RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection

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 shigellae, enterohemorrhagic E. coli, enteropathogenic E. coli. We are also searching for effective methods to protect or regulate bacterial infection by using knowledge accumulated.

1. Rho family GTPase Cdc42 is essential for the actin-based motility of *Shigella flexneri* in mammalian cells

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Shigella, the causative agent of bacillary dysentery, are capable of directing its movement within host cells by exploiting actin dynamics. The VirG protein expressed at one pole of the bacterium can recruit neural Wiskott-Aldrich syndrome protein (N-WASP), a downstream effector of Cdc42. Here, we show that Cdc42 is required for the actin-based motility of *Shigella*. Microinjection of a dominant active mutant Cdc42, but not Rac1 or RhoA, into Swiss 3T3 cells accelerated *Shigella* motility. In add-back experiments in *Xenopus* egg extracts, addition of a guanine-nucleotide dissociation inhibitor for Rho family, RhoGDI, greatly diminished the bacterial motility or actin assembly, which was restored by adding activated Cdc42. In N-WASP-depleted extracts, the bacterial movement almost arrested was restored by adding exogenous N-WASP but not H208D, a N-WASP mutant defective in binding to Cdc42. Actin polymerization induced by N-WASP-Arp 2/3 complex was stimulated by VirG, but further enhanced by adding of Cdc42 *in vitro*. The VirG interaction with N-WASP *in vitro* was slightly enhanced by Cdc42. Certainly, Cdc42 was accumulated at the site of initial actin cloud formation by invaded *Shigella* in mammalian cells. Furthermore, overexpression of H208D mutant in cells interfered with the actin assembly of infected *Shigella* and diminished the intra- and intercellular spreading. These results suggest that *Shigella* motility in epithelial cells requires Cdc42 activity and its regulation of N-WASP.

2. Profilin is Required for Sustaining Efficient Intra- and Intercellular Spreading of *Shigella flexneri*

Hitomi Mimuro, Toshihiko Suzuki, Shiro Suetsugu[1], Hiroaki Miki[1], Tadaomi Takenawa[1] and Chihiro Sasakawa

The ability of *Shigella* to mediate actin-based motility within the host cell is a prominent pathogenic feature of bacillary dysentery. The ability is dependent on the interaction of VirG with neural Wiskott-Aldrich syndrome protein (N-WASP) which in turn mediates recruitment of Arp2/3 complex and several actin-related proteins. In the present study, we show that profilin I is essential to the rapid movement of *Shigella* in epithelial cells, for which the capacity of profilin to interact with G-actin and N-WASP is critical. In COS-7 cells overexpressing either mutated profilin H119E, which failed to bind G-actin, or H133S, which is unable to interact with

poly-L-proline, *Shigella* motility was significantly inhibited. Similarly, depletion of profilin from *Xenopus* egg extracts resulted in a decrease in *Shigella* motility, that was completely rescued by adding back profilin I but not H119E or H133S. In COS-7 cells overexpressing a N-WASP mutant lacking the proline-rich domain (Δp) unable to interact with profilin, the actin tail formation of intracellular *Shigella* was inhibited. In N-WASP(depleted extracts, addition of Δp but not full-length N-WASP was unable to restore the bacterial motility. Furthermore, in a plaque formation assay with MDCK cell monolayers infected by *Shigella*, MDCK cells stably expressing H119E, H133S or Δp reduced the bacterial cell-to-cell spreading. These results indicate that profilin I associated with N-WASP is an essential host factor for sustaining efficient intra- and intercellular spreading of *Shigella*.

3. Helicobacter *pylori* CagA Protein Delivered into the Gastric Epithelial Cells can be Tyrosine Phosphorylated

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Attachment of *Helicobacter pylori* to gastric epithelial cells induces various cellular responses, including the tyrosine phosphorylation of an unknown 145-kD protein and interleukin 8 production. Here we showed that this 145-kD protein is the *cagA* product of *H. pylori*, an immunodominant, cytotoxin-associated antigen. Epithelial cells infected with various *H. pylori* clinical isolates resulted in generation of tyrosine-phosphorylated proteins ranging from 130 to 145 kD in size that were also induced *in vitro* by mixing host cell lysate with bacterial lysate. When epithelial cells were infected with [^{35}S] methionine-labeled *H. pylori*, a radioactive 145-kD protein was detected in the immunoprecipitates with antiphosphotyrosine antibody or anti-CagA (cytotoxin-associated geneA) antibody. Consistently, the 145-kD protein recognized by the anti-CagA and antiphosphotyrosine antibodies was induced in epithelial cells after infection of wild type *H. pylori* but not the *cagA*::Km mutant. Furthermore, the amino acid sequence of the phosphorylated 145-kD protein induced by *H. pylori* infection was identical to its own CagA sequence. These results reveal that the tyrosine-phosphorylated 145-kDa protein is *H. pylori* CagA protein, which may be delivered from attached bacteria into the host cytoplasm. The identification of the tyrosine-phosphorylated protein will thus provide further insights into understanding the precise roles of CagA protein in *H. pylori* pathogenesis.

4. Secondary type III secretion machinery of enterohemorrhagic and enteropathogenic *Escherichia coli*

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Determination of entire genome sequence of enterohaemorrhagic *Escherichia coli* led to the identification of a 17 kb chromosomal region, which contains type III secretion system gene cluster, at minute 64.5. This locus contains ORFs, whose amino acid sequences showed high similarity with proteins composing type III secretion apparatus, which are encoded by *inv/spa/prg* locus resides on a *Salmonella* SPI-1 pathogenicity island. Henceforth, this locus was designated as ETT2 (*E. coli* type III secretion system 2). The ETT2 was found in other pathogenic *E. coli* such as, EHEC O26, several serotypes of enteropathogenic *E. coli* (EPEC), and rabbit EPEC (REPEC-1). Whereas, all of *E. coli* except serotype O157, contains only a part of the sequence, which corresponds to the *spa/prg* homologous region but *inv* homologous region. The cloned ETT2 of EHEC O157 conferred secretion of EspB in *E. coli* K12 background, suggesting the ETT2-encoded type III secretion machinery was functional. Furthermore, transcription from promoters of ETT2 operons was regulated differentially from that of LEE-encoded type III secretion operon. These suggested that the second type III secretion system found in EHEC locus plays a role in secretion/ translocation of effector proteins during infection and is overlapping its function with type III secretion system encoded in LEE.

5. SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7

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The quorum-sensing system in bacteria is a well-known regulatory system that controls gene expression in a cell density-dependent manner. A transcriptional regulator (LuxR homologue), signal synthase (LuxI homologue) and autoinducer (acyl homoserine lactone) are indispensable for this system in most Gram-negative bacteria. In this study, we found that SdiA, an *Escherichia coli* LuxR homologue, is a negative regulator of the expression of virulence factors EspD and intimin in enterohaemorrhagic *E. coli* (EHEC) O157:H7. The expression of EspD and intimin was inhibited at the RNA level upon SdiA overexpression. SdiA has a DNA-binding motif in its C-terminal part and can bind to the promoter regions of the *esp* and *eae* genes *in vitro*. Extracellular factors, which accumulate in culture supernatants of O157:H7 at the stationary phase of growth and inhibit EspD and intimin synthesis, bind to the N-terminal part of SdiA *in vivo* and *in vitro*. O157:H7 overproducing the N-terminal part of SdiA exhibited hypertranscription of EspD and intimin, suggesting that the overproduced N-terminal part had inhibited the activity of intact SdiA through titration of the extracellular factors. These results indicate that a quorum-sensing system including the SdiA protein controls colonization by O157:H7.

