図０９

小括

1. HTLV-1感染細胞株を用いてHTLV-1感染系を構築した。

2. 日赤献血由来、HTLV-1抗体陽性血清でスクリーニングを行った。抗体陽性**献血者のPVL%が高く、env, gagの力価が高い抗体が感染抑制効果が高かった**。中にはPVL%が低いが感染抑制が認められる検体もあった。Pep180力価との相関は認められなかった。

3. シンシチウム形成を抑制する系を立ち上げた。30種類のHTLV-1陽性血漿を用いてスクリーニングを行った結果、**PVL%が高く、env, gagの力価が高い抗体がシンシチウム形成抑制効果が高かった**。中には、献血者のPVL%が低くても、シンシチウム形成抑制能が強い検体が認めらた。Pep180力価との相関は認められなかった。

4. 高力価グロブリン製剤作製の候補検体は、無症候性キャリアのうち、**PVL%が高い検体を候補とするのがよい**。

5. その他に注目する群が存在した。

9

図９：小括

図１０

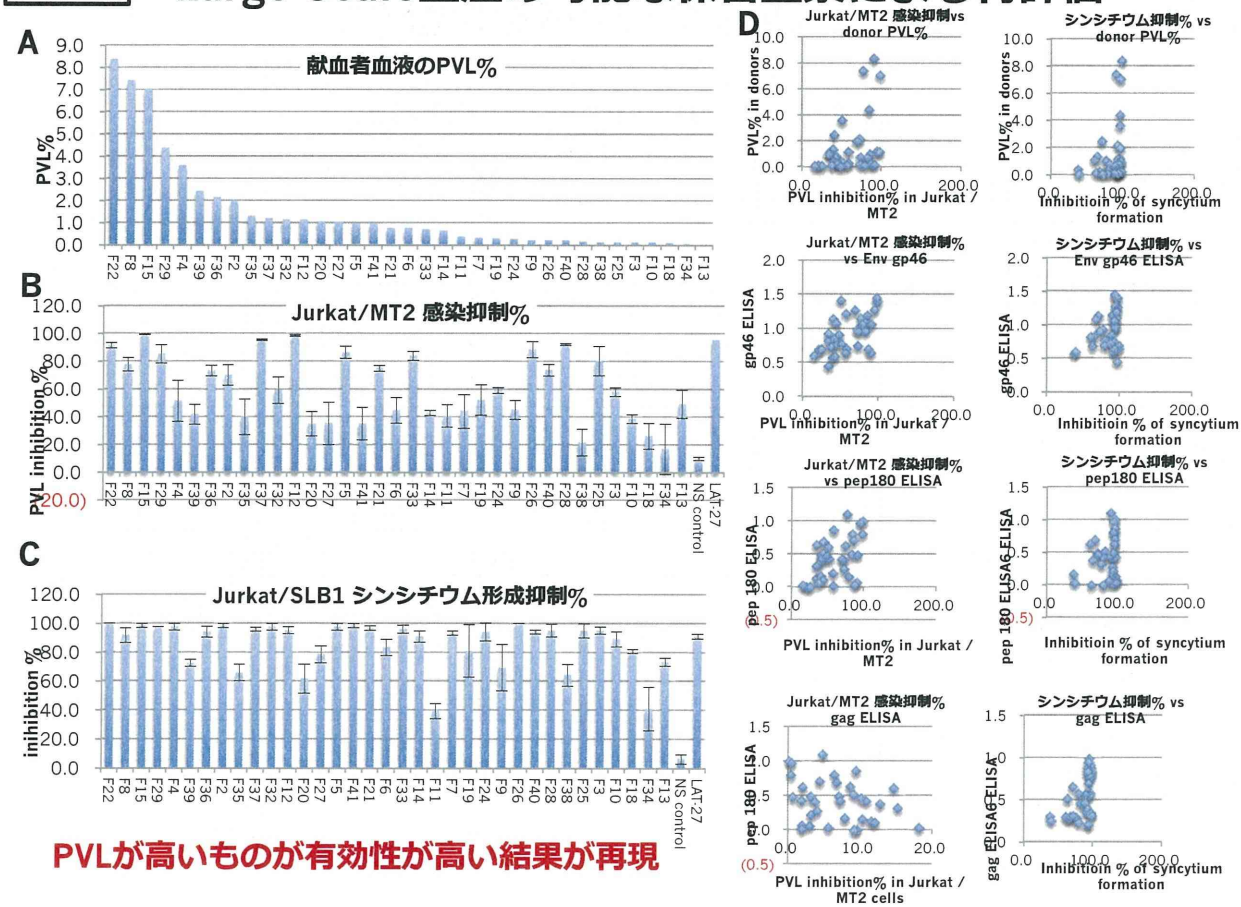
Large Scale生産の可能な保管血漿

ID	年齢	性別	CLEIA (Cutoff index)		WB (プロブロットHTLV-1)					Proviral load (%)	Peptide ELISA			syncytium形成阻害%		PVL抑制 %	
			CL4800	レミバル	判定結果	gp46	p53	p24	p19		env (OD)	pep180 (OD)	gag (OD)	%	sd	%	sd
1	52	F	10.0	N.T.	+	+	+	+	+	8.36	0.633	0.062	0.816	100.00	0.00	91.51	2.06
2	47	M	10.0	35.9	+	+	+	+	+	7.39	1.138	1.096	0.356	91.69	5.19	77.67	4.91
3	40	M	10.0	45.0	+	+	+	+	+	7.04	1.387	0.991	0.872	98.34	1.44	99.56	0.24
4	29	F	10.0	N.T.	+	+	+	+	+	4.37	1.092	0.795	0.808	97.51	0.00	85.17	6.51
5	36	F	10.0	45.0	+	+	+	+	+	3.57	1.408	0.607	0.578	97.51	2.49	51.48	14.79
6	28	M	10.0	N.T.	+	+	+	+	+	2.43	1.131	0.483	0.650	72.57	2.49	41.71	7.17
7	35	M	10.0	N.T.	+	+	+	+	+	2.13	0.979	0.408	0.386	94.18	3.81	73.16	3.80
8	57	F	10.0	N.T.	+	+	+	+	+	1.95	1.260	0.420	0.345	98.34	1.44	70.17	7.34
9	59	M	10.0	N.T.	+	+	+	+	+	1.31	0.816	0.375	0.272	65.92	5.76	40.02	12.88
10	47	F	10.0	45.0	+	+	+	+	+	1.17	1.268	0.964	0.986	95.84	1.44	95.54	0.57
11	43	F	10.0	45.0	+	+	+	+	+	1.13	1.211	0.861	0.759	97.51	2.49	58.95	9.50
12	51	F	10.0	N.T.	+	+	+	+	+	1.12	1.443	0.799	0.803	95.01	2.49	98.66	0.52
13	47	F	10.0	N.T.	+	+	+	+	+	1.05	0.817	0.627	0.295	61.76	10.08	35.04	8.70
14	35	F	10.0	N.T.	+	+	+	+	+	1.01	0.919	0.311	0.279	78.39	5.76	35.35	15.31
15	44	M	10.0	45.0	+	+	+	+	+	0.96	1.181	0.701	0.905	97.51	2.49	86.52	4.49
16	47	M	10.0	13.8	+	+	+	+	+	0.94	0.441	0.124	0.333	98.34	1.44	35.13	11.69
17	56	M	10.0	45.0	+	+	+	+	+	0.75	0.952	0.620	0.548	96.67	1.44	75.04	2.11
18	51	F	10.0	34.1	+	+	+	+	+	0.74	0.763	0.509	0.455	83.37	5.76	44.65	9.25
19	36	F	10.0	45.0	+	+	+	+	+	0.70	0.952	0.486	0.788	95.84	2.88	83.98	3.25
20	24	M	10.0	41.7	+	+	+	+	+	0.64	0.845	0.425	0.563	90.86	3.81	42.85	1.94
21	62	F	10.0	N.T.	+	+	+	+	+	0.36	0.565	0.165	0.299	39.32	5.19	40.72	8.03
22	56	F	10.0	27.3	+	+	+	+	+	0.32	0.585	0.102	0.874	93.35	1.44	44.02	12.17
23	53	M	10.0	17.9	+	+	+	+	+	0.29	0.764	0.420	0.225	80.88	18.04	52.27	10.94
24	39	M	10.0	39.9	+	+	+	+	+	0.27	0.699	0.011	0.830	94.18	6.28	59.09	2.03
25	55	F	10.0	N.T.	+	+	+	+	+	0.21	1.076	0.686	0.298	69.24	16.03	45.07	6.87
26	64	F	10.0	N.T.	+	+	+	+	+	0.21	0.685	0.027	0.310	100.00	0.00	88.50	5.68
27	37	F	10.0	N.T.	+	+	+	+	+	0.21	0.740	0.270	0.197	94.18	1.44	73.76	4.18
28	59	F	10.0	45.0	+	+	+	+	+	0.13	1.059	0.479	0.942	95.01	4.32	92.23	0.70
29	26	M	10.0	38.6	+	+	+	+	+	0.11	0.679	-0.005	0.511	64.26	7.62	21.73	9.71
30	53	F	10.0	N.T.	+	+	+	+	+	0.11	1.055	0.153	0.206	95.01	4.99	80.37	10.45
31	65	M	10.0	45.0	+	+	+	+	+	0.10	0.638	0.209	0.696	95.01	2.49	57.97	3.28
32	35	F	10.0	21.2	+	+	+	+	+	0.10	0.725	0.056	0.461	89.19	5.19	38.61	3.08
33	26	M	10.0	38.0	+	+	+	+	+	0.08	0.684	-0.008	0.569	80.88	1.44	26.12	9.28
34	56	M	10.0	45.0	+	+	+	+	+	0.04	0.590	0.025	0.247	40.98	15.03	16.91	18.27
35	35	F	10.0	N.T.	+	+	+	+	+	0.01	0.905	0.460	0.306	73.40	2.88	49.32	10.25

