

### 3. 結果

#### 1) 英語コーパスを用いた頻度解析

まず、LSD に対訳が収録されている 49,034 語の英単語および語句について、語尾変化も含めて英語コーパスでの出現頻度を計数した (図 4A)。全体の 84% がコーパスに 1 回以上出現したが、6,714 語 (16%) はコーパスで出現しない用語であった。内容としては大学や機関名などの固有名詞、希少な動植物の学名、化学や物理学など周辺分野の語句が多く見られた。スペースを含む複合語を除いた 31,218 語の英単語に限ると、29,473 語 (94%) が英語コーパス中に出現していた。

次に、英語コーパスの全単語の頻度解析を行った。語尾変化を考慮せずにすべてのユニークな文字列を抽出する手法を用いると、英文コーパスから 67 万種類の単語が抽出された。このうち、数字と記号のみからなる 5 万語を除外した 62 万語について出現頻度ランクごとに単語の種類を数え

ると、両対数プロットで負の直線的相関性 (Power law) が認められた (図 4B)。回帰直線の傾きは  $-0.67$  ( $r^2=0.99$ ) となった。出現頻度が低くなるにつれてカバー率は低下したが、ほぼ半数を占める 30 万語はコーパスで 1 回しか出現しない記号やスペルミス等であった。

さらに、LSD で対訳を規定した英語が実際に研究者の書く英語のどの程度をカバーしているかを評価するために、英語コーパスのテキストを LSD で訳せるかどうかを規則的な語尾変化に対応する逐語訳で検討したところ、1,000 回以上出現する英単語はほぼすべて変換された。英語コーパス全体に対して LSD 収録単語で解読できるテキストの割合は 88% と算出された。

#### 2) 日本語コーパスを用いた頻度解析

医学・生命科学領域の総説および教科書から作成した大規模な日本語コーパスを解析した例はこれまでにほとんど報告されていない。今回、まず日本語コーパスの漢字、カタカナ、ひらがな、英数字という文字種の割合を解析したところ、漢字の割合が 35%、英数字とカタカナで記述される割合が全テキストの 28% にも及ぶことが明らかになった (図 5A)。また、ひらがなのみからなる文字列を除去した上でコーパス中のユニークな単語の種類を数えたところ、全体で 26 万語が抽出されたが、20% に相当する 5.4 万語は英語ないし英数字記号であった。なお、LSD に対訳が収録されている 48,024 語の日本語について、日本語コーパスに 1 回以上出現している単語を数えると 44,286 語 (92%) であった。コーパスに出現しない単語は化学、物理学、数学などの周辺科学領域に多く見られた。

次に、コーパスに出現する漢字およびカタカナを含む 21 万語について出現頻度ランクごとに単語の種類を数えると、英語の場合と同様に両対数プロットで負の直線的相関性が認められ、回帰直線の傾きは  $-0.89$  ( $r^2=0.99$ ) であった (図 5B)。また、LSD に収録されている日本語について、漢字変換テーブルに収録されている語と対訳を有する語のそれぞれについて頻度を解析すると、頻度 5 以上ではいずれもほぼすべて収録されていた。しかし、対訳を有する日本語は頻度 100 未満に

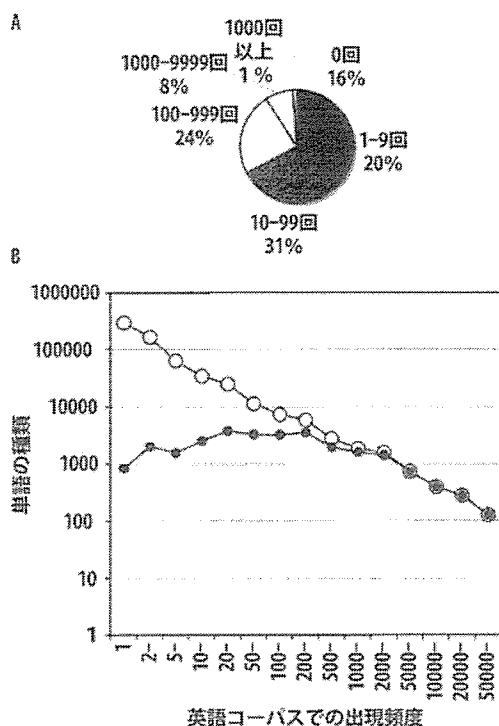


図 4 英語コーパスを用いた頻度解析

A: LSD 収録英語 (49,034 語) の頻度分布; B: 英語コーパスの全単語 (○) と LSD 収録英単語 (●) の出現頻度分布

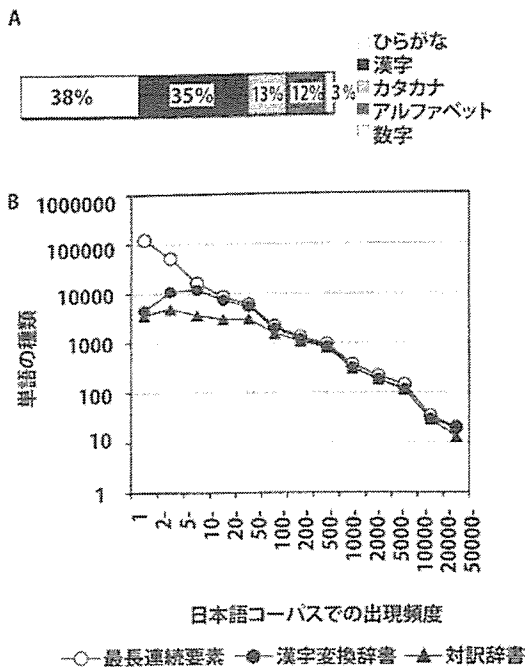


図5 日本語コーパスを用いた頻度解析

A: コーパスを校正する文字種の割合; B: 全単語 (○) と LSD 収録語 (●全日本語, ▲対訳あり) の頻度分布

において、出現頻度が低くなるにつれて収録率は低下していた (図 5B)。単純なテキストマッチングによって日本語コーパス中の漢字・カタカナ語に対して LSD 収録語がカバーする割合は 80%、さらに対訳が規定されている日本語に限定した場合は 66% と算出された。

3) MeSH ツリーへのマッピング

LSD 対訳テーブルで 70,622 対の訳語を定義している 49,034 語の英語に対して、自然な表記に変換した MeSH Term の照合を行ったところ (文末 図 7)、ツリーに帰属できる 65,733 語の MeSH Term のうち LSD と一致した共通語は 13,462 語 (20%) であった (表 1 最下段)。MeSH のうち、英語コーパスに 1 回以上出現した 33,195 語に限定すると、共通語は 12,971 語 (40%) となった。MeSH 収録語で LSD 未収録の用語としては、化合物名や生物学名などの事物を特定する固有の名称が多く見られた。一方、LSD 対訳のうち MeSH と照合できない英語は 35,572 語であり、その中には MeSH カテゴリーに掲

載されていない一般的な名詞 (例: increase, cooperation) や形容詞や動詞といった用言が特徴的に見られた。

次に MeSH カテゴリーおよび LSD 意味分類別に分けて詳細を検討した (表 1)。解剖学用語 (カテゴリー A) は LSD 収録数が MeSH を超えており、MeSH の 62% は LSD に収録されていた。LSD 収録語が多いのは、主として発生理学用語が多いためであった。生物学名 (カテゴリー B)、病名 (カテゴリー C)、化合物名 (カテゴリー D) について、MeSH の LSD 一致率はそれぞれ 22%, 31%, 13% と低かったが、英語コーパスに出現する語に限定すると、その割合は 37%, 57%, 29% に高まった。生物学名および病名では LSD 収録語のうちで MeSH 未収録の割合は比較的 low だったが、化合物名では日本国内でのみ用いられる薬物や生薬などで MeSH 未収録語の割合が高かった。技術・装置 (カテゴリー E) 以下の分類においては、LSD と MeSH の一致率はどちらから見ても

表 1 LSD と MeSH 収録語数と分類別比較

分類 [MeSH カテゴリー]	LSD英語 [A] <対訳数>	MeSH* [B] (2,024)	共通語* [C] =[A]∩[B]	カバー率* [D] =[C]/[B]	未マップ語 [E] =[A]-[C]
Anatomy [A]	4,102 (4,970)	2,576 (2,024)	1,604 (1,528)	62% (75%)	2,498
Organisms [B]	2,505 (3,101)	5,635 (3,254)	1,215 (1,193)	22% (37%)	1,290
Diseases [C]	5,403 (7,400)	12,095 (6,338)	3,700 (3,604)	31% (57%)	1,703
Chemicals & Drugs [D]	8,001 (9,806)	32,259 (13,835)	4,211 (4,015)	13% (29%)	3,790
Tecniq. & Equip. [E]	3,099 (4,214)	4,900 (2,740)	1,013 (965)	21% (35%)	2,086
その他の名詞 [F-2] および略語	14,648 (23,418)	8,268 (5,004)	1,719 (1,666)	21% (33%)	12,929
形容詞	7,984 (12,053)				7,984
動詞	2,144 (3,974)				2,144
副詞	1,148 (1,686)				1,148
合計	49,034 (70,622)	65,733 (33,195)	13,462 (12,971)	20% (40%)	35,572

\*丸カッコ内は英語コーパスで頻度 1 以上の語について集計した値

表 2 LSD を MeSH 収録語の平均語句長と頻度

	LSD英語 [A]	MeSH [B]	共通語 [C] =[A]∩[B]
語数	49,034	65,733	13,462
平均単語長	1.51	2.43	1.70
平均文字バイト数	12.7	19.5	14.7
平均頻度 (75% パーセントイル)	1,141 (257)	194 (16)	868 (328)

低く、LSD では現象や性質を表現する用語が多いのに対して、MeSH では事物の名称が多く収録されている対照的な違いが見られた。すべてのカテゴリについて略語を抽出すると、LSD に収録された 1,895 語のうち、MeSH に収録されているのは 325 語のみであった。

また、表 2 で示すように、MeSH と LSD 収録語について平均単語長と平均文字バイト数を比較すると、MeSH には複合語が多く LSD には単語が多い差異が明らかであり、共通語ではそれらの中間的な値を示した。英語コーパスでの出現頻度は MeSH より LSD で顕著に高かった。さらに、75% パーセンタイル値では共通語が最も高い値を示した。

#### 4. 考察

今回、LSD に対訳と意味情報が定義された英語および日本語について、それぞれ十分な大きさを有する専門文書コーパスを制作して出現頻度およびテキスト網羅率を検討し、さらに MeSH との照合により対訳シソーラス構築の第一歩を試みたことは、LSD の現状評価と将来の課題について、いくつかの重要な示唆を与える。

##### 1) 頻度解析による評価

まず、LSD は元来、PubMed 等の英文コーパスの頻度解析結果に基づいて収録すべき用語を選択しており、今回、新たに最近 10 年間に発表された PubMed 抄録や教科書を用いて頻度解析を行った結果、84% の語句がコーパス中に出現し、コーパステキスト中の全単語の 88% が LSD でヒットしたことによって、その方針の妥当性が裏付けられた。また、同様の解析を初めて日本語に対しても行ったが、LSD 収録語の 92% がコーパス中に出現したことは、LSD が生命科学の日本語を記述するために適切なボキャブラリーを有していることを意味している。しかし、単純なテキストマッチングでは日本語コーパスにある漢字・カタカナの 66% のみが LSD 収録語に一致する結果となった。このように英語に比べて網羅できる割合が低いのは「起こる」「明らかになる」等の漢字 1 文字とひらがなの組み合わせで構成される高頻度の用言が漢字 1 文字と解釈されて除外さ

れたことによるものであり、今後は形態素解析プログラムに LSD を辞書として組み合わせることによって、日本語辞書の正しい評価を行う必要がある。

##### 2) それぞれに含まれる用語

今回、研究者が公表する論文を対象にしたテキストマイニングを想定し、その抄録データベースである MEDLINE (あるいは PubMed) の統制語 MeSH と LSD のマッピングを行うことによって、広範な語彙で記述される生命科学の学術用語を英語・日本語を含めたシソーラスツリーに当てはめることを試みた。しかしながら、実際にその共通集合として得られた語数は、LSD と MeSH のいずれから見ても 20% 台に留まった。一方、前述したように LSD は PubMed を主体とする英語コーパスに対して 88% の網羅性を有している。この差異は、LSD がコーパスで高頻度に出現する用語を収録していることで説明される。

LSD と MeSH、さらに PubMed において特徴的に見られる語句の種類について、図 6 で概念的に示した。PubMed との重なりは LSD が MeSH より高く、LSD と MeSH の共通語にはコーパスに高頻度の語句が含まれている。LSD には動詞、形容詞、副詞といった用言や日本語独特の概念 (例:生活習慣病や再生医療に対応する訳語) が固有に収録されている一方、MeSH は化合物名や生物学名を網羅的に収録している。これらのシソーラスに含まれないのは主として低頻度の用語であり、特に遺伝子名のような記号や略語は多種

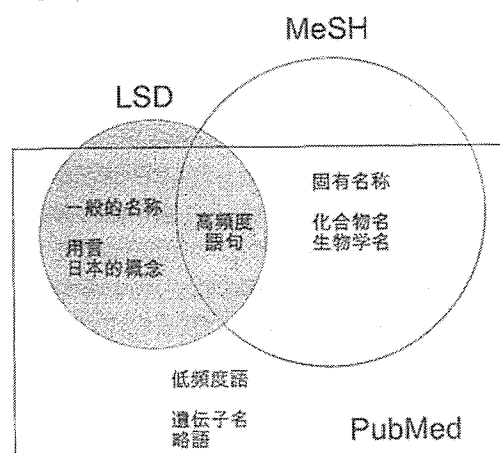


図 6 LSD, MeSH, PubMed 語彙の特徴

であり、特に遺伝子名のような記号や略語は多種多様である。これらについて、以下でより詳細に考察する。

### 3) LSD 未収録の英語

LSD 未収録語には非常に多様かつ低頻度の語句が残されており、中でも記号とも言える遺伝子名については、対訳を定義することは不可能に近く、実際に日本語コーパスにおいてもそれらは英語のまま記述されていた。同様に、化合物名や生物学名についても日本語でカタカナで表記される一般名(慣用名)を収録することは意味あるが、日本語でも英語のまま表記される化学名やラテン語学名をカタカナ表記で収録することはあまり意味がない(少なくともテキストマイニング辞書としては効果が低い)と考えられる。また、単なるカタカナへの変換は、日本語の視認性というメリットを低下させる要因でもあろう。むしろ日本語コーパスを用いた解析によって日本人が英語のまま記述しやすい用語を定量化かつ判別し、それらの用語については対訳シソーラスではなく英語で構築された Gene Ontology<sup>9)</sup> など他のシソーラスの応用を考えるのが現実的と考えられる。

