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 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. *toxB* Gene on pO157 of Enterohemorrhagic *Escherichia coli* O157:H7 Is Required for Full Epithelial Cell Adherence Phenotype

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Adherence of enterohemorrhagic *Escherichia coli* (EHEC) to the intestinal epithelium is critical for initiation of a bacterial infection. An *in vitro* infection study previously indicated that EHEC bacteria initially adhere diffusely and then proliferate to develop MC, a process that is mediated by various secreted proteins, such as EspA, EspB, EspD, Tir, and intimin, as well as other putative adherence factors. In the present study, we investigated the role of a large 93-kb plasmid (pO157) in the adherence of O157:H7 (O157Sakai) and found the toxB gene to be involved in the full adherence phenotype. A pO157-cured strain of O157Sakai (O157Cu) developed microcolonies on Caco-2 cells; however, the number of microcolonies was lower than that of O157Sakai, as were the production and secretion levels of EspA, EspB, and Tir. Introduction of a mini-pO157 plasmid (pIC37) composed of the toxB and ori regions restored full adherence capacity to O157Cu, including production and secretion of the proteins. In contrast, introduction of a pO157 mutant possessing toxB::Km into O157Cu could not restore the full adherence phenotype. Expression of truncated versions of His-tagged ToxB also promoted EspB production and/or secretion by O157Cu. These results suggest that ToxB contributes to the adherence of EHEC to epithelial cells through promotion of the production and/or secretion of type III secreted proteins.

2. *Shigella* invasion of macrophage requires the insertion of IpaC into the host plasma membrane. Functional analysis of IpaC

Asaomi Kuwae, Sei Yoshida, Koichi Tamano, Hitomi Mimuro, Toshihiko Suzuki, and Chihiro Sasakawa

Shigella infects residential macrophages via the M cell entry, after which the pathogen induces macrophage cell death. The bacterial strategy of macrophage infection, however, remains largely speculative. Wild type *Shigella flexneri* (YSH6000) invaded macrophages more efficiently than the noninvasive mutants, where YSH6000 induced large scale lamellipodial extension including ruffle formation around the bacteria. When macrophages were infected with the noninvasive *ipaC* mutant, the invasiveness and induction of membrane extension were dramatically reduced as compared with that of

YSH6000. J774 macrophages infected with YSH6000 showed tyrosine phosphorylation of several proteins including paxillin and c-Cbl, and this pattern was distinctive from those stimulated by Salmonella typhimurium or phorbolester. Upon addition of IpaC into the external medium of macrophages, membrane extensions were rapidly induced, and this promoted uptake of *Escherichia coli*. The exogenously added IpaC was found to be integrated into the host cell membrane as detected by immunostaining. The IpaC domain required for the induction of membrane extension from J774 was narrowed down within the region of residues 117-169, which contains a putative membrane-spanning sequence. Our data indicate that *Shigella* directs its own entry into macrophages, and the IpaC domain which is required for the association with its host membrane is crucial.

3. *Shigella* protein IpaH(9.8) is secreted from bacteria within mammalian cells and transported to the nucleus

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Various pathogenic bacteria such as Shigella deliver effector proteins into mammalian cells via the type III secretion system. The delivered *Shigella* effectors have been shown to variously affect host functions required for efficient bacterial internalization into the cells. In the present study, we investigated the IpaH proteins for their ability to be secreted via the type III secretion system and their fate in mammalian cells. Upon incubation in a medium containing Congo red, the bacteria secrete IpaH into the medium, but secretion of IpaH occurs later than that of IpaBCD. Immunofluorescence microscopy indicated that IpaH(9.8) is secreted from intracellular bacteria and transported into the nucleus. On microinjection of the protein, intracellular IpaH(9.8) is accumulated at one place around the nucleus and transported into the nucleus. This movement seems to be dependent on the microtubule network, since nuclear accumulation of IpaH(9.8) is inhibited in cells treated with microtubule-destabilizing agents. In nuclear import assay, IpaH(9.8) was efficiently transported into the nucleus, which was completely blocked by treatment with wheat germ agglutinin. The nuclear transport of IpaH(9.8) does not depend on host cytosolic factors but is partially dependent on ATP/GTP, suggesting that, like beta-catenin, IpaH(9.8) secreted from intracellular *Shigella* can be transported into the nucleus.

4. Role of bundle-forming pilus of enteropathogenic *Escherichia coli* in host cell adherence and in microcolony development

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Enteropathogenic *Escherichia coli* (EPEC) adheres to epithelial cells and forms microcolonies in localized areas. Bundle-forming pili (BFP) are necessary for autoaggregation and the formation of microcolonies. In this study, we show that BFP, expressed by EPEC on epithelialcells, disappeared with the expansion of the microcolony. Bacterial dispersal and the release of BFP from the EPEC aggregates were induced by contact with host cellular membrane extract. In addition, BFP-expressing EPEC adhered directly to cell surfaces, in preference to attaching to pre-formed microcolonies on the cells. These results suggested that BFP mediate the initial attachment of EPEC through direct interaction with the host cell rather than through the recruitment of unattached bacteria to microcolonies on the cell.

5. *Shigella* Spa32 is an essential secretory protein for functional type III secretion machinery and uniformity of its needle length

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The *Shigella* type III secretion machinery is responsible for delivering to host cells the set of effectors required for invasion. The type III secretion complex comprises a needle composed of MxiH and MxiI, and a basal body made up of MxiD, MxiG, and MxiJ. In *Shigella*, the needle length has a narrow range, with a mean of approximately 45 nm, suggesting that it is strictly regulated. Here we show that Spa32 encoded by one of the *spa* genes is an essential protein translocated via the type III secretion system, and is involved in the control of needle length as well as the type III secretion activity. When the *spa32* gene was mutated, the type III secretion complexes possessed needles of various lengths, ranging from 40 to1150 nm. Upon introduction of a cloned *spa32* into the *spa32* mutant, the bacteria produced needles of wild-type length. The *spa32* mutant overexpressing MxiH produced extremely long (>5 μm) needles. *Spa32* was secreted into the medium via the type III secretion system, but the secretion did not depend on activation of the system. The *spa32* mutant and the mutant overexpressing MxiH did not secrete effectors such

as Ipa proteins into the medium or invade HeLa cells. Upon introduction of *Salmonella invJ* encoding InvJ, which shares 15.4% amino acid identity with Spa32, into the *spa32* mutant, the bacteria produced type III needles of wild-type length and efficiently entered HeLa cells. These findings suggest that Spa32 is an essential secreted protein for functional type III secretion system of *Shigella* and is involved in the control of the needle length. Furthermore, its function is interchangeable with that of *Salmonella* InvJ.