6. Isolation and characterization of miniTn*5Km2* insertion mutants of enterohemorrhagic *Escherichia coli* O157:H7 deficient in adherence to Caco-2 cells

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Adherence of enterohemorrhagic *Escherichia coli* (EHEC) to intestinal epithelium is essential for initiation of the infection. To identify genes involved in the adherence, an EHEC O157:H7 strain (O157Sakai) was mutagenized by mini-Tn*5Km2*, and 4677 insertion mutants were screened for their ability to form microcolonies (MC) on Caco-2 cells. The less adherent mutants were divided into three groups: those with no adherent ability (designated as class 1 mutants, n=10), those less adherent than the wild type (class 2 mutants, n=16), and those unable to form MC but which adhered in a diffuse manner (class 3 mutants, n=1). The sites of insertion in class 1 mutants were all found within gene of the locus for enterocyte effacement (LEE) thought to required for type III protein secretion. Indeed, the class 1 mutants failed to secrete type III secreted proteins such as EspA and Tir into the culture medium. The insertions in class 2 mutants were outside the LEE and all the mutants except one were able to secrete type III proteins into the culture medium. The class 3 mutant had the insertion in the *tir* gene in LEE and was deficient in Tir and intimin expression, suggesting that in the absence of intimin-Tir, O157Sakai can still adhere to Caco-2 cells but in a diffused manner. This was confirmed by construction of a non-polar *eae* (encoding intimin) mutant. Examination of the *eae* mutant together with O157Sakai and one of the class-1 mutants for the ability to form MC revealed that EHEC initially adhered diffusely at 1.5 h after infection. Following washing out the non-adherent bacteria, while wild type EHEC bacteria developed MC for another 2 to 3 h on Caco-2 cells, the *eae* mutant diffusely adhered throughout the infection without forming MC. MC with O157Sakai but not the diffusely adherent *eae* mutant could evoke F-actin condensation beneath bacterium. Our results suggest that EHEC encode additional adherence associated loci, and that the type III secreted proteins are involved in the initial diffuse adherence, while intimin-Tir interaction is required for the subsequent development of MC.

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Department of Microbiology and Immunology

Division of Immunology (1)

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. Essential Role of Signal Transducer and Activator of Transcription (Stat) 5a for Interleukin-5-dependent IgH switch recombination

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Interleukin-5 (IL-5) plays an important role in IgM and IgG1 production of activated mouse splenic B (B-2) cells. IL-5 stimulation of CD38-activated splenic B cells induces μ-γ1 switch recombination in DNA level and production of high levels of lgG1 in IL-4-independent manner. Further addition of IL-4 in the system enhances IL-5-dependent μ-γ1 switch recombination and IgG1 production. Although IL-5 has been shown to induce STAT5 activation in mouse B-cell lines, role for STAT5 activation in IL-5-induced Ig switch recombination in primary B cells is still poorly understood. When we stimulated splenic B cells of wild type (WT) mice with anti-CD38 mAb, CS/2 and IL-5, tyrosine phosphorylation of STAT5a and STAT5b was induced. We stimulated STAT5a- and STAT5b-deficient B cells with CS/2 plus IL-5 and examined proliferative response, the expression of γ1 germline transcripts, and μ-γ1 switch recombination. STAT5a- and STAT5b-deficient B cells showed lower proliferative response and produced lower IgG1 than WT B cells. Moreover, almost no circular DNA, reciprocal products of μ-γ1 switch recombination, was detected in these mutant B cells. These data indicate that STAT5a and STAT5b are involved in IL-5R signaling and IL-5-dependent class switch recombination. Intriguingly, IL-4 partially rescued impaired production of IgG1 in STAT5b deficient B cells, but not in STAT5a deficient B cells. Our data support the notion that Stat5a and Stat5b are not redundant, but rather are at least partially distinctive in their function on B cell differentiation.

There are body of evidence that B cell differentiation and isotype switching is related to division cycle number. Thus, we examined division cycle number of STAT5 deficient B cells using 5-, 6-carboxyfluorescein diacetate, succinimidyl ester (CFSE). In WT B cells, μ-γ1 switch recombination and surface IgG1-positive cells appeared after five division cycles upon CS/2 and IL-5 stimulation. Stat5a deficient B and Stat5b deficient B cells were almost normally divided to 5 or 6 cell cycles, but they could express neither μ-γ1 switching nor surface IgG1. We infer from these results that Stat5a and Stat5b play pivotal roles in induction of IL-5-induced switch recombination.

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

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Ligation of CD38 on mouse B cells with CS/2, an anti-mouse CD38 mAb, induces B cell proliferation, IL-5Rα chain expression, and the expression of germline γ1 transcripts. We have also reported that IL-5 stimulation of CS/2-ligated B cells promotes both μ-γ1 switch recombination and lgG1 secretion in an IL-4 independent manner.

As we reported, CS/2 stimulation induces expression of germline γ1 transcripts that is required prior to switch recombination events. We examined nuclear factor (NF)-κB activation in CS/2-sitmulated mouse splenic B cells. Electrophoretic mobility shift assay (EMSA) revealed that CS/2 stimulation activated NF-κB complexes that were consisted of p50, p65, and c-Rel, but RelB was not activated and different from CD40 stimulation. To elucidate functions of c-Rel in CS/2-stimulated B cells, we examined proliferation and germline γ1 expression in c-Rel deficient B cells. Compared to WT B cells, c-Rel deficient B cells showed impaired proliferation and germline γ1 induction in response to CS/2. In contrast, CD40-stimulated c-Rel$^{-/-}$ B cells showed less severe impairment. IL-5-dependent IgG1 production was also severely decreased in CS/2-activated c-Rel deficient B cells. These results suggest a key and selective role for c-Rel in the expression of germline γ1 transcripts and Ig switch recombination. Our preliminary analyses by EMSA revealed that Btk is involved in CS/2-induced NF-κB activation and the expression of germline γ1 transcripts.

c. Molecular cloning of genes induced by IL-5 in mouse 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 the above kinases still remain unclear. To elucidate IL-5 signaling pathway in molecular levels, we prepared subtracted libraries between CD38- and IL-5-stimulated B cells and CD38-stimulated B cells to isolate genes induced by IL-5. The isolated genes thus obtained included genes encoding Ig γ1 heavy chain, cytoskeletal components, and molecules involved in DNA replication and cell cycle, cell metabolism, signal transduction, transcription, translation, and transport. We are currently analyzing function of some of genes on B cell differentiation and Ig switch recombination by using retrovirus transfection system.

2. Structural and functional analysis of IL-5 receptor

a. Regions in the cytoplasmic domain of IL-5Rα critical for B cell growth and IgG1 switch recombination

Byoung-Gon Moon, Toshimi Yoshida, Kazuki Nakao[3], Motoya Katsuki[3], Satoshi Takaki, and Kiyoshi Takatsu:[3]Division of DNA Biology and Embryo Engineering, Center for Experimental Medicine

IL-5 receptor (IL-5R) consists of two membrane proteins, IL-5Rα and βc. The IL-5Rα specifically binds IL-5 and the βc, which is shared among receptors for IL-3, IL-5 and GM-CSF, forms a high affinity receptor complex in combination with the IL-5Rα. We have demonstrated that the membrane-proximal proline-rich region (ppvp motif), conserved among cytoplasmic domains of IL-5Rα, IL-3Rα and GM-CSFRα, is critical to transduce signal for both cell growth and differentiation. Although the C-terminal region unique to IL-5Rα is not required for growth signaling, its function in signaling for cell differentiation remains to be elucidated. We tried to address the issue using chimeric receptors consisting of the extracellular domain of IL-3Rα fused with the cytoplasmic domain of IL-5Rα. BCL-B20 cells, that are able to differentiate into IgM-producing cells in response to IL-5, were transfected to express various chimeric receptors. Cells expressing the chimeric receptor with the intact cytoplasmic domain of IL-5Rα produced IgM upon IL-3 stimulation. On the other hand, cells expressing the chimeric receptor carrying a deletion in the C-terminal region did not. It is considered therefore that the C-terminal region plays an important role in IL-5-induced B cell differentiation.

