図4

HTLV-1陽性血漿30検体の性状解析

ID	Ab CL (COI)	Ab IF	provirus pX PVL (%)	Ab: プロブロットHTLV-1 (WB)					Ab: ELISA (OD)		
				p19	p24	p53	gp46	判定	p24(gag:capsid)	gp46-21(env)	pep180(env)
1	10	+	4.05	+	+	+	+	+	0.75605	0.88165	0.7462
2	10	+	4.11	+	+	+	+	+	0.79675	1.01025	0.8351
3	10	+	4.15	+	+	+	+	+	0.4602	1.06715	1.0436
4	10	+	4.16	+	+	+	+	+	1.21425	1.4073	0.8726
5	10	+	4.46	+	+	+	+	+	0.7252	0.99485	0.64805
6	10	+	4.50	+	+	+	+	+	0.72005	0.90995	0.35475
7	10	+	4.63	+	+	+	+	+	0.50625	0.72675	0.534
8	10	+	4.71	+	+	+	+	+	0.6374	0.5533	0.58585
9	10	+	4.93	+	+	+	+	+	0.557	0.66775	0.70095
10	10	+	5.00	+	+	+	−	?	1.29805	0.56185	0.06805
11	10	+	0.49	+	+	+	+	+	0.4	0.712	0.734
12	10	+	0.57	+	+	+	+	+	0.818	0.691	0.4555
13	10	+	0.61	+	+	+	+	+	0.708	0.8495	0.844
14	10	+	0.67	+	w+	w+	w+	+	0.0195	0.237	0.5055
15	8.8	+	0.79	+	w+	w+	−	?	0.2225	0.092	0.208
16	10	+	0.83	+	+	+	+	+	0.954	0.8815	1.25
17	10	+	0.90	+	+	+	w+	+	1.292	0.5765	0.009
18	10	+	1.10	+	+	+	+	+	0.19615	0.69255	0.71025
19	10	+	1.29	+	+	+	+	+	0.52795	0.7661	0.6331
20	10	+	1.39	+	+	+	+	+	0.95955	1.3135	0.8492
21	10	+	N.D.	+	+	+	−	?	0.05155	0.3895	0.0255
22	10	+	0.0003	w+	−	w+	w+	+	0.1305	0.553	0.3302
23	10	+	0.001	+	+	+	+	+	0.37445	0.67735	0.28295
24	10	+	0.002	+	+	+	±	?	0.0763	0.38335	0.2356
25	8.1	+	0.002	+	+	+	−	?	0.15695	0.44215	0.08495
26	9.5	+	0.01	+	w+	+	w+	+	0.22865	0.56825	0.02205
27	10	+	0.05	+	+	+	+	+	0.06615	0.659	0.4204
28	10	+	0.06	+	+	+	w+	+	0.0776	0.57475	0.00965
29	9.7	+	0.06	+	w+	w+	±	?	0.19255	0.41745	0.1353
30	10	+	0.07	+	+	+	+	+	0.8089	1.02475	0.62505

図 4：本研究課題の予備実験で用いた日本赤十字社の HTLV-1 陽性血漿のリスト。PVL 値、Westernblotting の結果、ELISA の結果をまとめた。

図5　HTLV-1陽性血漿の感染阻害効果 (*in vitro*モデル-1)

