range from 10^3 to 10^4 M^{-1} and from 10^5 to 10^7 M^{-1}, respectively (1).

We need the carbohydrate-binding molecules that distinguish a difference of carbohydrate structures for fundamental research and various applications of glycoconjugates. Display technology recently developed would be useful for identifying carbohydrate-binding molecules from libraries (11,12). During the period between 1992 and 2006, the reports that mention the selection against glycoconjugates numbered 77 papers, and many of them are studies on antibody library. In this review, we describe the studies on peptides, antibodies, and lectins that are selected from libraries and have affinity for carbohydrate moieties of glycoconjugates.

B. Phage Display Library

Phage display method was first reported by G. P. Smith and co-workers in 1985. This method provides an efficient selection (and screening) system to identify target-specific sequences from a large number of peptide and protein candidates (11,13). When the DNA coding foreign sequences is inserted into a coat protein region in the bacteriophage genome, the corresponding sequence is fused with the coat protein and is expressed on the phage particle. The foreign sequence is "displayed" on the phage particle, and is able

用での会合定数 (K_a) はそれぞれ 10^3–10^4、および 10^5–10^7 M^{-1} である (1)。

糖鎖の基礎研究や応用のためには、糖鎖構造の違いを見分ける糖鎖結合性分子が必要である。近年開発された提示技術は、ライブラリーからの糖鎖結合性分子の同定に役立つに違いない (11,12)。1992 年 –2006 年の期間において、複合糖質に対して選択する研究に関しての論文は 77 報あり、その多くは抗体ライブラリーによる研究である。このレビューでは複合糖質の糖鎖と親和性のあるペプチド、抗体、およびレクチンの選択を行っている研究について述べる。

B. ファージ提示ライブラリー

ファージ提示法は 1985 年に G. P. Smith らによって最初に報告された。この方法は多くのペプチドもしくはタンパク質候補の中から標的物質に特異的な配列を効率よく同定することのできる選択 (もしくはスクリーニング) 手法である (11,13)。バクテリオファージのゲノムの外殻タンパク質に外来配列の DNA を挿入すると、その配列は外殻タンパク質と融合されてファージ粒子上に発現される。この外来の配列はファージの表面に「提示」され、他の分子と相互作用できる。ファージクローンは 1 種類の配列を発現させるため、この

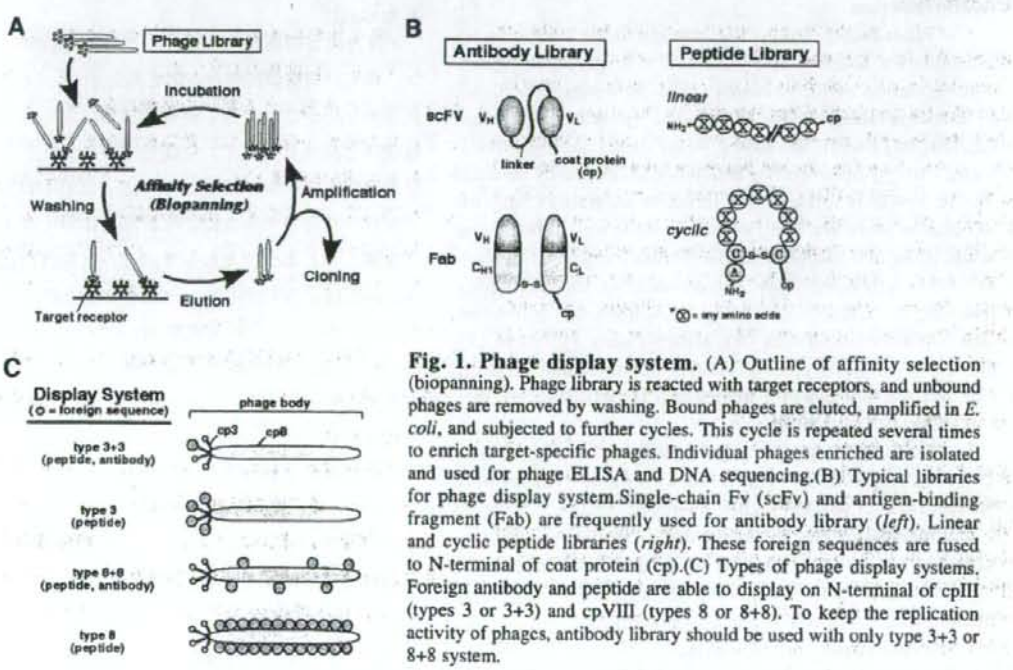

Fig. 1. Phage display system. (A) Outline of affinity selection (biopanning). Phage library is reacted with target receptors, and unbound phages are removed by washing. Bound phages are eluted, amplified in *E. coli*, and subjected to further cycles. This cycle is repeated several times to enrich target-specific phages. Individual phages enriched are isolated and used for phage ELISA and DNA sequencing.(B) Typical libraries for phage display system.Single-chain Fv (scFv) and antigen-binding fragment (Fab) are frequently used for antibody library (*left*). Linear and cyclic peptide libraries (*right*). These foreign sequences are fused to N-terminal of coat protein (cp).(C) Types of phage display systems. Foreign antibody and peptide are able to display on N-terminal of cpIII (types 3 or 3+3) and cpVIII (types 8 or 8+8). To keep the replication activity of phages, antibody library should be used with only type 3+3 or 8+8 system.

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to interact with other molecules. Single kind of sequence is expressed on a phage clone, so the assembly of the phage clone is library (phage library). We can easily identify the foreign sequence by DNA sequencing of the isolated phage genome. In addition to filamentous M13 series, T4, T7, and lambda (λ) have been used for this system, and peptide, antibody, protein libraries are available (14). Diversity of library is often 10^6–10^9, because culture volume (up to a few liters) of host *E. coli* is limited in lab-scale.

A set of procedures for isolating target-binding phage clones is referred to as affinity selection (or biopanning) (Fig. 1A). First, phage library interacts with target receptor, and unbound phages should be removed by washing. Second, the phages bound to target are eluted by incubation with acidic buffer (glycine-HCl buffer, pH2) or inhibitor-containing solution. The eluate is neutralized or substituted by flesh buffer (if required). Here the population of phages collected reduces (about ~10%). Finally, phages are amplified by infection of hosts (*E. coli*) to keep its population. By repeating these steps, target-binding phages are enriched. Isolation and DNA sequencing of individual phage clones give us alignment of amino acid sequences which have affinity for target receptor.

Peptide and antibody libraries are often applied for the selection (Fig. 1B) (12). In most cases, length of peptide library is 5–20 amino acids. Cyclic peptide library, the randomized region is surrounded by two cysteines (eg. CX_7C), is used to restrict peptide conformation through disulfide bridge. Single-chain Fv (scFv) and antigen binding fragment (Fab) antibody libraries are prepared from V_H and V_L (and C_{H1} and C_L) genes of lymphocytes (15). Several kinds of peptide libraries (Ph.D.™ phage display peptide library kits), and customizable phage vector (Ph.D.™ peptide display cloning system) and phagemid vector (recombinant phage antibody system) kit are commercially available.

Foreign sequences are fused to N-terminal of coat protein III (cpIII) or cpVIII (Fig. 1C). Small peptide is able to display all of cpIII (type 3 system) or cpVIII (type 8), because this fusion does not disrupt the viral infectivity and assembly of phages. However, since protein and antibody are bigger than peptides, they should coexist with normal coat proteins for phage propagation (eg. type 3+3 or 8+8 in Fig. 1C). Peptide and antibody libraries are often applied to types 3 and 3+3, respectively.

C. Random Peptide Library

First display of peptide fragment by phage system is the identification of single-binding domain (SBD) of ricin B chain (16,17). Heparin- and lactose-binding domains were also identified from vitronectin and galectin-3, respectively (18,19). On the other hand, using random peptide libraries, peptide sequences identified shared a high homology with natural

ファージクローンの集団はライブラリーとなる。クローンを単離してDNA配列を読むことでどのような配列が提示していたのか容易に同定できる。繊維状のM13系に加え、T4、T7およびλファージがこのシステムに用いられており、ペプチド、抗体、タンパク質ライブラリーが提示可能である(14)。ライブラリーの多様性は10^6–10^9が一般的であるが、これは実験室スケールでは大腸菌の現実的な培養容量(最大数リットル)の限界のよる。

標的物質に結合するクローンを単離する一連の操作を親和性選択(もしくはバイオパニング)と呼ぶ(図1A)。最初に、ファージライブラリーを標的受容体と相互作用させ、結合しないファージを洗浄して除去する。次に結合しているファージを酸性バッファーもしくは阻害剤で溶出する。必要なら溶出液は中和もしくは新しいバッファーに置換する。ここで溶出されたファージは元の数より少ない(~10%程度)。最後にファージを宿主(大腸菌)で増幅し、元の数に戻しておく。この操作を繰り返し行うことで、結合活性の強いファージが濃縮される。クローンの単離を行ってファージDNAの遺伝子を読むことで、標的物質に結合するアミノ酸配列を得ることができる。

選択に際してはペプチドおよび抗体ライブラリーがよく用いられる(図1B)(12)。多くの場合、ペプチドライブラリーの長さは5–20アミノ酸残基である。環状ペプチドライブラリーは2つのシステインでランダム部位を囲んでおり(CX_7Cなど)、ジスルフィド架橋によってペプチドのコンホメーションを制限する。単鎖可変領域フラグメント(scFv)やFabフラグメントのライブラリーはリンパ球のV_HやV_L遺伝子から調製される(15)。いくつかの種類のペプチドライブラリーや、独自にライブラリーを調製できるファージベクターやファージミドベクター(ヘルパーファージが必要)がキットとして市販されている。

外来配列は外殻タンパク質のcpIIIもしくはcpVIIIのN末端に融合される(図1C)。短いペプチドの場合はすべてのcpIII (type 3)やcpVIII (type 8)に提示させることができる。これはペプチドを融合してもファージの感染性や粒子の形成に影響を与えないからである。しかしタンパク質や抗体はペプチドよりも大きく、通常の外殻タンパク質を共存させないとファージの増殖を維持できない(例えばtype 3+3 or 8+8、図1C)。ペプチドライブラリーではtype 3、抗体ライブラリーでは3+3がよく使われている。

C. ランダムペプチドライブラリー

ファージ提示系によるペプチド断片の最初の研究はricin B鎖の単鎖結合ドメイン(SBD)の同定である(16,17)。ヘパリンやラクトース結合ドメインもビトロネクチンやガレクチン3から同定された(18,19)。一方、ランダムペプチドライブラリー

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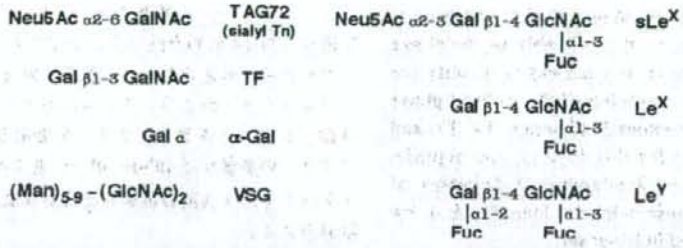

Fig. 2. Oligosaccharide structures of carbohydrate antigen. TF, Thomsen-Friedenreich; Tn, TF precursor; VSG, variant surface glycoprotein; Le^X, $Lewis^X$; sLe^X, sialyl Le^X; Le^Y, $Lewis^Y$.

carbohydrate-binding proteins (20). Other cases showed novel sequences were found. However, compared to antibody and lectin, the interaction of peptide with carbohydrate is considered to be weak. Therefore, in order to make the affinity selection a success, the target oligosaccharides are fixed to plates with high density (for oligosaccharides) or oriented immobilization (for glycolipids) in the selection process.

C-1. Peptides Bound to Carbohydrate Antigens

Many oligosaccharides of carbohydrate antigen are at nonreducing terminal, which provides easy access to carbohydrate-binding molecules (Fig. 2). Affinity selection has been carried out against carbohydrate antigen or oligosaccharide-linked protein (Table I, Fig. 3).