### 4) 多義性への対応

英語と日本語の対訳関係は 1 対 1 でない場合が多い。LSD でも複数の訳語を定義している語句が英和、和英いずれの方向でも 3 割程度存在しており、出現頻度の高い語句では一般的に見られる。例えば、discharge は生理学では「発火」、内分泌では「放出」「分泌」、臨床では「退院」を意味するため、単語レベルで意味を一義的に定めることは困難である。このような多義性の問題について、LSD では接続する単語を含めた複合語を収録することで対応する方針をとっていたが、今回、MeSH との比較によって平均単語長と文字数のいずれも短く、単語を中心に収録している事実が明らかになったことで、現状の LSD では意味を理解するための辞書としてはまだ不十分であることが指摘される。

略語も含めた多義性への対応に関しては、多くの推定手法が報告されている<sup>10)~12)</sup> が、その精度を高めるためにも多義性を有する語句の実例を多くカタログすることは価値がある。今後、LSD オ

ンラインサービスで実装している共起検索技術<sup>11)</sup> を応用して、全単語の接続する用語について頻度を調査し、一定値以上の共起関係が検出される複合語を網羅的に収録することで対応する必要があるだろう。また、生命科学は広範な学問領域を包含しているため、各々の専門領域に特化したコーパスに基づく用語の抽出<sup>13)</sup> も多義性の解決に有用と考えられる。

### 5) 日本語に特有な語彙と表記の多様性

LSD には約 7 万語の漢字変換辞書に対して、対訳を有するものは約 5 万語であるが、この差分は図 5B の結果から主として低頻度の日本語で構成されていることがわかる。今回、英語と日本語の頻度解析データを比較してみたところ、英訳のない(あるいは頻度の少ない)日本語には日本語独特の概念や事物(例:生薬、漢方薬など)が多く含まれていることがわかった。日本の科学を外国人だけでなくコンピュータに理解させるためには、これらの訳語を定義し、シソーラスにマッピングする新たな努力が必要であろう。

また、日本語の頻度解析からわかる日本語の特徴は、同一概念に対する表記の多様性である。典型的な例として「protein」に対して「タンパク質」「タンパク」「蛋白質」「蛋白」「たんぱく質」「たん白質」「プロテイン」等の訳語があり、事実これらが日本語コーパスから検出された。多様性の一因として異なる学問領域で異なる表記が推奨されているが、実際には隣接する語句によって用いられる表記には大きな偏りがある場合が多い。上記の例ではプリオン蛋白(prion protein)、プロテインキナーゼ(protein kinase)、蛋白尿(proteinuria)などが研究分野に関係なく慣用的な表記として用いられていた。したがって、対訳シソーラスにおいては接続語も含めて頻度の高い表記法を優先的に収録するのが好ましいように思われる。

### 6) コーパスの選択

今回用いた英語コーパスは PubMed 論文抄録を 80%、教科書テキストを 20% 含んでいる。抄録という限られたスペースで序論から結論までを記述する文章が主体である結果、定型的な表現が多くなる代わりに詳細な記述が少なく、頻度解析ブ

ロットにおいて傾斜が緩い、すなわち高頻度の用語（例：expression, suggest など）が多く用いられる結果が得られたと考えられる。研究者が記述する自然な文章を解析する目標を考えると、今後は英語についても論文の全文テキストを用いた解析が必要であろう。一方、日本語コーパスはほとんどが教科書の全文で詳細な記述が含まれる結果として、頻度解析プロットではより典型的な分布を示したと思われるが、コーパスサイズを考慮しても日本語の語彙数は英語のそれより小さかった。このことは、前述したように遺伝子名や学名などが翻訳し得ない事実を反映していると考えられる。

最近是对訳コーパスを用いて対訳を抽出する試みが数多く行われている。生命科学における対訳テキストは特許において最も典型的に見られるため、特許テキストを利用した評価系にも興味を持たれる。専門文書の日本語コーパスの構築にあたっては電子化や著作権といった現実的障害が横たわっているが、web における専門情報が著しく増大している現状からは、それらの有効な利用も考えられる。

#### 7) より良い対訳シソーラスへ

MeSH はカテゴリー A から D にわたる事物および病名の分類はマクロからミクロ的な視点、あるいは物質の構造等に基づいて分類学的かつ体系的に行われている。しかし、現象や概念を記述するその他のカテゴリーに関しては、必ずしも体系的に分類されていない印象を受けた。発生学や物理化学などの周辺領域についても MeSH は収録語彙が乏しい。さらに MeSH には名詞のみが収録されているが、LSD は略語、動詞、形容詞、副詞を収録しているため、これらは既存シソーラスとはリンクしない。しかしながら、シソーラスの応用先として実際のテキスト解析を考慮すると、これらを克服する方策を考案する必要がある。形容詞や副詞に対して、共起する名詞や動詞の統計値を収録することも有用であろう。

### 5. おわりに

本研究で制作した対訳シソーラスの実効性については、セマンティックウェブ検索ページや機

械翻訳辞書への実装を行って検証していきたいと考えている。また、今回明らかになった問題点や課題を踏まえて、他の様々なシソーラスも参考にしつつ、LSD を新しい機能的な対訳シソーラスとして体系化することを目指していきたいと考えている。

#### 謝 辞

本研究は、21 世紀 COE プログラム「ゲノム科学の知的情報基盤・研究拠点形成」、学術振興会科学研究費補助金 研究成果公開促進費、文部科学省科学研究費補助金 特定研究（応用ゲノム）、カシオ科学振興財団からの助成を得て行われた。

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英語表記	日本語コード	日本語表記	MeSHリンク
acute aortic dissection (n)	J041788	解離性大動脈瘤	T D
acute B-cell leukemia (n)	J051255	急性B細胞白血病	T T045626 B-Cell Leukemia, Acute D D015450 B-Cell Leukemia, Acute
acute brain injury (n*)	J053144	急性脳損傷	T T005495 Acute Brain Injuries D D001930 Acute Brain Injuries
acute cholecystitis (n)	J041369	急性胆嚢炎	T T521696 Acute Cholecystitis D
acute coronary occlusion (n)	J043680	急性冠閉塞	T D
acute coronary syndrome (n)	J041222	急性冠症候群	T D
acute coronary syndrome (n)	J052884	急性冠状動脈症候群	T D
acute disease (n*)	J041283	急性疾患	T T000603 Acute Disease D D000208 Acute Disease
acute disease (n*)	J051557	急病	T T000603 Acute Disease D D000208 Acute Disease
acute exacerbation (n)	J041070	急性増悪	T D
acute hemorrhagic conjunctivitis (n)	J071193	急性出血性結膜炎	T T009421 Conjunctivitis, Acute D D003232 Conjunctivitis, Acute
acute hemorrhagic leukoencephalitis (n)	J053359	急性出血性白質脳炎	T T014357 Leukoencephalitis, Acute D D004684 Leukoencephalitis, Acute
acute hepatic failure (n)	J046828	急性肝不全	T T051097 Hepatic Failure, Acute D D017114 Hepatic Failure, Acute
acute hepatitis (n)	J032500	急性肝炎	T D
acute infantile hemiplegia (n)	J031354	急性乳児片麻痺	T D
acute infection disease (n*)	J032501	急性伝染病	T D
acute inflammatory demyelinating (n)	J072348	急性炎症性脱髄性多発ニューロパチー	T T365254 Polyradiculoneuropathy D D011129 Polyradiculoneuropathy
acute intermittent porphyria (n)	J071727	急性間欠性ポルフィリン症	T T051121 Porphyria, Acute Intermittent D D017118 Porphyria, Acute Intermittent
acute leukemia (n)	J044723	急性白血病	T D

図6 LSD対訳とMeSHリンク例

## Germinal Center Marker GL7 Probes Activation-Dependent Repression of *N*-Glycolylneuraminic Acid, a Sialic Acid Species Involved in the Negative Modulation of B-Cell Activation<sup>∇†</sup>

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Received 2 November 2006/Returned for modification 9 January 2007/Accepted 30 January 2007

**Sialic acid (Sia) is a family of acidic nine-carbon sugars that occupies the nonreducing terminus of glycan chains. Diversity of Sia is achieved by variation in the linkage to the underlying sugar and modification of the Sia molecule. Here we identified Sia-dependent epitope specificity for GL7, a rat monoclonal antibody, to probe germinal centers upon T cell-dependent immunity. GL7 recognizes sialylated glycan(s), the  $\alpha$ 2,6-linked *N*-acetylneuraminic acid (Neu5Ac) on a lactosamine glycan chain(s), in both Sia modification- and Sia linkage-dependent manners. In mouse germinal center B cells, the expression of the GL7 epitope was upregulated due to the in situ repression of CMP-Neu5Ac hydroxylase (*Cmah*), the enzyme responsible for Sia modification of Neu5Ac to Neu5Gc. Such *Cmah* repression caused activation-dependent dynamic reduction of CD22 ligand expression without losing  $\alpha$ 2,6-linked sialylation in germinal centers. The in vivo function of *Cmah* was analyzed using gene-disrupted mice. Phenotypic analyses showed that Neu5Gc glycan functions as a negative regulator for B-cell activation in assays of T-cell-independent immunization response and splenic B-cell proliferation. Thus, Neu5Gc is required for optimal negative regulation, and the reaction is specifically suppressed in activated B cells, i.e., germinal center B cells.**

The germinal center is a special microenvironment which occurs in secondary lymphoid organs, mainly in response to T-cell-dependent antigen immunization. Mature B cells entering the germinal center edit their immunoglobulin gene through somatic hypermutation and class-switching recombination, differentiating into memory cells and plasma cells (30, 33). The activated B cells during the germinal center reaction in mice can be probed with peanut (*Arachis hypogaea*) lectin, peanut agglutinin (PNA) (8, 37, 46), or a rat monoclonal antibody (MAb), GL7 (5). GL7 was originally reported as a marker for polyclonally activated T and B cells (28) in mice. GL7 stains a subpopulation of T cells (19) and a subpopulation of the large pre-B-cell stage during differentiation in the bone marrow (38). Activated B cells express the GL7 epitope, but

mature B cells do not; thus, GL7 serves as a marker for germinal centers in the immunized spleen (18, 41, 52) or lymph nodes, and GL7<sup>high</sup> B cells have been shown to have higher functional activity for producing antibodies and presenting antigens (5). Despite growing knowledge about the use of this antibody as a marker for lymphocytes in various conditions, the molecular epitope of GL7 is poorly defined to date. In the original article characterizing GL7, Laszlo et al. (28) showed that GL7 could immunoprecipitate a 35-kDa cell surface protein from metabolically labeled activated B cells. However, no other studies have been published on this subject.

In the present study, we found that GL7 recognizes a glycan moiety containing terminal sialic acid (Sia) in both linkage- and modification-dependent manners. Sia is a family of acidic nine-carbon sugars that often occupies the nonreducing terminus of mammalian glycan chains (47), and Sia is essential for early development of mice (49). The localization of Sia-bearing glycan chains on the cell surface makes sialylated molecules seem to be likely targets for various cellular and molecular recognition molecules, such as the mammalian lectins that are abundant in the immune system (61). A family of enzymes, sialyltransferases, is responsible for the formation of the Sia linkage to the underlying glycan chains. To determine the

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<sup>∇</sup> Published ahead of print on 12 February 2007.

linkage specificity of GL7 recognition, we used the gene expression profiles of sialylation-related genes obtained by DNA microarray analysis to screen for a responsible sialyltransferase gene for the biosynthesis of the GL7 determinant.

Apart from the linkage variations, Sia also occurs in various molecular species as a result of modifications at its C-4, C-5, C-7, C-8, and C-9 positions; these modifications are spatially and temporarily regulated (60). We also found that the determinant recognition by GL7 is specific to a Sia modification at the C-5 position. In mice, Sia occurs in two main forms with respect to the moiety at the C-5 position: *N*-acetylneuraminic acid (Neu5Ac), which is a precursor form of the diverse Sia family, and its major modified form, *N*-glycolylneuraminic acid (Neu5Gc). The structural difference between Neu5Ac and Neu5Gc is a single oxygen atom in the C-5 position. The modification reaction that produces Neu5Gc is catalyzed at the sugar-nucleotide level in the cytosol by the enzyme CMP-Neu5Ac hydroxylase (*Cmah*) (24, 53). *Cmah* determines the cell surface expression ratio of these two Sia species, as the cytosolic *Cmah* reaction occurs prior to the sialyltransferase reaction, which takes place in the Golgi apparatus during the biosynthesis of glycoconjugates. We found that GL7 recognizes only Neu5Ac-bearing glycans and that the reduction of *Cmah* expression plays a major role in the formation of the GL7 epitope in activated B cells in the germinal center, which was in sharp contrast to the dominant expression of Neu5Gc in mouse lymphocytes.

To examine the *in vivo* function of Neu5Gc-bearing glycans, we disrupted the *Cmah* gene in mice. *Cmah* disruption is expected to modify the Sia-mediated Sia species-specific recognition event without affecting overall sialylation, which can affect the behavior of the protein in various ways. We primarily focused on the phenotypic consequences of *Cmah* disruption in B cells since *Cmah* is regulated in B cells, especially in response to activation. *Cmah*-null mice exhibited hyperresponsive B cell phenotypes in assays measuring B-cell functions, *i.e.*, antibody production and proliferation.

#### MATERIALS AND METHODS

**Materials.** Most of the materials used were obtained from Wako Chemical (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). The human immunoglobulin G1 (IgG1)-Fc fusion construct was provided by Paul Crocker and Ajit Varki. The Lec2 cells were provided by Pamela Stanley. The Plat-E cells were provided by Toshio Kitamura. Human B-cell lines were obtained from the Japanese Collection of Research Bioresources.

