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特集：ナノテクノロジーと医療

ナノレベルイメージングによる分子構造と機能の解析

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ナノレベルイメージングによる分子構造と機能の解析

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Nano-level imaging for analyzing protein structure and function

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Abstract

The present manuscript outlines the nano-level imaging project, which is under promotion by the three national research institutes and supported by a research grant from the Ministry of Health, Labor and Welfare (nano-001). This research project targets collecting fundamental information regarding comprehensive understanding of cardiovascular, neurological and the other disorders, developing new diagnostic and therapeutic methods by visualizing protein structure and function in atomic (sub-nano level) or molecular (nano-level) resolution. The results of the current projects will be extended into drug design, clinical diagnostic technology and medical materials in near future.

Key words: nano-technology, structural biology, drug design, protein crystallography, tailor-made medicine

はじめに

21世紀の医療の社会的課題として提唱されているテーラーメード医療の達成には，標的となる蛋白の構造を患者ごとに確定し(分子診断)，最適な薬剤の構造を選択し(分子治療)，薬剤と生体蛋白の相互作用を分子レベルで観察する(分子評価)などの医療基盤技術の育成が求められる．ナノレベルイメージングプロジェクトでは，蛋白分子の構造と機能の解析を通じてテーラーメード医療実現のための基盤技術の形成を目指している．

本稿では蛋白構造イメージングを中心に概説する．

1. 創薬に貢献した分子構造イメージング

近年，放射光を用いたX線回折法の発達により原子レベルの解像度で蛋白結晶の構造を決定できるようになった．構造に基づく薬剤設計の具体的な成功例として，AIDS治療薬(HIVプロ

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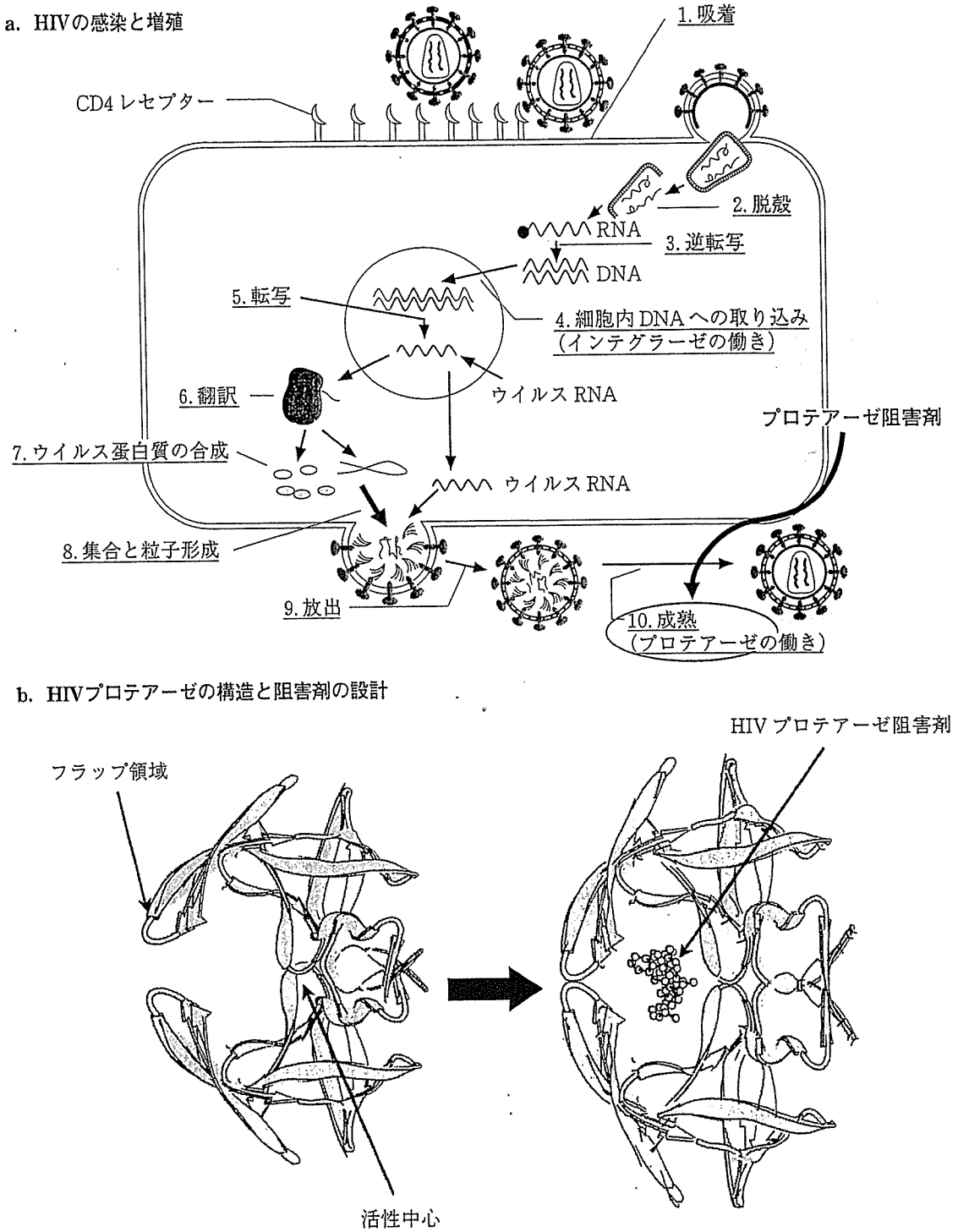


図1 AIDSウイルスの増殖過程と蛋白構造に基づくHIVプロテアーゼ阻害薬の作用機構

テアーゼ阻害薬), 白血病治療薬(グリベック)について以下に述べる.

AIDSウイルス, HIVは活性化外殻蛋白gp120によりCD4陽性Tリンパ球に感染し, 自己増殖をする. その際自己由来のプロテアーゼによって前駆体蛋白から活性化外殻蛋白を得る(図1-a). このHIVプロテアーゼの構造に基づいて設計され, その活性中心を選択的に阻害する目的で設計された薬剤がHIVプロテアーゼ阻害薬である(図1-b). 本剤はAIDSの発症を遅らせることに貢献した[1].

慢性骨髄性白血病ではフィラデルフィア染色

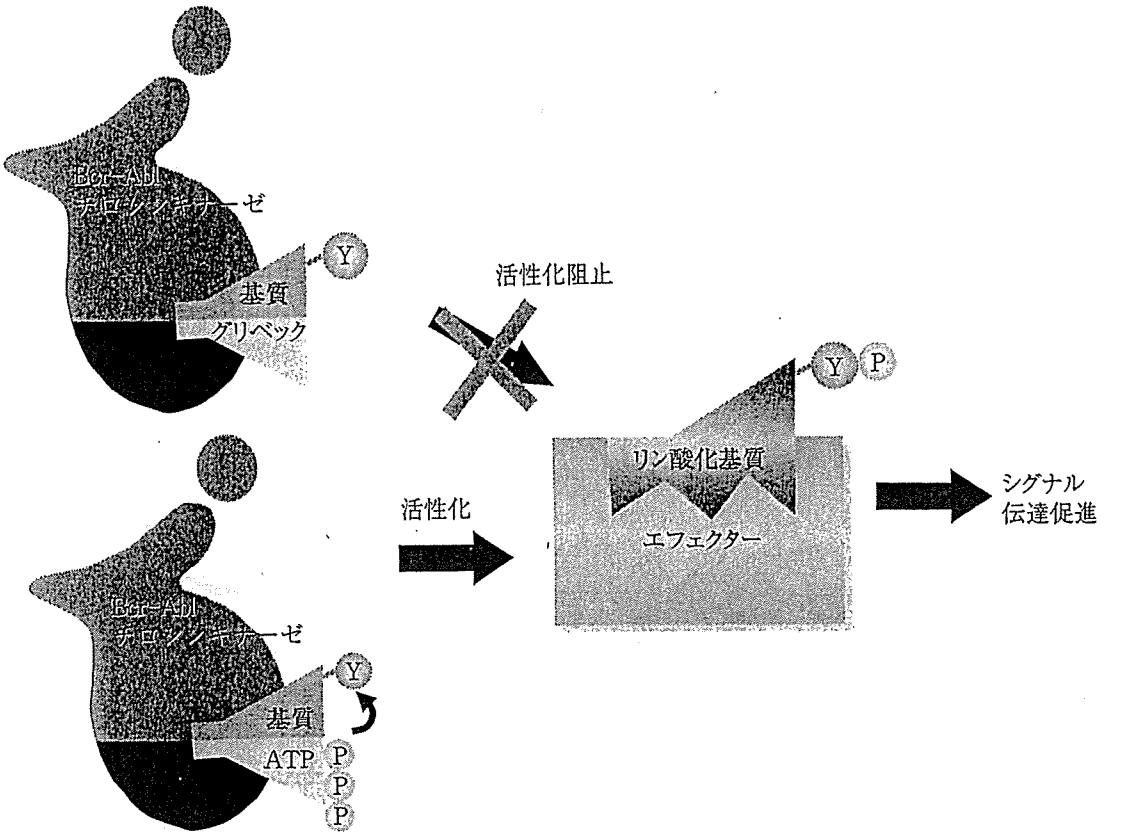


図2 慢性白血病治療薬(グリベック)の蛋白構造に基づく作用機構

体に由来するBcr-Ablチロシンキナーゼが恒常的な増殖シグナル伝達系の活性化を通じて慢性骨髄性白血病発症の原因になると考えられている．同酵素はATPと基質に結合し，ATPから切り離したリン酸基で基質のチロシン残基をリン酸化する．グリベックはBcr-Ablチロシンキナーゼの ATP結合部位の詳細な構造に基づいて設計され，基質のチロシンリン酸化を構造特異的に阻害して白血病化を防ぐ(図2)[2]．