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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. IL-5 induces IgG1 isotype switch recombination in CD38-stimulated murine B cells

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Interleukin-5 (IL-5) stimulation of CD38-activated murine splenic B cells induces μ-γ1 class switch recombination (CSR) at the DNA level leading to a high level of lgG1 production. Further addition of IL-4 in the system enhances IL-5-dependent μ-γ1 CSR. Although IL-5 has been shown to induce Stat5 activation in mouse B cell lines, role of Stat5 activation in IL-5-induced Ig class switch recombination in primary B cells is still remained unclear. Thus, we stimulated Stat5a- and Stat5b-deficient B cells with CD38 stimulation and IL-5 and examined proliferative response, the expression of γ1 germline transcripts, μ-γ1 CSR, and expression of the genes induced by IL-5. Compared with wild type B cells, Stat5a- and Stat5b-deficient B cells showed lower proliferative response and produced no IgM and IgG1. Moreover, these mutant B cells could not produce almost no circular DNA, reciprocal products of μ-γ1 CSR, despite of the intact induction of γ1 germline transcripts.

There are bodies of evidence that B cell differentiation and isotype switching are related to division cycle number. We examined division cycle number of STAT5 deficient B cells using 5-(and -6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE). In wild type B cells, surface IgG1 positive cells appeared after five division cycles in CD38 and IL-5 stimulation. Stat5-deficient B cells were almost normally divided to 5 or 6 times, but these B cells could not express surface IgG1. These results suggested that the defective IgM and IgG1 production in Stat5-deficient B cells is not due to their impaired proliferation and implied that Stat5 plays pivotal roles on induction of switch recombination machinery. We examined Stat5 target genes involved in B cell differentiation and switch recombination. RT-PCR analysis revealed equivalent levels of *AID* in wild type and Stat5b-deficient B cells, indicating that poor or absent *AID* expression is not responsible for the lack of switching to IgG1 and production of IgM and IgG1 in CD38 and IL-5 stimulated Stat5-deficient B cells. Interestingly, expression of the *Blimp-1* gene was impaired in Stat5b deficient B cells. We infer from these results that Stat5a and Stat5b are essential for IL-5-dependent μ-γ1 CSR and Ig secretion, however, their major target may not be AID. We are currently analyzing genes affected in Stat5b-deficient B cells upon CD38 and IL-5 stimulation.

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

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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. We have also reported that IL-5 stimulation of CD38-stimulated B cells promotes both μ-γ1 switch recombination and production of IgM and IgG1 in an IL-4 independent manner.

We examined NF-κB activation in CD38-stimulated mouse B cells. Electrophoretic mobility shift assay (EMSA) revealed that CD38 stimulation activated NF-κB complexes that were consisted of p50, p65, and c-Rel. To elucidate functions of c-Rel and p50 in CD38 stimulation, we examined proliferation and germline γ1 expression in c-Rel and p50 deficient B cells. Compared to control B cells, these mutant B cells showed impaired proliferation and the germline γ1 induction expression upon CD38 stimulation. These results suggest that c-Rel and p50 play pivotal roles in the B cell activation in response to CD38 ligation.

Next, we examined signaling molecules involved in NF-κB activation by CD38 stimulation. We reported that CD38 ligation induces the tyrosine phosphorylation of Btk. Btk deficient B cells showed the impaired proliferation to CD38 stimulation. The results by EMSA revealed that both CD38 and BCR stimulation could not induce NF-κB activation in Btk deficient B cells. These data suggest that Btk plays a pivotal role for NF-κB activation by CD38 and BCR, but CD38-induced signaling pathway to Btk activation is distinct from BCR-induced pathway.

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.

2. Structural and functional analysis of IL-5 receptor

a. IL-5/IL-5R system for maintaining B-1 cells in peritoneal cavity

Byoung-Gon Moon, Satoshi Takaki, and Kiyoshi Takatsu

IL-5 receptor (IL-5R) consists of two membrane proteins, IL-5Rα and βc. The IL-5Rα specifically binds IL-5 and the βc. We have demonstrated that the membrane-proximal proline-rich region (ppvp motif) is critical to transduce signal for both cell growth and differentiation. We defined functions of the ppvp motif and DC3 region, by generating mice each expressing a mutant form of an *IL-5Rα* transgene, alanine substitution to proline and lacking the DC3 region, on an IL-5Rα null background. We compared those with mice expressing the wild-type (WT) IL-5Rα transgene. Our results clearly indicate that the ppvp motif of IL-5Rα plays a role critical for IL-5-induced proliferation and differentiation of B cells *in vivo*. In contrast, the IL-5Rα C-terminal region, in particular the DC3 subregion, is important for both IL-5-induced IgM production and μ to γ1 switch recombination in B-2 cells, but is dispensable for

IL-5-induced B cell proliferation.

B-1 cells in peritoneal cavity constitutively express IL-5Rα. We previously reported that the number of B-1 cells was decreased in peritoneal cavity of *IL-5Rα*$^{-/-}$ mice. Furthermore, the size of B-1 cells was also reduced in *IL-5Rα*$^{-/-}$. In this study, we investigated critical cytoplasmic regions of IL-5Rα for development or maintenance of B-1 cells and the source of IL-5 in peritoneal cavity. To distinguish whether the abnormality of B-1 cells in *IL-5Rα*$^{-/-}$ is due to impaired development or maintenance of mature cells, anti-IL-5 was injected into WT mice. The number and size of B-1 cells were decreased within 6 days after anti-IL-5 injection. This indicates that the IL-5/IL-5R system is required to maintain the number and size of mature B-1 cells. To define critical cytoplasmic regions of IL-5Rα for maintaining B-1 cells, WT IL-5Rα expression was enforced as a transgene to rescue B-1 cells in peritoneal cavity of *IL-5Rα*$^{-/-}$. However, the abnormality was not recovered by IL-5Rα carrying mutation in the proline rich region, and was partially recovered by IL-5Rα lacking the C-terminal region. Then, B-1 cells from *nu/nu*, *TCRδ*$^{-/-}$, *TCRδ*$^{-/-}$*β*$^{-/-}$ or *c-KitW/W*^{v} were analyzed to identify IL-5 producing cells. Sizes of B-1 cells from those mice were normal in range, and anti-IL-5 injection resulted in reduction of B-1 cell in size. It is likely that αβ T cells, γδ T cells, mast cells or other unidentified cells simultaneously provide IL-5 to B-1 cells. In addition, the adoptive transfer of *IL-5Rα*$^{-/-}$ B-1 cells into the WT mice or *RAG*$^{-/-}$ mice demonstrated the importance of IL-5/IL-5R system for maintaining survival and homeostatic proliferation of B-1 cells in peritoneal cavity.

b. Transcriptional regulation of the murine IL-5Rα gene by E12/E47, C/EBPβ, Sp1 and Oct-2

Yuki Ashizawa, Satoshi Takaki, and Kiyoshi Takatsu

The IL-5 receptor (IL-5R) consists of two distinct membrane proteins, IL-5Rα and βc. While the βc is shared by IL-3R and GM-CSFR, IL-5Rα is specific for IL-5 and its expression is restricted in B cells, eosinophils, and basophils, it seems likely that IL-5 specific activities are largely due to the restricted expression of IL-5Rα on target cells.

