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区分	導入遺伝子	魚の種類	生体での機能等	研究・開発国	遺伝子組換え法	ベクター	プロモーター	ターミネーター	マーカー	文献
研究用	病原性をもつヒトAベータの42アミノ酸から成るペプチド	ゼブラフィッシュ	生後16月で異常な色素パターンが現れた	オーストラリア Univ. Adelaide			ゼブラフィッシュmitfa (nacre)遺伝子プロモーター			1
	EGFP	ゼブラフィッシュ	耳プラコード、耳胞、形成中の骨格構造において導入遺伝子が発現した	アメリカ Univ. Oregon		細菌人工染色体	亜鉛フィンガー転写因子sp7			2
	ゼブラフィッシュsod1遺伝子(変異型G93Rと野生型)と16kbの周辺の配列	ゼブラフィッシュ	変異型遺伝子を持つ魚は筋委縮性側索硬化症の主要な表現型を示した	アメリカ Ohio State Univ.			ゼブラフィッシュsod1遺伝子プロモーター			3
	GFP、RFP	メダカ	in vivoプロモーターアッセイによって3つのプロモーターの性質を調べた	日本 東京大			3種類のメダカのミオシン重鎖遺伝子プロモーター			4
	GFP	マス	体節形成から一生、速筋線維において導入遺伝子が発現した	フランス Natl Inst Agricultural Research			速ミオシン軽鎖2プロモーター			5
	DsRed、アカヒレ成長ホルモン遺伝子	アカヒレ	成長ホルモン遺伝子を導入すると成長が促進された	中国 Shanghai Ocean Univ.	受精卵へ顕微注入	pDsRed2-1, pTLA-GH	アカヒレベータアクチンプロモーター		DsRed	6
	Venus	メダカ	成魚の生殖腺を除いてすべての組織においてプロモーターは活性を示した	日本 東京大			メダカ熱ショックタンパクプロモーター(olphsp70.1)			7
	GFPとB型肝炎ウイルスXタンパクとの融合遺伝子	ゼブラフィッシュ	導入遺伝子は肝臓で発現した。やつれ、脂肪症、脂肪性肝炎が現れた	台湾 Natl. Taiwan Ocean Uni.						8
	EGFP	南アメリカナマズ	様々な処理を施して遺伝子導入の効率を調べた	ブラジル Federal Univ. of Pelotas	精子媒介遺伝子移動法	pEGFPプラスミド				9
バイオリアクター	ティラピアインシュリン様成長因子-1, 2	ゼブラフィッシュ	受精卵の細胞質で水溶性タンパクとして発現した	台湾 Institute of Cellular and Organismic Biology		pT2-ZP-tIGFs-IRES-hrGFP	卵母細胞特異的透明帯3プロモーター		hrGFP	10
研究用	ウォールアイ皮膚肉腫ウイルスrv-cyclin	ゼブラフィッシュ	rv-cyclin単独では腫瘍を作らない	アメリカ Cornell Univ.			CMVtkプロモーター			11
	成長ホルモン	ゼブラフィッシュ	抗酸化防御系と筋形成関連遺伝子の転写が減少して老化が進むらしい。	ブラジル Univ. Federal do Rion Grande						12
	EGFP	ゼブラフィッシュ	初期原腸期で内在性lhx1aと類似の発現パターンを示した	アメリカ Univ. Pittsburgh			lhx1a遺伝子プロモーター			13
	ヒトASCL1遺伝子座の近傍のエンハンサーと推定される9つの配列	ゼブラフィッシュ	ASCL1と類似の発現を示すクローンが得られた	アメリカ Johns Hopkins Univ. School of Medicine						14
	tau遺伝子	ゼブラフィッシュ	tauタンパクの凝集による病理を短時間で再現した	ドイツ Ludwig-Maximilians Univ.						15
	epinecidin-1/DsRed融合遺伝子	ゼブラフィッシュ	バクテリアの感染後24時間までその成長を阻害した	台湾 Institute of Cellular and Organismic Biology	Tol2トランスポゾンシステム		mylz2プロモーター			16