このような構造に基づいて薬剤設計を行うことで標的蛋白との結合の特異性を高め，副作用を減少させることを期待できる．

2. ヒト心筋トロポニンの構造解析とそれに基づく創薬の可能性

心筋収縮を調節する心筋トロポニンの中核部分(コアドメイン)の構造は分担研究者である武田と理化学研究所の前田らによって解析され，Nature誌に報告された(Vol 424, 2003)[3]．前田らの総説[4]に基づき，トロポニンの筋収縮調節メカニズムについて述べる．

筋収縮はアクチンとミオシンの滑り運動による．アクチンフィラメントはアクチン，トロポニン，トロポミオシンを含む複合体であり，それらの3分子は7：1：1の存在比をもつ．トロポニンの存在下でアクチンとミオシンはカルシウムイオン濃度に応じた収縮と弛緩を行う．

図3に心筋トロポニンのコアドメインの構造を示す．トロポニンはTnC，TnI，TnTと呼ばれる3つのポリペプチド鎖からなる．これまでの研究により，TnIは収縮抑制因子，TnCは脱抑制因子，TnTはTnCの脱抑制を弱める因子(カルシウム濃度依存性の付加因子)であることが示されている[5]．

トロポニンのコアドメインは更に調節頭部とITアームの2つのサブドメインに分かれる．調節頭部はカルシウムイオンとの結合を通じてトロポニンの構造変化とそれに基づくアクチンとミオシンの滑り運動に対するスイッチの役割を果たす．ITアームは剛性を有するコイルドコイ

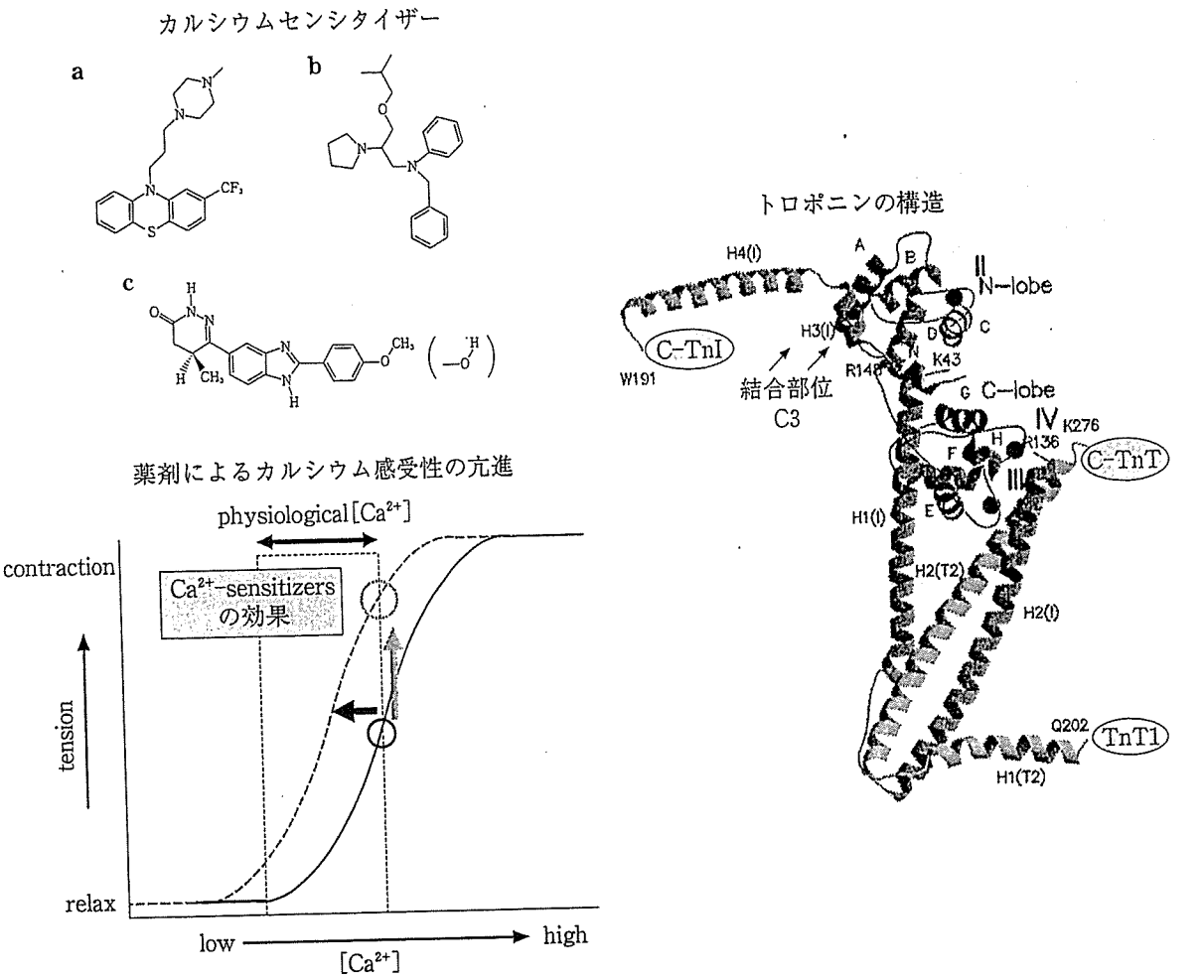
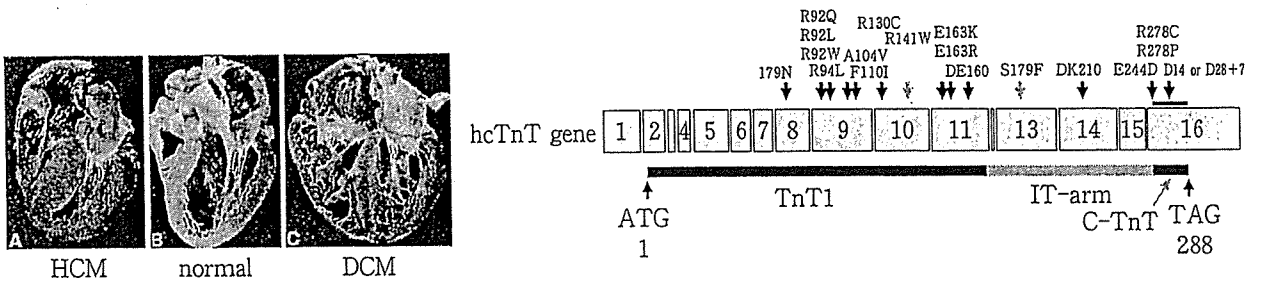


図3　トロポニンコアドメインの構造(文献[3]より改変引用)

ル構造からなる．TnCはN末端側とC末端側の2つの球状部がαヘリックスで連結された構造をもつ．カルシウム濃度にかかわらずC末側球状部はTnIに結合し，TnCをトロポニン分子内に常につなぎとめている．一方，TnCのN末端側球状部は細胞内カルシウム濃度が上昇した場合のみ構造が開き，TnIの第二結合部位(両親媒性αヘリックスH3)を結合する．これにより，TnIの調節領域全体がトロポミオシン/アクチンより解離し，アクチンとミオシンの滑り運動が始まる．

TnCのN末端側球状部にカルシウムセンシタイザーが結合すると，同球状部は開いた構造をとりTnIの第二結合部位を結合しやすくなる．すなわち，TnCによるTnIの脱抑制が起こりやすくなる．前述のようにTnTはTnCの脱抑制作用にカルシウム濃度依存性を付加することができるので，TnCとTnTの制御を組み合わせることで段階的な筋収縮の増強を実現できるかもしれない．近年循環器領域では血管作動性薬剤で優れた新薬が数多く開発されてきたが，ジギタリス以来，これを超える強心剤が生まれていない．従来の強心剤は細胞内カルシウムイオン濃度を高めて強心作用を誘導するために，細胞に対する負荷(カルシウムoverload)が不可避であった．1980年代後半に開発されたカルシウムセンシタイザーと呼ばれた薬剤群はカルシウムイオン濃度-張力関係を左方にシフトさせることにより，低い細胞内カルシウムイオン濃度で高い収縮力を得ることができる理想的な強心剤ではないかと期待された[6]．しかしながら，これらの薬剤の臨床使用経験から，短期的に心筋収縮力は高まるものの，心不全患者の長期予後の改善に役立つことはなかった．これらのカ



