表4　性器ヘルペスの治療

	薬剤と投与法	投与期間
初発	バラシクロビル500mg1日2錠分2， またはアシクロビル200mg1日5錠分5	5～10日間
	＜重症例＞	
	静注用アシクロビル5mg/kg 1日3回点滴静注	5～7日間 その後，経口で 追加計10日間
再発	バラシクロビル500mg1日2錠分2， またはアシクロビル200mg1日5錠分5	5日間
	＜軽症例＞	
	3%ビダラビン軟膏 5%アシクロビル軟膏 } 1日数回塗布	5～10日間
再発抑制療法	バラシクロビル500mg1日1回	3カ月～ 症例による

性器ヘルペスの治療にはアシクロビルまたはバラシクロビルの経口投与が用いられる。臨床型により量や期間が異なる。（文献7より）

1．初　発

一般に初発（特に初感染初発）は症状が強く，病変が広く，治癒までの時間もかかる。現在の薬剤は潜伏感染状態のHSVを排除することができないため，抗ヘルペスウイルス薬によって治療してもその後の再発は免れない。髄膜炎を合併したり，外陰の病変が広く，排尿痛が強く日常生活が困難な場合，末梢神経麻痺による尿閉などを合併する場合は入院し，経静脈投与が薦められる。

日本性感染症学会のガイドラインでは，VACV 500mg×2/日またはACV 200mg×5/日を5～10日間経口投与する。5日間でもよいが症状に応じて10日間まで延長することになっている。初発性器ヘルペスの投与期間について年代順に並べたものが**表5**であるが，年代とともに次第に長くなっているようである。CDC（米国疾病予防管理センター）のガイドライン[8]ではVACV 1,000mg×2/日と，わが国の倍量を経口投与し，期間も7～10日間としている。性器ヘルペスの世界的リーダーのひとりであるCoreyは，VACV 500～1,000mg×2/日の10～14日間の投与を薦めている[12]。

筆者は，一見，外陰症状が治癒しても10日間は投与している。その理由は，初感染例にACV 200mg×5/日を5日間またはVACV 500mg×2/日を5日間の投与におけるHSVに対する効果を経時的にHSVの培養を行って，その陰転化率を見たところ，第5病日では1型，2型感染例の10%以上がなお陽性であり，5日間の投与では不十分である例があった（**図5**）。もうひとつの理由は，仙髄神経節におけるHSVの増殖は外陰病変が治癒してからもなお続いている可能性があるため，これを抑えておくことは潜伏HSV量を減らすことになり，したがって，将来の再発の頻度を減らすことができるのではないかと考えているからである。

2．再　発

再発例は一般に症状が軽いため投与期間は5日間と短い。再発の治療は，発症してから1日以内，できれば6時間以内に投薬すると有意な治療効果

CDC（米国疾病予防管理センター）

表5　初発性器ヘルペスの抗ウイルス療法

報告者	年度	1日量	投与期間	報告誌
Europian guideline	2001	ACV 200mg×5 VACV 500mg×2	5日間	Inter J STD & AID
Patel R	2002	ACV 200mg×5 VACV 500mg×2	5～10日間	JID
Kimberlin D	2004	ACV 200mg×5 VACV 1,000mg×2	7～10日間	N Engl J Med
Corey L	2008	ACV 200mg×5 VACV 500～1,000mg×2	10～14日間	Sex, Trans, Dis, ed by Holmes KK
CDC	2010	ACV 200mg×5 VACV 1,000mg×2	7～10日間	MMWR
日本性感染症学会	2011	ACV 200mg×5 VACV 500mg×2	5～10日間	日本性感染症学会誌
筆者	2009	VACV 500mg×2	10日間	

初発性器ヘルペスに対する抗ウイルス薬の投与量と期間は少しずつ異なっている。
CDC：米国疾病予防管理センター，ACV：アシクロビル，VACV：バラシクロビル

（筆者作成）

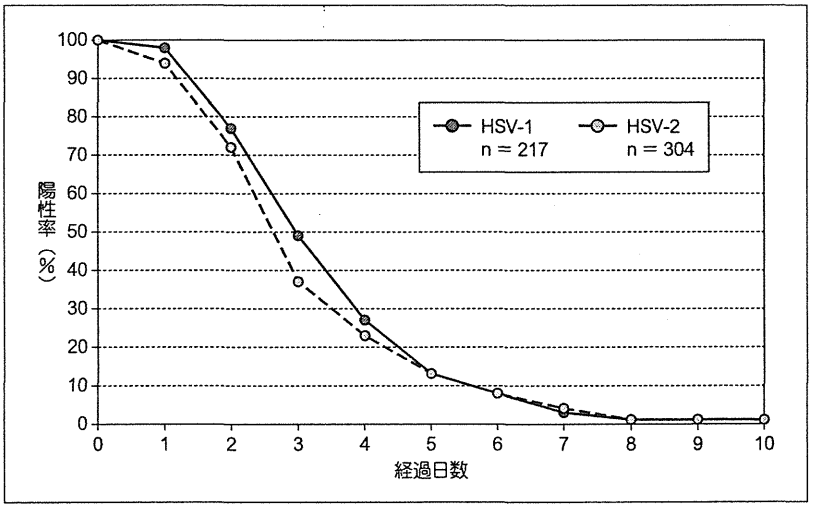

図5　ACVまたはVACV治療による経時的陰転率

100%陰転化には8日以上を要する。
ACV：アシクロビル，VACV：バラシクロビル
HSV-1：単純ヘルペスウイルス1型，HSV-2：単純ヘルペスウイルス2型

（筆者作成）

が得られるため，あらかじめ患者に薬剤を渡しておいて，再発の前兆があったときに服用させると発症しないことも多い（patient initiated treatment：先制療法）ので外国ではしばしば行われているが，わが国では保険の適用はない。

前述のCDCのガイドラインではVACA 500

mg×2/日を3日間の投与でもよいとしている。Coreyは500mg×2/日の3日間投与，さらに1,000mg×2/日を1日で投与する短期療法でもよいとしている。HSVの再活性化により発症する再発は外陰の病変は軽度でも，実はHSVの排泄は広い範囲におきているため抗ウイルス薬の経口投与による全身療法がよく，局所の軟膏療法は推奨されていない[13]。

3．再発抑制療法

くり返す再発は患者に肉体的負担だけでなく精神的に大きなストレスとなり，QOLが低下する[14]。症状は軽くても再発すると，パートナーや家族に感染させるのではないかという心配もある。そこで，再発を抑制するべく持続的に抗ウイルス薬を服用する抑制療法（suppressive treatment）が開発された。この療法を年6回以上，再発する症例に用いたところ，約3カ月間では再発しないものが約70%であったのに対し，プラセボ群ではわずかに約10%で有意な差があったとしている。また，再発までの平均日数がプラセボ群では20日であったのに対し112日以上であったと，再発抑制効果を示した[15]。抑制療法中でも再発することはあるが，その症状は一般的に軽い。さらに，抑制療法を行ったときにはHSVの排泄も抑えられる結果，パートナーへの感染率も約75%抑えられることが証明されている[16]。

すなわち本療法により，患者本人にとっては再発を減らすことによりQOLが改善されるだけでなく，他人へ感染させるのではないかという不安もある程度解消できる。副作用が心配になるが，現在のところ，長期に服用しても問題となる副作用は知られていない。

わが国では年6回以上（2カ月に1回）再発をくり返す症例について，VACV 500mg 1日1回服用する再発抑制療法が保険適用になっている。抑制療法中に再発する例があるが，この場合は1日2錠の治療量に3～5日間戻す。抑制療法中でも再発を頻繁にくり返す場合は1日の血中濃度をなるべく一定に保つことを目的として，1回250mg 1日2回または1回1,000mgを1日1回投与することが薦められている。

2006年9月から本療法は始まっているが市販後調査は終了しており，わが国でも諸外国と同様の治療効果と，長期にわたる服用による副作用の状況などの報告が待たれる。本療法が認可された当初は年6回以上の再発例にのみ保険が認められたが，このしばりは，外国で行われた本療法の有効性を証明した治験が年6回以上の再発例について行われたこと，わが国での臨床経験がなく副作用の発現を心配したこと，薬剤の乱用を防ぎ，本療法が適正に行われるようにすることなどを考慮した結果であった。