図１０：Large Scale 生産の可能な保管血漿感染ウイルス量（PVL）の高い血漿、中間のもの、低コピーのものと３つのグレードに分け、さらに各種抗原（p24 (gag:capsid), gp46-21(env), pep180(env)) ELISA 結果と western blot の結果(p19, p24, p53, gp46)を加味した。

図１１

Large Scale生産の可能な保管血漿による再評価

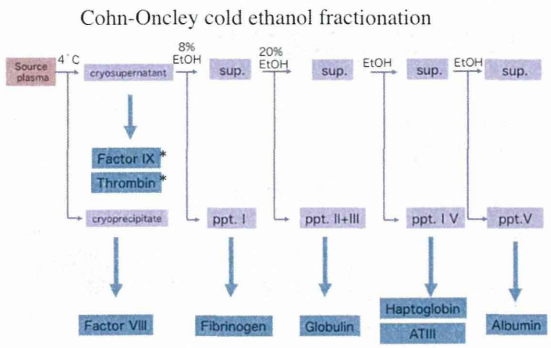


PVLが高いものが有効性が高い結果が再現

図１１：**Large Scale** 生産の可能な保管血漿による再評価　A) Large Scale 生産の可能な保管血漿における、PVL の値　B) それぞれの検体の Jurkat/MT2 感染抑制% C)それぞれの検体の Jurkat/SLB1 シンシチウム形成抑制% D)各種パラメーターとの比較

図１２

血漿からのグロブリンの精製

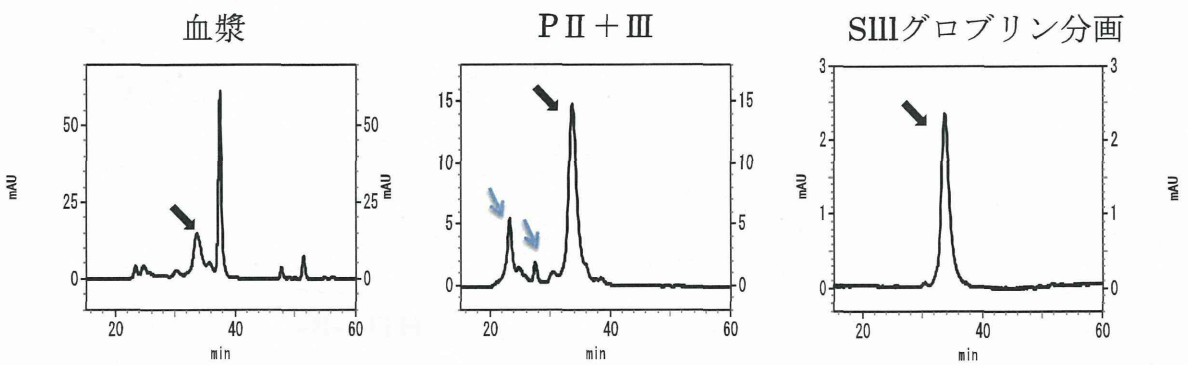


副作用の原因となる重合物の除去

[25%の場合]
Plasma
↓
(8%EtOH,pH7.47 (22.5℃))
↓
SI
↓
(25%EtOH,pH 6.73(22.4℃))
↓
PII+III
↓
(20%EtOH, pH6.66(20.5℃))
↓
PII+III−W (17%EtOH, pH5.48(22.0℃))
↓
SIII

精製効率　　37．4%
グロブリン含量　97．3%

カラム：G3000SWXL 2本
流速：　0.5ml/min
Buffer: 0.05% NaN3, 0.1M $NaSO_4$,
1/7.5M Phosphate buffer (pH7.0)



実験レベルとしては良質の免疫グロブリンの製造に成功した

図１２：血漿からのグロブリンの精製　精製の方法は Louie RE *et al.*,1994 に従い行った。その結果、副作用の芸院となる重合物の除去に成功し、グロブリン含有率 97.3%のサンプルを精製することに成功した

参考文献：Louie RE, Galloway CJ, Dumas ML, Wong MF, Mitra G. Inactivation of hepatitis C virus in low pH intravenous immunoglobulin. ***Biologicals***. 1994; 22: 13-19.

