厚生労働科学研究費補助金

エイズ対策研究事業

免疫不全に伴う脳内潜伏トキソプラズマ原虫再活性化の事前予想と再活性化原発局所における宿主遺伝子発現レベルの網羅的解析

平成18年度～20年度　総合研究報告書

研究代表者　高島　　康弘

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研究要旨　免疫不全に伴う潜伏中のトキソプラズマ原虫再活性化は致死性の脳炎を引き起こし、AIDS 患者の死亡原因の上位を占めている。しかしながら現在のところ再活性化の直接的引き金となる現象が把握できていない。本研究は脳内局所環境における原虫再活性化の直接的引き金となる分子を同定することを研究課題としている。本研究では H18 年度において新技術を確立し、本原虫の再活性化原発部位の同定をはじめて可能とした。この系を用いた実験により H19 年度には本原虫の潜伏様式について新たな考え方を提唱した。すなわち、spontaneous な活性化によってシストから脱出した虫体を宿主免疫系が速やかに排除することによる潜伏の維持という従来の考え方に変って、潜伏中の虫体に対する免疫反応が潜伏型虫体のステージ変換そのものを阻んでいるという解釈である。言い換えれば、潜伏中の原虫に対する免疫反応が宿主細胞の内部環境を「原虫の潜伏を維持できる状態」にしているということである。これを踏まえ、H20 年には本原虫の潜伏を許す宿主細胞内中の環境を同定することを目的とする。

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A. 研究目的

現在 AIDS 患者においては、末梢血 CD4 陽性細胞数が一定以下になった場合に日和見感染症のリスクが高いと判断されて予防的投薬が開始されている。しかし CD４陽性 T 細胞数は個々の日和見感染症病原体の活動レベルとは直接関係のない値である。したがって CD４陽性 T 細胞数を指標とすると日和見感染症発症リスクを見逃したり、それほど危険がない時期にも投薬を行ってしまったりする可能性が高い。日和見感染発症にいたる直接的な宿主側要因を同定しこれをモニターできるようになれば、ピンポイントに再活性化時期を予測することが可能となり、真にハイリスクな時期のみの短期間投薬が可能となろう。AIDS にともなう日和見感染症には、潜伏感染からの再活性化によって発症にいたるものが少なくない。そこで本研究ではこのような発症メカニズムをとる病原体のひとつであるトキソプラズマ脳炎をモデルとして、原虫の潜伏と再活性化を決める宿主側の要因を検索することを目的とした。このような要因、とりわけ再活性化原発部位で直接的に原虫の運命を決定する因子が同定できれば、潜伏からの再活性化を予想するための重要な因子となりうる。

B. 研究方法

１．原虫再活性化原発部位の同定

１－１．動物および寄生虫

C57BL/6J および ＭＡＬＢ/c マウスは Oriental Yeast Co., Ltd. (Tokyo, Japan)より購入し、岐阜大学実験動物委員会の承認のもと維持し実験に供した。トキソプラズマ原虫 PLK 株および、同株を親株として作成した組換え原虫は 7.5％ウシ胎児血清添加 RPMI1640 培地で培養した Vero 細胞に感染させて維持継代し必要に応じて実験に供した。

１－２．赤色蛍光発現組換え原虫の作成

dense granule protein 1 (GRA1) 遺伝子の３‘末領域および the surface antigen 1 (SAG1) 遺伝子の５’領域は同領域の配列を含むプラスミドをテンプレートとして、それぞれ以下のプライマーを用いて PCR にて増幅した。

5’-GAATTCCTGGCGAAATCAACGCACACCAAAA

ACTTG-3' (forward)
および
5'-GGATCCTATACAAATAATTAATTAAGACTACGA
CGA-3' (reveres)、
および
5'-AAGCTTTTACATCCGTTGCC-3' (forward)
5'-GGATCCACTCGTCAAAAAACCAGAAG-3'
(reverse)。
下線部はそれぞれ、*Eco* RI、*Bam*HI 、*Hind III*、*Bam*HI の認識サイトである。増幅された断片は pT7Blue T-Vector (Novagen, Darmstadt, Germany)にクローニングしたのち遺伝子配列を決定した。得られたプラスミドをそれぞれ *Bam*HI および *Eco* RI、*Bam*HI および *Hind III* で切断することにより GRA1 遺伝子３'末領域および SAG1 遺伝子５' 領域を得た。得られた GRA1 遺伝子３'末領域は *Bam*HI および *Eco* RI で消化した pUC19 プラスミドに挿入した。さらにこのプラスミドを *Bam*HI および *Hind III* 消化し、GRA1 遺伝子３'末領域を挿入した。得られたプラスミドは pUC/SAG-GRA とした。DsRed Express 遺伝子は pCMV-DsRed Express (Clontech, Palo Alto, CA) を *Nhe* I and *Not* I で切断することによって得、平滑末端化の後、pUC/SAG-GRA の *Bam* I サイトに挿入した。得られたプラスミドを pSAG-RED とした。DsRed Express 発現ユニットを含む pSAG-RED の *Eco* RI - *Hind* III 断片を pDHFR-TSc3 の *Spe* I サイトに挿入した。得られたプラスミドは pToxo-Red とする。Vero 細胞で維持したトキソプラズマ原虫 PLK 株に pToxo-Red をエレクトロポレーション法にて導入した。導入後のトキソプラズマ原虫を VERO 細胞に感染させた。ピリメタミンを含んだ培地を用いて数代経代したのち、赤色蛍光を発する原虫の濃縮がみられたサンプルについて原虫のクローニング作業を行った。得られた組換え原虫株を PLK/RED とした。

１－３．赤色蛍光発現の安定性確認

得られた組換え原虫 PLK/Red を in vitro にて薬剤選択圧のない環境で約１ヶ月経代した後、赤色蛍光を観察した。また C57BL マウスに感染させ、感染後数日後の肝臓・脾臓・脳を採材して感染したトキソプラズマ原虫タキゾイトを蛍光顕微鏡で観察し、赤色蛍光を発することを観察した。この際、コントロールとして少量の緑色蛍光蛋白発現株（PLK/GFP）を共感染させて緑色蛍光も観察した。