しかし，2006年のCDCのガイドラインでは，本療法は再発回数が年6回以内の例や再発の症状が軽い例でも有意義であるばかりでなく，HSVの排泄を抑制するため他人への感染のリスクを減らせることもあることから，これらも考慮した上で，再発時のみ服用する発症時療法にするか，抑制療法にするか，患者と相談することを薦めている。

抑制療法の適応は再発の頻度で決めるのではなく，再発時の重症度，再発による精神的ダメージの程度，パートナーへの感染に対する不安なども考慮すべきであろう。再発をくり返す患者は発症時治療よりも抑制療法を好むものが多いという報告もある[17]。ただ，年に6回以上，再発する患者と年1～2回しか再発しない患者では当然，状況が異なってくると考えられるため，一律に抑制療法を薦めるべきではないであろう。

本療法を行えば再発の頻度は激減するものの，いつまで続けるべきかを患者から尋ねられる。この判断においては，本療法を中止したときの再発の頻度がどのように変わるかが大切である。6年間のACVによる抑制療法を行った後の再発率を治療の前と比較検討した研究では，37%の症例が75%以上減少し，35%が35～75%減少したが，28%の例では変わらなかったと言う[18]。しかし，この報告の考察で述べているように，自然経過でもこのような変化があり得るため抑制療法の結果とは一概には言えない。現状では確立した見解はないようである。

筆者は，性器ヘルペスの再発のリスク因子には仙髄神経節に潜伏感染しているHSVの量と宿主

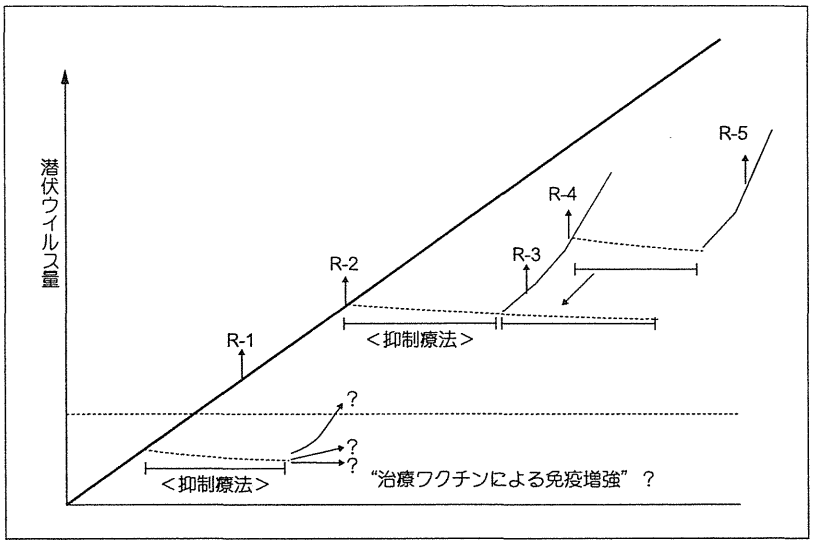

図6　再発抑制療法の病態に関する作業仮説（初感染）
再発抑制療法はHSVの量が増えないうちに開始したほうがよいのではないか。
（筆者作成）

の免疫力が関連していると考えている。現在の保険診療のように年6回以上の再発例を適応とすることは，言ってみれば，かなりの量のHSVの量が知覚神経節に潜伏感染するまで待ってから始めるということになっているのではないかと思う。潜伏感染しているHSVの排除はVACVではできないため抑制療法を中止すれば再活性化により再発をくり返すであろう。そこで，HSVの量が増えないうちに抑制療法を開始するほうがよいのではないか，特に，2型感染例は早い時期から始めるほうがよいのではないかと個人的に考えている。Handsfieldも筆者と同じように，早期に抑制療法を始めることを提案している[19]。

これを薦めるもうひとつの理由は，2型感染後，再発は1年以内に多く，ウイルスの排泄も多いという報告があるため，この時期にHSVの増殖を抑えておくのがよいと思うからである。

以上のようなことから筆者は，2型初感染例にはなるべく早い時期から抑制療法を始めることのほうが結果的にはより効果的ではないかと考えている。そして，将来的には特異的な免疫を高めるワクチン療法を同時に追加するとよいのではないかと考えている（図6）。

Ⅵ　性器ヘルペスの予防

性器ヘルペスの蔓延には無症候性ウイルス排泄者の存在が主役となっている。このような感染経路を遮断するには，性活動を始める前にワクチン戦略によりHSVの感染を予防することが賢明である。すでに2型の表面にあるglycoprotein Dを用いたコンポーネントワクチンが製造され，臨床治験が行われた[20]。その結果，HSVに対する抗体のない女性では約40%に2型の感染が予防できることが示された。この結果を受けて大規模な臨床研究が行われ，その結果が最近発表された[21]。今回の研究では残念ながら，前回の試験の追試はできず，1型の感染を35%防いだのみであった。この結果から，ワクチン戦略にはさらなる改良が必要であることが判明した。

Ⅶ　まとめ

現在の性器ヘルペスの診療において改善すべき点として，診断の面では核酸増幅法による病原診断と型特異的抗体検出の早急な保険適応が望まれる。治療の面では初感染においては10日以上の投薬が必要と考える。抑制療法については年6回

以上の症例に限るのではなく，再発症状の重いもの，再発に対しての精神的なストレスが強いものなど，患者のQOLの改善を目的にすべきであろう。抑制療法の開始時期は再発の感染病理に基づいた戦略を考えるべきではないか。

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Unique properties of memory B cells of different isotypes

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Summary: Memory antibody responses are typically seen to T-cell-dependent antigens and are characterized by the rapid production of high titers of high-affinity antigen-specific antibody. The hallmark of T-cell-dependent memory B cells is their expression of a somatically mutated, isotype-switched B-cell antigen receptor, features that are mainly generated in germinal centers. Classical studies have focused on isotype-switched memory B cells (mainly IgG isotype) and demonstrated their unique intrinsic properties in terms of localization and responsiveness to antigen re-exposure. However, recent advances in monitoring antigen-experienced B cells have revealed the considerable heterogeneity of memory B cells, which include unswitched IgM^+ and/or unmutated memory B cells. The IgM and IgG type memory B cells reside in distinct locations and appear to possess distinct origins and effector functions, together orchestrating humoral memory responses.

Keywords: memory B cell, class-switch, somatic hypermutation, follicular helper T cell, PLC-γ2

Introduction

One striking feature of the adaptive immune response is the generation of immunological memory. When the primary immunological challenge is resolved, several features of the immune response, such as the increased cellularity of secondary lymphoid tissues, subside. For many antigens, however, resolution of the immune response does not completely reset the immune system to the state prior to antigen exposure; the system retains a memory of the response, and it is long lasting, sometimes persisting for a lifetime in both rodents and humans (1, 2).

Immunological memory is characterized by qualitative and quantitative differences between the response that occurs after the primary exposure and the subsequent response that occurs after re-exposure to the same antigen. T-cell-dependent memory humoral responses are typically more rapid, have a greater magnitude, and contain antibodies of higher affinity (1). This improved responsiveness is thought to derive from a relatively small number of memory B cells and T cells that participated in the primary immune response and then survived in a quantitatively and qualitatively altered state. Thus, understanding

the mechanism by which memory B cells are generated and maintained, as well as defining the basis for their heightened responsiveness, is of fundamental interest to reveal the basis of immunological memory.

It is generally thought that memory B cells develop under T-cell help within the germinal center (GC), where somatic hypermutation and isotype switching of the immunoglobulin (Ig) heavy chain take place (3–5). The isotype-switched B-cell antigen receptor (BCR), for instance of IgG type, has been considered the 'gold standard' for identifying memory B cells. However, accumulating evidence has shown that unswitched and unmutated memory B cells exist in both rodents and humans, raising the question of whether these memory B cells have distinct or overlapping functions. The purpose of this review is to summarize the major recent advances in our understanding of how heterogeneous memory B-cell populations are generated in response to initial antigen exposure, mainly focusing on T-cell-dependent antigens, where these cells are located, and how they respond to antigen re-exposure, with specific emphasis on their intrinsic properties.

How to identify memory B cells

In this review, memory B cells are defined as quiescent antigen-experienced lymphocytes that promptly differentiate into effector cells after antigen re-exposure. Studies of memory B cells have been done in rodent and human systems, and both models have contributed to our understanding of the unique properties of memory B cells. Here, we first describe approaches to identify and isolate memory B cells from humans and mice, the most widely studied of the rodents, and then point out several advantages and disadvantages of each model.