**Antibodies and lectins.** The antibodies used were as follows: donkey F(ab')<sub>2</sub> against mouse IgM (Jackson ImmunoResearch, West Grove, PA); R-phycoerythrin (R-PE)-conjugated anti-mouse B220 (RA3-6B2); R-PE-conjugated goat F(ab')<sub>2</sub> anti-human IgG-Fc; R-PE-conjugated streptavidin (CALTAG Laboratories, Burlingame, CA); fluorescein isothiocyanate (FITC)-conjugated and purified GL7; FITC-conjugated anti-mouse B220 (RA3-6B2); R-PE-conjugated anti-mouse I-A/I-E (M5/114.15.2); biotin-conjugated anti-CD22 (Cy34.1) (BD Pharmingen, San Diego, CA); horseradish peroxidase (HRP)-conjugated goat anti-rat IgM; alkaline phosphatase-conjugated isotype-specific goat anti-mouse IgA, IgG1, IgG3, and IgM; unlabeled isotype-specific goat anti-mouse IgA and IgG3; R-PE-conjugated anti-mouse IgM (1B4B1); biotin-conjugated anti-mouse CD22 (2D6) (Southern Biotechnology Associates, AL); anti-mouse polyvalent Igs; HRP-conjugated PT-66 (an antiphosphotyrosine MAb; Sigma, St. Louis, MO); CD90 (Thy1.2) MicroBeads; anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany); rabbit anti-mouse CD22 serum (Chemicon, Temecula, CA); HRP-conjugated donkey F(ab')<sub>2</sub> anti-rabbit Ig (Amersham Life Science, Buckinghamshire, United Kingdom); antiactin (Santa Cruz Biotechnology, Santa Cruz, CA); HRP-conjugated goat anti-mouse IgG; HRP-conjugated rabbit anti-goat IgG (ZYMED Lab, South San Francisco, CA). Anti-CD22 MAb

(Cy34.1) was purified from the culture supernatant of hybridoma Cy34.1 (ATCC). Biotinylated *A. hypogaea* PNA was obtained from HONEN (Tokyo, Japan), and FITC-conjugated *Sambucus sieboldiana* agglutinin (SSA) was obtained from Seikagaku Kogyo (Tokyo, Japan).

**Preparation of Fc fusion proteins of sialoadhesin and CD22.** Recombinant soluble forms of the amino-terminal domains (domains 1 to 3) of mouse sialoadhesin/Siglec-1, mouse CD22/Siglec-2, and human CD22/Siglec-2 fused to the Fc region of human IgG1 (mSn-Fc, mCD22-Fc, and hCD22-Fc, respectively) were produced in stably transfected Lec2 cells, a cell line deficient in protein sialylation. The production of the Siglec (Sia-binding Ig superfamily lectin)-Fc fusion probe in the Lec2 cell line resulted in considerably enhanced binding to the ligand, which allowed the identification of changes in ligand expression. The Siglec-Fc probes were purified from the culture supernatant using protein A-Sepharose columns (Pierce, Rockford, IL).

**Flow cytometry.** Cell labeling was carried out in fluorescence-activated cell sorter buffer (1% bovine serum albumin [BSA] and 0.1% NaN<sub>3</sub> in phosphate-buffered saline [PBS]). Data were acquired using a FACScan (Becton Dickinson, Franklin Lakes, NJ) instrument and analyzed using FlowJo software (Tree Star, San Carlos, CA). For comparison with the microarray data, B lymphoma cells (1 × 10<sup>5</sup>) were stained with FITC-conjugated GL7 (dilution, 1:100) for 1 h. This staining condition was determined using the criterion that the strongest staining did not reach a plateau. Mean fluorescence intensity (MFI) of GL7 staining was acquired using a FACScan at settings under which unstained control cells gave a signal of around 5 on the FL-1 channel. The mean FL-1 signal of each stained sample was divided by that of the unstained sample to produce the relative staining profiles on flow cytometry to be compared with the cDNA microarray profiles of relative gene expression. For mSn-Fc, mCD22-Fc, and hCD22-Fc staining, these Fc fusion proteins were precomplexed with R-PE-conjugated goat F(ab')<sub>2</sub> anti-human IgG.

**Sialidase treatment.** Sialidase treatment was carried out in 100 mM sodium acetate (pH 5.2) for 30 min at room temperature prior to the staining for flow cytometry. Sialidase from *Arthrobacter ureafaciens* (Calbiochem, San Diego, CA) and sialidase from *Salmonella enterica* serovar Typhimurium (Takara, Kusatsu, Japan) were used.

**Immunoblotting with GL7.** The cells were sonicated in detergent-free lysis buffer (25 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, protease inhibitor cocktail [Nacalai Tesque]). The pellets (membrane fractions) were collected by ultracentrifugation and solubilized in NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM HEPES [pH 7.4], protease inhibitor cocktail). The extracts were subjected to immunoblotting with GL7 in the presence or absence of 100 mM Neu5Ac.

**Development of cDNA microarray for glycan-related genes.** The RIKEN Frontier Human Glyco-gene cDNA microarray, version 2, which was spotted by Takara, consisted of 888 genes, which included glycosyltransferase genes and genes related to sugar metabolism, glycan modification, glycan recognition, and lipid metabolism.

**Use of cDNA microarray for identification of glycan-related genes.** Poly(A)<sup>+</sup> RNA samples were isolated from mid-log-phase cells using the mTRAP system (Activemotif, Carlsbad, CA) and were quality checked using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). One microgram of poly(A)<sup>+</sup> RNA from the B-cell lines (rRNA contamination subtracted) and universal reference RNA (Clontech, Mountain View, CA) were labeled using a CyScribe first-strand cDNA labeling kit (Amersham). Competitive hybridization was performed on the microarray, and data were obtained using an Affymetrix 428 array scanner. To achieve a fair cross-cell line comparison, we fixed Cy3 as the signal for the universal reference RNA and Cy5 for the RNA from the B-cell lines. Microarray data were background corrected using a smoothing function and then Lowess normalized using linear models for microarray data. This readout was sigma normalized to avoid variation among microarray replicates. Then, the Cy5 signal from the B-cell lines was divided by the Cy3 signal to obtain the relative expression profile for each gene in the six cell lines as expression ratios relative to the universal reference RNA (1, 16, 29, 40). The gene expression profiles were compared with the GL7 staining profiles from flow cytometry. The similarity between the profiles was evaluated with Pearson's correlation coefficient, and probability values (*P* values) were calculated by the correlation coefficient test. For the correlation coefficient test of a sample size of six, a coefficient of 0.81 indicates a statistical significance level of 5%.

**Transfection.** CHO-K1 cells were stably transfected with pIRES (where IRES is internal ribosome entry site) vector (Clontech), either with or without rat cDNA for *Stgall1*. Transfected cells were selected with G418 (1 mg/ml), and multiple stable clones were established.

**Enzyme-linked immunosorbent assay (ELISA).** In 96-well assay plates, GL7 antibody was immobilized in wells coated with the capturing antibody, purified



anti-rat IgM. The wells were washed and incubated with streptavidin-conjugated sugar chain probes (50  $\mu$ M), prepared as previously reported (65). The captured probes were detected with biotinylated alkaline phosphatase (Vector Laboratories, Burlingame, CA) and *p*-nitrophenyl phosphate by measuring the absorbance at 405 nm.

**Spleen sectioning and immunohistochemistry.** Mice were immunized intraperitoneally with  $3 \times 10^8$  sheep red blood cells (SRBC) in 100  $\mu$ l of saline. Spleens were removed 8 or 10 days after immunization and embedded in Tissue-Tek OCT (22-oxycalcein) compound (Sakura Finetechnical, Tokyo, Japan). Spleen sections were cut at a 6- $\mu$ m thickness on a cryostat microtome (Leica Geosystems, Heerbrugg, Switzerland), thaw-mounted onto Matsunari adhesive silane-coated slides, and fixed in acetone. After rehydration in Tris-buffered saline and blocking in Tris-buffered saline with 5% BSA and 0.05% Tween 20, the sections were stained with GL7, PNA, or mCD22-Fc precomplexed with R-PE-conjugated anti-human IgG. The stained sections were analyzed under a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

**Magnetic sorting preparation of splenic B-cell-enriched fraction.** B-cell-enriched fractions were obtained by Thy1.2 depletion of splenocytes on a MACS (magnetic cell sorter) depletion column (Miltenyi Biotec). Thy1.2-depleted fractions were stained with B220 to confirm B-cell enrichment. To avoid Neu5Gc contamination in the experimental systems, RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% human serum (Uniglobe, Reseda, CA) or chicken serum (JRH Biosciences, Lenaxa, KS), rather than fetal bovine serum (FBS) (JRH), was used in most of the experiments. In addition, sodium pyruvate (Invitrogen), nonessential amino acids solution (Invitrogen), L-glutamine, and 2-mercaptoethanol were added to the medium.

**Germinal center B-cell analyses.** Splenic B cells from SRBC-immunized mice were incubated with FITC-conjugated GL7 and then with anti-FITC MicroBeads. The labeled cells were collected as germinal center B cells using a MACS LS column (Miltenyi Biotec). The germinal center, nongerminal center, and control (untreated) B cells were lysed by sonication in detergent-free lysis buffer (described above), and the lysates were separated by ultracentrifugation. The supernatant (cytosolic fraction) was used for immunoblotting with anti-Cmah antibody, and the pellet (membrane fraction) was used for the analysis of Sia species by high-pressure liquid chromatography (HPLC). Immunoblotting was performed using rabbit N8 antiserum against mouse Cmah, as previously reported (27). The ratios of Neu5Gc were determined by derivatizing Sia with 1,2-diamino-4,5-methylenedioxybenzene (DMB), a fluorescent compound for  $\alpha$ -keto acids, as previously described (27). In brief, Sia was released by incubating the pellet in 2 M acetic acid at 80°C, derivatized with DMB (Dojindo, Mashiki, Japan), and analyzed on a reverse-phase column (TSK-gel ODS-80Tm; Tosoh, Tokyo, Japan) using a Shimadzu LC10 HPLC system.

**Detection of Sia in tissues.** The ratios of Neu5Ac and Neu5Gc were determined as above. Sia was released by incubating tissues in 100 mM sulfuric acid (which also destroys the *O*-acetyl group often found on the C-7 to C-9 positions of Sia molecules), derivatized with DMB, and analyzed by HPLC.

**Real-time RT-PCR analysis.** Real time reverse transcription-PCR (RT-PCR) experiments were performed using a QuantiTect SYBR Green PCR kit (QIAGEN Japan, Tokyo, Japan) and an ABI 7700 sequence detection system (Applied Biosystems Japan, Tokyo, Japan). Total RNA was purified from untreated or lipopolysaccharide (LPS)-stimulated mouse splenic B cells, and 2  $\mu$ g was used for reverse transcription. The amplification cycle was as follows: 15 min at 95°C, followed by up to 40 cycles of 15 s at 94°C, 30 s at 58°C/50°C, and 30 s at 72°C. The PCR primers used for amplification were: ZP-5, 5'-AGATTAC AAGGATTCC-3'; ZP-E, 5'-CTTAAATCCAGCCCA-3' (*Cmah*); PS-mCD22-6, 5'-CCTCCACTCCTCAGGCCAGA-3'; PS-mCD22-E, 5'-GCCTATCCCATTG GTCCCT-3' (*Cd22*); PS-ST6Gal-1, 5'-TCTTCGAGAAGAATATGGTG-3'; PS-ST6Gal-A, 5'-GACTTATGGAGAAGGATGAG-3' (*St6gal1*); PS-GAPDH-1 (where GAPDH is glyceraldehyde-3-phosphate dehydrogenase), 5'-GTGGAGATTGTTGCC ATCAACG-3'; PS-GAPDH-A, 5'-TCTCGTGGTTCACCCATCAC-3' (*Gapdh*); PS-BACTIN-1, 5'-ACGATATCGTGTGCGTGGTCC-3'; and PS-BACTIN-A, 5'-CAT GAGGTAGTCTGTCCAGGT C-3' (*Actb*). Each sample was analyzed in more than three wells. Relative mRNA abundance was calculated using the comparative cycle threshold method and expressed as a ratio to the nonstimulated sample.

**Retrovirus preparation and infection.** *Cmah* cDNA was cloned into the modified mouse stem cell virus vector, which expresses *Cmah* and the extracellular domain of human *CD4* by means of an internal ribosome entry site. Plasmids were transiently transfected into Plat-E packaging cells (35), and retrovirus-containing supernatants were collected. After stimulation with LPS for 12 to 14 h, splenic B cells were spin infected (at 32°C for 90 min) with the retrovirus in the presence of *N*[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Roche Diagnostics, Mannheim, Germany). The retrovirus-infected B cells were cultured in the presence of 30  $\mu$ g/ml LPS for 2 to 2.5

days, and then human CD4-positive cells were enriched with a MACS system using MACSelect 4 MicroBeads (Miltenyi Biotec). The sorted cells were subjected to flow cytometry or a proliferation assay (described below).

**Targeting construct and embryonic stem (ES) cells.** The *Cmah* targeting vector was assembled from a 129/Sv genomic clone containing exons 4 and 5 of this gene and a neomycin resistance gene driven by the phosphoglycerate kinase 1 promoter (PGK-neoR) as well as a diphtheria toxin A gene fragment driven by the MC1 promoter (DT-A) as positive and negative selection markers, respectively. The construct was created by inserting the PGK-neoR cassette into the NspV site of exon 5 of the *Cmah* gene. The DT-A cassette was then ligated adjacent to the 3' terminus of the construct.

**Generation of mutant mice.** Gene targeting and generation of mutant mice were performed essentially as described previously (23). In brief, E14 cells were electroporated with a Bio-Rad Gene Pulser (0.8 kV; 3  $\mu$ F) using 30  $\mu$ g of NotI-linearized targeting vector. The electroporated cells were selected in medium containing G418 (125  $\mu$ g/ml) and screened for homologous recombination by Southern blot analysis of genomic DNA digested with BglI, using both radiolabeled 5' internal and 3' external probes. The mutant cells were microinjected into 3.5-day-old C57BL/6J blastocysts, and the embryos were transferred into the uteri of pseudopregnant ICR mice. Mice were used for the determination of immunological features after more than seven backcrosses to the C57BL/6J strain. All mice examined in this study were housed in a specific-pathogen-free facility.

**Serum isotype-specific antibody measurement.** Serum samples from nonimmunized mice at 8 to 12 weeks of age were subjected to isotype-specific ELISAs. Isotype-specific capturing antibodies were coated onto 96-well ELISA plates, and nonspecific binding was blocked with 1% BSA-supplemented PBS. A serially diluted standard MAb of each isotype (Ansell, Bayport, MN) and diluted serum samples were captured on the wells. The captured Abs were detected with alkaline phosphatase-conjugated isotype-specific goat IgG using a 1420 ARVO SXC (Wallac, Turku, Finland) luminometer.