表2　非食用GM魚を作成した研究報告

区分	導入遺伝子	魚の種類	生体での機能等	研究・開発国	遺伝子組換え法	ベクター	プロモーター	ターミネーター	マーカー	文献
	gata1 cDNA	ゼブラフィッシュ	赤血球の生成が阻害された変異体の胚においてその機能が回復した	日本 筑波大	Tol2トランスポゾンシステム	Tol2供与プラスミド	gata1プロモーター			17
	GFP	ゼブラフィッシュ	内耳と側線の有毛細胞で導入遺伝子が発現した	アメリカ The Rockfeller Univ.	meganuclease		parvalbumin 3a, 3b遺伝子プロモーター			18
	EGFP	ゼブラフィッシュ	心臓のエンハンサートラップトランスジェニックゼブラフィッシュが18系統得られた	シンガポール Insitute of Molecular and Cell Biology	トランスポゾン					19
	ウォールアイ皮膚肉腫ウイルスrv-cyclin遺伝子	ゼブラフィッシュ	発癌性物質で処理したときに肝臓癌を抑制した	シンガポール Natl Univ. of Singapore			肝脂肪酸結合タンパクプロモーター			20
	発癌性ヒトHRAS遺伝子	ゼブラフィッシュ	黒色腫において多くの癌遺伝子は発現が抑制され、細胞サイクル遺伝子のみ発現が上昇した	イタリア Institute of Molecular Oncology						21
	GFP	ゼブラフィッシュ	レーザーポインターを使用して時空的にconditionalに制御して導入遺伝子を発現させた	アメリカ Univ. of Massachusetts			hsp71プロモーター			22
	prothymosin alpha type a 遺伝子	ゼブラフィッシュ	上皮細胞で発現させると増殖を促進して、UVBに誘導されるアポトーシスを抑制するが、皮膚癌を起こさない	台湾 Tamkang Univ.			keratin18 遺伝子プロモーター		RFP	23
	GFP	メダカ	GnRH2神経細胞において発現し、その細胞を電気生理学的に記録するとペースメーカーの活動が観察された	日本 東京大						24
	発癌性Kras(V12)	ゼブラフィッシュ	神経前駆細胞の広範なアポトーシスが誘導されて、脳に重傷な浮腫が起きた	韓国 Seoul Natl. Univ.			nestinプロモーター		mCherry、EGFP	25
	ヒト発癌性NRAS(Q61K)	ゼブラフィッシュ	p53機能喪失とNRAS発現が協力して黒色腫ができる	アメリカ Children's Hospital, Boston			メラニン細胞に限定されたmitfaプロモーター			26
	ウシラクトフェリン-GFP融合遺伝子	ゼブラフィッシュ	ゼブラフィッシュにGM胚を食べさせると抗バクテリア活性が得られた	台湾 National Taiwan Univ.	顕微注入		ゼブラフィッシュβ-アクチンプロモーター		GFP	27
	大腸菌ニトロリダクターゼ-eGFPの融合遺伝子	ゼブラフィッシュ	メトロニダゾールで処理すると精巣が小さくなり、精子が形成されなくなった	台湾 Tri-Service Hospital			ゼブラフィッシュ精巣に特異的なAsp、Odf、Sam遺伝子プロモーター			28
	GFP	ゼブラフィッシュ	胚の周皮、成魚の舌とひれにおいて発現した	アメリカ North Carolina Central Univ.			ゼブラフィッシュケラチン5プロモーター			29
	Cdt1とgeminのゼブラフィッシュホモログ遺伝子	ゼブラフィッシュ	胚において細胞周期の進行を観察した	日本 理研						30
	EGFP	ゼブラフィッシュ	胚と成魚でオリゴデンドロサイトとシュワン細胞において発現した	韓国 Chungnam National Univ.			mbpプロモーター			31
	GFP	ゼブラフィッシュ	mRNAは心臓、皮膚、椎骨において検出された	台湾 Institute of Cellular and Organismic Biology			長さの異なるゼブラフィッシュFSH betaプロモーター			32

表2 非食用GM魚を作成した研究報告（続き）

区分	導入遺伝子	魚の種類	生体での機能等	研究・開発国	遺伝子組換え法	ベクター	プロモーター	ターミネーター	マーカー	文献
	優性阻害型のmyostatin同等物遺伝子	メダカ	成魚では骨格筋線維の生産が増加したが、全体の筋肉量は変わらなかった	日本 名古屋大			OlMA1プロモーター			33
	GFP	メダカ	高濃度の4-nonylphenolを卵内でナノインジェクションすると高死亡率になり胚発生が阻害された	日本 九州大			メダカvasa遺伝子プロモーター			34
中国の研究	GFP	ゼブラフィッシュ	17-alpha-ethynylestradiolを投与すると蛍光が観察された	中国 Fudan Univ.			zvtg1プロモーター			35
中国の研究	GFP	ゼブラフィッシュ	foxj1b遺伝子に類似の発現パターンを示した	中国 Tsinghua Univ	Tol2トランスポゾンに基づいたジーントラッピング					36
研究用	Gal4、UAS	ゼブラフィッシュ	Gal4-UAS法によってジーントラップ、エンハンサートラップが可能である	日本 遺伝研	メダカTol2トランスポゾン					37
		ゼブラフィッシュ	導入遺伝子を1コピーだけ正確にゲノムに組み込んだ	日本 遺伝研	トランスポゾンTol2	バクテリア人工染色体				38
	GFP	ゼブラフィッシュ	原腸胚の段階で異所性の細胞を移植して観察した	アメリカ Smith College						39
	エンハンサートラップカセット	ゼブラフィッシュ	ゼブラフィッシュゲノムにカセットを挿入したクローンを338個作った	シンガポール Institute of Molecular and Cell Biology	Tol2トランスポゾン					40
	GFP	ゼブラフィッシュ	多能性前駆細胞の最後の分裂後数時間すべてのアマクリンと水平細胞で一過性にmRNAが発現した	イギリス Univ. of Cambridge			膵臓転写因子1aプロモーター			41
	GFP	ゼブラフィッシュ	E2で胚を処理すると受精後25時間で脳に蛍光が観察された	フランス Univ. de Rennes			cyp19a1bプロモーター			42
	EGFP	メダカ	メダカ卵巣には2種類の蛍膜細胞があることを示した	日本 基礎生物学研			cyp19a1プロモーター			43
	myeroperoxidase遺伝子	ゼブラフィッシュ	oxazoloneを投与して全腸炎を誘導して炎症を評価した	オランダ Erasmus Medical Center						44
	EGFP	ゼブラフィッシュ	イントロンを入れる、環状プラスミドを使うと発現が強くなる	シンガポール Genome Institute of Singapore	顕微注入		krt4プロモーター			45
	myostatinアンチセンス	ゼブラフィッシュ	筋肉が2倍になった	台湾 Natl Taiwan Univ.	顕微注入					46
	GFP、troponin Cアンチセンス	ゼブラフィッシュ	アンチセンスRNAをコンディショナルに発現させて遅延型心筋症のモデル動物を作った	台湾 Natl. Taiwan Univ. Hospital			cardiac myosin light chain 2 プロモーター			47
	ヒトsurvival motor neuron (smn)	ゼブラフィッシュ	smnの機能を調べた	アメリカ Ohio State Univ.			ゼブラフィッシュhb9プロモーター			48
	GFP、GAL4	ゼブラフィッシュ	初期胚でのGFPの発現は限定される	シンガポール Natl Univ. of Singapore			UAS、ゼブラフィッシュhsp70プロモーター			49
	DsRed	ゼブラフィッシュ	赤血球の発生における状態と血液の形成の進行を非侵襲的に調べた	オーストリア Inst. of Zoology			GATA-1			50