図１３

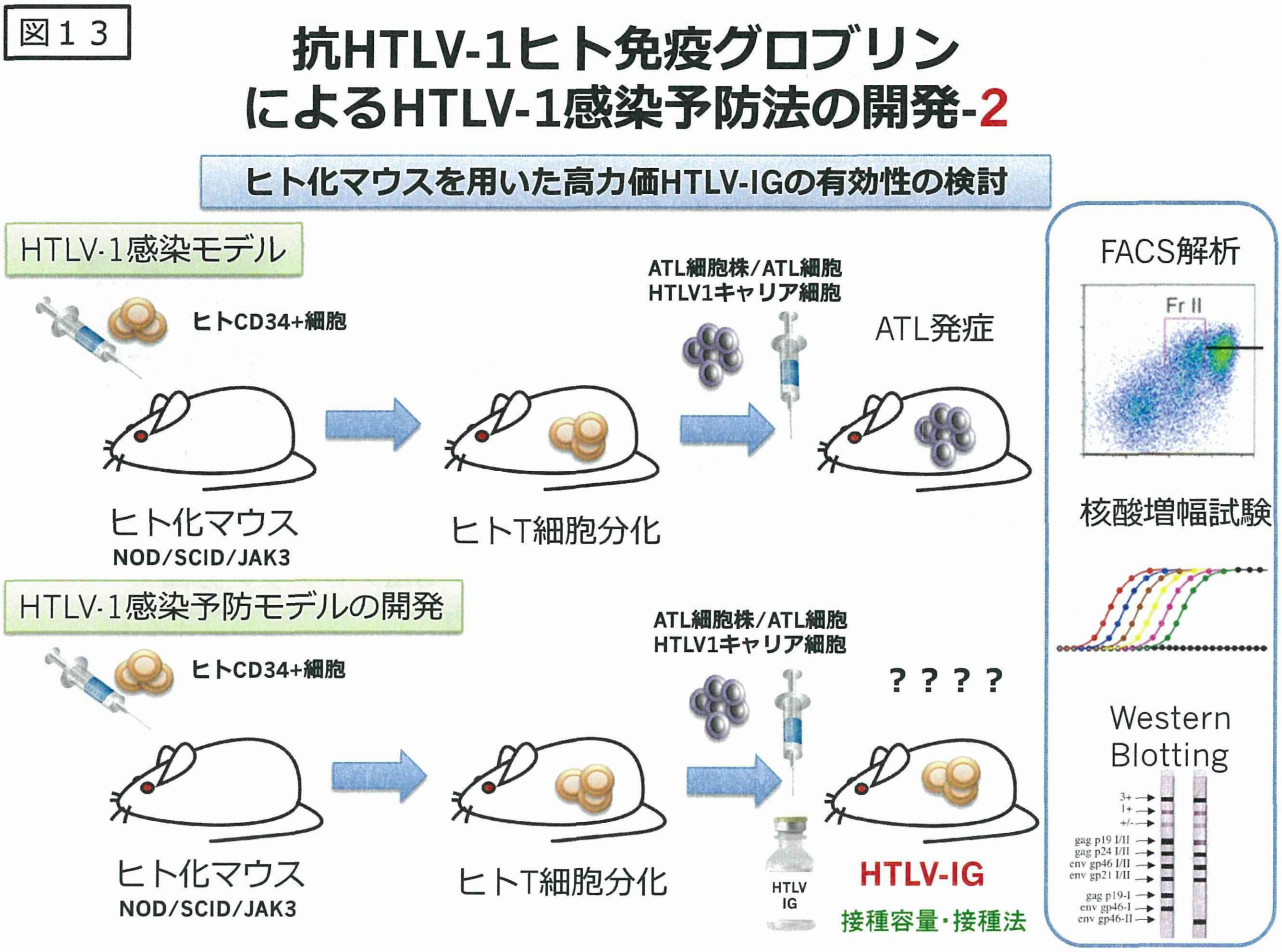


図１３：抗 HTLV-1 ヒト免疫グロブリンによる HTLV-1 感染予防法の開発－２　ヒト化マウスを用い、感染実験系を構築し、免疫グロブリンの有効性を検討する。

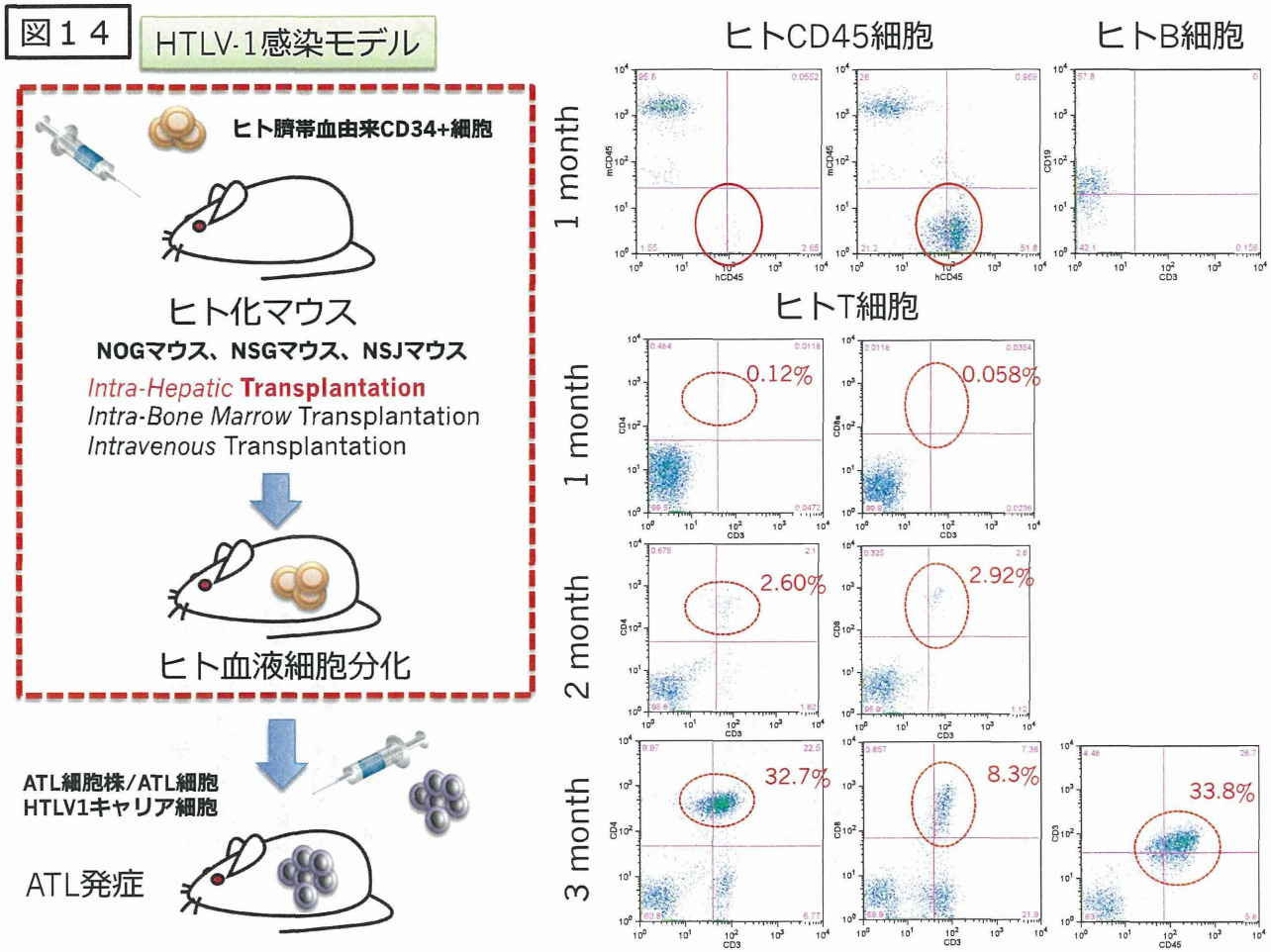


図１４：ヒト化マウスの構築　超免疫不全マウスである NOG マウス (NOD.Cg-Prkdcscid Il2rgtm1Sug/Jic)および NOJ マウス (NOD/SCID/JAK3null)にヒト臍帯血由来 CD34 陽性細胞を intra-Hepatic, Intra-Bone Marrow, Intravenous Transplantation 法で移植し、１ヶ月、２ヶ月、３ヶ月後の抹消血中の human CD45, CD19, CD3, CD4, CD8, CD25 の割合をフローサイトメトリー(JSAN, Baybioscience 社) で調べ、ヒト化マウスを作成した。