Gui *et al.* identified hexa- and decapeptide sequences that bind to human tumor-associated TAG72 antigen from random library (21,22). The carbohydrate epitope (glycotope) of this antigen is Neu5Acα2–6GalNAcα, another name is sialyl Tn antigen (Fig. 2). Biotinylated HYVSIELPDH peptides showed the binding to human colonic cancers that expresses TAG72 antigen. However, there is no evidence these peptides bind only to glycotope of this antigen.

Peletskaya *et al.* selected Thomsen-Friedenreich (TF) antigen-binding pentadecapeptides (20,23). In these cases, Galβ1–3GalNAcα structure-linked bovine serum albumin (BSA) was subjected to the selection (Fig. 3). They found a consensus sequence, WAY(W/F)SP, which is highly homologous with carbohydrate-binding proteins. The peptides, HGRFILPWWYAFSPS and GSWYAWSPLVPSAQI, bind to glycotope on asialofetuin with dissociation constant (K_d) of 0.1 μM and 1.2 μM, respectively. In addition, they prepared the consensus sequence-containing library at central part (X_5-WYAWSP-X_4, X=any amino acids) and further

による選択では、天然の糖鎖結合性タンパク質と相同性がある配列が得られている。その他のケースでは新規なアミノ酸配列が得られている。しかしながら抗体やレクチンと比較して、ペプチドは糖鎖との間の相互作用が弱いことが予想される。そこで親和性選択を成功させるために、オリゴ糖鎖を高い密度（オリゴ糖の場合）、もしくは配向固定（糖脂質の場合）して基板に固定化して選択処理が行われている。

C-1. 糖鎖抗原に結合するペプチド

糖鎖抗原のオリゴ糖鎖の多くは非還元末端側にあり、糖鎖結合性タンパク質が近づきやすい（図 2)。親和性選択は糖鎖抗原をそのまま用いるか、抗原となるオリゴ糖鎖を修飾したタンパク質を用いている（表 I, 図 3)。

Gui らはヒト腫瘍関連 TAG72 抗原に結合する 6 残基および 10 残基のペプチドをランダムライブラリーから同定した (21、22)。この抗原の糖鎖エピトープ（glycotope）は Neu5Acα2-6GalNAcα であり、シアリル Tn 抗原とも呼ばれる（図 2)。ビオチン化 HYVSIELPDH ペプチドは TAG72 抗原を発現しているヒト結腸ガンへの結合を示した。しかしながら、このペプチドが糖鎖のみに結合しているかどうかは定かではない。

Peletskaya らは TF 抗原に結合する 15 残基のペプチドを得ている (20、23)。この実験では Galβ1–3GalNAcα 構造が結合している BSA が用いられた（図 3)。彼らは共通配列 WAY(W/F)SP を同定し、この共通配列は糖鎖結合タンパク質と高い相同性があった。また HGRFILPWWYAFSPS および GSWYAWSPLVPSAQI ペプチドがアシアロフェツインの糖鎖エピトープにそれぞれ解離定数 (K_d)0.1 μM および 1.2 μM で結合した。さらにこの共通配列を中央に含むライブラリー (X_5-WYAWSP-X_4 X はすべてのアミノ酸）を作製し、更なる選択を行っ

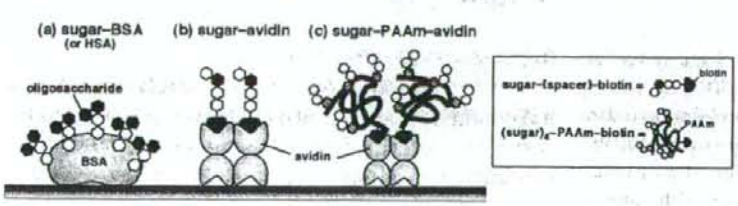

Fig. 3 Oligosaccharide immobilization for biopanning. Oligosaccharide-linked bovine (or human) serum albumin (BSA)(or HSA) fixed on microtiter plate (a). Mono- or multivalent oligosaccharide is linked to biotin to form the avidin-biotin complex through spacer (b and c). PAAm, polyacrylamide polymer.

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Table I. Phage-display selection with oligosaccharides

library type	target receptor	N-terminal sequence (identified)	library source (immunogen)	carbohydrate structure of target [1]	reference
peptide library	TAG72 (sialyl Tn) antigen	X_{10}, X_6 (HYVSIELPDH, ARTLRF)	random	TAG72 antigen (Neu5Acα2–6GalNAcα-O-Ser/Thr)	(21,22)
	TF antigen	X_{15} (HGRFILPWWYAFSPS, GSWYAWSPLVPSAQI)	random	Galβ1–3GalNAcα-BSA, asialofetuin	(20,23)
	TF antigen	X_3-WYAWSP-X_4 (YYAWHWYAWSPKSV)	random	Galβ1–4GalNAcα-HSA	(24)
	Le^x, sLe^x, LN, SLN	X_{12} (YNPLPQPSVTTS, AHWIPRYSSPAT, NFMESLPRLGMG, HSTLDRRSTPPI)	random	Le^x-PAA-avidin, sLe^x-BSA, LN-BSA, SLN-PAAm-avidin	(25,26)
antibody library	Le^x	Fab	mouse spleen (GM1 antigen)	Le^x-BSA	(45)
	α-galactosyl epitope	Fab	BMCs of healthy donor	Galα1–3Galβ1–4GlcNAc-BSA	(50)
	sLe^x	scFv	PBLs of cancer patients	sLe^x-BSA	(48)
	TF antigen	scFv	mouse spleen [TF-carrying asialoglycophorin (aGP) antigen]	Galβ1–3GalNAcα-PAAm-avidin	(49)
	variant surface glycoprotein (VSG)	heavy-chain antibodies (HCAbs)	dromedary lymphocyte (VSG antigen)	VSG [$(Man)_{3-9}$-$GlcNAc_2$-Asn]	(51)
	Le^x, Le^y	scFv	lymph nodes, PBMCs, spleen, tonsil	Le^x-avidin, Le^y-avidin	(46,47)

[1], carbohydrate epitopes are underlined.Abbreviations: TF, Thomsen-Friedenreich; Tn, TF precursor; scFV, single-chain Fv antibody; Fab, antigen binding fragment; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; BMC, blood mononulear cell; $Lewis^x$ (Le^x), Galβ1–4(Fucα1–3)GlcNAc; sialyl Le^x (sLe^x), Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc; $Lewis^y$ (Le^y), Fucα1–2Galβ1–4(Fucα1–3)GlcNAc; LN, Galβ1–4GlcNAc; SLN, sialyl LN; PAAm, polyacrylamide

selection resulted in obtaining improved peptides (24). A YYAWHWYAWSPKSV peptide binds to TF antigen with K_d of 60 nM. This maturation also improved specificity of peptides for glycotope of TF antigen.

Hyun *et al.* used dodecamer peptide library for selection of peptides against $Lewis^x$ (Le^x), sialyl Le^x (sLe^x), *N*-acetyl lactosamine (LacNAc), and sialyl LacNAc (Fig. 2)(25). These glycotopes were linked to BSA or avidin through polyacrylamide (PAAm)-biotin (Fig. 3). Peptides selected have highly homologous with natural proteins and are specific to target glycoproteins (Table I). To enhance the binding affinity of peptides, dimer or tetramer peptides were synthesized (tentacle type peptides)(26). A dimeric YNPLPQPSTTS peptide had affinity for Le^x with K_d of 20 μM.

Binding affinities of peptides and their derivatives were measured by fluorescence quenching method (Peletskaya *et al.*) or surface plasmon resonance (SPR) method (Hyun *et al.*). Since the K_d value depends on measurement protocols, it is unclear which peptide has the highest affinity. However, it's lower than antibody affinity, but we could say that these peptides are as same affinity as lectin.

てより高い親和性のものが得られている(24)。YYAWHWYAWSPKSV ペプチドの K_d は 60 nM であった。このマチュレーションにより、ペプチドの糖鎖エピトープへの特異性が改善された。

Hyun らは $Lewis^x$ (Le^x)、sialyl Le^x (sLe^x)、*N*-acetyl lactosamine (LacNAc) および sialyl LacNAc に対して 12 残基のペプチドライブラリーから選択を行った(図 2)(25)。これらの糖鎖エピトープは BSA またはポリアクリルアミド (PAAm)-ビオチンを介してアビジンに結合させている(図 3)。得られたペプチドは天然のタンパク質と高い相同性があり、またそれぞれの標的糖タンパク質に対して特異的であった(表 I)。ペプチドの結合親和性を高めるために、二量体もしくは四量体を合成している(触手型ペプチド)(26)。YNPLPQPSTTS ペプチドの二量体の Le^x への結合の K_d は 20 μM であった。

ペプチドやペプチド誘導体の結合親和性は蛍光消光法 (Peletskaya *et al.*) もしくは表面プラズモン共鳴 (SPR) 法 (Hyun *et al.*) によって測定されている。解離定数の値は測定方法によって異なるので、どのペプチドの結合活性が高いか比較することはできない。しかしながら、抗体には及ばないものの、これらのペプチドはレクチンと同じ程度の親和性を有しているようである。

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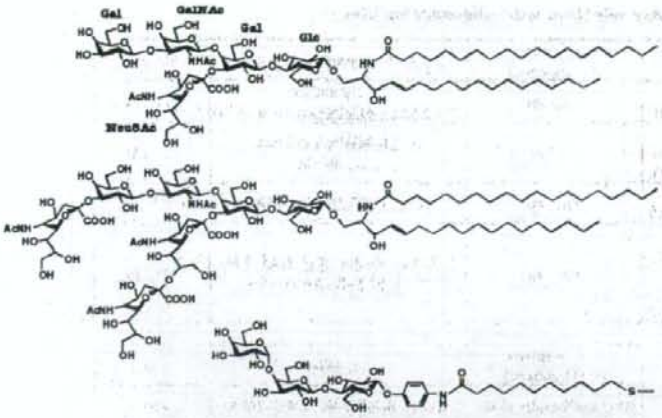

Fig. 4. Structures of glycolipids. GM1, Galβ1–3GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1'Cer; GT1b, Neu5Acα2–3Galβ1–3GalNAcβ1–4(Neu5Acα2–8Neu5Acα2–3)Galβ1–4Glcβ1–1'Cer; Gb3, Galα1–4Galβ1–4Glc.

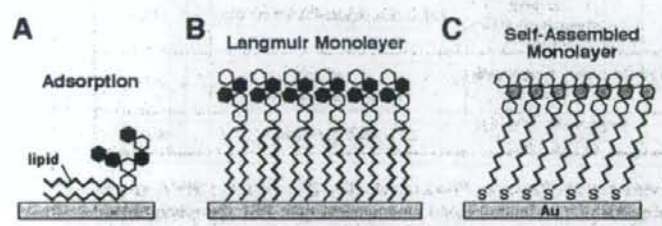

Fig. 5. Glycolipid immobilization for biopanning. (A) Adsorption of glycolipid on microtiter plate. (B) Oriented immobilization of glycolipid by lipid monolayer. Langmuir glycolipid monolayer is prepared at air-water interface, and then transferred onto a solid support. (C) Oriented immobilization of glycolipid by self-assembled monolayer (SAM). Thiol-containing synthetic glycolipid is assembled onto gold substrate. Monolayers are well-ordered glycolipid assemblies, so the high-density glycocluster would be suitable for obtaining sugar-specific phages.

C-2. Peptides Bound to Glycolipids

Glycolipids are composed of carbohydrate and lipid moieties (Fig. 4). It is easy to fix to mictotiter plate through lipid part by hydrophobic interaction, however, it is not possidie to control which part is recognized by phages (Fig. 5A). And unspecific binding of phages onto glycolipid-immobilized plate might interfere with the effective affinity selection. Matsubara *et al.* and Miura *et al.* exposed carbohydrate moiety by oriented immobilization of glycolipid (Table II, Fig. 5B–C)(27,28).