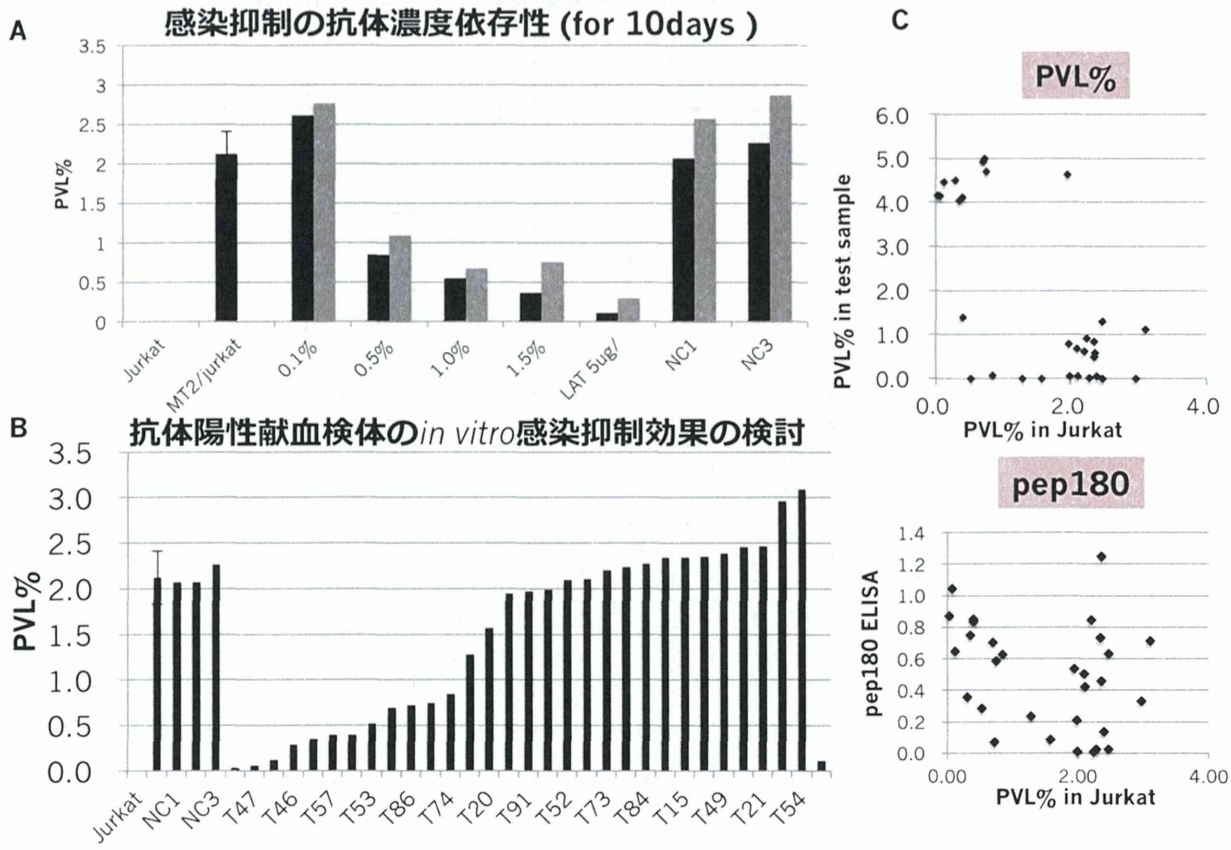


図 5：培養細胞を用いた in vitro 感染モデルの作成 1 。A) 感染抑制の濃度依存性の検討　0.5%が至適濃度であることが明らかとなった　B) 抗体陽性献血検体の in vitro 感染抑制効果の検討 C) 各種パラメーターとの相関関係

図6　HTLV-1陽性血漿の感染阻害効果 (*in vitro*モデル-2)