To understand transcriptional regulation of the murine IL-5R gene, we characterized its promoter regions. We identified the second transcriptional start site at the position +1002 with respect to the previously identified start site (+1) using 5'RACE. Reporter constructs carrying various fragments from the promoter regions were generated and transfected into IL-5Rα-positive (WEHI231, BCL_1) or IL-5Rα-negative (EL-4, FDC-P1) cell lines. The -250 ~ +160 region showed promoter activity only in IL-5Rα-negative cell lines. Nucleoproteins that bind to the -250 ~ -115 region were detected in IL-5Rα-negative cell lines and splenic B cells activated by CD38 cross-linking, and they reacted with antibodies against E12/E47, C/EBPβ, Sp1 or Oct-2. The promoter activity was greatly impaired by the mutations of binding motifs for E12/E47, C/EBPβ, Sp1 or Oct-2 in the -250 ~ -115 region. Our data indicate that E12/E47, C/EBPβ, Sp1 and Oct-2 participate in coordinate transactivation of IL-5Rα gene in 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, Hatsue Morita, Yoshinari Tezuka, and Kiyoshi Takatsu

Lnk is a 68-kDa-adaptor protein containing a conserved proline-rich region, SH2 and PH domains, and a possible tyrosine phosphorylation site. Lnk is a representative of a multigene adaptor protein family conserved from drosophila to mammalian in which APS and SH2-B are included. In *lnk*$^{-/-}$ mice, pre-B and immature B cells accumulated in the spleens, whereas T cell development was unaffected. In the bone marrow, B-lineage cells were proportionately increased, reflecting enhanced production of pro-B cells that 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.

Hypersensitivity of *lnk*$^{-/-}$ B cell precursors to SCF prompted us to study functions of hematopoietic stem cell (HSC) in *lnk*$^{-/-}$ mice. Hematopoietic stem cells (HSCs) give rise to variety of hematopoietic cells via pluripotential progenitors and lineage-committed progenitors, and are responsible for blood production throughout adult life. Amplification of HSCs or progenitors represents a potentially powerful approach to the treatment of various blood disorders and to applying gene therapy by bone marrow transplantation. We revealed that Lnk is also expressed in hematopoietic progenitors in bone marrow, and that in the absence of Lnk, the number and the hematopoietic ability of progenitors are significantly increased. Augmented growth signals through c-Kit partly contributed to the enhanced hematopoiesis by *lnk*$^{-/-}$ cells. Lnk was phosphorylated by and associated with c-Kit, and selectively inhibited c-Kit-mediated proliferation by attenuating phosphorylation of Gab2 and activation of MAPK cascade. These observations indicate that Lnk plays critical roles in the expansion and function of early hematopoietic progenitors, and provide useful clues for the amplification of hematopoietic progenitor cells.

b. Molecular cloning and characterization of the mouse APS, a member of the Lnk adaptor protein family

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Lnk adaptor protein family is a recently identified family whose members contain a proline-rich region, PH and SH2 domains, and a putative tyrosine phosphorylation site at the C-terminal. Lnk, one of the family members, controls production of B-lineage cells by negatively regulating c-Kit signals. SH2-B, another member of the family, becomes phosphorylated upon stimulation with various growth factors including insulin, IGF, growth hormone and NGF. However, its physiological roles are not elucidated yet.

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. 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. To explore the functions of APS *in vivo*, we generated *APS*$^{-/-}$ mice. In *APS*$^{-/-}$ mice, development of conventional B-2 cells was normal. Splenic B cells responded normally upon stimulation with various mitogenic stimuli. However, B-1 cell number in peritoneal cavity was significantly increased in *APS*$^{-/-}$ mice. In addition, antibody production against thymus-independent antigen type-2 (TI-2) antigen, TNP-Ficoll was augmented. These results indicate the regulatory function of APS in maintenance or production of B-1 cells and in controlling the function of B-1 cells.

c. Growth retardation and impaired fertility in mice lacking SH2-B

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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 function in signal transduction pathways of those growth factors and its physiological roles are poorly understood.

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*. *SH2-B-/-* mice did not show any gross anomalies. However, *SH2*$^{-/-}$ mice were smaller in size than +/+ or +/- littermates in their younger age. Lymphocyte development and activation were not perturbed in *SH2-B*$^{-/-}$ mice. Both male and female *SH2-B*$^{-/-}$ mice showed slight growth retardation and impaired fertility. Female knockout mice possessed small, anovulatory ovaries with reduced numbers of follicles. Male *SH2-B*$^{-/-}$ mice had small testes with a reduced number of sperm. *SH2-B*$^{-/-}$ cumulus cells did not respond to both follicle-stimulating hormone (FSH) and insulin-like growth factor (IGF)-I. The data suggest that SH2-B plays a critical role in the IGF-I-mediated reproductive pathway in mice.

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. Formation of the pre-B cell receptor (pre-BCR) complex, 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 cross-linking induces differentiation to the pre-B phenotype the CD79b expressed on developmentally arrested pro-B cells in RAG2-deficient mice.

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 cross-linking 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. We are currently trying to find target genes controlled by Btk dependent pathway in proB/preB transition using this unique induced differentiation system mimicking early B-cell development.

b. Mtβ, a transcriptional regulator for the *lck* proximal promoter

Satoshi Takaki, Atsuko Yamada, and Kiyoshi Takatsu

The *lck* gene encodes a protein tyrosine kinase that plays a key role in signal transduction mediated through T cell receptor (TCR) and pre-TCR complex. Transcription of the *lck* gene is regulated by two independent promoter elements; the proximal and distal promoters. Previous studies employing transgenic mice demonstrated that the sequence between -584 and -240 from the transcription start site in the mouse *lck* proximal promoter is required for its tissue-specific expression in the thymus. In this study, we demonstrate that a Krüppel-like zinc finger protein, mtβ (BFCOL1, BERF-1, ZBP-89, ZNF148), previously cloned as a protein that binds to the CD3δ gene enhancer, binds to the -365 to -328 region of the *lck* proximal promoter. Mtβ is ubiquitously expressed in various cell lines and mouse tissues. Overexpressed mtβ is more active in T-lineage cells than B-lineage cells for transactivating an artificial promoter consisting of the mtβ binding site 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 antisense mRNA. Our results indicate that mtβ activity is regulated in a tissue-specific manner, and that mtβ is a critical transactivator for the *lck* proximal promoter.

c. 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), 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 be attractive model to elucidate a novel mechanism to transmit signals in B lymphocytes.

