addition of antibodies against IFN-γ, IL-12 and IL-18 during the culture, suggesting that Peptide-25-dependent Th1 development from naive CD4$^+$ T cells of TCR-Tg mice can be induced in the absence of IFN-γ, IL-12 and IL-18. Interaction between Peptide-25/I-A^b complex and TCR may determineTh1 development. We are currently analyzing differentiation mechanisms of naive CD4+ T cells into Th1 cells by using these TCR transgenic mice.

c. Enhancing effect of Peptide-25 priming on the induction of cytotoxic T cell response

Shu-I-chiro Uehara, Toshiki Tamura, Takeshi Kikuchi, Xu Wen, Ai Kariyone, and Kiyoshi Takatsu

CD8$^+$ cytotoxic T cells (CTLs) play an important role in the protection against tumor growth. Tumor cells are thought to express an array of antigens recognizable by CTLs that principally contribute tumor

rejection. It is still unclear whether $CD4^+$ helper T cells together with CTLs mediate efficient immune responses leading to tumor rejection. We have been reported that the immunization with Peptide-25 emulsified in IFA is able to induce Th1 response. In this study, we examined effect of concomitant immunization of Peptide-25 with model tumor antigen on anti-tumor immune responses. We confirmed that *in vitro* stimulation of lymph node cells from ovalbumin (OVA)-primed C57BL/6 mice with attenuated E.G7-OVA cells (EL4 thymoma transfected with cDNA encoding chicken ovalbumin) induces a potent CTL against E.G7-OVA cells. Potent and reproducible OVA-specific CTL generation was enhanced when mice were immunization with OVA plus Peptide-25 at the same site. Immunization of mice with Peptide-25 and OVA at different sites of body, enhancing effect of Peptide-25 was not observed. The enhancing effect of Peptide-25 was dependent upon $CD4^+$ T cells and IFN-γ. Co-immunization of Peptide-25 with OVA was also shown to delay *in vivo* growth of E.G7-OVA cells and to prolong survival. Nearly half of mice received immunization of Peptide-25 and OVA survived even 60 days after EG.7 cell challenge, while all mice died within 40 days with tumor in OVA-immunized mice. These results indicate that Peptide-25 immunization provides efficient help for CTL induction against neo-tumor antigen when concomitantly immunized. Questions still remained are whether Peptide-25 can enhance induction of CTL against Class I binding peptide from tumor.

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Department of Microbiology and Immunology

Division of Immunology (2)

免疫調節分野 (2)

Associate Professor Tsuneatsu Mori, M.D., D.M.Sc.　助教授　医学博士　森　庸　厚

We have extensively promoted the analysis of immunomolecular mechanism in mammalian gamete selection, fertilization and implantation. Furthermore, we have developed anti-cancer compounds from human placental cells.

1. Programmed cell death (apoptosis) in mammalian ovary and testis

Tsuneatsu Mori, Maowu Guo, Aishun Jin, Yunlong Qi, Etsuko Mori[1] and Seiichi Takasaki[2]: [1,2]Division of Biochemistry

We have demonstrated that the expressive levels of Fas protein in MRL/lpr murine ovary were significantly lower than those in MRL/+ murine ovary. The administration of anti-Fas mAb *in vivo* or the stimulation of Sf9-FasL cells *in vitro* could induce the apoptosis of oocytes / eggs from MRL/+ mice in contrast with no generation of apoptosis of them from MRL/lpr mice depending on the defect of Fas death domain signaling to caspase cascade. Furthermore, we found the activation of caspase-3 and genomic DNA fragmentation in MRL/lpr murine oocytes stimulated with anti-Fas mAb but not in MRL/+ oocytes. Therefore, it was strongly suggested that ovarian adenopathy in old MRL/lpr mice was caused by the increase of follicles due to the dysfunction of Fas in the ovary.Thus, we concluded that Fas -FasL system display an important role to promote ovarian atresia through apoptosis. In murine testis, we have revealed that Fas is expressed in testicular germ cells and FasL is expressed in sertoli cells indicating their molecular interactions during the spermatogenesis. Thus, we concluded that Fas/Ligand system works as a primary machinary in gamete selection.

2. Fertilization

Tsuneatsu Mori, Maowu Guo, Aishun Jin, Yunlong Qi, Etsuko Mori[1] and Seiichi Takasaki[2]: [1,2]Division of Biochemistry

Based on the analysis of all or partial sugar structures of porcine or murine zona pellucida (ZP), it is suggested that the sugar chains are of bi-, tri-, and tetrae antennary complex type with a fucosylated trimannosyl core containing sialic acid and / or sulfate residue as acidic sugar chains. Among these sugar moieties of ZP, we found that murine or porine sperm protein binding to β-Galactose rather than α-Galactose residue and/or Le^X residue on egg ZP is an ADAM family one.

3. Implantation

Tsuneatsu Mori, Maowu Guo, Aishun Jin, Yunlong Qi, Etsuko Mori[1] and Seiichi Takasaki[2]: [1,2]Division of Biochemistry

The CD57^{+}HLA-DR^{bright} natural suppressor (57.DR-NS) cell line, which was established from human first trimester decidual tissue and maintained in our laboratory, releases a series of active factors into the culture to generate the apoptosis of human malignant cells and trophoblast cells. Actually, 57.DR-NS cell line generated the apoptosis in human leukemia (Molt4/K562) and gastric , chorionic, esophageal, prostate or mammary carcinoma (GCIY/ BeWo/T.Tn/PC-3/MDA-MB-435) cells but not in human diploid normal (WI-38) cells. The active factors released from 57.DR-NS cells were finally isolated by HPLC and their chemical structures were determined by the combination of NMR and MS as a series of modified nucleosides which were collectively termed as "apoptosis inducing nucleosides (AINs)". They could generate the apoptotic cell death of Molt4/K562 and GCIY/ BeWo/T.Tn/PC-3/MDA-MB-435 malignant cells following by DNA strand breaks and caspase-3 activation, but not that of WI-38 normal cells at all. The administration of AINs to GCIY/Molt4/MDA-MB-435 tumor bearing SCID mice culminated in the drastic suppression of tumor growth followed by the decrease in tumor size due to the occurrence of apoptosis in tumor tissues. It was speculated that 57.DR-NS cells derived from placental decidua might contribute to the regulation of embryonic malignancy and trophoblastic invasion at the feto-maternal interface via AINs. Thus, we found the true tumor immunity in the site of implantation as mother nature's experiments.