図１５

MT-2移植ヒト化NOGマウス

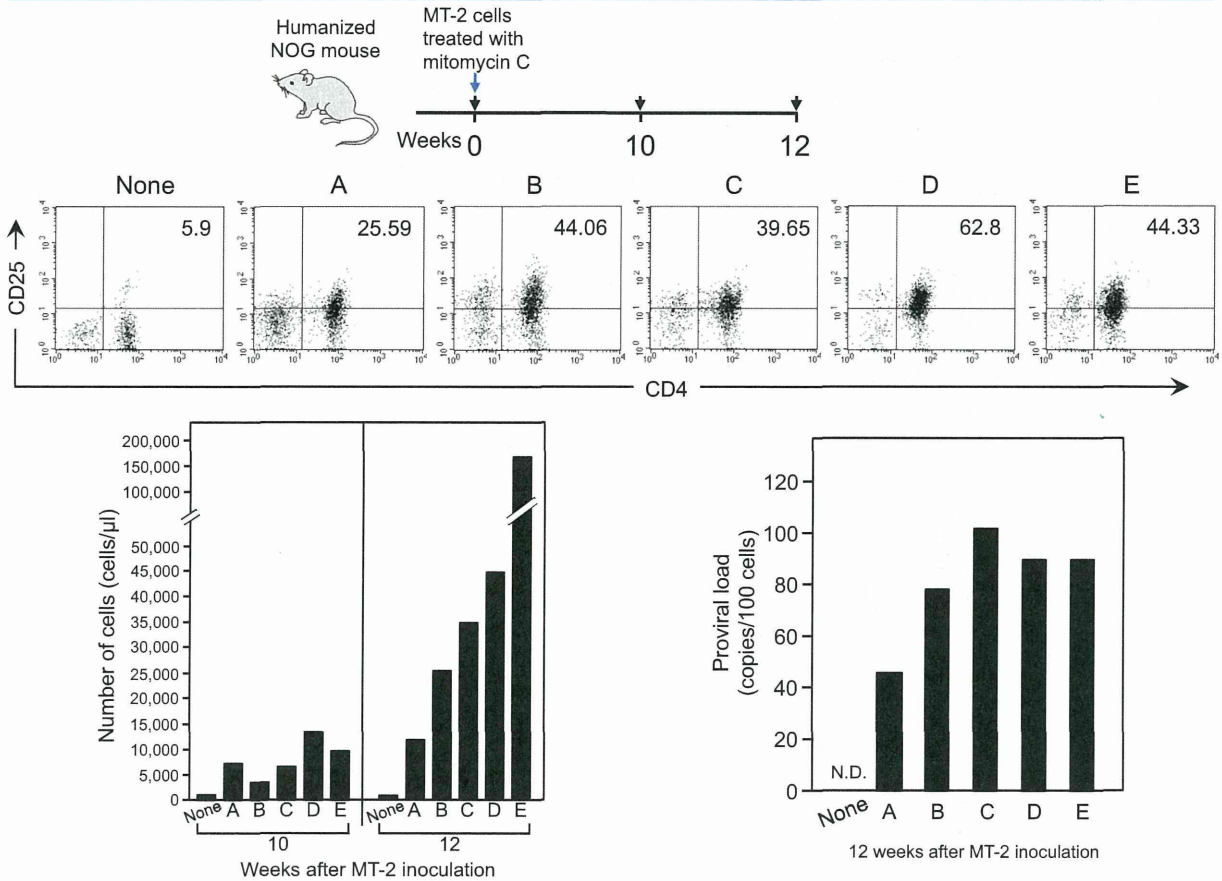


図１５：ヒト化マウスを用いた HTLV-1 感染モデルの作成　これらのヒト化したNOJマウスにMMC処理をしたMT-2を腹腔内に移植し、移植後１２週のマウスの病理解剖を行った。その結果、抹消血中にATL細胞と考えられるCD4陽性CD25陽性細胞の増加が移植したマウス全てで認められた。

図１６

MT-2移植ヒト化NOGマウス

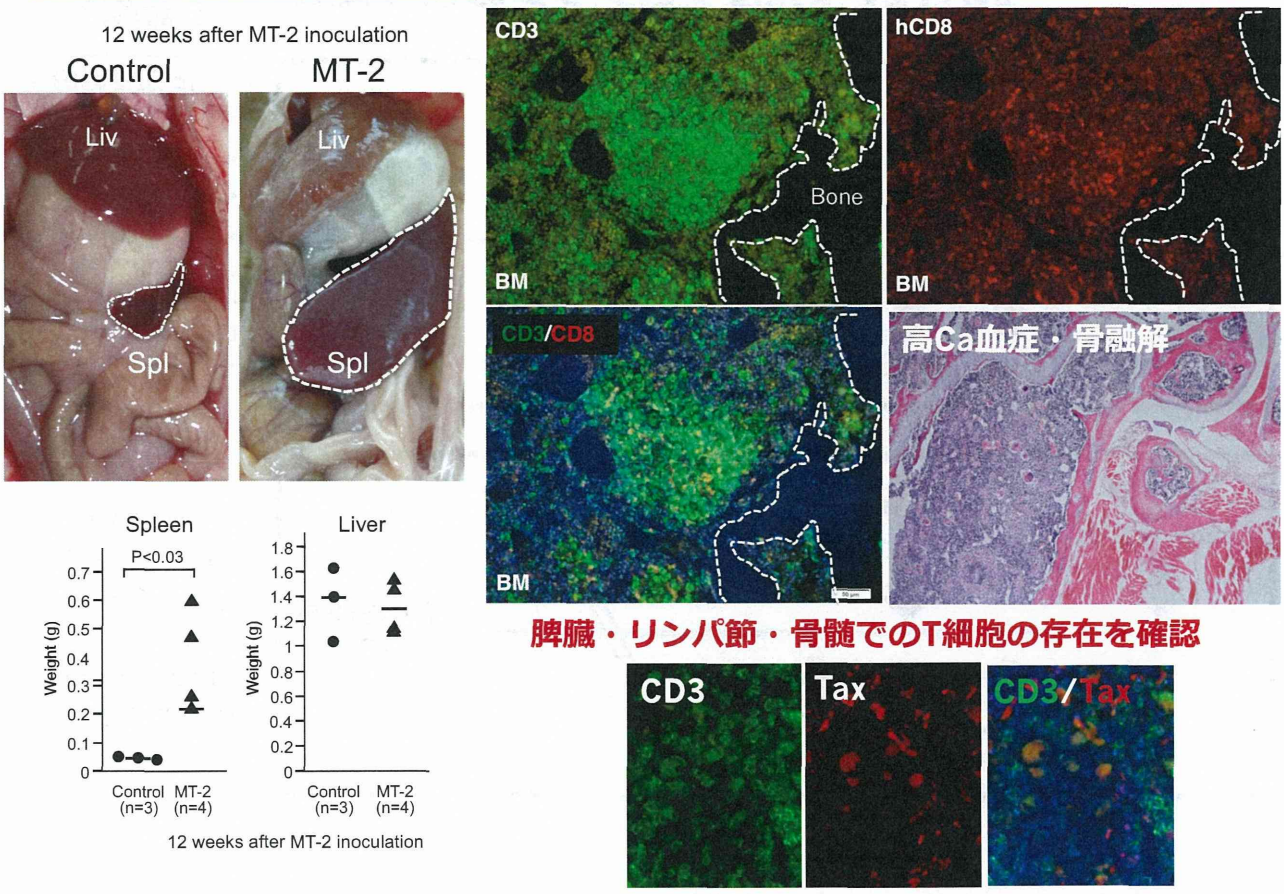


図１６：MT-2 移植ヒト化 NOG マウスの感染病理　MMC 処理 MT2 を移植したマウスでは、巨脾が認められた。また肝臓への浸潤も肉眼レベルで確認できた。脾臓、骨髄、リンパ節、肺、肝臓で多数の ATL 細胞と思われる CD3 陽性細胞が集簇していることが明らかとなった。骨髄では骨梁の融解が認められ、血清中の Ca 濃度が高い事も確認され、HTLV-1 感染により高カルシウム血症が起こっている事が示唆された。