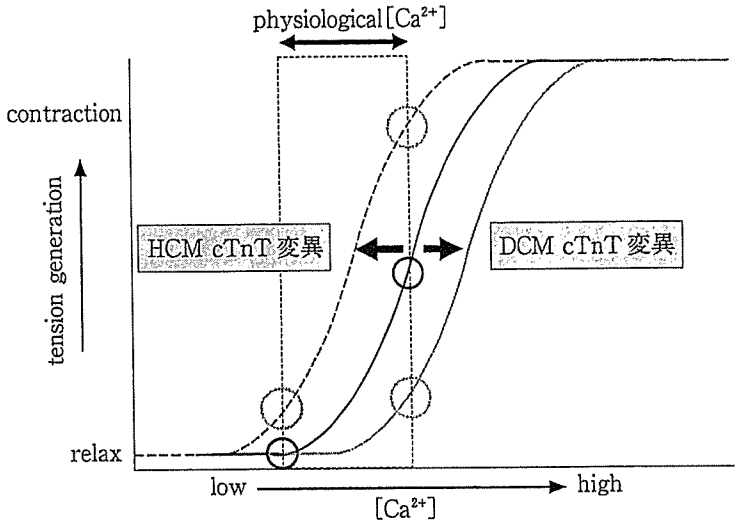


図4 心筋症におけるトロポニンの遺伝子変異と筋カルシウム感受性
心筋症の遺伝子変異はTnT1，C-TnTに多く，筋カルシウム感受性を修飾する．

ルシウムセンシタイザーはphoshodiesteraseの阻害作用も併せてもっており，細胞内cyclic-AMPの増加によって筋小胞体からのカルシウムイオン放出が増加し，ついにはカルシウムoverloadとなる可能性や[7]，構造が類似した他の蛋白と相互作用があるなど，薬剤としての標的特異性が低いことが原因として考えられる．拡張型心筋症例では，少なくとも一部の症例でカルシウム感受性の低下と収縮不全の関連が示唆されている．これらの事実はTnCやTnTを特異的に制御する化合物の設計により，新たな強心剤の開発の可能性を示している．

一方，肥大型心筋症(HCM)ではトロポニンの遺伝子変異によりカルシウム感受性が亢進することが発病に関連する可能性が示唆されている．同患者の遺伝子解析によると，約15%の患者にTnTの遺伝子変異が認められる．大槻らによれば[5]トロポニンがアクチン/トロポミオシンと直接接触する部分(TnT1，C-TnT，TnI調節領域)に変異が多く認められ，コアドメインには変異は少ないという(図4)．変異TnTの交換導入を行った心筋スキンドファイバーを用いた研究で，カルシウムイオン濃度-張力関係の左方シフト，すなわちカルシウム感受性の亢進が認められた．この結果からTnTの変異により，カルシウム感受性が亢進し，収縮増加と弛緩不全という肥大型心筋症に特有の症状が発症するという有力な仮説が生まれる．TnTの変異によるカルシウム感受性亢進のメカニズムを原子構造で解明すると，肥大型心筋症に特異的に作用する薬剤の設計を期待できる．原因となる遺伝子変異ごとに構造が異なる薬剤設計が求められる可能性もある．言い換えれば，心筋トロポニンの変異に基づく肥大型心筋症の治療法の開発はテーラーメード医療のモデルケースとなる可能性がある．

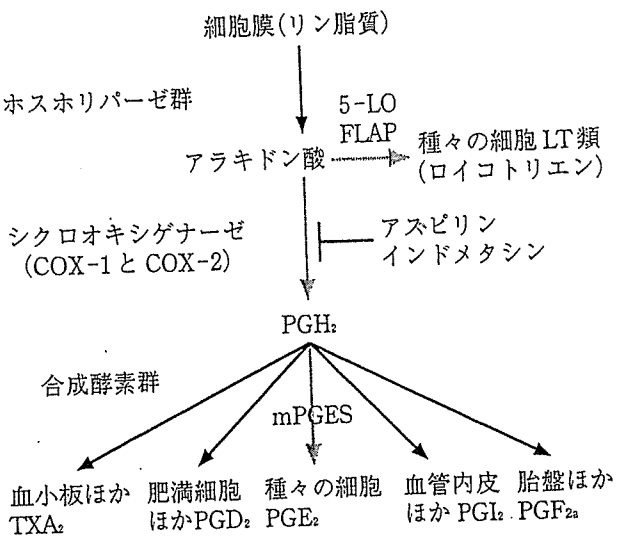


図5 プロスタグランジン産生系

3. 創薬の標的として注目されているプロスタグランジン合成酵素群の構造解析

シクロオキシゲナーゼ(COX)はプロスタグランジン(PG)を生合成する律速酵素として知られている(図5)2種類のアイソザイムが存在する．COX-1はconstitutive enzymeと呼ばれ，ほとんどの細胞で常時発現しており，生体の安定性を維持する役割を果たす．一方，COX-2はinducible enzymeとして，単球，線維芽細胞，滑膜細胞などの炎症にかかわる細胞で発現し，炎症性サイトカインなどによって誘導される．従来の非ステロイド系抗炎症剤は，COX-1とCOX-2の両方を阻害するために炎症巣のPGだけでなく，胃粘膜や腎でのPG(特にPGE_2)産生を抑制し胃や腎の副作用を合併する．そこで，炎症に深く関与していると考えられるCOX-2だけを選択的に阻害する薬剤の開発が進められてきた．このようにして開発されたCOX-2阻害薬は胃潰瘍を起こしにくい鎮痛剤として好んで投薬されていた．しかしながら，2004年末，米政府は，これらのCOX-2選択的阻害薬の3剤を心筋梗塞や脳梗塞の危険性を高める恐れがあるとして，心臓病患者への処方や多量の長期使用を避けるよう勧告した．COX-2の下流に位置するプロスタサイクリン合成酵素の作用も抑制するために，同酵素に由来する抗血栓性作用や血流増加作用が損なわれることが原因ではないかと考えられている[8]．図5に示したようにCOX-2の下流には多くの合成酵素があってそれぞれの作用を有する蛋白を合成している．個々の合成酵素を選択的に阻害する薬剤の開発が次世代の創薬の標的として注目される．PGE_2の産生にかかわるmPGESを阻害する薬物の開発は血管内血栓形成を伴わない理想的な抗炎症剤となる可能性がある．TXA_2産生を阻害する薬剤の開発は血管内血栓形成の予防，局所血流増加作用を通じて脳梗塞，心筋梗塞の予防薬や治療薬として期待できる．PGI_2は既に難病といわれた原発性肺高血圧症の治療に有効であることが知られている．PG関連薬剤の開発は構造に基づく創薬の最大の標的の一つになっており，ナノメディシンプロジェクトでも複数の関連酵素の構造解析に取り組んでいる．

4. ナノメディシンプロジェクトのそのほかの研究

本プロジェクトでは分子構造イメージングに関連して上記のほかに，細胞内イオン環境や，血管新生にかかわる蛋白など幾つかの蛋白構造についても研究を進めている(国立循環器病センター研究所)．国立精神神経センターではin-silicoスクリーニング法によるParkinson病の治療薬探索に蛋白構造情報を応用する研究を進めている．国立医薬品食品衛生研究所では原子間力顕微鏡を用いて蛋白表面の詳細な構造を解析することなどを通じて，医用材料作成に向けた応用研究に取り組んでいる．

一方，分子機能イメージングの領域では，国立循環器病センターの望月らが増殖因子(EGF)刺激に伴うRas分子の活性化をFRET法で可視化できることをNature誌に報告した[9]．ナノメディシンプロジェクト開始後も血管内皮の走化運動にかかわるRap1蛋白の可視化に関する研究などにFRET法による分子イメージングを展開している．国立精神神経センターの研究グループでは分子機能イメージング技術を応用してシナプス機能，プリオン蛋白質の機能の評価に

取り組みProc Natl Acad Sciなどの雑誌に研究成果を報告している[10].