１－４．ステージ特異的発現の確認

作成した赤色蛍光発現組換え原虫 PLK/RED と、原虫のステージにかかわらず常に緑色蛍光を発する既存の原虫（PLK/GFP）を in vitro にて VERO 細胞に混合感染させ、pH8.2 の培地をもちいてエアーインキュベーター内で培養しタキゾイトからブラディゾイトへのステージ変換を促す刺激とした。刺激前と 24 時間後で緑色蛍光と赤色蛍光の強度を比較した。また、PLK/RED と PLK/GFP を C57BL マウスに感染させ、感染後６週間以上たってから脳内のシストを蛍光顕微鏡にて観察した。

２．原虫の潜伏動態の把握

２－１．動物および寄生虫

BALB/c マウスは Oriental Yeast Co., Ltd. (Tokyo, Japan)より購入し、岐阜大学実験動物委員会の承認のもと維持し実験に供した。トキソプラズマ原虫 PLK 株および、同株を親株として作成した組換え原虫は 7.5%ウシ胎児血清添加 RPMI1640 培地で培養した Vero 細胞に感染させて維持継代し必要に応じて実験に供した。

２－２．ステージ特異的蛍光発現組換え原虫 PLK/DUAL の作成

トキソプラズマ原虫のタキゾイト期（活性化期）に特異的に活性を有する SAG1 プロモーター支配下に赤色蛍光タンパク（DsRedExpress）遺伝子をつないだ発現ユニット、ならびにブラディゾイト期（潜伏期）に特異的に活性を有する BAG1 プロモーター支配下に緑色蛍光タンパク（GFP）遺伝子をつないだ発現ユニットをトキソプラズマ原虫 PLK 株に組み込んだ。その後、H18 年度に開発した手法に従って組換え原虫 PLK/DUAL を得た。

２－３．トキソプラズマ原虫感染様式の確認

BALB/c マウスに PLK/DUAL 株を感染させ、急性期および潜伏期に脳を採材して観察した。観察は脳組織の小片をスライドグラス上で圧潰した標本か、H18 年度に開発したプラスチック抱埋による薄切標本を用いた。再活性化した原虫を観察するため、潜伏感染中のマウスに H18 年度と同じプロトコールで免疫不全を誘発し発症の前後で脳を観察した。

３．原虫の潜伏維持に必要な宿主側因子の検索

宿主細胞 P38αMAPK の関与：P38αMAPK 欠損マウス胎児から樹立した繊維芽細胞（KOP）とそのリバータント（RKOP）に PLK/DUAL 株を感染させ、細胞外からの潜伏誘導刺激がない状態で培養する。原虫の発す

る蛍光色を経時的に観察することで宿主細胞 P38α MAPK 情報伝達系の欠如が原虫のステージ転換に影響を及ぼすか否か調べる。

（倫理面への配慮）
岐阜大学または日本大学の動物実験委員会の指針に従い承認のもとですべての動物実験を実施。

C. 研究結果
1．原虫再活性化原発部位の同定
1－1．赤色蛍光を発する組換え原虫の作成
タキゾイトからブラディゾイトにステージ変換した際に赤色蛍光を減衰させることを目的とし、タキゾイト期にのみ活性を有する SAG1 プロモーター下流に赤色蛍光蛋白 DsRed-Express の遺伝子をつないだものを PLK 株のタキゾイトに挿入した。得られた原虫（PLK/Red）のタキゾイトは in vitro 、in vivo の双方で強い赤色蛍光を発した。PLK/RED 感染マウスの脳をシャーレ上に置き、倒立型蛍光顕微鏡で観察したところ、脳中央部に感染する 1～数個のタキゾイトの発する赤色蛍光を検出できた（図3）。また、PLK/RED をタキゾイトの状態で約 1 ヶ月 in vitro にて培養したが赤色蛍光は安定して観察された。
1－2．赤色蛍光発現のステージ特異性
今回得られた組換え原虫である PLK/RED と、ステージにかかわらず常に緑色蛍光を発する原虫である PLK/GFP を VERO 細胞に混合感染させ、in vitro にてブラディゾイトへステージ変換させた。ステージ変換の誘導から 24 時間後の時点で赤色蛍光のみ減衰が起こり、緑色蛍光はステージ変換後も安定して観察された。このことから PLK/RED による赤色蛍光の発現はタキゾイト期特異的なものであると考えられた。また、マウス脳内で形成された PLK/GFP のシストに含まれるブラディゾイトは強い緑色蛍光を発していたが、PLK/Red のシストに含まれるブラディゾイトは蛍光を示さなかった。

2．原虫の潜伏動態の把握
PLK/DUAL を In vitro で培養したところ、タキゾイト培養条件では赤色蛍光を、ブラディゾイト誘導条件では緑色蛍光を示した。本原虫をマウスに感染させたところ、急性期には赤色のタキゾイトが潜伏期には緑色蛍光を示すシストが脳内に見られた。潜伏維持中にシスト外に存在するタキゾイトを認めたが、これらは緑色蛍光は示さず赤色蛍光のみを示した。残存する GFP が認められなかったことから、これらのタキゾイトは少なくとも最近シストから再活性化してきたものではないことが推測される。

3．原虫の潜伏維持に必要な宿主側因子の検索
PLK/DUAL 株を P38α MAPK 欠損株である KOP 細胞に感染させたところ、外部からの潜伏誘導刺激をくわえなくても、感染後 2－3 日で緑色蛍光を発し始めた。このような現象は P38α MAPK 遺伝子を導入したリバータント（RKOP）では見られなかった。