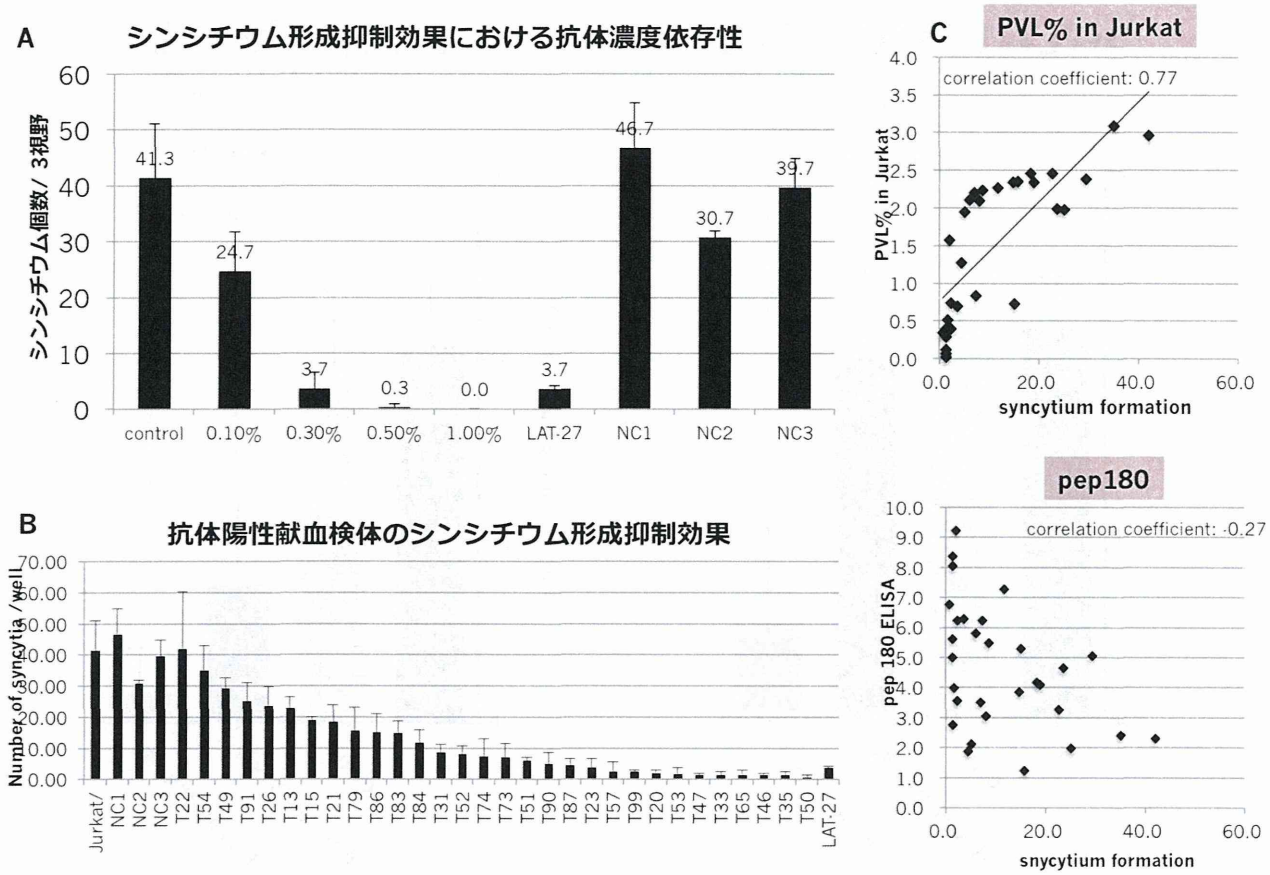


図 6 : A) 抗体による Syncytium の抑制。Jurkat 細胞と SLB-1 を共培養するとシンシチウムが形成される。この時、抗体陽性血漿を添加するとシンシチウムの形成は抑制される。シンシチウム形成抑制効果における A) 抗体濃度依存性の検討を行った。B)抗体陽性献血検体のシンシチウム形成抑制効果　C) 各種パラメーターとの相関関係。

図7　HTLV-1陽性血漿の感染阻害結果 (*in vitro*モデル1&2)

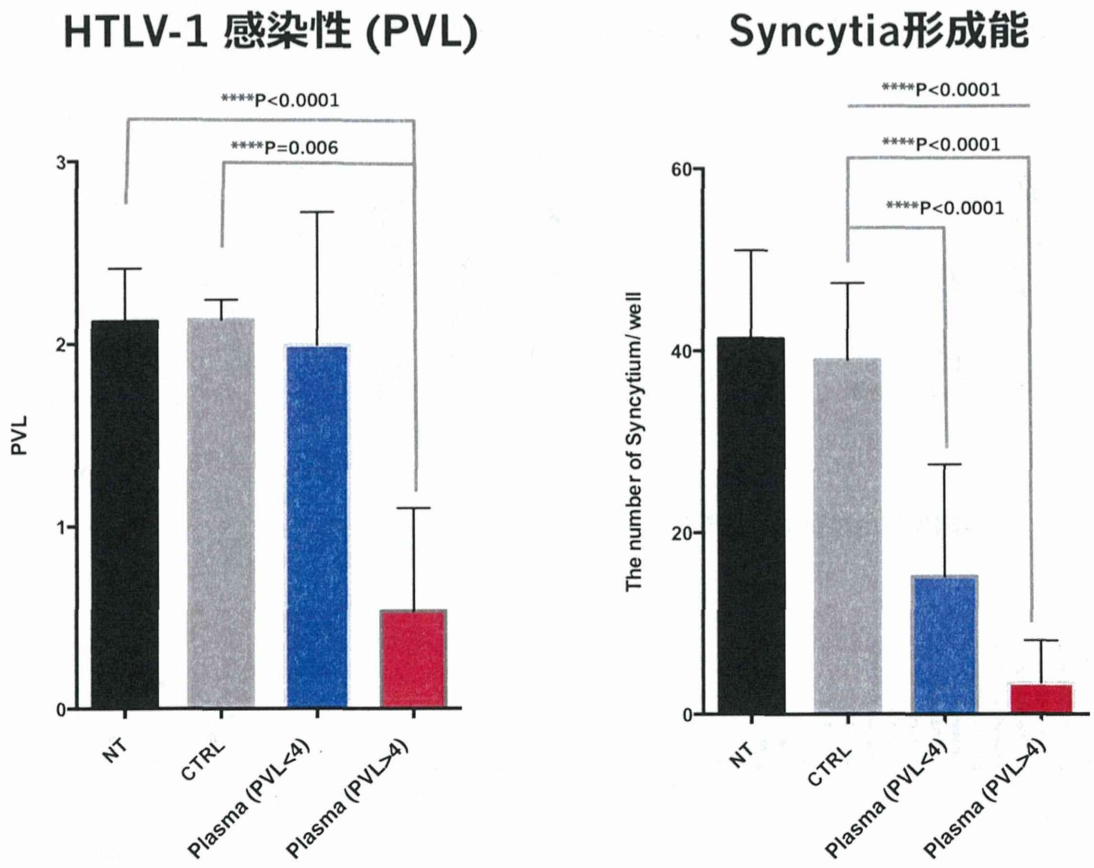
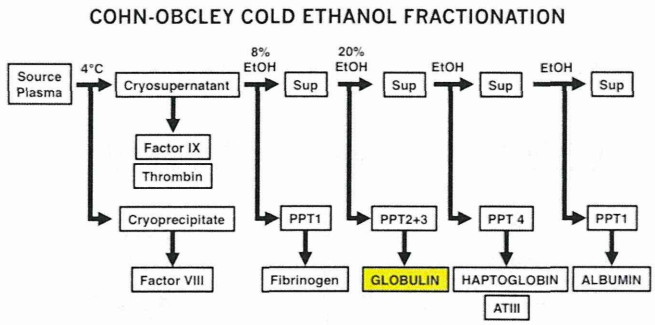