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

a. Amino acid residues of T cell epitope of the α antigen of Mycobacteria

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 α antigen-specific Vβ11$^+$ T cells as the 15-mer peptide, covering amino acid residues 240-254 of α antigen that contains the I-A^b binding motif. We then determined amino acid residues of Peptide-25 critical for TCR recognition by using Peptide-25-reactive Vβ11$^+$ Th1 clones and substituted mutants of Peptide-25. Results revealed 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 I-A^b contact residues.

Peptide-25 was found to be immunogenic and to induce production of IFN-γ and TNF-α and development of V_β11$^+$CD4$^+$ T cells. We also observed that active immunization of C57BL/6 mice with Peptide-25 can lead to decreased bacterial load in the lungs of

M. tuberculosis H37Rv-infected mice. These results should provide us with a useful tool for delineating the regulation of $V_\beta11^+$ Th1-cell development during *M. tuberculosis* infection and for developing a vaccine inducing a Th1-dominant immune response. 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 mice. Peptide-25 could also induce the activation of $V\beta11^+$ $CD8^+$ T cells that may also contribute to immune protection against *M. tuberculosis* infection. Roles of $V\beta11^+CD8^+$ T cells in the protection against *M. tuberculosis* infection are currently investigated.

b. Role of antigen-TCR interaction in the Th 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 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 α antigen or to Peptide-25 expressed highly restricted TCR repertoire, Vβ11. We isolated TCR Vα5 gene expressed in peptide-25 reactive Th1 clones and confirmed to reconstruct functional TCR reactive to peptide-25/I-A^b complex leading to produce Th1 cytokine by transfection into TCR deficient T cell hybridoma, TG40 with Vα5 and Vβ11 gene. These results led us to examine whether the antigen itself is able to determine the Th subset development. For this purpose, we generated transgenic mice for TCR that reacts to Peptide-25. We are currently analyzing differentiation mechanisms of naive $CD4^+$ T cells into Th1 cells by using these TCR transgenic mice.

c. Adjuvanticity of Peptide-25 in anti-tumor immunity

Shu-I-chiro Uehara, Toshiki Tamura, 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. On the other hand, it is unclear whether $CD4^+$ helper T cells actually 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.

Based on this retionale, we examined the adjuvanticity of Peptide-25 in anti-tumor immune responses. It was found that co-immunization of C57BL/6 mice with OVA and Peptide-25 *in vivo* can induce more potent and reproducible OVA-specific CTL responses than immunization with OVA. Co-immunization of OVA and Peptide-25 was also shown to prevent *in vivo* growth of E.G7-OVA cell line (EL4 thymoma transfected with cDNA encoding chicken ovalbumin). These results suggest that Peptide-25 provide efficient help for CTL induction against tumor. We are currently analyzing enhanced mechanisms of anti-tumor immune responses induced by Peptide-25.

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Department of Microbiology and Immunology

Division of Immunology (2)

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

Immunomolecular mechanins in mammalian 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, Walther Flemming reported the detailed observation on cellular degeneration of the rabbit ovary in the earliest description of programmed cell death (Archiv fur anatomie und Entwickelungsgeschichite, 221-244, 1885). On the otherhand, we have recently demonstrated that the definite expression of Fas was found in 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. Furthermore, the direct interaction between zona pellucida (ZP) free oocytes/eggs as the target cells 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/Ipr 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/Ipr 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/Ipr murine oocytes stimulated with anti-Fas mAb but not in MRL/+ oocytes. Therefore, it was evidenced that ovarian adenopathy in old MRL/Ipr 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 primary 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 by RT/PCR-Southern blot hybridization, further 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 45kDa 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 ZP to adhere to the egg plasma membrane with its postacrosomal membrane followed 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 rersidue and/or Le^X residue on egg ZP. Furthermore, we are now cloning the gene of β-Galactose binding protein from porcine testis. 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/esophageal/prostatic/chorionic carcinoma(GCIY/T.Tn/PC-3/BeWo) cells but not in 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/T.Tn/PC-3/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 Host-Parasite Interaction

Cellular mechanisms for the surveillance and exclusion of expression by DNA parasites such as provirus 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 and cellular gene expression (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 regenerative medicine.

1. Epigenetical regulation and SWI/SNF chromatin remodeling complex

Taiji Ito, Mai Yamauchi, Mitsue Nishina, Nobutake Yamamichi, Taketoshi Mizutani, Motoyasu Ui, Masao Murakami, and Hideo Iba

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. This year, we clearly showed mechanistic links between transcriptional factor AP-1 and chromatin remodeling factor SWI/SNF complex and their importance in transcriptional activation of AP-1 (a). We next showed a clear example that this chromatin remodeling complex is involved in the maintenance of retroviral gene expression (b).

a. Discovery of mechanistic link between AP-1 and chromatin remodeling -Identification of SWI/SNF complex subunit BAF60a as a determinant of transactivation potential of Fos/Jun dimers-

The transcriptional factor, AP-1 (Activator Protein-1) complex comprises members of the Fos (c-Fos, Fra-1, Fra-2 and FosB) and Jun (c-Jun, JunB and JunD) protein families. Jun family members can form low-affinity dimers among themselves and high-affinity heterodimers with the Fos family components, whereas Fos-related proteins do not form stable dimers among themselves. Although these hetero- and homodimers bind to similar DNA binding sites (TGAC/GTCA, AP-1 binding sites), we have previously shown that each kind of heterodimer has distinct the transcriptional activity. For example, transcriptional activity of c-Fos/c-Jun complex is much higher than Fra-2/c-Jun complex. While functional interactions between some members of Fos/Jun family proteins and adaptor proteins such as CBP or TBP were reported crucial proteins that recognize the dimer specificity and/or facilitate the induction of transcriptional initiation were largely unknown. Here we show BAF60a, a subunit of SWI/SNF chromatin remodeling complex, is a determinant of transactivation potential of Fos/Jun dimers. BAF60a was initially shown to specifically

bind to c-Jun among Jun family proteins. Interestingly it was turn out to also bind specifically to c-Fos among Fos family proteins. Fur thermore,BAF60a binds to a specific subset of Fos/Jun heterodimers using the two different interfaces for c-Fos and c-Jun, respectively. Only when functional SWI/SNF complex is present, c-Fos/c-Jun (high affinity to BAF60a) but not Fra-2/JunD (marginal affinity to BAF60a) can induce the endogenous AP-1-regulated genes such as collagenase and *c-met*. These results indicate that a specific subset of Fos/Jun dimers recruits SWI/SNF complex via BAF60a to initiate transcription.

b. 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

Silencing of transgenes, transposons and retroviruses is often considered as a host defense system in cellular nuclei. A DNA methylation-dependent mechanism is known to be involved in this process, but additional mechanisms remain largely unknown. This year, we show 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 level, 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 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.