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Department of Microbiology and Immunology

Division of Host-Parasite Interaction
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Cellular mechanisms for the surveillance and exclusion of expression by DNA parasites such as retoroviruses and transposons are now being recognized as an important host cell defense system in the cell nuclei. Obviously, viruses would have their own strategy to escape from the defense system. Our goal is to elucidate molecular mechanisms involved in host-parasite interaction by analyzing epigenetical regulation of viral gene silencing or activation observed in the infected cells. The results would give us new ideas for latent infection observed in many viruses and also for the design of unique retroviral vectors that would support long-term transgene expression providing strong tools for human gene therapy and regeneration medicine.

1. Epigenetical regulation and SWI/SNF chromatin remodeling complex

In multicellular organisms, epigenetic regulation of transcription supports distinct cell type-specific gene expression. Therefore, to understand viral strategies to proliferate and cause specific pathological effects in certain host cells, epigenetical analysis on both viral and host gene expression would be essential in the post-genome project. While DNA methylation, histone acetylation and chromatin remodeling are expected to play major roles in these epigenetical regulations, their interaction with transcriptional factors as well as their mutual relationship remain largely unsolved.

We have been concentrated on the analysis of the major chromatin remodeling complex, SWI/SNF, which is composed of 10 protein subunits in mammmalian. The catalytic subunits, BRG1 and Brm, have ATPase activity with the helicase motif. Each SWI/SNF complex contain a single molecule of either BRG1 or Brm, but not both. We previously showed mechanistic links between chromatin remodeling factor SWI/SNF complex and transcriptional factor AP-1, which is known to play important roles in wide variety of biological function, such as host and viral immediate early responses, cellular growth, differentiation and tumor formation. Our results showed that a specific subset of Fos/Jun dimers (that constitute AP-1) recruits SWI/SNF complex via BAF60a to initiate transcription from the promoters that are in a relatively inactive context of chromatin. We further presented evidences that this SWI/SNF complex subunit, BAF60a is the major determinant of AP-1 transactivating activity.

This year, we first showed that this chromatin remodeling complex is involved in the maintenance of retroviral gene expression (a). Here, we propose that SWI/ SNF complex should be considered as "a trithorax-G complex essential for cellular and viral memory", which is counteracting Polycomb-G complexes. In this respect, it is quite interesting that SW13, a human adrenal adenocarcinoma cell line, has been reported to be deficient in the expression of both *BRG1* and *Brm* genes. Can this cell line maintain "cellular memory" by lacking functional SWI/SNF complex? To answer this question, we intensively analyzed SW13 and found that this cell line encode functional *BRG1* and *Brm* and transcribes both genes constitutively. We further show that in a subtype of SW13, mRNA expression of *BRG1* and *Brm* genes was tightly suppressed at the post-transcription level (SW13(vim-)). We have identified another subtype of SW13, SW13(vim+), which has acquired to express

both *BRG1* and *Brm* mRNA spontaneously. Consistent with our previous observation that functional SWI/SNF complex is essential for transactivation through AP-1, the endogeneous *vimentin*, *CD44*, c-*met* and *collagenase* genes that are know to be under the control of AP-1, were not expressed in SW13(vim-) but were induced in SW13(vim+). We will describe this unique epigenetical transition between two subtypes and discuss its biological meanings (b).

a. Maintenance of integrated proviral gene expression requires Brm, a catalytic subunit of SWI/SNF complex

Taketoshi Mizutani, Taiji Ito, Mitsue Nishina, Nobutake Yamamichi, Akiko Watanabe, and Hideo Iba

This year, we showed that MuLV-based retrovirus vector transgene expression is rapidly silenced in human tumor cell lines lacking expression of Brm, a catalytic subunit of the SWI/SNF chromatin remodeling complex, even though these vectors can successfully enter, integrate, and initiate transcription. We detected this gene silencing as a reduction in the ratio of cells expressing the exogenous gene rather than a reduction in the average expression levels, indicating that down-regulation occurs in an all-or-none manner. Retroviral gene expression was protected from silencing and maintained in Brm-deficient host cells by exogenous expression of Brm but not BRG1, an alternative ATPase subunit in the SWI/SNF complex. Introduction of exogenous Brm to these cells suppressed recruitment of protein complexes containing YY1 and histone deacetylase (HDAC) 1 and 2 to the 5'-LTR region of the integrated provirus, leading to the enhancement of acetylation of specific lysine residues (Lys 5 and Lys 8) in histone H4 located in this region. Consistent with these observations, treatment of Brm deficient cells with HDAC inhibitors but not DNA methylation inhibitors suppressed retroviral gene silencing. These results suggest that the Brm-containing SWI/SNF complex subfamily (trithorax-G) and a complex including YY1 and HDACs (Polycomb-G) counteract each other to maintain transcription of exogenously introduced genes.

b. SW13 cells can transition between two distinct subtypes by switching expression of *BRG1* and *Brm* genes at the post-transcriptional level

Mitsue Yamamichi-Nishina, Taiji Ito, Taketoshi Mizutani, Nobutake Yamamichi, Hirotaka Watanabe, and Hideo Iba

The human adrenal carcinoma cell line, SW13, has been reported to be deficient in both *BRG1* and *Brm* expression and therefore is considered to lack a functional SWI/SNF complex. We found that the original cell line of SW13 is composed of two subtypes: one that expresses neither BRG1 nor Brm (SW13(vim-)) and the another which does express both (SW13(vim+)). The presence of BRG1 and Brm in SW13 correlates completely with the cellular ability to express such genes as *vimentin*, *collagenase*, *c-met* and *CD44* that were under the control of a transcription factor, AP-1, which was previously shown to require a functional SWI/SNF complex for its transactivating activity. Transient treatment with inhibitors of histone deacetylase induced a stable transition of SW13(vim-) to a cell type indistinguishable from SW13(vim+), suggesting that these two subtypes are epigenetically different. Run-on analysis indicated that, unlike these four genes driven by AP-1, transcription of the *BRG1* and *Brm* genes are initiated in SW13(vim-) at a frequency comparable to SW13(vim+). No block in transcriptional elongation of either *BRG1* or *Brm* gene was detected in SW13(vim-) cells, indicating that their expression was completely suppressed at the post-transcriptional level in SW13(vim-) cells. We would like to propose that SW13 can spontaneously transition between two subtypes by switching expression of *BRG1* and *Brm* at the post-transcriptional level.