D. 考察
当初計画では潜伏維持中にみられる spontaneous な再活性化像（発症に至らない）の周辺と免疫抑制群に見られる再活性化原発部位（発症にいたると思われる）周辺の比較を予定していたが、潜伏維持中のマウスからは再活性化像が得られなかった。一方で、免疫抑制群からは再活性化像が得られた。この結果から「spontaneous な活性化」という概念に疑義が生じ、「spontaneous な活性化によってシストから脱出した虫体を宿主免疫系が速やかに排除することによる潜伏の維持」という従来の考え方だけで潜伏現象を説明するのは無理があると考えられた。すなわち、潜伏中の虫体に対する免疫反応が潜伏型虫体のステージ変換そのものを阻んでいるため、免疫正常群では再活性化した赤色原虫が見られないと考えるほうが実験結果を解釈しやすいということである。それでは「潜伏中の虫体に対する免疫反応が潜伏型虫体のステージ変換そのものを阻んでいる」宿主側の因子は何であるかという疑問が湧くわけであるが、本研究においてｐ３８αMAPK 分子がその候補として上がった。

E. 結論
本研究によりトキソプラズマ原虫の潜伏感染は従来考えているものよりも静的なものであると示唆された。「潜伏中も常に散発的な再活性化がおこっていて宿主免疫系が再活性化した原虫を順次排除している」という従来のモデルよりも「宿主体内で潜伏型のまま維持されていた原虫が AIDS 発症をきっかけに再活性化する」と捉えたほうが現実に即しているということである。すなわち、健常な宿主は本原虫を潜伏型のまま維持する能力を有していることになるが、この現象に P38α MAPK が関与していそうである。今後、本原虫の再活性化ひいては脳炎を根本的に防止するための研究として原虫と P38α MAPK 分子の相互作用

についてさらに検証すべきであろう。

F. 健康危険情報
特になし。

G. 研究発表
1）Unno A., Suzuki K., Batanova T., Cha S. Y, Jang H. K., Kitoh K., Takashima Y. 2009. Visualization of *Toxoplasma gondii* stage conversion by expression of stage-specific dual fluorescent proteins. Parasitology. *In press*
2）Unno A, Suzuki K., Xuan X., Nishikawa Y., Kitoh K., Takashima Y. 2008. Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow. Parasitol. Int. 57(4): 515-8.
3）Ota H., Takashima Y., Matsumoto Y., Hayashi Y., Matsumoto Y. 2008. Pretreatment of macrophages with the combination of IFN-gamma and IL-12 induces resistance to Leishmania major at the early phase of infection. J Vet Med Sci. 70(6):589-93.
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H. 知的財産権の出願・登録情報
なし。

研究成果による特許権等の知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Unno A., Suzuki K., Batanova T., Cha S. Y, Jang H. K., Kitoh K., Takashima Y	Visualization of *Toxoplasma gondii* stage conversion by expression of stage-specific dual fluorescent proteins.	Parasitology	印刷中	印刷中	2009
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CAMBRIDGE UNIVERSITY PRESS Parasitology

Visualization of Toxoplasma gondii stage conversion by expression of stage-specific dual fluorescent proteins

Journal:	*Parasitology*
Manuscript ID:	PAR-2008-0241.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	27-Dec-2008
Complete List of Authors:	Unno, Akihiro; Gifu University, Department of Veterinary Parasitological Diseases Suzuki, Kazuhiko; Nihon University, Laboratory of Veterinary Epizootiology Batanova, Tatiana; Gifu University, Department of Veterinary Parasitological Diseases Cha, Se-Yeoun; Chonbuk National University, Department of Infectious Diseases and Avian Diseases Jang, Hyung-Kwang; Chonbuk National University, Department of Infectious Diseases and Avian Diseases Kitoh, Katsuya; Gifu University, Department of Veterinary Parasitological Diseases Takashima, Yasuhiro; Gifu University, Vetrrinary Parasitological diseases
Key Words:	Toxoplasma gondii, stage conversion, dual fluorescent proteins

Visualization of *Toxoplasma gondii* stage conversion by expression of stage-specific dual fluorescent proteins

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Running title: Visualization of *Toxoplasma gondii* stage conversion

SUMMARY

To recognize the stage conversion of *Toxoplasma gondii* between tachyzoite and bradyzoite in live host cells, a transgenic *T. gondii* line, which expressed stage-specific red and green fluorescence, was constructed. *T. gondii* PLK strain tachyzoites were stably transformed with genes encoding red fluorescent protein (DsRed Express) and green fluorescent protein (GFP) under the control of tachyzoite-specific SAG1 and bradyzoite-specific BAG1 promoters, respectively. The resulting transgenic parasite was designated PLK/DUAL. When PLK/DUAL was cultured under in pH 7.0 media, the PLK/DUAL zoites expressed red fluorescence, but no detectable levels of green fluorescence were observed. The PLK/DUAL zoites reacted with anti-SAG1 antibody, but not anti-BAG1 antiserum. When PLK/DUAL was cultured under high pH conditions, or in the presence of the p38 MAPK inhibitor SB202190, a small number of zoites expressed green fluorescence and were BAG1 positive. C57BL/6J mice were infected with PLK/DUAL tachyzoites. During the acute and reactivating phase, zoites expressed red fluorescence. However, green fluorescence was not detectable. By contrast, latent cysts expressed green fluorescence. The stage-specific dual fluorescence of PLK/DUAL facilitates identification of the parasitic stage in live cells, with the advantage that fixation or immunostaining is not required.

Key words

Toxoplasma gondii, stage conversion, dual fluorescent proteins

Introduction

Toxoplasma gondii is an intracellular coccidian with a complex life cycle, belonging to the phylum Apicomplexa. Although *T. gondii* infects a wide range of intermediate hosts, such as mammal and bird species, as well as humans, the sexual cycle of this parasite occurs only in the feline intestinal epithelium, thereby yielding sporozoite-containing oocysts in the feces (Innes, 1997; Montoya and Liesenfeld, 2004). In the body of the intermediate host, two asexual forms, namely acute lytic tachyzoites and latent bradyzoite tissue cysts, are found (Lyons *et al.* 2002). Infection of intermediate hosts with *T. gondii* occurs by ingestion of either tissue cysts (bradyzoites) or oocysts (sporozoites). After infection, bradyzoites and sporozoites rapidly differentiate into tachyzoites and disseminate throughout the body; this is referred to as an acute infection. Although the majority of tachyzoites are usually eliminated by the host's immune response, a small percentage of tachyzoites survive and differentiate into bradyzoites, primarily in the brain and muscle. The bradyzoites persist for the life of the host, and are essentially dormant and harmless (Carruthers and Suzuki 2007).