表2　非食用GM魚を作成した研究報告（続き）

区分	導入遺伝子	組換えタンパクの発現状況等	研究・開発国	遺伝子組換え法	ベクター	プロモーター	ターミネーター	マーカー	備考	文献
研究用	EGFP、リバーステトラサイクリン制御性トランス活性化因子	doxycyclineによって発現誘導できる	韓国 Catholic Univ. of Daegu School of Medicine		レトロウイルス	テトラサイクリンによって誘導がかかるプロモーター、PGKプロモーター				1
	EGFP	G2世代の卵管の管状腺で特異的に発現した	韓国 Natl. Inst. of Animal Sciences	stageXでウイルスを感染させた	レンチウイルス	ニワトリオブアルブミンプロモーター				2
	galactosyltransferase 1	magnum細胞のゴルジ分画で活性が検出された	日本 名古屋大		レトロウイルス				ガラクトシル化された組換えタンパクが生産できる	3
新しい遺伝子導入法	EGFP	DMSOグループの1羽で発現した	ブラジル Campus Univ.	N,N-dimethylacetamideまたはDMSOと精子媒介遺伝子転位	pEGFP-N1	CMVプロモーター	SV40ポリA			4
	EGFP	胸の筋肉中から導入遺伝子が検出された	日本 農業生物資源研	sonoporation	pCAG-EGFPac					5
その他	eGFP		韓国 Bio-Organ Research Center	骨髄細胞を雄性生殖細胞に分化転換して精巣に注入した						6
	インフルエンザウイルスのポリメラーゼを阻害するshRNA		イギリス Univ. of Cambridge						鳥インフルエンザを広がりが抑制された	7

表3　非食用GMニワトリを作成した研究報告

区分	導入遺伝子	生体での機能等	研究・開発国	遺伝子組換え法	ベクター	プロモーター	備考	文献
臓器移植用		ブタα1,3GT遺伝子座の1つを破壊した	アメリカ PPL Therapeutics Inc.	相同組換え、体細胞核移植				1
		4頭の健康なα1,3GTダブルノックアウトブタが作られた	アメリカ PPL Therapeutics Inc.					2
	ヒトCD55、CD59、alpha 1,2-fucosyltransferase	CD55、CD59はすべての組織で強く発現した。alpha 1,2-fucosyltransferaseの発現は比較的弱くalphaGalはあまり減少しなかった	オーストラリア St. Vincent's Hospital					3
		alpha-1,3-galactosyltransferase遺伝子をダブルノックアウトした	オーストラリア Univ. of Adelaide	交配、体細胞核移植				4
		ブタα1,3GT遺伝子座をノックアウトし、ヒトCD46遺伝子を導入した		交配、体細胞核移植				5
	水溶性ニト腫瘍壊死因子受容体1-Fc融合遺伝子	血清はヒトTNF-αで刺激されるブタ内皮細胞のケモカイン、E-セレクチンの誘導を阻害した	韓国 Seoul Natl Univ.	体細胞核移植				6