図 7：HTLV-1 陽性血漿の感染阻害効果。 PVL が４％以上のサンプルはウイルス感染価・シンシチウム形成を有意に阻害する 左) MT-2 と Jurkat 細胞の共培養によるウイルス感染阻害実験結果　右) SLB-1 と Jurkat 細胞の共培養によるシンシチウム形成阻害実験

図8

HTLV-1陽性血漿からのグロブリンの精製

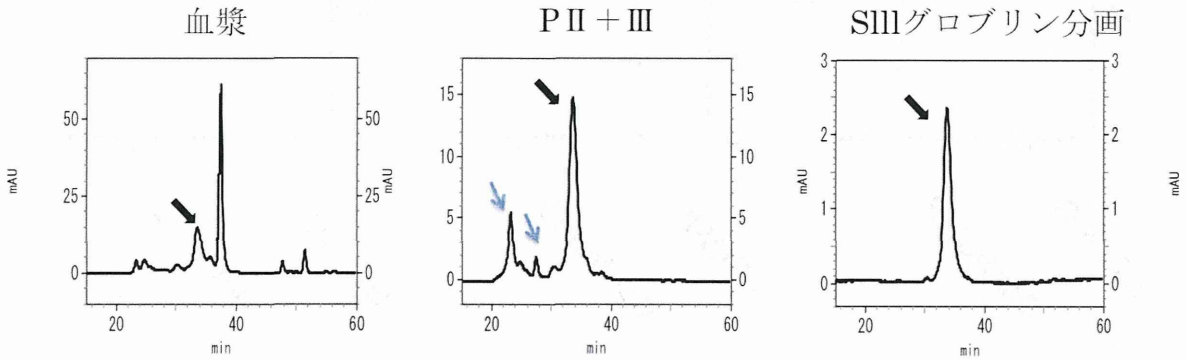


[25%の場合]
Plasma
↓
(8%EtOH,pH7.47 (22.5°C))
↓
SI
↓
(25%EtOH,pH 6.73(22.4°C))
↓
PII+III
↓
(20%EtOH, pH6.66(20.5°C))
↓
PII+III-W (17%EtOH, pH5.48(22.0°C))
↓
SIII

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)

副作用の原因となる重合物の除去



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 8：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、副作用の原因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9