2. Function of oncogenes and anti-oncogenes in epithelial cells

Rous sarcoma virus (RSV) is known to form exclusively sarcomas although it does not cause carcinomas (tumors originated from epithelium). But the molecular mechanisms supporting this as well as v-*src* function in the epithelium are largely unknown. We have recently developed recombination organ culture systems which enabled us to transfer genes specifically into primary epithelial cells of the developing chicken glandular stomach (proventriculus). This year, we concentrated how v-src expression affects the fate of epithelial cells both genetically and epigenetically. Our findings would explain at least in part why RSV does not apparently form exclusively carcinomas (a).

We previously established a unique system in which high titer stocks of VSV-G pseudotyped retrovirus vector can be stably produced stringently after the introduction of Cre-recombinase. We further showed that this vector can introduce exogenous genes into the entire population of most human tumor cell lines by a single transduction. Making use of this vector, we had been constructed vectors carrying a representative natural tumor suppressor gene, p53 and anti-oncogene supjunD-1 which is designed by ourselves. When these vectors were introduced into human tumor cell lines, very efficient suppression of their oncogenic potential was observed. This year we used this vector for biological analysis of a putative tumor suppressor gene, *patched* in human squamous cell carcinoma (b).

a. Epithelial-mesenchymal transition induced by Rous sarcoma virus in developing glandular stomach

Yasuhito Shimizu, Nobutake Yamamichi, Kanako Saitoh, Akiko Watanabe, Taiji Ito, Mitsue Nishina, Toshihiko Suzuki[1], Chihiro Sasakawa[1], Sadao Yasugi[2], Masao Ichinose[3], Hideo Iba: [1]Department of Microbiology and Immunology, Division of Bacterial Infection, Institute of Medical Science, University of Tokyo, [2]Department of Biology, Faculty of Science, Tokyo Metropolitan University, Hachioji, Tokyo, [3]Second Department of Internal Medicine, Wakayama Medical College, 811-1 Kimiidera, Wakayama

The oncogene function in primary epithelial cells is largely unclear. Recombination organ cultures in combination with the stable and transient gene transfer techniques by retrovirus and electroporation, respectively, enabled us to transfer oncogenes specifically into primary epithelial cells of the developing avian glandular stomach (proventriculus). In this system, the epithelium and mesenchyme are mutually dependent upon each other for their growth and differentiation. This system therefore offers an environment closed to *in vivo*. We report here that either stable or transient expression of v-*src* in the epithelium causes budding and migration of epithelial cells into mesenchyme. In response to the transient expression of v-Src or a constitutive active mutant of MEK, we observed immediate down-regulation of the *Sonic hedgehog* gene and subsequent elimination of *E-cadherine* expression in migrating cells, suggesting the involvement of MAP kinase signaling pathway in these processes. v-*src*-expressing cells that were retained in the epithelium underwent apoptosis (anoikis) and detached from the culture. Continuous expression of v-*src* by for example, Rous sarcoma virus was required for the epithelial cells to acquire the ability to express *type I collagen* and *fibronectin* genes (mesenchymal markers) and finally to establish the epithelial-mesenchymal transition. These observations would partly explain why RSV does not apparently cause carcinoma formation but induces sarcomas exclusively.

b. Introduction of wild-type patched gene suppresses the oncogenic potential of human squamous cell carcinoma cell lines including A431

Chika Koike, Taketoshi Mizutani, Yasuhito Shimizu, Nobutake Yamamichi, Taiji Ito, Takashi Kameda, Eiji Michimukai[4], Naoya Kitamura[4], Tetsuji Okamoto[4] and Hideo Iba: [4]Department of Molecular Oral Medicine and Maxillofacial Surgery 1, Faculty of Dentistry, Hiroshima University, Hiroshima

Defects in a developmental signaling pathway involving the mammalian homologue of the *Drosophila* segment polarity gene, *patched* are associated with human tumors such as basal cell carcinoma, medulloblastoma and squamous cell carcinoma. Loss of heterozygosity (LOH) in some of these tumor cells suggests that *patched* functions as a tumor suppressor gene. To evaluate the biological significance of *patched* mutations in human sporadic tumor cells, we constructed a VSV-G pseudotyped retrovirus vector carrying the wild-type *patched* gene and transduced it into two human squamous cell carcinoma (SCC) cell lines, A431 and KA, that express only mutant *patched* mRNA. When SSC cells were transduced with Ptc virus, colony forming activity in soft agar was drastically reduced and these cells recovered anchorage independent growth when Sonic hedgehog (Shh), the ligand of Patched (Ptc), was added into the soft agar culture. Expression of exogenous *patched*, however, had no effect on anchorage independent growth of *Ras*-transformed NIH3T3 cells or SCC cell line, NA, which expresses wild-type *patched* mRNA. Cyclopamine, a specific inhibitor of the Shh/Ptc/Smo signaling pathway, efficiently suppressed anchorage independent growth of A431 and KA cells. These results indicate that loss of *patched* function plays a major role the in acquisition of oncogenic potential in these SCCs and further that Ptc virus would be an effective reagent for suppressing tumorigenicity of such SCCs.

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水谷壮利、伊庭英夫：長期間にわたって遺伝子発現を安定して維持するレトロウイルスベクターの開発　血液･免疫・腫瘍　7:137-143, 2002

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Viruses can cause devastating diseases. The long-term goal of our research is to understand the molecular pathogenesis of viral diseases, using influenza and Ebola virus infections as models. Interactions between viral and host gene products during viral replication cycles determine the consequences of infection (i.e., the characteristics of disease manifestation, whether limited or widespread); hence, our research has centered on such interactions in these viral infections.

1. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture

Neumann G, Feldmann H, Watanabe S, Lukashevich I, Kawaoka Y.

Ebola virus, a prime example of an emerging pathogen, causes fatal hemorrhagic fever in humans and in nonhuman primates. Identification of major determinants of Ebola virus pathogenicity has been hampered by the lack of effective strategies for experimental mutagenesis. Here we exploit a reverse genetics system that allows the generation of Ebola virus from cloned cDNA to engineer a mutant Ebola virus with an altered furin recognition motif in the glycoprotein (GP). When expressed in cells, the GP of the wild type, but not of the mutant, virus was cleaved into GP1 and GP2. Although posttranslational furin-mediated cleavage of GP was thought to be an essential step in Ebola virus infection, generation of a viable mutant Ebola virus lacking a furin recognition motif in the GP cleavage site demonstrates that GP cleavage is not essential for replication of Ebola virus in cell culture.

2. Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles

Watanabe T, Watanabe S, Neumann G, Kida H, Kawaoka Y.

Despite the success of influenza virus vaccines in reducing severe illness, their efficacy is suboptimal. We describe here the immunogenicity and protective capacity of replication-incompetent influenza virus-like particles (VLPs) which were generated entirely from cDNAs and lacked either the entire NS gene (encoding both the NS1 and NS2 protein) or only the NS2 gene. In mammalian cells infected with NS gene-deficient VLPs, the nucleoprotein, but not other viral proteins including hemagglutinin (HA) and neuraminidase (NA), was detected. In contrast, cells infected with VLPs expressing NS1 but not NS2 (NS2 knockout) expressed multiple viral proteins, including HA and NA. When challenged with lethal doses of an antigenically homologous mouse-adapted influenza virus, 94% of mice vaccinated with the NS2-knockout VLPs survived, compared with less than 10% of those given the NS-deficient VLPs. These results demonstrate the potential of replica-

tion-incompetent NS2-knockout VLPs as novel influenza vaccines and perhaps also as vectors to express genes from entirely unrelated pathogens.

3. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP

Noda T, Sagara H, Suzuki E, Takada A, Kida H, Kawaoka Y.

Using biochemical assays, it has been demonstrated that expression of Ebola virus VP40 alone in mammalian cells induced production of particles with a density similar to that of virions. To determine the morphological properties of these particles, cells expressing VP40 and the particles released from the cells were examined by electron microscopy. VP40 induced budding from the plasma membrane of filamentous particles, which differed in length but had uniform diameters of approximately 65 nm. When the Ebola virus glycoprotein (GP) responsible for receptor binding and membrane fusion was expressed in cells, we found pleomorphic particles budding from the plasma membrane. By contrast, when GP was coexpressed with VP40, GP was found on the filamentous particles induced by VP40. These results demonstrated the central role of VP40 in formation of the filamentous structure of Ebola virions and may suggest an interaction between VP40 and GP in morphogenesis.

4. Human influenza A viral genes responsible for the restriction of its replication in duck intestine

Hatta M, Halfmann P, Wells K, Kawaoka Y.

Although influenza A viruses are occasionally transmitted from one animal species to another, their host range tends to be restricted. Currently circulating human influenza A viruses are thought to have originated from avian viruses, yet none of these strains replicate in duck intestine, a major site of avian virus replication. Although the hemagglutinin (HA) and neuraminidase (NA) genes are known to restrict human virus replication in ducks, the contribution of the other viral genes remains unknown. To determine the genetic basis for host range restriction of the replication of human influenza A virus in duck intestine, we first established a reverse genetics system for generating A/Memphis/8/88 (H3N2) (Mem/88) and A/mallard/New York/6750/78 (H2N2) (Mal/NY) viruses from cloned cDNAs. Using this system, we then attempted to generate reassortant viruses with various combinations of candidate genes. We were able to generate single-gene reassortants, which possessed PB2, NP, M, or NS from Mem/88, with the remainder from Mal/NY. Despite unsuccessful production of other single-gene reassortants from Mem/88, we did generate reassortant viruses comprising both the HA and NA, all three polymerase genes (PB2, PB1 and PA) or all polymerase genes and NP gene from Mem/88, with the rest derived from Mal/NY. Among these reassortants, only those possessing the M or NS gene from Mem/88 and the remainder from Mal/NY replicated in duck intestine. These results indicate incompatibility between the genes of avian and human influenza A viruses, and that all genes other than the M and NS restrict replication of human influenza A virus in duck intestine.

5. Influenza A virus with defective M2 ion channel activity as a live vaccine

Watanabe T, Watanabe S, Kida H, Kawaoka Y.

We propose a rational approach to the design of live virus vaccines against influenza infection by alteration of the influenza A virus M2 protein, which is responsible for ion channel activity. Previously we demonstrated that a mutant A/WSN/33 (H1N1) influenza virus with defective M2 ion channel activity did not show appreciable growth defects in cell culture, although its growth was attenuated in mice. Here, we show that this M2 ion channel defective mutant virus, the M2del29-31, protected mice against challenge with lethal doses of influenza virus, indicating the potential of incorporating this M2 alteration in a live influenza vaccine as one of the attenuating mutations.

6. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice

Ninomiya A, Ogasawara K, Kajino K, Takada A, Kida H.

Mucosal immunity is critical for protection from viral infections. We attempted to activate mucosal cytotoxic T lymphocytes (CTLs) specific for influenza A virus nucleoprotein (NP) which play an important role in protective immunity. It has been shown that dendritic cells (DCs) activated by signaling via CD40-CD40 ligand (CD40L) interaction are required for the differentiation of naive CD8(+) T cells into antigen-specific CTLs in a non-mucosal environment. We herein inoculated mice intranasally with an anti-CD40 monoclonal antibody (anti-CD40 mAb) and NP366-374 peptide, corresponding to a CTL epitope on NP, encapsulated in liposome (liposomal NP366-374) to induce protective CTL responses against influenza A virus. Intranasal but not subcutaneous immunization with liposomal NP366-374 effectively induced mucosal immunity to

reduce virus replication in the lung, suggesting that anti-CD40 mAb also functioned as a mucosal adjuvant. Interestingly, neither MHC class I- nor class II-deficient mice immunized intranasally with these materials were resistant to the infection. Since anti-CD40 mAb was considered to help replace CD4(+) T cells, another help of CD4(+) T cells are presumably required for the induction of CTL activity in the lung. This approach may prove promising for developing vaccines to induce mucosal CTL responses, and seems to highlight differences between mucosal and non-mucosal immunity.

