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分担研究報告書

Mycobacterium bovis BCG の亜株の自然免疫誘導活性の解析

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研究要旨　入手可能な 14 株の BCG 亜株（Russia, Moreau, Japan, Sweden, Birkhaug, Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia, Pasteur）を用いて自然免疫誘導活性の差異について感染宿主細胞（ヒト肺胞上皮細胞株 A549，ヒト骨芽球系細胞株 THP-1，マウスマクロファージ細胞株 Raw264.7，マウス初代培養骨髄細胞）からの一酸化窒素(NO)、及び炎症性サイトカイン(IL-1β, IL-6, IL-8, IL-12, TNF-α)を測定した。Pasteur 研究所からの分与時期の早い株（Russia, Moreau, Japan, Sweden, Birkhaug）にこれらの自然免疫活性化の指標を上げる活性が見られた。この結果は、株は metoxy ミコール酸の合成に係わる酵素*(mma3)*に変異と相関していた。初期株（Japan）、後期株(Connaught)から抽出した trehalose-dimycolate (TDM) を用いて実験したところ同様の結果が得られた。以上のことから、初期株に含まれる TDM に優れた自然免疫誘導活性があることが推察された。

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A．研究目的

現在使用されている結核予防ワクチン BCG (bacille Calmette-Guérin)は現在実用化されている唯一のワクチンであり、BCG はフランスの医師 Calmette と獣医 Guérin が *Mycobacterium bovis*（ウシ型結核菌）を 13 年間 231 代にわたって継代培養し、1921 年に完成された弱毒化生ワクチンである。わが国への導入は、志賀潔が 1925 年にパスツール研究所より持ち帰ったものとされる。1937 年にその安全性と有効性が国内で確認され、1942 年から集団接種が行われるようになった。1943 年からは凍結乾燥が試みられるようになり、1950 年から BCG-Tokyo として凍結乾燥ワクチンが用いられた。

現在は世界各国で導入され、年間約 1 億人に投与されている BCG の原株はパスツール研究所由来であるが、世界各国に広まり継代されていく間に、やや異なる性質が固定されていったと考えられている。BCG は変異を起こしやすく、Calmette と Guérin が継代培養を開始してわずか 15 代目にはコロニー形態の異なる変異株が得られ、その株の仔ウシやモルモットに対する病原性は低下していたと言われている。継代による菌の変異をできるだけ少なくするため、現在は菌体の凍結乾燥によるシードロット制が取り入れられ、常に一定の性質を持った「BCG ワクチン」を提供できるシステムとなっている。現在世界では日本の Tokyo-172 の他、Glaxo、Copenhagen、Pasteur が主なシードとしてワクチン製造に使われているが、それらの細菌学的・免疫学的性質は異なることが知られている。また、近年の分子生物学の進歩によって、これら BCG 亜株間の差異が遺伝子レベルで明らかとなり、1998 年に *M. tuberculosis* $H_{37}Rv$ 株の全ゲノム配列が明らかにされた。この配列を基準として *M. bovis* や BCG を DNA マイクロアレイ法で調べたところ、少なくとも 11 の領域が *M. bovis* には見られず、さらに *M. bovis* には見られる 5 つの領域が、大半の BCG では欠失していることが 1999 年に報告された。

BCG ワクチンは、小児の結核性髄膜炎や粟粒結核などの発病・重症化を極めて有効に阻止するが、成人の結核に対する評価は不定で、有効性は 50%程度、免疫持続期間は 10～15 年と言われており、現在 BCG の成人肺結核に対する有効性は疑問視されている。しかし、高い安全性が示され、唯一の結核予防ワクチンである BCG はこれからも継続して投与されていくことが予想される。より効果的に BCG ワクチンを利用するためには、各国の BCG 亜株を同一条件で再評価することが必要である。

そこで本研究では、現在ワクチンとして使用されている BCG 亜株の自然免疫系に対する in vitro における比較解析を行った。

B．研究方法

菌株： *Mycobacterium bovis* BCG 亜株（Australia ATCC 35739、Birkhaug ATCC 35731、Connaught ATCC 35745、Danish ATCC 35733、Glaxo ATCC 35741、Mexico ATCC 35738、Montreal ATCC 35735、Pasteur ATCC 35734、Phipps ATCC 35744、Russia ATCC 35740、Tice ATCC 35743）、Australia vaccine seed、Sweden 株は国立感染症研究所の山本三郎博士より供与された。*Mycobacterium tuberculosis* $H_{37}Rv$ ATCC 25618、Cololado State University より供与された。*Mycobacterium bovis*, *M. bovis* BCG Moreau,は

結核研究所より供与された。各菌株は Middlebrook 7H9 Broth／0.25 % Tween 80／10% ADC 培地にて培養した。

Trehalose dimycolate (TDM)の抽出：培養した細菌菌体に 20 倍量のクロロフォルム・メタノール(2:1)を加えて破砕機で破砕し、水－有機層分配系で有機層を得、薄層クロマトグラフィー(TLC)にて TDM を分離する。TLC より TDM のスポットを掻きとり、ヘキサンで抽出する。これを定量後、培養プレートやガラスビーズにコートした。

一酸化窒素（NO）の測定：ヒト肺胞上皮細胞株 A549、ヒト骨芽球系細胞株 THP-1、マウスマクロファージ細胞株 Raw264.7 細胞、マウス初代培養骨髄細胞に BCG を Multiplicity of infection(MOI)=10 で感染させた。もしくは TDM でコートした培養プレートに細胞をまくか、TDM でコートしたガラスビーズを細胞あたり 10 個加えた。48 時間後に培養上清を回収し、ろ過滅菌後 diaminonaphtalen(DAN)を用いた蛍光法で測定した。