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

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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 epitherium 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). In this primary organ cultures, the epithelium and mesenchyme are mutually dependent upon each other for growth. This system therefore offers an environment closed to *in vivo*. We report here RSV infection into the epithelium causes budding and migration of epithelial cells into mesenchyme through activation of MAP kinase. We identified down-regulation of the *Shh* gene as an earliest gene response to RSV infection. These epithelial cells subsequently eliminated *E-cadherin* expression during migration. Other v-*src*-expressing cells that were retained in the epithelium underwent apoptosis (anoikis) through MAP kinase-independent pathway and detached from the culture. These RSV infected-cells further acquired the ability to express type I collagen and fibronectin (mesenchymal markers) to establish the epithelial-mesenchymal transition. These observations would explain at least in part why RSV does not apparently form carcinomas. Transient expression of v-*src* into epithelial cells by electroporation further indicated that the ontogeny of v-*src* introduced epithelial cells was irreversibly abrogated, confirming the importance of epigenetical regulation in viral pathology.

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

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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 the *patched* gene. The *patched* gene encodes the transmembrane protein Patched (Ptc), which acts as a receptor for Sonic hedgehog (Shh), a secreted molecule essential for formation of a wide variety of organs during embryonic development. In the absence of Shh, Ptc forms an inachive complex with Smoothened (Smo), another transmembrane protein. When Shh binds to Ptc, the complex changes conformation, and Smo become activated to transduce the signal to the nucleus.

Germline mutations in the human homologue of *patched* cause Gorlin syndrome and somatic mutations in the *patched* gene have been identified in sporadic basal cell carcinomas at a frequency of 20-30%. It was shown recently that several established cell lines derived from human SCCs possessed *patched* mutations. Such cell lines include A431, which was derived from vulva and was shown to have a point mutation in both alleles of the *patched* gene.

To evaluate the biological significance of *patched* mutations in human sporadic tumor cells more clearly, we here 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. We demonstrate that the anchorage independent growth of several human SCC cell lines with *patched* mutations was suppressed efficiently by Ptc virus suggesting the potential therapeutic importance of this viral vector. 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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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. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses

Hatta M, Gao P, Halfmann P, Kawaoka Y

In 1997, an H5N1 influenza A virus was transmitted from birds to humans in Hong Kong, killing 6 of the 18 people infected. When mice were infected with the human isolates, two virulence groups became apparent. Using reverse genetics, we showed that a mutation at position 627 in the PB2 protein influenced the outcome of infection in mice. Moreover, high cleavability of the hemagglutinin glycoprotein was an essential requirement for lethal infection.

2. Adaptation of influenza A viruses to cells expressing low levels of sialic acid leads to loss of neuraminidase activity

Hughes MT, McGregor M, Suzuki T, Suzuki Y, Kawaoka Y

Influenza A viruses possess two virion surface proteins, hemagglutinin (HA) and neuraminidase (NA). The HA binds to sialyloligosaccharide viral receptors, while the NA removes sialic acids from the host cell and viral sialyloligosaccarides. Alterations of the HA occur during adaptation of influenza viruses to new host species, as in the 1957 and 1968 influenza pandemics. To gain a better understanding of the contributions of the HA and possibly the NA to this process, we generated cell lines expressing reduced levels of the influenza virus receptor determinant, sialic acid, by selecting Madin-Darby canine kidney cells resistant to a lectin specific for sialic acid linked to galactose by alpha(2-3) or alpha(2-6) linkages. One of these cell lines had less than 1/10 as much N-acetylneuraminic acid as its parent cell line. When serially passaged in this cell line, human H3N2 viruses lost sialidase activity due to a large internal deletion in the NA gene, without alteration of the HA gene. These findings indicate that NA mutations can contribute to the adaptation of influenza A virus to new host environments and hence may play a role in the transmission of virus across species.

3. Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity

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

Ion channel proteins are common constituents of cells and have even been identified in some viruses. For example, the M2 protein of influenza A virus has proton ion channel activity that is thought to play an important role in viral replication. Because direct support for this function is lacking, we attempted to generate viruses with defective M2 ion channel activity. Unexpectedly, mutants with apparent loss of M2 ion channel activity by an *in vitro* assay replicated as efficiently as the wild-type virus in cell culture. We also generated a chimeric mutant containing an M2 protein whose transmembrane domain was replaced with that from the hemagglutinin glycoprotein. This virus replicated reasonably well in cell culture but showed no growth in mice. Finally, a mutant lacking both the transmembrane and cytoplasmic domains of M2 protein grew poorly in cell culture and showed

no growth in mice. Thus, influenza A virus can undergo multiple cycles of replication without the M2 transmembrane domain responsible for ion channel activity, although this activity promotes efficient viral replication.

4. Plasminogen-binding activity of neuraminidase determines the pathogenicity of influenza A virus

Goto H, Wells K, Takada A, Kawaoka Y

When expressed *in vitro*, the neuraminidase (NA) of A/WSN/33 (WSN) virus binds and sequesters plasminogen on the cell surface, leading to enhanced cleavage of the viral hemagglutinin. To obtain direct evidence that the plasminogen-binding activity of the NA enhances the pathogenicity of WSN virus, we generated mutant viruses whose NAs lacked plasminogen-binding activity because of a mutation at the C terminus, from Lys to Arg or Leu. In the presence of trypsin, these mutant viruses replicated similarly to wild-type virus in cell culture. By contrast, in the presence of plasminogen, the mutant viruses failed to undergo multiple cycles of replication while the wild-type virus grew normally. The mutant viruses showed attenuated growth in mice and failed to grow at all in the brain. Furthermore, another mutant WSN virus, possessing an NA with a glycosylation site at position 130 (146 in N2 numbering), leading to the loss of neurovirulence, failed to grow in cell culture in the presence of plasminogen. We conclude that the plasminogen-binding activity of the WSN NA determines its pathogenicity in mice.