Stage conversion between tachyzoites and bradyzoites is a sporadic phenomenon, rather than occurring en masse (Ferreira *et al.* 2008). The co-existence of tachyzoites and bradyzoites in one vacuole was demonstrated in an *in vitro* experimental study (Bohne *et al.* 1993). Although such a vacuole, containing both tachyzoites and bradyzoites, has not yet been observed in animal tissue, latent cysts containing both SAG1-positive and -negative zoites have been observed in the mouse brain (Silva *et al.* 1998; Bohne *et al.* 1998). The co-existence of latent cysts and tachyzoites in the brain has also been shown in animal and human studies (Ferguson *et al.* 1989; Takashima *et al.* 2008). Thus, *T. gondii* stage conversion seems to be a gradual and progressive event.

Time-lapse analysis is a powerful tool for studying gradually progressing events. In the field of virology, for example, simultaneous tracking of capsid, tegument, and envelope proteins of herpes simplex virus-1 characterized how virus particles are produced, providing the first evidence that these three proteins are assembled in the trans-Golgi network of the host cell (Sugimoto *et al.* 2008). *T. gondii* stage conversion is also a gradually progressing event, like the assembly of a virus particle. However, to further understand this phenomenon, the behavior of stage-converting zoites needs to be characterized over time. To date, *T. gondii* stage conversion and the detection of stage-specific antigens has been demonstrated utilizing immunohistochemistry (Ferguson. 2004; Lüder *et al.* 1999; Gross *et al.* 1995; Bohne *et al.* 1994; Bohne *et al.*

1993). The necessity of a fixation procedure for immunohistochemistry makes it impossible to observe living parasites during stage conversion. To observe temporal changes in gene-expression patterns in stage-converting *T. gondii* zoites, Lyons, R. et al. (2001) harvested mRNA from cell cultures infected with stage-converting zoites once every 24 hr. They clearly described the stage conversion process in a mass of parasites in terms of the changes in stage-specific gene expression. However, even with this method, it is impossible to fully characterize the mechanism underlying the stage conversion of an individual parasite.

To identify the mechanism underlying the stage conversion of an individual parasite, it is necessary to ascertain the stage the observed parasite is in without killing it. That is, it is necessary to utilize live-cell imaging. Fluorescent proteins have proven to be powerful tools for live-cell imaging (Day and Schaufele 2008). In the present study, a transgenic *T. gondii* line expressing stage-specific red and green fluorescent proteins (DsRed Express and GFP, respectively) was constructed. This transgenic parasite allows for live imaging of the stage conversion in *T. gondii*.

Materials and Methods

Mice

Female, 6-8-week-old, C57BL/6J and BALB/c mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The C57BL/6J mice were infected with parasites and their organs excised after euthanasia by cervical dislocation. BALB/c mice were used for the production of polyclonal antibody. Experiments were performed in accordance with the Gifu University Animal Care and Use Committee guidelines.

Cells and Parasites

The African green monkey-kidney cell line Vero was cultured in RPMI1640 medium supplemented with 7.5% FCS at 37°C in a 5% CO_2 incubator. *T. gondii* tachyzoites of the following strains were propagated for routine use in Vero cells: *T. gondii* P(LK) HXGPRT-strain (Roos *et al.* 1994; Pfefferkorn and Borotz 1994), GFP-expressing stable transgenic *T. gondii* (PLK/GFP) (Nishikawa *et al.* 2008; Zhang *et al.* 2007), DsRed Express-expressing stable transgenic *T. gondii* (PLK/RED) (Takashima *et al.* 2008), and the dual fluorescent protein-expressing stable transgenic *T. gondii* (PLK/DUAL), engineered as described below. The PLK/GFP line expresses GFP during the tachyzoite and bradyzoite stages (Nishikawa *et al.* 2008; Zhang *et al.* 2007). PLK/RED expresses DsRed Express only during the tachyzoite stage (Takashima *et al.* 2008). PLK/Dual expresses DsRed Express and GFP as described below.

Construction of the transfer vector

The promoter region of the bradyzoite-specific antigen 1 (BAG1) gene was PCR amplified from the plasmid Bag/cat (kindly provided by Dr. Bohne, Georg-August University) using the following primer set: 5'-AAGCTTCTTCCAGTTGCCCGGCTCTGGGTACCTTCT-3' and 5'-GGATCCCTTTTTTGAATATCATACGGGACCTGGGCT-3'.

The amplified fragment was cloned into the pT7Blue T-Vector (Novagen, Darmstadt, Germany), and the resulting plasmids were designated as pT7/BAGp. The BAG1 gene promoter was obtained from pT7/BAGp by digestion with *Bam*HI and *Hind* III, followed by ligation with the *Bam*HI- and *Hind* III-digested plasmid pUC/GRA (Takashima *et al.* 2008). The resulting plasmid was designated pUC/BAG-GRA. The gene for enhanced GFP was obtained from the plasmid, pCX-EGFP (Niwa *et al.* 1991) (kindly provided from Dr. J. Miyazaki, Osaka University). The obtained GFP gene was blunt-ended with *Klenow* fragments and inserted into the blunt-ended *Bam* HI site of the plasmid pUC/BAG-GRA. The resulting plasmid was designated pBAG-GFP. GFP expression units, under the control of the BAG1 promoter, and DsRed Express (Clontech, Palo Alto, CA), under the control of the major surface antigen 1 (SAG1) promoter, were obtained from the plasmids pBAG-GFP and pSAG-RED (Takashima *et al.* 2008). The obtained GFP and DsRed Express expression units were inserted into the *Hind* III and *Eco* RI sites of the plasmid pminiHXGPRT (kindly provided by Dr. Roos, Stanford University) (Roos *et al.* 1994; Pfefferkorn *et al.* 1994), respectively, as shown in Fig. 1A. The resulting plasmid was designated pminiHXGPRT/DUAL.