We used Langmuir monolayer of glycolipid to expose only carbohydrate moiety for affinity selection (Fig. 5B). Ganglioside GM1 is spread at an air-water interface in Langmuir trough, and the GM1 monolayer was transferred onto hydrophobic plate. We identified GM1-binding pentadecapeptide sequences from random peptide library through five rounds of panning cycles (27). The peptide sequences identified shared the consensus sequence (W/F)RxL(xP/Px)xFxx(Rx/xR)xP, and peptide VWRLLAPPFSNRLLP (p3) was found to inhibit the cholera toxin B subunit (CTB) binding to GM1 with IC_{50} of 1 μM. This peptide is specific to GM1, and the consensus sequence is important for GM1 recognition (29). Furthermore, terminal galactose and Neu5Ac of GM1 are required for binding

C-2. 糖脂質糖鎖に結合するペプチド

糖脂質は糖鎖と脂質で構成されている(図4)。脂質部分の疎水性を利用して固定化は容易であるが、ファージがどの部分を認識するのか制御できない(図5A)。さらにファージ粒子の糖脂質固定プレートへの非特異的な結合が効率的な親和性選択を邪魔する可能性もある。Matsubara および Miura らは糖脂質を配向固定し、糖鎖部分のみを露出させる工夫を行っている(表II, 図5B–C)(27,28)。

我々は糖鎖部分のみを露出させるため、糖脂質のラングミュア単分子膜を調製して親和性選択を行った(図5B)。ガングリオシドGM1をラングミュア水槽の気-水界面に展開させ、GM1単分子膜を疎水性基板に写し取った。我々はランダムペプチドライブラリーから5回のパニングサイクルによって、GM1に結合する15残基のペプチド配列を選択した(27)。同定されたペプチドは共通配列を有しており、ペプチドVWRLLAPPFSNRLLP (p3)はコレラ毒素Bサブユニット(CTB)のGM1への結合を IC_{50} = 1μMで阻害した。このペプチドはGM1に特異的であり、共通配列(W/F)RxL(xP/Px)xFxx(Rx/xR)xPがGM1認識に重要であることがわかった(29)。さらにGM1の末端ガラクトースとNeu5Acがp3との結合には必要であった。

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Table II. Phage-display selection with glycolipids

library type	target receptor	N-terminal sequence (identified)	library source (immunogen)	structure of target	reference
peptide library	GM1	X_{15} (VWRLLAPPFSNRLLP)	random	Galβ1–3GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1'Cer	(27,29)
	GM1	X_3, CX$_5$C, X_{12} (NPPSPLSVSHRT, RSSTKPPLSPLG)	random	Galβ1–3GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1'Cer	(31)
	Gb3C$_{10}$	X_{12} (FHENWPS)	random	Galα1–4Galβ1–4Glcβ–C$_{10}$SH	(28)
	GT1b	X_{12} (HLNILSTLWKYR)	random	Neu5Acα2–3Galβ1–3GalNAcβ1–4(Neu5Acα2–8 Neu5Acα2–3)Galβ1–4Glcβ1–1'Cer	(32)
antibody library	asialo GM1	Fab	mouse spleen (GM1 antigen) and L3 and H3 of clone 10 randomized	Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1'Cer	(52)
	N-glycolyl GM3	Fab	14F7 hybridoma cells	Neu5Gcα2–3Galβ1–4Glcβ1–1'Cer	(53)

of p3. This recognition by p3 is reasonable, because these carbohydrate residues are glycotope of CTB. These residues are exposed on the GM1 surface, and would provide easy access to GM1-binding molecules.

Atomic force microscopy studies of GM1/glucosylceramide mixed monolayers indicated that p3 had affinity for only high-density GM1 (80 mol% or more) (Fig. 6A) (29). On the other hand, the binding of CTB to GM1 increased rapidly at low-density GM1 (10–20 mol%) and then became saturated at higher densities. These results indicate the binding mode of p3 is different from that of CTB, which is very interesting to think about carbohydrate recognition. Shimizu *et al.* identified the conformation of p3 in solution by two-dimensional nuclear magnetic resonance studies (30). This

これらの糖残基はCTBの糖鎖エピトープであり、p3によるこの認識は妥当である。これらの残基はGM1表面に露出するため、GM1結合分子の接近を容易にしているに違いない。

GM1/グルコシルセラミド混合単分子膜の原子間力顕微鏡観察により、p3は高密度GM1 (80 mol%以上)にのみ結合することが示された(図6A)。一方でCTBの結合は低密度(10–20 mol%)で急激に増加し、それ以降の高い密度においては飽和した。これらの結果はp3の結合様式はCTBとは異なっていることを示し、糖鎖認識を考えるうえで大変興味深い。Shimizuらは、このp3の溶液中の構造解析を二次元核磁気共鳴研究によって行った(30)。このペプチドはフリーの状態では中央で折れ曲がっているのみだが、GM1と結合する時にはコンホメー

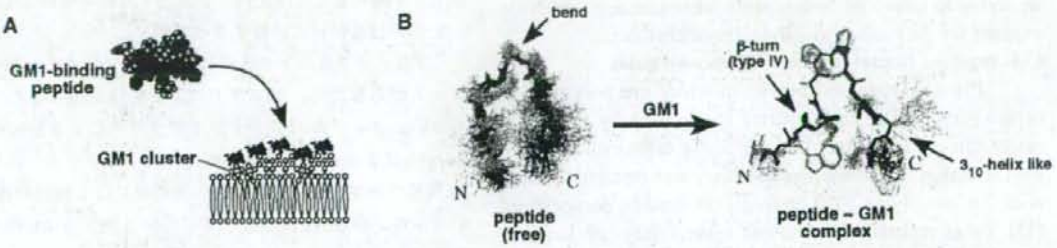

Fig. 6. Characterization of carbohydrate recognition of GM1-binding peptide. (A) High affinity for high-density GM1 membrane (GM1 cluster). (B) Conformational change before and after GM1 recognition determined by two-dimensional NMR analysis. Images of possible twenty structures superimposed.

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peptide is bending at free state in solution, but conformational change is found when binding to GM1 (Fig. 6B). This peptide takes a more stable conformation containing β-turn and helical structure. This peptide, of only 15-amino acids, takes a suitable conformation for carbohydrate recognition.

Montaner *et al.* selected GM1-binding peptides for applying as adjuvants of antigens (31). Interestingly, the consensus sequence PLS they proposed is different from our consensus sequence. Since GM1 was adsorped onto the plate (Fig. 5A), their peptides may be able to recognize not only carbohydrate moiety but also lipid moiety. It may be that the binding to lipid moiety is required for getting adjuvant action.

Miura *et al.* used self-assembled monolayer (SAM) of synthetic glycolipid for affinity selection (28). Gb3, Galα1–4Galβ1–4Glc, is a Shiga toxin (Stx) receptor, and the peptides which inhibit the Stx–Gb3 interaction may be applied for inhibitors. Gb3 was linked to *p-N*-undecanoylamidophenyl group and SAM of the Gb3C10 disulfide (Fig. 4) obtained was allowed to self-assemble on gold substrate (Fig. 5C). After three rounds of affinity selection, the Gb3-specific sequence, FHENWPS, was found in the peptide sequences selected. We also obtained Gb3-binding pentadecamer peptides by the selection with Gb3Cer monolayer (unpublished data). The peptide had the inhibitory activity on the binding of Stx1B to Gb3 with IC_{50} of 0.63 mM. Glycolipid thin layer prepared by using oriented immobilization such as Langmuir monlayer and SAM is very useful to obtain oligosaccharide-specific sequences.

For identifying GT1b-binding dodecamer peptide sequences, Liu *et al.* eluted phages by tetanus toxin C fragment (rTTC) at the affinity selection (32). A Tet1 peptide selected showed the binding to PC12, primary motor neuron, and dorsal root ganglion cells. Tet1 was internalized into cytoplasm of PC12 cells, this internalization was accelerated by nerve growth factor-induced differentiation. The peptide suggests a potential utility for neurotherapeutic strategy.

Besides GM1-binding peptides, we had identified 7-mer and 15-mer amino acid sequences that have affinity for GM2, GM3, GD1a, and GT1b (unpublished data). Now we are trying to obtain the amino acids (or sequences) which are required for the binding to sialyl oligosaccharides.

C-3. Peptides Bound to Other Glycoconjugates

Proteoglycan and polysaccharides are polymers of carbohydrates. The carbohydrate recognition by peptide is easily achieved because there are many carbohydrate units. First selection of random peptide library was reported by Burg *et al.* for identifying NG2 proteoglycan-binding decapeptides (33). Other reports are selections against only two kinds of glycoconjugates, hyaluronan (HA) (35) and lipopolysaccharide (LPS) (36–40).

HA is an extracellular matrix, and is known to interact

ション変化を起こす (図 6B)。このペプチドはβ- ターンやヘリックス構造を含んだより安定な構造を形成する。このペプチドはわずか 15 残基であるにも関わらず、糖鎖認識のために適切なコンホメーションを形成する。

Montaner らはアジュバントとして用いるために GM1 に結合するペプチドを選択している (31)。興味深いことに、彼らは我々とは異なる共通配列 PLS を提唱している。彼らは GM1 をプレートに吸着させているため (図 5A)、彼らのペプチドは糖だけではなく脂質部分も認識している可能性がある。もしかしたらアジュバント作用を得るためには脂質部分への結合が必要なのかもしれない。

Miura らは合成脂質の自己組織化単分子膜 (SAM) を親和性選択に用いた (28)。Gb3(Galα1–4Galβ1–4Glc) は志賀毒素 (Stx) の受容体であり、Stx–Gb3 間相互作用を抑えるペプチドは阻害剤としての応用が考えられる。Gb3 を *p-N*-undecanoylamidophenyl 基に結合し、Gb3C10 ジスルフィド (図 4) を金基板に自己集合させた (図 5C)。3 回の親和性選択で、Gb3 特異的な配列 FHENWPS が選択されたペプチド配列から見いだされた。筆者らも Gb3 に結合する 15 残基のペプチドを Gb3Cer 単分子膜に対する親和性選択によって得ている (未発表データ)。このペプチドは Stx1B の Gb3 への結合を IC_{50} = 0.63 mM で阻害する活性を有していた。ラングミュア単分子膜や SAM のような配向固定化による糖脂質の薄膜はオリゴ糖鎖特異的な配列を得るのに大変有用である。

GT1b に結合する 12 残基のペプチド配列を得るために、Liu らは親和性選択において破傷風毒素 C 断片 (rTTC) でファージを溶出している (32)。選択された Tet1 ペプチドは PC12、初期運動神経細胞、後根神経節細胞に結合した。Tet1 は PC12 細胞の細胞質に入り、この取り込みは神経成長因子誘導による分化で促進された。このようなペプチドは神経治療の展開が期待できるであろう。

我々は GM1 結合ペプチド以外に、GM2、GM2、GD1a、GT1b などに結合する 7 残基および 15 残基のアミノ酸配列を同定している。現在はシアリルオリゴ糖に結合するのに必要なアミノ酸 (もしくは配列) を同定する研究を行っている。

C-3. 他の複合糖質に結合するペプチド

プロテオグリカンや多糖は糖鎖高分子である。多くの糖ユニットがあるため、ペプチドによる糖鎖認識はそれほど難しくない。ランダムペプチドライブラリーによる最初の選択は Burg らによるものであり、NG2 プロテオグリカンに結合する 10 残基のペプチドである (33)。その他には 2 種類の複合糖質、ヒアルロン酸 (HA)(34、35) とリポ多糖 (LPS)(36–40) のみ報告されている。

HA は細胞外マトリックスであり、様々な分子と相互作用することが知られている。Mummert らは 12 残基のペプチド

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with many kinds of molecules. Mummert *et al.* identified dodecamer peptide sequences to inhibit HA–CD44 interaction, and the peptide showed the inhibition of the CD44-related behaviors of leukocytes and tumor cells (34,35,41). LPS-binding peptides were selected from 7-mer and 12-mer libraries by four groups (36–40). However, although same library was used, the peptide sequences selected does not share consensus sequence. This would be because epitopes recognized by peptides are different, because LPS is composed of various kinds of constituent sugars.