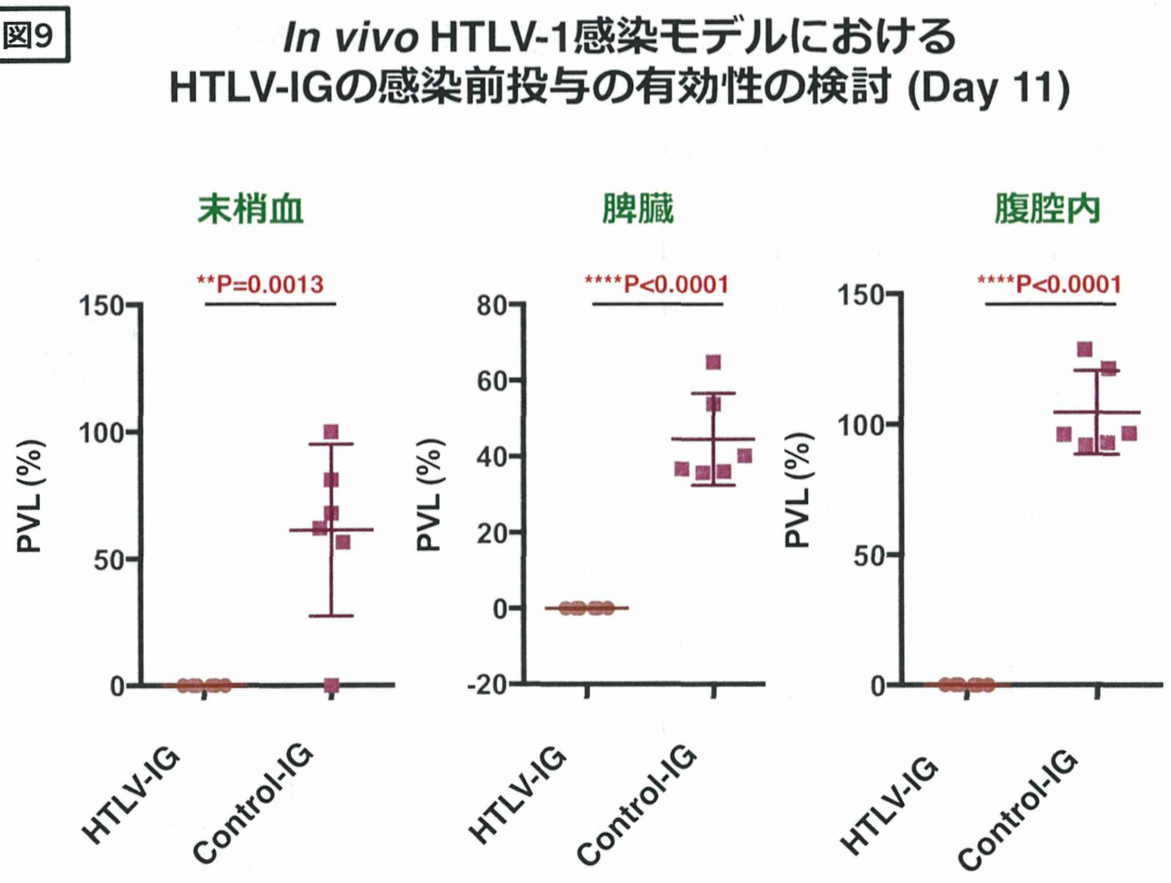


図 9：ヒト化マウスを用いた **HTLV-1** 感染実験及び **HTLV-IG** の有効性の検討。HTLV-1 感染 5 日前より Control-IG 及び HTLV-IG を投与したヒト化マウスの HTLV-1 感染の有無の確認。感染後 11 日目の解析。末梢血、脾臓細胞、腹腔内細胞より genomic DNA を分離・精製し、PVL を定量 PCR 法で測定した。その結果、何れの組織においても HTLV-1 感染を 100%阻止している事が明らかとなった。