Parasite transfection and selection

A total of 100 μg of the plasmid pminiHXGPRT/DUAL was electroporated into P(LK) HXGPRT–tachyzoites as described previously (Soldati and Boothroyd 1993), and the transfected parasites were then added to Vero cell cultures. Forty-eight hours after electroporation, medium containing 25 μg/ml mycophenolic acid and 40 μg/ml xanthine was added to the Vero cell culture. Stable transgenics were selected for in the presence of mycophenolic acid and xanthine for 10 days. Following selection, cloning of red fluorescent tachyzoites was performed, and the resulting clone was designated PLK/DUAL.

In vitro stage conversion

Stage conversion of tachyzoites into bradyzoites was induced by high pH or the p38 MAPK inhibitor SB202190, as previously described (Soete *et al.* 1993, Radke *et al.* 2006) with minor modifications. For induction of stage conversion by high pH, Vero cells that were infected with free PLK/DUAL or PLK/RED tachyzoites were cultured in D-MEM medium (pH 8.1), supplemented with 2.0% FCS and 20 μg/ml gentamicin, at 37°C in a humidified incubator for four or seven days. For induction of stage conversion by the p38 MAPK inhibitor SB202190, Vero cells were pre-incubated in D-MEM medium containing 1.0% FCS and 8 nM SB202190 (In Solution™ SB202190, Calbiochem, U.S.A) for 3 hr prior to tachyzoite infection, and washed with D-MEM medium containing 1.0% FCS. Following incubation with PLK/DUAL tachyzoites suspended in D-MEM medium containing 1.0% FCS for 4 hr, supernatant was removed and fresh medium containing 1.0% FCS and 8 nM SB202190 was added. The infected Vero cell cultures were incubated at 37°C in a 5% CO_2 incubator for 6 days.

Flow cytometry

Flow cytometry was performed on a FACSCalibur (BD Biosciences PharMingen, San Diego, CA) to detect green and red fluorescence in purified free tachyzoites from Vero cells, as described previously (Unno *et al.* 2008). GFP and DsRed Express expression were detected using Fl-1 and Fl-2, respectively.

***In vivo* stage conversion**

To observe parasites during the acute phase, C57BL/6J mice were infected intraperitoneally with 1×10^6 PLK/DUAL tachyzoites. The brains of the infected mice were excised 6 days later, and fixed and embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany). Thin brain sections were directly observed using fluorescent microscopy, as previously described (Takashima *et al.* 2008). To prepare latently infected mice, C57BL/6J mice were intraperitoneally infected with 20-1000 PLK/DUAL or PLK/GFP tachyzoites and observed for 6 weeks. Mice that survived more than 6 weeks without symptoms were labeled as latently infected mice and used in subsequent experiments. To observe parasites during the latent phase, the brains of latently infected mice were excised and chopped into small pieces. The brain pieces were then crushed onto glass slides and observed under fluorescence microscopy and confocal laser microscopy. Red fluorescence from the crushed samples was detected by short (1/3.0 sec) and long (1.5 sec) time exposure. Green fluorescence from the crushed samples was detected by just

long-time exposure.

Reactivation of latent brain cysts

Latently infected mice were prepared as described above. Dexamethasone (dexamethasone 21-phosphate disodium salt; Sigma) was dissolved at a concentration of 10 mg/liter in the drinking water, and the latently infected mice were allowed free access to the dexamethasone-containing water. After 28 days of dexamethasone treatment, the brains of treated mice were excised and chopped into small pieces. The pieces of brain were crushed onto glass slides and observed under fluorescence microscopy.

Anti-BAG1 antiserum

The truncated BAG1 gene (aa 94-229,) with introduced *Bam* HI and *Sma* I sites, was synthesized and ligated with the pGEX-4T-3 vector (Amersham Pharmacia Biotech, PA), which was digested with *Bam* HI and *Sma* I. *E. coli* (BL21) was transformed with the resulting plasmid and cultured in LB medium containing 100 μg/ml ampicillin at 37°C until OD_{600} reached 0.3. Isopropyl-β-D(-)-thiogalactopyranoside was then added to the medium (final concentration, 1 mM). Following 3 hr of incubation, the transgenic *E. coli* were pelleted by centrifugation at 3,000 rpm for 10 minutes at room temperature to remove the supernatant. Soluble, bacterial proteins were eluted using B-PER™ Reagent (PIERCE, USA) according to manufacturer's instructions. From the soluble proteins, glutathione *S*-transferase (GST) fusion protein (GST-BAG1)-was purified using Glutathione Sepharose™ 4B (Amersham Bioscience, Sweden) according to manufacture's instructions. A total of 50 μl of purified GST-BAG1 solution (containing 16.3 μg/μl GST-BAG 1) was mixed with 100 μg of adjuvant, TiterMax Gold (TiterMax USA. INC., GA). A total of 100 μl of the mixture was subcutaneously injected into each of three 6-week-old female BALB/c mice. Thirty-two days after the first immunization, a second immunization was performed by injecting 200 μl of the mixture. Serum (anti-BAG1 antiserum) was harvested from immunized mice 20 days after the second immunization, and was stored at -20°C until further use.

Immunostaining

Free PLK/DUAL tachyzoites, prepared as previously described (Unno *et al.* 2008), were incubated for 45 min at 37°C in an air incubator with anti-SAG1 mAb (3T × 19 MAb TP3, HyTest Ltd, Turku, Finland) or anti-BAG1 serum 50× diluted in PBS

containing 3% FCS. After washing them three times with PBS containing 3% FCS, the tachyzoites were incubated for 45 min at 37°C in an air incubator with Alexa Fluor® 350-labeled goat anti-mouse IgG (H+L) (Invitrogen). After washing them three times with PBS containing 3% FCS, the reacted tachyzoites were mounted with ProLong® Gold antifade reagent (Invitrogen) onto glass slides and covered with a coverslip. The glass slides were incubated for 30 min at room temperature in the dark and observed by fluorescence microscopy.