The above approach has limitations regarding functions of IL-5Rα cytoplasmic domain subregions on distinct primary B cell subpopulations and their responsiveness to IL-5. In the present study, we generated mice each expressing a mutant form of an IL-5Rα transgene ligated with μ enhancer and V_H promoter, lacking the cytoplasmic DC3-region or substituting two proline residues for alanine (ApvA) in the membrane-proximal ppvp motif of the cytoplasmic domain, on IL-5Rα null background. The ppvp motif, which mediates activation of JAK2/STAT5 and Btk, contributes to *c-fos*, *c-jun*, and *c-myc* expression. IL-5Rα null mutant mice showed impaired B-1 cell development, reduced serum IgG3 and IgM, no IL-5-induced enhancement of B cell proliferation and IL-5-induced switch recombination from μ to γ1 gene; these are not recovered following the expression of ApvA mutant. In contrast, lack of the DC3 region affects the IL-5-induced switch recombination from μ to γ1 gene and B-1 cell development, while IL-5-induced proliferation and IgM production are of levels similar to these of B cells

expressing WT IL-5Rα transgene. Our results clearly indicate that the ppvp motif and the DC3 region of IL-5Rα play a distinct role in B cell proliferation and differentiation. Thus, our present approach offers new insights into the functions of the cytoplamic subregions of IL-5Rα, particularly of its carboxy-terminal region.

b. Characterization of a nuclear protein complex binding to the murine IL-5Rα gene

Yuki Ashizawa, Satoshi Takaki, and Kiyoshi Takatsu

The IL-5 receptor (IL-5R) consists of two distinct membrane proteins, IL-5Rα and βc. Although the βc is shared by IL-3R and GM-CSFR, IL-5Rα is specific to IL-5 and its expression is restricted in B cells, eosinophils, and basophils. This restricted expression of IL-5Rα appears to be crucial for specific activities of IL-5 on IL-5-responsive cells.

To understand how the restricted IL-5Rα expression is achieved, we characterized the promoter regions of the murine IL-5Rα gene. By employing 5' RACE, the second transcriptional start site is identified at the position +1002 with respect to the previously identified start site (+1), indicating the mIL-5Rα gene carries multiple promoters. We then characterized regulatory regions in the promoters by making luciferase reporter plasmids and transfecting them into various cell lines. The region from -250 ~ +160 showed a promoter activity in WEHI231, IL-5Rα$^+$ B cell line but not in EL4 and FDC-P1, IL-5Rα$^-$ cell lines. In contrast, +577 ~ +1105 region showed a promoter activity in all tested cell lines. A complex binding to the -250 ~ +160 region was found only in nuclear extracts obtained from IL-5Rα$^+$ B cell lines. The complex contained Sp1, C/EBPβ, and Oct-2 as components. Mutations of Sp1, C/EBPβ, and Oct-2 binding sites in the -250 ~ +160 region resulted in reduction of its promoter activity. Our results demonstrate a part of the transcriptional regulation responsible for the restricted expression of IL-5Rα in mouse B cells.

3. Regulatory role of adapter protein in the immune system

a. Lnk, an adapter protein that controls B cell production and stem cell function

Satoshi Takaki, Yoshinari Tezuka, and Kiyoshi Takatsu

Lymphocytes differentiate from hematopioetic precursor cells through a process whereby the coordinate regulation of cell proliferation, differentiation and death directs development of functional cells. Significant advances have been made in understanding the signal transduction pathways regulating both lymphocyte development and activation, however, the mechanisms underlying homeostasis in the lymphocyte compartment remain poorly defined. Recently, growing evidence supporting the importance of adaptor proteins has been presented in numerous signal transduction cascades.

Lnk is an SH2 domain-containing adaptor protein expressed preferentially in lymphocytes. To illuminate the importance of Lnk, we generated *lnk*$^{-/-}$ mice. Whereas T cell development was unaffected, pre-B and immature B cells accumulated in the spleens. In the bone marrow, B-lineage cells were proportionately increased, reflecting enhanced production of pro-B cells which resulted in part from hypersensitivity of precursors to SCF (stem cell factor), the ligand for c-kit. Hence Lnk ordinarily acts to regulate B cell production. Further characterization of *lnk*$^{-/-}$ mice also revealed that full-length Lnk is a 68 kDa protein containing a conserved proline-rich region and a PH domain. Lnk is a representative of a multigene adaptor protein family conserved from drosophila to mammalian whose members presumably act, by analogy with Lnk, to modulate intracellular signaling mediated by tyrosine kinases.

Hypersensitivity of *lnk*$^{-/-}$ B cell precursors to SCF prompt us to study functions of hematopoietic stem cell (HSC) in *lnk*$^{-/-}$ mice. Fairly amounts of *lnk* mRNA are expressed in HSC fractions of normal mice. Competitive repopulation assay revealed an augmented ability of *lnk*$^{-/-}$ HSC for reconstituting hematopoietic cells in irradiated hosts. Lnk also regulates HSC functions and controls hematopoiesis.

b. Isolation and characterization of APS, a Lnk family adaptor protein expressed in mature B cells

Masanori Iseki, Kiyoshi Takatsu, and Satoshi Takaki

Engagement of cell-surface receptors leads to the activation of protein tyrosine kinases, which in turn phosphorylate various downstream enzymes and adaptor proteins. Lnk is an adaptor protein that appears to be involved in signal transduction in lymphocytes, and forms an adaptor protein family with SH2-B. We tried to identify another member of the adaptor protein family and isolated the mouse APS (adaptor molecule containing PH and SH2 domains). APS contains a proline-rich region, PH and SH2 domains, and a putative tyrosine phosphorylation site at the C-terminal end, and the overall structure resembles to those of Lnk and SH2-B. APS is expressed in brain, kidney, muscle, and in mature B cells in spleen. Mouse *APS* gene consists of 8 coding exons and deduced to be mapped on the chromosome 5. APS is tyrosine phosphorylated at the C-terminal phosphorylation site conserved among the Lnk family adaptor proteins by the stimu-

lation of IL-5 or IL-3 as well as by crosslinking of B cell receptor complex. These results suggest that APS is a member of the Lnk family adaptor protein, and likely to play a role in signaling in B cells.

c. Growth retardation in mice lacking SH2-B, a member of Lnk family adaptor proteins

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SH2-B, a member of the Lnk-family adaptor proteins was originally isolated by its ability to associate with phosphorylated ITAM motif of FcεRIγ chain. Recent studies demonstrated that SH2-B is phosphorylated by the stimulation of various growth factors such as insulin, IGF, NGF and growth hormone. However, its functions in signal transduction pathways of those growth factors and its physiological roles are poorly understood. First, we re-evaluated expression of *SH2-B* transcripts in various mouse tissues, and found *SH2-B* is ubiquitously expressed in wide range of tissues including thymus and spleen. We generated *SH2-B*$^{-/-}$ mice to investigate the functions of SH2-B *in vivo*. The exons encoding the N-terminal region to SH2 domain of SH2-B protein were replaced by a neomycin phosphotransferase cassette via homologous recombination in mouse ES cells. Successful homologous recombination and germline transmission were confirmed via genomic blot analysis of mouse tail DNA from offspring of heterozygous *SH2-B*$^{+/-}$ mice. *SH2-B*$^{-/-}$ mice did not show any gross anomalies, and were produced in mendelian ratios. However, it became apparent at 2 week-old of their age that *SH2-B*$^{-/-}$ mice were smaller in size than +/+ or +/- littermates. Lymphocyte development is not perturbed as assessed by flow cytometric analysis. The cause of growth retardation in *SH2-B*$^{-/-}$ mice is currently being investigated.