おわりに

本ナノメディシンプロジェクトでは循環器治療の中核施設である国立循環器病センター内に構造生物学ラボを立ち上げ，分子特異的な治療薬の開発を目指している．ナノDDS技術や分子機能イメージング技術に関する研究を併せて推進することで，特異的分子治療薬の分子輸送技術開発と他の分子との相互作用の可視化技術を推進することが可能となる．これにより，分子診断・分子治療・分子評価を包含するテーラーメード医療の基盤形成に貢献したい．

謝辞　本原稿の執筆内容は本研究グループの成果を元にしております．国立循環器病センター研究所若林繁夫分子生理部長およびユーセフ・ベン・アマー同研究員，増田道隆循環器形態部室長，柴田洋之心臓生理部同室員，五十嵐智子同研究員，松原孝宜同研究員，大阪大学月原富武教授，理化学研究所宮野雅司主任研究員に感謝いたします．また，本原稿編集と英文作成に協力していただいた東本弘子女史，松尾千重女史に感謝します．

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Review

Importance of Considering Injured Microorganisms in Sterilization Validation

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Disinfection or sterilization treatment by heating, irradiation, or chemicals can cause injury to microorganisms at sublethal levels. Microbial injury is the inability to grow under conditions suitable for the uninjured microorganisms. This inability of injured microorganisms to grow is explained in terms of more complex or different nutritional requirements or in terms of increased sensitivity to environmental conditions such as incubation conditions (time or temperature) or to chemical agents such as halogen compounds. Injured microorganisms can be distinguished from those that are dead or mutated by their ability to regain normal physiological activity when placed in appropriate conditions for cultivation. The return to normal physiological function has been termed repair. The extent and severity of sublethal injury, the mechanisms of injury, and the mechanisms and degree of recovery vary with the sterilization procedures, the species, the strains, the condition of the microorganism, and the methods of repair. Injury to spore formers has been detected at different stages of the spore cycle. The sites of injury include damage to enzymes, membrane disruption, and/or damage to DNA or RNA. Information on the sublethal injury and recovery of microorganisms is very important in evaluating sterilization/disinfection procedures. This paper supplies academic as well as practical information dealing with the repair, and detection of injured microorganisms for performing reproducible sterilization validation.

Key words : Injured microorganisms/Injury/Repair/Damage/Sterilization validation

INTRODUCTION

The injury to microorganisms by various sterilization procedures is a common occurrence. The detection of injured microorganisms is of significance for evaluating the sterilization process in health care products, and attainment of sterility assurance and reproducible sterilization validation (Busta, 1978; Hurst, 1977; Hurst, 1984a). Inadequate detection of injured microorganisms in health care products can lead to potential spoilage and hazards if the injured microorganisms undergo repair and proliferate during product storage. Therefore it is important to employ techniques that will optimize the detection and enumeration of injured microorganisms. Ignorance or poor recovery of injured microorganisms may lead to an overestimation of lethal effects, which can result in faulty sterilization validation. This must be avoided. An understanding of injury can supply valuable information to optimize the proliferation and maintenance of microbial cultures.

It is important to understand the resistance, injury, repair, and detection of the bioburden to attain satisfactory and reproducible sterilization validation.

1. Importance of inactivating spore formers

The spore cycle is described in Figure 1. Spores are formed in the vegetative cell during sporulation and are released into the environment during cell lysis. Spores can withstand high doses of heat, irradiation, and chemicals.

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The typical structure of bacterial endospore consists, from the inside to the outside, of a protoplasmic core, a spore wall, a cortex, the spore coats, and exosporium (Figure 2). The spore coats protect the spores and respond to germinants. The cortex and the cell wall are comprised of peptidoglycan (Figure 3). The spore cell wall forms the cell wall of the germinated cell. The dehydrated core consists of DNA, ribosomes, enzymes, and other cellular components including calcium (Barach et al., 1976), magnesium, manganese, and dipicolinic acid (Denyer et al., 2004). The chemical structure of dipicolinic acid is shown in Figure 4. Bacterial spores in the injured state are not considered as threats unless they regain metabolic activity.

The objective of health care product sterilization is to destroy bacterial spores, rendering the product safe while retaining acceptable quality for long periods of time. The repair of injured spores may lead to product quality loss and/or safety problems to people. Thus, it is quite important to detect undamaged as well as injured spores in products to avoid any risks concerned. The recovery and enumeration of normal or injured spores which are susceptible to injury and repair involve the completion of the various stages in the spore cycle in Fig. 1 (Gould, 1984).

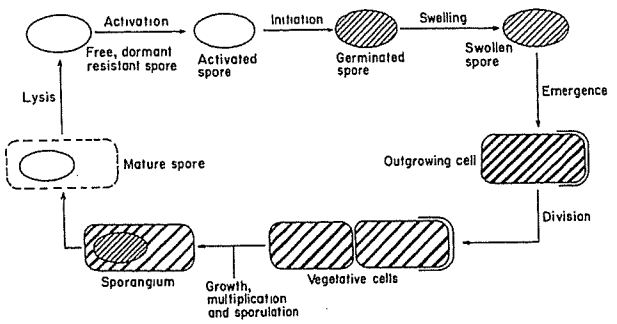

FIG. 1. Diagrammatic representation of the cycle of bacterial endospore formation, germination and outgrowth

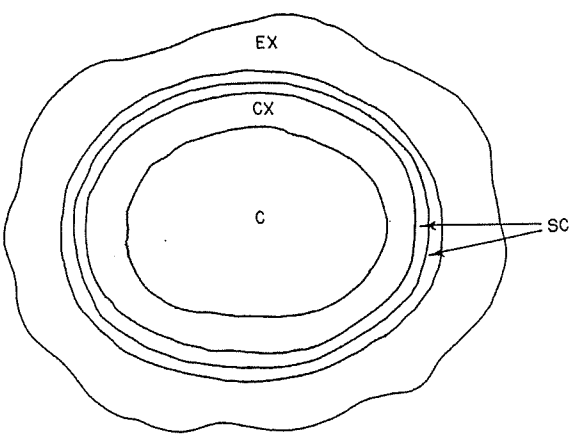

FIG. 2. A typical bacterial spore
EX, exosporium; SC, spore coats; CX, cortex; C, core

2. Definition

The representative cycle of bacterial endospore activation, germination, outgrowth and growth is presented in Fig. 1.

2-1. Activation

Activation is generally accomplished by sublethal levels or other treatments (heating, irradiation, chemical agents exposure and so on) which do not cause significant changes in the properties of the dormant spore, but accelerates the germination process. This treatment also inactivates the vegetative cells in the medium.

2-2. Germination

The sequence of major changes accompanying endospore formation and germination is presented in Table 1. During the stage of germination, the dormant state of the spore is irreversibly terminated. When spores germinate they lose their characteristic resistance to heat, radiation, chemicals, and other stresses. Germination is initiated by several agents. They include nutrients (e.g., amino acids and sug-

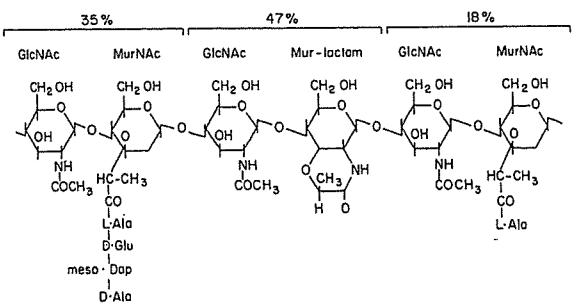

FIG. 3. Chemical structure of peptidoglycan

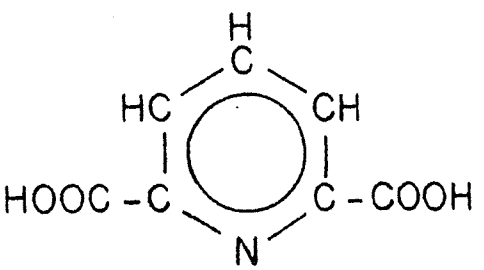

FIG. 4. Chemical structure of dipicolinic acid

TABLE 1. Sequence of major changes accompanying endospore formation and germination

Spore formation (timescale commonly 8 h)	Spore germination (timescale commonly 5 min)
End of vegetative growth	Addition of germinants
Chromatin filament formed	Heat resistance lost
Spore protease excreted	
Forespore septum formed	Calcium and dipicolinic acid excreted
Forespore protoplast engulfed	Temporary rise in resistance to ultraviolet irradiation
Heat resistant catalase formed	
Peptidoglycan cortex synthesized	Refractility loss observable by phase contrast microscopy
Spore becomes refractile	
Dipicolinic acid synthesized	
Calcium taken up	Resistance to stains lost
Proteinaceous spore coats assembled	Release of fragments of hydrolysed peptidoglycan
Resistance to organic solvents acquired	
Resistance to heat acquired	Fall in extinction of spore suspensions
Mother cell lyses to release mature spore	Onset of metabolism

ars), nonnutrient germinants (e.g., metal ions, bicarbonate, and calcium dipicolinate), enzymes (e.g., lysozyme), and physical treatments (Adams, 1973; Adams, 1974; Alderton et al., 1974). According to the present author's experiment, Ca, vitamin mixtures, L-alanine, puruvate and glucose are appropriate agents to promote germination (Shintani, 2006). Mg ion does not successfully promote germination contrary to reported results (Cazemier et al., 2001; Busta et al., 1976).