**Determination of antibody production in immunized mice.** Eight-week-old mice were immunized after preimmune serum was obtained. Freund's complete adjuvant containing 100  $\mu$ g of dinitrophenyl (DNP)-keyhole limpet hemocyanin (KLH) was used for primary T-dependent immunization by intraperitoneal injection, and a second boost was performed with the antigen in incomplete adjuvant. For T-independent immunization, 10  $\mu$ g of DNP-Ficoll in PBS was injected. The anti-DNP titer was measured essentially as above, except that DNP-BSA was used for antibody capture, and a mixed pool of DNP-KLH-immunized serum was used as the standard. The value relative to that of the pooled serum was used to normalize the values obtained from different plates.

**B-cell proliferation analysis.** In 96-well plates, 100- $\mu$ l aliquots of B cells at  $1 \times 10^5$  cells/ml were stimulated in RPMI 1640 medium containing the indicated concentrations of stimulation reagents. After 24 h of incubation, bromodeoxyuridine (BrdU) was added, and the incubation was continued overnight. Incorporated BrdU was detected using a chemiluminescent ELISA system (Roche Diagnostic GmbH) with an 1420 ARVO SXC luminometer.

**Immunoblotting and immunoprecipitation of CD22.** Splenic B cells were adjusted to  $5 \times 10^6$  cells/50  $\mu$ l in RPMI 1640 medium. After preincubation at 37°C, the B cells were stimulated with F(ab')<sub>2</sub> anti-mouse IgM (10  $\mu$ g per  $5 \times 10^5$  cells) at 37°C. To detect the pattern of tyrosine phosphorylation, cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (50 mM Tris-HCl [pH 7.6], 2% sodium dodecyl sulfate, 0.1% pyronin G, 10% glycerol, 2-mercaptoethanol). For immunoprecipitation studies, the stimulated B cells were lysed in NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM HEPES [pH 7.4], 5 mM NaF, 2 mM sodium orthovanadate, protease inhibitor cocktail [Nacalai Tesque]), and CD22 was immunoprecipitated with anti-CD22 (Cy34.1) antibody and protein G-Sepharose beads (Amersham Biosciences). In the CD22 immunoprecipitation studies, after a probing step with PT-66, the membrane was probed with anti-CD22 polyclonal antibody.

**Experimental animals.** The studies presented here were performed in accordance with animal care guidelines and were approved by the animal experimental committee of Kyoto University Graduate School of Biostudies.

**Microarray data accession numbers.** The GEO platform (GPL3465) and experimental results are registered in the Gene Expression Omnibus database under accession number GSE4407.

## RESULTS

**Sia involvement in GL7 staining of B-cell lines.** During B-cell development in mice, the epitope of the MAb GL7 appears and disappears in multiple maturation steps (5, 18, 32,

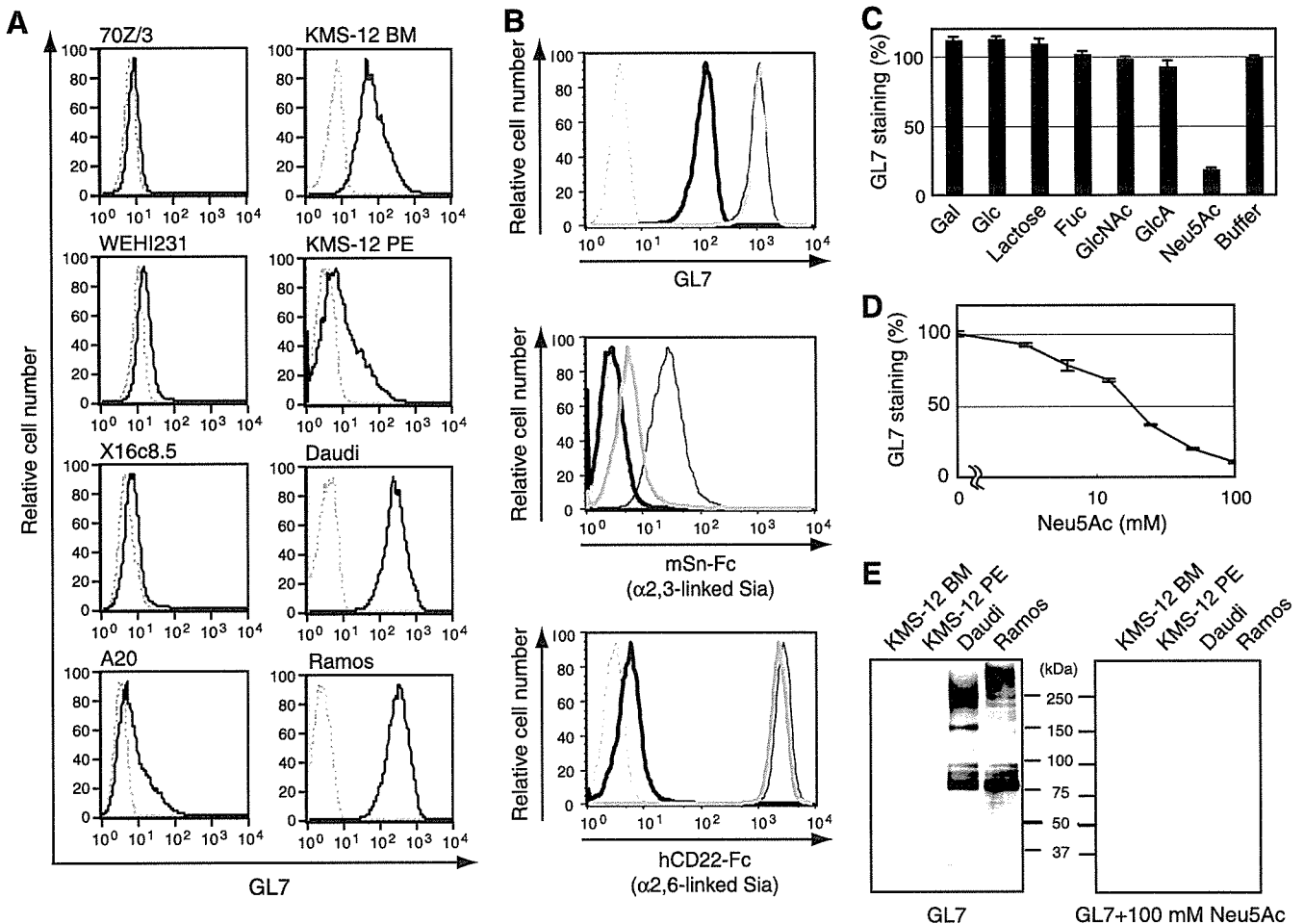


FIG. 1. Involvement of Sia in the GL7 epitope. (A) GL7 staining in flow cytometry. Mouse B-cell lines (70Z/3, WEHI231, X16c8.5, and A20) and human B-cell lines (KMS-12 BM, KMS-12 PE, Daudi, and Ramos) were stained with FITC-conjugated GL7. Black solid lines indicate staining with GL7, and gray dashed lines indicate nonstaining controls. (B) The effect of sialidase treatment on GL7 staining. Daudi cells were treated with sialidase before staining with FITC-conjugated GL7, mSn-Fc, or hCD22-Fc. Gray dashed lines indicate negative controls (nonstaining for GL7 and R-PE-conjugated anti-human IgG for the others), and black thin lines indicate the results without sialidase treatment. Black bold lines indicate the results with *A. ureafaciens* sialidase treatment, and gray bold lines indicate results with *S. enterica* serovar Typhimurium sialidase treatment. Sialidase from *A. ureafaciens* releases  $\alpha$ 2-3,6,8-linked Sia, whereas sialidase from *S. enterica* serovar Typhimurium is specific to the  $\alpha$ 2-3 linkage. To confirm the effect of sialidase treatment, changes in cell surface expression of  $\alpha$ 2,3-linked Sia and  $\alpha$ 2,6-linked Sia were detected with mSn-Fc and hCD22-Fc chimeric probes precomplexed with R-PE-conjugated anti-human IgG, respectively. (C and D) Effect of free sugars on GL7 binding. Daudi cells were stained with FITC-conjugated GL7 in the presence of 50 mM free sugars (C) or the indicated concentrations of Neu5Ac (D). The data are shown as the relative MFI of each staining. Gal, galactose; Glc, glucose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; GlcA, glucuronic acid. (E) GL7 blotting of human B-cell lines. Membrane fractions of human B-cell lines (KMS-12 BM, KMS-12 PE, Daudi, and Ramos) were analyzed by GL7 immunoblotting. The addition of 100 mM Neu5Ac during incubation with GL7 reduced most of the staining on blotted membranes.

38). We were interested in the change of GL7 epitope expression, and thus we first assessed the reactivity of this antibody with various B-cell lines, including human germinal center-like Burkitt lymphomas. GL7 showed stronger reactivity toward human B-cell lines than toward mouse B-cell lines (Fig. 1A). The GL7 epitope has been shown to be sensitive to sialidase treatment, although the type of sialidase used in the study reporting this finding was not specified (19). To understand the relationship of GL7 epitopes present on human B-cell lines and mouse activated B cells, we further characterized the determinant on human B-cell lines. The GL7 epitope on Daudi cells was similar to that on mouse activated B cells, as GL7 staining of Daudi cells was also inhibited by sialidase treatment when a broad-range sialidase, *A. ureafaciens* sialidase, was used

(Fig. 1B). In contrast, *S. enterica* serovar Typhimurium sialidase, which is specific to  $\alpha$ 2,3-linked Sia, had no effect (Fig. 1B). To assess the role of Sia and other sugars in GL7 reactivity, we analyzed the inhibitory effects of sugar on GL7 binding. The results clearly showed specificity of Neu5Ac for inhibition (Fig. 1C), and the inhibition was dependent on the Neu5Ac concentration (Fig. 1D). Neu5Ac is a major form of Sia in human cells. GL7 binding was decreased with a metabolic *N*-glycosylation inhibitor, tunicamycin (see Fig. S1 in the supplemental material). Multiple bands were detected in immunoblotting experiments using the membrane fraction of Daudi cells (Fig. 1E). Thus, it is likely that GL7 recognizes some glycan epitopes, including Sia, rather than some specific protein(s).

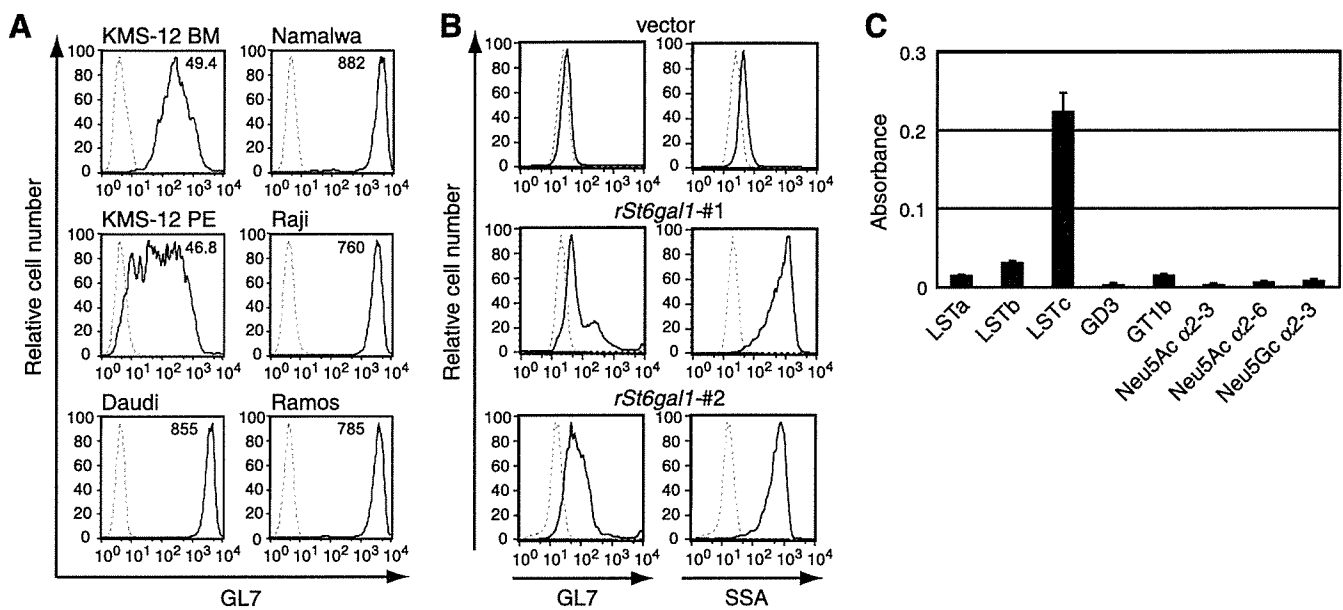


FIG. 2. Involvement of  $\alpha$ 2,6-linked Neu5Ac in the GL7 epitope. (A) Numerical comparison of GL7 staining among human B-cell lines. The results of GL7 staining of human B-cell lines were numerically compared using MFI values in flow cytometry. To normalize the binding in different cells, the endogenous fluorescence of sample cells (gray dashed lines) was adjusted to an MFI of around 5. For comparison with the gene expression profile, GL7-stained MFI values were divided by the background value. The relative values indicated on the top of each staining were used as the GL7 determinant expression profile. (B) Appearance of the GL7 determinant by ST6GAL1 expression. CHO-K1 clones stably transfected with rat *St6gal1* or an empty vector (as a control) were stained with FITC-conjugated GL7 or FITC-conjugated SSA. The results from two such clones are shown. (C) Carbohydrate binding assay of GL7. Carbohydrate binding was measured using ELISA. Data are shown as the means of triplicate samples, and the bars represent standard errors of the mean. LSTa, Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc; LSTb, Gal $\beta$ 1-3(Neu5Ac $\alpha$ 2-6)GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc; LSTc, Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc; GD3, Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc; GT1b, Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\beta$ 1-4(Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-3)Gal $\beta$ 1-4Glc; Neu5Ac  $\alpha$ 2-3, Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc; Neu5Ac  $\alpha$ 2-6, Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4Glc; Neu5Gc  $\alpha$ 2-3, Neu5Gc $\alpha$ 2-3Gal $\beta$ 1-4Glc.