図１７

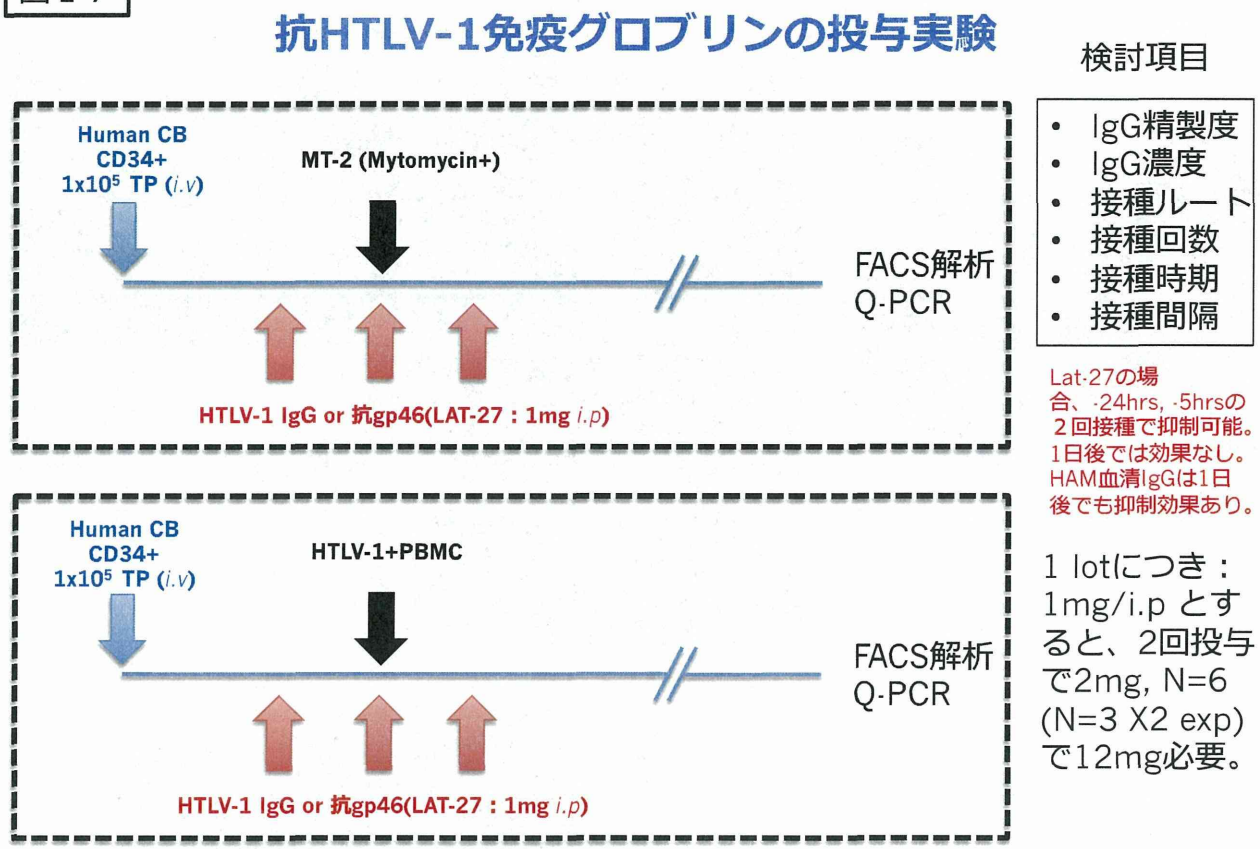


図１７：抗 HTLV-1 免疫グロブリンの投与実験　精製した高力価免疫グロブリンの投与実験を計画し、接種ルート、回数、時期、間隔の検討を開始している。

図１８　本研究の意義と期待される成果・厚生行政への貢献

新規感染予防法	HTLV1感染について、**HTLV-IGの感染阻止に関する有効性**を示す
ヒト臨床へのTRS	ヒト化マウスを用いる事で、ヒト細胞の実際の生体内での感染予防を確認でき、**ヒトへの外挿**が可能となる
新薬開発/国際貢献	HTLV-1抗体陽性血漿は**日本でしか入手できないため**、**高品質のHTLV-IGは日本発で開発・製造**可能。世界的には数千万人のキャリアがおり、**世界的な規模での利用**が期待されている。
厚生行政	HTLVワクチン（H23年度 厚生労働科学研究費　長谷川秀樹主任研究者）が成功すれば、**HTLV-IG+Vaccineとの組み合わせ**でHBVの母子感染予防のような感染予防策が実施可能となり、新規感染は抑制可能となる。
献血の有効利用	貴重な**献血血液の有効利用**に繋がる

研究班

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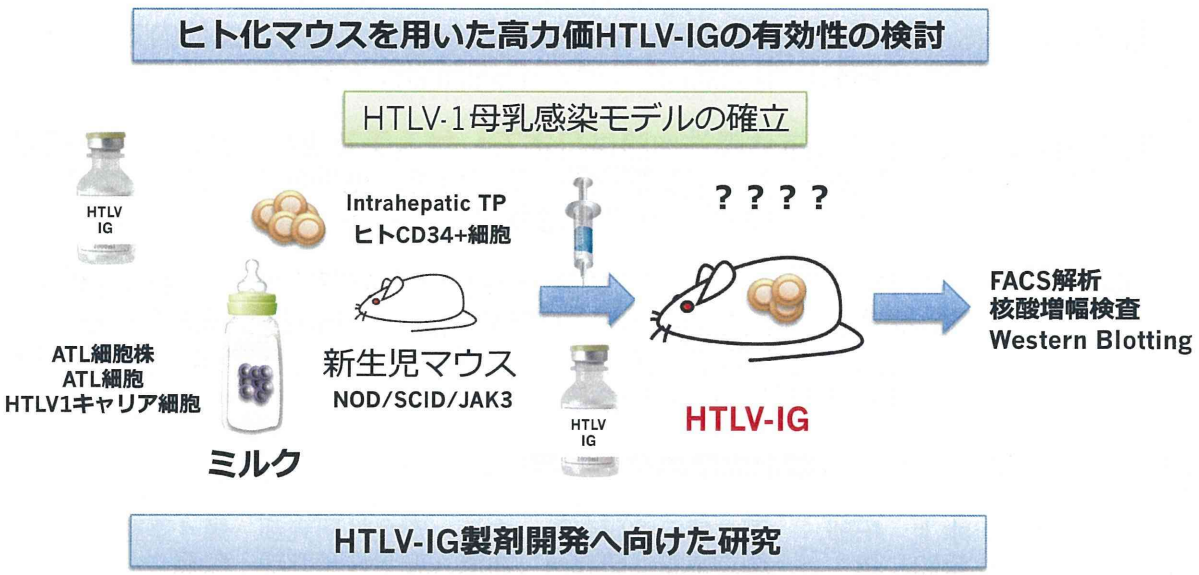
図１８：本研究の意義と期待される成果・厚生行政への貢献