D. Antibody Library

The repertories of antibody fragments displayed on phage are single-chain Fv fragments (scFv) and Fab fragments, the heavy (V_H) and light (V_L) chain V genes are derived from RNA extracts of lymphocytes (42). To make further diverse repertories, V genes are modified using randomized synthetic nucleotides (43) or by error-prone polymerase chain reaction (PCR) (44). The affinity selection of phage library is achieved *in vitro*, so there is a possibility of obtaining carbohydrate-specific antibodies, even if a weak immunogenicity of target glycoconjugates (4).

D-1. Antibodies against Oligosaccharides and Glycolipids

The carbohydrate antigens such as Le^X (45–47), sLe^X (48), $Lewis^Y$ (Le^Y)(46,47), TF (49), and αGal (50) were subjected to the affinity selection (Table I). Oligosaccharides of these antigens were linked to BSA, HSA, or avidin (Fig. 3). Variant surface glycoprotein (VSG) of *Trypanosoma brucei brucei* has a conserved glycotope, *N*-linked high mannose oligosaccharide (Man_{5-9}–$GlcNAc_2$) (51). The scFvs and Fabs against these antigens were found to have affinity for carbohydrate moiety of their antigens.

Qiu *et al.* selected anti-asialo GM1 antibody Fab fragment (Table II) (52). Primary antibody library was prepared from total RNA of mouse spleen immunized against GM1. The affinity selection resulted in isolating of clone 10, and the H3 and L3 region of clone 10 was mutated and further selection was performed. Fab fragments identified were found to be soluble and be specific to asialo GM1. Rojas *et al.* constructed light-chain shuffling Fab libraries, anti-*N*-glycolyl GM3 antibodies were identified (53).

D-2. Antibodies against Glycosaminoglycans and Polysaccharides

Surface of microorganism is surrounded by polysaccharides. Lipopolysaccharide of Gram-negative bacteria is composed of lipid A and polysaccharide. Toxicity and immunogenicity are associated with lipid A and polysaccharide, respectively. Anti-*Salmonella* *O*-polysaccharide antibodies were identified from semi-synthetic scFv library that is derived from Se 155-4 antibody (43,44,54,55). Fab fragments against immunogenic capsular

配列を同定し、HA–CD44 間相互作用を阻害させた。このペプチドは白血球やガン細胞の CD44 が関連する挙動を阻害した (34,35,41)。LPS 結合ペプチドは 4 つのグループによって、7 残基もしくは 12 残基のライブラリーから選択されている (36–40)。しかしながら、同じライブラリーを使っているにも関わらず、共通配列が見いだされなかった。これはペプチドが認識するエピトープが異なっていると考えられ、LPS は様々な糖で構成されていることに起因するのだろう。

D. 抗体ライブラリー

ファージ上に提示する抗体フラグメントの種類には単鎖可変領域フラグメント (scFv) や Fab フラグメントがあり、重鎖 (V_H) および軽鎖 (V_L) の V 遺伝子は白血球の RNA 抽出物から得られる (42)。さらなる多様性を得るために、V 遺伝子はランダム合成ヌクレオチド (43) や変異性 PCR によって変異が導入された (44)。ファージライブラリーによる親和性選択は *in vitro* で行うので、免疫原性が低い複合糖質であっても糖鎖特異的な抗体が得られる可能性がある (4)。

D-1. 糖脂質糖鎖に結合する抗体

糖鎖抗原では、Le^X (45–47)、sLe^X (48)、$Lewis^Y$ (Le^Y)(46,47)、TF (49)、および αGal (50) に対して選択されている (表 I)。これらの抗原のオリゴ糖鎖は BSA、HSA、およびアビジンに結合させて固定化している (図 3)。アフリカ睡眠病トリパノソーマの変異性表面糖タンパク質 (VSG) は糖鎖エピトープとして、*N* 結合型高マンノースオリゴ糖鎖 (Man_{5-9}-$GlcNAc_2$) が保存されている (51)。これらの糖鎖抗原に対する scFv や Fab は糖鎖部分に結合することが明らかにされた。

Qiu らは抗アシアロ GM1 抗体 Fab 断片を選択した (Table II) (52)。最初の抗体ライブラリーは GM1 で免疫したマウスの脾臓から得た全 RNA から調製された。親和性選択でクローン 10 が単離でき、この H3 と L3 の領域を変異させてさらに選択を行った。同定された Fab 断片は溶解性でアシアロ GM1 に特異的であった。Rojas らは軽鎖シャッフリング Fab ライブラリーを構築し、抗 *N*-glycolyl GM3 抗体を得ている (53)。

D-2. グリコサミノグリカンや多糖に結合する抗体

微生物の表面には多糖で覆われている。グラム陰性菌のリポ多糖はリピド A と多糖で構成されており、毒性と免疫原性はそれぞれ、リピド A と多糖が関係している。抗サルモネラ *O*- 多糖抗体は、Se 155-4 抗体由来の半合成 scFv ライブラリーから同定された (43,44,54,55)。抗原性のヘモフィルス・イン

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polysaccharide of *Haemophilus influenzae* type b (56) and mannan of *Candida albicans* (yeast) (57) were also identified.

Heparan sulfate (HS)-binding antibodies have been strenuously studied by van Kuppevelt *et al.* (58–65). Anti-HS antibodies inhibited the interaction of HS with a basic fibroblast growth factor and vascular endothelial growth factor (58,64).

Pectin, cell wall of plants, is composed of three major polysaccharide types homogalacturonan (HG), rhamnogalacturonan (RG)-I, and RG-II. HG- and RG-II-binding antibodies were selected by Willats *et al.* (66) and Williams *et al.* (67), respectively.

E. Mutation Library of Lectin

Until now, surprisingly, mutation library of lectin has not been constructed except for a legume lectin. Yamamoto *et al.* prepared *Bauhinia purpurea* lectin (BPA), galactose-binding lectin, mutation library on λ phage (68). The carbohydrate-binding loop of BPA was randomized and affinity selection was performed against mannose-linked BSA. BPA mutants obtained were found to have affinity for mannose, and already to lose affinity for galactose. However, selection of this library against GlcNAc did not result in GlcNAc-binding mutants. They concluded that longer carbohydrate-binding loop might be required for GlcNAc recognition. Without using the display technology, Yim *et al.* randomly mutated *Maackia amurensis* hemagglutinin and obtained various lectins that have affinity for other sugars (69).

Another approach might be more effective instead of lectin mutation to get carbohydrate-binding molecules. The carbohydrate-binding domains of some glycosidases are apart from its catalytic site. The carbohydrate-binding domains of glycosidase have been used as independent molecules for carbohydrate recognition. A xylan-binding module of xylanase was mutated by error-prone PCR, and used for the selection with xylan (70). After two rounds of selection, unfortunately, binding activity of mutants isolated decreased more than wild type. Verhaert *et al.* selected amylase mutants that bind to starch at low pH (71).

F. Perspective

To identify glycoconjugate-binding molecules by phage display technology, random libraries of antibody, lectin, and peptide have been used for the affinity selection. Carbohydrate-binding antibody and lectin are necessary for the detection of the presence of carbohydrates in etiological diagnosis (4). Peptide–carbohydrate interaction is not stronger than protein–carbohydrate interaction, and affinity and specificity of peptide have no advantage over proteins. However, the multivalent binding effect led to the peptide binding more efficiently to carbohydrates (20–26). In addition

フルエンザb型菌の莢膜多糖類 (56)、およびカンジダ・アルビカンス (酵母) (57) に対する Fab 断片も同定されている。

ヘパラン硫酸 (HS) 結合抗体は van Kuppevelt らによって精力的に研究されている (58–65)。抗 HS 抗体は HS と塩基性線維芽細胞成長増殖因子や血管内皮細胞増殖因子との相互作用を阻害した (58、64)。

植物細胞壁のペクチンは3つの主要な多糖、ホモガラクツロナン (HG)、ラムノガラクツロナン (RG)-I、および RG-II で構成されている。HG および RG-II に結合する抗体がそれぞれ、Willats ら (66)、および Williams ら (67) によって選択されている。

E. レクチンの変異ライブラリー

これまでに意外にも、マメ科レクチンの1例を除いて、レクチンの変異ライブラリーの構築は行われていない。Yamamoto らはガラクトースに結合する *Bauhinia purpurea* レクチン (BPA) の変異ライブラリーをλファージで調製した (68)。BPA の糖鎖結合ループをランダム化し、親和性選択をマンノース修飾 BSA に対して行った。得られた BPA 変異体はマンノースに結合し、ガラクトースに対する結合性を失っていた。しかし GlcNAc に対しての選択では、GlcNAc に結合する変異レクチンは得られなかった。彼らは GlcNAc 認識にはもう少し長い糖鎖結合ループが必要なのではないかと考察している。提示技術を使ってはいないが、Yim らは *Maackia amurensis* ヘマグルチニンをランダム変異させ、異なる糖鎖に結合性を有した様々なレクチンを同定している (69)。

レクチン変異ではなく、他のアプローチでも糖鎖結合分子を得ることが可能であろう。あるグリコシダーゼの糖鎖結合ドメインは触媒部位から離れている。グリコシダーゼの糖鎖結合ドメインが独立した糖鎖認識を行う分子として利用されている。キシラナーゼのキシラン結合モジュールを変異性 PCR によって変異させ、キシランとの選択に用いられた (70)。2ラウンド終了後、残念なことに単離された変異体の結合活性は野生型よりも減少していた。Verhaert らは、低い pH でスターチに結合するアミラーゼの変異体を選択している (71)。

F. 展　望

ファージ提示技術を使って複合糖質に結合する分子を得るために、抗体やレクチン、およびペプチドのランダムライブラリーが親和性選択に用いられてきた。糖鎖を認識する抗体やレクチンは、病因診断における糖鎖の存在を確認に欠かせない (4)。これに対してペプチド－糖鎖間相互作用はタンパク質－糖鎖間相互作用ほど強くなく、また親和性や特異性においてもタンパク質に劣る。しかしペプチドであっても多価

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to multivalent effect of peptides, the bottom-up approach by the fusion of these peptide sequences with liposomes and polymer carriers might be effective for molecular design (72).

The increase of the range of applications for these carbohydrate-binding molecules would encourage the growth of this area of research. For example, the increasing interest in peptide drugs might indicate the future direction of this area (73). Protein–carbohydrate interaction is widely related to various diseases such as cancer and infectious diseases. Detection of causative agents and discovery of inhibitors are one of the main purposes of the phage-display studies. To reach the ultimate goal, we need development of technology to overcome some weak points of peptide drugs: immunogenicity and degradation loss of peptides in our body, etc. As the meaning of carbohydrate functions within the living body is clarified, we will attach importance to the development of carbohydrate-binding molecules to replace antibody and lectin.