図10

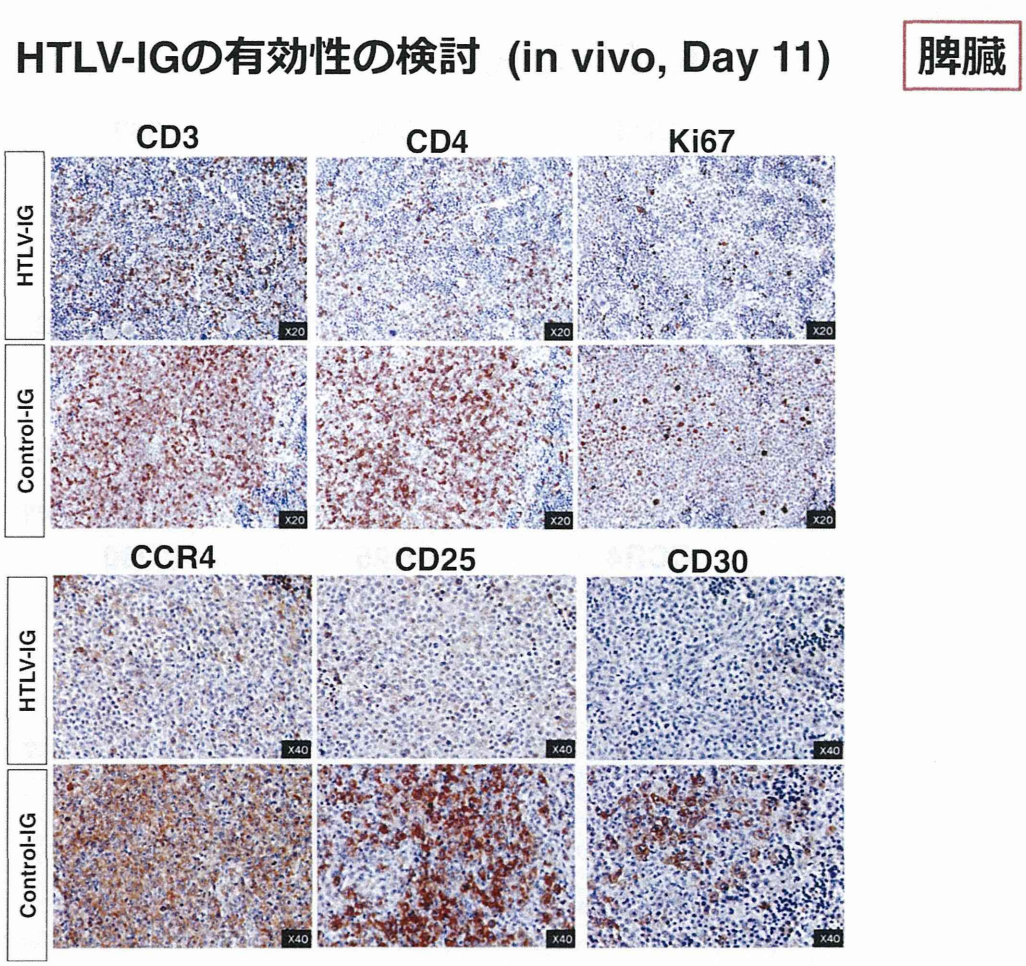


図 10：HTLV-IG 投与後の 11 日目の組織変化. ヒト化マウス(NOG マウス) における HTLV-1 感染モデルにおける HTLV-IG の有効性の検討。Control-IG 投与群においてはリンパ球様の細胞が多数、脾臓内に認められるが、HTLV-IG 投与群において、リンパ球様細胞の集簇は小さい。

Control-IG 投与群においては CD3, CD4, Ki67 陽性細胞が多数、脾臓内のリンパ球様細胞付近に認められ、集簇しているが、HTLV-IG 投与群において、CD3, CD4, Ki67 陽性細胞は分散して存在している。また、Control-IG 投与群においては CCR4, CD25, CD30 の発現が脾臓内のリンパ球様細胞に認められ、集簇しているが、HTLV-IG 投与群において、リンパ球様細胞における CCR4, CD25, CD30 の発現は殆ど認められない。

図11

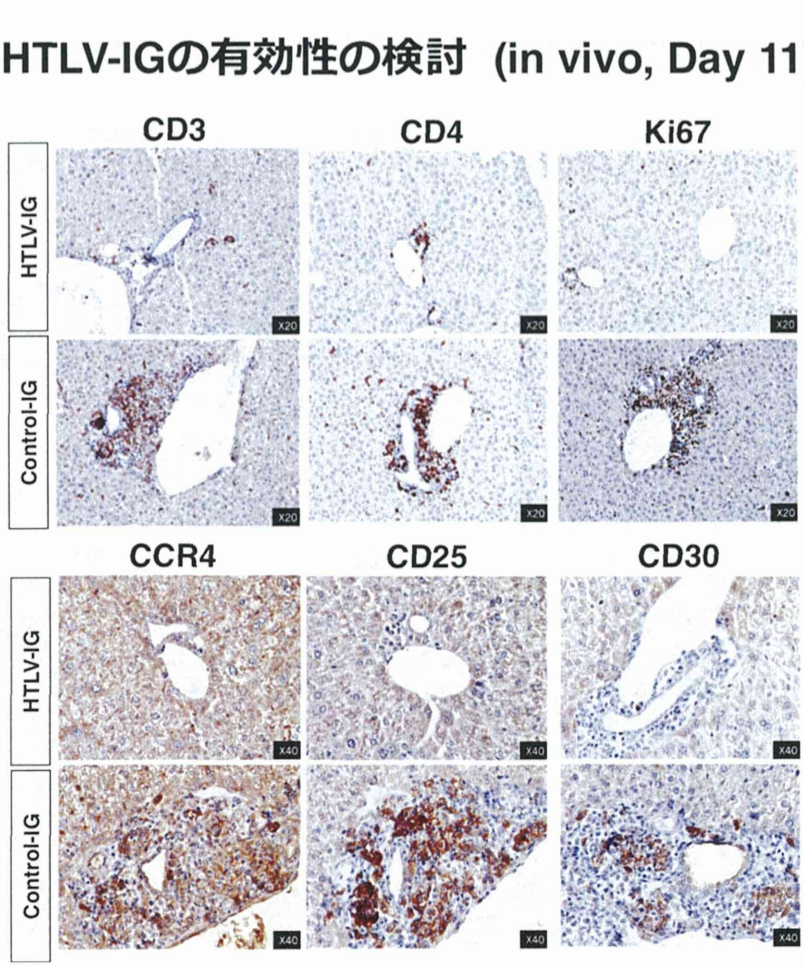


図 11：HTLV-IG 投与後の 11 日目の肝臓組織変化. ヒト化マウス(NOG マウス) における HTLV-1 感染モデルにおける HTLV-IG の有効性の検討。Control-IG 投与群においてはリンパ球様の細胞が多数、血管付近に認められるが、HTLV-IG 投与群において、リンパ球様細胞の集簇は少ない。

Control-IG 投与群においては CD3, CD4, Ki67 陽性細胞が多数、肝臓の血管付近に認められ、集簇しているが、HTLV-IG 投与群において、CD3, CD4, Ki67 陽性細胞は余り認められない。また、Control-IG 投与群においては CCR4, CD25, CD30 の発現がリンパ球様細胞に認められ、集簇しているが、HTLV-IG 投与群において、リンパ球様細胞における CCR4, CD25, CD30 の発現は殆ど認められない。