7. Polymorphisms and the differential antiviral activity of the chicken Mx gene

Ko JH, Jin HK, Asano A, Takada A, Ninomiya A, Kida H, Hokiyama H, Ohara M, Tsuzuki M, Nishibori M, Mizutani M, Watanabe T.

The nucleotide sequence of chicken Mx cDNA was reported earlier using the White Leghorn breed in Germany, but it showed no enhanced resistance to viruses. In this study, the nucleotide sequences of chicken Mx cDNA were determined in many breeds. A total of 25 nucleotide substitutions, of which 14 were deduced to cause amino acid exchanges, were detected, suggesting that the chicken Mx gene is very polymorphic. Transfected cell clones expressing chicken Mx mRNA were established after the Mx cDNA was constructed with an expression vector and introduced into mouse 3T3 cells, and the Mx genes from some breeds were demonstrated to confer positive antiviral responses to influenza virus and vesicular stomatitis virus. On the basis of the comparison among the antiviral activities associated with many Mx variations, a specific amino acid substitution at position 631 (Ser to Asn) was considered to determine the antivirally positive or negative Mx gene. Thus, a single amino acid substitution influences the antiviral activity of Mx in domesticated chickens.

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Department of Microbiology and Immunology

Division of Virology (2)

ウイルス感染分野 (2)

Associate Professor Yoshiaki Yogo, Ph.D.

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We have been studying various aspects of the human polyomavirus JC virus (JCV). This virus is ubiquitous in humans, infecting children asymptomatically, then persisting in the kidney. In most adults, renal JCV is not latent but replicates to excrete progeny in urine. In immunocompromised patients, however, JCV causes a fatal demyelinating disease in the central nervous system, known as progressive multifocal leukoencephalopathy (PML). The following studies were performed in 2002.

1. PML diagnosis using PCR

Yoshiaki Yogo, Tomokazu Takasaka and Huai-Ying Zheng[1]: [1]Graduate School of Medicine, The University of Tokyo

We recently established a nested PCR that could efficiently amplify the JCV regulatory region from cerebrospinal fluid (CSF) (Sugimoto et al., *Arch. Virol.* 143:249-262, 1998). Since the structures of PML-type JCV regulatory regions are unique to individual patients, our PCR, if the amplified fragments are sequenced, can eliminate false positives that may arise from contamination. Using this method, we have been performing PML-diagnosis service to hospitals throughout Japan. In 2002, we tested the CSF samples in 31 cases for which PML was suggested by clinical symptoms and radiographic observations, and detected PML-type regulatory regions in six cases. Underlying diseases of JCV DNA-positive patients were acquired immunodeficiency syndrome (n=3), adult T-cell leukemia (n=1), chronic myelocytic leukemia (n=1), and systemic lupus erythematosus (n=1).

2. Asian genotypes of JC virus in Japanese-Americans suggest familial transmission

Makoto Suzuki[1,2], Huai-Ying Zheng[1], Tomokazu Takasaka, Chie Sugimoto, Tadaichi Kitamura[1], Ernest Beutler[2], and Yoshikai Yogo: [2]The Scripps Research Institute

It was previously proposed that JCV is mainly transmitted within the family during long-term cohabitation. To examine the proposed mode of JCV transmission, we collected urine samples in Los Angeles from 2nd and 3rd generation Japanese-Americans whose parents and grandparents were all Japanese. Control urine samples were collected from general patients in La Jolla near Los Angeles. The Japanese-American and control urine samples were used to amplify and sequence a 610-bp region (IG region) of the JCV DNA. From the obtained sequences, together with reference sequences reported previously, a neighbor-joining phylogenetic tree was constructed to classify the detected JCV strains into distinct genotypes. Two subtypes (CY and MY) that predominantly occurred in homeland Japanese accounted for about 90% of the isolates detected in each generation of the Japanese-Americans. In contrast, the major JCV genotype (EU) in Europe and various genotypes in Old-World and New-World populations were detected in the control

samples. We confirmed the validity of the IG-region based classification by a phylogenetic analysis using the whole-genome approach. The present findings provided support for the hypothesis that JCV is transmitted mainly within the family through long-term cohabitation.

3. Generation and transmission of JC virus variants carrying nucleotide substitutions in the coding region

Huai-Ying Zheng[1], Tomokazu Takasaka, Qin Chen[1], Tadaichi Kitamura[1], Yasuda Yukiharu[3], and Yoshiaki Yogo: [3]Tokai University School of Medicine

To understand the mode of JCV evolution, here we studied how often JCV mutants emerge in parents and how often they are transmitted to offspring. To this end, we selected five parent -child pairs having the same 610-bp IG . We cloned and sequenced many full-length JCV DNAs in each family. We detected nucleotide substitutions in all families. The frequency of nucleotide substitution increased with age. Phylogenetic analysis of the detected complete sequences was performed in each family, and the results obtained suggested that in four of the six families examined, the original strains were transmitted from parents to children, and that in one family, a variant strain that evolved within the parent was transmitted to the child. The current observation should provide the basis for understanding JCV evolution. In addition, we found that JCV in the brain of a PML patient rarely underwent nucleotide substitutions in the coding region.