図１９

抗HTLV-1ヒト免疫グロブリン によるHTLV-1感染予防法の開発-3



- HTLV-IGの性状・用法・容量の設定
- 抗HTLV-1ヒト免疫グロブリンの認識抗原の同定
- 製造工程中のウイルス混入リスク解析（核酸増幅試験）
- 製造工程中のウイルスクリアランス
- 国家検定に準じた品質管理試験（安全性・物理化学試験）の実施

図１９：抗 HTLV-1 ヒト免疫グロブリンによる HTLV-1 感染予防法の開発－３　ヒト化マウスの母乳感染モデルを構築することを試み、感染予防ツールとしての免疫グロブリンの有効性を検討する。また製剤化へ向けた品質管理に関わる予備的検討を行う。

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	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	巻号	ページ	出版年
Kasama Y, Mizukami T, Kusunoki H, Peveling-Oberhag J, Nishito Y, Ozawa M, Kohara M,Mizuochi T, Tsukiyama-Kohara K. B-cell-intrinsic	Hepatitis C virus expression leads to B-cell-lymphomagenesis and induction of NF-κB signalling.	***Plos one, accepted***			2014
Tsukasaki K, Imaizumi Y, Tokura Y, Ohshima K, Kawai K, Utsunomiya A, Amano M, Watanabe T, Nakamura S, Iwatsuki K, Kamihira S, Yamaguchi K, Shimoyama M.	Meeting report on the possible proposal of an extranodal primary cutaneous variant in the lymphoma type of adult T-cell leukemia-lymphoma.	***J Dermatol. 2014; 41: 26-28.***	41	26 - 28	2014
Terada C, Mori J, Okazaki H, Satake M, Tadokoro K.	Effects of riboflavin and ultraviolet light treatment on platelet thrombus formation on collagen via integrin αIIbβ3 activation.	***Transfusion. 2014 Feb 8.***			2014
Takizawa K*, Nakashima T*, Mizukami T*, Kuramitsu M, Endoh D, Kawauchi S, Sasaki K, Momose H, Kiba Y, Mizutani T, Furuta RA, Yamaguchi K, Hamaguchi I. *Equally contributed	Degenerate polymerase chain reaction strategy with DNA microarray for detection of multiple and various subtypes of virus during blood screening.	***Transfusion.***	53	2545 - 2555	2013

Odaka C, Kato H, Otsubo H, Takamoto S, Okada Y, Taneichi M, Okuma K, Sagawa K, Hoshi Y, Tasaki T, Fujii Y, Yonemura Y, Iwao N, Tanaka A, Okazaki H, Momose SY, Kitazawa J, Mori H, Matsushita A, Nomura H, Yasoshima H, Ohkusa Y, Yamaguchi K, Hamaguchi I.	Online reporting system for transfusion-related adverse events to enhance recipient haemovigilance in Japan: a pilot study.	***Transfus Apher Sci.***	48	95 - 102	2013
Krayukhina E, Uchiyama S, Nojima K, Okada Y, Hamaguchi I, Fukui K.	Aggregation analysis of pharmaceutical human immunoglobulin preparations using size-exclusion chromatography and analytical ultracentrifugation sedimentation velocity.	***J Biosci Bioeng.***	115	1014 - 110	2013
Ohsugi T, Wakamiya M, Morikawa S, Matsuura K, Kumar JM, Kumasaka T, Yamaguchi K.	Invasion of histiocytic sarcoma into the spinal cord of HTLV-1 tax transgenic mice with HTLV-1-associated myelopathy/tropical spastic paraparesis-like disease.	***Oncol Res.***	20	403 - 410	2013
Asanuma S, Yamagishi M, Kawanami K, Nakano K, Sato-Otsubo A, Muto S, Sanada M, Yamochi T, Kobayashi S, Utsunomiya A, Iwanaga M, Yamaguchi K, Uchimaru K, Ogawa S, Watanabe T.	Adult T-cell leukemia cells are characterized by abnormalities of Helios expression that promote T cell growth.	***Cancer Sci.***	104	1097 - 1106	2013
Furui Y, Satake M, Hoshi Y, Uchida S, Suzuki K, Tadokoro K.	Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors.	***Transfusion.***	53	2190 - 2197	2013
Saito F, Shimazu T, Miyamoto J, Maemura T, Satake M.	Interstitial fluid shifts to plasma compartment during blood donation.	***Transfusion. 2013 Feb 26.***			2013
Taira R, Satake M, Momose S,	Residual risk of	***Transfusion.***	53	1393	2013

Hino S, Suzuki Y, Murokawa H, Uchida S, Tadokoro K.	transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan.			- 1404	

III. 研究成果の刊行物・印刷

Degenerate polymerase chain reaction strategy with DNA microarray for detection of multiple and various subtypes of virus during blood screening

Kazuya Takizawa, Tatsuo Nakashima,* Takuo Mizukami,* Madoka Kuramitsu, Daiji Endoh, Shigeto Kawauchi, Kohsuke Sasaki, Haruka Momose, Yoshiharu Kiba, Tetsuya Mizutani, Rika A. Furuta, Kazunari Yamaguchi, and Isao Hamaguchi*

BACKGROUND: The risk of transferring blood-borne infections during transfusion is continually increasing because of newly emerging and reemerging viruses. Development of a rapid screening method for emerging viruses that might be transmitted by transfusion is required to eliminate such pathogens during blood donor screening. Owing to increased use of human materials in organ transplants and cell therapy, the risk of donor-transmitted viral infections is also increasing. Although nucleic acid amplification technology (NAT) is dedicated to blood screening, a small, convenient detection system is needed at the laboratory and hospital level.

STUDY DESIGN AND METHODS: We developed a new pathogen detection system that can detect multiple viruses simultaneously, using originally designed degenerate polymerase chain reaction primers to amplify a wide range of viral genotypes. Amplified samples were identified using a DNA microarray of pathogen-specific probes.

RESULTS: We detected very low copy numbers of multiple subtypes of viruses, such as human hepatitis C virus (HCV), human hepatitis B virus (HBV), human parvovirus B19 (PVB19), and West Nile virus (WNV), using a single plate. We also detected all genotypes of human immunodeficiency virus (HIV) but sensitivity was less than for the other viruses.

CONCLUSION: We developed a microarray assay using novel primers for detection of a wide range of multiple pathogens and subtypes. Our NAT system was accurate and reliable for detection of HIV, HBV, HCV, PVB19, and WNV, with respect to specificity, sensitivity, and genotype inclusivity. Our system could be customized and extended for emerging pathogens and is suitable as a future NAT system.

Quality and safety in blood products are major public health concerns. In addition to general quality control (QC) testing, introduction of good manufacturing practice and routine screening of blood material and products have assured consistency and quality in production and increased blood transfusion safety in recent decades. Newly developed serologic tests and nucleic acid technology (NAT) have markedly reduced the risk of transmitting human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) from infected blood.[1] Currently, several Food and Drug Administration (FDA)-licensed NAT assays are available to screen blood donors for HIV, HCV, HBV, and West Nile virus (WNV). However,

ABBREVIATIONS: DLC-chip = diamond-like carbon-coating microarray chip; dPCR = degenerate polymerase chain reaction; IC = internal control; NIBSC = National Institute for Biological Standards and Control; OE-PCR = overlap-extension polymerase chain reaction; PVB19 = parvovirus B19; TMA = transcription-mediated amplification; WNV = West Nile virus.