Human system

Due to the inherent obstacles to experimentation in humans, isolation of antigen-specific memory B cells, either before or after their activation *in vivo*, is not as easy as in mice. Therefore, human memory B cells of unknown antigen-specificity have been isolated solely by cell surface markers. Due to the lack of any positive markers, human memory B cells were first isolated by negative selection (IgD^-CD38^-) from total tonsillar B cells (6, 7). However, later studies suggested that CD27, a tumor necrosis factor receptor (TNF-R) family member of unknown function, could be used as a marker of human memory B cells (8, 9). The idea that $CD27^+$ B cells represent memory B cells was supported by several observations. $CD27^+$ B cells responded to several stimuli more robustly than naive B cells *in vitro*, their numbers were very low in the antigen inexperienced newborn but increased with age, and more importantly, most of the mutated and class-switched B cells were $CD27^+$ (8–12). It is impractical to study the *in vivo* behavior of human $CD27^+$ B cells during the course of primary and secondary responses, however recent adoptive transfer studies using immune-compromised host mice showed that human memory B cells could at least differentiate into plasma cells after antigen re-challenge (13).

The biggest advantage of using human rather than murine memory B cells is the greater abundance of the cells. Around 0.01% of total B cells in the spleen of an immunized mouse are antigen-specific memory B cells. However, based on CD27 expression, close to 40% of the B cells in adult human venous blood are considered to be memory cells, although they are polyclonal (8). Many of the phenotypic features and several intrinsic properties of memory B cells in terms of their responsiveness to various *in vitro* stimuli were in fact first described in studies of humans. Many of these findings have proven to be true for both humans and mice, but there are notable exceptions, for example, dependency on Toll-like receptors, as described in detail below. A drawback to studies of the phenotypes and intrinsic properties of human memory B cells is the number of variables that cannot be controlled or even determined, for example the form of initial antigen exposure, its delivery route, and the age of the memory cells. Therefore, human $CD27^+$ memory B cells are clearly composed of several subsets that likely have different features and possibly functions, as described in the next section.

Murine system

There are two major advantages for studying the memory B-cell responses in mice rather than humans. First, by using model antigens and BCR-transgenic mice in the study of immune responses, identifiable precursor cells can be tracked over the course of the response. Second, because synchronous activation of the antigen-specific cells is feasible *in vivo* and because antigen-experienced cells can be permanently labeled by using methods such as insertion of tamoxifen-inducible Cre into an *Aicda* locus [encoding activation-induced cytidine deaminase (AID)] (14, see below), the age, behavior, and function of memory B cells can be tracked *in vivo* (Fig. 1).

Classically, many groups have isolated murine memory B cells as antigen-binding, isotype-switched B cells that also express or not a combination of other markers that are presumed to discriminate memory B cells from other antigen-experienced B-cell populations, such as GC B cells and plasma

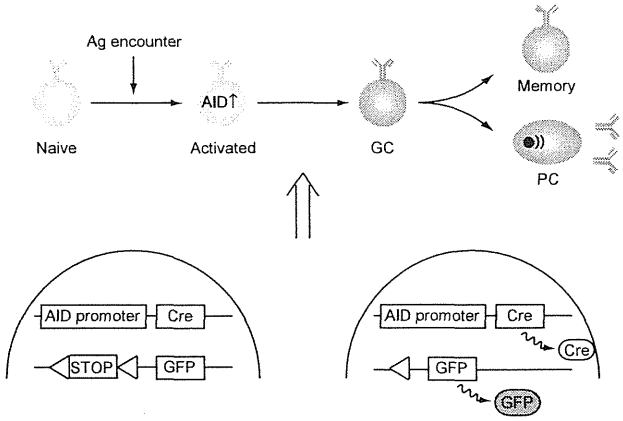

Fig. 1. Fate mapping for memory B cells. In the mice expressing Cre recombinase under the control of activation-induced cytidine deaminase (AID) promoter, antigen-experienced B cells upregulate Cre recombinase which can mediate excision of the loxP-flanked stop cassette to allow the permanent expression of green fluorescence protein (GFP). Germinal center B cells, memory B cells, and isotype-switched plasma cells are irreversibly labeled by GFP even after Cre expression is ceased. (triangles: loxP sites)

cells (15, 16). Plasma cells are easily marked by several surface receptors (e.g. CD138) (17) or transcription factors (e.g. Blimp-1) (18), and GC B cells can be separated from memory B cells by virtue of their binding to the GL-7 mAb (19) or peanut agglutinin (PNA) (20) and because they have down-regulated CD38 (15).

While the above approach has enabled us to isolate isotype-switched memory B cell, unswitched memory B cells are not captured by this type of analysis. Recently, antigen-experienced cells including both switched and unswitched, have been successfully labeled using a Cre/loxP system. AID was discovered as an indispensable enzyme for isotype-switching and somatic hypermutations of the BCR (21). As it turns out, B cells are activated by antigen exposure *in vivo*, almost all of them upregulate AID at the transcriptional level, thereby making AID expression a signature of antigen encounter. Indeed, mice bearing an *Aicda* locus with a knocked-in tamoxifen-inducible Cre recombinase effectively turned on enhanced green fluorescence protein in the cells that had expressed AID during the course of an immune response (14). Importantly, this strategy does not depend on the phenotype of the cells at the moment of analysis, but rather on their previous antigen-experience or that of their precursors. Because a memory B cell is by definition antigen-experienced, these mice provide a novel tool to identify memory B cells and to trace their functions *in vivo*, regardless of whether they have isotype-switched or not.

Although an excellent model system, the above fate mapping strategy using AID has a potential weak point, because not only memory B cells but also GC B cells are labeled. In this regard, it would definitely be desirable to find specific markers, analogous to FoxP3 for regulatory T cells (22–24), that would allow the specific isolation of the memory population. Now that global gene expression data from antigen-binding murine memory B cells is available and several genes that are upregulated on memory B cells have been found (25, 26), this approach should become a feasible one for the identification of novel memory B-cell markers.

Heterogeneity of memory B cells

There is now accumulating evidence of heterogeneity of memory B cells. This heterogeneity is particularly evident in humans because, by using $CD27^+$ as an isolation marker for memory B cells, a broad range of B cells in terms of antigen-specificity, isotype expression, and ages can be identified. Indeed, in addition to isotype-switched $CD27^+$ memory cells, about half of $CD27^+$ B cells in blood expressed IgM, and these cells can be further subdivided into IgM^+IgD^+ and IgM-only compartments (8, 12). Both of these populations primarily contain somatically mutated V regions, although unmutated cells also exist (8, 27). The origin of somatically mutated $CD27^+IgM^+$ memory B cells has recently become a topic of debate (28, 29). Three major mechanisms have been postulated. First, the cells are generated independently of GC processes and in fact undergo somatic hypermutation during diversification of the pre-immune repertoire (30, 31). However, in terms of the accepted definition that memory B cells are antigen experienced, such cells cannot be considered as *bona fide* memory B cells. Second, the cells are generated by exposure to T-independent antigens, such as bacterial polysaccharides, probably through a GC-independent manner (12, 31). Indeed, $CD27^+IgM^+$ human B cells have been reported to play a role in immunity against encapsulated bacteria by producing anti-polysaccharide IgM (12). Finally, the cells are generated in the GC by canonical T-dependent antigens. Supporting this third mechanism, $CD27^+IgM^+$ memory B cells, like class-switched memory cells, have somatic mutations in the Bcl-6 gene, a genetic footprint of GC experience, demonstrating that many, if not all of the $CD27^+IgM^+$ cells, derive from a GC reaction during a T-cell-dependent response (32).

Due to the low precursor frequency of murine antigen-specific memory B cells, their phenotypic characterization has lagged behind that possible with the human polyclonal memory cells. The usage of BCR-transgenic and knock-in mice has somewhat circumvented this problem. Through such analysis, Shlomchik and his colleagues (33) have recently found that (4-hydroxy-3-nitrophenyl)acetyl (NP)-binding memory B

cells could be separated into subpopulations based on CD80 and CD35 expression. Intriguingly, 70% of the $CD35^+$ fraction of $CD80^+$ memory B cells had unmutated V genes, whereas almost all the $CD35^-CD80^+$ memory B cells were mutated. These data clearly indicate that unmutated memory B cells are generated and maintained for a long period as distinct subsets expressing unique surface markers. These unmutated memory B cells may originate from a pathway that is independent of GCs as discussed in detail later.