Enzyme Linked Immuno-Sorbent Assay（ELISA 法）による培養上清中のサイトカインタンパクの測定：ヒト肺胞上皮細胞株 A549、ヒト骨芽球系細胞株 THP-1、マウス初代培養骨髄細胞に BCG を Multiplicity of infection(MOI)=10 で感染させ、48 時間後に培養上清を回収し、ろ過滅菌後 Enzyme Linked Immuno-Sorbent Assay（ELISA）法で測定した。Human IL-1β ELISA Set（BD Biosciences）、Human IL-6 ELISA Set（BD Biosciences）、Human IL-8 ELISA Set（BD Biosciences)、IL-12 ELISA Set(BD Biosciences)、Human TNF-α ELISA Set(BD Biosciences)を使用し、使用法は添付の方法に従って行った。

D．考察

自然免疫系の活性化の指標として、宿主細胞からの一酸化炭素(NO)の産生と炎症性サイトカイン（IL-1β, IL-6, IL-8, IL-12, TNF-α）の産生を測定した。初期分与株(Russia, Moreau, Japan, Sweden, Birkhaug)の方が後期分与株（Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia, Pasteur）と比べて高い傾向が見られた。この結果は、*mma3* 変異とよく相関していた。初期分与株の代表として Japan 株、後期分与株の代表として Connaught 株から抽出した TDM を用いて NO と IL-12, TNF-α の産生誘導活性を検討した結果、誘導活性は全体的に弱いながら、菌体そのもので刺激した場合と同様の結果が得られた。IL-1β の産生については、生菌と死菌で逆の結果が得られた。以上のように初期分与株の生菌が自然免疫系の優れた誘導活性があることが明らかとなった。今後、自然免疫を強く誘導する BCG がメモリー誘導活性や結核防御活性を持つか、否か、検討していく必要がある。

E．結論

本研究の結果、初期分与株(Russia, Moreau, Japan, Sweden, Birkhaug)の方が後期分与株(Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia, Pasteur）と比べて自然免疫系をよく活性化することが明らかとなった。この結果は、*mma3* 変異とよく相関していた。活性化の機構の一部にメトキシミコール酸の関与が示唆された。本研究の成果は BCG 亜株間の in vitro での性質の一部を明らかにした基礎的な知見である。今後、免疫記憶や有効性について検討を重ねていくことで、よりよいワクチンの選択と接種の施行方法に関する今後のワクチン行政に貢献できると考えられる。

F．健康危険情報

本研究の遂行に当たり健康被害報告等の危険情報はない。

G．研究発表

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2009 年 3 月 27 日(京都)

H. 知的財産権の出願・登録状況

1. 特許出願中： 抗結核化合物、及びその利用
(特願 2008-059903)

2. 特許出願中：抗結核薬及びその用途
(特願 2009-21026)

US-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM
43rd Tuberculosis and Leprosy Research Conference
AGENDA

MONDAY, JULY 7

6-8 p.m. **WELCOME RECEPTION**

Location: Harbor View (on the first floor of the Hyatt hotel)

Beverages and snacks will be served. This will be a convenient time to meet and socialize with conference attendees.

TUESDAY, JULY 8

7:30 a.m. **REGISTRATION**

8:15 a.m. **OPENING REMARKS:**
Bill Bishai, JHU Host
Gyanu Lamichhane, JHU Host
David McMurray, Chair, US Tuberculosis and Leprosy Panel

SESSION 1
Co-chairs: Isamu Sugawara; William Bishai

8:30 a.m. The molecular mechanism of the *Mycobacterium tuberculosis* NDH-1-mediated inhibition of host cell apoptosis involves NOX2 and TNF-α.
Volker Briken

8:50 a.m. Protection of DNA by mycobacterial DNA-binding protein 1 (MDP1) by preventing the iron-induced Fenton reaction.
Sohkichi Matsumoto

9:10 a.m. Live *Mycobacterium tuberculosis* inhibits phagolysosome biogenesis in macrophages by modulating localization of Rab GTPase proteins on its phagosome.
Shintaro Seto

9:30 a.m. Identification of tuberculosis susceptibility genes with human macrophage gene expression profiles.
Nguyen Thuong

9:50 a.m. **BREAK**

10:10 a.m. A subset of *Mycobacterium tuberculosis* clinical isolates within a defined genetic lineage differentially interact with human macrophages due to a truncated lipoarabinomannan and reduced higher-order phosphatidyl-*myo*-inositol mannosides.
Larry Schlesinger

10:30 a.m. RD1 region in mycobacterial genome is involved in the induction of necrosis in infected macrophages via mitochondrial membrane damage and ATP depletion.
Ikuo Kawamura

10:50 a.m An Approach to Identify Susceptibility Genes in Non-HIV-Related Pulmonary *Mycobacterium Avium* Complex Infection.
Naoto Keicho

11:10 a.m. Mce transporters contribute to mycobacterial persistence by facilitating the acquisition of host cholesterol.
Chris Sassetti

11:30 a.m. **LUNCH / POSTER SESSION 1**

SESSION 2
Co-chairs: Yasuhiko Suzuki; David McMurray

1:30 p.m. Molecular epidemiology of multi-drug resistant tuberculosis including extensively drug resistant tuberculosis in Osaka, Japan.
Tomoshige Matsumoto

1:50 p.m. Phylogenetic Information Derived from Variable Number of Tandem Repeats (VTNR) of the *Mycobacterium tuberculosis* Beijing Family
Takayuki Wada