4. Molecules involved in the lymphocyte activation

a. Bruton's tyrosine kinase (Btk) is required for signaling the CD79b-mediated in Pro-B to Pre-B transition

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Bruton's tyrosine kinase (Btk) is a cytoplasmic tyrosine kinase expressed in B-lineage, myeloid and erythroid cells. Btk is required for B cell development and activation since mutations of the *btk* gene cause X-linked agammaglobulinemia (XLA) in human and X-linked immunodeficiency (XID) in mouse. The developmental steps affected by Btk deficiency are, however, discrepant; pre-B cells are reduced in most XLA patients, whereas the number of pre-B cells is normal and functional defects are observed in peripheral B cells in XID mice. Formation of the pre-B cell receptor (pre-BCR) complex, which consists of productive Ig heavy chain, Vpre-B, λ5, CD79a and CD79b, is a critical checkpoint during B cell development, and induces the transition of pro-B to pre-B cells. CD79b (Igβ) is a signaling component in the pre-BCR complex, since differentiation to the pre-B phenotype is induced by crosslinking the CD79b expressed on developmentally arrested pro-B cells from recombination activating gene (RAG)-2-deficient mice *in vivo*. However, little is known about the signaling cascades activated by CD79b cross-linking in pro-B/pre-B transition. We established the system of crosslinking surface CD79b on pro-B cells and trigger signals to differentiate into Pre B cells in RAG-2$^{-/-}$ mice *in vivo*.

To examine whether Btk functions in CD79b-mediated signaling for the pro-B/pre-B transition, we utilized RAG2/Btk double knockout (DKO) mice. The pro-B/pre-B transition visualized by up-regulation of CD25, BP-1 and CD2 expression and cell size reduction of pro-B cells was not induced at all by CD79b cross-linking in RAG2/Btk-DKO mice. In contrast, tyrosine phosphorylation of cellular proteins as well as phosphorylation of Erk1/2 and PLC-γ2 were induced normally. BTK is phosphorylated after crosslinking of CD79b on RAG2-deficient pro-B cells. These findings suggest that BTK-dependent pathways downstream of CD79b are critical for the pro-B/pre-B transition, and BTK-independent signaling pathways are also activated via the pre-BCR complex. Further analysis could be required to find our target molecules downstream of BTK in early cell development.

b. Mutual regulation between BTK and BAM11, a 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), exists 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 be attractive model to elucidate a novel mechanism to transmit signals in Blymphocytes.

c. Mtβ, a transcriptional regulator for the lck proximal promoter

Atsuko Yamada, Satoshi Takaki and Kiyoshi Takatsu

The *lck* gene encodes a protein tyrosine kinase that plays key roles in signaling mediated through T cell receptor (TCR) or pre-TCR complexes. Transcription of the gene from the *lck* gene is regulated by two independent promoter elements; the proximal promoter and the distal promoter. Previous studies employing transgenic mice demonstrated that the sequences between -584 and -240 with respect to the transcription start site in the mouse *lck* proximal promoter are required for its tissue-specific expression in the thymus. We demonstrated that a Krüppel-like zinc finger protein, mtβ (BFCOL1, BERF-1, ZBP-89, ZNF148) that has been cloned as a binding protein to the CD3δ gene enhancer binds to the -365 to -328 region of the *lck* proximal promoter. Mtβ was ubiquitously expressed in various cell lines and mouse tissues. Overexpressed mtβ was more active in T-lineage cells than B-lineage cells for transactivating an artificial promoter consisting of mtβ binding sites and a TATA box. Activity of the *lck* proximal promoter was significantly impaired by mutating the mtβ binding site or by reducing mtβ protein expression level by using its antisense mRNA. Our results indicate that mtβ activity is regulated in tissue-specific manner, and that mtβ is a critical transactivator for the *lck* proximal promoter.

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

a. Amino Acid Residues of T cell epitope of the α Antigen of Mycobacteria Critical for Vβ11⁺ T-cell Epitope

Ai Kariyone, Hideaki Kano, and Kiyoshi Takatsu

α Antigen is one of the major antigens from *Mycobacterium tuberculosis* and *M. Bovis* BCG. We have shown that stimulation of *M. tuberculosis*-primed lymph node cells of C57BL/6 mice with α antigen (also known as Antigen 85B and MPT59) induces TCRVβ11⁺ CD4⁺ Th1 cells in conjunction with antigen-presenting cells in an I-A^b-restricted manner. We identified the antigenic epitope (peptide-25) for α antigen-specific Vβ11⁺ T cells as the 15-mer peptide, covering amino acid residues 240-254 of α antigen {peptide-25 (AA240-254)} which contains the motif that is conserved for I-A^b, and requires for processing by antigen-presenting cells. We then examined amino acid residues of peptide-25 critical for TCR recognition by using peptide-25-reactive Vβ11+ T cell clones and substituted mutants of peptide-25. Results revealed that the amino acid residues at positions 245, 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 I-A^b contact residues.

Peptide-25 was found to be immunogenic and to induce production of protective cytokines, IFN-α and TNF-α and development of $V_\beta11^+CD4^+$ T cells. Active immunization of C57BL/6 mice with peptice-25 can lead to decreased bacterial load in the lungs of *M. tuberculosis* H37Rv-infected mice. We found that substitution mutants of peptide-25, whose amino acids critical for I-A-binding motif were substituted to amino acids capable of binding to I-A^k and I-A^d, could induce the Th1 development in C3H/HeN and BALB/c.

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

Toshiki Tamura, Ai Kariyone, Shuichiro Uehara and Kiyoshi Takatsu

Activated CD4⁺ Th cells can be classified into at least two subsets, Th1 and Th2, on the basis of cytokine production profiles. The development of each one of Th subsets is regulated by cytokines in environment, such as IL-4 and IL-12, during the primary antigen response of naive T cells. In addition to cytokines, other stimuli such as type of APC, costimulation and genetic background could also be involved in differentiation of naive CD4⁺ T cells into Th1 and Th2 cells.

As we demonstrated, $CD4^+$ T cells reactive to α antigen or to peptide-25 expressed highly restricted TCR repertoire, Vβ11. We isolated TCRVα5 gene expressed in peptide-25-reactive Th1 clones and confirmed by transfection into TG40 cells that TCR-Vα5 can interact with TCRVβ11 to reconstruct functional TCR reactive to peptide-25 and APC leading to produce Th1 cytokine. These results lead us to examine whether the antigen itself could determine the Th subset development. For this purpose, we are currently generating transgenic mice expressing TCR that reacts to peptide-25. We also examine the property of peptide-25 for vaccine and adjuvant that stimulate Th1 immunity.

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Department of Microbiology and Immunology

Division of Immunology (2)

1. Immunomolecular mechanism in mammalian fertilization and implantation

Tsuneatsu Mori, Maowu Guo, Aishun Jin, Xiang Li, Etsuko Mori[1] and Seiichi Takasaki[1]:Division of Immunology and [1]Division of Biochemistry

We have extensively promoted the analysis of immunomolecular mechanism and its application in mammalian gamete selection, fertilization and implantation

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

The ovarian follicular atresia is one of typical phenomenons in programmed cell death. Actually, We have demonstrated that Fas was definitely expressed in B6C3 F1 murine ovarian oocytes, ovulated eggs and granulosa cells in maturated follicle, while, Fas ligand (L) was restrictedly expressed in granulosa cells under the hormonal regulation of gonadotropins showing by the facts that the administration of PMSG resulted substantially in decline of Fas and increase of FasL in atretic follicles. Furthermore, the direct interaction between zona pellucida (ZP) free oocytes/eggs and granulosa/Sf9-FasL cells *in vitro* resulted in the induction of apoptosis in ZP free oocytes/eggs. In addition, 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. 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 a key role to promote ovarian atresia through apoptosis, most likely depending on local gonadotropin levels in the ovary. 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. We demonstrated the positive expression of Fas in testicular germ cells and of FasL in testicular cells by RT/PCR-Southern blot hybridization, supporting by the findings with *in situ* hybridization that Fas was localized in germ cells, whereas, FasL was localized in sertoli cells of murine testis. A specific band at 45 kDa was obtained in the lysates from testis and germ cells with Western blot analysis. The co-incubation of germ cells with Sf9-FasL cells *in vitro* resulted in the induction of apoptosis in germ cells detected by the TUNEL method. Furthermore, DNA fragmented ladders were also demonstrated in germ cells co-incubated with Sf9-FasL cells.