5. Amino acids responsible for the absolute sialidase activity of the influenza a virus neuraminidase: relationship to growth in the duck intestine

Kobasa D, Wells K, Kawaoka Y

The 1957 human pandemic strain of influenza A virus contained an avian virus hemagglutinin (HA) and neuraminidase (NA), both of which acquired specificity for the human receptor, N-acetylneuraminic acid linked to galactose of cellular glycoconjugates via an alpha2-6 bond (NeuAcalpha2-6Gal). Although the NA retained considerable specificity for NeuAcalpha2-3Gal, its original substrate in ducks, it lost the ability to support viral growth in the duck intestine, suggesting a growth-restrictive change other than a shift in substrate specificity. To test this possibility, we generated a panel of reassortant viruses that expressed the NA genes of human H2N2 viruses isolated from 1957 to 1968 with all other genes from the avian virus A/duck/Hong Kong/278/78 (H9N2). Only the NA of A/Singapore/1/57 supported efficient viral growth in the intestines of orally inoculated ducks. The growth-supporting capacity of the NA correlated with a high level of enzymatic activity, comparable to that found to be associated with avian virus NAs. The specific activities of the A/Ann Arbor/6/60 and A/England/12/62 NAs, which showed greatly restricted abilities to support viral growth in ducks, were only 8 and 5%, respectively, of the NA specific activity for A/Singapore/1/57. Using chimeric constructs based on A/Singapore/1/57 and A/England/12/62 NAs, we localized the determinants of high specific NA activity to a region containing six amino acid substitutions in A/England/12/62: Ser331 → Arg, Asp339 → Asn, Asn367 → Ser, Ser370 → Leu, Asn400 → Ser, and Pro431 → Glu. Five of these six residues (excluding Asn400) were required and sufficient for the full specific activity of the A/Singapore/1/57 NA. Thus, in addition to a change in substrate specificity, a reduction in high specific activity may be required for the adaptation of avian virus NAs to growth in humans. This change is likely needed to maintain an optimal balance between NA activity and the lower affinity shown by human virus HAs for their cellular receptor.

6. Ebola virus glycoprotein: proteolytic processing, acylation, cell tropism, and detection of neutralizing antibodies

Ito H, Watanabe S, Takada A, Kawaoka Y

Using the vesicular stomatitis virus (VSV) pseudotype system, we studied the functional properties of the Ebola virus glycoprotein (GP). Amino acid substitutions at the GP cleavage site, which reduce glycoprotein cleavability and viral infectivity in some viruses, did not appreciably change the infectivity of VSV pseudotyped with GP. Likewise, removal of two acylated cysteine residues in the transmembrane region of GP showed no discernible effects on infectivity. Although most filoviruses are believed to target endothelial cells and hepatocytes preferentially, the GP-carrying VSV showed greater affinity for epithelial cells than for either of these cell types, indicating that Ebola virus GP does not necessarily have strong tropism toward endothelial cells and hepatocytes. Finally, when it was used to screen for neutralizing antibodies against Ebola virus GP, the VSV pseudotype system allowed us to detect strain-specific neutralizing activity that was inhibited by secretory GP (SGP). This finding provides evidence of shared neutralizing epitopes on GP and SGP molecules and indicates the potential of SGP to serve as a decoy for neutralizing antibodies.

7. Infectivity-enhancing antibodies to Ebola virus glycoprotein

Takada A, Watanabe S, Okazaki K, Kida H, Kawaoka Y

Ebola virus causes severe hemorrhagic fever in primates, resulting in mortality rates of up to 100%, yet there are no satisfactory biologic explanations for this extreme virulence. Here we show that antisera produced by DNA immunization with a plasmid encoding the surface glycoprotein (GP) of the Zaire strain of Ebola virus enhances the infectivity of vesicular stomatitis virus pseudotyped with the GP. Substantially weaker enhancement was observed with antiserum to the GP of the Reston strain, which is much less pathogenic in humans than the Ebola Zaire and Sudan viruses. The enhancing activity was abolished by heat but was increased in the presence of complement system inhibitors, suggesting that heat-labile factors other than the complement system are required for this effect. We also generated an anti-Zaire GP monoclonal antibody that enhanced viral infectivity and another that neutralized it, indicating the presence of distinct epitopes for these properties. Our findings suggest that antibody-dependent enhancement of infectivity may account for the extreme virulence of the virus. They also raise issues about the development of Ebola virus vaccines and the use of passive prophylaxis or therapy with Ebola virus GP antibodies.

8. Identification of the porcine cytomegalovirus major capsid protein gene

Rupasinghe V, Iwatsuki-Horimoto K, Sugii S, Horimoto T

A major capsid protein (MCP) gene homologue of porcine cytomegalovirus (PCMV) was identified. Sequence analysis indicated that the PCMV MCP gene is 4,026 nucleotides in length encoding a protein of 1,341 amino acid residues. The predicted molecular weight of the PCMV MCP is 151,456 Da, equivalent to those of other herpesvirus MCP counterparts. Phylogenetic analysis using herpesviral MCP gene sequences confirmed that PCMV is a betaherpesvirus with higher homology with human herpesvirus-6 and -7 than human and mouse cytomegaloviruses. The serum of pig experimentally infected with PCMV did not react with bacterially expressed MCP, suggesting that the PCMV MCP may not be related to the humoral immune response in the course of PCMV infection. Also, we established polymerase chain reaction (PCR) protocols using primers corresponding to MCP gene sequences for detection of PCMV infection. The PCR protocol would be effective for the diagnosis of slow-growing PCMV infection, for which traditional methods involving virus-isolation are not useful.

9. Capsid protein gene variation among feline calicivirus isolates

Horimoto T, Takeda Y, Iwatsuki-Horimoto K, Sugii S, Tajima T

We amplified the capsid protein gene fragments of 30 Japanese isolates of feline calicivirus (FCV), including the C, D, and E regions, by reverse transcription-polymerase chain reaction (RT-PCR), followed by direct sequencing. Alignment of the predicted amino acid sequences, together with other published sequences from the isolates obtained in other countries, demonstrated a marked heterogeneity among the isolates, confirming the current definition of hypervariable regions within the capsid protein: these regions give rise to the antigenic variations seen in FCV isolates. Phylogenetic analysis of the nucleotide sequences could not identify significant geographically or temporally separated clusters of FCV isolates, supporting the theory of a single genotype.