2:10 p.m. A PCR-RFLP technique for SNP-typing expedites the discrimination of *Mycobacterium leprae* genotypes in Philippine leprosy populations and reveals association with specific short tandem repeat alleles.
Vara Vissa

2:30 p.m. Quantitation and evaluation of *Mycobacterium leprae* viability found in water in a leprosy endemic area.
Masanori Matsuoka

2:50 p.m. **BREAK**

3:10 p.m. Distribution analysis of mycobacteria in soils with molecular- and culture-method
Kazumasa Fukuda

3:30 p.m. Clinical, bacteriological and immunological follow-up of household contacts of leprosy patients from a post-elimination area-Antioquia, Colombia.
Nora Cardona-Castro

3:50 p.m. Exosomes from Mycobacteria-infected macrophages: Activation of the innate and acquired immune responses and their potential use as *M. tuberculosis* vaccines.
Jeff Schorey

4:10 p.m. **LATE BREAKING / SHORT TALKS**

HIV-1 Replication is Differentially Regulated by Distinct Clinical Strains of *Mycobacterium Tuberculosis*
Shahin Ranjbar

The secretion and virulence mechanisms of the ESX-1 Type VII secretion system in *M. tuberculosis* and *M. marinum*
Lian-Yong Gao

Characterization of a novel pathogenicity island in *M. tuberculosis*
Adel M. Talaat

Free-living pathogenic amoebae as potential reservoirs/vectors in leprosy transmission
James Krahenbuhl

6:00 p.m. **DINNER**

US-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM
43rd Tuberculosis and Leprosy Research Conference
AGENDA

WEDNESDAY, JULY 9

SESSION 3
Co-chairs: **Masahiko Makino; Linda Adams**

8:00 a.m. Diabetes and Hyperlipidemia Impair TB Defense
Hardy Kornfeld

8:20 a.m. Novel IL-17-producing memory cells are key to vaccine-based protective immunity against *M. tuberculosis* challenge.
Shabaana Khader

8:40 a.m. Mycobacterium leprae infection in $CD4^{-/-}$ and $CD8^{-/-}$ mice
Linda Adams

9:00 a.m. Human TLR2/1-mediated antimicrobial response is vitamin D-dependent and requires induction of the inflammasome and antimicrobial peptides.
Philip Liu

9:20 a.m. **BREAK**

9:40 a.m. Human innate *Mycobacterium tuberculosis*-reactive αβTCR+ thymocytes.
Marielle Gold

10:00 a.m. Host-derived oxidized phospholipids and high density lipoprotein regulate innate immunity in mycobacterial infections.
Daniel Cruz

10:20 a.m. CD8+ T cells require perforin to provide protection against *Mycobacterium tuberculosis* infection
Samuel Behar

10:40 a.m. Defining immunodominance for the human CD8+ T cell response to *Mycobacterium tuberculosis*
David Lewinsohn

11:00 a.m. **LUNCH / POSTER SESSION 2**

SESSION 4
Co-chairs: **Masashi Okada; Samuel Behar**

1:00 p.m. CD4 T cells are critical to control initial *M. tuberculosis* infection but not latent infection in the non-human primate model.
Philana Lin

1:20 p.m. $CD4^+$ T cell activation by antigen-presenting cells infected with urease-deficient recombinant BCG.
Masahiko Makino

1:40 p.m. Immunological response in foot pads and lymph nodes of vaccinated mice following challenge with live *Mycobacterium leprae*.
Ramanuj Lahiri

2:00 p.m. **BREAK**

2:20 p.m. New Antigens and Adjuvants for the Next Generation TB Vaccines
Steve Reed

2:40 p.m. Intranasal delivery of BCG secreting GM-CSF increases the number of dendritic cells, anti-mycobacterial T cell responses and protection against *Mycobacterium tuberculosis*.
Warwick J Britton

3:00 p.m. Protective efficacy of recombinant BCG Tokyo (Ag85A) in rhesus monkeys (Macaca mulatta) infected intratracheally with H37Rv *Mycobacterium tuberculosis*
Isamu Sugawara

3:20 p.m. Update on new approaches for tuberculosis vaccines.
Yasir Skeiky

3:40 p.m. ~~The treatment of MDR-TB infected mice with DNA vaccines or in combination with medicine~~
~~**Xueqiong Wu**~~ * WITHDRAWN

4:00 p.m. **LATE BREAKING / SHORT TALKS**

TGFβ targeting siRNA intrapulmonary therapy increases the antimicrobial activity in mice chronically infected with *Mycobacterium tuberculosis*
Mercedes Gonzalez-Juarrero

Mice deficient in B7 costimulation are resistant to acute *Mycobacterium tuberculosis* infection but lose their ability to control chronic infection
Kamlesh Bhatt

4:30 p.m. **BASEBALL GAME / DINNER**

US-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM
43rd Tuberculosis and Leprosy Research Conference
AGENDA

THURSDAY, JULY 10

SESSION 5
Co-chairs: **Hatsumi Taniguchi; Jacques Grosset**

8:00 a.m. **LATE BREAKING / SHORT TALKS**

Guinea pig model of *Mycobacterium tuberculosis* dormant infection
Antonio Campos-Neto

Reassessing the diagnosis of residual pulmonary tuberculosis cavities among healing TB patients
Zhaoqin Zhu * NEW

A Guinea Pig Model of TB Chemotherapy?
Petros C. Karakousis

8:40 a.m. Immunotherapy of Latent Tuberculosis
Zhongming Li * NEW

9:00 a.m. Isoniazid resistance associated with mutations in the *M. tuberculosis katG* gene has multiple origins.
Richard Magliozzo