表4　非食用GMブタを作成した研究報告

区分	導入遺伝子	生体での機能等	研究・開発国	遺伝子組換え法	ベクター	プロモーター	備考	文献
研究用		κ軽鎖定常領域をダブルノックアウトした	アメリカ Revivicor Inc.	ジーンターゲッティング、体細胞核移植			治療用ヒトポリクローナル抗体の生産を目指す	7
	tet-controlled transactivator、transactivator response element、ブタCTLA-4Ig、RANKL	doxycyclineの投与量に依存して発現量が変動した				CAGプロモーター	ブタにおける外来遺伝子発現誘導の最初の例	8
	嚢胞性線維症膜貫通コンダクタンス制御因子(F508/F508)	甲状腺上皮においてcAMPに刺激されるCl電流を媒介する	アメリカ Kansas State Univ. College of Veterinary Medicine					9
		染色体の相互転移が検出された	スペイン Univ. de Murcia					10
	GFP	遺伝子導入サイトの近傍で転写の低下、DNAの高度なメチル化、ヒストンH3、H4のアセチル化の欠損が観察された	中国 College of Life Science	体細胞核移植				11
	4種類の蛍光タンパク遺伝子	ウイルスの2Aペプチドを利用して4種類の蛍光タンパクを共発現させた	中国 Chinese Academy of Sciences	トランスフェクション、体細胞核移植				12
	GFP	胎児線維芽細胞をtrichostatin Aと5-Aza-2'-deoxycytidineで処理してサイレンシングされた外来遺伝子の発現再活性化させた	中国 Northeast Agricultural Univ. of China			CMVプロモーター		13
	EGFP	胚で発現した	中国 Southern Medical Univ.	リポソーム、体細胞核移植	pOGN2	ブタOct-4プロモーター		14
	APOBEC3G、YFP-Cre	Creリコンビナーゼまたは遺伝的分離によって選抜マーカーを除去した	アメリカ Univ. of Minnesota	前核注入			Sleeping Beautyトランスポゾン系を使用	15
	ヒトカタラーゼ、eGFP	へその緒の内皮細胞においてmRNAとタンパクの発現が確認された	アメリカ Univ. of Missouri	トランスフェクション、核移植		Tie2プロモーター		16
	EGFP	生殖細胞、胚盤胞の内部細胞塊と栄養外肺葉でのみ発現した	ドイツ Friedrich-Loeffler-Inst.	体細胞核移植		Oct4プロモーター		17
	ヒトhemo-oxygenase-1、CD39、CD73	3つの導入遺伝子を含む胚盤胞を1ステップで作った	イタリア Univ. of Milano-Bicocca	精子に媒介される遺伝子転移				18
	ヒトDAF、ヒトTFPIのK1、K2ドメインとヒトCD34のD3、D4ドメインの融合遺伝子	50%ヒト血清の存在下で耳細胞の80%以上が生存した	韓国 Inje Univ.	体細胞核移植		PCMVIEプロモーター		19
	合成した脂肪酸不飽和化酵素-1	mRNAの発現を確認した	中国 Chinese Academy of Agricultural Sciences	リポソーム、体細胞核移植				20
新しい遺伝子導入法	EGFP	前者の遺伝子導入法で4分の1の胎児がトランスジェニックだった	日本 明治大	細胞質間精子注入を介した遺伝子転位、前核への顕微注入				21
		発現は1年以上続いた	ドイツ Friedrich-Loeffler-Inst.	受精卵へ顕微注入			Sleeping Beautyトランスポゾン系を評価した	22
病態モデル	ヒトApoCIII	肝臓と腸で発現した。血漿中でトリグリセリド濃度が上昇した	中国 Jilin Univ.他	トランスフェクション、体細胞核移植				23
	elongation of very long chain fatty acids-4 gene(5bp除去、270ストップ変異)	網膜の応答が低下した	アメリカ North Carolina State Univ.	前核への顕微注入と体細胞核移植			Stargardt-like macular dystrophyタイプ3の病態モデルを作った	24

表4　非食用GMブタを作成した研究報告

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Masuda, K., Kajikawa, A., and Igimi, S.	Establishment and evaluation of an in vitro M cell model using C2BBe1 cells and Raji cells.	Bioscience and Microflora.	30	37-44	2011
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Establishment and Evaluation of an *in vitro* M Cell Model using C2BBe1 Cells and Raji Cells

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***In vitro* M cell models, consisting of co-cultures of Caco-2 cells and lymphoid cells, were developed and examined to observe bacterial transport. However, under our experimental conditions, the differentiation of Caco-2 cells into M cell-like cells could not be induced efficiently. To obtain a functionally stable M cell model based on human cells, C2BBe1 cells were screened and co-cultured with human Raji cells. In our co-cultures, increased sialyl Lewis A antigen expression and decreased *Ulex europeaus* agglutinin 1 binding were observed. Regarding the functional properties of the model, microsphere and lactic acid bacteria transport across the C2BBe1 co-cultures were increased compared with the levels seen in monocultures. The C2BBe1 monolayers that were co-cultured with Raji cells exhibited some M cell features; therefore, we consider our M cell model to be useful for investigating the interactions of bacteria with M cells.**

Key words: M cell; C2BBe1; Raji; co-culture

INTRODUCTION

Immunization via the oral route offers several important advantages. In particular, unlike parenteral routes, specific immune responses to vaccine antigen are induced in the mucosa *(1)*. Therefore, a variety of oral vaccines that were generated from genetically modified bacteria have been reported *(2)*. We generated recombinant lactic acid bacteria (LAB) for use in an oral vaccine. These recombinants induced protective immunity and exhibited adjuvant properties *(3, 4)*. However, no practical oral vaccines that have used LAB as an antigen delivery vehicle have been established. The first step in the induction of protective intestinal immune responses is the uptake and transport of antigens to gut-associated lymphoid tissue (GALT). Hence, it is thought that efficient recombinant LAB transport to immuno-competent cells is necessary for effective vaccination.

It is generally thought that M cells, which are located in the follicle-associated epithelium (FAE) of Peyer's patch, play a major role in the uptake of luminal antigens *(5)*. M cells have a characteristic morphology and different functions compared with other intestinal enterocytes. M cells lack a well-organized brush border, have a thick glycocalyx, and display low levels of digestive enzymes, such as alkaline phosphatase and sucrase-isomaltase *(6–9)*. In addition, M cells have intraepithelial pockets containing lymphocytes, macrophages, and dendritic cells. The antigens internalized by M cells are transferred to these underlying immune cells, and antigen-specific immune responses are initiated *(10)*. Therefore, it is considered that these processes are key triggers of the induction of intestinal mucosal immunity. In addition, M cells are targeted by invasive pathogens, which exploit their uptake mechanisms to gain access to the body *(11)*. However, the uptake mechanisms of M cells are little known except for those of a few pathogens such as *Yersinia* and type-I-piliated bacteria *(12, 13)*. Due to the low number of M cells in the human intestine and the difficulty in culturing M cells, the characterization of M cells including their antigen uptake mechanisms has not advanced very far in *in vivo* or *in vitro* studies *(14, 15)*.