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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 2001.

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 (Sugimoto et al., J. Virol. 72:5335-5342, 1998). To examine whether JCV T-antigen is involved in the replication of JCV in COS 7 cells, we constructed a chimeric JCV DNA in which the T-antigen gene was totally replaced with the luciferase gene (JCV/Luc) or a 3'-terminal segment of the T-antigen gene was left undeleted (JCV/Luc+). COS-7 cells were transfected with these chimeric JCV DNAs and cultured for weeks to allow viral growth. JCV/Luc did not induced viral growth even 6 weeks post transfection, but JCV/Luc+ induced a viral growth 4 weeks post-transfection. In the JCV DNA recovered from the JCV-positive cultures, most part of the luciferase gene was deleted but a major part of the 3'-segment of T-antigen gene was retained. We are currently examining the role of the 3'-segment of T-antigen gene in the replication of JCV in COS-7 cells.

2. PML diagnosis using PCR

Yoshiaki Yogo, Tomokazu Taksaka 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 performed PML-diagnosis service to hospitals throughout Japan. In 2001, we tested the CSF samples in 34 cases for which PML was strongly suggested, and detected PML-type regulatory regions in three cases. Brain biopsy was performed in four cases where the CSF was negative for JCV DNA, and we detected PML-type regulatory regions in three cases. Although PCR-amplification of JCV DNA sequences is widely used as an effective way to diagnose PML, our results indicate that JCV DNA may not be detected from the CSF in some PML cases. We emphasize the necessity for brain biopsy, when the CSF is negative for JCV in cases where PML is strongly suggested for these case by their clinical features, underlying disease and lesions in the white-matter. It remains to be elucidated the proportion of the PML cases at which the CSF is negative for JCV DNA.

3. JC Virus strains indigenous to northeastern Siberians and Canadian Inuits

Chie Sugimoto, Masami Hasegawa[2], Huai-Ying Zheng[1], Vladimir Demenev[2], Yoshiharu Sekino[4], Kazuo Kojima[5], Takeo Honjo[6], Hiroshi Kida[7] and Yoshiaki Yogo:[2]The Institute of Statistical Mathematics; [3]Administration of Khabarovsk Territory, Russia; [4]The Great Journey;[5]The Last Great Expedition on the Earth; [6]Himalayan Veterinary Hospital; [7]Graduate School of Veterinary Medicine, Hokkaido University

Urine samples were collected from the following Siberian/Arctic populations: Nanais living in the lower Amur river region, Chukchis and Koryaks living in extreme northeastern Siberia and Inuits living in Arctic Canada. Partial DNA sequences of JCV in the urine samples were determined, and subjected to phylogenetic analysis using the neighbor-joining method. According to the resultant phylogenetic tree, the Siberian/Arctic JCV isolates were mainly classified as belonging to Type-A, the major type of JCV prevalent in Europe and Mediterranean areas. To evaluate the evolutionary relationships among Siberian/Arctic and European/Mediterranean Type-A isolates, we adopted the whole-genome approach with which a highly reliable phylogeny of JCV isolates can be reconstructed, and used two phylogenetic methods, the neighbor-joining and maximum-likelihood methods. In analysis using the neighbor-joining method, Type-A JCVs worldwide diverged into three subtypes, EU-a, -b and -c, with 100% bootstrap probabilities. EU-a included not only European/Mediterranean but also Siberian/Arctic isolates. The Siberian/Arctic EU-a isolates were mainly derived from Chukchis, Koryaks and Inuits, and formed a distinct cluster (EU-a/Arc) within the EU-a subtype, with a 98% bootstrap probability. EU-b included only European/Mediterranean isolates. EU-c included only Siberian/Arctic isolates, mostly derived from Nanais. These patterns of Type-A divergence were confirmed by phylogenetic analysis using the maximum-likelihood method. Furthermore, the grouping of EU-a and -b with EU-c as an outgroup was strongly indicated by analysis using the maximum likelihood method. From these findings, we concluded that a novel subtype belonging to Type-A and a unique cluster within subtype EU-a occur in northeastern Siberians and Canadian Inuits. From the present findings, we inferred ancient human migrations, accompanied by Type-A JCVs, across Asia and to Arctic areas of North America.

4. Insights into the peopling of the Philippines based on JC virus genotypes

Jasmin Jiji Miranda[8], Chie Sugimoto, Tomokazu Takasaka, Huai-Ying Zheng[1] and Yoshiaki Yogo: [8]University of the Philippines, the Philippines

The Philippines is generally believed to have been established by various peoples who migrated from neighboring areas. To gain new insights into the peopling of the Philippines, we used the JCV genotyping approach. We collected about 50 urine samples in each of two representative islands of the Philippines, Luzon and Cebu. DNA was extracted from the urine samples and used to amplify the 610-bp region (IG region) of the viral genome. For each island, we determined about 20 IG sequences, from which a neighbor-joining phylogenetic tree was constructed to classify detected JCV isolates into distinct genotypes. The predominant genotype detected was SC, the southeastern-Asian genotype. Minor JCV genotypes were SC/Phi, B1-a and B3. SC/Phi was a sub-cluster of SC and has not been detected in areas other than the Philippines. The other minor genotypes were detected previously in the following areas: B1-a, mainland China, Pamalican Island (Palawan, Philippines) and Taiwan (an aboriginal tribe); B3, China and Thailand. These findings suggested that the modern Filipino population was formed not only by Southeastern Asians carrying SC but also by a few distinct ethnic groups carrying SC/Phi, B1-a or B3.

5. JC virus genotypes in northwestern China: implications for its population history

Zheng Guo[9], Shu Ping Zheng[10], Chie Sugimoto, Yue Ling Wang[9], Huai-Ying Zheng[1], Tomokazu Takasaka, Tadaichi Kitamura[1], Jing Guo[11] and Yoshiaki Yogo:[9]the First Affiliated Hospital Hubei, China; [10]The Fifth Hospital of Wuhan, China; [11]University of Alberta, Canada

We previously reported JCV genotypes in eastern China and Mongolia (Guo et al., *J. Gen. Virol.* 77:919-927, 1998). To gain a comprehensive picture of JCV genotypes in China, we collected urine samples at three northwestern sites along the Silk Road: X'ian, Lanzhou and Urumqi. DNA was extracted from urine samples, and used to amplify the 610-bp IG region of the viral genome. For each geographical site, we determined 16 to 24 IG sequences, from which a neighbor-joining phylogenetic tree was constructed to classify detected JCV isolates into distinct genotypes. (1) The northeastern-Asian subtype (CY) was mainly detected at X'ian and Lanzhou. This finding suggested that these two sites were colonized mainly by northeastern Asians. (2) The northeastern-Asian (CY) and central/western-Asian subtype (B1-b) were mainly detected at Urumqi. This suggested that Urumqi was colonized by both northeastern and central Asians. (3) In addition, several minor JCV subtypes were detected at these sites. These included a subtype (B1-c) prevalent in Europe and western

Asia and a subtype (Af2) prevalent in Africa and western Asia. Significant admixture of human populations may have occurred in areas along the Silk Road that was used in ancient times to transport goods between China and Europe.