9:20 a.m. Leveraging chemistry in the assault on *Mycobacterium tuberculosis*: battling drug resistance and persistence.
Joel Freundlich

9:40 a.m. ~~Discovery of a new lead compound active on indole-3-glycerol phosphate synthase of *Mycobacterium tuberculosis*.~~
~~**Honghai Wang**~~ * WITHDRAWN

10:00 a.m. **BREAK**

10:30 a.m. **LATE BREAKING / SHORT TALKS**

P38 and PI-3 kinase signaling pathways reciprocally regulate matrix degrading enzymes in tuberculosis and may be key therapeutic targets
Jon S. Friedland

Diagnostic test for TB based on immunodominant epitopes of *M. tb* complex specific cell-wall proteins
Suman Laal

Mycobacterium tuberculosis: direct from sputum identification and determination of drug resistance within hours for all antimycobacterial agents
Nicole Parrish

Rapid and sensitive detection of tuberculosis using LAMP and a novel sputum processing method
Mark Perkins

11:30 a.m. **LUNCH / POSTER SESSION 3**

SESSION 6
Co-chairs: **Masanoro Matsuoka; Steven Reed**

1:30 p.m. A regimen of PA-824 (Pa), moxifloxacin (M) and pyrazinamide (Z) is more active than the first-line regimen in the mouse model of tuberculosis (TB).
Eric Nuermberger

1:50 p.m. Gene-compound synthetic lethality *Mycobacterium Tuberculosis* drug discovery
Shichun Lun

2:10 p.m. Increasing the potency of rifamycin-containing drug regimens for tuberculosis with low doses of daily rifapentine or high doses of daily rifampin?
Jacques Grosset

2:30 p.m. Therapeutic effect of multiple T cell epitope-based DNA vaccine combined with chemotherapy in *Macaca fascicularis* chronically infected with MDR-*Mycobacterium tuberculosis*
Wei Xu (PRESENTED BY DR. LI)

2:50 p.m. **BREAK**

3:10 p.m. Initial evaluation of a bioluminescence assay for rapid antimicrobial susceptibility testing of *Mycobacterium tuberculosis*
Peter Chun

3:30 p.m. A survey of *Mycobacterium Tuberculosis* complex from clinical samples in Bangladesh by a species distinguishable multiplex PCR
Yasuhiko Suzuki

3:50 p.m. Antigen selection and test development for rapid leprosy diagnosis.
Greg Ireton

4:10 p.m. Construction of *ureC*-disrupted BCG which expressing *M. leprae* MMP II antigen.
Tetsu Mukai

4:30 p.m.	Eosinophilic Skin Hypersensitivity to Glycolipids in Mycobacteria-infected Guinea Pigs **Masahiko Sugita, M.D., Ph.D.**
5:00 p.m.	**CLOSING REMARKS:** **Isamu Sugawara, Chair, Japan Tuberculosis and Leprosy Panel**

Meeting Summary
U.S.-Japan Cooperative Medical Sciences Program
Diagnostics Workshop: Development and Regulatory Issues
Sponsored by the Acute Respiratory Infections, Parasitic Diseases and Tuberculosis/Leprosy Panels
Baltimore, MD
July 11, 2008

Introduction and Background

Dr. Masamichi Goto, panel member, Japan Tuberculosis and Leprosy Panel, and Dr. David McMurray, Chair U.S. Tuberculosis and Leprosy Panel welcomed the workshop participants. They noted that the workshop was co-sponsored by three panels of the U.S.-Japan Cooperative Medical Sciences Program: the Acute Respiratory Infections, Parasitic Diseases and Tuberculosis/ Leprosy Panels. They thanked Drs. William Bishai and Gyanu Laminchhane of The Johns Hopkins School of Medicine for providing the local support to the meeting and Drs. Gail Jacobs and Karen Lacourciere, Tuberculosis and Other Mycobacterial Diseases Section, Respiratory Disease Branch, NIAID, NIH for organizing the workshop program.

They also acknowledged support from a number of organizations and entities including: the Department of Health and Human Services (DHHS), the National Institutes of Health (U.S., {NIH]), the National Institute of Allergy and Infectious Diseases (NIAID), the Johns Hopkins School of Medicine, the Bill and Melinda Gates Foundation (BMGF), Aeras, the Foundation for Innovative New Diagnostics (FIND), Otsuka, Becton Dickenson (BD), Cellestis, Oxford Immunotech, Roche.

They stated that in additional to medical and scientific issues, regulatory issues are very important in development and deployment of diagnostics. Diagnostics are needed not only to identify infected persons for treatment, but they also underpin areas like vaccine development. They noted that there is the potential for phase III trials of tuberculosis vaccines in the near future, and that the availability of diagnostics will play an important role in identifying and monitoring the participants in such trials.

SESSION 1: Intellectual Property Considerations for Developing Diagnostics

US vs. WIPO patent process and implications for development:
***George Elliott*, Director, Technology Center 1600, US Patent and Trademark Office**