The human colon carcinoma cell line Caco-2 is widely used as a model of intestinal epithelial cells in studies of bacterial adhesion, invasion, and drug absorption *(16–18)*. In 1997, Kernéis et al. co-cultured Caco-2 cells with isolated murine Peyer's patch lymphocytes and proposed an *in vitro* human FAE model *(19)*. In this model, Caco-2 cells showed similar features to M cells, such as apical microvilli disorganization, the disappearance digestive enzymes, and the ability to transport microspheres and *Vibrio*. Based on this model, a human intestinal M cell model was established using co-cultures of Caco-2 cells and human Raji B cells instead of murine cells *(20)*. Subsequently, further M cell models with improved culture conditions have been developed, for example

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using Caco-2 subclones, and used to evaluate the transport of several pathogens and proteins *(21–23)*.

C2BBe1 cells were cloned from Caco-2 cells, and this cell line shows a more homogeneous brush border expression than the parental Caco-2 cells *(24)*. C2BBe1 cells have also been co-cultured with murine Peyer's patch lymphocytes in order to establish an M cell model *(25)*. In this study, to obtain a functionally stable M cell model based on human cells, we attempted to establish an M cell model by co-culturing C2BBe1 cells with Raji B cells.

MATERIALS AND METHODS

Bacterial strain and culture

Recombinant *Lactobacillus casei* IGM393 harboring pLPEmpty was grown in MRS broth (Difco) containing 5 μg/ml of erythromycin at 37 °C *(3)*.

Cell culture conditions

C2BBe1 cells were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10 % fetal bovine serum (FBS; JRD), 1 × Glutamax I (Gibco BRL), 1 × nonessential amino acids (Gibco BRL), penicillin (100 U /ml), and streptomycin (100 μg/ml) (Gibco BRL). The human Burkitt's lymphoma cell line Raji (RCB1647) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. The Raji cells were cultivated in RPMI1640 (Sigma) supplemented with 10 % FBS, 1 × nonessential amino acids, 1 × Glutamax I, penicillin (100 U /ml), and streptomycin (100 μg/ml) (Gibco BRL). All cells were grown in a humidified 5 % CO_2 atmosphere at 37 °C.

Induction of M cell features in C2BBe1 cells co-cultured with Raji cells

The induction of M cells from C2BBe1 cells was performed according to the methods of Corr et al. *(25)*. C2BBe1 cells were seeded (1×10^5 cells) onto transwell membranes (12-mm membrane diameter, 3.0-μm pore size, Corning) and cultured until they had fully differentiated. The medium was changed every 2 days. The transepithelial electrical resistance (TEER) of the C2BBe1 cells was measured with a Millicell-ERS (MILLIPORE) to confirm their differentiation and the integrity of the monolayer. After the TEER value of the C2BBe1 monolayer had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartment (Fig. 1). The co-cultures were maintained for 3–6 days. The upper medium was changed every day.

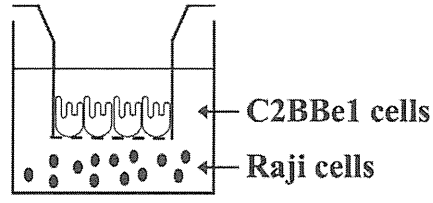

Fig. 1. Schematic of C2BBe1 cell and Raji cell co-culture model. Raji cells were added to basolateral side of C2BBe1 cell monolayers and co-cultured for 3–5 days.

Immunofluorescence

For immunofluorescence microscopy, samples were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS. After fixation, the samples were quenched with 50 mM NH_4Cl for 10 min and washed with PBS. The samples were then blocked with 2% bovine serum albumin in PBS for 60 min, before being incubated with a sialyl Lewis A (SLAA) antibody, β1 integrin antibody, or FITC-conjugated *Ulex europeaus* (UEA-1) for 60 min at room temperature. Then, the samples were washed and incubated for 60 min with an Alexa Fluor 488 goat anti-mouse IgG1 antibody (dilution: 1:2000). Transwell membranes were removed with a scalpel and mounted on glass slides. The slides were observed by fluorescence microscopy (Biozero; KEYENCE), and the stained area was measured by imageJ software *(26)*.

Fluorescent microsphere transport

Microsphere transport was observed in Hank's Balanced Salt Solution (HBSS) buffered to pH 7.4. After equilibration, the HBSS on the donor side was replaced with 500 μl of prewarmed microsphere suspension. The number of particles transported across the cell monolayer was then quantified in a Fluorescent Activated Cell Scan (FACScan, Becton-Dickinson).

Measurement of Lactobacillus casei IGM393 transport

L. casei IGM393 that had been cultured overnight were collected by centrifugation, washed three times with PBS, and resuspended in DMEM, before the bacterial cell concentrations were adjusted to 2×10^8 CFU/ml. A 500-μl volume of the bacterial suspension was added to the apical side of the C2BBe1 monolayers and incubated for 3 h. The basolateral media were then sampled and spread onto MRS-agar plates to estimate the number of colony-forming units.