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TABLE 1. Pathogen-specific dPCR primers for microarray detection*

Pathogens	Primer sequence (5′-3′)	GenBank Accession Number	Position (NT)	Gene name	Amplicons (bp)
HIV	RARAGGGGGGATTGGGGGGTA	NC_001802	4336-4356	Integrase	129
	YTGTCYCTGWAATAAACCCGA		4444-4464		
HCV	GAAAGCGYCTAGCCATGGCGT	D90208	59-327	5′-UTR	269
	TGCACGGTCTACGAGACCTCC		307-327		
HBV	AYTAYCAAGGTATGTTGCCCG	X70185	450-470	S	266
	GGAAAGCCCKRCGMACCACTG		695-715		
PVB19	AGTGGTGGTGAAAGCTCTGAA	NC_000883	2148-2168	NS1	122
	TCTCCTGAACTGGTCCCG		2252-2269		
WNV	GGHTGTTGGTATGGNATGGA	NC_009942	3451-3590	NS1	141
	CTCCTGGGTGRCCAAGAAC		3573-3591	NS2A	
IC	TCGAAGACGATCAGATACCGT	M10098	1147-1157	18S rRNA	129
	ATACTCCCCCCGGAACC		1259-1275		

* Code base description: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; B, C/G/T; N, A/T/C/G.

the continuous development of a highly sensitive screening system is a challenging task for NAT. The focus is mainly on assay sensitivity rather than the range and diversity of viral species detected; therefore, the current NAT systems only ensure detection of a restricted range of viruses and their subtypes and not newly diverged, emerging, or reemerging viruses.[2] It has been reported that the NAT sensitivity for HCV detection differs slightly in relation to virus subtype.[3] Recent advances in organ transplantation and cell therapy have also increased the risk of donor-transmitted viral infections, such as cytomegalovirus, Epstein–Barr virus, WNV, and lymphocytic choriomeningitis virus.[4] Further development of multiple virus detection systems is required to increase coverage of a range of virus strains and subtypes. We have experienced pandemics, such as WNV in the United States in 2003 and the chikungunya virus on Reunion Island in 2006; thus, there is a need to develop a rapid virus detection system that uses a more flexible blood-testing platform and meets the safety requirements for transfusion.

HBV is one of the most geographically widespread viruses and is subdivided into eight main genotypes (A–H),[5,6] causing liver cirrhosis and hepatocellular carcinoma. Although most infectious blood units are removed by screening for hepatitis B surface antigen (HBsAg), there is clear evidence that transmission by HBsAg-negative components occurs during the serologically negative window period and late stages of infection.[7] In addition to the window period of infection, HBV blood screening is required to detect all virus genotypes. Similar to HBV, several false-negative results in minipool NAT screening were reported after the introduction of WNV NAT because of the low viral load. Moreover, WNV continues to diverge rapidly from the originally isolated strain.[8,9] Multiplex NAT assays have become the modern method for detecting several viruses, and in conjunction with automated systems, they have the potential to improve processes that ensure blood safety. Candotti and colleagues[10] have reported the feasibility of a multiplex real-time quantitative reverse-transcriptase polymerase chain reaction (PCR) for HBV, HCV, and HIV-1, suggesting that simultaneous amplification of multiple pathogens is an effective approach for improving pathogen detection methods. The flexibility provided by multiplex PCR is limited, however, because the PCR primers are designed in commonly preserved regions of the viral genomes. To increase the detectable range for multiple pathogens, PCR using degenerate primers has been developed. Recently, bioinformatics has strongly improved the design of degenerate primers, allowing the coverage of a wide range of virus subtypes. We have developed a new method for designing degenerate primers.[11] Here, we used the diamond-like carbon-coating microarray chip (DLC-chip) to reduce background noise and increase the detection sensitivity of the system.[12,13] We combined two newly developed technology platforms for a multiple pathogen detection system using a degenerate PCR–based NAT system (dPCR-NAT).

MATERIALS AND METHODS

Design of dPCR primers and microarray oligoprobes

We designed dPCR primers that hybridized with HIV, HCV, HBV, human parvovirus B19 (PVB19), and WNV genomic sequences (Table 1). We used the CoCoMo (Coordination of Common Motifs) algorithm (www.geneknot.info/cocomo)[14] for dPCR primer design. CoCoMo determines primer regions in commonly conserved nucleotide regions in the assembled nucleotide sequences of virus strains. In each case, all viral sequences were identified from GenBank and EMBL, and a low degeneracy primer set was selected as a candidate using the CoCoMo algorithm. We collected data on 2072 HIV-1 nucleotide sequences and selected 14 complete genome sequences that corresponded to each genotype of HIV-1. We designed dPCR primer sets for detecting 14 complete genome sequences, resulting in approximately 3897

TABLE 2. Oligonucleotide probe sequences of DNA microarray assay

Virus	Probe name	Sequence (5′-3′)	Tm (°C)*
HIV	IR1-1	ACTATTCTTTCCCCTGCACTGTACCCCCCAATCC	78
	IR1-2	TCTGTTGCTATTATGTCTACTATTCTTTCCCC	66
	IR1-3	CTTTAGTTTGTATGTCTGTTGCTATTATGTCTAC	63
	IR1-4	GTAATTTGTTTTTGTAATTCTTTAGTTTGTATGTCTG	66
	IR3-1	GGGATTGTAGGGAATTCCAAATTCCTGCTTGATT	76
	IR3-3	CTTTAATTCTTTATTCATAGATTCTACTACTCCTTGACTTTG	69
HCV	CF1-1	AACCGGTGAGTACACCGGAATTGCCAGGAC	77
	CF1-2	TTTCTTGGATCAACCCGCTCAATGCCTGGAGATTTGGGCG	88
	CF1-3	TGCCCCCGCAAGACTGCTAGCCGAGTAGTGTTGGG	85
	CF2-1	AGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGA	86
	CF2-2	CTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTG	81
	CF2-3	GCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCT	82
	CR2-1	TCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCT	86
HBV	BF4-1	CTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGG	75
	BF4-2	CTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCCTC	78
	BF4-3	TGTCCTCTAATTCCAGGATCAACAACAACCAGTAC	73
	BF4-4	ATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACC	84
	BF4-5	CCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTC	84
	BF4-6	GTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAG	80
	BR4-1	CCAGAAGAACCAACAAGAAGATGAGGCATAGCAG	75
PVB19	PVB19F-1	GGCGCCTGGAACACTGAGACCCCGCGCTCTAGTAC	85
	PVB19F-2	GGCGCCTGGAACACTGAAACCCCGCGCTCTAGTAC	84
	PVB19F-3	GAACTCAGTGAAAGCAGCTTTTTCAACCTCATCACTCC	78
	PVB19R-1	GTACTAGAGCGCGGGGTCTCAGTGTTCCAGGCGCC	85
	PVB19R-2	GTACTAGAGCGCGGGGTTTCAGTGTTCCAGGCGCC	84
	PVB19R-3	GGAGTGATGAGGTTGAAAAAGCTGCTTTCACTGAGTTC	78
WNV	WNVF-1	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-2	ATGATTGACCCTTTTCAGTTGGGCCTTCTGGTCG	80
	WNVF-3	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-4	ACGCCGACATGATTGATCCTTTTCAGTTGGGCCT	81
	WNVR-1	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-2	CGACCAGAAGGCCCAACTGAAAAGGGTCAATCAT	80
	WNVR-3	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-4	AGGCCCAACTGAAAAGGATCAATCATGTCGGCGT	81
IC	IC-1	GTCGTAGTTCCGACCATAAACGATGCCGACCGG	81
	IC-2	GGCGATGCGGCGGCGTTATTCCCATGACCC	86
	IC-3	CCGCCGGGCAGCTTCCGGGAAACCAAAGTCTTTG	87
	IC-4	TCGAAGACGATCAGATACCGTCGTAGTTCCGACC	78
QC	QC	TTGGCAGAAGCTATGAAACGATATGGG	69