Dr. Elliott provided an overview of the U.S. patents process and of the pitfalls that face new inventors. He indicated that the two roles of a patent are: (1) as a property-right reward for the effort required to invent new and useful things; the goal is to encourage innovation and encourage development to bring an invention to market; and (2) for rapid disclosure of new scientific and technical information to the public so as to ensure continuing expansion of the public knowledge base. The foundation for patents is found in Article 1, Section 8, clause 8 of the U.S. Constitution which has been codified in Title 35 of the United States Code (U.S.C.), Sections (§) 101, 102, 103 and 112, which set forth the major requirements for patentability. In contrast to regulatory situations, such as FDA approval, where efficacy has to be proven, an inventor is entitled to a patent unless his or her invention is not: patent eligible subject matter, and useful (35 U.S.C. § 101); new (35 U.S.C. § 102); nonobvious (35 U.S.C. § 103) or adequately described and distinctly claimed (35 U.S.C. § 112). Patent-eligible subject matter does not include: abstract ideas, laws of nature or natural phenomena. The invention should have real

world utility and be technically available. It cannot be speculative. Section 102 addresses whether an invention is "new," that is, if it is not described in a single publication or document in all of its detail and if it hasn't been for sale or marketed. The invention has to differ by a single element from that which has been previously done. Section 112 requires that the invention, and the manner and process of making and using it, must be described in such "full, clear, concise, and exact terms" as to enable a person skilled in the art to which it pertains to make and use the invention. The second paragraph of Section 112 requires that the invention be defined in one or more "claims" that tell the public, with a reasonable degree of clarity and particularity, what the boundaries of the patented invention are. There has to be sufficient clarity so that a potential competitor would know if he were infringing on the patent.

The patent application process sets the invention in a moment of time. New findings would necessitate a new application. There are three types of patent applications: provisional, non-provisional and international. Provisional Applications are fast (few formal requirements), inexpensive, and establish a priority date for invention. The disclosure must meet the requirements of the first paragraph of 35 U.S.C. § 112 with respect to any invention later claimed in a non-provisional application. These applications are not examined and are automatically abandoned after 1 year if not pursued by the inventor; the abandonment results in the loss of the priority date. Non-provisional Applications are the mechanism that one thinks of in saying patent application. These applications must describe, enable and claim an invention. They can claim priority to a Provisional Application if they are filed within 1 year of the Provisional Application. Non-provisional Applications are examined. International Applications are filed under the Patent Cooperation Treaty (PCT). They allow for later filing in any of many countries and result in a preliminary search and patentability report. (This report may affect the decision of an inventor about whether to go forward on national stage.) The International Application can provide a 30 month delay before filing a U. S. application and entering the examination queue. This aspect of the International Application may be useful for diagnostics which have to go to the FDA for approval.

Dr. Elliott provided a number of hints for inventors to keep in mind. These included the need to keep good records; records should be dated, signed and, if possible, witnessed. Inventors should not publish a description of the invention more than 1 year before filing an application for a patent. Most countries don't have the 1 year allowance that the U.S. does and do it is important to set a priority date before publication for international work. Inventors should not sell or offer the invention for sale more than 1 year before filing an application for a patent. Inventors should not allow public use of the invention for sale more than 1 year before filing an application for a patent. If an inventor wishes to have other laboratories test the invention, they need to be cautious about how this is arranged so as not to be considered as public use.

Dr. Elliott noted than it is common for many patent applications to be rejected at least once. The Patent Office provides for an interview process. While the interview is usually conducted between the patent examiner and the inventor's attorney, the inventor may also be present. Interviews are often very useful for resolving misunderstandings or misconceptions and can frequently lead to the identification of patentable subject matter, or resolve other issues raised by the examiner.

Dr. Elliott noted that it is important for inventors to get help in applying for a patent. If the inventor is at an academic institution, the technology transfer office can be a good resource. For non-academic inventors, a patent attorney can provide assistance. Inventors should develop a needs based strategy for their invention. For example, if trials will be needed for FDA approval, the outlook for the invention should be long-term. If the inventor is seeking venture capital, the outlook may be short term. It is important to establish priority for the invention and to do it quickly, especially if there have been publications or if publications are planned. As part of their strategic planning, inventors should also consider whether they will seek

international protection. Inventors should seek a Provisional Application if they are if their needs are short-term and if in the process of publishing, or already have. A Non-provisional U. S. application will allow inventors to begin the examination process and to take advantage of Accelerated Examination. (There are substantial hurdles to the Accelerated Examination process and the inventor will have to do some of the work that the PTO ordinarily does in researching the subject of the patent.) The International Application (PCT) establishes priority in the U.S. and abroad and as noted provides for 30 months from the priority date before the application enters the U.S. examination queue as well as a preliminary report.

Inventors also need to consider the reality of the patent situation. A patent is not a license to make, use or sell an invention, it is only a right to exclude those unauthorized by the patent owner from making, using or selling the subject matter of the patent. Moreover, one patent may dominate another. If, for example, there are foundational technology patents in the subject area, the inventor may get a patent, and still be stopped from practicing his invention by someone whose patent dominates theirs or the inventor may be forced to pay license fees to the holder of the broad-based patent.

In response to questions, Dr. Elliott indicated that disclosure on a scientific manuscript occurs when the journal is published. NIH grant applications are confidential. Only the abstracts of awarded NIH grants appear on the NIH web-site. In terms of talks at scientific meetings, if the presentation is detailed enough to enable the invention to be made, the presentation could be considered disclosure.

Prior to 2000, U.S. patent applications were considered confidential until it was determined that the invention could be patentable. In 2000, the U.S. adopted the international policy which allowed for publication 18 months after the application was filed. Although a patent applicant can request confidentiality, doing so prevents filing an international application. Published patent applications can be considered prior art against inventions that are applying for a patent. The public can now access all of the documents associated with a patent application, but they need the patent number to do so.

Court decisions have enjoined the Patent Office from applying limits to continuation applications or from applying limits on the number of claims. The latter decision is being appealed.