Statistical analysis

Data were evaluated with Student's t-test and p values of less than 0.01 were considered statistically significant.

RESULTS

Monitoring the transepithelial electrical resistance of C2BBe1 monolayers during growth on a transwell membrane

The differentiation of C2BBe1 cells and the integrity of the monolayers were confirmed by measuring their transepithelial electrical resistance. The TEER values of the C2BBe1 cells had reached 300 $\Omega \times cm^2$ at 21 days (Fig. 2). After the C2BBe1 cells had been co-cultured with Raji cells, the TEER values of the co-cultures were similar to those of the monocultures (Fig. 3).

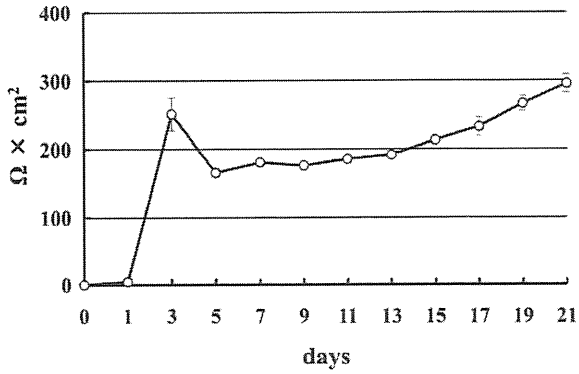

Fig. 2. TEER values of C2BBe1 monolayers grown on transwell membranes.

Expression of M cell markers

To investigate the effects on the C2BBe1 monolayer of co-culture with Raji cells, the expression levels of characteristic phenotypic markers of human M cells were examined. The expression of SLAA was increased by approximately 3-fold in the co-cultures compared with the monocultures (Fig. 4), and the binding of UEA-1 was decreased in apical membrane of the co-cultures (Fig. 5). There was no clear difference in the apical localization of β1 integrin in the C2BBe1 monolayers between the monoculture and co-culture conditions.

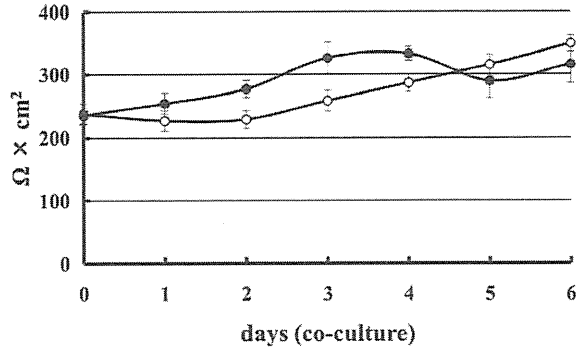

Fig. 3. Comparison of the TEER values of C2BBe1 monocultures and co-cultures. After the TEER values of the monolayer had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartment. The TEER values of co-cultures were measured everyday (closed circles). Monocultures of C2BBe1 monolayers were used as controls (open circles).

Transport of fluorescent microspheres

In order to confirm that the C2BBe1 cells had acquired M cell functional features, the number of transported fluorescent microspheres was measured. The transport of particles was increased 100-fold in the co-cultures compared to the C2BBe1 monocultures (Fig. 6).

Quantification of L. casei IGM393 transport across C2BBe1 monolayers

The ability of the *in vitro* M cell model to translocate *L. casei* IGM393 was examined. *L. casei* IGM393 were added to the apical side of the C2BBe1 monolayers. The C2BBe1 monolayers cultured with Raji cells had transported 10^3 CFU *L. casei* IGM393 after 3 h incubation at 37°C (Fig. 7). On the other hand, little bacterial transport was observed in the C2BBe1 monolayers cultured alone.

DISCUSSION

Observations of the internalization of the bacteria into non-phagocytic cells have mainly been performed using epithelial cell monolayers. However, in the intestine, a number of bacteria invade the host through M cells, and the morphology and function of M cells are markedly different from those of epithelial cells. Hence, a simple epithelial cell monolayer is insufficient as an M cell model, and a model system resembling M cells is necessary to observe bacterial internalization *in vitro*.

In vitro M cell models have been generated by co-culturing a variety of Caco-2 subclones with mouse Peyer's patch or human B cells. We attempted to establish an M cell model using Caco-2 cells in a preliminary study. However, as the Caco-2 monolayer was unstable during co-culture, we found it difficult to establish an M cell model using this technique. Therefore, Caco-2 clones were screened to see if they could be used to produce a stable model.