Memory B-cell development

Where are memory B cells generated?

By using rodent systems, information about the behavior of naive B cells during immune responses has been accumulated, thereby providing insights into where memory B cells are generated. The following general scenario has emerged from these types of studies. After initial exposure to antigen, antigen-binding IgM^+ B cells migrate from random locations within the B cell follicles to the border between the follicles and the T cell-rich area, where cognate interactions with antigen-specific CD4 T cells take place (34, 35). Next, B cells proliferate and foci of antibody (Ab)-secreting plasmablasts, some of which contain isotype-switched antibodies, appear at the border between the T cell area and the red pulp of the spleen, 6–8 days after primary immunization. Shortly after these foci appear, clusters of isotype-switched cells such as $IgG1^+$ B cells that are stained with PNA and GL-7 mAb appear in GCs within the areas of the follicles occupied by follicular dendritic cells (FDCs) (36–38).

In situ detection of isotype-switched B cells at early times after the switch had been difficult, because of the rarity of these antigen-specific B cells. However, now this problem has been circumvented by employing B cells harboring antigen-specific BCR knocked-in the endogenous heavy chain locus and therefore capable of class switching, thereby allowing us to track the genesis and fate of antigen-experienced IgM as well as class-switched B cells. By using this approach, Jenkins and his colleagues traced the behavior of IgM and IgG type B cells after primary immunization (39). They observed that antigen-specific isotype-switched B cells (in their experimental setting, IgG2a type) appeared on day 2 in the follicles rather than in foci of plasmablasts in the red pulp and medullary cords, as expected from previous work (40, 41). Thus, it now appears more likely that, after receiving signals from helper T cells at the T–B border, IgM B cells migrate into follicles where they proliferate and initiate class switching. On day 4, some of the antigen-experienced IgM and IgG2a B cells appeared in the pre-GC region, where antigen was concentrated, presumably on FDCs. On day 10, many of the surviving antigen-experienced B cells in these regions expressed IgG2a, whereas IgM^+ cells were located outside the GCs. At least two explanations are possible for the failure of antigen-experienced IgM B cells to persist in GCs on day 10. First, antigen-specific IgM B cells might promptly undergo isotype-switching after they enter this location due to abundance of antigen-specific follicular type T cells. Thus, by day 10, no more precursor IgM type B cells would remain in the GC region. Alternatively, although some IgM type progeny are generated in the GC region, IgG2a type B cells might have a competitive survival advantage. As suggested in this paper, the antigen-experienced IgG2a B cells located in the GC region on day 10, some of which expressed CD38, can be considered as memory or memory-precursor B cells. As mentioned above, CD38 is a good marker for distinguishing memory versus GC B cells among class-switched B cells. The antigen-specific IgM type B cells scattered in the follicles on day 10 were likely memory or memory-precursor cells, because they had undergone several rounds of proliferation. Thus, this study not only identifies the initial site of class switching, but also provides significant insight into where IgM and IgG2a type memory B cells are initially generated.

Potential mechanisms for development of memory B cells

In terms of the developmental pathways of memory B cells, published studies have only focused on the IgG type cells. Most models of post-GC B cell development posit that cells destined to become plasma cells and memory B cells develop separately (bifurcative differentiation model) (Fig. 2). However, an alternative possibility is that there is a single developmental path, beginning with memory cells and progressing to plasmablast and plasma cells (linear differentiation model). Although there is little data at present to validate either model, the early appearance of $Blimp^+$ plasmablasts in the GC somewhat favors the bifurcative model (42). Assuming that the bifurcative model is correct, does this plasma versus memory decision come about as a result of different progeny receiving different cytokine, chemokine, and/or niche cues from their environment? Or is the decision the result of differences in the duration or strength of antigen stimulation? Or is it instead a stochastic event that occurs owing to cell-intrinsic unequal segregation of lineage-determining factors to daughter cells, as has been proposed for T-cell fate determination? These important questions have not been adequately addressed so far, but some fragmentary evidence has accumulated.

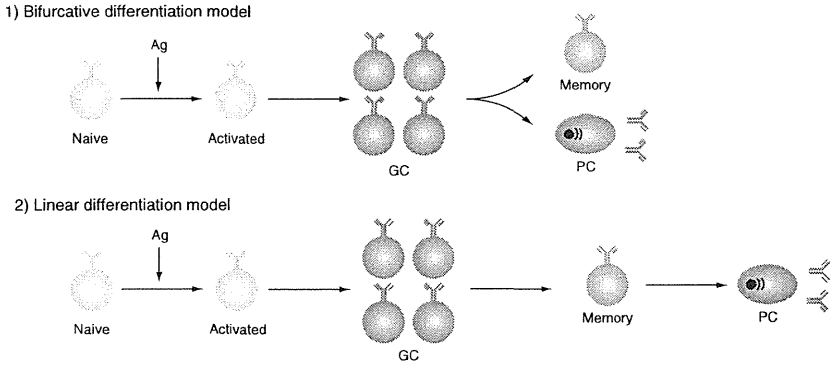

Fig. 2. Two possible models for the generation of memory B cells and plasma cells from germinal center (GC) B cells. Model-1 'Bifurcative differentiation model': Naive B cells are activated and differentiated into GC B cells. GC B cells develop both memory B cells and plasma cells. Model-2: 'Linear differentiation model': GC B cells first differentiate into memory B cells, and subsequently plasma cells are generated from memory B cells.

BCR affinity

As for the BCR-antigen affinity issue, by using B cells harboring a knockin anti-hen egg lysozyme (HEL) BCR, Brink and his colleagues (43) have tried to determine whether *in vivo* plasma cell differentiation of IgG1 GC B cells occurs in an affinity-based selection manner. Indeed, the IgG1 GC B cells that had acquired high affinity for the immunizing antigen were apt to differentiate into plasma cells, suggesting that stronger BCR signaling favors plasma cell differentiation. Therefore, it is possible that the BCR signal could play a direct role in facilitating plasma cell differentiation, for example by inducing Bcl6 degradation and thereby releasing Blimp-1 from its repressed state (44, 45). Alternatively, responding GC B cells may stochastically commence plasma cell differentiation but require the strong BCR signal to survive beyond the very earliest stages of this process. Although the study of Brink and his colleagues did not examine the affinity of anti-HEL IgG1 memory B cells, given the evidence that IgG1 memory B cells in the NP-hapten system do not necessarily have higher affinity for antigen (46), it seems reasonable to anticipate that IgG GC B cells require a relatively permissive BCR signal strength to enter the memory pool, in contrast to the high stringency needed for entering the plasma cell pool.

Cytokine requirements

While analysis of mice with germline deletion of cytokine and cytokine receptor genes provides some insights into memory B-cell biology, interpretation of these data needs to be done with caution because the phenotype is obviously the sum of effects on both B and T cells, as well as other cell types. In this regard, recent studies of B-cell-specific deletion of Stat3 and Stat5 genes are informative. B-cell-specific deletion of Stat3 resulted in profound defects in T-dependent IgG responses after primary immunization (47). Because GC formation, isotype switching, and generation of antigen-specific IgG B cell appeared to be intact in these mice, Stat3 was proposed to play a major role in differentiation of IgG GC B cells to plasma cells. Given that IL-6, IL-2, IL-10, and IL-21 activate Stat3 (48–51), these cytokines, singly or in combination, are likely to utilize Stat3 for plasma cell differentiation. The function of Stat3 in memory B cell formation and maintenance remains to be determined. By *in vitro* gain-of-function experiments, Stat5 has been shown to control the differentiation and self-renewal potential of human memory B cells (52). By contrast, when the Stat5 gene was deleted in murine B cells *in vivo*, memory B cell formation as well as plasma cell differentiation occurred normally (53).

In the case of human GC B cells, the requirement for different cytokines in memory versus plasma cell formation has been defined by using *in vitro* experiments. In response to IL-4, human tonsillar GC B cells (roughly 50% are IgG positive) acquired a memory B cell phenotype (54). These cells express high levels of Bcl-2, CD80, and CD86 mRNA, a hallmark of *in vivo* memory B cells (7), and, with appropriate stimulation by IL-10, develop rapidly into plasma cells, also consistent with the memory features mentioned above. When GC B cells are grown with IL-10, they develop into cells with a plasma cell phenotype (54). Thus, exposure to IL-4 or IL-10 appears to bias GC B cells toward memory or plasma development, respectively. Despite the importance of IL-4 for *in vitro* human memory differentiation, its *in vivo* role in the mouse is unclear. The numbers of $IgG1^+$ GC B cells and memory B cells were equally reduced in IL-4-deficient mice, suggesting that IL-4 is required for GC formation but not for development of

memory B cell from GC B cells (55). However, IL-13, a closely related cytokine that shares similar biological activities and signaling pathways, could compensate for of IL-4 (56).