Issues to Consider for Licensing of Diagnostics:
***Mojdeh Bahar*, Technology Licensing Specialist, Office of Technology Transfer, NIH, DHHS**

Dr. Bahar addressed the great diversity of diagnostics and how that impacts the licensing of them. The statutory definition of a diagnostic is an "instrument, apparatus, machine, *in vitro* reagent, test, kit, etc., intended for use in diagnosis of disease." Some medical devices are classified as diagnostics and others are classified and regulated as devices. For the latter, the definitions in Section 201 (k) of Food, Drug and Cosmetics Act apply. The statutory definition of a medical device is: "an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent or other similar or related article, including any component, part or accessory that: is recognized in the official National Formulary, the US Pharmacopeia, or supplements to them; is intended for diagnosis, cure, mitigation or treatment of disease; is intended to affect the structure or any function of the body; and does not achieve any of its primary purposes through a chemical reaction on or within the body, and is dependent on being metabolized to achieve any of its principal intended purposes."

There are many types of diagnostic devices and they are used in a variety of settings. For example, there are i*n vitro* reagents, genomic-based tests; and proteomic-based tests. Some

diagnostics can be simply bought at a pharmacy and some diagnostic tests are done through the advice of a medical care provider. Diagnostic tests can be done in different settings, e.g., home, a physician's office, a hospital, a laboratory, etc. The licensing of them can be different because of the settings in which they are to be used.

Given its public health mission, the NIH best practices for genomic inventions suggest that if significant R&D is not needed, consideration should be given to NOT patenting the invention. An example of this is the finding of a receptor for which the ligand is not known. If the invention is to be patented, consideration should be given to a non-exclusive licensing strategy. This would avoid the ability of an organization to corner the market and would allow for the development of a confirmatory test by other organizations or groups. If the invention is to be exclusively licensed, then the scope of the license should be limited so as to ensure expeditious development to address public health needs. The NIH position is that if the invention is for pure research, it should not be patented. The underlying concept is to consider the long-term implications of the process. An example of the types of problems that can be caused by patent and license agreements is the events surrounding BRCA-1, a breast cancer risk gene, which was elucidated at UCLA, but the function of which was found by the University of Utah. The University of Utah granted an exclusive license to Myriad Company. Myriad Company then sued an individual investigator at the University of Pennsylvania who had used a different research approach and through that approach had also found BRCA-1.

Dr. Bahar summarized NIH's Licensing Principles. These are: granting only the appropriate scope of rights; reserving the right to grant research-use licenses; refraining from asserting IP rights against non-profit research; having a preference for non- or partial exclusivity; having specified fields of use; having enforceable milestones and benchmarks (e.g. providing a business plan, a defined lead scientist. Without benchmarks, there is potential for the product to be shelved by a licensee.); maximizing public health interests (products, uses); and ensuring appropriate return on public investment. There are government-wide statutes and regulations covering exclusivity. These are covered in 37 Code of Federal Regulations (CFR) 404, Government-Owned Inventions.

NIH's genomic licensing principles involve distinguishing diagnostics from drug/vaccine products, and for diagnostics, most often choosing non-exclusive licensing. A higher level of justification is needed for exclusivity for diagnostics; exclusivity is more justifiable for therapeutics because of the higher cost of product development. NIH permits multiple points of testing and of laboratory developed tests, e.g. confirmatory testing by clinical reference laboratories. In order for improvements to me made for a test, there is a need for competition.

Exclusive licensing of genomic diagnostics is considered for products regulated by the FDA, such as clinical diagnostics and analyte-specific reagents. Exclusivity provides an incentive for development of new products for unmet diagnostic needs, e.g. for rare diseases and for products with a narrow field of usage. NIH also reserves the right to grant non-exclusive licenses for internal and research uses. There is a statutory period for public comments (60 days).

NIH had asked the National Research Council to provide recommendations on proteomic and genomic diagnostics. The NRC provided 13 recommendations and the NIH and adopted them in their policies. Dr. Bahar noted besides the FDA and PTO roles in diagnostic tests, there is a third party, the Health Care Financing Agency (HCFA). HCFA assigns a code to a diagnostic that is used for billing purposes. If the diagnostic is not cost-effective, it will not be reimbursed, and thus, may not be used in practice.

Dr. Bahar noted that the licensing of diagnostic devices is quite different from that for diagnostic tests. Devices are generally multi-component and the different components may all be subject to various parties' IP rights (e.g., the manufacturer of each of the individual

components and the medical device company). Thus, the ownership becomes complicated and the control of patent prosecution can be an issue. It may be necessary to license some of the components. It is also not clear who owns the device, the group that assembled it or the entity who found the key element. Enforcement of rights/litigation may also be an issue when multiple entities are involved in a device. The contractual relationship between the manufacturers and the device company becomes important (whether the device company is a licensor or the licensee). There is the potential that one of the participant companies might decide to abandon their part of the device, and thus, put the entire device in jeopardy.

SESSION 2: Regulatory Issues for Diagnostics

US FDA Devices Policies and Advice:
***Sally Hojvat*, Director, Division of Microbiological Devices, US Food and Drug Administration (FDA)**

Dr. Hojvat indicated that she would provide a general overview of regulatory pathways and boundaries from the FDA perspective. The FDA's mission is to promote the public health by promptly and efficiently reviewing clinical research and taking appropriate action on the marketing of regulated products in a timely manner and to protect the public health by ensuring a reasonable assurance of the safety and effectiveness of devices intended for human use. In approaching its mission, the FDA needs to balance the risks and benefits of each device. The FDA has a 90 day review period for 510(k) diagnostics and a 180 day review period for Pre Market Approval (PMA) diagnostics.