2-3. Outgrowth

In this stage, the germinated spore is transformed into the first vegetative cell. It is a metabolic step involving the synthesis of RNA, proteins, DNA, and the cell wall and membrane, which differentiate the spore into a vegetative cell (Adams, 1978).

2-4. Growth

The stage involves metabolic growth which can be observed as an increase in the number of vegetative cells. The inhibition of the growth or the doubling process will prevent detection of the organism.

3. Spore injury

Injury is defined as damage incurred during exposure to sublethal environmental stresses. Injury may cause a longer lag period, increased sensitivity or decreased tolerance to a variety of chemical agents and the inability to multiply until repair of the injury has been attained. Repair is characterized as a return to normality and is defined as the correction of the injury-mediated damage and the process involved therein.

One major difference between bacterial spores and vegetative cells is the high resistance of the spore to environmental stresses. Several types of injury have been reported for bacterial spores (Adams, 1978). Spore injury is more complex than injury to vegetative cells (Hurst, 1984b) because any of the several steps in the spore cycle can be independently affected (Fig. 1). The injury and repair mechanisms of bacterial spores are different from those of vegetative cells due to the dormant and resistant state of spores. Several stages are involved in the transformation of a spore into a vegetative cell, and there is the possibility that a spore is defective in nature during sporulation (Gould, 1984). Some treatments may injure spores by mechanisms different from those involved in the injury to the vegetative cells. Treatments such as freezing and drying, which injure vegetative cells, are not usually implicated in the injury of spores. The physical treatments involved in damage to bacterial spores include heating, ionizing and UV radiation, and hydrostatic pressure. Chemical injury to spores occurs due to changes in the pH, ion exchange treatments, and disinfectants such as hypochlorite, phenol, hydrogen peroxide, peracetic acid, and ethylene oxide. Enzymatic treatments can cause spore characteristics and spore coat permeability is altered with lysozyme and hydrolytic enzymes. Metabolic factors may be active during sporulation and include the lack of important nutrients and altered levels of divalent cations of Ca, Mg, Mn and so on. Injury towards bacterial spores is expressed in various forms. There is a need for nonnutrient germination stimulants by the injured spores, modified optimum incubation temperatures for the enumeration of survivors, an increased sensitivity of the survivors to inhibitors and selective agents, and altered nutritional requirements by the survivors (Adams, 1978). Other factors required for recovery includes changes in pH, the oxidation-reduction potential, the recovery medium, the

incubation period and so on.

3-1. Injury by heating

Elevated temperatures may cause stress and injure bacterial spores (Foegeding and Busta, 1981; Gould, 1984). Bacterial spore injury by heating has been observed in the temperature range of 50 to 170℃ and on spores of both aerobic and anaerobic bacteria (Adamas, 1978). Heat-injured spores are unable to grow under conditions that are optimal for unheated spores. They exhibit sensitivities to antibiotics and curing agents, such as sodium chloride (Briggs and Yazdany, 1970), fastidious growth requirements, the need for nonessential agents, such as starch, activated charcoal, lysozyme, and other lytic enzymes, altered incubation temperature and an extended lag phase (Gould, 1984).

3-2. Radiation injury by ionizing and UV irradiation

Spore injury caused by irradiation has been reported (Farkas et al., 1995; Foegeding and Busta, 1981). It is an interesting phenomenon that spores injured by irradiation can generally initiate germination at rates even faster than those of unirradiated controls. This appears different from the injury caused by heat, which is expressed as damage to germination (Gould, 1984). An explanation for this is that irradiation damages spore DNA that is not needed for the initiation of germination. From this, irradiation injury is usually expressed during outgrowth (Rowley et al., 1983).

The significant difference between UV and gamma-ray irradiation is that the former involves base dimer formation (e.g., thymine-thymine, thymine-cytosine, cytosine-cytosine mostly pyrimidine dimers) leading to messenger disorder while the latter consists mainly disruption of single strand or double strand of DNA. In both cases, disorder of DNA messenger to produce proteins has occurred and dinfection can be evaluated from the death or survival of targeted microorganisms.

3-3. Injury by several sorts of chemicals

Several chemicals used in disinfection and/or sterilization can injure bacterial spores, especially through their action on spore coats (Gould, 1984). Spores with damaged spore coats are more sensitive than intact organisms to chemicals. Bacterial spore injury through the action of chemicals has been reported with adverse pH conditions (Gould, 1984), ethylene oxide (Futter and Richardson, 1970a, b, Davis et al., 1978), hydrogen peroxide (Wallen and Walker, 1979), hypochlorite (Foegeding and Busta, 1983a,b), alcohols (Craven and Blankenship, 1985) and/or nitrite (Gould, 1984).

Hypochlorite injures bacterial spores such as *Clostridium botulinum* (*C. botulinum*) (Foegeding and Busta, 1983a, b). Hydrogen peroxide has injured spores of *Bacillus atroaeus* (*B. atrophaeus*) (Wallen and Walker, 1979), *B. cereus* and *C. sporogenes* (Neal and Walker, 1977). Ethylene oxide injures bacterial spores (Roberts, 1970). They are, for example, *C. perfringens* (Futter and Richardson, 1970a,b), *B. subtilis*, and *Geobacillus stearothermophilus* (*G. stearothermophilus*) (Davis et al., 1978). Alcohol treatment can reduce the activation of *C. perfringens* spore, and higher levels injure them. Ozone has been reported as an effective sporicide, especially at low pH and higher humidity at more than 90% (Sakurai et al., 2003). The removal of spore coat proteins in *Bacillus* and *Clostridium* spores enhanced their inactivation by ozone (Foegeding, 1985). Spores lacking intact coats were significantly more sensitive to inactivation by chlorine dioxide than strains with intact spore coats (Foegeding et al., 1986). Copper increases spore sensitivity to hydrogen peroxide (Bayliss and Waites, 1976; Waites et al., 1979).

3-4. Injury by combination

The phenomenon of combined chemical and/or physical treatments causing bacterial spore injury is reported by Waites and Bayliss (1984). Combination effects have also been observed between gamma-irradiation and heating. Irradiation reduced heat resistance (Gomez et al., 1980) and preheating reduced radiation resistance (Ma and Maxcy, 1981). The presence of free radical producing compounds (e.g., iodide, iodate, or iodoacetate) during radiation treatment results in strong synergistic effects expressed as the inhibition of spore germination. This is thought to be due to inactivation by the free radicals of spore proteins or enzymes involved in germination (Gould, 1984; Waites et al., 1979). Irradiated spores are more sensitive to subsequent heat treatment than unirradiated spores. The increased heat sensitivity was not observed when the spores were heated in the presence of sucrose or glycerol (Foegeding and Busta, 1981; Gomez et al., 1980). One reason is that sucrose or glycerol served as a radical scavenger with their OH functional groups and another is the damage of an osmoregulatory mechanism of rehydration involved in the development of heat sensitivity in irradiated spores. Spore damage is greater in the presence of both heat and chemical treatments and either one of these treatments can be used to sensitize the spores to the other (Waites and Bayliss, 1984). The pretreatment of spores with hydrogen

peroxide and/or peracetic acid makes them more susceptible to damage by heat. Heated spores are more sensitive to inhibition by chemicals such as hydrogen peroxide or glutaraldehyde.

4. Injury during outgrowth

The outgrowth stage of spore formers may also involve injury by several sorts of sterilization treatments. Shifts in optimal temperatures to lower levels for the recovery of heated spores have been suggested as favoring outgrowth because germination can occur in a wider temperature range (Prentice and Clegg, 1974; Busta, 1967). Injury during outgrowth may involve any of several structures and metabolic pathways (Adams, 1978). Injury may involve damage to the spore membrane, DNA or other vital components and structures of the cell. After injury, the spores were germinated with lysozyme but they retained their injury because they continued to be sensitive to chemicals. The results suggested that injury had occurred in the outgrowth stage of the life cycle of spore formers.

5. Vegetative cell injury

The injury of spores is practically more important than that of vegetative cells, because spores are more tolerant to sterilization procedures. Research is needed on the sporulation of injured cells to determine any inherent changes in the resulting spores that may influence subsequent destruction, injury, and repair processes (Hurst, 1984b). The injury of vegetative cells may be expressed as sensitivity to several sorts of chemicals, modified metabolic activity, and leakage of intracellular material (Tsuchido, 2003).