効果により、糖鎖への効率的に結合した(20–26)。ペプチドの多価の効果に加えて、これらのペプチド配列とリポソームと高分子担体との融合による、ボトムアップ型の分子設計も効果的かもしれない(72)。

これら糖鎖結合性分子の活用の幅を広げることは、この研究分野の成長を促すことになろう。例えばペプチド医薬への関心の高まりは、この分野の将来の方向性を示しているかもしれない(73)。タンパク質－糖鎖間相互作用はガンや感染症など、多くの疾病と関係している。原因物質の検出や阻害剤の探索が、ファージライブラリー法の研究の主要な目的の1つとなっている。目的の達成にはペプチドのもつ弱点、抗原性や体内での分解による損失などを克服する技術開発も必要である。生体内における糖鎖の機能が明らかになるのに従って、抗体やレクチンに代わる糖鎖認識分子の開発は重要になってくると考えられる。

References

1. "Lectin" 2nd edition (2003)(Sharon, N. ,and Lis, H. eds.) Springer Netherlands
2. Lis,H., and Sharon, N. (1998) Chem. Rev. **98**, 637–674
3. Weis, W.I., and Drickamer, K. (1996) Annu. Rev. Biochem. **65**, 441–473
4. Kannagi, R., and Hakomori, S. (2001) Adv. Exp. Med. Biol. **491**, 587–630
5. Weintraub, A. (2003) Carbohydr. Res. **338**, 2539–2547
6. Yuki, N. (2001) Lancet Infect. Dis. **1**, 29–37
7. Kaida, K., Kanzaki, M., Morita, D., Kamakura, K., Motoyoshi, K., Hirakawa, M., Kusunoki, S.(2006) J. Neurol. Neurosurg. Psychiatry **77**, 1043–1046
8. Said, G. (2006) Neuromuscul. Disord. **16**, 293–303
9. Nobile-Orazio, E. (2001) J. Neuroimmunol. **115**, 4–18
10. Quiocho, F.A. (1988) Curr. Top. Microbiol. Immunol. **139**, 135–148
11. Smith, G.P. (1985) Science **228**, 1315–1317
12. Smith, G.P., and Petrenko, V.A. (1997) Chem. Rev. **97**, 391–410
13. Scott, J.K., and Smith, G.P. (1990) Science **249**, 386–390
14. Garufi, G., Minenkova, O., Lo Passo, C. Pernice, I., and Felici, F. (2005) Biotechnol. Annu. Rev. **11**, 153–190
15. Rader,C., and Barbas 3rd, C.F. (1997) Curr. Opin. Biotechnol. **8**, 503–508
16. Swimmer, C., Lehar, S.M., McCafferty, J., Chiswell, D.J., Blattler, W.A., and Guild, B.C. (1992) Proc. Natl. Acad. Sci. U. S. A. **89**, 3756–3760
17. Jolly, C.L., Beisner, B.M., and Holmes, I.H. (2000) Virology **275**, 89–97
18. Liang, O.D., Rosenblatt, S., Chhatwal, G.S., and Preissner, K.T.(1997) FEBS Lett. **407**, 169–172
19. Moriki, T., Kuwabara, I., Liu, F.T., and Maruyama, I.N. (1999) Biochem. Biophys. Res. Commun. **265**, 291–296
20. Peletskaya, E.N., Glinsky, G., Deutscher, S.L., and Quinn, T.P. (1996) Mol. Divers. **2**, 13–18
21. Gui, J., Moyana, T., Malcolm, B., and Xiang, J. (1996) Proteins **24**, 352–358
22. Gui, J., Moyana, T., and Xiang, J. (1996) Biochem. Biophys. Res. Commun. **218**, 414–419
23. Peletskaya, E.N., Glinsky, V.V., Glinsky, G.V., Deutscher, S.L., and Quinn, T.P. (1997) J. Mol. Biol. **270**, 374–384
24. Landon, L.A., Peletskaya, E.N., Glinsky, V.V., Karasseva, N., Quinn, T.P., and Deutscher, S.L. (2003) J. Protein Chem. **22**, 193–204
25. Hyun, S., Kim, J., Kwon, M., and Yu, J. (2007) Bioorg. Med. Chem. **15**, 511–517
26. Kwon, M., Jeong, S., Lee, K.H., Park, Y.K., and Yu, J. (2002) J. Am. Chem. Soc. **124**, 13996–13997
27. Matsubara, T., Ishikawa, D., Taki, T., Okahata, Y., and Sato, T. (1999) FEBS Lett. **456**, 253–256
28. Miura, Y., Sasao, Y., Kamihira, M., Sakaki, A., Iijima, S., and Kobayashi, K. (2004) Biochim. Biophys. Acta **1673**, 131–138
29. Matsubara, T., Iijima, K., Nakamura, M., Taki, T., Okahata,Y., and Sato, T. (2007) Langmuir **23**, 708–714
30. Fujitani, N., Shimizu, H., Matsubara, T., Ohta, T., Komata. Y., Miura. N., Sato, T., and Nishimura, S.I. (2007) Carbohydr. Res., *in press*
31. Montaner, A.D., De Nichilo, A., Elias, F., Rodriguez, J.M., Flo, J.M., Lopez, R.A., Zorzopulos, J., and Frank, R. (2006) Vaccine **24**, 1889–1896
32. Liu, J.K., Teng, Q., Garrity-Moses, M., Federici, T., Tanase, D., Imperiale,M. J., and Boulis, N.M. (2005) Neurobiol. Dis. **19**, 407–418
33. Burg, M.A., Pasqualini, R., Arap, W., Ruoslahti, E., and Stallcup, W.B. (1999) Cancer Res. **59**, 2869–2874
34. Takashima, A., Mummert, M., Kitajima, T., and Matsue, H. (2000) Ann. N. Y. Acad. Sci. **919**, 205–213
35. Mummert, M.E., Mohamadzadeh,M., Mummert,D.I., Mizumoto, N., and Takashima, A. (2000) J. Exp. Med. **192**, 769–779
36. Noda, K., Yamasaki, Hironaka,Y., and Kitagawa, A. (2001) FEMS Microbiol. Lett. 205, 349–354
37. Zhu, Y., Ho, B., and Ding, J.L. (2003) Biochim. Biophys. Acta **1611**, 234–242

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38. Kim, Y.G., Lee, C.S., Chung, W.J., Kim, E.M., Shin, D.S., Rhim, J.H., Lee, Y.S., Kim, B.G., and Chung, J. (2005) Biochem. Biophys. Res. Commun. **329**, 312–317
39. Kim, Y.G., Lee, C.S., Chung, W.J., Kim, E.M., Shin, D.S., Kim, J.H. Lee, Y. S., Chung, J., and Kim, B.G. (2006) Biotechnol. Lett. **28**, 79–84
40. Guo, X., and Chen, R.R. (2006) Biotechnol. Prog. **22**, 601–604
41. Mummert, M.E., Mummert, D.I., Ellinger, L., and Takashima, A. (2003) Mol. Cancer Ther. **2**, 295–300
42. Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D., and Winter, G.(1994) EMBO J. **13**, 692–698
43. Deng, S.J., MacKenzie, C.R., Hirama, T., Brousseau, R., Lowary, T.L., Young, N.M., Bundle, D.R., and Narang, S.A. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 4992–4996
44. Deng, S.J., MacKenzie, C.R., Sadowska, J., Michniewicz, J., Young, N.M., Bundle, D.R., and Narang, S.A. (1994) J. Biol. Chem. **269**, 9533–9538
45. Dinh, Q., Weng, N.P., Kiso, M., Ishida, H., Hasegawa, A., and Marcus, D.M. (1996) J. Immunol. **157**, 732–738132
46. Soderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A.M., Nilsson, A., Jansson, B., Ohlin, M., Wingren, C., Danielsson, L., Carlsson, R., and Borrebaeck, C.A. (2000) Nat. Biotechnol. **18**, 852–856
47. Johansson, R., Ohlin, M., Jansson, B., and Ohlson, S. (2006) J. Immunol. Methods **312**, 20–26
48. Mao, S., Gao, C., Lo, C.H., Wirsching, P., Wong,C.H., and Janda, K.D.(1999) Proc. Natl. Acad. Sci. U. S. A. **96**, 6953–6958
49. Ravn, P., Danielczyk, A., Jensen, K.B. Kristensen, P., Christensen, P.A., Larsen, M., Karsten, U., and Goletz, S. (2004) J. Mol. Biol. **343**, 985–996
50. Wang, L., Radic, M.Z., Siegel, D., Chang, T., Bracy, J., and Galili, U. (1997) Mol. Immunol. **34**, 609–618
51. Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L., Senter, P., Revets, H., De Baetselier, P., Muyldermans, S., and Magez, S. (2004) J. Biol. Chem. **279**, 1256–1261
52. Qiu, J.X., Kai, M., Padlan, E.A., and Marcus, D.M. (1999) J. Neuroimmunol. **97**, 172–181
53. Rojas, G., Talavera, A., Munoz, Y., Rengifo, E., Krengel, U., Angstrom,J., Gavilondo, J., and Moreno, E. (2004) J. Immunol. Methods **293**, 71–83
54. MacKenzie,R., and To, R. (1998) J. Immunol. Methods **220**, 39–49
55. Mertens, P., and Walgraffe, D., Laurent, T., Deschrevel, N., Letesson, J.J., De Bolle, X. (2001) Int. Rev. Immunol. **20**, 181–199
56. Reason, D.C., Wagner, T.C., and Lucas, A.H. (1997) Infect. Immun. **65**, 261–266
57. Zhang, M.X., Bohlman, M.C., Itatani, C., Burton, D.R., Parren, P.W., St Jeor, S.C., and Kozel, T.R. (2006) Infect. Immun. **74**, 362–369
58. van Kuppevelt, T.H., Dennissen, M.A., van Venrooij, W.J., Hoet, R.M., and Veerkamp, J.H. (1998) J. Biol. Chem. **273**, 12960–12966
59. Jenniskens, G.J., Oosterhof, A., Brandwijk, R., Veerkamp, J.H., van Kuppevelt, T.H. (2000) J. Neurosci. **20**, 4099–4111
60. van Kuppevelt, T.H., Jenniskens,G.J., Veerkamp, J.H., ten Dam, G.B., and Dennissen, M.A. (2001) Methods Mol. Biol. **171**, 519–534
61. van de Westerlo, E.M., Smetsers, T.F., Dennissen, M.A., Linhardt, R.J., Veerkamp,J.H., van Muijen, G.N., and van Kuppevelt, T.H. (2002) Blood **99**, 2427–2433
62. Dennissen, M.A., Jenniskens, G.J., Pieffers, M., Versteeg, E.M., Petitou, M., Veerkamp, J.H., and van Kuppevelt, T.H. (2002) J. Biol. Chem. **277**, 10982–10986
63. Smetsers, T.F., van de Westerlo, E.M., ten Dam, G.B., Clarijs, R., Versteeg, E.M., van Geloof, W.L., Veerkamp, J.H., van Muijen, G.N., and van Kuppevelt, T.H. (2003) Cancer Res. **63**, 2965–2970
64. Smits, N.C., Robbesom, A.A., Versteeg, E.M., van de Westerlo, E.M., Dekhuijzen, P.N., and van Kuppevelt, T.H. (2004) Am. J. Respir. Cell Mol. Biol. **30**, 166–173
65. Lensen, J.F., Rops, A.L., Wijnhoven, T.J., Hafmans, T., Feitz, W.F., Oosterwijk, E., Banas, B., Bindels, R.J., van den Heuvel, L.P., van der Vlag, J., Berden, J.H., and van Kuppevelt, T.H. (2005) J. Am. Soc. Nephrol. **16**, 1279–1288
66. Willats, W.G., Gilmartin, P.M., Mikkelsen, J.D., and Knox, J.P. (1999) Plant J. **18**, 57–65
67. Williams, M.N., Freshour, G., Darvill, A.G., Albersheim, P., and Hahn, M.G. (1996) Plant Cell **8**, 673–685
68. Yamamoto, K., Maruyama, I.N., and Osawa, T. (2000) J. Biochem. (Tokyo). **127**, 137–142
69. Yim, M., Ono, T., and Irimura, T. (2001) Proc. Natl. Acad. Sci. U. S. A. **98**, 2222–2225
70. Miyakubo, H., Sugio, A., Kubo, T., Nakai, R., Wakabayashi, K., and Nakamura, S. (2000) Nucleic Acids Symp. Ser. 165–166
71. Verhaert, R.M., Beekwilder, J., Olsthoorn, R., van Duin, J., and Quax, W.J. (2002) J. Biotechnol. **96**, 103–118
72. Takikawa, M., Kikkawa, H., Asai, T., Yamaguchi, N., Ishikawa, D., Tanaka, M., Ogino, K., Taki, T., and Oku, N. (2000) FEBS Lett. **466**, 381–384
73. Marx, V. (2005) Chem. Eng. News **83**, 17–24

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Characterization of Monocyte-Macrophage–Lineage Cells Induced from CD34+ Bone Marrow Cells In Vitro