4. Phylogenetic relationships among JC virus strains in Japanese/Koreans and Native Americans speaking Amerind or Na-Dene

Huai-Ying Zheng[1], Chie Sugimoto, Masami Hasegawa[4], Nobuyoshi Kobayashi, Akihiro Kanayama[5], Antonieta Rodas[6], Mildred Mejia[6], Jesus Nakamichi[7], Jing Guo[8], Tadaichi Kitamura[1], and Yoshiaki Yogo: [4]The Institute of Statistical Mathematics, [5]Yokohama City Institute of Health, [6]University of San Carlos of Guatemala, Guatemala, [7]Torreon Coahuila, Mexico, [8]University of Alberta, Canada

Many genetic studies using human mtDNA or the Y-chromosome have been conducted to elucidate the relationships among the three Native American groups speaking Amerind, Na-Dene or Eskimo-Aleut. Human polyomavirus JCV may also help to gain insights into this issue. JCV isolates are classified into more than ten geographically distinct genotypes (designated here as subtypes), which were generated by splits in the three superclusters, Types A, B and C. A particular subtype of JCV (named MY) belonging to Type B is spread in both Japanese/Koreans and Native Americans speaking Amerind or Na-Dene. In this study, we evaluated the phylogenetic relationships among MY isolates worldwide, using the whole-genome approach with which a highly reliable phylogeny of JCV isolates can be reconstructed. Thirty-six complete sequences belonging to MY (ten from Japanese/Koreans, twenty-four from Native Americans and two from others), together with fifty-four belonging to other subtypes around the world, were aligned and subjected to phylogenetic analysis using the neighbor-joining and maximum likelihood methods. On the resultant phylogenetic trees, the MY sequences diverged into two Japanese/Korean and five Native American clades with high bootstrap probabilities. Two of the Native American clades contained isolates mainly from Na-Denes and the others contained isolates mainly from Amerinds. The Na-Dene clades were not clustered together, nor were the Amerind clades. In contrast, the two Japanese/Korean clades were clustered at a high bootstrap probability. We concluded that there is no distinction between Amerinds and Na-Denes in terms of indigenous JCVs, although they are linguistically distinguished from each other.

5. Peopling of Myanmar as demonstrated by genotyping of urinary JC virus DNA

Lei Saruwatari[9], Huai-Ying Zheng[1], Tomokazu Takasaka, Chie Sugimoto, Eiichi Sakai[9], Bo Bo[10], Nwe Nwe Aung[10], Tadaichi Kitamura[1], Yoshiaki Yogo and Norikazu Ohno[9]: [9]Aichi-Gakuin University Japan, [10]Institute of Dental Medicine, Myanmar

The genotyping of urinary JCV DNAs is a novel means of elucidating the origin of ethnic populations. We adopted this method to gain insights into the peopling of Myanmar. JCV genotype profiles at two sites of Myanmar, Yangon facing the Andaman Sea and Peinnebeen located in the central part of Myanmar, were reported previously. In this study, we elucidated JCV genotype profiles at three other sites of Myanmar: Chaungtha Beach facing the Bay of Bengal, Myitkyina located near China and Tiddim located near India. From the JCV genotype profiles at the five sites elucidated here and previously, it is suggested that SC, the southeastern-Asian/southern-Chinese subtype, mainly occurrs throughout Myanmar, and that a few minor subtypes occur at southern and northern sites (Yangon, Chaungtha Beach and Myitkyina). Furthermore, using the whole-genome approach, we evaluated the phylogenetic relationships among various SC isolates detected in Myanmar and other countries. The results of this analysis revealed that SC diverged into various subgroups. Most were unique to Myanmar,

while one was widespread in South China and southeastern Asia, including Myanmar. The present findings are consistent with the view that Myanmar was established by waves of human migration from neighboring regions.

6. Phylogenetic analysis of JC virus DNAs detected in Ainus: An attempt to elucidate the origin and diversity of the Ainu

Yoshiaki Yogo, Huai-Ying Zheng[1], Masami Hasegawa[4], Chie Sugimoto, Shintatu Tanaka[11], Takeo Honjo[12], Nobuyoshi Kobayashi, Nobutaka Ohta[1], Tadaichi Kitamura[1]: [11]Antique & Coffee Shop Agapansas, [12]Himalayan Veterinary Hospital

To elucidate the origin and diversity of the Ainu people, an indigenous population living on a northern island (Hokkaido) of Japan, we collected urine samples from thirty Ainus at three sites (Urakawa, Shiraoi, and Asahikawa), Hokkaido, Japan. We detected five genotypes of JCV: two (MX and MY-x) first identified in the Ainu and related to those prevalent in Japanese and Native Americans, two (EU-a/Arc and EU-c) previously identified in northeastern Siberians and an Arctic tribe, and one (MY-b) widespread among Hondo-Japanese (i.e. contemporary Japanese excluding Ainus). The following inference was made based on the present findings. (1) Multiple populations that migrated from the Asian Continent established the modern Ainu. (2) An ancestral population of the native northeastern Siberians who are closely related to Europeans formed the core of the modern Ainu. (3) Populations that formed Jomonese and novel northeastern populations also contributed to the formation of modern Ainu.

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Department of Microbiology and Immunology
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感染遺伝学分野

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Our research mainly focuses on a molecular mechanism underlying lipopolysaccharide (LPS) recognition. LPS is a membrane component of Gram-negative bacteria that potently activates the innate immune system. Endotoxin recognition molecules have been recently identified as Toll-like receptor 4 (TLR4) and MD-2. We have cloned MD-2 that is associated with the extracellular domain of TLR4. MD-2 association imparts LPS responsiveness to TLR4. TLR4-MD-2, but not TLR4 alone, recognizes LPS. MD-2 is a potential target for therapeutic intervention of endotoxin shock.

1. TLR4/MD-2 is downregulated upon LPS stimulation

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MD-2 is a small extracellular molecule that is associated with the extracellular domain of TLR4 and is important for LPS responses by TLR4. We previously showed that human MD-2 changed the fine specificity of mouse TLR4, suggesting that MD-2 directly regulates LPS recognition by TLR4. To further understand a role for MD-2 in LPS recognition, we focused on the previously reported phenomenon that cell surface TLR4/MD-2 on peritoneal macrophages is rapidly downregulated upon LPS stimulation. To further study the molecular mechanism, we used Ba/F3 cells expressing TLR4/MD-2/CD14.The downregulation was apparent in those expressing TLR4/MD-2/CD14 but not those expressing TLR4/MD-2. It was induced by LPS but not by peptidoglycan. Immunoprecipitation assay showed that LPS induced TLR4/MD-2 down regulation was not TLR4/MD-2 dissociation, but it was TLR4/MD-2 conformation change. Interestingly, the downregulation does not require a signal through TLR4, since it was similarly observed in Ba/F3 cells expressing TLR4 whose cytoplasmic portion was deleted. And LPS antagonist was able to block the downregulation, suggesting that the antagonist acts upstream of the TLR4/MD-2 downregulation. These results suggest that the downregulation of TLR4/MD-2 is directly linked to LPS recognition.