$CD4^+$ T cells and NKT cells produce IL-21 (57, 58). Among the $CD4^+$ T cells, IL-21 is expressed at the highest levels by T follicular helper (Tfh) cells (59). Its receptor is expressed on T cells, B cells, NK cells, macrophages, and dendritic cells (DCs) (60). Therefore, in the context of TD humoral responses, the *in vivo* function of IL-21 is thought to be mediated by its dual effects on, at least, two types of cells, Tfh and B cells. In terms of GC B cell maintenance, initial reports proposed a dominant role for IL-21 on Tfh cell formation, which in turn affects B-cell biology, rather than any direct action on B cells (61, 62). Indeed, in naive T cells, IL-21 leads to upregulation of Bcl6, the transcriptional regulator of Tfh cells (63–65). However, more recent reports by using mixed bone marrow chimeras have shown that IL-21 acts directly on B cells, thereby manifesting the defects of B-cell responses in IL-21-deficient mice (55, 66). In contrast to its effect on GC B-cell maintenance, IL-21 is dispensable for the generation of memory B cells (55). In addition, extrafollicular antibody responses, as seen with Salmonella species, are not affected in the absence of IL-21 (66).

Non-classical pathways for memory B-cell development

Immunoglobulin V genes of T-cell-dependent memory B cells carry somatic hypermutations which are introduced by GC reaction. However, unmutated V genes have been repeatedly observed in IgG type memory B cells by several groups (16, 33, 55), raising the idea that memory B cell development does not necessarily require GCs. Supporting this, immunization of mice having Bcl-6-deficient B cells with NP-hapten coupled to a carrier protein failed to generate GCs and somatic hypermutations, however it elicited IgG type memory B cells lacking somatic hypermutations (67). They persisted for a long period and promptly differentiated into plasma cells after secondary challenge, indicating that they represent functional memory B cells. The idea that unmutated memory B cells could develop via a GC-independent pathway was further supported by a different experimental setup in which memory B-cell development within GCs was blocked by treatment with an anti-inducible co-stimulator (ICOS) mAb (68). Consistent with the results from Bcl-6-deficient mice, the frequency of unmutated memory B cells was highly elevated in the treated mice. Thus, these data clearly indicate that even in T-cell-dependent responses, unmutated memory B cells can be generated independently of GCs (Fig. 3).

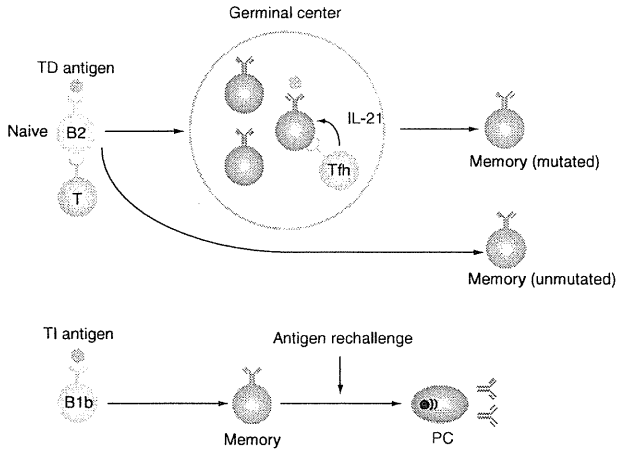

Fig. 3. Multiple pathways for memory B-cell development. In response to T-dependent (TD) antigen, naive B cells differentiate into memory B cells through germinal centers (GCs) or independently of GCs. Within GCs, the precursors for high-affinity memory B cell are generated by somatic hypermutations, while GC-independent pathway generates only low-affinity memory B cells. The maintenance of GC B cells partly depends on IL-21 from Tfh cells. In response to T-independent (TI) antigens, B1b cells differentiate into low-affinity memory B cells, which are maintained at resting state for a long period and promptly differentiate into plasma cells without T cell help after antigen re-exposure.

One of the classical features of memory B cells is that they require T-cell help both for their development and re-activation. T-cell-dependent memory B cells have long been the major targets of memory B-cell studies, especially in the mouse. However, there are several reports describing the appearance of memory B cells during T-cell independent responses in rodents. Antigen-specific B cells persisted for a long period in rats that had been immunized with trinitrophenyl-lipopolysaccharide (TNP-LPS), a T-cell independent type 1 antigen and these cells showed enhanced responsiveness following antigen re-exposure (69). More recently, two papers have described the development and persistence of memory B cells in response to T-cell independent type II antigens (70, 71). In one report, memory B cells specific for a T-cell independent antigen on the surface of *Borrelia hermsii* were shown to originate from B1b cells ($CD11b^+CD21^-CD23^-$), which had been thought to behave as 'innate' rather than inducible memory cells (70). These B1b type memory B cells persisted as quiescent cells and promptly differentiated into plasma cells following bacterial re-challenge. Additionally, T-cell independent memory B cells generated in response to NP-Ficoll expressed unswitched IgM BCR with no somatic hypermutations (71). Thus, T-cell independent memory B cells indeed exist and differ from canonical T-cell-dependent memory B cells in their origins and phenotypes (surface markers and immunoglobulin genes).

Localization of memory B cells

Since memory B cells are believed to be long-lived irrespective of whether they express IgM or a switched isotype, the question arises of whether the initial lodging sites for IgG2a and IgM type memory B cells, identified by Jenkins and his colleagues, can function as niches for longer time periods, or do these sites function simply as transit stations for memory B cells prior to their joining the recirculating pool. These questions have been answered, in part, by tracing their lodging sites over longer periods.

By using a transgenic mouse line harboring a rearranged V_H186.2 IgM type heavy chain gene, Shlomchik and his colleagues (33) demonstrated that long-lived IgM type memory B cells reside not just in the marginal zone (MZ), as had been observed in the rat system, but also scattered in splenic follicles on 12 weeks after immunization with NP-CGG. By using non-transgenic mice and similar immunization protocols, we have recapitulated their results; IgM type memory cells were seen scattered in splenic follicles on day 60 after immunization. Moreover, we observed that IgG1 type memory B cells preferentially localized adjacent to contracted GCs (Aiba *et al.*, manuscript in preparation). Together, these data favor the idea that the initial lodging sites for IgG and IgM type memory B cells can function like niches for their long-term survival, rather than as a transit station. Persistence of the GC-like structures on day 60 after injection of NP-CGG is consistent with the recent report demonstrating that GC-like structures can be detected for up to 8 months after challenge with sheep red blood cells (14). Thus, it is reasonable to anticipate that the GC-like structures persist longer than previously thought, and that the duration of such GC-like structures is dependent, at least partly, on the nature of the immunogen and adjuvants.

Although many experimental systems have shown that memory T cells reside in peripheral non-lymphoid organs, which are likely to be the first line of defense (73), there is limited data so far regarding the presence of memory B cells in these sites. Virus infection in the lower respiratory tract generates lymphoid tissue in the lungs named induced bronchus-associated lymphoid tissue (iBALT), which resembles secondary lymphoid organs with distinct B and T-cell areas and HEV (74). iBALT developed by day 7 after infection, concomitantly with the formation of GL-7^+ GC structures. Moreover, virus-specific plasma cells persist in the lungs for a long period even after viral clearance, although the origin and lifespan of these plasma cells remain elusive (75, 76). Sangster's group (76) has recently enumerated memory B cells in infected lungs by an approach similar to that utilized for counting human memory B cells with a particular antigen-specificity. After stimulation of lung B cells under limiting dilution conditions, these investigators detected considerable numbers of virus-specific plasma cells of IgG and IgA isotypes, and concluded that virus-specific class-switched memory B cells reside in the infected lungs. Notably, IgA^+ B cells preferentially localized to the lungs, whereas IgG^+ B cells were equivalently dispersed in the draining LN and lungs. Although we have to await the isolation and further characterization of those antigen-experienced B cells in the lungs, these data suggest that, in analogy with memory T cells, memory B cells with distinct functions may persist in non-lymphoid organs and confer protection against recurrent infections.