* The melting temperature (Tm) was calculated using NetPrimer (PREMIER Biosoft International, http://www.premierbiosoft.com/).

primer sets. For HCV, we collected 978 sequences and selected 167 complete genome sequences to design dPCR primer sets, generating 31 primer sets. For HBV, 1461 sequences were collected and 1344 complete genome sequences were selected to generate the dPCR primer sets, generating approximately 29 primer sets. For WNV, we collected 17,172 sequences and used 111 complete genome sequences to design the dPCR primer sets, generating 1649 primer sets. For PVB19, we collected 1145 sequences and selected seven complete genome sequences to design the dPCR primer sets, generating 2517 primer sets. Selected primers are listed in Table 1. The sequences of the oligonucleotide detection probes on the DLC-chip are indicated in Table 2. The probes were manually designed from regions amplified by the degenerate primers. Thirty to 42 oligomers that had a GC content between 50 and 60% were selected. The resultant melting temperature values were 62 to 88°C. The hybridization stability of the PCR fragments was biased according to strand; therefore, we designed probes on each strand of the PCR products (sense strand—same sense as forward primer).

Synthesis of genotype panel oligomers for screening primers

Genotype panel oligomers of HIV-1, HBV, PVB19, and WNV were prepared by overlap-extension PCR (OE-PCR;[15] Fig. 1A). Regions for OE-PCR were selected according to the nucleotide sequences amplified with our primers for each virus genotype. The nucleotide sequences of HBV subtypes B and C panel oligomers had the same sequence. The joining oligonucleotides listed in Supplemental Table S1 (available as supporting information in the online version of this paper) were designed using DNA works (http://helixweb.nih.gov/dnaworks/).[16] The nucleotide sequences of each genotype panel oligomers are listed in Supplemental Table S2 (available as supporting information in the online version of this paper). OE-PCR

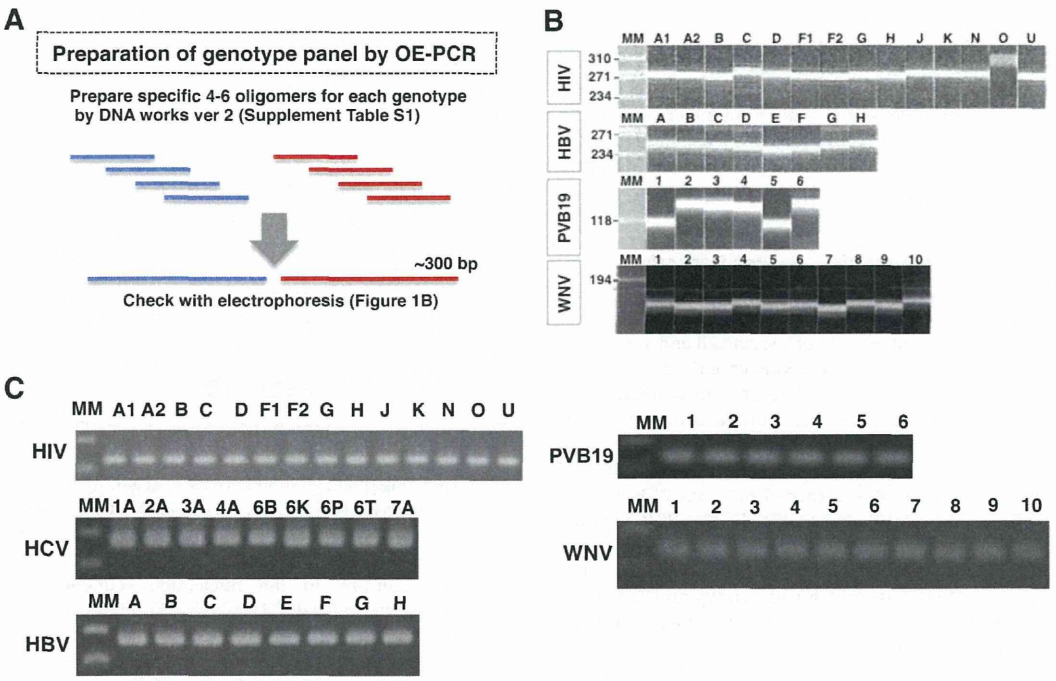

Fig. 1. Synthesis of genotype panel oligomers for HIV, HCV, HBV, PVB19, and WNV. (A) Preparation of genotype panel oligomers by PCR-based gene synthesis, OE-PCR. dPCR primers were validated with genotype panel oligomers. (B) Genotype panel oligomers were synthesized against 14 subtypes of HIV-1, eight genotypes of HBV, nine genotypes of HCV, six genotypes of PVB19, and 10 genotypes of WNV. (C) Each of the HIV-1 subtype oligomers was amplified by dPCR primers for HIV-1. The top panel shows the agarose gel electrophoresis analysis of PCR products to identify the 14 HIV-1 subtypes. Similar results were obtained from HCV, HBV, PVB19, and WNV genotype panel oligomers with specific dPCR.

was carried out according to a two-step reaction method by using a PCR kit (Prime Star, Takara-bio, Otsu, Japan; Fig. 1). The first reaction was carried out using a mixture of OE-PCR oligomers in 30 cycles of 98°C for 15 seconds, 55°C for 10 seconds, and 72°C for 15 seconds. One microliter of the first PCR products was transferred to the second PCR solution, which included 5 pmol/μL each of the 5′- and 3′-end primers. The second reaction consisted of 30 cycles at the temperature conditions used in the first reaction. The molecular weights of the OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu, Kyoto, Japan; Fig. 1B). HCV genotype panel oligomers were synthesized and obtained from Invitrogen (Carlsbad, CA; custom DNA oligonucleotide synthesis service).

Viral samples for dPCR-based NAT

For more accurate analysis, we purchased PVB19 NAT-based assays genotype panel (First International Standard; Category Number, 09/110 National Institute for Biological Standards and Control [NIBSC], UK); HIV-1 RNA genotype panel (Category Number 08/358 NIBSC); HCV RNA genotype panel (Category Number 08/264 NIBSC); HCV for NAT (Fourth WHO International Standard; Category Number 06/102 NIBSC); HBV for NAT (Third WHO International Standard; Category Number 10/264 NIBSC); and PVB19 DNA NAT assays (Second International Standard; Category Number 99/802 NIBSC). To evaluate the specificity of our dPCR-NAT system, we diluted each genotype panel with defibrinated plasma (Basematrix 53, SeraCare BBI Diagnostics, Milford, MA) to give a final concentration of 151 to 9722 copies/mL (HIV), 500 to 1500 IU/mL (HCV), 5754 to 123,027 IU/mL (HBV), and 870,964 to 954,933 IU/mL (PVB19), respectively. To evaluate the sensitivity of our dPCR-NAT system, we diluted each international standard with defibrinated plasma to give a final concentration of 1 to 10,000 IU/mL. Samples containing the New York strain of WNV RNA (NY 2001-6263; NATtrol, Category Number NATWNV-0005,