b. Fertilization

In mammalian fertilization, the sperm at first binds to a carbohydrate moiety of the extracellular glycoprotein matrix of the egg ZP and then penetrates through it to adhere to the egg plasma membrane with its postacrosomal membrane fol-

lowed by fusion of the two gametes. We have approached to the molecular mechanisms for multifarious cross-talks of sperm and egg in mammalian fertilization. Based on the analysis of all or partial sugar structures of porcine or murine ZP 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 bound to β-Galactose rather than α-Galactose residue and/or Le^X residue on egg ZP. At the fusion step of sperm and egg in the fertilization, we have further confirmed the expression and function of CD4/$p56^{lck}$ complex on murine egg plasma membrane corresponding to the presence of MHC class II molecule at the posterior region of sperm by the electron microscope.

c. Implantation

We elucidated the critical role of CD56 (NK) and CD57 (NS) positive cells expanded in pregnant decidual tissue promoting the placental formation and embryonal development. $CD57^+HLA\text{-}DR^{bright}$ natural suppressor (57.DR-NS) cell line, which was cloned from human 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 or chorionic carcinoma (GCIY/ BeWo) cells but not in a human diploid normal (WI-38) cells. The 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 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 into GCIY/Molt4 tumor bearing SCID mice resulted in the drastic suppression of tumor growth followed by the decrease in tumor size due to the occurrence of apoptosis in tumor tissues. Thus, we found the real tumor immunity in the implantation site of feto-maternal interface as mother nature's experiments.

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Department of Microbiology and Immunology
Division of Virology (1)

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. Plasmid-deriven formation of influenza virus-like particles

Gabriele Neumann, Tokiko Watanabe, and Yoshihiro Kawaoka

We established a plasmid-based system for generating infectious influenza virus-like particles entirely from cloned cDNAs. Human embryonic kidney cells (293T) were transfected with plasmids encoding the influenza A virus structural proteins, together with a plasmid that encodes an influenza-like vRNA containing an antisense copy of the cDNA for green fluorescence protein (GFP) flanked by an RNA polymerase I promoter and terminator. Intracellular transcription of the latter construct by RNA polymerase I generated GFP vRNA that was packaged into influenza virus-like particles. This system, which produced more than 10^4 infectious particles per ml of supernatant, would be useful in studies of influenza virus replication and particle formation. It might also benefit efforts in vaccine production and in the development of improved gene therapy vectors.

2. Balanced hemagglutinin-neuraminidase activities are critical for efficient replication of influenza A virus

Lyndon J. Mitnaul, Maria R. Castrucci, Mikhail N. Matrosovich, Alexander B. Tuzikov, Nikolai V. Bovin, Darwyn Kobasa and Yoshihiro Kawaoka

The SD0 mutant of the A/WSN/33 (WSN) influenza A virus, characterized by a 24 amino acid deletion in the neuraminidase (NA) stalk, does not grow in embryonated chicken eggs because of defective NA function. Continuous passage of SD0 in eggs yielded 10 independent clones that replicated efficiently. Characterization of these egg-adapted viruses showed that five of the viruses contained insertions in the NA gene from the PB1, PB2, or NP gene, in the region linking the transmembrane and catalytic head domains. The other five viruses did not contain insertions in this region, but displayed decreased binding affinity toward sialylglycoconjugates, as compared with binding properties of the parental virus. Sequence analysis of one clone revealed mutations in the hemagglutinin (HA) gene, at sites in close proximity to the sialic acid receptor-binding pocket. These mutations appear to compensate for reduced NA function due to stalk deletions. Thus, balanced HA-NA functions are necessary for efficient influenza virus replication.

3. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice

Mark T. Hughes, Mikhail Matrosovich, M. Elizabeth Rodgers, Martha McGregor, Yoshihiro Kawaoka

Influenza A viruses possess both hemagglutinin (HA), which is responsible for binding to the terminal sialic acid of sialyloligosaccarides on the cell

surface, and neuraminidase (NA), which contains sialidase activity that removes sialic acid from sialyloligosaccharides. Interplay between HA receptor-binding and NA receptor-destroying sialidase activity appears important for replication of the virus. Previous studies by others have shown that influenza A viruses lacking sialidase activity can undergo multiple cycles of replication if sialidase activity is provided exogenously. To investigate the sialidase requirement of influenza viruses further, we generated a series of sialidase-deficient mutants. Although their growth was less efficient than that of the parental NA-dependent virus, these viruses underwent multiple cycles of replication in cell culture, eggs, and mice. To understand the molecular basis of this viral growth adaptation in the absence of sialidase activity, we investigated changes in the HA receptor-binding affinity of the sialidase-deficient mutants. The results show that mutations around the HA receptor binding pocket reduce the virus' affinity for cellular receptors, thus compensating for the loss of sialidase. Thus, sialidase activity is not absolutely required in the influenza A virus life cycle, but appears necessary for efficient virus replication.

4. Recognition of *N*-glycolylneuraminic acid linked to galactose by the α2,3 linkage is associated with intestinal replication of influenza A virus in ducks

Toshihiro Ito, Yasuo Suzuki,Takashi Suzuki, Ayato Takada, Taisuke Horimoto, Krisna Wells, Hiroshi Kida, Koichi Otsuki, Makoto Kiso, Hideharu Ishida, and Yoshihiro Kawaoka

The hemagglutinin (HA) of H3 human influenza viruses does not support viral replication in duck intestine despite its avian origin. Leu-to-Gln mutation at position 226 and Ser-to-Gly at position 228 in the HA of human A/Udorn/307/72 (H3N2) permits a reassortant virus [human Udorn HA with all other genes from A/mallard/New York/6750/78 (H2N2)] to replicate in ducks. To understand the molecular basis of this change in host range restriction, we investigated the receptor specificity of duck influenza viruses as well as human-duck reassortants. The results indicate that the recognition of a glycoconjugate moiety possessing *N*-glycolylneuramic acid (NeuGc) linked to galactose by the α2,3 linkage (NeuGcα2,3 Gal) is associated with viral replication in duck intestine. Immunofluorescence assays with NeuGcα2,3 Gal-specific antiserum detected this moiety primarily on the crypt epithelial cells of duck colon. Such recognition, together with biochemical evidence of NeuGc in crypt cells, correlated exactly with the ability of the virus to replicate in duck colon. These results suggest that recognition of the NeuGcα2,3 Gal moiety plays an important role in the enterotropism of avian influenza viruses.

5. Early alterations of the receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals

Mikhail Matrosovich, Alexander Tuzikov, Nikolai Bovin, Alexandra Gambaryan, Alexander Klimov, Maria R. Castrucci, Isabella Donatelli, and Yoshihiro Kawaoka

Interspecies transmission of influenza A viruses circulating in wild aquatic birds occasionally results in influenza outbreaks in mammals, including humans. To identify early changes in the receptor binding properties of the avian virus hemagglutinin (HA) after interspecies transmission and to determine the amino acid substitutions responsible for these alterations, we studied the HAs of the initial isolates from the human pandemics of 1957 (H2N2) and 1968 (H3N2), the European swine epidemic of 1979 (H1N1), and the seal epidemic of 1992 (H3N2), all of which were caused by the introduction of avian virus HAs into these species. The viruses were assayed for their ability to bind the synthetic sialylglycopolymers 3′SL-PAA and 6′SLN-PAA, which contained, respectively, 3′-sialyllactose (the receptor determinant preferentially recognized by avian influenza viruses) and 6′-sialyl(N-acetyllactosamine) (the receptor determinant for human viruses). Avian and seal viruses bound 6′SLN-PAA very weakly, whereas the earliest available human and swine epidemic viruses bound this polymer with a higher affinity. For the H2 and H3 strains, a single mutation, 226Q → L, increased binding to 6′SLN-PAA, while among H1 swine viruses, the 190E → D and 225G → E mutations in the HA appeared important for the increased affinity of the viruses for 6′SLN-PAA. Amino acid substitutions at positions 190 and 225 with respect to the avian virus consensus sequence are also present in H1 human viruses, including those that circulated in 1918, suggesting that substitutions at these positions are important for the generation of H1 human pandemic strains. These results show that the receptor-binding specificity of the HA is altered early after the transmission of an avian virus to humans and pigs and, therefore, may be a prerequisite for the highly effective replication and spread which characterize epidemic strains.