2. Essential role of MD-2 in LPS responsiveness and TLR4 distribution

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Toll-like receptor 4 (TLR4) mediates lipopolysaccharide (LPS) signaling in a variety of cell types. MD-2 is associated with the extracellular domain of TLR4 and augments TLR4-dependent LPS responses *in vitro*. However, it remains controversial to what degree MD-2 is required for LPS responses *in vivo*. To address this issue, we developed mice lacking MD-2 and show that these mice do not respond to LPS, survive endotoxin shock, and are susceptible to *Salmonella typhimurium* infection. Furthermore, to address a role for MD-2 in intracellular distribution of TLR4, we established wild-type or MD-$2^{-/-}$ embryonic fibroblasts (EF cells) and introduced TLR4 with the FLAG epitope at the c-terminus into these cells. In MD-$2^{-/-}$ EF cells, TLR4 is not able to reach the plasma membrane and predominantly resides in the Golgi apparatus, whereas TLR4 is distributed to the leading edge surface of cells in wild type EF cells. Thus, MD-2 is indispensable in LPS responses *in vivo*, and essential for correct intracellular distribution and LPS recognition of TLR4.

3. Analysis of the mouse B lymphocyte activation via RP105/MD-1

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RP105, which has leucine-rich repeat motif similar to Toll like receptors (TLRs), expressed on the cell surface of mouse B lymphocyte. This molecule exists on cell surface as the complex form with MD-1, which is a homologous protein to the binding molecule (MD-2) to TLR4. The studies using RP105 deficient mice indicated that RP105/MD-1 complex recognized bacterial lipopolysaccharide(LPS) and regulated the LPS signaling in B lymphocyte. Some evidences indicated that RP105/MD-1 associated the innate immune responses. The cross-linking of RP105/MD-1 on B lymphocytes with anti-RP105 monoclonal antibodies (mAb) results in the proliferation of these cells and increasing of CD86 expression on the cell surface. These B lymphocyte responses are similar to those when the cross-linking of CD40 with mAb against it. However, the following stimulation by anti-IgM mAb induce apoptosis of pretreated cells with anti-RP105 mAb, on the other hand, induce further proliferation of pre-stimulated cells via CD40 that is essential molecule in acquired immune responses of B lymphocytes. These diametrically opposite reactions of B lymphocytes may indicate the differences between innate and acquired immune responses. We analyzed the differences of gene expressions between in mouse splenocytes stimulated via RP105/MD-1 (RP blast) and in those stimulated via CD40 (CD40 blast), utilizing DNA microarray and RT-PCR. We found some kinds of genes specifically expressed in RP blast or in CD40 blast. Interestingly, the expressions of some of CD40-spcific genes were depressed in RP blast than not only in CD40 blast, but also in untreated splenocytes. We are analyzing the expression of these genes in B lymphocyte and those functions in detail.

4. Kinetics of nucleoside triphosphate hydrolase of *Toxoplasma gondii* in mice with acute and chronic toxoplasmosis

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We had made a monoclonal antibody (6C6) against the enzyme, a nucleoside triphosphate hydrolase (NTPase) which has a wide specificity toward NTP. 6C6 demonstrated that this molecule is located on the surface membrane of *Toxoplasma gondii* tachyzoites in immuno-EM study. 6C6 could inhibit NTPase activity *in vitro* and invasion of the parasites to host cells.

These data suggested the molecule recognized by 6C6 might be considered a potential candidates antigen for vaccine against *T. gondii* tachyzoites and a target for diagnosis.

The kinetics of the NTPase of *T. gondii* was examined an avidin-biotin sandwich-ELISA (ABS-ELISA) based on an anti-NTPase monoclonal antibody, 6C6. The RH and ME49 strains of the parasite were used to produce acute and chronic infections in mice, respectively. In the acute model, detectable serum concentrations of NTPase were observed from day 1 post-infection and gradually increased until the death of the mice. They were associated with parasitaemia (as estimated by bioassay). No anti-*T.gondii* antibody could be detected at any time. In the chronic model, in which 20 *T. gondii* ME49 cysts were given to each mouse *per os*, the NTPase concentration generally increased from day 3, peaked between days 7 and 14 and then declined. However, one of the four mice used still had a high serum concentration of NTPase on day 35. Again, detectable NTPase concentrations occurred when the mice had parasitaemias. Antibody to *T. gondii* was detected from day 7 (IgM) or 10 (IgG) and brain cysts were observed from day 14. Since detectable serum concentrations of NTPase appear to be associated with parasitaemia in both acute and chronic toxoplasmosis, the ABS-ELISA of the enzyme may make a useful diagnostic tool.

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Department of Microbiology and Immunology

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炎症免疫学分野

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The mucosal surface provides a first line of defence for the host. The goal of our research is to understand the molecular and cellular aspects of the mucosal immune system and their contribution for the host defense against infectious diseases, inflammation and immunological disorders. Further, it is important to apply our fundamental findings of the mucosal immune system for the development of mucosal vaccines and mucosal immunotherapy with all haste.

1. Mucosal intranets: epithelial cell and intraepithelial lymphocyte interactions

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Numerous environmental antigens enter through the mucosal epithelium which consists with intraepithelial αβ and γδ T cells, and epithelial cells. Thus, it is important to examine cell-to-cell interaction among αβ and γδ T cells, and epithelial cells for the induction of first line of immunity. Thus, molecular mechanisms for the triangular celluar interaction among these three types of cells in epithelium via cytokine(s), receptor(s) and adhesion molecule(s) are currently under intensive investigation in our laboratory. To this end, we have found that IL-7 and IL-7R mediated signaling cascade between epithelial cells and intraepithelial γδ T cells is involved in the generation of mucosal barrier. In addition, our most recent results have suggested that IL-15 is a key cytokine for intraepithelial NK cell medaited epithelial cell turn over at villus epithelium.