Electron microscopic observations have indicated holes and fractures in the membranes of heated B. cereus cell (Hurst, 1977). The disappearance of ribosome and the coagulation of cytoplasmic protein were also observed. Studies with *B. subtilis* and *B. cereus* have indicated reversible damage to cell membranes, DNA, and RNA.

6. Factors causing injury

The factors affecting bacterial spore destruction can also affect injury (Foegeding and Busta, 1981). The conditions influencing resistance to stress, injury, mechanisms of injury, and repair vary with the genera, species, and strains of spore formers. Spore of *B. subtilis* is less resistant to irradiation injury than that of *C. botulinum*. It is interesting that the mechanism of DNA damage by heat was different for the spore and vegetative cell of *B. subtilis* (Uchida and Kadota, 1979). Cells in the log phase are more susceptible to injury than spores or cells in the stationary phase (Mackey, 1984; Mossel and van Betten, 1984; Manas and Mackey, 2004). Important properties of the substrate include the water content, the concentration and type of ions and pH, the oxidation-reduction potential, the gas atmosphere, the presence of protective agents, and contamination with other microorganisms. Handling, storage, and treatment of the injured spore suspension after exposure to stress will also affect recovery. Important considerations include the recovery enriched media, the germinants, the nutrients, the selective agents, the antimetabolites, the oxidation-reduction potential, the gas atmosphere, the pH, the water activity, the incubation temperature, and the prolonged cultivation period (Foegeding and Busta, 1981).

Thermally injured spores have demonstrated sensitivities to sodium chloride (Briggs and Yazdany, 1970) and other curing salts (i.e., sodium nitrate or sodium nitrite), antibiotics, other chemical inhibitors, certain culture media, varying lots of culture media, the pH of the recovery medium, the oxidation-reduction potential, and the gas atmosphere during recovery. In addition, thermally injured spores have demonstrated delayed germination, a sensitivity of the germination process to media components, the need for a modified incubation temperature, and the need for a longer incubation period for optimum colony formation (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981). The spore injury by irradiation has led to a sensitivity to sodium chloride and other components, pH, certain gas atmospheres, dilution and incubation temperature, and a requirement for a longer incubation period for recovery (Foegeding and Busta, 1981). Spores injured by chemicals are sensitive to pH, gas atmosphere, sorts of media, and incubation temperature. In that meaning, to attain successful and reproducible sterilization validation, these factors must be seriously studied and a scientific rationale attained to avoid falsified validation results.

Recovery has been improved with lysozyme, high L-alanine levels, lactate, and malate, which improved germination with ferrous sulfate ($FeSO_4$), manganous sulfate ($MnSO_4$), yeast extract, glucose, and vitamin-free casamino acids, and with a longer incubation period (Foegeding and Busta, 1981, Shintani, 2006).

7. Sites and mechanisms of spore injury and repair

Sites and mechanisms of spore injury are presented in Table 2 (Waites and Baylis, 1984). Sites and mechanisms of spore injury by heat include as follows: damage to germination systems, the loss of

TABLE 2. Site of bacterial spore injury by combined treatment

Site of injury	Treatments
Coat	Heat + chlorine
	Heat + hydrogen peroxide
	Glutaraldehyde + formaldehyde
Membrane	Heat + neomycin and polymyxin
	Glutaraldehyde + formaldehyde
Cortex	Heat + hydrostatic pressure
	Heat + irradiation (γ)
	Heat + chlorine
	Heat + hydrogen peroxide
	Heat + Cu^{2+}
	Irradiation (γ) + hydrostatic pressure
	Ultrasonic waves + glutaraldehyde
Exosporium	Ultrasonic waves + hydrogen peroxide
Proteases	Heat + Cu^{2+}
Calcium removal	Glutaraldehyde + ionizing cation
DNA	Heat + irradiation (γ)
	Heat + hydrogen peroxide
	Heat + ethidium bromide
	Irradiation (γ) + hydrostatic pressure
	Irradiation (UV) + hydrogen peroxide

Cited from Waites and Baylis (1984), pp.223

cortex lytic activity through enzyme inactivation or inactivation of the mechanism involved in enzyme release, damage to the spore membrane (plasma or cortical) structures related to the need for suitable osmolarity or water activity during recovery, and damage to DNA.

Thermal injury does not cause single-strand DNA breaks. Chemical injury has been associated with the inactivation or alteration of spore-germination systems, while injury by irradiation is almost associated with single-strand or double-strand DNA breaks. This means depending on the sort of sterilization procedure, sites of injury differ, which means recovery mechanisms also differ.

7-1. Damage to germination enzymes

The germination of bacterial spores may be inactivated or altered by heat and chemical treatments (Hurst, 1984a; Gould, 1984). Damage to germination systems by heat has often been characterized by a need for specific germinants or enzymes in the medium (Foegeding and Busta, 1981; Gould, 1984). For example, Ca, glucose or alanine are considered as germinants and thus inclusion of them into the culture medium is indispensable to attain reproducible and successful sterilization validation (Shintani, 2006; Shintani et al., 2000; Sasaki et al., 2000). Damage to the germination system has been demonstrated with the use of calcium dipicolinate (Edwards et al., 1965a,b), lactate (Foegeding and Busta, 1983a), lysozyme (Busta and Adams, 1972; Adams and Busta, 1972; Barach et al., 1974; Adams, 1974; Duncan et al., 1972; Alderton et al., 1974), and mixtures of amino acids (Uchida and Kadota, 1979; Gurney and Quesnel, 1981; Gurney and Quesnel, 1980) and so on. As most chemicals that promote improved recovery (e.g., lysozyme, other lytic enzymes, calcium dipicolinate, and amino acids) are involved in promoting spore germination (Gould, 1984), it is speculated that they act by helping the spores bypass the injured L-alanine germination system (Gould, 1984).

The mechanism of injury to germination involves the inactivation of lytic enzyme systems, or the mechanism that releases these lytic enzymes, which are involved in degradation during germination (Adams, 1978). This is supported by the effect of lysozyme, other lytic enzymes, and dipicolinate in allowing the injured spores to bypass the damage and germinate. The inactivation of germination enzymes has been supported by large losses of cortex lytic enzyme activity in thermally injured spores, and by thermodynamic values for the inactivation of the L-alanine germination system that were consistent with protein denaturation (Adams and Busta, 1972). Increased recovery by the use of complex amino acid mixtures may be effective through the generation of NADH and ammonium, since these could be unavailable in the L-alanine germination system through the inactivation of alanine dehydrogenase involved in the alanine

cycle (Gurney and Quesnel, 1981; Gurney and Quesnel, 1980). Treatment with chemical agents may render the germination process sensitive to lysozyme, which enhances the germination of spores (Hurst, 1977). One example is that the treatment with alkali removes spore coat proteins and renders the germination process sensitive to lysozyme. The other example is that chlorine treatment also removes coat proteins from spores (Wyatt and Waites, 1975).

In the mechanism of the injury to germination systems, lytic enzymes, e.g., lysozyme, are speculated to enhance germination by hydrolyzing the β,1-4 linkage of peptidoglycan in the spore cortex (Fig. 3), which then permits the hydration of the core of the spore (Gould, 1984). It may involve interference with membrane function as indicated by the increased sensitivity of heated spores (Flowers and Adams, 1976) to surface active agents including antibiotics (Gould, 1984).

7-2. Damage to the spore membrane

The spore membrane and structure have been proposed as the site of injury (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981; Gould, 1984). Even when lysozyme is needed to germinate injured spores, the actual repair may be taking place during the outgrowth stage. This could indicate that injury might be associated not with a germination system, but with the membrane (Foegeding and Busta, 1981; Barach et al., 1974; Barach et al., 1975). Evidence to support the membrane damage theory includes the sensitivity of heated C. perfringens spore to several antibiotics and chemicals with surface-active properties (Barach et al., 1974; Flowers and Adams, 1976). Damaged membrane is also sensitive to sodium chloride, nitrate, nitrite, and fatty acids (Flowers and Adams, 1976; Chumney and Adams, 1980). The lack of repair during germination occurred when transferred in a medium supporting outgrowth (Barach et al., 1974; Flowers and Adams, 1976) and at the completion of repair during outgrowth. It occurred even in the presence of inhibitors of RNA, proteins, DNA, and cell wall synthesis (Flowers and Adams, 1976; Chumney and Adams, 1980). The injured spores were osmotically fragile (Gomez et al., 1980). It was observed by electron microscopy that in heat-treated spores, the plasma membrane was separated from the core of *C. botulinum*. From this it can be speculated that heat causes damage to the plasma and cortical membrane of the spore, which become the vegetative cell membrane and cell wall, respectively (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981).