6. Sialic acid species as a determinant of the host range of influenza A viruses

Yasuo Suzuki, Toshihiro Ito, Takashi Suzuki, Robert E. Holland Jr., Thomas M. Chambers, Makoto Kiso, Hideharu Ishida, and Yoshihiro Kawaoka

The distribution of sialic acid (SA) species varies among different animal species, but the biologic role

of this variation is largely unknown. Influenza viruses differ in their ability to recognize SA-galactose (Gal) linkages, depending upon animal hosts from which they are isolated. For example, human viruses preferentially recognize SA linked to Gal by theα2,6 (SAα2,6Gal) linkage, while equine viruses favor SAα2,3Gal. However, whether a difference in relative abundance of specific SA species (*N*-acetylneuraminic acid [NeuAc] and *N*-glycolylneuraminic acid [NeuGc]) among different animals affects the replicative potential of influenza viruses is uncertain. We therefore examined the requirement of the hemagglutinin (HA) for support of viral replication in horses, using viruses whose HAs differ in receptor specificity. A virus with an HA recognizing NeuAcα2,6Gal, but not NeuAcα2,3Gal or NeuGcα2,3Gal, failed to replicate in horses, while one with an HA recognizing the NeuGcα2,3Gal moiety replicated in horses. Furthermore, biochemical and immunohistochemical analyses and a lectin-binding assay demonstrated the abundance of the NeuGcα2,3Gal moiety in epithelial cells of horse trachea, supporting that recognition of this moiety is critical for viral replication in horses. Thus, these results provide evidence of a biologic effect of different sialic acid species in different animals.

7. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1

Gabriele Neumann, Mark T. Hughes, and Yoshihiro Kawaoka

For nuclear export of proteins, the formation of a ternary export complex composed of the export substrate, a cellular export factor, and Ran-GTP is crucial. CRM1 is a cellular export factor for proteins containing leucine-rich nuclear export signals (NESs). Although the NES sequence is crucial for nuclear export, its exact role in the formation of the ternary export complex is controversial. Here we demonstrate an interaction between human CRM1 (hCRM1) and influenza A virus NS2 protein which contains an NES motif in its N-terminal region. Replacement of the hydrophobic amino acids in the NES motif did not abolish NS2's interaction with hCRM1. Using our recently established systems for the generation of influenza virus or virus-like particles from cloned cDNAs, we found that NS2 is essential for nuclear export of influenza virus ribonucleoprotein (RNP) complexes, and that alteration of the NS2-NES abrogated this event and influenza virus generation. These findings suggest that the NS2-NES is not crucial for the interaction of this protein with hCRM1 but is for the formation of the ternary export complex with Ran-GTP.

8. Immunological and PCR analyses for Borna disease virus in psychiatric patients and blood donors in Japan

Koji Fukuda, Kazuo Takahashi, Yasuhide Iwata, Kenji Gonda, Norio Mori, Shin-ichi Niwa, Taisuke Horimoto, Masato Tashiro, and Shiro Shigeta

Seroepidemiological and PCR studies have suggested a possible involvement of Borna disease virus (BDV) in psychiatric diseases in humans. However, there is much controversy surrounding this issue, since conclusive evidence is lacking. Herein, for the first time, we describe T cell proliferative responses to BDV proteins in psychiatric patients and healthy individuals in Japan. In addition, we evaluate the extent of BDV infection using Western blot with inhibition tests and PCR analyses. Positive proliferative responses to both BDV p24 and p40 proteins were detected in 6% (3/49) of mood disorder patients, 4% (2/48) of schizophrenic patients, and 2% (1/49) of blood donors. BDV p40 antibody was detected in only 2% (1/49) of patients with mood disorders, while BDV p24 antibody was detected in 8% (4/48) of schizophrenic and 2% (1/49) of mood disorder patients. No plasma reacted with both BDV proteins. BDV RNA was detected in only 3% (1/39) of mood disorder patients. In these three assays, there was no significant difference in prevalence among the three groups. However, in 6 patients who were positive for either the BDV p40 or p24 antibody, one of two schizophrenic patients and one of four mood disorders were positive for proliferative responses to both BDV proteins. Although the association between BDV infection and human psychiatric disorders was not established, the present study suggests that certain individuals manifesting psychiatric illness are also infected with BDV or a BDV-related virus.

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Department of Microbiology and Immunology
Division of Virology (2)

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 (CNS), known as progressive multifocal leukoencephalopathy (PML). The following studies were performed in 2000.

1. Characterization of the *in vitro* system that supports the efficient growth of JC virus

Chie Sugimoto and Yoshiaki Yogo

The renal/urinary JCV DNAs carry the archetype regulatory region that generates various rearranged regulatory regions (PML-type regulatory regions) occurring in JCVs derived from the brain of PML patients. It is thought that archetype JCV is circulating in human populations. Although tissue cultures that support the growth of archetype JCV were not available, we reported that archetype JCV can efficiently replicate in COS-7, simian cells expressing the SV40 T antigen (Hara et al., *J. Virol*. 72, 5335-5342, 1998). To elucidate the role of the JCV T-antigen in the replication of JCV in COS-7 cells, we analyzed the capacity of various mutant and chimeric JCV DNAs to induce the growth in COS-7 cells. The results of these experiments suggested that a short DNA segment locating at the 3'-terminus of the JCV T antigen gene plays an important role in the replication of JCV in COS-7 cells. We are currently examining how this segment enhance the replication of JCV in COS-7 cells.

2. Rearrangements of the JCV regulatory region in the CNS of PML patients

Yoshiaki Yogo and Chie Sugimoto

We recently established a nested PCR system that can 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 system, if the amplified fragments are sequenced, can eliminate false positives that may arise from contaminations. Using this system, we have been performing a PML- diagnosis service to hospitals throughout Japan. Owing to this service we had opportunities to analyze JCV DNAs in brain tissues autopsied from two PML patients. In each case, we detected multiple JCV regulatory regions generated from the archetype through a common intermediate carrying only deletions. Interestingly, each rearranged regulatory region showed a distinct regional distribution pattern in the CNS. These findings offer partial support for "dual rearrangement model " that we have recently proposed (Yogo and Sugimoto, in press).