図12

HTLV-IGの有効性の検討 (in vivo, Day 11)

肺組織

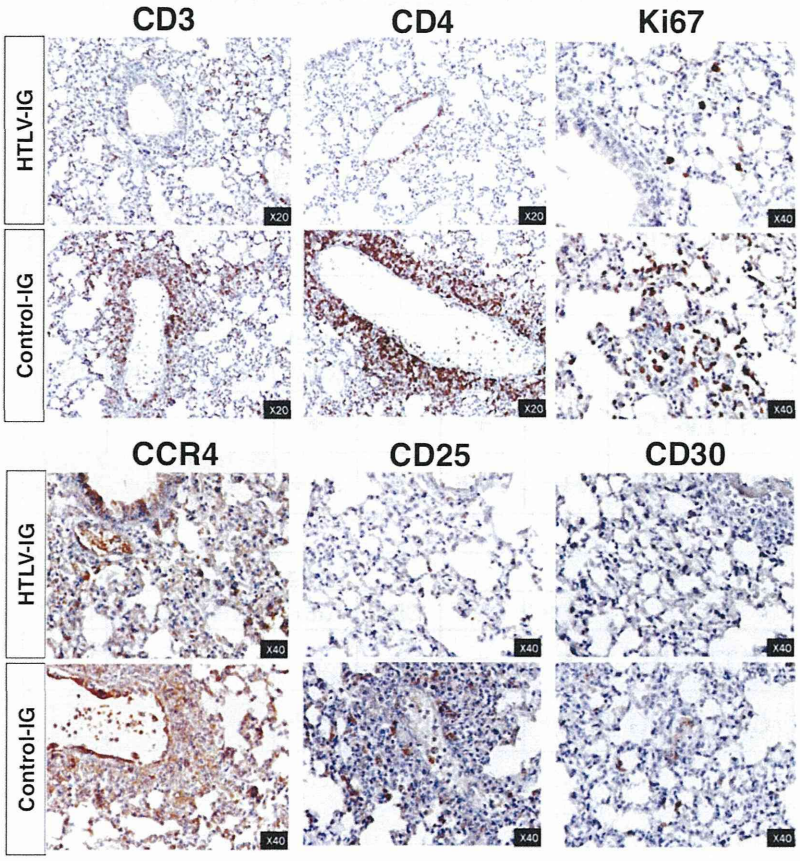


図 12：HTLV-IG 投与後の 11 日目の肺組織変化. ヒト化マウス(NOG マウス) における HTLV-1 感染モデルにおける HTLV-IG の有効性の検討。Control-IG 投与群においてはリンパ球様の細胞が多数、肺胞付近、血管付近に認められるが、HTLV-IG 投与群において、リンパ球様細胞の集簇は小なく、分散している。Control-IG 投与群においては CD3, CD4, Ki67 陽性細胞が多数、肺胞や血管付近に認められ、集簇しているが、HTLV-IG 投与群において、CD3, CD4, Ki67 陽性細胞は余り認められない。また、Control-IG 投与群においては CCR4, CD25, CD30 の発現が脾臓内のリンパ球様細胞に認められ、集簇しているが、HTLV-IG 投与群において、リンパ球様細胞における CCR4, CD25, CD30 の発現は殆ど認められない

図13

In vivo HTLV-1感染モデルにおける HTLV-IG 有効性の検討 (Day 11)

	Spleen						
	CD3	CD4	CD8	Ki67	CCR4	IL-2Ra	CD30
HTLV-IG	+	+	+	+	-	-	-
Control-IG	+++	+++	+	+++	+++	+++	+++

	Liver						
	CD3	CD4	CD8	Ki67	CCR4	IL-2Ra	CD30
HTLV-IG	+	+	+	+	-	-	-
Control-IG	+++	+++	+	+++	+++	+++	++

	Lung						
	CD3	CD4	CD8	Ki67	CCR4	IL-2Ra	CD30
HTLV-IG	+	+	+	+	-	-	-
Control-IG	+++	+++	+	+++	+++	++	++

図 13： NOG マウスを用いたヒト化マウスにおける HTLV-1 感染モデル。HTLV-IG の有効性をまとめた。HTLV-IG は正常の T 細胞の各種組織への定着には影響を与えないが、HTLV-1 感染を抑え、Control-IG 投与群で認められる HTLV-1 感染 T 細胞における CCR4、CD25 (IL-2Ra)、CD30 の発現上昇を抑えると共に脾臓・肝臓・肺への浸潤も抑える。

図14

In vivo HTLV-1感染モデルにおける HTLV-1 IgGの感染前投与の有効性の検討 (Day 38)