Maintenance of memory B cells

Humans with loss-of-function mutation in genes encoding CD40/CD40L, NEMO (NFκB essential connector), ICOS, signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), and CD19 have dramatic reductions in the frequencies and absolute numbers of memory B cells (30, 77–80). These patients are essentially devoid of isotype-switched memory B cells and lack the majority (more than 60%) of IgM type memory B cells. However, these reports have struggled to distinguish whether a gene is required for GC formation, memory differentiation, or memory maintenance. Such problems are now being resolved by the generation of mouse strains containing inducible forms of Cre recombinase, such as ERT2-Cre, or Cre driven by promoters that either become active after B cell activation or are expressed late in development (81). These include Cγ1-Cre, in which deletion of floxed genes is restricted to B cells initiating CSR to IgG1, and an AID-Cre strain in which Cre is expressed from a bacterial artificial chromosome (BAC) transgene under the control of the AID promoter and thus restricted to B cells after *in vivo* activation (82, 83).

To address the importance of BCR signaling in formation and/or maintenance of memory B cells, we had chosen the PLC-γ2 gene, because PLC-γ2 is well known to function downstream of the BCR and to be required for calcium/PKCβ activation following antigen stimulation (84, 85). By crossing PLC-$\gamma2^{f/f}$ mice with Cγ1-Cre or ERT2-Cre mice, we demonstrated that PLC-γ2 is critical for GC and memory B-cell formation and, more importantly, that it is also required for memory B-cell maintenance (86). Antigen-dependency of memory B-cell survival is still debated (87, 88). For instance, it had been shown that antigen could be retained on FDCs for many months, and possibly years after primary immunization

(89). It was therefore hypothesized that memory B cells needed to interact with this retained antigen in the form of immune complexes possibly trapped by Fc and/or complement receptors expressed on FDCs for long-term survival. In contrast, using an elegant *in vivo* system in which BCR specificity could be changed after memory B cells had been generated, Maruyama *et al.* (90) found that memory B cells that could no longer bind the immunizing antigen had a lifespan comparable to that of memory B cells that had not switched receptor specificity. Although Maruyama's data suggest that antigen occupancy is dispensable for the maintenance of memory B cells, they do not necessarily mean that BCR signaling is not required. Thus, based on our observations using the PLC-$\gamma 2^{f/f}$ mice, we proposed that survival receptors, probably the BCR, use PLC-γ2 for the maintenance of memory B cells.

In addition to BCR, the involvement of TLR receptors, TNF-receptor family, and cytokine receptors in maintenance of memory B cells has been postulated. The idea that memory B cells can be polyclonally activated by TLR ligands was proposed based on *in vitro* responsiveness of human memory B cells and the observed linear correlation of persistent serum Ab titers and memory B-cell frequencies in humans (91). However, in mice, deletion of key signaling molecules for TLR signaling [MyD88 and TIR domain containing adaptor-inducing interferon-beta (TRIF), together] did not significantly affect the secondary antibody responses (92). Although the number of antigen-specific memory B cells during extended time periods such as 3 months was not analyzed in these studies, they do raise the question of whether the TLR-dependent maintenance mechanism operates *in vivo* during typical TD-antigen-induced immune responses. As for the TNF-receptor and cytokine receptor families, so far there is no convincing genetic evidence demonstrating their involvement in maintenance of memory B cells at least in the mouse model systems.

Reactivation of memory B cells

The cellular and molecular basis for the faster and more robust humoral responses after antigen re-exposure still remains a mystery, although some suggestive data has been gleaned as described below. In terms of this important issue, at least, three layers of regulation are worth considering. First, not only the B cells, but also other types of cells, such as T cells, might be changed during primary immune responses. For instance, memory type Tfh cells may be formed during primary immune responses, and they may be specialized for helping memory B cells upon antigen re-exposure. Second, assuming that such other cell types are required for activating memory B cells, spatial reorganization of these cells might be occurring in the host, which could then contribute to the faster and more robust humoral memory responses. Finally, in regard to B cells, the increased frequency of antigen-specific memory B cells and the intrinsic properties that memory B cells acquire after primary antigen experience could contribute to the features of memory responses.

Contribution of other cell types and their spatial reorganization to rapid memory responses

It is now clear that antigen-specific T-helper cell development can proceed along multiple paths depending upon the nature of the antigen assault. In this context, Tfh cells have been considered a separable T-helper cell subset specialized for regulating the evolution of primary and memory B-cell responses (93). McHeyzer-Williams and his colleagues (94) demonstrated the existence of a memory compartment of antigen-specific effector Tfh cells that persists locally to regulate accelerated memory B-cell responses. Importantly, the local retention of Tfh memory cells in draining lymphoid tissues appears to require cell-associated cognate peptide-MHCII, suggesting that the primary antigen is not completely eliminated in the periphery, but still remains, at least to some extent, for maintenance of the memory Tfh cells. Assuming that memory Tfh cells are a major player in T-dependent memory humoral responses, clarifying the exact location of the memory Tfh cells in the secondary lymphoid tissues and their activation mechanisms is very important to understand why humoral memory responses are rapid and robust. In regard to localization of memory Tfh cells, we have recently shown that memory Tfh cells reside close to IgG type memory B cells surrounding the contracted GCs in the spleen (72), allowing us to propose that such close proximity of memory B and Tfh cells can explain, at least partly, the more rapid kinetics of humoral memory responses (Fig. 4).

What is the intrinsic difference between naive and antigen-experienced memory B cells?

Because of the relative ease of obtaining large numbers of memory B cells, the intrinsic properties in the *in vitro* reactivation of these cells have been extensively analyzed in humans. Most studies have utilized purified $CD27^+$ cells and then compared their responsiveness to $CD27^-$ naive B cells. Activation of splenic $CD27^+$ memory B cells with CD40L, IL-2, and IL-10 induced terminal differentiation into plasma cells, more efficiently than with naïve cells (95). Additionally, after

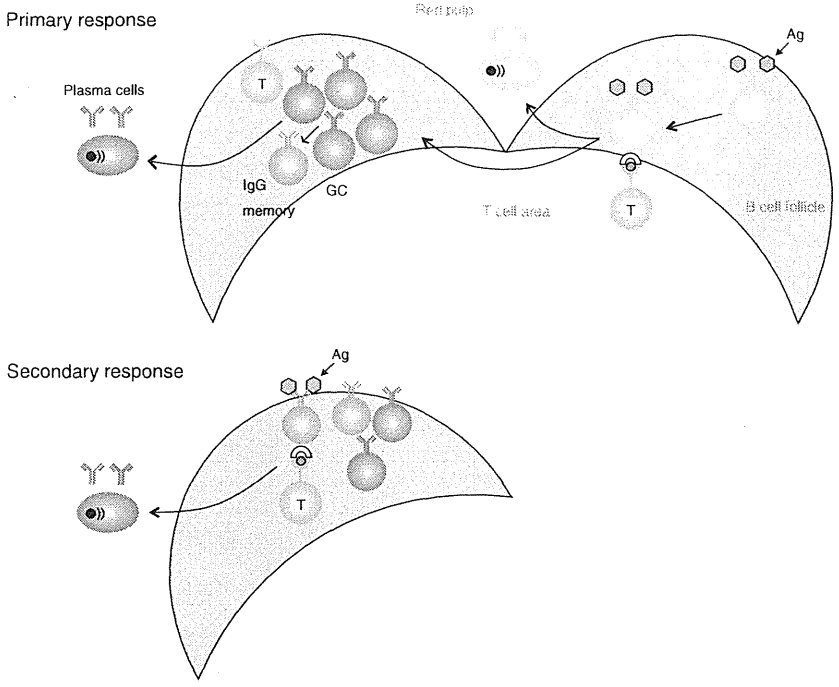

Fig. 4. Unique localization of IgG type memory B cell allows the prompt memory response. After primary antigen exposure, antigen-reactive B cells move to T–B boundary and interact with helper T cells. B cells activated by helper T cells differentiate into plasma cells, germinal center (GC) B cells or memory B cells. IgG type memory B cells preferentially localize adjacent to contracted GCs and promptly interact with Tfh cells proximal to them and rapidly differentiate into plasma cells, which appear to explain, at least partly, the rapid kinetics of secondary IgG responses.

treatment with T-cell-dependent (CD40L+anti-Ig) and T-cell independent (CpG+anti-Ig) stimuli, $CD27^+$ memory B cells entered the cell cycle earlier and underwent greater numbers of division than naive B cells (96). Some of these studies carefully examined the *in vitro* properties of non-class-switched and class-switched $CD27^+$ memory B cells, showing that both possess similar characteristics. These *in vitro* properties support the idea that there are intrinsic differences between naïve and memory B cells, irrespective of which isotype is expressed, and explain partly why memory B cells are able to respond faster upon secondary exposure to antigen. Global gene expression analysis of human memory B cells identified several candidate genes and signaling pathways that may underlie the rapid secondary response (97). Compared to naive B cells, both IgM^+ and isotype-switched memory B cells expressed reduced levels of negative cell cycle regulators, but increased levels of positive regulators. To address the role of these molecules in the rapid entry of memory B cells into cell cycle, Good and Tangye (96) have focused on the KLF4, KLF9, and promyelocytic leukemia zinc finger (PLZF) transcription factors, which are negative regulators of the cell cycle that are down-regulated in memory B cells. They observed that enforced expression of these genes in memory B cells delayed their entry into cell cycle, concluding that the reduced expression of these molecules in memory B cells allows their rapid response to external stimuli. In contrast to these recent clues about potential mechanisms for rapid entry into cell cycle, the molecular basis for why human memory B cells are prone to differentiate into plasma cells remains elusive (98).