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Abstract

We characterized the expression of cell surface antigens and cytokine-secreting ability of monocyte-macrophage–lineage cells induced in vitro from CD34+ bone marrow cells. After cultivation for 3 weeks, we observed 2 distinct cell fractions: a floating small, round cell fraction and an adherent large, protruding cell fraction. Both cell fractions expressed myelocyte-monocyte–lineage antigens, but mature-macrophage markers such as CD206 were expressed only by the adherent cells. An assessment of cells cultured for 5 weeks revealed spontaneous secretion of interleukin 8 (IL-8) and IL-6, and lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF-α) secretion in both fractions, but only the adherent cell fraction secreted IL-10 after LPS stimulation. In contrast, both fractions of cells cultured for 3 weeks spontaneously secreted low levels of IL-8, but none of the other cytokines. Upon LPS stimulation, the cells secreted IL-6 and TNF-α, but not IL-10. We also assessed the effect of granulocyte colony-stimulating factor (G-CSF) pretreatment on TNF-α secretion by each cell fraction and found that G-CSF reduced TNF-α secretion only in the adherent fraction of cells cultured for 3 weeks. Monocyte-macrophage–lineage cells induced in vitro should provide an ideal model for functional analysis of monocyte-macrophage cells.
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Key words: Monocyte-macrophage lineage; Antigen; Cytokine; Expression

1. Introduction

The mononuclear phagocyte system includes a widely distributed family of related cells (such as peripheral blood monocytes, macrophages, Kupffer cells, dendritic cells, osteoclasts, and microglia) that exhibit highly specialized functions. Macrophages resident in a number of tissues act as professional phagocytes and remove pathogens or apoptotic cells [1]. Dendritic cells are specialized to capture and present antigens, initiating the immune response through naive T-cell activation [2]; dendritic cells are also implicated in maintaining tolerance to self antigens [3]. Osteoclasts, multinucleated bone-resorbing cells found in the vicinity of bone, play an essential role in bone remodeling as well as in regulating calcium homeostasis [4]. Microglia represent a unique category of mononuclear phagocytes distributed throughout the central nervous system [5], and in addition to their role as the immune effectors of the central nervous system, they perform nonimmunologic functions, including the production of neurotrophic factors and glutamate uptake [6,7].

The mononuclear phagocytic cells are believed to originate from hematopoietic stem cells in the bone marrow (BM). In the conventional view, monocytes that develop in the BM are released into the circulation and then enter the tissues to become resident macrophages and other mononuclear phagocytic cells [8,9]. Consistent with this view, Kennedy and Abkowitz demonstrated in a mouse transplantation system that more mature monocytes give rise to tissue macrophages, including alveolar macrophages in the lung and Kupffer cells in the liver [10]. The results of a number of studies have suggested, however, that the mechanism of mononuclear phagocytic cell development is more complicated. One intriguing possibility is that less mature marrow-derived cells, such as macrophage colony-forming units, enter

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the tissues and differentiate into macrophages [11]. Prior studies have shown that tissue macrophages divide in situ, indicating that these cells are responsible for the renewal and expansion of this population [12-14].

The functional and phenotypic heterogeneity within the phagocyte system may be evidence of the differentiation plasticity of a common progenitor, but the details of the developmental pathways leading to the maturation of mononuclear phagocytic cells are still unclear. In vitro culture systems in which mature mononuclear phagocytic cells are induced from hematopoietic stem cells or monocyte precursors have been employed in a number of studies to clarify the molecular mechanism of phagocytic cell development. For example, monocyte-macrophage–lineage cells can be induced from CD34⁺ cord blood hematopoietic stem cells by liquid culture with cytokines [15] and by cocultivation with BM stromal cell lines in the presence of cytokines [16]. BM progenitors have recently been identified by their ability to differentiate into dendritic cells or osteoclasts, depending on whether they are exposed to RANKL in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) or M-CSF [17].

To evaluate the usefulness of monocyte-macrophage–lineage cells as a source for an in vitro model for the functional analysis of a monocytic phagocyte system, we analyzed the immunophenotype of and cytokine production by monocyte-macrophage–lineage cells induced from CD34⁺ BM cells in vitro. In this study, we showed that several distinct developmental stage–related subpopulations are present in monocyte-macrophage–lineage cells induced from CD34⁺ BM cells in vitro.

2. Materials and Methods

2.1. Cells and Reagents

Human BM CD34⁺ cells from Cambrex Bio Science Walkersville (Walkersville, MD, USA) were used. The cells had been isolated from human tissue after informed consent had been obtained. Recombinant human cytokines were purchased from PeproTech (London, UK). Fluorescently conjugated monoclonal antibodies were purchased from Beckman Coulter (Westbrook, MA, USA). Unless otherwise indicated, all other chemical reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cultures

Monocyte-macrophage–lineage cells were induced by incubating human BM CD34⁺ cells (1×10^5 cells/well of a 6-well plate) at 37°C under 5% carbon dioxide in 5 mL of 10% (vol/vol) fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA) containing RPMI 1640 medium (Sigma-Aldrich) supplemented with a cytokine mixture consisting of interleukin 3 (IL-3) (20 ng/mL), IL-6 (20 ng/mL), M-CSF (100 ng/mL), GM-CSF (20 ng/mL), and Flt-3 ligand (100 ng/mL) [15,18]. Every week, half of the medium was replaced with fetal calf serum–containing medium supplemented with M-CSF alone. After 3 weeks of cultivation, the medium was completely replaced with the medium supplemented with M-CSF alone, and the cells were cultured for another 2 weeks. At the end of 5 weeks of cultivation, the floating cells in the medium were collected, and the adherent cells were harvested with 0.25% trypsin plus 0.02% EDTA (Immuno-Biological Laboratories Co, Gunma, Japan). These 2 cell fractions were used for further examination.

For the histology studies, cells were harvested and immobilized on glass slides with Cytospin 2 (Shandon, Pittsburgh, PA, USA). After Giemsa staining, cell morphology was assessed by light microscopy (BX-61; Olympus, Tokyo, Japan). Cells were tested for cytokine secretion by exposing the cells to G-CSF and stimulating them with lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 hours, as described previously [19].

2.3. Reverse Transcriptase–Polymerase Chain Reaction Analysis

Total RNA was extracted from cultured human BM cells, and complementary DNA (cDNA) was generated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and a First-Strand cDNA Synthesis Kit (Pfizer, Uppsala, Sweden). cDNA synthesized from 150 ng of total RNA was used as a template for one amplification reaction. The following sets of primers were used: 5′-ttattacccccctccttcagacac-3′ (sense) and 5′-aagtctggaaacatctggagagag-3′ (antisense), for amplification of a 347-bp fragment of human tumor necrosis factor α (TNF-α) cDNA; 5′-aagtggtgttctccatgtcc-3′ (sense) and 5′-gagcgaatgacagagggttt-3′ (antisense), for amplification of a 664-bp fragment of human IL-1β cDNA; and 5′-gctggaggactttaagggtt-3′ (sense) and 5′-cccagatccgattttggaga-3′ (antisense), for amplification of a 394-bp fragment of human IL-10 cDNA. The set of primers for amplification of human glyceraldehyde-3-phosphate dehydrogenase was obtained from Stratagene (La Jolla, CA, USA). The polymerase chain reaction (PCR) was repeated for 30 cycles of heating at 94°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 2 minutes; the PCR products were then separated on a 1.5% agarose gel.

2.4. Immunofluorescence Study and Cytokine Measurement

A multicolor immunofluorescence study was performed with a combination of fluorescein isothiocyanate, phycoerythrin, and phycoerythrin–Cyanine 5 (PC-5). Cells were stained with fluorescently labeled monoclonal antibodies and analyzed by flow cytometry (Epics XL; Beckman Coulter), as described previously [19]. The concentrations of cytokines and chemokines in culture supernatants were determined with a Cytometric Bead Array (CBA) (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

3. Results

3.1. Differentiation into Monocyte-Macrophage–Lineage Cells of Human BM CD34⁺ Cells Cultured with a Combination of Cytokines

We first characterized the morphology and surface-antigen expression of human BM CD34⁺ cells cultured with the

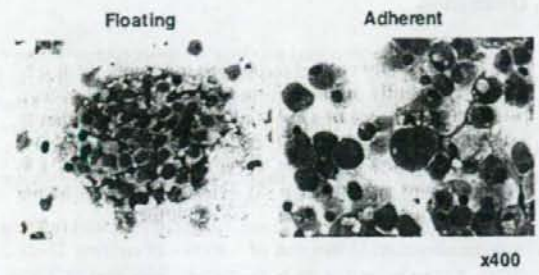


Figure 1. Morphology of monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Human BM CD34⁺ cells were cultured for 5 weeks in the presence of a cytokine mixture, as described in "Materials and Methods." Floating and adherent cell fractions were subsequently collected separately and cytocentrifuged on a glass slide. Morphology was assessed after Giemsa staining. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown (original magnification ×400).

combination of cytokines indicated in "Materials and Methods." During the second week of culture, microscopical observation revealed that a portion of the cells had started to adhere to the bottom of the culture dish. After 3 weeks of cultivation, the cells were clearly divided into floating and adherent fractions. When 1×10^5 CD34⁺ cells were cultured, approximately 7×10^6 adherent cells and 3×10^6 floating cells were obtained after 5 weeks of cultivation. At the end of the 5-week cultivation period, the floating and adherent fractions were collected separately, and their morphologies were assessed after May-Giemsa staining. As shown in Figure 1, the cells in the floating fraction were small and round and contained little cytoplasm. In contrast, the cells in the adherent fraction were large and contained abundant foamy cytoplasm with protrusions.

We also used flow cytometry to examine the cells for expression of monocyte-macrophage–lineage markers (Figure 2). Most floating cells expressed CD11b, CD31, CD33, and CD97, but no other mature-macrophage markers. The adherent cells, on the other hand, expressed markers of the myelocyte-monocyte lineage, such as CD13, CD14, CD36, CD54, CD64, CD85k, and CD105. It is noteworthy that the adherent cells expressed the mature-macrophage marker CD206, which was not expressed by the peripheral blood monocytes examined as a control (Figure 3).

3.2. Cytokine Secretion by Monocyte-Macrophage–Lineage Cells Induced from Human BM CD34⁺ Cells

Next, we assessed the cytokine-secreting ability of human BM CD34⁺ cells cultured with the cytokine combination. At the end of 5 weeks of cultivation, the floating and adherent fractions were collected separately, and cytokine secretion was assessed with the CBA system with and without LPS stimulation. Figure 4 shows that both the floating and adherent cell fractions spontaneously secreted IL-8 and IL-6 without LPS stimulation. After 24 hours of LPS stimulation, IL-6 secretion was enhanced in both fractions, but IL-8 secretion by adherent cells was decreased. TNF-α secretion, on the other hand, was induced in the 2 fractions only after LPS stimulation. It is noteworthy that LPS stimulation induced IL-10 secretion only in the adherent cell fraction and not in the floating cell fraction. Neither IL-1β nor IL-12 secretion was induced by LPS stimulation in either fraction.

We also used reverse transcriptase–polymerase chain reaction analysis to assess the effect of LPS stimulation on the expression levels of cytokine messenger RNA (mRNA). Consistent with the results of the CBA analysis, LPS stimulation enhanced the expression of TNF-α mRNA in both the adherent and floating cell fractions (Figure 5). IL-10 mRNA expression, however, was already detectable in both cell fractions in the unstimulated state, and LPS stimulation did not enhance expression. In addition, LPS stimulation reduced IL-10 mRNA expression in the floating cell fraction. It is interesting that although no secretion of IL-1β protein was detected by the CBA assay, LPS stimulation significantly increased IL-1β mRNA expression in both cell fractions.