3. Evolution of JCV: implications for the population history of humans

Chie Sugimoto, Atsushi Kato[1], Huai-Ying Zheng[1], Tadaichi Kitamura[1] and Yoshiaki Yogo: [1]Graduate School of Medicine, The University of Tokyo

By phylogenetic analysis using a 610-bp region (IG region) of the viral genome, we identified 12 JCV subtypes that occupy unique domains in Europe, Africa and Asia (Sugimoto et al., *Proc. Natl. Acad. Sci. USA*. 94, 9191-9196, 1997). The IG region encompass-

es the 3'-terminal regions of both T-antigen and VP1 genes, and is relatively rich in nucleotide differences among JCV strains, and is useful to roughly classify strains into subtypes. Nevertheless, the information (i. e. nucleotide differences) in this region was not sufficient to elucidate the evolutionary relationships among JCV strains. Recently, phylogenetic analysis of complete JCV DNA sequences was used to reconstruct a highly reliable phylogeny of JCV strains. Therefore, we decided to adopt this whole genome approach to establish the evolutionary relationships among JCV strains on a global scale. Based on 65 complete JCV DNA sequences derived from various sites of the world and belonging to 11 of the 12 known JCV subtypes, we constructed phylogenetic trees using neighbor-joining and maximum parsimony methods. The resultant trees showed that ancestral JCVs were divided into three superclusters, designated as Types A, B and C. These findings suggested that early divisions in the ancestral human population gave rise to 3 populations carrying prototypes of Types A, B and C. From the geographic distributions of the descendants of these prototype JCVs, it was inferred that human populations carrying prototypes of Types A, B and C mainly produced European, Asian and African populations, respectively. A split in Type A generated two clusters. The first and second splits in Type B generated the major African subtype and a minor European subtype, respectively. The third split in Type B generated two clusters, one including 3 Asian subtypes and the other including 4 Asian subtypes. Type C remains undivided. These findings have wide implications for the population history of Europe, Africa and Asia.

4. Detection of the same genotype (MY) of JCV from two major native American groups, Amerind and Na-Dene

Yoshiaki Yogo, Chie Sugimoto, Huai-Ying Zheng[1], Tadaichi Kitamura[1], Jing Guo[2] and Luc G. Berthiaume[2]: [2]University of Alberta

Greenberg et al. classified Native Americans into three major groups, Amerind, Na-Dene and Eskimo-Aleut. We showed that three populations belonging to Amerind carried MY (one of the two major JCV genotypes in Japan), and that Inuits belonging to Eskimo-Aleut carried EU (the major genotype in Europe and Mediterranean areas). In this study, we collected urine samples from two groups, Dene Tha' and Beaver, belonging to Na-Dene and living in the northern part of Alberta, Canada. From urine samples, we amplified the 610-bp IG region of the JCV DNA. We sequenced the amplified IG regions, and the resultant sequences, together with those previously identified in various regions of the Old World, were used to construct a neighbor-joining phylogenetic tree. According to the tree, all JCV strains from Dene Tha' and Beaver fell into the genotype MY. Agostini et al. reported that Navaho, a group of Na-Dene living in the United Sates, carried a genotype of JCV named 2A/2C which were later found to correspond to the genotype MY. Thus, the detection of MY from the Canadian Na-Denes showed that MY is the indigenous JCV genotype not only in Amerind but also in Na-Dene. It is likely that Amerind and Na-Dene both carrying MY constitute a single family that can be distinguished from Eskimo-Aleut carrying EU.

5. Transmission of the Japanese JCV genotypes to the 2nd and 3rd generations of Japanese Americans

Makoto Suzuki[1], Huai - Ying Zheng[1], Chie Sugimoto, Tadaichi Kitamura[1] and Yoshiaki Yogo

JCV is horizontally transmitted among humans. However, there is a close correlation between JCV genotypes and human populations, as indicated by the geographic distributions of JCV genotypes in the Old World. To solve how this correlation was generated, we hypothesized that JCV is transmitted from parents to children through long-term cohabitation. To test this hypothesis, we examined the JCV genotypes in Japanese Americans. About 100 urine samples were collected in Nikkei Medical Center at Little Tokyo in Los Angeles from the 2nd and 3rd generations of Japanese Americans whose parents and grandparents were all Japanese. Control samples were obtained from general patients at the Scripps Clinic in La Jolla near Los Angeles. The Japanese Americans mainly carried two JCV genotypes, CY and MY, predominating in Japan. The detection ratio for CY, however, was significantly higher than that for MY. This finding appeared to be a reflection of the historical fact that western Japanese emigrated to the United Sates more frequently than eastern Japanese (CY occurs more frequently in western Japan than in eastern Japan). The control Americans carried the European genotypes and several genotypes from the world. These findings support the hypothesis that JCV is transmitted from parents to children through long-term cohabitation.

6. JC virus genotype: a novel means of tracing the origins of unidentified cadavers

Hiroshi Ikegaya[1], Hirotarou Iwase[1], Chie Sugimoto and Yoshiaki Yogo

There is no reliable means of tracing the origins of unidentified cadavers, although the increases in number of such cases is a growing problem throughout the world. The recent finding that JCV serves as a means of elucidating human migrations suggested

that this virus may also be useful to trace the origins of unidentified cadavers. We found that a 610-bp IG region of the JCV genome, usually used for the identification of JCV genotype, can efficiently be amplified by PCR from the kidneys or urine of cadavers even a week after death, and that genotype profiles of JCV DNAs recovered from cadavers are consistent with those expected from their geographic origins. Thus, we concluded that the JCV genotype is a novel marker useful for tracing the origins of unidentified cadavers.

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Our major research interest is to elucidate host-parasite defense mechanism of infectious diseases using in Vivo and in Vitro models. For this purpose, we established a monoclonal antibody against Toxoplasma gondii NTPase which can reduce NTPase activity and prevent parasite growth in vitro, and the results obtained from the studies indicated that the NTPase play an important role for T. gondii infection. To examine the pathogenicity of T. gondii, we also established a new animal model for T. gondii infection using squirrel monkeys and reported the usefulness of this model.

1. Membrane localization and demonstration of isoforms of nucleoside triphosphate hydrolase from *Toxoplasma gondii*

Takane Kikuchi, Takahisa Furuta and Somei Kojima: Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo

Toxoplasma gondii has a unique enzyme, a NTPase, which has a wide specificity toward NTP. In the present study, we produced a monoclonal antibody (IgG1, 6C6) against the enzyme which could recognize NTPase isozymes among several strains of *T. gondii*. Three avirulent strains of *T. gondii*, ME49, Beverley and Nakayama, were found to have one NTPase (63 kDa, pI 6.0), while a virulent strain RH and an avirulent strain Fukaya had two isozymes (63 kDa) with different pIs (pIs 6.0 and 6.5 for the former, and pIs 6.2 and 6.4 for the latter, respectively), suggesting that this monoclonal antibody recognizes a common epitope of NTPase among *T. gondii* strains. Furthermore, 6C6 could inhibit NTPase activity in the presence of dithiothreitol in a dose-dependent manner, and immuno-EM study of NTPase revealed that this molecule is located on the surface membrane of *T. gondii* tachyzoites. When Vero cells were co-cultured with tachyzoites pretreated with 6C6, the number of infected cells significantly decreased, suggesting that 6C6 inhibits invasion of the parasites to host cells. These data suggested the molecule recognized by 6C6 might be considered a potential candidate antigen for vaccines against *T. gondii* tachyzoites.

2. Horizontal transmission of Toxoplasma gondii in squirrel monkeys (Saimiri sciureus)

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The possibility of horizontal transmission of *T. gondii* was examined in squirrel monkeys. After three monkeys were inoculated perorally with 1.1-2.1x10^3 cysts of the *T. gondii* ME49, the animals were divided into two cages and maintained with one normal monkey for each cage as a cagemate. Two out of three *T. gondii*-inoculated monkeys were dead, and remaining one monkey was sacrificed in a moribund state at one week later because of acute toxoplasmosis, and many *T. gondii* tachyzoites were recovered from broncho-alveolar lavages and found histo-

pathologically in the lung, liver, spleen, kidney and lymph nodes and impression smears of tissues in three *T. gondii*-inoculated monkeys by Giemsa staining. Anti-*T. gondii* antibody was examined by immunoblot assay in these animals, and the antibody to T. gondii major surface membrane protein (p30) could be detected after the start of experiment. Furthermore, a specific band of *T. gondii* NTPase gene was observed by PCR in the liver and lung of infected and cagemate monkeys, and the sequence of the second PCR products obtained from the cagemates which were clinically normal but gave a positive result in immunoblotting assay, was exactly the same as the sequence of NTPase gene of *T. gondii* ME49. These findings suggested that transmission of *T. gondii* from the infected monkeys to cagemates was easily occurred, and since many *T. gondii* tachyzoites were recovered from the broncho-alveolar lavages of three *T. gondii*-inoculated monkeys, it suggested the possibility that aerosol infection plays an important role for the enzootic toxoplasmosis in the colony of squirrel monkeys.