Results

Construction of transgenic *T. gondii* expressing dual fluorescent proteins

To produce transgenic parasites expressing stage-specific dual fluorescent proteins, we constructed a transfer vector, pminiHXGPRT/DUAL. The transfer vector contained genes coding for DsRed Express and GFP under the control of SAG1 and BAG1 promoters, respectively (Fig.1A). *T. gondii* P(LK) HXGPRT- strain tachyzoites were transformed with the transfer vector, pminiHXGPRT, and the stably transformed clone was designated as PLK/DUAL. The PLK/DUAL tachyzoites showed red fluorescence under pH 7.0 culture conditions (Fig.1 B, upper panel and Fig.1 C, upper panel). Although 293 zoites were observed, the number of red fluorescence negative zoites was only a few (6/293). During *in vitro* culture in pH 7.0 media for one month, with repeated passages, the proportion of fluorescent negative zoite did not increase. When PLK/DUAL tachyzoites were cultured under a stage conversion-inducing condition (pH 8.1 medium), a number of green fluorescence-positive tachyzoites were observed (Fig.1 B, lower panel and Fig.1 C, lower panel). Green fluorescence-positive tachyzoites were not detected in the pH 7.0-culture condition (Fig.1 B upper panel and Fig.1 C, upper panel). To confirm that the green fluorescence expression under the pH 8.1 condition was to the result of autofluorescence observed only under high pH conditions, but was rather caused by specific GFP expression in PLK/DUAL zoites, a transgenic *T. gondii* strain, PLK/RED, which expressed DsRed Express but no GFP, was also cultured in the pH 8.1 media. As shown in Fig. 1 D, green fluorescence-positive zoites were detected in Vero cell cultures infected with PLK/DUAL, but not in those infected with PLK/RED. These data suggest that PLK/DUAL expressed GFP during the bradyzoite stage.

Stage-specific expression of fluorescent proteins by PLK/DUAL

To investigate whether DsRed Express and GFP were expressed stage specifically,

PLK/DUAL zoites were stained with anti-SAG1 antibody or anti-BAG1 antiserum. Under the pH 7.0-condition, before the antibody staining procedure, almost all parasites expressed Ds Red Express but no zoite expressed GFP (Fig.1 B, C). When the parasites cultured in pH 7.0-condition, all zoites were stained by anti-SAG1 antibody but specific staining was not revealed by anti-BAG1 antiserum (Fig.2 A). SAG1-/DsRed Express + zoite was not observed. Although some parasites looked like SAG1+/DsRed Express – (Fig. 2 A, arrows), it must be a result of artificial reduction of fluorescent intensity by the staining procedure because almost all zoites showed strong red fluorescence before the staining procedure (Fig. 1 B). These results indicate that the DsRed Express-positive zoites, cultured under the pH 7.0-condition, were in the tachyzoite stage.

When PLK/DUAL zoites were cultured under stage conversion-inducing conditions, either high pH or the presence of a p38 MAPK inhibitor, a number of zoites expressed not only DsRed Express, but also GFP (Fig.1 B, C, D, and Fig.2 B). The intensity of GFP expression varied between individual zoites (Fig. 2 B). Zoites cultured in medium containing a p38 MAPK inhibitor were stained with anti-BAG1 antiserum (Fig.2 B). Fig. 2 B demonstrates a zoite that was strongly expressing GFP and was BAG1 positive, as well as a zoite showing weak GFP expression that was weakly BAG1 positive. Although only a few zoites are shown in Fig. 2 B, all observed zoites in this culture demonstrated a similar trend (data not shown). These results indicate that BAG1 and GFP expression patterns were synchronized; in other words, GFP expression indicated bradyzoite stage-specific expression.

DsRed Express and GFP *in vivo* expression in the PLK/DUAL strain

To confirm *in vivo*, stage-specific expression of DsRed Express and GFP by the PLK/DUAL strain, C57BL/6J mice were infected with PLK/DUAL tachyzoites. The brain samples were observed at acute, latent, and reactivating phases. In the acute phase, disseminated red fluorescence-positive zoites were observed in the brain samples (Fig.3 A and Fig.3 D, left panel). However, GFP expression was not detected (Fig. 3 A). During the latent phase, green fluorescence-positive cyst and green fluorescence-positive bradyzoites within the cyst were observed in the brain samples (Fig.3 B and Fig.3 D, center panel). As shown in Fig. 3 B, red fluorescence was not detected in these cysts by short-time exposure (1/3.0 sec). Whole brain tissues from one latently infected mouse were examined by short-time exposure. Analysis resulted in neither red fluorescence-positive cysts nor tachyzoites. The expression of fluorescent proteins was detected in the parasites but secretion into the

parasitophorous vacuole was not observed (Fig.3 D). To investigate whether reactivated tachyzoites from latent cysts also expressed DsRed Express, latent cysts were reactivated by administration of dexamethasone to the latently infected mice. After 28 days, red fluorescence-positive zoites were observed in the brain; however, green fluorescence-positive zoites were not detected (Fig.3 C). These results indicate that stage specificity could be identified in the PLK/DUAL living zoites by fluorescence.

Low-level DsRed Express expression in PLK/DUAL cysts

As described above, red fluorescence-positive PLK/DUAL latent cysts were not detected by short-time exposure (Fig.3 B). To determine whether PLK/DUAL bradyzoites in the latent cysts express a trace amount of DsRed Express or none at all, PLK/DUAL latent cysts were observed using a long time exposure. The results demonstrated that a portion of the bradyzoites in latent cysts weakly showed red fluorescence (Fig. 4 left panel). Bradyzoites in latent PLK/GFP cysts did not show red fluorescence (Fig. 4 right panel). These results indicate that the weak red fluorescence in the PLK/DUAL bradyzoites was not because of GFP bleed-through into the red fluorescent channel, but rather was caused by the presence of DsRed Express molecules in the bradyzoites.