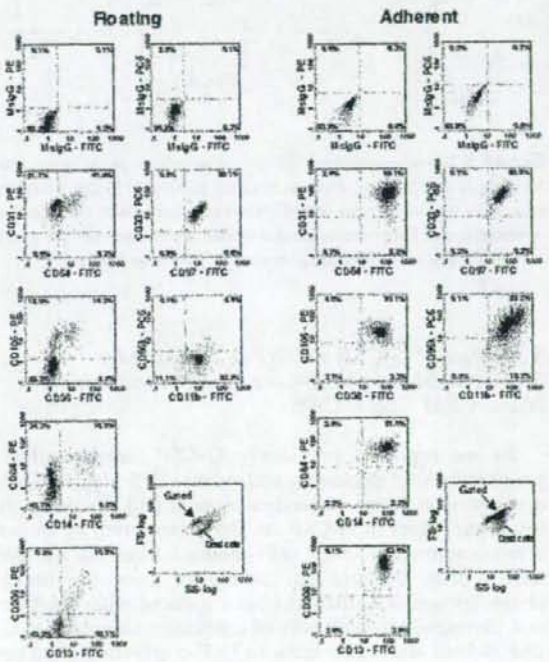


Figure 2. Immunophenotypic analysis of monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Human BM CD34⁺ cells were cultured for 5 weeks (as in Figure 1). At the end of the culture period, the floating and adherent cell fractions were collected separately, stained with combinations of fluorescently labeled antibodies as indicated, and examined by flow cytometry. The experiments were repeated 3 times, and reproducible results were obtained. Representative histogram data are shown. MsIgG, mouse immunoglobulin G; PE, phycoerythrin; PC-5, PE-Cyanine 5; FITC, fluorescein isothiocyanate; FS, forward light scatter; SS, side light scatter.

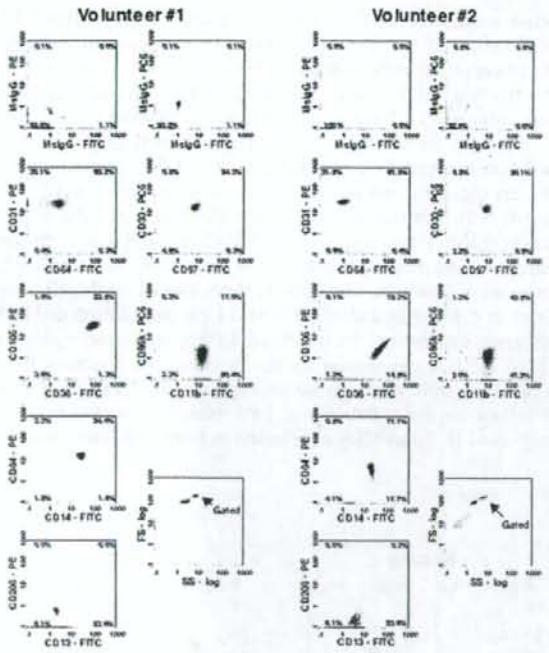


Figure 3. Immunophenotypic analysis of peripheral blood monocytes. Mononuclear cells were obtained from the peripheral blood of healthy volunteers via Ficoll-Paque centrifugation and stained with the indicated combinations of fluorescently labeled antibodies. Monocytes were gated, and the expression of each antigen was analyzed as in Figure 2.

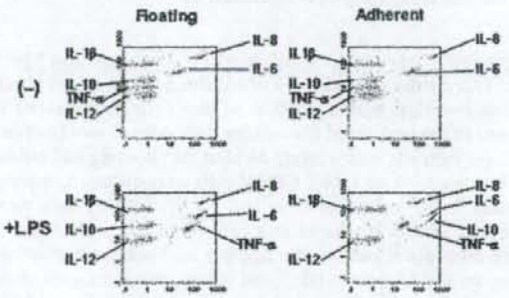
3.3. Effect of G-CSF on TNF-α Secretion by Monocyte-Macrophage–Lineage Cells Induced from Human BM CD34⁺ Cells

As we reported previously, G-CSF directly affects peripheral blood monocytes and reduces LPS-induced TNF-α secretion in a time-dependent manner [20]. We therefore tested the effect of G-CSF on TNF-α secretion by monocyte-macrophage–lineage cells induced from human BM CD34⁺ cells. Because our testing of monocyte-macrophage–lineage cells that had been induced with cytokines and harvested after 5 weeks of cultivation showed that G-CSF did not affect LPS-induced TNF-α secretion (data not shown), we tested cells harvested at different time points. Our assessment of LPS-stimulated cytokine secretion by adherent cells (Figure 6) (but not floating cells; data not shown) collected after 3 weeks of cultivation revealed cytokine-secretion patterns different from those of cells collected after 5 weeks of cultivation. Figure 6 shows that cells cultured for 3 weeks spontaneously secreted low levels of IL-8, but not other cytokines. LPS stimulated the cells to secrete IL-6 and TNF-α and an increased level of IL-8. Pretreatment with G-CSF reduced LPS-induced TNF-α secretion in a time-dependent manner.

4. Discussion

This study has shown that monocyte-macrophage–lineage cells were efficiently induced from CD34⁺ BM cells in liquid culture in the presence of a cocktail of cytokines and that the monocyte-macrophage–lineage cells induced in vitro were capable of cytokine secretion upon stimulation with LPS. Several different subsets of monocyte-macrophage–lineage cells were induced during the course of culture.

For example, 2 distinct fractions, adherent cells and floating cells, were observed at the end of 5 weeks of culture. These 2 fractions were distinctive in both morphology and immunophenotype. Adherent cells were large, had a macrophage-like appearance, and expressed the mature-macrophage markers CD14, CD105, and CD206. In contrast, the floating cells were relatively small and contained little cytoplasm. Only some of them expressed mature-macrophage markers, whereas most



		Floating, ng/mL	Adherent, ng/mL
IL-8	(-)	9.24	26.13
	LPS	12.03	3.90
IL-1 β	(-)	0.07	0.07
	LPS	0.07	0.10
IL-6	(-)	0.87	2.03
	LPS	7.35	20.30
IL-10	(-)	0.02	0.02
	LPS	0.02	34.62
TNF-α	(-)	<0.00	<0.00
	LPS	2.55	0.87
IL12	(-)	<0.00	<0.00
	LPS	<0.00	<0.00

Figure 4. Cytometric Bead Array (CBA) analysis of lipopolysaccharide (LPS)-stimulated cytokine secretion by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells by cultivation for 5 weeks, as described for Figure 1. At the end of the culture period, the floating and adherent cells were stimulated with and without LPS for 24 hours. Subsequent cytokine secretion was assessed with the CBA system. The histograms obtained (upper panels) and calculated concentrations of each cytokine (table at bottom; <0.00 indicates undetectable) are shown. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. IL-1β indicates interleukin 1β; TNF-α, tumor necrosis factor α.

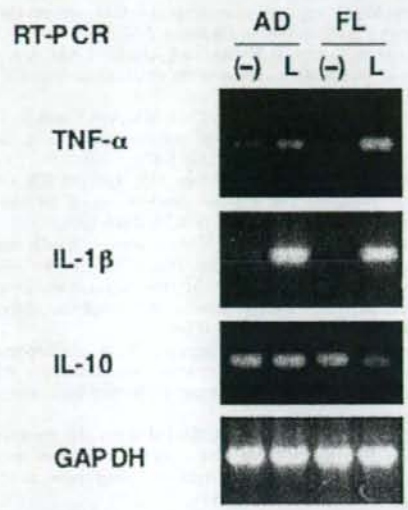


Figure 5. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of lipopolysaccharide (LPS)-stimulated cytokine production by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells as described for Figure 1, and then floating (FL) and adherent (AD) cell fractions were collected separately. After stimulation with (L) or without (–) LPS for 24 hours (as described for Figure 4), total RNA was extracted, and the indicated messenger RNA molecules were analyzed by the RT-PCR after complementary DNA synthesis. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. TNF-α indicates tumor necrosis factor α; IL-1β, interleukin 1β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells expressed myelomonocytic antigens, including CD31, CD33, and CD97. It is interesting that these 2 fractions exhibited different cytokine-secretion abilities. Figure 4 shows that both fractions spontaneously secreted IL-8 and IL-6, and they both secreted TNF-α upon stimulation with LPS. Only the adherent cell fraction secreted IL-10 after LPS stimulation, however. These characteristics suggest that the adherent cell fraction represents mature macrophages, whereas the floating cell fraction may be related to immature monocytes. Evidence that further cultivation of the floating cell fraction induced an adherent cell fraction (data not shown) supports this idea.

It is noteworthy that both cell fractions contained more IL-1β mRNA after LPS stimulation but that no IL-1β secretion at the protein level was detected in either fraction. The data indicate that monocyte-macrophage–lineage cells induced in vitro are capable of producing IL-1β upon stimulation with LPS but that the stimulation is insufficient to induce secretion of IL-1β.

On the other hand, the adherent cells exhibited a profile of cytokine secretion after 3 weeks of cultivation that was distinct from that obtained after 5 weeks. At 3 weeks, the adherent cell fractions displayed almost the same immunophenotype as monocyte-macrophage–lineage cells cultured for 5 weeks; however, the cells spontaneously secreted only low levels of IL-8, and not other cytokines. Although the cells secreted IL-6 and TNF-α after LPS stimulation, they did not secrete IL-10. Thus, our data indicate that different culture conditions induce different monocyte-macrophage–lineage subsets or monocyte-macrophage–lineage cells with different degrees of maturity.

Several studies have shown the induction of monocyte-macrophage–lineage cells by in vitro culture of cells from different cell sources. For example, Akagawa reported that M-CSF–induced monocyte-derived macrophages (M-Mphi) and GM-CSF–induced Mphi (GM-Mphi) differ in morphology, cell surface antigen expression, and function, including Fcγ receptor–mediated phagocytosis, hydrogen peroxide production and sensitivity, catalase activity, susceptibilities to human immunodeficiency virus type 1 and *Mycobacterium tuberculosis*, and suppressor activity [21]. She therefore concluded that the characteristics of GM-Mphi resemble those of human alveolar macrophages. Servet-Delprat et al also

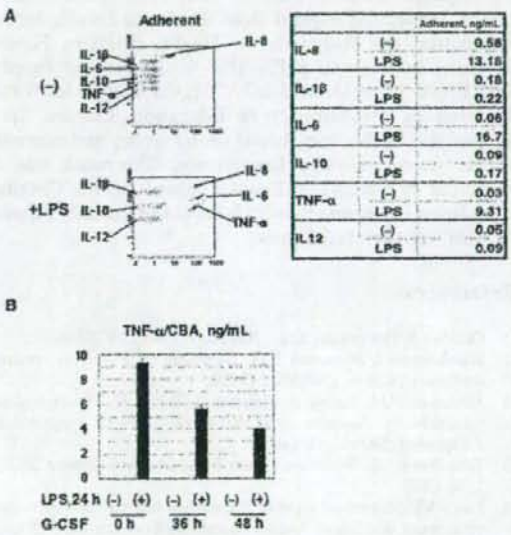


Figure 6. Effect of granulocyte colony-stimulating factor (G-CSF) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor α (TNF-α) secretion by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. A, Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells after 3 weeks of cultivation in the presence of the mixture of cytokines described in "Materials and Methods." At the end of the culture period, cells were stimulated with LPS for 24 hours. Subsequent cytokine secretion was assessed as in Figure 4. B, Induced monocyte-macrophage cells pretreated and not pretreated with G-CSF were stimulated with (+) and without (–) LPS for 24 hours, and subsequent cytokine secretion was assessed as in (A). The experiment was performed in triplicate, and the data are presented as the mean + SD. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. IL-1β indicates interleukin 1β.

reported that a variety of monocyte-macrophage–lineage cells, including macrophages, osteoclasts, dendritic cells, and microglia, can be induced from murine BM cells by ex vivo culture with different combinations of cytokines [22]. These reports further support our hypothesis that different culture conditions can induce different subsets of monocyte-macrophage–lineage cells.

In conclusion, the results of this study indicate that monocyte-macrophage–lineage cells induced from CD34⁺ BM cells in vitro can be used for functional assays, at least in terms of cytokine secretion. Further investigation is clearly necessary, however; the establishment of culture conditions that enable the induction of different subsets of monocyte-macrophage–lineage cells should provide an ideal experimental model for the analysis of monocyte-macrophage–lineage cell function.