**Strong correlation between expression of the GL7 epitope and expression of the *ST6GAL1* gene in human B-cell lines.** Sia clearly plays an important role in GL7 epitope expression. Interestingly, the GL7 staining of a panel of human B-cell lines was not uniform but, instead, exhibited different intensities (Fig. 1A). Given that a number of bands were detected in immunoblotting experiments, the differences in GL7 epitope expression seemed to be caused by differences in the expression level of an enzyme(s) involved in the biosynthesis of the GL7 epitope glycan rather than differences in carrier protein expression. Therefore, we analyzed the correlation of GL7 epitope expression with the relative level of Sia-related gene expression. The reason to expect such a correlation was that glycosyltransferase activity tends to be regulated through the control of gene expression and substrate accessibility rather than through posttranslational modifications. Six human B-cell lines were stained with GL7 (Fig. 2A), and the relative MFI from flow cytometry was compared with the gene expression profile of the same set of B-cell lines obtained from a newly developed cDNA microarray that can be used to analyze the expression of glycan-related genes. To perform cross-sample comparisons of gene expression among cell lines, we compared poly(A)<sup>+</sup> RNA from each B-cell line and commercially available universal reference RNA. The relative gene expression was obtained by dividing the cDNA microarray fluorescence signal from cellular RNA by that of the universal reference (see Table S1 in the supplemental material). From among the genes spotted on the microarray, various genes for sialyltrans-

ferases and Sia-metabolizing enzymes were picked to examine their possible relationships to the degree of GL7 staining, because it has been shown that sialyltransferase gene expression might correlate with the surface phenotype of lectin binding (2). We calculated the Pearson's correlation coefficient. Among the sialyltransferase and other Sia-metabolizing enzyme genes, *ST6GAL1* showed the strongest correlation between its expression profile and the GL7 staining profile (Table 1). This result indicates that *ST6GAL1* expression could be responsible for the biosynthesis of the GL7 epitope in these human B-cell lines. ST6GAL1 transfers Sia onto a Gal residue of terminal *N*-acetylglucosamine (LacNAc; Gal  $\beta$ 1-4GlcNAc) with an  $\alpha$ 2,6 linkage (42), and B cells have been shown to express this enzyme (20, 64). This indicates that the terminal transferase reaction by ST6GAL1, but not the supply of the substrate, is the rate-limiting step in GL7 epitope biosynthesis in these cells. Interestingly, a negative correlation was found between GL7 staining and the expression of *SIAE*, a gene encoding Sia 9-*O*-acetyltransferase (Table 1). Although Sia 9-*O*-acetyltransferase cleaves the *O*-acetyl group of Sia, *SIAE* is expressed in cell types expressing its substrate, 9-*O*-acetylated Sia (57). If the degree of 9-*O* acetylation were to correspond with the level of *SIAE* expression, GL7 binding might be negatively affected by 9-*O*-acetyl modification, similar to CD22 (56).

**Effect of ST6GAL1 overexpression on GL7 epitope expression.** Data from the correlation index calculation suggest that GL7 recognizes  $\alpha$ 2,6-linked Sia on N-glycan and that the expression of the GL7 epitope on human B cells depends mainly

TABLE 1. Pearson's correlation index analysis of Sia-related genes<sup>a</sup>

Index	P value	Gene name	Encoded enzyme
0.937	5.87E-3	<i>ST6GAL1</i>	ST6Gal I
0.806	5.30E-2	<i>ST3GAL3</i>	ST3Gal III
0.551	2.57E-1	<i>CMAH</i>	Pseudogene for CMP-Neu5Ac hydroxylase
0.473	3.44E-1	<i>ST3GAL2</i>	ST3Gal II
0.215	6.82E-1	<i>SLC35A1</i>	CMP-Sia transporter
0.173	7.43E-1	<i>ST8SIA1</i>	ST8Sia I
0.142	7.89E-1	<i>ST3GAL6</i>	ST3Gal VI
0.137	7.96E-1	<i>PGM3</i>	GlcNAc-6-P mutase
0.096	8.56E-1	<i>GMPBB</i>	GDP-Man pyrophosphorylase
0.052	9.22E-1	<i>ST6GALNAC2</i>	ST6GalNAc II
-0.103	8.47E-1	<i>ST8SIA3</i>	ST8Sia III
-0.196	7.10E-1	<i>ST8SIA4</i>	ST8Sia IV/PST
-0.210	6.89E-1	<i>GNE</i>	UDP-GlcNAc-2-epimerase/ ManNAc kinase
-0.283	5.87E-1	<i>ST6GALNAC6</i>	ST6GalNAc VI
-0.442	3.80E-1	<i>ST3GAL5</i>	ST3Gal V
-0.448	3.72E-1	<i>ST6GALNAC1</i>	ST6GalNAc I
-0.452	3.68E-1	<i>ST8SIA5</i>	ST8Sia V
-0.508	3.04E-1	<i>SAS</i>	Neu5Ac-9-P synthase
-0.639	1.72E-1	<i>ST6GALNAC4</i>	ST6GalNAc IV
-0.678	1.39E-1	<i>NEU3</i>	Membrane sialidase
-0.696	1.25E-1	<i>NEU1</i>	Lysosomal sialidase
-0.739	9.30E-2	<i>ST8SIA2</i>	ST8Sia II/STX
-0.742	9.12E-2	<i>ST3GAL4</i>	ST3Gal IV
-0.898	1.52E-2	<i>ST3GAL1</i>	ST3Gal I
-0.938	5.62E-3	<i>SIAE</i>	Sia-9-O-acetyltransferase

<sup>a</sup> Pearson's correlation coefficient (index) values of relative gene expression in the microarray against relative GL7 staining MFI among six B-cell lines were calculated for sialyltransferase genes and Sia metabolism-related genes. A positive value indicates the presence of a correlation between gene expression and staining. A negative value indicates the presence of a negative correlation. Index values are also expressed as *P* values.

on *ST6GAL1* expression. To evaluate these findings, we explored the *ST6GAL1* expression dependence of GL7 epitope expression. CHO-K1 cells are known to lack  $\alpha$ 2,6-linked Sia on their cell surfaces. As expected, the parental CHO-K1 cells were GL7 negative (data not shown), as were vector-transfected CHO-K1 cells (Fig. 2B). In contrast, rat *ST6GAL1* (*rSt6gal1*)-transfected CHO-K1 cells showed a marked increase in GL7 staining (Fig. 2B). The increase in GL7 staining upon *rSt6gal1* expression coincided with the increase in staining by SSA, a plant lectin which reacts with Sia  $\alpha$ 2,6-Gal/GalNAc on glycans. As CHO-K1 cells are nonimmune cells, GL7 seemed to recognize  $\alpha$ 2,6-linked Neu5Ac-containing sugar chains on various proteins. Immunoblotting analysis of these stable clones further clarified that the introduction of *rSt6gal1* was sufficient to give rise to bands on the blot. The membrane fractions of both CHO-K1 stable clones and human B-cell lines resulted in multiple bands (data not shown).

**Glycan-binding assay of GL7.** To confirm that GL7 is an antiglycan antibody that recognizes  $\alpha$ 2,6-linked Sia and also to determine the fine specificity of the epitope, we examined GL7 binding to various glycan probes (65) by ELISA. GL7 bound to LSTc (Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) but not to its structural isomer with  $\alpha$ 2-3 linked Neu5Ac, LSTa (Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc) (Fig. 2C). Interestingly, GL7 did not bind to Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4Glc (sialyllactose) in spite of the existence of  $\alpha$ 2,6-linked Neu5Ac in the probe. The glucose (Glc) of the reducing terminal was destroyed during probe preparation for coupling with strepta-

vidin. Thus, it is likely that the structure of Neu5Ac $\alpha$ 2-6Gal is not sufficient for GL7 binding but that the binding requires at least a trisaccharide for optimal recognition or GlcNAc in the underlying lactosamine. Taking all of the results into consideration, we concluded that GL7 recognizes  $\alpha$ 2,6-linked Sia-containing glycan chains that are often found on N-glycans of various proteins.

**A shift in the major Sia species, Neu5Gc to Neu5Ac, in the mouse germinal center reaction.** It was still not clear why GL7 failed to react with mouse mature B cells, given that these cells abundantly express  $\alpha$ 2,6-linked sialoglycans, as *St6gal1* is also expressed in these cells (20, 64). The dominant difference in sialylation between mice and humans occurs in the Sia modification at the C-5 position (60). Humans predominantly express Neu5Ac, whereas the major Sia in mice is Neu5Gc (Fig. 3A). It is possible that the change in GL7 reactivity could be a consequence of the change in sia modification. Neu5Gc modification in biosynthesis is regulated by the *Cmah* reaction in the cytosol, which metabolically gives rise to the donor, CMP-Neu5Gc, for a subsequent sialyltransferase reaction(s) in the Golgi apparatus (Fig. 3B) (24, 25). We therefore asked whether mouse B cells undergo a change in Sia species, from Neu5Gc to Neu5Ac, in GL7-positive cells. We first stained the germinal centers with GL7 and the lectin domain of mouse CD22 (mCD22-Fc), because mouse CD22 demonstrates a marked preference for Neu5Gc-bearing over Neu5Ac-bearing  $\alpha$ 2,6-linked sialoglycan ligands (26, 44, 50). As shown in Fig. 3C, in the SRBC-immunized mouse spleen, GL7-positive germinal centers were specifically excluded by mCD22-Fc recognition. This complementarity of staining appeared to be the result of the probe preferences, Neu5Ac for GL7 and Neu5Gc for mCD22-Fc, respectively. We then assessed *Cmah* expression and the Neu5Ac-Neu5Gc ratio in GL7-positive germinal center B cells. Germinal center (GL7-bound) cells showed severely reduced expression of *Cmah*, and this reduction coincided with the loss of Neu5Gc in the membrane fraction of the cells (Fig. 3D). In contrast, GL7-negative SRBC-immunized B cells were not significantly different from nonimmunized splenic B cells. Thus, the gain of GL7 staining reflected the loss of the CD22 ligand in germinal center B cells due to the repression of *Cmah*.

**Real-time PCR analysis during mouse B cell activation.** LPS stimulation induces the GL7 epitope in B cells (28). Therefore, we adopted this system to assess the enzyme (gene) responsible for GL7 epitope expression. *Cmah* is responsible for Sia species change, and *St6gal1* is responsible for Sia linkage biosynthesis. We examined the expression of *Cmah* and *St6gal1* to determine whether changes in the expression of these genes could account for the GL7 epitope induction detected in B-cell activation events. In real-time RT-PCR experiments, *Cmah* expression showed an 80% reduction in LPS-stimulated B cells compared with unstimulated splenic B cells after 48 h of incubation (Fig. 4A). This reduction was already detectable after 3 h of culture. Despite the slightly enhanced expression level of  $\alpha$ 2,6-linked Sia-containing glycan probed with SSA, *St6gal1* expression showed a subtle reduction in activated B cells after 48 h (Fig. 4A and B). *Cmah* reduction appears to play a prominent role in the appearance of the GL7 epitope in activated B cells. Retrovirus-mediated ectopic *Cmah* expression consistently reduced the expression of the GL7 epitope in

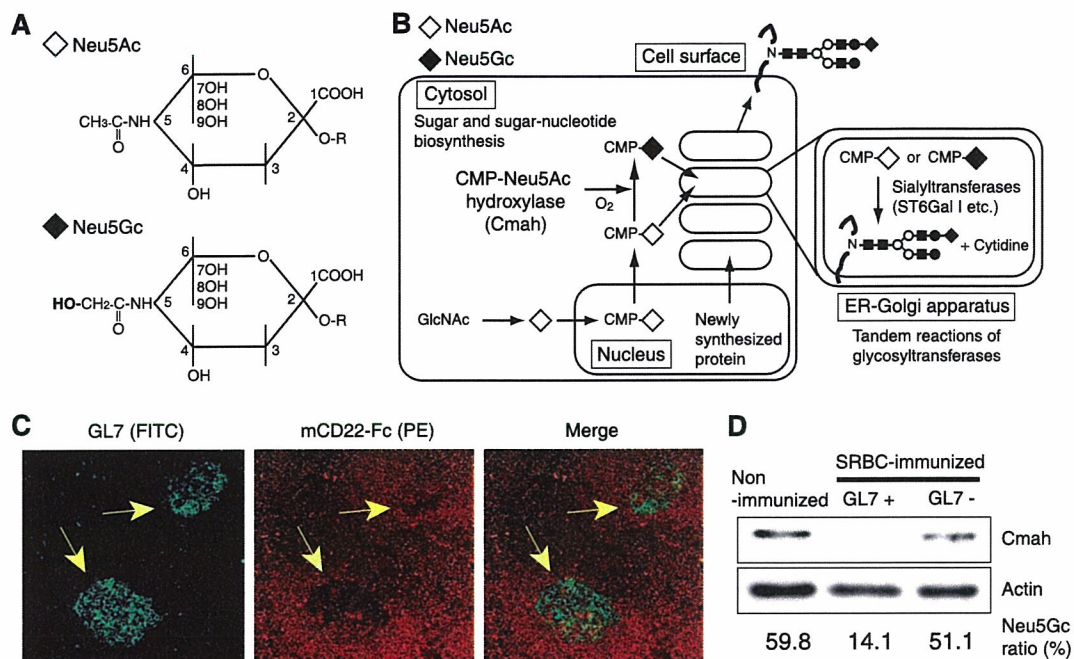


FIG. 3. Change in Sia species in germinal centers. (A) Structural differences between two major molecular species of Sia. The metabolic precursor Neu5Ac and its modified form Neu5Gc differ only by an oxygen atom at the C-5 position. The conversion of CMP-Neu5Ac to CMP-Neu5Gc is catalyzed by the enzyme Cmah. (B) Biosynthesis of sialylated glycoproteins destined for the cell surface. Cytosolic metabolism of Sia is responsible for the abundance of the molecular species of Sia on the cell surface, as a given ratio of cytosolic CMP-Sia is imported into the Golgi apparatus and then used by the sialyltransferases for the biosynthesis of glycoproteins en route to the plasma membrane. (C) Loss of CD22 ligand in germinal centers. Spleen sections of SRBC-immunized mice (10 days after immunization) were costained with FITC-conjugated GL7 and mCD22-Fc precomplexed with R-PE-conjugated anti-human IgG. The mCD22-Fc is a chimeric probe that binds to the CD22 ligand. Arrows indicate germinal centers. (D) Downregulation of Cmah expression in germinal center B cells. GL7-positive germinal center cells and GL7-negative cells were prepared from a B-cell-enriched fraction derived from the spleen of a mouse 12 days after immunization with SRBC. Ultracentrifugation supernatant fractions (cytosolic fractions) of untreated mouse B cells (nonimmunized; control), GL7-positive B cells (GL7+), and GL7-negative B cells (GL7-) were subjected to immunoblotting with anti-mouse Cmah antibody and antiactin antibody (to demonstrate equal loading of samples). The Neu5Gc/(Neu5Ac+Neu5Gc) ratio of the ultracentrifugation pellets (membrane fractions) of each cell type was measured by HPLC.