2. Mucosal vaccine

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For the prevention of mucosal infections including HIV, influenza, *E.coli*, O157 and *Salmonella*, it is essential to build effective immunity in the mucosa-associated tissues. As a result, mucosal administration has been an effective and practical immunization route for the induction of antigen-specific immune responses in mucosal and systemic compartments. Thus, mucosal vaccine can induce two layers of im-

munity against different infectious agents. Our present effort is aimed at elucidating antigen-specific mucosal Th1 and Th2 αβ T cell and sIgA$^+$ B cell responses to mucosally-administered vaccine antigens. In addition, our current efforts are aimed at the development of novel mucosal adjuvant which provides an optimal stimulation signal for the induction of protective immunity. Thus, mutant cholera toxin (mCT), the chemira between mCT-A and LT-B, IL-12 and IL-15 are considered as new generation mucosal adjuvants. Finally, our recent effort is aimed toward the generation of MHC class II tetramer with toxin associated peptide for the elucidation of vaccine and pathogen antigen-specific Th cell responses in both mucosal and systemic compartments.

3. Inflammatory bowel disease (IBD)

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Recent adaptation of gene manipulation technology has allowed the development of numerous murine models for intestinal inflammation. These murine IBD models exhibit the common feature of disrupting a T-cell-dependent regulatory system which includes alterations in the T-cell subpopulations or T-cell selection, as well as those with a targeted disruption of the cytokine genes and cytokine receptor genes. Results obtained from these experimental IBD models strongly indicate that disturbance of homeostasis in the mucosal immune system due to a lack of regulatory T cells or an emergence of forbidden CD4$^+$ T cells plays a crucial role in the development of intestinal inflammation. We have shown that a population of CD4$^+$ T cells with TCR β-chain without TCR α-chain (CD4$^+$, ββ$^+$ T cells) producing Th2-type cytokines play an essential role for the development of IBD. Analysis of TCR-β immunoprecipitates by two-dimensional electrophoresis and RT-PCR revealed TCR of the CD4$^+$ T cells was a homodimer of TCR-β-chains. PCR-SSCP analyses of TCR Vβ-chain transcripts of the ββ$^+$ T cells revealed monoclonal to oligoclonal accumulation of the cells in the colon but not small intestine, suggesting clonal expansion of the mucosal ββ$^+$ T cells upon the stimulation with gut-derived antigens. The homodimer of TCR β-chains on the ββ$^+$ T cells was a biologically functional receptor which transduced activation signals provided by MHC-class II-associated peptidic antigens and superantigens. Treatments of the mutant mice with mAb against TCR β or IL-4 suppressed the onset of IBD. These findings suggest that the generation of oligoclonal Th2-type ββ$^+$ T cells plays a critical role for the development of IBD.

4. Mucosal IL-5R$^+$ and IL-15R$^+$, B-1 Cells for the induction of CMIS independent IgA response

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It was shown that IL-5R and IL-15R are essential for the development of localized B-1a and B-1b cells in mucosal effector sites, while gut associated lymphoid tissue (GALT)- and nasopharyngeal associated lymphoid tissue (NALT) derived B-2 cells are exempted from IL-5R and IL-15R dependency. In addition, IL-5/IL-5R and IL-15/IL-15R signaling pathways are essential for the development of sIgA$^+$ B-1 but not B-2 cells in mucosal effector sites. It was also shown that sIgA$^+$ B-1 cells arise from the common mucosal immune system (CMIS)-independent pathway, while sIgA$^+$ B-2 cells arise from IgA inductive tissues (e.g., GALT and NALT). Since the distribution of B-1a, B-1b and B-2 cells differed in several mucosa-associated tissues, it would be interesting and important to examine the contribution of these different sources of sIgA$^+$ B cells for the induction of antigen-specific mucosal immune responses against TD, TI-1 and TI-2 antigens and their specific requirements of Th1 and Th2 cytokines. These investigations are currently ongoing in our laboratory.

5. Uniqueness of NALT immune system

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Initiation of NALT development is independent of the programmed cytokine cascade necessary for the formation of Peyer's patches(PP) and peripheral lymphonodes(PLN), a cytokine cascade which consists of IL-7R, LTα1β2/LTβR, and NIK. However, the subsequent organization of NALT seems to be controlled by these cytokine singaling cascades since the maturation of NALT structure is generally incomplete in those cytokine cascade-deficient mice. NALT as well as PP and PLN are completely absent in Id2$^{-/-}$ mice. NALT organogenesis is initiated following the adoptive transfer of CD3$^-$CD4$^+$CD45$^+$ cells into Id2$^{-/-}$ mice, constituting direct evidence that CD3$^-$CD4$^+$CD45$^+$ inducer cells can provide an IL-7R-, LTα1β2/LTβR-, and NIK-independent tissue organ-

ogenesis pathway for secondary lymphoid tissue development.

6. Mucosally-induced allergic diarrhea

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Systemically primed mice develop severe diarrhea following repeated oral administration of ovalbumin(OVA). This murine diarrhea model was used to clarify the underlying mechanism of intestinal hypersensitivity. Histological analysis demonstrated that dramatic infiltration of eosinophils and mast cells selectively occurred in the large intestine. Large intestinal $CD4^+$ αβ T lymphocytes elicited a brisk synthesis of IL-4, and IL-13 but little or no IFN-γ synthesis, whereas small intestinal $CD4^+$ αβ T lymphocytes produced no detectable levels of antigen-induced cytokines. As would be expected from the high levels of Th2-type cytokines, brisk levels of IgE were detected in sera and IgE antibody-producing cells were detected in the large but not small intestine of mice with diarrhea. Strikingly, identically treated signal transducers and activators of transcription 6 (STAT6) gene-disrupted mice failed to develop OVA-induced diarrhea. These results strongly suggest that antigen-specific Th2 type cells of the large intestine play a critical role in the onset of diarrhea, and that further STAT6 signaling transduction is involved in these Th2-derived intestinal allergic desorders upon repeated administration of oral antigen.

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