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References

1. Gordon S. The macrophage. *Bioessays*. 1995;17:977-986.
2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245-252.
3. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med*. 2000;191:411-416.
4. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000;289: 1504-1508.
5. Perry VH. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. *J Neuroimmunol*. 1998;90:113-121.
6. Elkabes S, DiCicco-Bloom EM, Black IB. Brain microglia/ macrophages express neurotrophins that selectively regulate microglial proliferation and function. *J Neurosci*. 1996;16:2508-2521.
7. Noda M, Nakanishi H, Nabekura J, Akaike N. AMPA-kainate subtypes of glutamate receptor in rat cerebral microglia. *J Neurosci*. 2000;20:251-258.
8. Crofton RW, Diesselhoff-den Dulk MM, van Furth R. The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. *J Exp Med*. 1978;148:1-17.
9. Thomas ED, Ramberg RE, Sale GE, Sparkes RS, Golde DW. Direct evidence for a bone marrow origin of the alveolar macrophage in man. *Science*. 1976;192:1016-1018.
10. Kennedy DW, Abkowitz JL. Mature monocytic cells enter tissues and engraft. *Proc Natl Acad Sci USA*. 1998;95:14944-14949.
11. Takahashi K, Naito M, Takeya M. Development and heterogeneity of macrophages and their related cells through their differentiation pathways. *Pathol Int*. 1996;46:473-485.
12. Yamamoto T, Naito M, Moriyama H, et al. Repopulation of murine Kupffer cells after intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. *Am J Pathol*. 1996;149: 1271-1286.
13. Wijffels JF, Hendrickx RJ, Steenbergen JJ, Eestermans IL, Beelen RH. Milky spots in the mouse omentum may play an important role in the origin of peritoneal macrophages. *Res Immunol*. 1992; 143:401-409.
14. Lawson LJ, Perry VH, Gordon S. Turnover of resident microglia in the normal adult mouse brain. *Neuroscience*. 1992;48:405-415.
15. Sawano A, Iwai S, Sakurai Y, et al. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood*. 2001;97:785-791.
16. Taghon T, Stolz F, De Smedt M, et al. HOX-A10 regulates hematopoietic lineage commitment: evidence for a monocyte-specific transcription factor. *Blood*. 2002;99:1197-1204.
17. Miyamoto T, Ohneda O, Arai F, et al. Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood*. 2001;98:2544-2554.
18. Xu MJ, Tsuji K, Ueda T, et al. Stimulation of mouse and human primitive hematopoiesis by murine embryonic aorta-gonad-mesonephros-derived stromal cell lines. *Blood*. 1998;92: 2032-2040.
19. Kiyokawa N, Kokai Y, Ishimoto K, Fujita H, Fujimoto J, Hata JI. Characterization of the common acute lymphoblastic leukaemia antigen (CD10) as an activation molecule on mature human B cells. *Clin Exp Immunol*. 1990;79:322-327.
20. Saito M, Kiyokawa N, Taguchi T, et al. Granulocyte colony-stimulating factor directly affects human monocytes and modulates cytokine secretion. *Exp Hematol*. 2002;30:1115-1123.
21. Akagawa KS. Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Int J Hematol*. 2002;76:27-34.
22. Servet-Delprat C, Arnaud S, Jurdic P, et al. Flt3⁺ macrophage precursors commit sequentially to osteoclasts, dendritic cells and microglia. *BMC Immunol*. 2002;3:15.

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Experimental Hematology

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Interleukin-7 contributes to human pro–B-cell development in a mouse stromal cell–dependent culture system

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Objective. **The role of interleukin (IL)-7 in human B lymphopoiesis is still controversial. We used an in vitro culture system to verify involvement of IL-7 in development of human pro-B cells from hematopoietic stem cells.**

Materials and Methods. **Human $CD34^+$ bone marrow cells were cultured for 4 weeks on MS-5 mouse stromal cells to induce pro-B cells. Expression of IL-7 receptor α or other B-cell differentiation marker genes on cultured human $CD34^+$bone marrow cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Colony assay of human $CD34^+$ bone marrow cells was also performed to determine the effect of IL-7 on colony-forming ability. Neutralizing antibody or reagent that eliminates the effect of IL-7 was added to the culture system, and the number of pro-B cells induced was estimated by flow cytometry.**

Results. **RT-PCR analysis revealed mRNA expression of IL-7 receptor α as well as B-cell differentiation marker genes in not only $CD19^+$ pro-B cells but also $CD19^-$ $CD33^-$ cells induced from $CD34^+$ bone marrow cells after cultivation for 4 weeks on MS-5 cells. Addition of anti-mouse IL-7 antibody, anti-human IL-7 receptor α antibody, or JAK3 kinase inhibitor reduced the number of pro-B cells induced, demonstrating that elimination of IL-7 reduces pro–B-cell development. Addition of anti-mouse IL-7 antibody emphasized the colony-forming ability of burst-forming unit erythroid cells.**

Conclusions. **IL-7 produced by MS-5 cells is required for human pro–B-cell development from $CD34^+$bone marrow cells in our culture system, and IL-7 appears to play a certain role in early human B lymphopoiesis.** © **2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Interleukin (IL)-7 is a cytokine that was first cloned from a murine bone marrow (BM) stromal cell line and is involved in the regulation of lymphopoiesis [1]. Several studies have shown that IL-7 is crucial to proliferation and development of murine B cells. For example, injection of mice with recombinant IL-7 has been shown to greatly increase the number of B cells [2], whereas injection of anti–IL-7 antibodies severely represses B-cell development [3,4]. Study of the effect of IL-7 on fractionated B-lineage cells from normal mouse BM in a stromal-cell–dependent culture system revealed that IL-7 is required for effective differentiation of pro-B cells into pre-B cells [5]. IL-7 is sufficient to induce differentiation of murine common lymphoid progenitors into pro-B cells in cultures under stromal-cell–free conditions [6].

The requirement for IL-7 in B-lymphocyte development in mice was further demonstrated by experiments in which components of the IL-7 signal transduction pathways were deleted by gene targeting [7–10]. Results showed that B-cell development is severely arrested at the common lymphoid progenitor stage in the BM of adult IL-7 receptor (R) α and common γ-chain–deficient mice, leading to a striking paucity of peripheral B cells.

In contrast to murine B-cell development, however, human B-cell development does not appear to require IL-7

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[11]. Unlike the mouse common γ knockouts, patients with human X-linked severe combined immunodeficiency, who lack a functional common γ chain, produce normal numbers of B cells [12]. Immunodeficiency patients with autosomal recessive mutations in either IL-7Rα chain or JAK3 tyrosine kinase, a downstream signaling molecule of IL-7R, also have normal numbers of peripheral B cells [13–15]. All of this evidence indicates that IL-7 is not always required for B-cell development in humans.

Nevertheless, some studies found that IL-7 affects human B-cell development in some way. For example, it was found that IL-7 transduces signals that lead to specific changes in gene expression during human B-cell development. IL-7 stimulation induces a specific increase in CD19 on the surface of human pro-B cells and decrease in RAG-1, RAG-2, and TdT messenger RNA levels [16]. Proliferation of $CD19^+CD34^+$ pro-B cells on human BM stromal cells is enhanced by inclusion of exogenous IL-7 in the culture [17]. Therefore, if not essential, IL-7 may play an integral role in some aspects of human B-cell development.

In an attempt to clarify the effect of IL-7 on human B-cell development, we used an in vitro culture system in which human hematopoietic stem cells are cocultured with murine BM stromal cells that induce pro–B-cell differentiation. In this article, we report finding that IL-7 is essential for the differentiation of human $CD34^+$ BM cells into pro-B cells in our culture system, and we discuss the possible role of IL-7 in early human B-cell development.

Materials and methods

Reagents

Monoclonal antibodies used were phycoerythrin (PE)-conjugated anti-CD33, from Becton Dickinson Biosciences (San Diego, CA, USA), and PE-cyanine (PC)-5–conjugated anti-CD19, from Beckman/Coulter Inc. (Westbrook, MA, USA). Goat polyclonal anti-mouse IL-7 antibody (Ab) and goat anti-human IL-7Rα Ab were obtained from R&D Systems (Abingdon, UK) and used in the cultures at concentrations of 1 to 5 μg/mL, as indicated. Recombinant human IL-2, -4, -7, -9, and -11 were obtained from PeproTech EC Ltd. (London, UK) and recombinant human IL-15, -21, and both human and mouse thymic stromal lymphopoietin (TSLP) were obtained from R&D Systems.

4-[(3′-Bromo-4′-hydroxyphenyl) amino]-6,7-dimethoxyquinazoline, a potent specific inhibitor of JAK3 kinase (IC_{50} = 5.6 μM) was obtained from Calbiochem-Novabiochem Co. (San Diego, CA, USA) and used in the cultures at a concentration of 5 μM. The specificity of this chemical compound as a JAK3 kinase inhibitor has been examined by Goodman et al. [18] and Sudbeck et al. [19]. They demonstrated that this compound exhibited detectable inhibitory activity only against recombinant JAK3, but not JAK1 or JAK2, in immune complex kinase assays and also inhibited IL-2–induced JAK3-dependent signal transducers and activators of transcription (STAT) activation, but not inhibited IL-3–induced JAK1/JAK2-dependent STAT activation in 32Dcl1-IL2R cells. Unless otherwise indicated, all chemical reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cells, cultures, and colony assay

Human BM $CD34^+$ cells used were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). BM cells were isolated from human tissue after obtaining informed consent. A cloned murine BM stromal cell line, MS-5, was kindly provided by Dr. A. Manabe (St. Luke's International Hospital, Tokyo, Japan) and Dr. K. Mori (Nigata University, Nigata, Japan), and maintained in RPMI-1640 medium (Sigma-Aldrich Fine Chemical Co., St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich) at 37°C under a humidified 5% CO_2 atmosphere.

To induce pro-B cells, MS-5 cells were plated at a concentration of 1×10^5 cells in 12-well tissue plate (Asahi Techno Glass Co., Chiba, Japan) 1 day prior to seeding human BM $CD34^+$ cells. $CD34^+$ cells were plated 4×10^4 cells/well/2 mL onto the MS-5 cells in RPMI-1640 supplemented with 10% fetal calf serum and

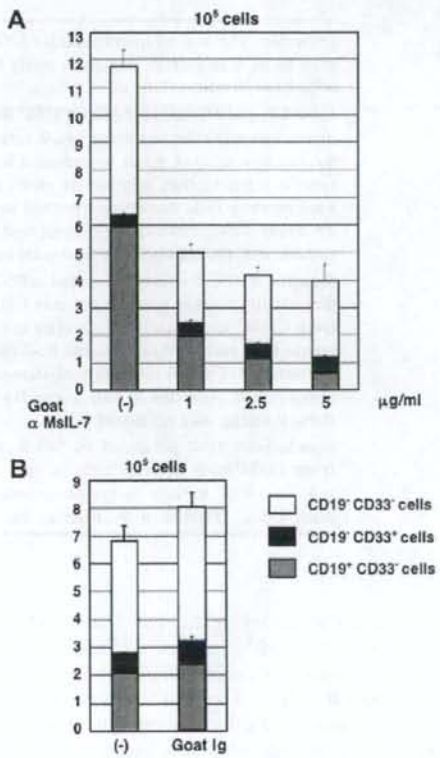

Figure 1. Effect of anti-mouse interleukin (IL)-7 antibody on human pro–B-cell development. (**A**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks in the presence or absence [(–)] of different concentrations of goat polyclonal anti-mouse IL-7 antibody. The subsequent $CD19^+CD33^-$ cell number (lower light gray column), $CD19^-CD33^+$ cell number (middle dark gray column), and $CD33^-CD19^-$ cell number (upper white column) of cultured $CD34^+$ cells were calculated by flow cytometry. (**B**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks in the presence or absence of goat immunoglobulin (goat Ig) as a negative control.