As described above, it is now possible to label not only IgG type but also IgM type memory B cells by utilizing tamoxifen-inducible Cre inserted into *Aicda* locus. This method, combined with adoptive transfer of these memory subsets into mice, has revealed unappreciated differences between IgM and IgG type memory B cells (14) (Fig. 5). After antigen re-exposure, most transferred IgG^+ memory B cells differentiated into IgG-secreting plasma cells, similar to the biased terminal differentiation of human tonsillar memory B cells into plasma cells *in vitro* (98). Furthermore, this behavior is very consistent with a recent report showing that IgG type memory B cells have little capacity to reinitiate a GC response (99). Intriguingly, however, IgM type memory B cells predominantly gave rise to IgM and IgG GC B cells rather than differentiating into plasma cells, disclosing their very different effector properties in comparison to IgG type memory B cells. This *in vivo* property of IgM type memory B cells differs markedly from their *in vitro* characteristics mentioned above. One interpretation that reconciles these data is that these IgM type

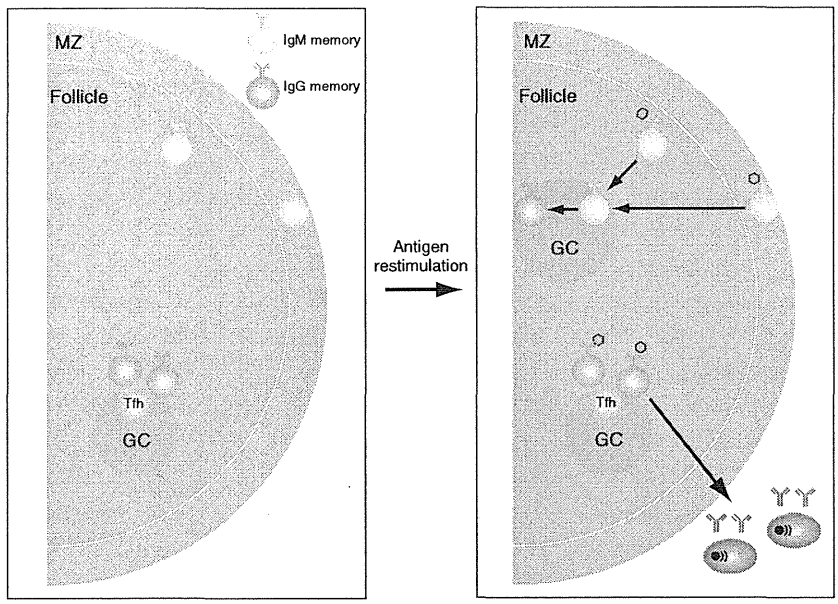

Fig. 5. Different effector functions of IgM and IgG type memory B cells. After priming with T-cell-dependent antigens, IgM type memory B cells emerge not only in marginal zone (MZ), but also scatter in splenic follicles. IgG type memory B cells, however, preferentially localize adjacent to contracted germinal center (GC) together with Tfh cells. After antigen re-exposure, IgM type memory B cells preferentially differentiate into GC B cells, while IgG type memory B cells terminally differentiate into plasma cells, exerting different effector functions.

memory B cells, despite having a greater potential to differentiate into plasma cells than naive B cells, are in an appropriate microenvironment where sufficient cytokines for GC B-cell differentiation are provided *in vivo*.

The molecular mechanisms underlying these differential effector properties of IgM and IgG type memory B cells are currently unknown, but their differential properties are likely to have a significant biological impact; IgM type memory B cells contribute to replenishment of the memory pool by their rapid mobilization into GC and switching to IgG, while IgG type memory B cells promptly differentiate into isotype-switched plasma cells to immediately confer protective functions. More detailed analysis of IgM and IgG type memory B cells in both mice and humans is definitively required for dissecting the molecular mechanisms underlying the differential effector properties of these B cells.

Future perspectives

Many intrinsic properties of memory B cells have been elucidated by using human memory B cells, however, recent advances that have allowed visualization of murine memory B cells, defined as antigen-experienced, long-lived B cells, after synchronous activation with antigen have provided new insights into the overall picture of memory B-cell responses. Consistent with the human studies, murine memory B cells are heterogeneous in their phenotypes, isotypes, and mutational status of their immunoglobulin V genes. Moreover, through the analysis of *in vivo* dynamics of murine memory B cells, it has been elucidated that considerable diversification exists in their localization and effector functions, issues that were not possible to directly address in the human studies. At present, we do not know where and how this heterogeneity of memory B cells originates, but it appears to be generated by the complexity of precursors and pathways for memory B-cell development. Because the choice for each developmental pathway is determined in part by immunization methods, we may need to use several types of antigens and delivery routes to visualize and dissect as many memory components as possible. In particular, many antigens that have great biological impacts are not pure protein antigens, but also include sugars and complex carbohydrates with unique structures. Dissection of memory B-cell response against these non-conventional antigens will be necessary for the translation of basic information into vaccine design, or treatment of allergy and autoimmune diseases.

In addition to these newly arising questions, two long-standing fundamental questions about the intrinsic properties of memory B cells still remain unanswered. How is the survival of memory B cells achieved? What determines the robust and prompt responsiveness of memory B cells after antigen re-exposure? Because PLC-γ2 has been identified as a key

molecule for memory B-cell survival, and the responsiveness of re-stimulated memory B cells can now be monitored in vivo, it will be possible in the near future to finally answer these questions.

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Inactivated and adjuvanted whole-virion clade 2.3.4 H5N1 pre-pandemic influenza vaccine possesses broad protective efficacy against infection by heterologous clades of highly pathogenic H5N1 avian influenza virus in mice

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ABSTRACT

In this study, we evaluated the immunogenicity and protective efficacy of a candidate attenuated H5N1 pre-pandemic influenza vaccine of clade 2.3.4, rgAnhui, which was reverse genetically generated from highly virulent A/Anhui/01/2005 (H5N1) wild-type virus. When a low-dose antigen (0.3 μg HA) vaccine was combined with aluminum hydroxide adjuvant, virus neutralization and anti-HA IgG antibodies induced in the sera of vaccinated mice showed similar levels as those in mice vaccinated with non-adjuvanted high-dose antigen (3 μg HA) vaccine. Serum antibodies had broad reactivity against highly pathogenic H5N1 viruses of both homologous and heterologous clades. All mice vaccinated with adjuvanted and non-adjuvanted rgAnhui vaccines at low and high antigen doses survived, without any significant weight loss, lethal challenge infection with homologous clade 2.3.4 viruses, including antigenic variant virus and heterologous clade 2.1.3. Mice vaccinated with low-dose antigen without adjuvant, however, exhibited 20% and 60% survival rates against clade 1 and clade 2.2 viruses, respectively; but, addition of adjuvant improved these rates to 80% and 100%, respectively. The data strongly suggest that aluminum hydroxide-adjuvanted rgAnhui vaccine can elicit broad cross-reactive and protective immunities against homologous and heterologous clades, and that the rgAnhui vaccine is a useful pre-pandemic H5N1 vaccine.