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

Takane Kikuchi, Takahisa Furuta and Somei Kojima:Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo

The kinetics of NTPase of *T. gondii* was examined by avidin-biotin sandwich-ELISA (ABS-ELISA) with an anti-NTPase monoclonal antibody, 6C6. In the acute model, serum levels of NTPase by ABS-ELISA and parasitemia by bioassay were examined simultaneously with antibody responses to *T. gondii* after *T. gondii* RH infection. NTPase was detected in mice from day 1, its level continued to increase until the death of mice, and the findings were consistent with the parasitemia examined by bioassay. No anti-*T. gondii* antibody was detected throughout the experiment. In the chronic model, 20 *T. gondii* ME49 cysts were inoculated per os, the amount of NTPase, antibody titres to *T. gondii*, and the number of cysts in the brains were examined after the infection. NTPase increased from day 3 and peaked at day 7 and 14, thereafter they were declined. This early appearance of NTPase was consistent with the findings of parasitemia. On the contrary, antibody to *T. gondii* occurred at day 7 for IgM and day 10 for IgG antibodies, the cyst formation was also observed at day 14. From the above findings, the results of ABS-ELISA and bioassay were correlated in acute and chronic toxoplasmosis, suggesting that appearance of NTPase by ABS-ELISA might suggest the presence of ongoing toxoplasmosis.

4. FR901469, a novel antifungal antibiotic from an unidentified fungus No. 11243. II. *In Vitro* and *In Vivo* activities

Akihiko Fujie[1], Toshiro Iwamoto[1], Hideyuki Muramatsu[1], Terumi Okudaira[1], Ikuko Sato[1], Takahisa Furuta[2], Yasuhisa Tsurumi[1], Yasuhiro Hori1, Motohiro Hino[1], Seiji Hashimoto[1] and Masakuni Okuhara[1]:[1]Experimental Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., and [2]Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo

FR901469 is a water-soluble macrocyclic lipopeptidelactone ($C_{17}H_{116}N_{14}O_{23}$) that has inhibitory activity against 1,3-b-glucan synthase and exhibits *in vitro* and *in vivo* antifungal activity against *Candida albicans* and *Aspergillus fumigatus*. The MICs of FR901469 against *Candida albicans* FP633 and *Aspergillus fumigatus* FP1305 in macro-broth dilution test were 0.63 and 0.16 mg/ml, respectively. FR901469 showed excellent efficacy by subcutaneous injection against both *Candida albicans* and A*spergillus fumigatus* in a murine systemic infection mode, with ED_{50}s of 0.32 and 0.2 mg/Kg, respectively. This compound also showed potent anti-*Pneumocystis* activity in the nude mice model with experimental *Pneumocystis* pneumonia. the hemolytic activity of FR901469 towards mouse red blood cells in about 30-fold weaker than that of amphotericin B.

5. Improvement of growth of *Plasmodium falciparum* fresh clinical isolates by using an established serum-free medium, GIT.

Takahisa Furuta[1], Mikio Kimura[2], Takafumi Tsunoda[3], Takane Kikuchi[1] and Somei Kojima[1]: [1]Division of Infectious Genetics and [2]Department of Infectious Diseases and Applied Immunology, The Institute of Medical Science, The University of Tokyo and [3]Department of Infectious Diseases, Tokyo Metropolitan Ebara General Hospital

In the present study, we have tried to establish continuous cultures of fresh clinical isolates of *P. falciparum* by using a serum-free meduim, GIT. To examine the ability of GIT to support the parasite growth, the growth of various *P. falciparum* isolates including two laboratory strains of *P. falciparum*, FCR3 and k-1 was compared in both of GIT and RPMI 1640 medium supplemented by 10% human serum (RPMI-HS). Growth rates of various *P. falciparum* expressed as fold increases were compared in GIT and RPMI-HS, and the maximum growth rates of *P. falciparum* was 72 in GIT and 35 in RPMI-HS during the culture for 8 days. Growth rate of the clinical isolates varied individually in both culture media, with average growth rates of parasites being 15.9 in GIT and 8.8 in RPMI-HS, respectively (not significant). Growth rates of FCR 3 and K-1 strains were

28.0 and 6.6 in GIT, and 10 and 7.5 in RPMI-HS. After 30 days culture of *P. falciparum* in GIT, 9 of 12 clinical isolates still continuously propagated but other three isolates disappeared. Despite variation of the *P. falciparum* isolates in their abilities to multiply in GIT, our experiments suggested that GIT is useful for culturing of fresh clinical isolates of *P. falciparum* that are derived from geographically distinct areas as well as laboratory strains used commonly in laboratory research.

6. *Pneumocystis* pneumonia in cavalier king charles spaniel

Yoshimi Hagiwara[1], Shunsuke Fujiwara[1], Hirotake Takaki[1], Koichi Ohno[1], Kenichi Masuda[1], Takahisa Furuta[2], Hiroyuki Nakayama[3], Kunio Doi[3], and Hajime Tsujimoto[1]:[1]Department of Veterinary Medicine, Graduate School of Agricultural and Life Science, [2]Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo and [3]Department of Veterinary Medicine, Graduate School of Agricultural and Life Science

Pneumocystis pneumonia was diagnosed by postmortem examination of a 1 year old Cavalier king Charles Spaniel with four weeks history of dyspnea. Cytologic and histologic evaluation from lung tissues revealed numerous *P. carinii* trophozoites and cysts, and *P. carinii* specific DNA was detected by polymerase chain reaction. The dog showed by hypogammaglobulinemia and extremely low levels of serum IgG. It was considered that *P. carinii* pneumonia in this case was associated with an immunodeficient condition which has been reported in miniature dachshunds.

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The mucosal surface provides a first line of defence for the host. To this end, a large number of infectious agents, allergens and foreign proteins enter the inside of our bodies via the oral region, nasal and upper respiratory tracts, and intestinal and reproductive tracts. The total area of these mucosal surfaces, which cover the tube-like tissues of the host, is at least two hundred times larger than that of skin. In order to provide an optimal first line of defense for these large surface areas, the mucosal immune system including secretory immunoglobulin A (S-IgA), mucosal αβ and γδ Tcells, antigen presenting cells, B cells and epithelial cells forms mucosal inter- and intranets and play an essential role for the induction and regulation of an appropriate immunity. 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 vaccine and mucosal immunotherapy.

1. Mucosal intranet: epithelial cell and intraepithelial lymphocyte interactions

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 investigatio 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 involve in the generation of mucosal barrier.

2. Mucosal vaccine

For the prevention of mucosal infections including HIV, influenza, *E.coli*, O157 and *Salmonella*, it is essential to build effective immunity in the mucosal 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 immunity 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.

3. Inflammatory bowel disease (IBD)

Recent adaptation of gene manipulation technology 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 ($CD4^+$, $\beta\beta^+$ 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 $\beta\beta^+$ T cells revealed monoclonal to oligoclonal accumulation of the cells in the colon but not small intestine, suggesting clonal expansion of the mucosal $\beta\beta^+$ T cells upon the stimulation with gut-derived antigens. The homodimer of TCR β-chains on the $\beta\beta^+$ 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 $\beta\beta^+$ 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

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 GALT- and 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) of the CMIS. 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. Mucosally-induced allergic diarrhea

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 $CD4^+$ αβT lymphocytes produced no detectable levels of antigen-induced cytokines. As would be expected given 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 signal transduction is involved in these Th2-derived intestinal allergic disorders upon repeated administration of oral antigen.

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