LPS-stimulated B blasts (Fig. 4C), further confirming the responsibility of *Cmah* for the repression of the appearance of the GL7 epitope. After 48 h of stimulation with LPS, *Gapdh* expression increased by about 30% (Fig. 4A). This may be attributable to the blastic transformation of LPS-stimulated proliferating B cells (B blasts), which produce much more cytosolic space and subsequent metabolism than resting B cells. GL7 staining of LPS-stimulated B cells showed heterogeneity in the degree of staining. Thus, cells used to prepare RNA for this real-time PCR experiment were a mixture of GL7<sup>high</sup> and GL7<sup>low</sup> cells. When these findings are taken into consideration, the reduction of *Cmah* expression in GL7<sup>high</sup> germinal center B cells could be more drastic. The expression of *Cd22*, an  $\alpha$ 2,6-linked Neu5Gc binding protein, on B cells was reduced to around 40% after 48 h, even though its cell surface expression was still comparable to that of unstimulated cells in flow cytometry (Fig. 4A and B).

**Targeted disruption of the *Cmah* gene in mice.** To further examine the *in vivo* function of Neu5Gc-bearing glycans, we targeted the *Cmah* gene in mice by inserting the neomycin resistance gene cassette into the second coding exon (Fig. 5A and B). Biochemical analysis of mouse tissues made it clear that gene inactivation was achieved, as homozygous null mice lacked enzyme expression in the liver ultracentrifugation su-

pernatant, as shown by immunoblotting using antiserum against the N terminus of Cmah (Fig. 5C). We also did not detect a signal with a different molecular mass from the *Cmah*-disrupted allele. We further analyzed the effect of the enzyme deficiency on the level of its product by HPLC. *Cmah*-null tissues lacked detectable production of Neu5Gc throughout the normal adult mouse body (Fig. 5D). We concluded that the *Cmah* gene is indispensable for most of the cellular biosynthesis of Neu5Gc, as previously suggested in humans (6, 22). The development of the null mice appeared to be grossly normal; however, the numbers of null and heterozygote mutant offspring derived from F<sub>1</sub> crosses were subtly reduced from wild-type littermates in the rate expected from Mendelian rules (wild-type:heterozygote:null, 508:881:449), even though the mice were bred in a specific-pathogen-free mouse facility.

**Normal B-cell maturation in *Cmah*-deficient mice.** We found that Neu5Gc expression was severely repressed during B-cell activation in germinal centers, and thus we examined the development of the immune system in *Cmah*-null mice. In null mice, the values from blood counts and blood chemistry analyses were normal in every category examined (white blood cell, red blood cell, blood hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular

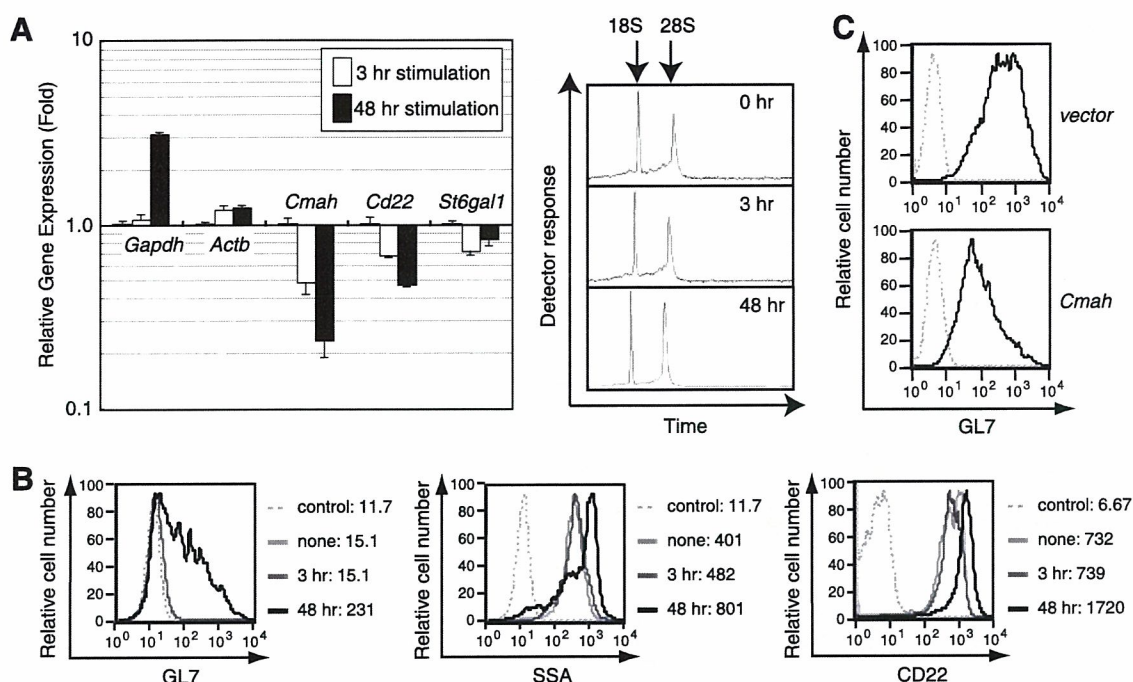


FIG. 4. Downregulation of *Cmah* mRNA in primary cultured B cell blasts, causing GL7 epitope expression. (A and B) *Cmah* repression caused by in vitro B-cell activation. Splenic B cells were stimulated with 30  $\mu$ g/ml LPS for the indicated times. Reverse-transcribed cDNAs prepared from total RNA of these cells were subjected to real-time PCR analysis. The right box shows capillary electrophoresis analysis results indicating the lack of RNA degradation in the RNA used for cDNA synthesis. The expression levels of the mRNA of *Gapdh*, *Actb* (beta actin), *Cmah*, *Cd22*, and *St6gal1* are shown as the relative change compared with the mRNA expression in untreated B cells (A). The same set of cells that was used to prepare total RNA was stained with FITC-conjugated GL7, SSA, and anti-CD22 (B). The MFI of each stain is indicated at the right of each panel. (C) Reduced expression of the GL7 epitope by ectopic *Cmah* expression. *Cmah* was ectopically expressed in LPS-stimulated splenic B blasts using retrovirus. Retrovirus-infected cells were sorted and stained with FITC-conjugated GL7.

hemoglobin concentrate, and platelet). The development of immune cells in *Cmah*-null mice appeared to be grossly normal for T-cell and B-cell maturation, as indicated by routine flow cytometric analysis profiles. The indicators analyzed included the ratio of B1 to B2 cells, the ratio of marginal zone to follicular B cells, and the expression level of surface IgM, major histocompatibility complex class II (MHC-II), and CD22 (Fig. 5E; also see Table S2 in the supplemental material). We also examined the staining profile of activation markers for B cells. The only probe with a significant change in the null B cells was GL7 (Fig. 5F), which recognizes  $\alpha$ 2,6-linked Neu5Ac on LacNAc (Fig. 2C). Serum Ig measurements using the sandwich ELISA method revealed a significant ( $P = 0.074$ ) increase in the serum IgG1 level of the *Cmah*-null population (Table 2).

**Hyperreactive B cells in *Cmah*-deficient mice.** We examined the mouse phenotype after immunization. When mice were immunized with the T-dependent antigen DNP-KLH or the T-independent (II) antigen DNP-Ficoll, the response to the T-independent antigen (serum titer against the hapten, DNP conjugated to BSA, by ELISA) was enhanced in null mice compared with controls, most prominently for IgM but also significantly for IgG3 (Fig. 6A). In contrast, the T-dependent response of the null group to DNP-KLH with potent complete Freund's adjuvant was not significantly different from that of the control group (Fig. 6B). Thus, the Neu5Gc deficiency in B cells resulted in a hyperresponsive phenotype to the T-independent antigen, indicating the importance of Neu5Gc-mediated negative regulation of B-cell activation. To further study

the regulatory mechanism of the B-cell response by Neu5Gc-bearing glycans, mature splenic B cells were isolated and used in an in vitro proliferation assay with various stimuli. In this assay, compared with the cells from littermate controls, *Cmah*-null B cells proliferated robustly in response to the F(ab')<sub>2</sub> fragment against BCR (anti- $\mu$  chain), regardless of interleukin-4 (IL-4) addition (Fig. 6C). The FBS routinely used to support the cell culture contains around 5% Neu5Gc and represents a possible supply for *Cmah*-null cells. Therefore, we also examined the difference in proliferation using serum from chickens and humans, which contain only Neu5Ac as a Sia source (as determined by HPLC analysis [data not shown]). Under such conditions, *Cmah*-null B cells also showed augmented proliferation compared with control cells, although the degree of overall proliferation was much stronger in medium with FBS, perhaps because of differences in the growth factor(s) contained in each type of serum (data not shown). When anti-CD40 was used as the stimulus in a model mimicking T-dependent stimulation, B cells with both genotypes proliferated equally (data not shown); thus, Neu5Gc glycan-mediated regulation appeared to be stimulation dependent, and the effect seemed to be more related to T-independent activation. When T-cell proliferation was assessed using anti-CD3 as the stimulant, both *Cmah*-null and control splenic T cells proliferated to the same extent (see Fig. S2A in the supplemental material). No obvious bias toward either Th1 or Th2 was found in the cytokine production pattern of anti-CD3-stimulated *Cmah*-null T cells; however, a significant reduction of gamma

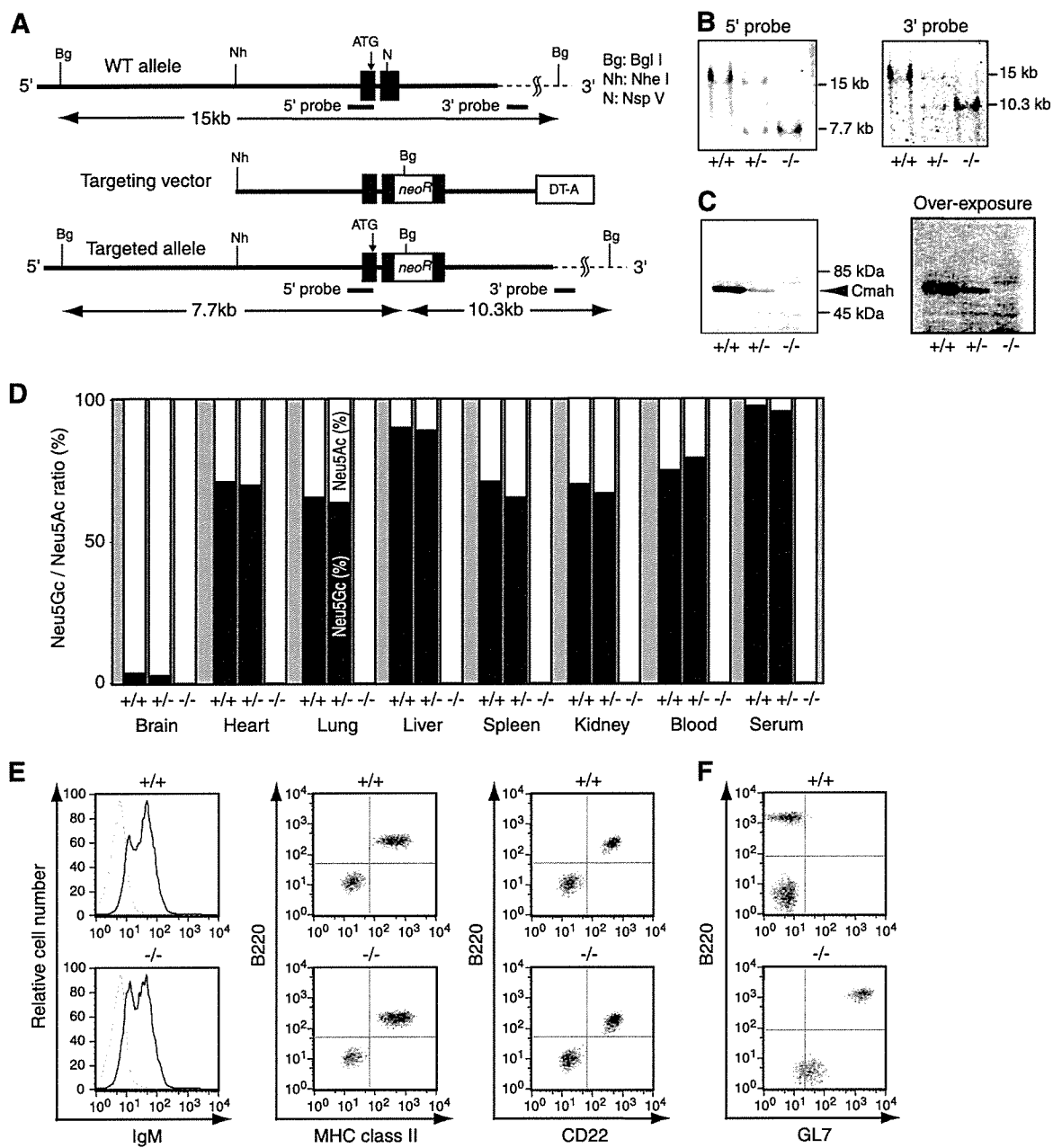


FIG. 5. Generation and biochemical analyses of *Cmah* knockout mice. (A) Allele for targeted *Cmah*. A targeting vector was created by inserting the *PGK-neoR* cassette into the *NspV* site of the second coding exon (exon 5) of the *Cmah* gene. (B) Genotype of homologous recombination of selected ES cell lines. The genotypes of G418-selected cell lines were determined by Southern blotting analysis of genomic DNA digested with *Bgl*I, using both radiolabeled 5' internal and 3' external probes. The genetic status of the *Cmah* allele is indicated as follows: +/+, wild type; +/-, heterozygote; and -/-, null (B to F). (C) Loss of *Cmah* enzyme demonstrated by immunoblotting analysis of liver cytosolic fractions. Ultracentrifugation supernatant fractions of livers were assessed for the expression of *Cmah* using anti-mouse *Cmah* immunoblotting. Staining of a ~67-kDa band (arrowhead) in wild-type and heterozygote livers represents the signal of *Cmah*, which is not detectable in *Cmah*-null liver samples. (D) Loss of Neu5Gc production throughout the body in mutated mice. Acid-hydrolyzed Sia from the indicated tissues was derivatized using DMB, and the ratios of Neu5Ac and Neu5Gc to total Sia were measured by reverse-phase HPLC. Solid columns represent the percentage of Neu5Gc in various tissues, and open columns represent the percentage of Neu5Ac. The detection limit for Neu5Gc in this assay was around 0.1%. (E) Flow cytometry profile of *Cmah*-null mice splenocytes. The expression of IgM, MHC-II (I-A and I-E), and CD22 on splenocytes from wild-type and *Cmah*-null mice was detected by flow cytometry. In anti-MHC-II and anti-CD22 staining, splenocytes were costained with anti-B220, a marker for B cells. (F) Strong expression of the GL7 epitope on *Cmah*-null mice B cells. Splenocytes from wild-type and *Cmah*-null mice were costained with anti-B220 and GL7 and subjected to flow cytometry.

interferon and IL-4 secretion was found in these cells (see Fig. S2B in the supplemental material). Based on these findings, we conclude that B cells from *Cmah*-deficient mice acquire hyperresponsiveness to stimuli, and thus the null animals show

hyperresponsiveness (hyperproduction of antibodies) to the T-independent antigen.