1. Introduction

H5N1 highly pathogenic avian influenza virus (H5N1 HPAIV) has become enzootic in some countries and has the potential to cause an influenza pandemic. The direct avian-to-human transmission of H5N1 HPAIV, with a high mortality rate, was first reported in Hong Kong in 1997 [1,2]. Subsequently, the number of reported human infections has increased in various countries around the world, particularly in Southeast Asia, the Middle East and Africa. As of August 2, 2011, the World Health Organization (WHO) has confirmed 563 cases of human infections and 330 deaths in 15 countries [3]. Most cases of H5N1 HPAIV infection in humans appear to be caused by direct avian transmission. However, several suspected cases of human-to-human transmission have also been reported [4–6], and these viruses have the potential to become human-adapted viruses due to the accumulation of mutations in their genome. Although there have been no reports of human infections in Japan, the isolation of H5N1 HPAIV from wild birds and outbreaks at poultry farms have been intermittently reported since 2003. In particular, 30 cases of virus detection in wild birds and 24 outbreaks in poultry farms were confirmed to be caused by clade 2.3.2 viruses in 2010 and 2011 [7]. Furthermore, anti-H5N1 HPAIV antibodies were detected in workers exposed to H5N1 HPAIV-positive poultry, although they showed no symptoms suggestive of viral infection [8]. Thus, sustained measures against H5N1 HPAIV by more rigorous monitoring of both wild birds and poultry, and by the national stockpiling of pre-pandemic H5N1 vaccines remain a high priority for pandemic preparedness.

The development of attenuated H5N1 vaccines from H5N1 HPAIVs isolated from wild birds, poultry and humans is performed by reverse-genetics (rg) to modify the virulent H5 hemagglutinin (HA) gene and to reassort the six backbone genes of A/Puerto Rico/8/1934 (H1N1) virus [9–11]. These rg H5N1 virus vaccines generally have low immunogenicity without adjuvant [12]. Conse-

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quently, we [13] and other groups [14,15] have assessed the effects of aluminum hydroxide adjuvant (alum), which is the most widely used licensed adjuvant for human use [16], and have confirmed positive results with respect to strengthened immunogenicity in mouse models and human clinical studies. However, other oil-in-water adjuvants, such as MF-59 [17] and AS03 [18], have recently been licensed and are now used in human H5N1 vaccines [19–22].

The H5N1 HPAIVs isolated worldwide have diversified both genetically and antigenically, and the current major isolates are clade 2.3.2 viruses detected in Hong Kong (SAR), China, Korea and Japan and clade 2.2.1 viruses detected in Egypt, although clade 1 viruses, which were the major isolates in 2004 [23], are still sporadically detected in Cambodia [24]. Therefore, multiple H5N1 vaccine candidate viruses from these clades, or a vaccine virus that can elicit broad reactive and protective immunities against various clades of H5N1 HPAIVs, must be prepared for pandemic preparedness.

In the present study, we assessed the immunogenicity and protective efficacy of an inactivated H5N1 whole virus vaccine, rgAnhui, with or without alum adjuvant against homologous and heterologous clades of H5N1 HPAIVs using a mouse model.

2. Materials and methods

2.1. Vaccine virus and adjuvant

Recombinant avirulent A/Anhui/01/05-PR8-IBCDC-RG5 (rgAnhui01/05) virus generated from the virulent A/Anhui/01/05 strain by reverse genetics (rg) was obtained from the United States Centers for Disease Control and Prevention (USCDC, Atlanta, USA). Virus was grown in the allantoic cavity of 10-day-old embryonated chicken eggs and purified by velocity density gradient centrifugation through a 10–50% linear sucrose gradient. The purified virus pellet obtained by ultracentrifugation was re-suspended in phosphate buffered saline (PBS) and inactivated by 0.05% formalin, as described previously [13].

A portion of the purified virus was separated by SDS-PAGE using a 12.5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue, and the gel image was captured using a CS cool saver (ATTO, Tokyo, Japan). The digitized gel image was then analyzed with a CS analyzer (ATTO), and the percentage HA content (%HA) was calculated by (HA1 + HA2)/(HA1 + NP + M1 + HA2) × 100. Protein concentration of the purified virus was determined using a DC protein assay kit (Bio-Rad Laboratories K.K., Tokyo, Japan) based on the modified Lowry method. HA content (μg/mL) was calculated by (%HA) × (protein concentration). Purified virus was appropriately diluted and mixed with aluminum hydroxide gel or PBS in order to obtain a final concentration of 0.3 mg/mL.

2.2. Animals, immunization and H5N1 HPAIV challenge

Eight-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Procedures involving mice were performed in accordance with the institutional guidelines for animal care.

Five mice per group were subcutaneously immunized twice at a 3-week interval with 100 μl of formalin-inactivated virus vaccine containing 0.3 or 3 μg HA with or without alum adjuvant. Mice in the control group were immunized according to the same schedule with alum adjuvant only. Three weeks after the last immunization, mice were anesthetized and then intranasally inoculated with 20 μl of virus solution containing 20× 50% mouse lethal dose (MLD_{50}) of H5N1 HPAIV. The H5N1 HPAIVs used in this study were: A/Viet Nam/JP1203/2004 (VN) [clade 1], A/Indonesia/5/2005 (Indo) [clade 2.1.3], A/Turkey/12/2006 (Tk) [clade 2.2], A/Japanese White Eye/HK/1038/2005 (JWE) [clade 2.3.4], A/Laos/JP127/2007 (Laos) [clade 2.3.4] and A/Myanmar JPA007-07/2007 (Myan) [clade 2.3.4]. For 2–3 weeks after lethal H5N1 HPAIV challenge, mice were monitored daily for their survival and weight. During monitoring, mice losing 30% of their body weight were euthanized.

2.3. Hemagglutination inhibition (HI) assay

Post-infection ferret anti-sera raised against JWE, rgAnhui and Laos viruses were provided by the USCDC. Sera were treated with RDE (Denka Seiken, Niigata, Japan) for 18–20 h at 37 °C and were then inactivated by incubation for 30 min at 56 °C. Sera were treated with packed turkey red blood cells for 60 min at room temperature in order to remove non-specific hemagglutinating factors in the sera. Using U-bottom 96-well microtiter plates (AGC Techno Glass Co., Ltd., Chiba, Japan), 25 μl of sera were serially diluted 2-fold with PBS and mixed with an equivalent volume of test antigens containing 8 hemagglutinating units (HAU) of virus. The mixture of diluted sera and virus was incubated for 30 min at room temperature. Fifty microliters of 0.5% turkey red blood cells was added to the antigen/serum mixture and incubated for 45 min at room temperature. After determination of HI (positive or negative), endpoint antibody titers were expressed as the reciprocal value of the last dilution at which complete inhibition of hemagglutination was observed.

2.4. Titration of antigen-specific antibodies in mouse sera

Virus-neutralizing antibody titers were determined as follows. Sera were collected from mice at 21 days after the last vaccination, and were treated with RDE for 18–20 h at 37 °C, followed by inactivation via incubation for 30 min at 56 °C. Sera were serially diluted and mixed with an equivalent volume of H5N1 HPAIV containing 200× 50%-tissue-culture infectious dose ($TCID_{50}$) of virus. The mixture of diluted sera and virus was incubated for 30 min at 37 °C. Confluent monolayers of MDCK cells in 96-well microtiter plates were washed with PBS, and serum/virus mixtures were transferred into the wells. After incubation for 4 days at 37 °C under a humidified atmosphere containing 5% CO_2, cells were fixed with 10% formalin and then stained with NB staining solution (0.1% naphthol blue black, 0.1% sodium acetate and 9% acetic acid). Stained cells were eluted with 100 mM NaOH, and optical densities at 655 nm were measured using a microplate reader (Model 550; Bio-Rad Laboratories, Tokyo, Japan). Endpoint antibody titers were expressed as the reciprocal value of the last dilution with an optical density that was at least a half unit greater than the optical density of the uninfected control.

Viral HA-specific antibody titers were determined according to the method described by Takahashi et al. [25]. Briefly, baculovirus-produced recombinant HA proteins were used as the coating antigen, and viral HA-specific IgG1 and IgG2a antibodies in the sera of mice were measured by ELISA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 and IgG2a antibodies (Southern Biotechnology, Birmingham, AL, USA) were used as detection antibodies.

2.5. Statistical analysis

For comparison among virus-neutralizing antibody titers against various HPAIVs, we performed Steel's method for multiple comparison. The virus-neutralizing antibody titers against 3–4 virus strains were compared with anti-Myan or anti-VN virus-neutralizing antibody titers. The p-value of each comparison under 0.05 was regarded as statistically significant difference.