Significant changes in pH may also cause damage associated with spore membranes (Gould, 1984). Alkali treatment dissolves protein components in the spore coat, which increases permeability and allows lysozyme to act on the cortex peptidoglycan. This also happens after treatments that rupture disulfide bonds (Gould, 1984). Milder alkali treatments increase the sensitivity of spores to various germinants (Vary, 1973) by improving access of the germinants to receptor sites (Gould, 1984). The effect of alkali treatment on spore coat permeability may also be responsible for the increased susceptibility of the spores to disinfectants and other chemicals, some of which (e.g., hypochlorite) also remove coat protein (Gould, 1984). Reduced pH may activate spores and enhance germination, while drastically low pH values may induce dormancy (Gould, 1984; Vary, 1973). High acidity is linked with the removal of cations, especially calcium, from the spores (Blocher and Busta, 1985; Rode and Foster, 1966), which may cause a reversible injury of the germination mechanism (Gould, 1984) because calcium is an indispensable agent for spore germination (Shintani, 2006). The exchange of cations caused by acid treatment also reduces the heat resistance of the spores (Alderton, 1964), which is also reduced by irradiation resistance (Gomez, 1980). Reduced heat resistance catalyzed by acid is reversed when the spores are reloaded with cations (Gould, 1984). The mechanism for the acid-catalyzed loss of heat resistance may be related to changes in spore hydration caused by changes in the ionic state of the spore cortex, which affects its osmotic and contraction state (Gould, 1984; Alderton, 1963).

7-3. Injury to DNA and RNA

Injury by irradiation or heat may also be due to changes in the genetic materials of the cell, especially DNA (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981; Gould, 1984). Breaks in the single-strand of the spore DNA have been identified in irradiated spores (Grecz and Grice, 1978). The spore DNA is more resistant to single- and double-strand breaks than the DNA of vegetative cells. The ionizing and UV-radiation resistances of DNA isolated from cells and spores are similar. The increased resistance of spore DNA appears to be due to either increased structural integrity and/or its ability to repair single-strand DNA breaks after the initiation of spore germination (Grecz and Grice, 1978).

The recovery of DNA appears to take place in dormancy. The repair of DNA during dormancy is apparently catalyzed by an enzyme which appears to be a magnesium-dependent DNA ligase (Gould, 1984; Durban et al., 1974). Spore injury by UV irradiation

also involves DNA but it appears to be different from the DNA breaks caused by ionizing radiation as mentioned in 3.2.

Damage of spore DNA by UV irradiation appears to be different from that of vegetative cells. Spore damage is thought to be due to changes in the configuration of DNA (Gould, 1984). Injury and repair by thermorestoration is speculated to occur at the site of DNA (Tanooka, 1978). The synergistic effect of irradiation and heat treatments, which is expressed as an increased heat sensitivity of pre-irradiated spores (Gombas and Gomez, 1978), is probably due to the increased fragmentation of spore DNA, and the inactivation of DNA repair enzymes by heat (Gould, 1984). This indicated that spores treated only with irradiation were active (Gould, 1984). The increased heat sensitivity of preirradiated spores may be due to damaged peptidoglycan in the spore cortex, which can allow the hydration of the core and result in reduced heat resistance (Hurst, 1984a; Gould, 1984). In addition to germination enzymes and membrane damage, DNA has also been suggested as a target of spore injury by heat (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981; Gould, 1984; Gombas, 1983).

Some of the more complex nutritional requirements of heat-injured spores have been attributed to heat-induced mutations (Adams, 1981). The need for amino acid supplementation to induce the recovery of heat-injured spores has suggested that heat may damage spore DNA (Uchida and Kadota, 1979). The alteration of amino acid metabolism or amino acid-stimulated germination may be another effect of thermal injury (Gould, 1984). Dry heat also caused mutations which were expressed as reduced sporulation (Gould, 1984). Chemical damage may also occur on DNA and result in lower heat resistance (Hanlin et al., 1981).

8. Repair of injured spores

Several papers have been published on this subject so far (Hurst, 1984a; Gould, 1984; Ray and Adams, 1984; Waites and Baylis, 1984; Mossel and van Netten, 1984; Mackey, 1984, Gilbert, 1984a,b; Johnson and Busta, 1984). The role of medium constituents in the recovery of injured spores has been discussed (Blocher and Busta, 1982; Shintani, 2006; Shintani et al., 2000; Sasaki et al., 2000). They described the effects of inhibitors on recovery and the modified metabolic requirements of injured spores. Their conclusion is the constituents to enrich the culture medium are required by injured microorganisms. This means an enriched culture medium is more desirable than the selective medium to avoid falsified results in a sterilization validation study.

Injury was initially recognized in terms of cultural inadequacies observed in microorganisms exposed to various stresses. Most of these inadequacies need to be addressed before the organisms present any evidence of vitality through cell division. Restoration to the original undamaged condition is accomplished through the process known as repair or recovery (Busta, 1978). There is a need for the development of standardized methods for the recovery and enumeration of both injured and uninjured spores (Johnson and Busta, 1984). There are several complex steps (i.e., activation, germination, outgrowth, and growth) to be completed before the dormant spores can grow and be enumerated (Fig. 1). The same set of events should be completed for the detection and enumeration of injured spores.

The culture media and environmental conditions employed should support these events for appropriate spore recovery. Injured spores may require modified conditions for their recovery and enumeration compared with uninjured entities. The sublethal damage of spores results in various sensitivities and additional requirements for the injured spores to repair their injury and grow. Factors to be considered include sensitivity to activation treatments, germinants, selective agents, chemical additives, other inhibitors, oxidation-reduction potential, pH, water activity, osmolarity, nutritional requirements, and incubation requirements including gas atmosphere, temperature, and incubation period.

The repair and recovery of injured cells usually requires richer culture media, the removal of inhibitors, and optimum incubation conditions, including pH, relatively lower temperatures for cultivation, and an extended incubation period. The influence of these factors in specific situations appears to be dependent on the type of microorganism, the nature of the stress, and the type and extent of injury (Johnson and Busta, 1984). An activation treatment usually consists of heating at 80℃ for 10 min, but specific requirements may vary with the strain, the injury, the suspending medium, and the recovery medium (Johnson and Busta, 1984). Some specific factors to be monitored during recovery are the condition of the population before its exposure to stress, handling and storage of the sample before enumeration, the composition of the recovery medium (e.g., the type of medium, nutrients, and inhibitors), the dilution procedures and diluents, the mode of inoculation, the enumeration procedure (e.g., plating or MPN), and the conditions of incubation (Mossel and van Netten, 1984). The recovery requirements may be different with the type and extent of injury, the species, strains,

and individual spore suspensions (e.g., the conditions of sporulation and handling before and after exposure to stress), storage and handling after exposure to stress and before recovery, and the conditions of recovery. Research on specific recovery media and constituents for injured spores is limited and not systematic (Table 3). The responses may be different with varying strains and the types of injury (Blocher and Busta, 1982; Farkas and Roberts, 1982) Thus, specific media may be appropriate for recovery under certain conditions, and different individual components may be needed for enumeration under different conditions of injury. Variations in media, their constituents, purity, and storage period may be influential in the repair of injured spores. Media components that may enhance recovery under certain conditions include potential nutrients such as sugars, yeast extract, glucose, and amino acids, absorbants such as starch, reducing agents such as thioglycollate and cysteine, lysozyme and egg yolk, bicarbonate, cultural filtrates and divalent cations (Blocher and Busta, 1982). In addition to the media and their constituents, the appropriate pH, gas atmosphere, oxidation-reduction potential, incubation temperature, and incubation period should be selected for the individual strains to recover from specific types of injury.

Injured spore formers have significant sensitivity to sodium chloride (Feehery et al., 1987), nitrate and nitrite (Chumney and Adams, 1980), surface-active agents including antibiotics (Chumney and Adams, 1980), pH (Feehery et al., 1987), water activity and osmolarity (Mattick et al., 2001), gas atmosphere (Feehery et al., 1987), culture media, media brand and their lots (Pflug et al., 1979; Pflug et al., 1981; Sasaki et al., 2000; Shintani and Akers, 2000; Shintani et al., 2000), and incubation temperature and period (Feehery et al., 1987; Shintani et al., 2000). The type and extent of these sensitivities vary with the species and the type of injury.

Irradiation-injured spores in general were less affected by pH than by heat-damaged spores (Futter and Richardson, 1970). It was indicated that only two modifications have been recommended widely and adopted in the specific detection of injured and uninjured spores (Ray and Adams, 1984). They include the use of starch as a binder of potential inhibitors in the recovery medium and extension of the incubation period for the detection of survivors. Other potential requirements and modifications for injured spore

TABLE 3. Effect of added agents to culture media on the recovery of injured bacterial spores

Ingredients	*Clostridium*	*Bacillus*
Nutrients		
Glucose	0,+	0,+
Fructose		+
Sucrose	0	+
Galactose		+
Maltose		0,+
Egg yolk	+	+
Yeast extract	+	0,+
Casamino acids		+
Lactate	+	
Absorbants		
Starch	0,+	+
Charcoal	+	+
Serum albumin	+	
Reducing agents		
Thioglycollate	−,+	0
Cysteine	+	0
Cations		
Magnesium		+
Calcium	+	+
Iron		+
Others		
Bicarbonate	+	
Lysozyme	+	0
Cultural filtrates	+	+

0: No effect, −: Reduced recovery, +: Increased recovery
Cited from Blocker, J.C. and Busta, F. F. (1982).

formers to recover are discussed in the following.