**Retrovirus-mediated rescue of hyperproliferative B-cell response in null mice.** The LPS stimulation-dependent prolifer-

TABLE 2. Serum Ig isotype levels of nonimmunized *Cmah*-null mice

Isotype	Serum Ig level ( $\mu\text{g/ml}$ ) <sup>a</sup>		
	Wild type	Heterozygote	<i>Cmah</i> null
IgM	169.6 $\pm$ 24.7	205.3 $\pm$ 38.9	190.0 $\pm$ 33.3
IgG1 <sup>b</sup>	115.5 $\pm$ 14.9	151.3 $\pm$ 19.5	197.4 $\pm$ 41.2
IgG3	20.4 $\pm$ 2.3	23.9 $\pm$ 3.1	19.6 $\pm$ 3.8
IgA	242.7 $\pm$ 9.7	280.0 $\pm$ 29.2	260.2 $\pm$ 10.6

<sup>a</sup> Serum Ig levels were measured in nonimmunized mice at 7 to 13 weeks of age (at least 20 per genotype). Values are expressed as the means  $\pm$  standard errors of the means.

<sup>b</sup> The serum IgG1 level was slightly increased in *Cmah*-null mice (Student's *t* test; *P* = 0.074 for wild type versus *Cmah* null).

ative response is also related to the T-independent response. In *Cmah*-null B cells, LPS stimulation caused enhanced proliferation (Fig. 7A). Given that LPS induces a considerable percentage of cells to progress through the cell cycle, retroviral infection-mediated gene rescue is possible. To determine whether the B-cell hyperreactivity was caused by the *Cmah* mutation, we expressed *Cmah* ectopically in LPS-stimulated proliferating *Cmah*-null B cells and found that the introduction of *Cmah* did result in repression of the hyperproliferation of *Cmah*-null B cells (Fig. 7B). This rescued hyperproliferative phenotype produced by ectopic *Cmah* expression in *Cmah*-null B cells indicates that the phenotypes in *Cmah*-null mice are caused by the loss of *Cmah* expression and probably not by effects on the expression of other genes owing to the insertion of the neomycin-resistance cassette during ES cell-based mutagenesis. This conclusion is also supported by the consistent phenotype resulting from the *Cmah*-disrupted allele in an extensively backcrossed C57BL/6J background. Moreover, our RT-PCR results confirmed equal expression levels of *Lrrc16* and *6330500D04Rik*, the genes located adjacent to the *Cmah* gene in the genome, in splenocytes of wild-type and *Cmah*-null mice (data not shown). To infect control and *Cmah*-encoding retrovirus, we used the same *Cmah*-null B-cell fractions. Since attenuated proliferation was found in *Cmah*-infected B-cell blasts, the augmented proliferation found in the *Cmah*-null B cells compared to the wild type (Fig. 6C) was not due to any subtle population difference in the B-cell fraction. Thus, we conclude that *Cmah* expression determines the proliferation of B cells when activated and that the difference in the in vivo response to the T-independent antigen is caused by differential expression of Neu5Gc in B cells.

**Normal germinal center formation in the *Cmah*-deficient spleen.** As shown in Fig. 5F, *Cmah*-null B cells strongly express the GL7 epitope, and GL7 has been used to detect the germinal center reaction in mice (5, 17, 41, 55). GL7-negative mature B cells turn GL7 positive during germinal center reactions upon T-dependent immunization. Germinal center B cells further develop to CD79b-positive memory B cells, which are no longer stained by GL7 (52). Therefore, it was of interest to assess whether these *Cmah*-null mice could undergo normal germinal center formation. PNA binds to glycan moieties with a terminal  $\beta$ -galactose residue at the core-1 branch of O-linked glycans, and it has been used as a marker for germinal center B cells (8). We compared the staining profiles of the two germinal center probes using spleen sections of wild-type and *Cmah*-null mice, either with or without SRBC immunization.

In the wild-type spleen without immunization, PNA showed some staining in the marginal zone area, whereas GL7 did not (Fig. 8A). As expected from flow cytometric staining, GL7 widely stained the B-cell zone of the *Cmah*-null spleen even without immunization (Fig. 8A). When wild-type mice were immunized with SRBC, in addition to the marginal zone staining, intense PNA-positive germinal center follicles were observed. When PNA and GL7 staining results were compared on merged images, PNA appeared to stain a larger number of cells in the germinal center than did GL7, which stained a limited number of cells in the area, most probably centrocytes (Fig. 8B). In SRBC-immunized *Cmah*-null spleen, the staining pattern of GL7 was not different from that of the nonimmunized spleen section. These results confirmed that the appearance of GL7 epitope via the conversion of Neu5Gc to Neu5Ac is an activation-dependent event in the wild-type spleen, whereas *Cmah*-null mice lose Neu5Gc throughout; thus, *Cmah*-null spleen was stained by GL7 regardless of the immunization. In contrast, with GL7 staining, the *Cmah*-null spleen formed PNA-positive follicles that resembled the germinal centers of wild-type sections (Fig. 8B). These results suggest that *Cmah*-null mice could develop germinal centers upon SRBC immunization, which is consistent with the normal T-dependent antigen response found in *Cmah*-null mice.

#### Change in ligand expression for Siglecs in *Cmah*-null mice.

The cell surface change in Sia species (Neu5Gc to Neu5Ac) by *Cmah* disruption could potentially cause a global change in sialylated glycan recognition throughout the body, as Neu5Gc is the predominant form of Sia in the mouse body, except in the neural system (Fig. 5D). In the immune system, various members of the Siglec family of Sia-binding lectins are expressed in a variety of immune cells. The counter-receptors for sialylated glycans affected by the C-5 position oxygen atom include sialoadhesin (Siglec-1, or CD169), which requires  $\alpha$ 2,3-linked Neu5Ac on galactose as a ligand (10), and CD22 (Siglec-2), which has a strong preference for Neu5Gc over Neu5Ac in the  $\alpha$ 2,6 linkage to LacNAc in mice (3, 26, 44, 50). To explore the change in ligand expression for Siglecs in *Cmah*-null mice, we prepared Siglec-Fc fusion probes that were free from intramolecular sialylation. In null B cells, the expression of the CD22 ligand was reduced roughly 20-fold compared with that in wild-type cells (Fig. 9A). We also histochemically examined the expression of the CD22 ligand on spleen sections from *Cmah*-null mice. Regardless of immunization, the mCD22-Fc probe failed to detect any staining in the sections of *Cmah*-null spleen, as in the germinal centers of immunized wild-type mice (Fig. 9B). Therefore, *Cmah* disruption caused the reduction of the optimal ligand for CD22. At the same time, ligand expression for sialoadhesin was greatly increased in *Cmah*-null mice (Fig. 9A). Sialoadhesin is expressed on macrophages, whereas CD22 is expressed on B cells. Ligand(s) for Siglec-G, another Siglec molecule presumably expressed on B cells, was not detected on B cells (data not shown); thus, the Siglec-related effects in *Cmah*-null B cells could be a loss of CD22 ligand.

**Normal tyrosine phosphorylation upon BCR cross-linking in *Cmah*-null B cells.** In addition to its biochemical activity as a lectin, CD22 also contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail (4, 48). These ITIMs are phosphorylated as part of the phosphorylation



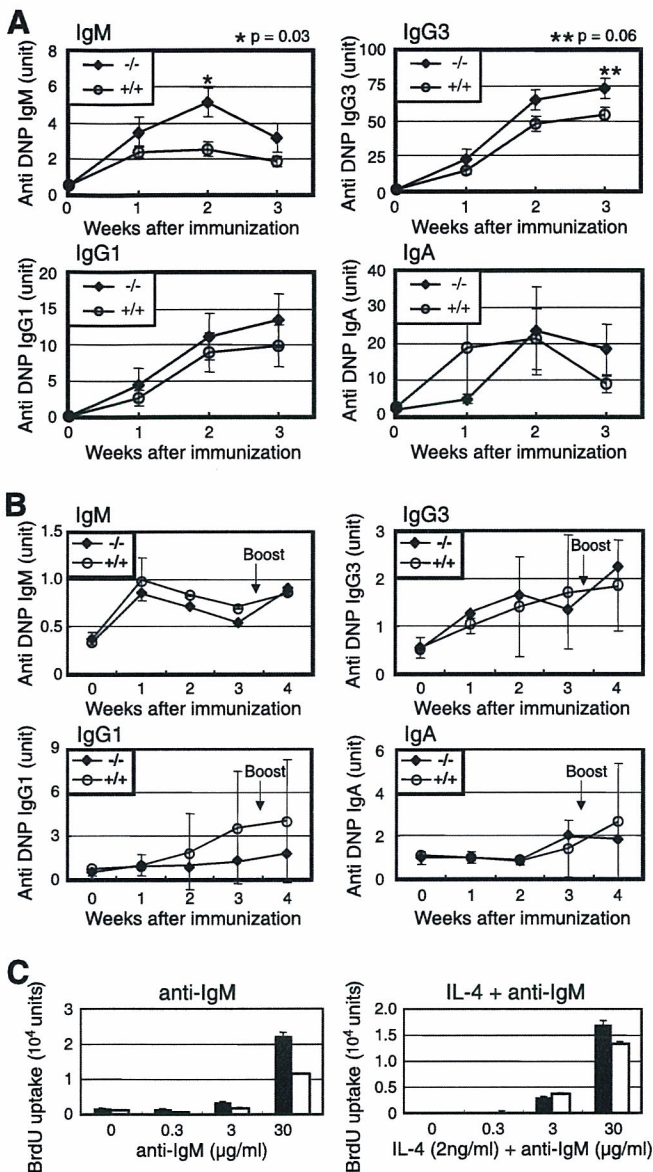


FIG. 6. Hyperresponsive phenotypes of *Cmah*-null mice. (A) T-independent hyperresponse of *Cmah*-null mice. DNP-Ficoll was used to immunize 8-week-old mice. Serum was collected each week and analyzed for reactivity with DNP-conjugated BSA coated on ELISA plates. The titer of hapten-reacting mouse Igs from each animal was determined by isotype-specific ELISA. The measured optical density at 405 nm was normalized to anti-DNP units by comparison with the value from standard pooled serum against DNP on the same plate. The results are presented as the mean responses of 10 animals for each genotype measured in two sets of experiments. The bars represent standard errors of the means. Open circles indicate the responses of wild-type mice, and filled diamonds indicate the responses of *Cmah*-null mice for each isotype. Genotypes are indicated as follows: +/+, wild-type; -/-, *Cmah*-null (A and B). (B) Normal T-dependent immune response of *Cmah*-null mice. DNP-KLH in complete Freund's adjuvant was used to immunize 8-week-old mice. The titers of hapten-reacting mouse Igs from each animal were determined by isotype-specific ELISA as above. Arrows indicate the time of secondary immunization with DNP-KLH. Open circles indicate the responses of wild-type mice, and filled diamonds indicate the responses of *Cmah*-null mice for each isotype. (C) In vitro hyperproliferation response of *Cmah*-null B cells. Splenic B cells from wild-type (open columns) and *Cmah*-null (filled columns) mice were assessed for proliferation using the F(ab')<sub>2</sub> fragment of anti-mouse IgM ( $\mu$  chain) or anti-IgM plus

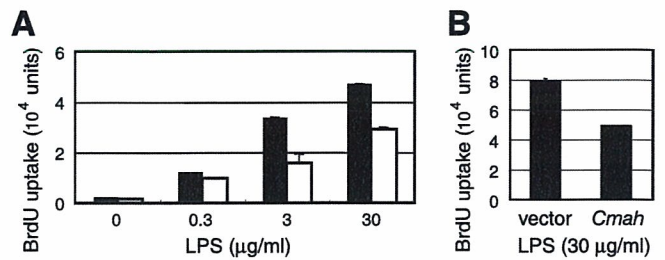


FIG. 7. Rescue of augmented proliferation of *Cmah*-null B cells by *Cmah* expression. (A) In vitro hyperproliferation response of *Cmah*-null B cells to LPS. Splenic B cells from wild-type (open columns) and *Cmah*-null mice (filled columns) were assessed for proliferation using LPS from *S. enterica* serovar Enteritidis as the stimulating reagent. Proliferation assays were performed as described in the legend of Fig. 6C. Data are shown as the means of triplicate cultures, and the bars represent standard errors of the means. (B) Reduction of B-cell proliferation by retrovirus-mediated *Cmah* expression. *Cmah* was ectopically expressed by mouse stem cell virus in *Cmah*-null splenic LPS B blasts. After being cultured for 2.5 days in the presence of 30  $\mu\text{g/ml}$  LPS, the virus-infected B cells were subjected to a proliferation assay. As a control, cells were infected with an empty vector. Data are shown as the means of triplicate cultures, and the bars represent standard errors of the means.

cascade after BCR cross-linking. CD22 recruits SHP-1 tyrosine phosphatase to negatively regulate BCR signaling (11, 39). Given that CD22 is believed to be a regulator of BCR signaling and B-cell apoptosis (7, 13, 34, 58, 63) and that the level of BCR in *Cmah*-null mice was not different from that of the wild-type control (Fig. 5E), we analyzed the immediate-early CD22 phosphorylation status of mature B cells upon activation by BCR ligation. The overall tyrosine phosphorylation profile of B cells was not different for the two types of mice when the F(ab')<sub>2</sub> fragment of the anti-IgM (anti- $\mu$  chain) was used as a stimulant (Fig. 9C), although this may not be an optimal stimulant for CD22 phosphorylation (21). We further confirmed the tyrosine phosphorylation of CD22, possibly by Lyn kinase at the ITIM motif, upon BCR ligation. Consistently, the phosphorylation profile of CD22 assessed after immunoprecipitation by immunoblotting with an anti-phosphotyrosine antibody was almost identical in *Cmah*-null B cells and controls (Fig. 9D). In contrast, *Cmah*-null B cells showed augmented proliferation when a combination of tetradecanoyl phorbol acetate and ionomycin was used as a stimulant to directly activate classical protein kinase C(s). Thus, a downstream event of protein kinase C activation probably affects the hyperproliferative phenotype of *Cmah*-null B cells (Fig. 9E).