21 CFR 809.3 defines in vitro diagnostics (IVD) as "reagents instruments, and systems intended for use in the diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae in man. ... for use in the collection, preparation, and examination of specimens from the human body." These can be used in clinical laboratories or other settings (e.g., point-of-care [POC] or over-the counter [OTC]). The FDA has regulatory authority over in vitro diagnostics that are used for diagnosis, screening, epidemiology/surveillance and for first response (i.e., on-site screening of persons for contamination by biohazardous material). The FDA regulates devices that use human specimens, but does not regulate devices used for environmental detection of agents.

Dr. Hojvat summarized the legislation that governs the FDA's regulatory authority on diagnostics. These legislative authorities include the Federal Food, Drug, and Cosmetic Act of 1938 (The Act); the Medical Device Amendments of May 28, 1976 (which classified all existing IVDs); the Clinical Laboratory Improvement Act (CLIA) 1988; the Safe Medical Devices Act of 1990; and the FDA Modernization Act (FDAMA) of 1997 and of 2007. She noted that FDA does not regulate laboratory developed tests (known as "home-brew,") except for the Analyte Specific Reagent (ASR) component of LDTs manufactured under the FDA QS Regulations.

Under FDAMA, the FDA's goals with respect to IVDs are for: risk-based regulation, decreased time to market, use of the least burdensome route, having early collaboration with manufacturers, providing FDA advice on valid scientific evidence and having a focus on clinical trial design. Using the risk-based approach, IVDs are categorized into three groups (I, II, or III) depending on risk/risk mitigation. The overall concept is that a diagnostic test should be reliable and that the health care professional/patient should be able to understand its value and limitations. Class I and II IVDs have lower likelihoods of harm and are governed by the 510(k) regulatory process. Class III IVDs have a higher likelihood of harm or have a harm that is unknown. They are subject to the PMA regulatory process.

Review and clearance of an IVD requires the assessment of safety (Are there reasonable

assurances, based on valid scientific evidence that probable benefits to health from use of the device outweigh any probable risks?) and of a demonstration of effectiveness/clinical utility (Is there reasonable assurance based on valid scientific evidence that the use of the device in the target population will provide clinically significant results?) Dr. Hojvat noted that there is a statutory process for CLIA Waivers.

There are a number of regulatory tools to support rapid IVD clearance. These include Pre-IDE Meetings. Multiple meetings are possible for this free consultative service by FDA review staff. Such meetings allow for a non-binding agreement between the FDA and the sponsor. The goal is to have a well-prepared submission with a shortened review time. Another regulatory tool relates to the transparency of the process. This includes the posting on the FDA web-site of decision summaries so as to allow a new sponsor to observe what other sponsors have done. Another aspect of agency transparency is the availability of FDA guidance documents.

In addition to PMAs, there are other pathways to clearance including: (1) Automatic Classification (De Novo). This is an alternative to the PMA for lower risk devices where there is no predicate, or when it is ancillary to other well-accepted methods. The appropriateness is determined on a case-by-case basis and is always risk based; (2) Reclassification Petition, Compassionate Use ([IDE] which permits the distribution and diagnostic use of reagents and instruments not cleared for use by FDA and facilitates collection of data on safety and efficacy to support future submissions. It requires IRB review and informed consent (IC). 21 CFR 50(g) (3) (D) was promulgated at CDC's request and allows for exemption from IC in emergency situations); (3) Humanitarian Device Exemption (HDE); and (4) Emergency Use Authorization (EUA) is for the use of an unapproved product during a declared emergency, such as, a life-threatening or serious condition or where no alternative is available.

Dr. Hojvat provided an overview of the key elements of a submission. These include: the intended use and/or indications for use; a description of the device; analytical validation; clinical validation/clinical utility; instrument and software validation, if applicable; proposed labeling (package insert); and manufacturing, design controls, quality system requirements ([QSRs/cGMP], the latter are for the PMA only). She noted that the intended use (the intended population, indication for use, and the analyte) is the driving force of the review. Products are now more complex; previously they measured one disease, now there are products that measure multiple diseases. Essentially, the manufacturer provides his claims and has to provide data showing that the propose usage can be achieved. The data should be derived not only from use of the device within the company, but also from use in the field.

The major issues in the review process include the analytical performance (How reliably and correctly the test measures the analyte, e.g., issues of cross-contamination, carryover, and interference and cross-reactivity), the clinical performance (How reliably the test measures the clinical condition.) and the labeling (The intended use, directions for use, warnings, limitations, interpretation of results, performance summary.) With respect to the instrument, documentation and hazard analysis is required and claims for use on multiple amplification /detection platforms need to be substantiated. Assays need to be validated and cleared for each platform. With respect to clinical evaluation, the samples/populations should represent the intended-use population. Samples should be prospectively collected with appropriate collection/storage (fresh versus frozen) and with clearly defined inclusion and exclusion criteria. The number of positive specimens should be statistically appropriate. A minimum of three sites should be involved for specimen collection.

Dr. Hojvat briefly addressed some issues related to the regulation of influenza, TB and malaria diagnostics. She indicated that rapid assays for detection of influenza antigens are in Class I and subject to the 510(k) review process. Reagents for the detection of novel influenza strains

go through the De Novo process and are considered Class II. She noted that the currently available rapid influenza assays are not capable of detecting novel strains such as H5 and H7. She indicated that there are a number of challenges for developing rapid tests for novel influenza strains including: obtaining positive novel influenza human specimens; making devices robust and simple enough for POC testing, having production surge manufacturing capacity, since there is not a clear market; having the ability to rapidly adapt to a newly mutated strain; achieving sensitivities of 80-90+% and achieving specificities of >95%.
The FDA's pandemic influenza preparedness strategy includes: early collaboration with developers/future applicants; encouraging coordination across disciplines; providing input on evaluation protocols and study design; encouraging guidance and standards development; and encouraging partnerships with DOD, CDC, NIH, Academia and with commercial device sponsors.