Discussion

The present study demonstrated the use of a transgenic *T. gondii* strain, PLK/DUAL, as a tool to investigate stage conversion in *T. gondii*. The transgenic parasites express DsRed Express during the tachyzoite stage and GFP during the bradyzoite stage. Several transgenic parasite-expressing fluorescent proteins have been reported as tools for investigation for stage conversion in *T. gondii* and/or visualization of parasites in host tissue (Vanchinathan *et al.* 2005; Takashima *et al.* 2008; Nishikawa *et al.* 2008). This method is a powerful tool with which to observe one-way stage conversion, from tachyzoite to bradyzoite, or from bradyzoite to tachyzoite. However, none of these methods have allowed for the observation of stage conversion in both directions. Vanchinathan P. *et al.* (2005) constructed a transgenic parasite that fluoresced only during the bradyzoite stage. Using this transgenic parasite, stage conversion from tachyzoites into bradyzoites could easily be detected as an emission of fluorescence. However, it was impossible to detect reactivation of the parasite (stage conversion from bradyzoite to tachyzoite) using fluorescence. We have also

reported a transgenic parasite that fluoresced only during the tachyzoite stage (Takashima *et al.* 2008), and have visualized *T. gondii* reactivation in brain tissue. However, it is difficult to observe entrance into a latent state (stage conversion from tachyzoite to bradyzoite), because bradyzoites in brain cysts do not show bright fluorescence. To better understand the life cycle of *T. gondii* in the intermediate host, it is important to study the mechanisms of stage conversion between tachyzoites and bradyzoites in both directions. Although much knowledge regarding stage conversion has been independently provided by several research groups (Takashima *et al.* 2008, Vanchinathan *et al.* 2005), various strains of *T. gondii* were used for these studies. Because of the diverse properties among parasite strains (Grigg *et al.* 2001; Gross *et al.* 1997), an assortment of independent reports does not directly result in an overall understanding of the *T. gondii* life cycle. The PLK/DUAL strain, in which stage conversion is visualized in both directions by dual fluorescence, could provide a greater understanding of the *T. gondii* life cycle in the intermediate host. *T. gondii* cysts are primarily formed in brain and muscle tissue of the host (Turner 1978). Latent cysts have also been reported in the placenta (Dubey 1987). This indicates that *T. gondii* tachyzoites can convert to bradyzoites in the placenta. In addition, it was reported that there is lagged-time between *T. gonii* infection in the placenta and the fetus in some pregnant mouse models (Shiono *et al.*, 2007). There is a possibility that the bradyzoites existing in the cysts in the placenta could reactivate and reach the fetus later. Thus to understand the behavior of *T. gondii* in the placenta, observation of stage conversion in both directions is necessary. Therefore, the PLK/DUAL strain could contribute to further understanding of the mechanisms of *T. gondii* vertical infections.

Stage conversion between tachyzoite and bradyzoite is associated with morphological and molecular biological changes, including stage-specific antigen expression, many of which have been identified (Lyon *et al.* 2002). To construct transgenic *T. gondii* stage-specifically expressing fluorescent proteins, the promoters of the SAG1 and BAG1 genes were utilized, thereby specifically targeting expression in tachyzoites and bradyzoites, respectively (Ferguson. 2004). In addition, it has been reported that stage-specific expression of SAG1 and BAG1 is controlled at the transcriptional level (Cleary *et al.* 2002; Bohne *et al.* 1997). SAG1 gene transcription occurs during the tachyzoite stage, and decreases drastically during stage conversion from tachyzoite to bradyzoite (Cleary *et al.* 2002). By contrast, the upregulation of BAG1 mRNA occurs early during differentiation from tachyzoite to bradyzoite (Bohne *et al.* 1997). To confirm stage-specific expression of fluorescent proteins by

PLK/DUAL zoites, immunostaining was performed. Although zoites showing weak and no red fluorescence were observed after SAG-1 staining (Fig. 2 A), this loss of red fluorescence of zoite may be artifact by staining procedure because almost all PLK/DUAL showed red fluorescence strongly before staining (Fig. 1 B). DsRed Express positive and GFP negative zoites exhibited SAG1-expression, but no BAG1-expression (Fig. 2 A). When PLK/DUAL zoites were cultured medium containing SB202190, a number of zoites expressed BAG1, as well as GFP (Fig. 2 B). Strongly BAG1-positive zoites exhibited brighter GFP expression compared with BAG1-negative or BAG1-slightly positive zoites (Fig.2 B). These results indicate that DsRed Express and GFP expression patterns coincided with SAG1 and BAG1, respectively; in other words, tachyzoites were DsRed Express and SAG1 positive, and bradyzoites were GFP and BAG1 positive. *In vivo* analysis demonstrated that zoites in the acute and reactivating phases, most likely tachyzoites, exhibited DsRed Express expression; however, GFP expression was undetectable (Fig.3 A and C) By contrast, zoites within latent cysts, most likely bradyzoites, exhibited GFP expression, and DsRed Express expression was undetectable (Fig.3 B). Therefore, *in vivo* PLK/DUAL zoite stages could also be identified by fluorescence. Although it is necessary to consider the effect of expressed fluorescent proteins to immune reactions and parasites' behaviors, the PLK/DUAL strain could be a tool for investigation of in vivo stage-conversion of *T. gondii*. The intensity of the DsRed Express expressed by PLK tachyzoites was strong enough to detect zoites in the brain tissue from the outside (Takashima *et al.*, 2008). However, despite of the usage of strong bradyzoite-specific BAG1 promoter (Bohne *et al.* 1997), the intensity of green fluorescence of GFP expressed by bradyzoites was not enough to detect latent cysts in that manner. Trying out other fluorescent proteins and bradyzoite specific promoters, it might be possible to construct more useful recombinant parasites, of which latent cysts can be identified by strong fluorescence without either squashing tissue or making thin sections.