8-1. Culture medium

The culture medium used to detect injured spore formers will influence their recovery (Johnson and Busta, 1984; Pflug et al., 1979; Wallen and Walker, 1979; Shintani et al., 2000; Sasaki et al., 2000; Shintani and Akers, 2000; Shintani, 2006). Several media have been used for the recovery of injured spores (Table 4). The recovery of *B. pumilus* spores injured by UV irradiation was greater in double-strength soybean casein digest broth (SCDB) than in soybean casein digest agar (SCDA) (Abshire et al., 1980). Heat-injured *C. sporogenes* P.A. 3679 spore recovered better in Anderson's pork-pea infusion than in trypticase peptone agar, yeast extract agar, pork infusion agar, and T-Best agar (Polvino and Bernard, 1982). This suggested that recovery differed when medium differed and additionally suggested that the pour-plate procedure was not adequate for the recovery of heat-stressed spores. The repair rate of *C. perfringens* was much higher in trypticase yeast extract broth than in 0.1% peptone (Traci and Duncan, 1974). The recovery of heat-stressed *C. perfringens* was also better on tryptone-sulfite-cycloserine medium without egg yolk than on sulfite-polymyxin-sulfadiazine medium (Orth, 1977). More heat-stressed *C. sporogenes* recovered on modified P.A. 3679 agar than on yeast extract agar and peptone trypticase agar (Grischy et al., 1983). Treatment of *B. megaterium* with chlorhexidine resulted in a sensitivity to potassium chloride during recovery in SCDA, but recovered in SCDB (Nadir and Gilbert, 1982). Recovery is influenced not only by the kind and constituents of the culture medium, but also by the condition of the medium (Pflug et al., 1981; Pflug et al., 1979), the quality of the constituents (Johnson and Busta, 1984; Shintani et al., 2000; Sasaki et al., 2000), and the brand and the lot of the medium (Pflug et al., 1981; Pflug et al., 1979; Shintani et al., 2000; Sasaki et al., 2000). Different lots of soybean casein digest, even from the same manufacturer, resulted in different rates of recovery of heated *G. stearothermophilus spores* (Pflug et al., 1981; Shintani et al., 2000; Sasaki et al., 2000; Shintani and Akers, 2000; Shintani, 2006). This lot to lot variation

TABLE 4. Culture media used for the recovery of injured spore formers

Treatment	Organism	Medium
Heat	*Clostridium sporogenes*	Yeast extract starch bicarbonate agar
		Yesair's pork infusion+thioglycollate
		Pork-pea agar
		Pork-pea or beef infusion extract
		Thioglycollate milk+bicarbonate
		Beef infusion or yeast extract agar
		Anderson's pork-pea infusion
		Modified P.A. 3679 agar
	C. botulinum	Yesair's pork infusion+starch
		Yeast extract starch bicarbonate agar
		Yeast extract agar
	C. perfringens	Tryptone-sulfite-cycloserine
		Shahidi-Ferguson perfringens agar
		Trypticase yeast extract broth
		Sulfite-polymyxin-sulfadiazine medium
	Bacillus cereus	Mannitol egg yolk polymyxin
	B. stearothermophilus	Antibiotic assay medium A
		Eugon broth
	B. subtilis	Beef infusion
		Columbia broth
	Desulfotomaculum niginficans	Soytone-sulfite agar
Irradiation	*C. sporogenes*	Eugon agar cystine
		Pork-pea infusion
	C, botulinum	Pork-pea infusion
	B. pumilus (UV)	Double-strength trypticase soy broth
Ethylene oxide	*B. subtilis*	Columbia broth
Hydrogen peroxide	*B. subtilis*	Yeast extract, glucose, casamino acid agar + $FeSO_4$+$MnSO_4$

Cited from Blocher and Busta (1982).

and brand to brand variation may be due to the differences in the nutrient composition among the same culture medium. New lots or brand of media and components should be validated to avoid falsified sterilization validation because consecutive success in validation studies is indispensable (Johnson and Busta, 1984; Shintani et al., 2000; Sasaki et al., 2000; Shintani and Akers, 2000). In that meaning equivalency between the old culture medium and the new culture medium must be confirmed in sterilization validation studies (Shintani et al., 2000; Sasaki et al., 2000; Shintani, 2006).

8-2. Nutritional requirements

Injured spores have fastidious requirements and may need nutrient supplementation in the recovery medium (Mackey, 1984; Shintani et al., 2000; Sasaki et al). Nutritional requirements vary with species and strains of microorganisms and are variable with respect to the injured state (Johnson and Busta, 1984). The recovery of heated *Bacillus* spores was improved when glucose or blood was added to the nutrient agar (Johnson and Busta, 1984). Several compounds (yeast extract, liver extract, glucose, fructose, mannose, galactose, sucrose, maltose, soluble starch, pyruvate, glycerol phosphate, amines, amino acids, vitamins and divalent cations) increased the recovery and apparent heat resistance of *B. subtilis* spore (Johnson and Busta, 1984). Other compounds (xylose, arabinose, trehalose, lactose, glycerol, mannitol, glycogen, lactate, acetate, and succinate) did not significantly improve the recovery of injured *B. subtilis* spore (Johnson and Busta, 1984).

Several individual and some combinations of amino acids (glycine, alanine, homoserine, threonine, valine, glutamine, arginine, isoleucine, aspartic acid, and methionine) also improved the recovery of injured *B subtilis* spore (Johnson and Busta, 1984). The recovery of hydrogen peroxide-damaged *B. atrophaeus* spore was improved with yeast extract, glucose, and casamino acids (Johnson and Busta, 1984; Wallen and Walker, 1979). In every case of injury, addition of glucose, pyruvate, alanine and divalent cations (Ca and Mg) will cause significant recovery (Shintani, 2006).

8-3. Germinants

Useful agents to initiate germination are lysozyme, egg yolk emulsion, initiation protein, and calcium dipicolinate. Among them, lysozyme is the most common additive that has improved the recovery of heat-injured *C. perfringens* (Barach et al., 1974) and *C. botulinum* spores (Alderton et al., 1974). An initiation protein produced by *C. perfringens* has also increased the heat resistance (Duncan et al., 1972). An initiation factor was also detected during the growth of *G. stearothermophilus* in certain media, and promoted the recovery of thermally injured *G. stearothermophilus* spore (Labbe, 1979). This factor appeared to be influential on outgrowth.

Calcium dipicolinate has also improved recovery, and increased the heat resistance of *B. subtilis* spores (Busta and Adams, 1972).

8-4. Detoxifying agents

The stressed microorganisms indicate the increased sensitivity to several inhibitors. Traces of inhibitory agents (e.g., unsaturated fatty acids such as linoleic acid and linolenic acid) which may be present in the culture media are speculated to be absorbed and neutralized through the addition of starch, activated charcoal, and serum albumin to the medium. Increased recovery with the inclusion of such compounds in the medium has been recognized with various spore formers (Labbe, 1979; Labbe and Change, 1995). Another study has reported no improved recovery with starch in the culture medium (Blocher and Busta, 1982). The speculated discrepancy between the results is due to the presence of inhibitors not absorbed by starch, or the presence of inhibitor concentrations higher than those absorbed by starch (Blocher and Busta, 1982).

8-5. Inhibitors

Damaged spores are sensitive to several agents, such as antibiotics and surface-active agents (e.g., sodium lauryl sulfate, sodium deoxycholate, and quaternary ammonium compounds), chloride, nitrite, nitrate, acids, alkali, unsaturated fatty acids, and so on (Barach et al., 1974; Flowers and Adams, 1976; Chumney and Adams, 1980; Tsuchido et al., 1983; Tsuchido et al., 1987). Injured vegetative cells presented a reduced recovery in media with selective agents.

However undamaged cells were perfectly tolerated and were not influenced the recovery rate. The recovery substrate should be applied to support the recovery of injured organisms (Blocher and Busta, 1982).

Modifications may include adjustments in pH, the removal of inhibitory agents, the use of different selective agents, or the use of chemicals that will support recovery (Blocher and Busta, 1982). These modifications will depend on the microbial strains and the type of injury (Gould, 1984). Injured spores are often sensitive to pH variations in the recovery medium (Futter and Richardson, 1970a). Heat-injured spores in general recovered optimally at a neutral pH (Cook and Brown, 1965; Yokoya and York, 1965). In