M. Tuberculosis IVDs are regulated based on their intended use and risk. Under 21 CFR 866.3370, IFA reagents for direct detection from culture isolates are Class I and are covered by 510(k). Antibiotic susceptibility devices are Class II and also governed by 510(k). Nucleic Acid amplification systems are Class III and require the PMA process.

She closed by noting that the key to success in the FDA regulatory process is having scientifically designed and well executed studies with appropriate statistical analysis of data in a well-written submission that is based on scientific principles. Good communication with the FDA throughout the entire process along with pre-IDE or pre-EUA meetings is highly recommended.

View of Regulatory Affairs through Industry's Lens:
***Pamela Angell,* Vice President of Regulatory Affairs, Inverness Medical Innovations**

Dr. Angell addressed regulatory issues from the perspective of industry. Inverness Medical Innovations is involved in the global manufacture of medical devices. They were involved with the Walter Reed Army Institute of Research (WRAIR) in the development of Binax NOW®, a rapid assay for malaria. Dr. Angell indicated that product realization, that is, the time from having an idea to having the product in hand, averages 3-5 years for an infectious disease product; the malaria diagnostic took 7 years. The time frame for a diagnostic varies with the complexity of the disease as well as whether the reagents for the product need to be developed or are being purchased. Other considerations in product development are regulatory compliance issues, the regulatory pathway, and the clinical studies.

Among the key regulations for companies wishing to manufacture a product is the Quality System Requirements (QSR). QSR are a set of objectives that are governed by 21 CFR 820. The quality plan varies with the product and focuses on the procedures needed to achieve the objective and not on specific processes. There are 15 QSR elements which are enforceable by the FDA. Entities wishing to make a product and not having a QSR have to build it themselves, hire a consultant, or partner with someone who has expertise in this area. The quality system should include a quality policy and a quality manual and provide procedures for dealing with all possibilities. An organization only has to address those QSR elements that are needed, but it also should justify the omission of any elements. Design controls are one element of the QSR that is needed to assure that design requirements are met.

In choosing to develop a product, it is important to consider user needs as well as R& D feasibility. The chosen design should be one that allows the manufacturer to be competitive. The product should be defined in terms of yielding an acceptable test result. Consideration must also be given as to where the product will be marketed. During the design process, it is important to monitor progress along the projected pathway and to determine if it is viable to go

forward.

Dr. Angell noted that in developing a product, it is essential to identify the regulatory pathway for it, that is, Exempt, 510(k) or PMA. One way to ascertain this is to look at the clearance of products with similar design, technology and intended use. It is also essential to determine the need for and scope of clinical trials as early as possible. Clinical trials are needed to substantiate the proposed intended use of the device and to establish its performance in the intended use setting. Typically prospective studies are needed at a minimum of three sites, with at least one site being in the U.S. It is possible to use retrospective studies if the disease is of low incidence. The trials must be conducted in a manner than is compliant with CCP, and require prior IRB approval, informed consent, and monitoring.

The risk category of the proposed product must also be determined. Most in vitro devices fit the "non-significant risk" category. Implants are an example of "significant risk" products. A diagnostic may be exempt if the testing is non-invasive.

Dr. Angell summarized by stating that it is important to develop regulatory and clinical plans early in the development process. The FDA should be engaged early and presented with a well-thought-out plan. The company's expertise should be assessed. Use should be made of available tools, guidance documents and consultants. When funding is an issue, a company should seek suitable interested partners to share the expense of product realization.

Regulatory Policies for Japan:
***David West*, Vice President, Medical Device Development,**
Quintiles Consulting

Dr. West indicated that his area of specialization was in the U.S. and Europe and that Ms. Natsuko Hosoda Senior Consultant, RA & MW, Clinical Development, Quintiles Transnational Japan K.K had provided him with much of the information for his presentation. He noted that there was a great commonality in the regulatory philosophy between the U.S. and Japan and that the differences were in procedures and environment.

In Japan, the term "in vitro diagnostic reagent" used in the Pharmaceutical Affairs Law (PAL) refers to a drug exclusively used in the diagnosis of disease. In Japan, the purpose of an IVD should be one of the following: (1) diagnosis of internal organs (vital functions, immunity, hemostasis, etc.); (2) diagnosis of disease, (3) diagnosis of method or effect of treatment; (4) diagnosis of pregnancy; (5) diagnosis of blood type or cell type. IVDs are to detect or assay the following substances or items in specimen for the diagnosis: (1) amino acid, peptide, protein, sugar, fat, nucleic acid, electrolyte, mineral, liquid, etc.; (2) hormone, enzyme, vitamin, coenzyme, etc.; (3) chemical or its metabolite, etc.; (4) antigen, antibody, etc.; (5) virus, microbe, protozoa or its nit, etc.; (6) pH, acid degree, etc.; (7) cells, tissues or their constituents.

IVDs have a risk-based classification. The risk is related to the risk of missed or erroneous diagnosis.
For Class I, there is self-certification. No approval is necessary. This is similar to an Exemption in the U.S. For Class II, there is third-party certification to a standard. For Class III, there is approval from the Ministry of Health Labor and Welfare (MHLW) based on an approval standard. This is like the FDA's PMA process. Class III IVD include: new IVDs to detect or assay new items, IVDs without an approval standard, IVDs that are in conformity to the approval standard, and IVDs that are not in conformity to the
Approval/Certification/No-approval Standard. Approval standards are essentially acceptance criteria that apply to specific products.

The information required in an application for approval is similar to that required by the FDA