C2BBe1 cells form a polarized monolayer with an

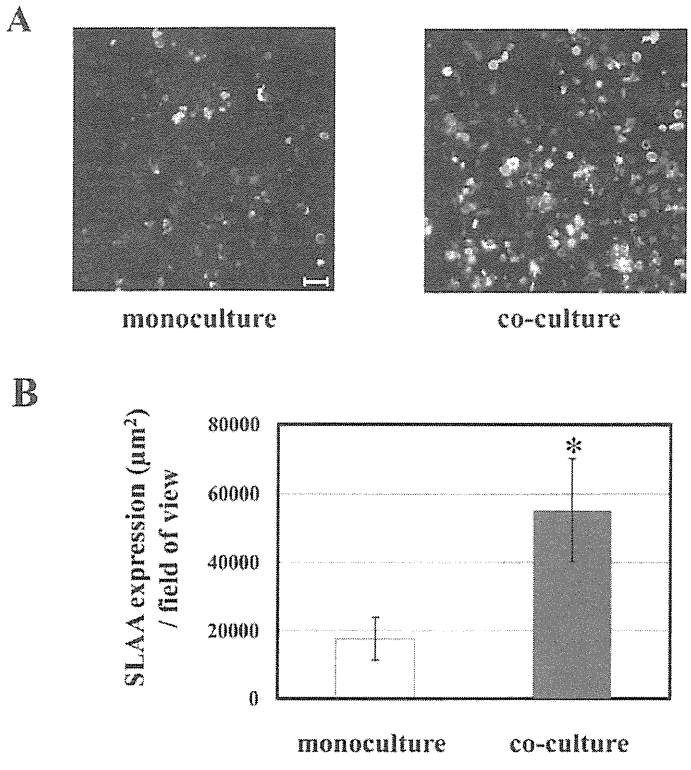

Fig. 4. Observation and quantification of sialyl Lewis A antigen expression. A) Immunohistochemistry of SLAA expression in C2BBe1 monocultures and co-cultures was observed by fluorescence microscopy. Scale bar: 50 μm. B) SLAA expression in the apical membranes of co-cultures compared with that observed in monocultures.

apical brush border that is morphologically comparable with that of the human colon and have been used to investigate bacterial adhesion and invasion *(27, 28)*. In the present study, we investigated whether human Raji B cells can induce C2BBe1 cells to differentiate into M cell-like cells.

First, the TEER values of C2BBe1 cells cultured on transwell membranes were measured as an indicator of cell monolayer integrity because we consider careful monitoring to be important for the establishment of a stable and reproducible model *(29)*. The TEER value increased rapidly within 3 days of the cells being seeded on the transwell membranes and gradually increased thereafter (Fig. 2). The C2BBe1 cells grew slowly and more stably over the long-term than other Caco-2 clones (data not shown). After the TEER value had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartments of the C2BBe1 monolayers. Monolayers of other Caco-2 clones could not be used because the TEER values of their co-cultures were extremely low, and the integrity of the differentiated monolayers was lost (data not shown). On the other hand, the TEER values of C2BBe1 co-cultures were between 250 and 300 $\Omega \times cm^2$, which was similar to that of the C2BBe1 monocultures (Fig. 3). The reduction in the Caco-2 cell co-culture TEER has been suggested to be due to the conversion of Caco-2 cells into M cells, whereas the C2BBe1 co-cultures seemed to maintain their integrity *(21)*.

In order to investigate the effects of Raji cells on C2BBe1 monolayers, the expression of M cell markers was examined. Several M cell markers have been reported, and in our experiment we observed that the apical expression of SLAA was significantly increased in co-cultures compared to monocultures (Fig. 4). The binding of UEA-1, which is a mouse and rabbit M cell marker, was decreased in the apical membranes of the co-cultures (Fig. 5). These results were also observed in a number of human M cell models *(20, 30)*. On the other hand, we were not able to find clear differences in the localization of β1 integrin between the co-cultures and

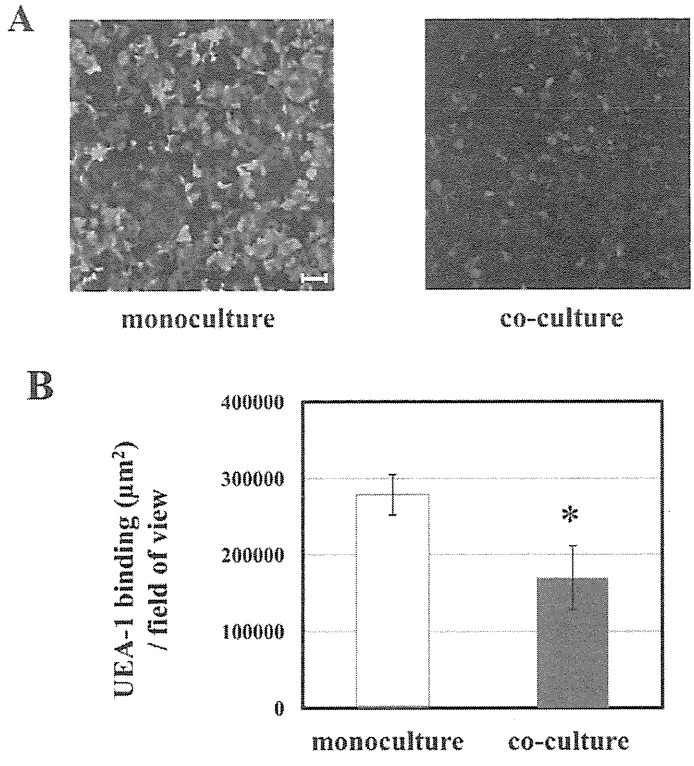

Fig. 5. Binding of UEA-1 lectin to C2BBe1 cells in monocultures and co-cultures. A) The binding of UEA-1 conjugated with FITC to C2BBe1 cell monolayers was observed by fluorescence microscopy. Scale bar: 50 μm. B) UEA-1 binding in C2BBe1 co-cultures compared with that observed in monocultures

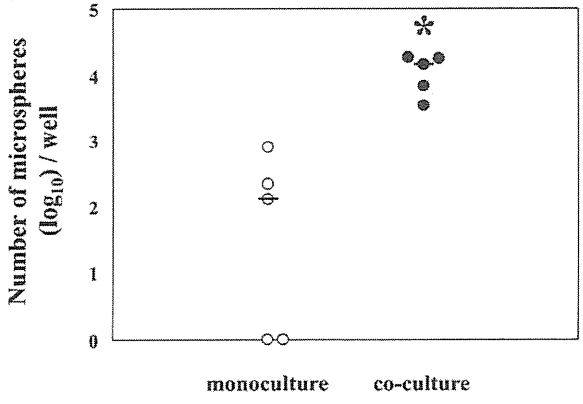

Fig. 6. Transport of microspheres across C2BBe1 monocultures and co-cultures. Mono- and co-cultures were incubated with microspheres for 3 hr at 37°C. The number of transported microspheres was evaluated by FACS.