Bohne W. et al. (1993) observed a parasitophorous vacuole containing both tachyzoites and bradyzoites during the *in vitro* stage-converting phase. It was also reported that there is a point when parasites are expressing both SAG1 and BAG1 (Ferguson, 2004). We also observed a PLK/DUAL cyst containing both of red fluorescence +/green fluorescence - and red fluorescence +/green fluorescence + zoites in the brain on the 28 days after PLK/DUAL infection (data not shown). It suggested that PLK/DUAL also has SAG1/BAG1 double positive phase during stage-conversion. Although SAG1 is regarded as a tachyzoite-specific antigen, latent

cysts of the *T. gondii* ME-49 strain have been shown to contain some SAG1-slightly positive zoites (Silva *et al.* 1998). The PLK strain is a clonal derivative of ME-49 (Bohne *et al.* 1998). Therefore, we attempted to detect low levels of red fluorescence in the PLK/DUAL latent cysts using a long time exposure. As shown in Fig. 4, weak red fluorescence was detected in PLK/DUAL latent cysts. Considering that weak expression was detected not only in the cyst shown in Fig. 4, but also in all observed PLK/DUAL cysts (data not shown), it is unlikely that the weak red fluorescence indicates reactivation. We have previously reported that latent cysts of the PLK/RED strain exhibit much brighter red fluorescence at the point of inception of reactivation (Takashima *et al.* 2008). Our present results suggest low-level SAG1 promoter activity during the latent phase in the PLK strain.

It has been reported that *T. gondii* modifies many signaling cascades within the host cell (Seaij et al., 2007, Fouts and Boothroyd 2007). It has also been demonstrated that tachyzoites and bradyzoites can induce host transcriptional changes (Fouts and Boothroyd 2007). However, it is not yet known what biological activity exists between tachyzoite- and bradyzoite-infected cells to induce various host transcriptional changes in different manners. To elucidate the difference in the mechanisms of biological activity, that is, their reactions to exogenous stimuli, it is necessary to identify the stages of the infected parasites without killing them. As well, it is necessary to observe the parasite and/or host cells over a period of time. The PLK/DUAL strain could be an effective tool for investigating the biological activities of tachyzoite- and/or bradyzoite-infected host cells.

Acknowledgments

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. David Roos: *Toxoplasma gondii* Host Strain P(LK) HXGPRT and *Toxoplasma gondii* Selection Plasmid (pminiHXGPRT). The plasmid, Bag/cat, was provided by Dr. Wolfgang Bohne. We thank Dr. David Roos and Dr. Wolfgang Bohne for supplying these materials. We thank Dr. Fumie Aosai for discussion. This work was supported by the Health Labor Sciences Research Grant, Research on HIV/AIDS from the Ministry of Health, Labor and Welfare of Japan.

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Figure Legends

Fig.1 Construction of transgenic *T. gondii* expressing DsRed Express and GFP. (A) Schema of the transfer vector, pminiHXGPRT/DUAL. Genes coding DsRed Express and GFP are under the control of the SAG1 and BAG1 promoter, respectively. (B) Vero cells, infected with PLK/DUAL tachyzoites, were cultured in pH 7.0 medium (upper panels) and pH 8.1 medium (lower panels). Left and center

panels show red and green fluorescence detected by fluorescence microscope, as detected by 1/3.0 sec and 1.5 sec exposure, was shown respectively. Right panels show overlay images of red and green fluorescence. Scale bar = 50.0 μm. Arrows in the expanded Fig. indicate a green fluorescent zoites. (C) Fluorescent PLK/DUAL zoites in a vacuole. PLK/DUAL tachyzoites cultured in pH 7.0 medium (upper panels) and pH 8.1 medium (lower panels) were observed by large magnification. Scale bar = 10.0 μm. (D) PLK/DUAL or PLK/RED zoites released from infected Vero cells cultured in pH 8.1 medium (left and light panels, respectively). GFP and DsRed Express were detected by Fl-1 and Fl-2, respectively.

Fig.2 Stage-specific expression of DsRed Express and GFP by PLK/DUAL. (A) Purified free zoites from PLK/DUAL-infected Vero cells cultured in pH 7.0-medium. The purified free zoites were stained with anti-SAG1 antibody (upper panels) or anti-BAG1 antiserum (lower panels). Goat anti-mouse IgG (H+L)-Alexa 350, secondary antibodies were used for both experiments. Transmitted light images, red fluorescence images, green fluorescence images, overlay images of red and green fluorescence, and blue fluorescence images (-SAG1 or -BAG1) are shown. The blue fluorescence images were converted into gray scale. Arrow in the upper panels indicates zoites of wich red fluorescence had reduced by the staining procedure. (B) Purified free zoites from PLK/DUAL-infected Vero cells cultured in medium containing SB202190. The purified free zoites were stained with anti-BAG1 antiserum. Goat anti-mouse IgG (H+L)-Alexa 350 secondary antibody was used. Transmitted light images, red fluorescence images, green fluorescence images, overlay images of red and green fluorescence, and blue fluorescence images (□-BAG1) are shown. Arrowhead and arrow indicate red and green fluorescent zoites, respectively. The blue fluorescence images were converted into gray scale. Bar = 20.0 μm.

Fig.3 Red and green *in vivo* fluorescence in the PLK/DUAL strain. (A-C) PLK/DUAL fluorescence in the brains of infected C57BL/6J mice during the acute phase (A), latent phase (B) and reactivating phase (C). Red fluorescence was detected by short exposure (1/3.0 sec.). Green fluorescence was detected by long exposure (1.5 sec). Transmitted light images, red fluorescent images, green fluorescent images, and overlay images of transmitted, red fluorescent, and green fluorescent images are shown. (D) Area shown by the dotted frames in Fig.3 A (left pannel), B (center pannel) and C (right pannel) were observed by confocal laser microscopy. Bar = 5.0

μm. Note that images in Fig. 3 D is shown as mirrored images of Fig. 3 A, B and C, because of the difference in structure of fluorescent microscope and confocal laser microscope.

Fig.4 Expression of DsRed Express in latent cysts of the PLK/DUAL strain. Latent cysts of the PLK/DUAL (left panels) and PLK/GFP (right panels) strains in the brains of infected C57BL/6J mice are shown. Red fluorescence was detected by long exposure (1.5 sec). Green fluorescence was detected under the conditions described in Fig. 3. Bar = 20.0 μm

Fig. 1