## DISCUSSION

**Change in *Sia* species in the germinal center.** In the present study, we showed that activated B cells undergo a dramatic

2 ng/ml IL-4 as stimulating reagents. After stimulation for 24 h, BrdU was added. Following incubation overnight, incorporated BrdU was detected by ELISA. Data are shown as the means of triplicate cultures, and the bars represent standard errors of the means. The results shown here were obtained in one of the experiments using 10% FBS-containing medium.

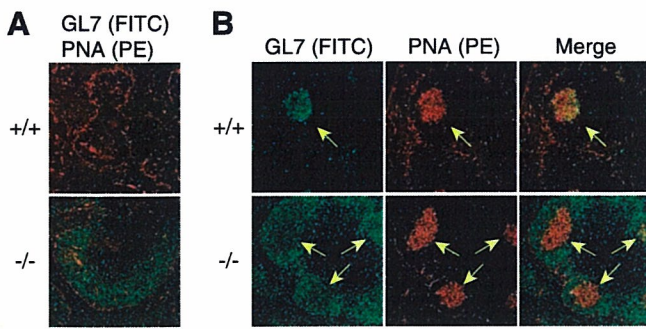


FIG. 8. Changes in staining of germinal center markers in normal or SRBC-immunized *Cmah*-null mice. (A) Histochemical analyses of spleen sections without immunization. Spleen sections from wild-type and *Cmah*-null mice were costained with FITC-conjugated GL7 and biotin-conjugated PNA visualized by R-PE-conjugated streptavidin. (B) Histochemical analyses of spleen sections after T-dependent immunization. Wild-type and *Cmah*-null mice were immunized with SRBC, and the spleens were removed 8 days after immunization. The frozen spleen sections were costained with FITC-conjugated GL7 and biotin-conjugated PNA followed by R-PE-conjugated streptavidin. Arrows indicate germinal centers. Genotypes are indicated as follows: +/+, wild type; -/-, *Cmah* null.

alteration of surface-sialylated glycans and that this alteration of Sia species from Neu5Gc to Neu5Ac can be probed with GL7. This is the first report regarding the epitope identification of GL7, which is routinely used to stain germinal center B cells in mice. We demonstrated that the GL7 epitope is the Neu5Ac $\alpha$ 2-6LacNAc-containing N-glycan, which is prominently expressed in activated B cells upon the repression of *Cmah*. Gain of GL7 epitope expression coincided with the loss of optimal ligand expression of CD22 in germinal center B cells, presumably centrocytes. Considering the rather strong degree of GL7 positivity in germinal center B cells in comparison with *in vitro* stimulated B-cell blasts, the degree of *Cmah* reduction might have been severe in these cells. In general, it is thought that Neu5Gc is easy to accumulate but difficult to turn over in cells. This is attributable to the one-way direction of the metabolic pathway; Neu5Gc is biosynthesized by *Cmah* from Neu5Ac (24, 36, 54), whereas no conversion activity was found to biosynthesize Neu5Ac from Neu5Gc. Therefore, the reduction of Neu5Gc found in the GL7-enriched germinal center cells is remarkable. Such rapid clearance of Neu5Gc could be attributable to several characteristics of germinal center cells. Most importantly, as shown in Fig. 3D, these cells repressed *Cmah*, the enzyme responsible for the *de novo* biosynthesis of Neu5Gc. Moreover, because lymphocytes are small cells with limited cytosolic space, the cytosolic pool of Sia in these cells is likely limited and easily turned over. In addition, centrocytes undergo extremely fast cell cycles (66), which probably leads to rapid passive dilution of the cytosolic pool in these cells. At the same time, new protein synthesis should be a primary event that happens in germinal center B cells, as shown by cDNA microarray analysis (51). The transcriptional repression of *Cmah*, together with these features of germinal center cells, could contribute to the efficient conversion of the major Sia species from Neu5Gc to Neu5Ac.

**Negative regulation of B-cell activation by *Cmah* and its product, Neu5Gc.** To clarify the biological role of Neu5Gc in

*vivo*, we disrupted the *Cmah* gene in mice and examined their B-cell activation phenotypes. *Cmah*-null mice showed a hyperreactive B-cell phenotype to T-independent stimulation. In contrast, the T-dependent immunization response was similar to that in wild-type mice. This is consistent with the findings that *Cmah* expression is severely repressed in the germinal centers of wild-type spleen upon T-dependent immunization and that *Cmah*-null mice could develop follicles stained with PNA, another marker for germinal centers. Forced expression of *Cmah* caused repression of the proliferative response of *Cmah*-null B cells, indicating that Neu5Gc-containing sialoglycan functions to suppress B-cell reactivity through the mechanism is still unknown. This suppression via Neu5Gc-containing sialoglycan appears to be canceled by *Cmah* repression in germinal center B cells that are "activation committed" or "activation competent." The hyperreactive B-cell phenotypes observed in *Cmah*-null B cells could mirror differences in cellular reactivity between germinal center and nongerminal center B cells, as indicated by differential cell surface expression of the GL7 epitope (5).

**Possible change in sialoglycan-receptor interaction in *Cmah*-null mice.** As *Cmah* disruption results in a single oxygen atom change in these mice, it is expected that this mutation leaves both the Sia amount and Sia linkage intact in terms of sialoglycans, which could change the stability or turnover of the proteins modified with Sia (14). Although only limited information is available, sialyltransferases that biosynthesize sialylated glycans in the Golgi apparatus do not show strong preferences for CMP-Neu5Ac or CMP-Neu5Gc as substrates (59). When we probed linkage-specific protein sialylation by using  $\alpha$ 2,6-linked Sia-binding plant lectins such as *Sambucus nigra* agglutinin, we did not observe a change (data not shown). Thus, the molecular event affected in *Cmah*-null mice is likely to be lectin recognition of a single oxygen atom on sialoglycans expressed on the cell surface, although a single responsible lectin may not explain the phenotype. One of the candidate lectins as the receptor of sialoglycans is the Siglec family (9, 12, 62), though a yet-to-be-characterized Sia-binding molecule could be affected.

When ligand expression for Siglecs was detected using Siglec-Fc probes, *Cmah*-null mice lost optimal ligand expression for CD22 (Siglec-2). The ligand function of CD22 in a mouse model has been addressed in two different ways. One study was done using *St6gal1*-knockout mice (20), and another study analyzed gene-targeted mice expressing mutant CD22 molecules that do not interact with ligands (43). The phenotypes found in *Cmah*-null mice are considerably different from these two previous studies; therefore, *Cmah*-null phenotypes might be caused by the combination of loss/gain of a Sia-mediated interaction. Additional studies using a combination of various knockout strains related to sialoglycan recognition are required to address such possibilities.

Apart from the phenotypic contribution of CD22 to the assays in the present study, CD22 ligand expression is not static but is, instead, a regulated event during *in vivo* B-cell activation. We showed that mCD22-Fc probe staining was downregulated in germinal centers. Moreover, it was reported that *in vitro* activated human B cells unmask CD22 from a *cis*-ligand (45). Thus, the regulation of CD22 ligand expression

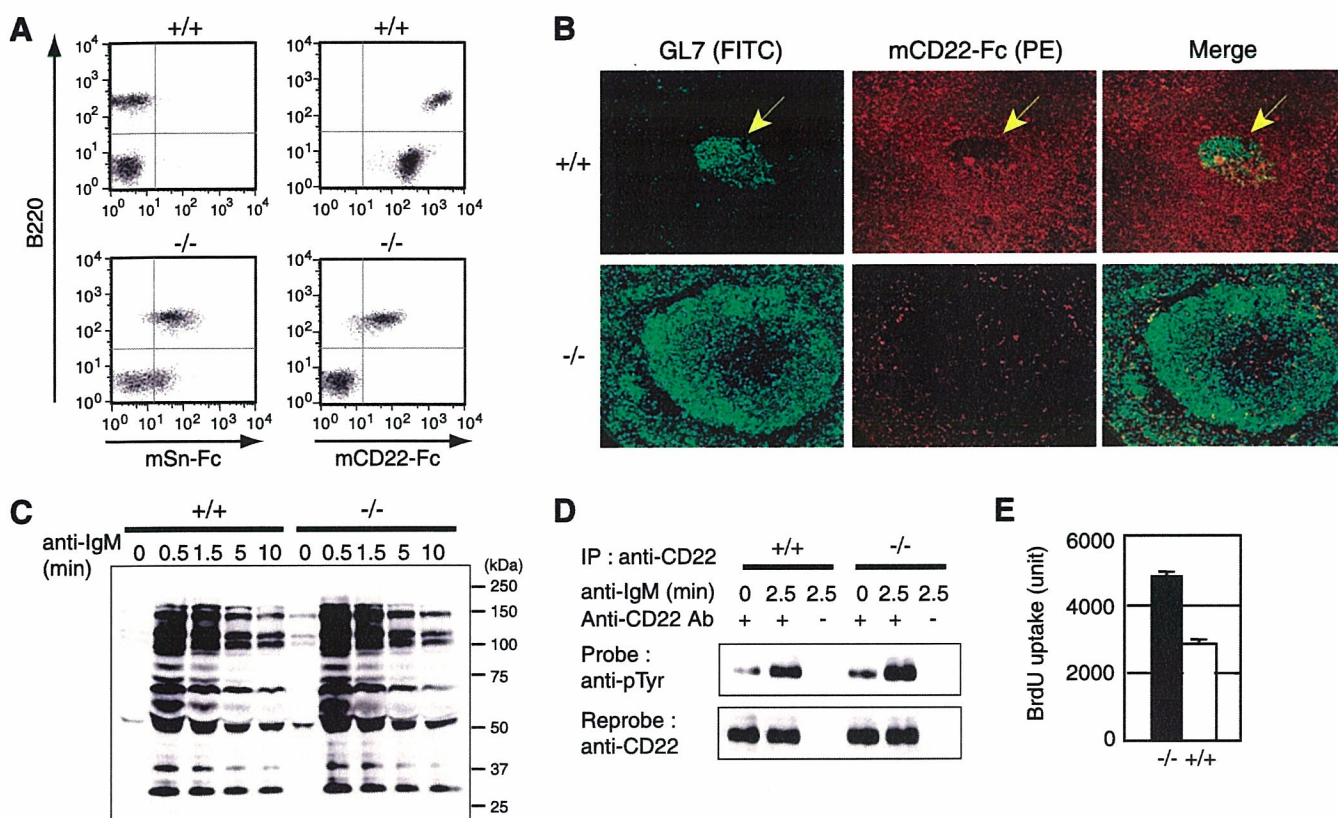


FIG. 9. Loss of optimal CD22 ligand and normal immediate response upon BCR cross-linking in *Cmah*-null mice. (A) Loss of optimal ligand for CD22 in *Cmah*-null mice. The expression of surface ligands for sialoadhesin and CD22 was detected by flow cytometry. Splenocytes from wild-type and *Cmah*-null mice were costained with FITC-conjugated anti-B220 and mSn/mCD22-Fc precomplexed with R-PE-conjugated anti-human IgG. Wild-type B cells were strongly stained with mCD22-Fc. In contrast, the level of mCD22-Fc staining showed a marked decrease in *Cmah*-null mice. The weak signal found on *Cmah*-null splenocytes was detected only with the chimeric probe mCD22-Fc prepared from Lec2 cell culture medium and not with the probe prepared from COS7 cells, possibly because of the autoglycosylation. (B) Histochemical analyses of CD22 ligand expression in spleen sections. Spleen sections from wild-type and *Cmah*-null mice 8 days after SRBC immunization were costained with FITC-conjugated GL7 and mCD22-Fc precomplexed with R-PE-conjugated anti-human IgG. Arrows indicate germinal centers. (C) Overall tyrosine phosphorylation upon anti-IgM stimulation. Splenic B cells from wild-type and *Cmah*-null mice were stimulated with the F(ab')<sub>2</sub> fragment of anti-mouse IgM ( $\mu$  chain) for the indicated times. Whole-cell lysates were subjected to immunoblotting with antiphosphotyrosine antibody (PT-66). (D) Phosphorylation of CD22. Splenic B cells were stimulated with the F(ab')<sub>2</sub> fragment of anti-mouse IgM ( $\mu$  chain) for the indicated times. The cell lysates were subjected to immunoprecipitation with anti-CD22 antibody (Cy34.1). The precipitated proteins were immunoblotted with antiphosphotyrosine (pTyr) antibody (PT-66) and then reprobated with anti-CD22 polyclonal antibody. (E) In vitro hyperproliferation response of *Cmah*-null B cells to calcium signaling. Splenic B cells were assessed for proliferation using tetradecanoyl phorbol acetate (10 ng/ml) plus ionomycin (5  $\mu$ g/ml) as stimulating reagents. The proliferation assay was performed as described in the legend of Fig. 6C. The open column represents the mean proliferation of wild-type B cells, and the filled column represents the mean proliferation of *Cmah*-null B cells. The bars represent the standard errors of the mean for triplicate cultures. +/+, wild type; -/-, *Cmah* null; IP, immunoprecipitation.

could be an important event to modulate B-cell activation in vivo.

**Loss of Neu5Gc in relation to human deficiency for the *CMAH* gene.** *Homo sapiens* is the sole mammalian species that lacks Neu5Gc expression throughout the body; indeed, Neu5Gc is antigenic to humans (31). This is a striking difference between humans and chimpanzees, which express Neu5Gc as the major species of Sia throughout their bodies. Recently, it was shown that, unlike gene expression in the extant great apes, the *CMAH* gene is inactivated in humans (6, 22). Here, we demonstrated that *Cmah* is the sole enzyme responsible for the production of Neu5Gc in cells since our mouse model reproduced the human-like deficiency in Neu5Gc biosynthesis. This result confirmed that a genetic mu-

tation in the human lineage caused the lack of Neu5Gc in humans.

Sia is commonly used in the host recognition system of microbes, and human-specific microbes are reported to recognize epitope(s) containing Neu5Ac on human cells. The mouse described here is thus the first mammalian line that could be used as an animal model system to assess Sia-targeted human infectious diseases (15).

#### ACKNOWLEDGMENTS

We thank Motomi Osato and Yoshiaki Itoh for the blood chemistry and blood counting experiments. We also thank Ajit Varki, Takeshi Tsubata, and Reiji Kannagi for helpful discussions during the preparation of the manuscript.

This work was supported by CREST, Japanese Science and Technology; a grant-in-aid program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and RIKEN.

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