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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. An endotoxin recognition molecule had been enigmatic but was recently identified as Toll-like receptor 4 (TLR4). 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.

1. Human MD-2 confers on mouse Toll-like receptor 4 species-specific lipopolysaccharide recognition

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Toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS). MD-2 is associated with TLR4 and imparts LPS responsiveness to it. Little is known, however, as to whether MD-2 directly regulates LPS recognition by TLR4. To address the issue, we took advantage of a species-specific pharmacology of lipid IVa, an analogue of lipid A. Lipid IVa acted agonistically on mouse (m) TLR4/MD-2 but not on human (h) TLR4/MD-2. Lipid IVa antagonized the agonistic effect of lipid A on hTLR4/MD-2. We examined the chimeric complex consisting of mTLR4 and hMD-2 to ask whether species specificity is conferred by TLR4 or MD-2. hMD-2 was clearly distinct from mMD-2 in the way of influencing LPS recognition by mTLR4. hMD-2 conferred on mTLR4 responsiveness to lipid A but not to lipid IVa. Moreover, lipid IVa acted as a lipid A antagonist on mTLR4 that is associated with hMD-2. Collectively, MD-2 directly influences the fine specificity of TLR4.

2. Requirement for MD-1 in Cell Surface Expression of RP105/CD180 And B Cell Responsiveness to Lipopolysaccharide[1]

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RP105 is a B cell surface molecule that has been recently assigned as CD180. RP105-ligation with an antibody induces B cell activation in humans and mice, leading to proliferation and upregulation of a costimulatory molecule B7.2/CD86. RP105 is associated with an extracellular molecule MD-1. RP105/MD-1 has structural similarity to Toll-like receptor 4 (TLR4)/MD-2. TLR4 signals a membrane constituent of Gram-negative bacteria, lipopolysaccharide (LPS). MD-2 is indispensable for TLR4-dependent LPS responses, since cells expressing TLR4/MD-2, but not TLR4 alone, respond to LPS. RP105 also has a role in LPS responses, since B cells lacking RP105 show hyporesponsiveness to LPS. Little is known, however,

as to whether MD-1 is important for RP105-dependent LPS responses, as is MD-2 for TLR4. To address the issue, we made mice lacking MD-1 and mAbs to it. MD-1-null mice showed impairment in LPS-induced B cell proliferation, Ab production, and B7.2/CD86 upregulation. These phenotypes are similar to those of RP105-null mice. The similarity was attributed to the absence of cell surface RP105 on MD-1-null B cells. MD-1 is indispensable for cell surface expression of RP105. A role for MD-1 in LPS responses was further studied with anti-mouse MD-1 mAbs. In contrast to highly mitogenic anti-RP105 mAbs, the mAbs to MD-1 were not mitogenic but antagonistic on LPS-induced, B cell proliferation and B7.2 upregulation. Collectively, MD-1 is important for RP105 with respect to B cell surface expression and LPS recognition/signaling.

3. *Pneumocystis carinii* infection in red-bellied tamarins and cynomolgus monkeys, and the characterization of mitochondrial large subunit ribosomal RNA gene of *Pneumocystis carinii*

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Pneumocystis carinii (P. carinii) is a causative agent of opportunistic infection and a widely distributed organism among a variety of animal species including human beings and causes fatal pneumocystosis in immunocompromised hosts like those with AIDS. It has been reported on cases of fatal and systemic pneumocystosis in rhesus monkeys *(Macaca mulatta)* infected with simian immunodeficiency virus and activation of latent *P. carinii* infection in these monkeys. The recorded data implies that this is a common opportunistic infection in monkeys. *P. carinii* infection has been reported in various new and old world monkey species, but there has been no report in red-bellied tamarins *(Saguinus labiatus)*. Recently, we had an opportunity to examine *P. carinii* -infected red-bellied tamarins and cynomolgus monkeys and then found a high incidence of *P. carinii* infection in these animals by histopathologic examination. We also examined mitochondrial large subunit ribosomal RNA gene (mt LSU rRNA) of *P. carinii* derived from these two species and then compared their sequences with those of *P. carinii* derived from other mammalian hosts.

At autopsy, the lungs showed almost normal findings, and *P. carinii* cysts were detected in 6 of 10 tamarins and 1 of the 66 cynomolgus monkeys on histopathologic examination although the number of cysts was not so high. *P. carinii* has been isolated from the lungs of a variety of mammalian hosts, and the morphology is indistinguishable, although immunological and genetic studies indicate that these organisms are not identical. To examine the genetic diversity of this pathogen, we sequenced PCR products of mt LSU rRNA of *P. carinii* derived from tamarin and cynomolgus monkey-*P carinii*, and the number of nucleotide is 315 bp and 307 bp, respectively. Genetic diversity in *P. carinii* DNA was found in both monkeys, indicating that *P. carinii* derived from different hosts is genetically distinct. Furthermore, the tamarin and cynomolgus monkey-*P. carinii* DNA sequences were aligned with those derived from 6 different mammalian hosts. Tamarin and cynomolgus monkey-*P.carinii* sequence contained regions with significant homology to *P. carinii* sequences isolated from other hosts, in addition to regions that were divergent. Phylogenic trees were constructed from each alignment using the neighbor joining method, in the form of a bootstrapped unrooted tree. The results of the analysis suggested that cynomolgus monkey-*P. carinii* is closer to tamarin-*P. carinii* than that of rhesus monkey, although cynomolgus and rhesus monkeys are taxonomically in the same group of *Macaca* monkeys. On the other hand, the rhesus monkey-*P. carinii* is close to human-*P. carinii*, and then, rat- and mouse-*P. carinii*. The results show that tamarin- and cynomolgus monkey-*P. carinii* seem to be different from groups of other *P. carinii*, and that each *P. carinii* had its own type of organism with specific mt LSU rRNA gene, although these data are still preliminary. More specimens from those simians will be sequenced in future to reconfirm the results obtained here and we will also analyze other genes of *P. carinii* to examine the differences among *P. carinii* derived from different hosts.

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 potentiol 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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