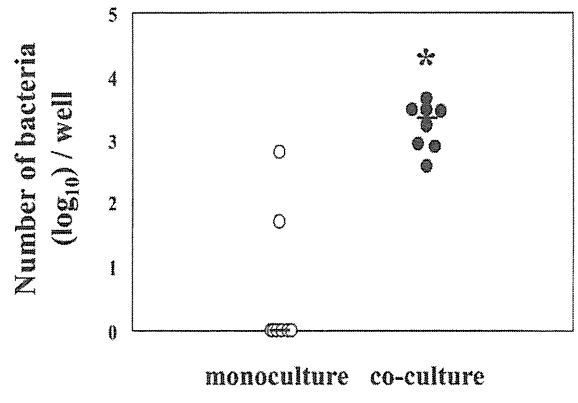

Fig. 7. Transport of *Lactobacillus casei* IGM393 across C2BBe1 monocultures and co-cultures. Mono- and co-cultures were incubated with *L. casei* IGM393 for 3 hr at 37°C. The number of transported bacteria was measured by CFU.

mono cultures.

Furthermore, to investigate the transport function of our model, microsphere transport was examined in both the co-cultures and monocultures. The number of transported particles was significantly increased in the co-cultures (Fig. 6). Before and after the particle transport

assay, the TEER values of each monolayer were not change, indicating that the integrity of the monolayers was maintained during the transport assay. Increased particle transport is a typical feature of M cell models *(20, 31)*. These results suggest that Raji cells induce C2BBe1 cell differentiation.

Finally, to investigate whether the co-cultures are able to transport non-invasive bacteria, we observed *L. casei* IGM393 transport across the monolayers. The number of transported *L. casei* was significantly increased in the basolateral compartments of the co-cultures compared to those of the monocultures (Fig. 7). Consequently, it was shown that the co-cultures were capable of incorporating even non-pathogenic and non-invasive lactic acid bacteria. However, in a few monocultures, a similar level of *L. casei* transport was found. It was reported that the differentiation of Caco-2 cells into M cell like-cells occurred without lymphocyte treatment, and a similar phenomenon was also seen in our experiment *(22)*.

Recently, it has been suggested that the induction of M cell features in Caco-2 monolayers is mediated by direct contact between Caco-2 and Raji cells, soluble factors such as those found in the Raji cell culture supernatant, and/or macrophage migration inhibitory factor (MIF) *(21, 30, 32)*. However, neither the Raji cell culture supernatant nor MIF efficiently induced the differentiation of C2BBe1 monolayers in our experiment. The differences in the results between the above studies and ours might have been due to the different Caco-2 subclones and culture conditions used including differences in the FBS used. At the very least, the presence of Raji cells is important in our C2BBe1 model.

C2BBe1 cells co-cultured with murine Peyer's patch lymphocytes showed M cell-like features such as disordered apical membrane brush borders and bacterial transport *(25)*. That model was constructed with an established human cell culture and primary mouse cells which were isolated from mouse Peyer's patch. On the other hand, our model was based entirely on established human cell lines. Hence, it is thought that our model is a homologous co-culture like the *in vitro* human M cell model compared with previous murine Peyer's patch model reported by Corr et al. in 2006 (*25*).

However, the LAB transport efficiency of our model is lower than that of the murine Peyer's patch model. This difference might be due to the induction efficiency of C2BBe1 differentiation during co-culture because Peyer's patches contain a variety of immunocompetent cells. To obtain an efficient differentiation model, improvements in the culture conditions such as ensuring the close contact of C2BBe1 cells and Raji cells will be necessary *(21)*. Alternatively, there might be differences between the abilities of *L. salivarius* and *L. casei* to adhere to intestinal epithelial cells and Peyer's patch cells *(33, 34)*. However, it remains to be determined whether the uptake of LAB by M cells is a specific or non-specific response.

In this study, to establish a more homologous co-culture model using C2BBe1 cells, C2BBe1 cells were co-cultured with Raji B cells. We demonstrated that Raji cells induced C2BBe1 cells to differentiate in a manner similar to Caco-2 cells that had been co-cultured with murine Peyer's patch cells and the cells used in a number of other *in vitro* M cell models. Therefore, we consider that our C2BBe1 co-cultured model is a useful M cell model. As the interactions between M cells and LAB are poorly understood, investigations of these interactions would help to elucidate the mechanisms of immunostimulation by lactic acid bacteria. Furthermore, our M cell model might contribute not only to examinations of the factors that affect the adhesion and uptake of lactic acid bacteria by M cells but also to studies selecting M cell targeted bacterial strains as vehicles for mucosal vaccine delivery.

ACKNOWLEDGMENT

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