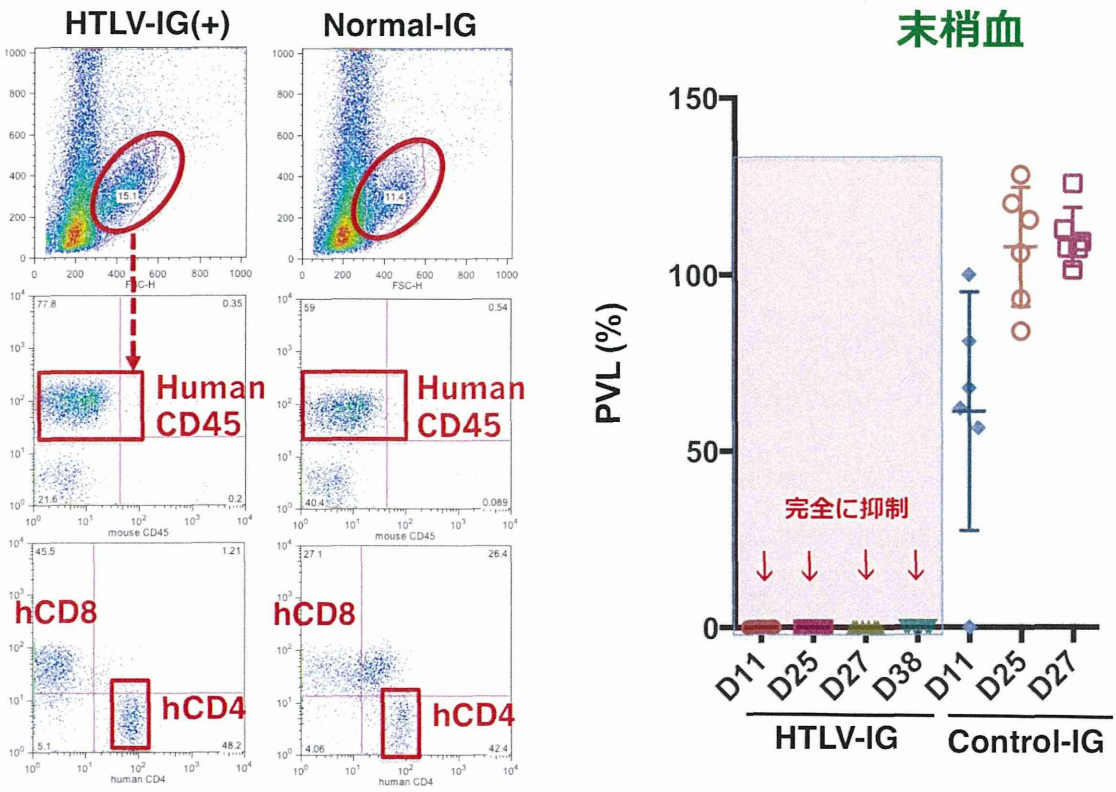


図 14：　ヒト化マウスを用いた HTLV-1 感染実験及び HTLV-IG の有効性の検討。HTLV-1 感染 5 日前より Control-IG 及び HTLV-IG を投与したヒト化マウスの HTLV-1 感染の有無の確認。更に、接種後 25 日，27 日、38 日目に末梢血を解析し、ヒト化に異常がない事を確認した。Control-IG 接種群においては引き続き PVL が増加傾向にあるが、HTLV-IG 投与群では HTLV-1 感染細胞はまったく認められなかった。

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
Kuramitsu M, Okuma K, Yamagishi M, Yamochi T, Firouzi S, Momose H, Mizukami T, Takizawa K, Araki K, Sugamura K, Yamaguchi K, Watanabe T, Hamaguchi I.	Identification of TL-Om1, an adult T-cell leukemia (ATL) cell line, as reference material for quantitative PCR for human T-lymphotropic virus 1.	***J Clin Microbiol.***	53	587-596	2015
Satake M, Yamada Y, Atogami S, Yamaguchi K.	The incidence of adult T-cell leukemia/lymphoma among human T-lymphotropic virus type 1 carriers in Japan.	***Leuk Lymphoma***	21	1-7	2015
Mizukami T, Momose H, Kuramitsu M, Takizawa K, Araki K, Furuhata K, Ishii KJ, Hamaguchi I, Yamaguchi K.	System vaccinology for the evaluation of influenza vaccine safety by multiplex gene detection of novel biomarkers in a preclinical study and batch release test.	***PLoS One***	9	e101835.	2014
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***	9	e91373.	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***	54	1808-1816	2014

Satake M.	Risk of transfusion-transmitted infection in Japan. (Japanese)	***Rinsho Ketsueki.***	55	2152 - 2161	2014
Sobata R, Shinohara N, Matsumoto C, Uchida S, Igarashi S, Hino S, Satake M, Tadokoro K.	First report of human immunodeficiency virus transmission via a blood donation that tested negative by 20-minipool nucleic acid amplification in Japan.	***Transfusion***	54	2361 - 2362	2014
Owada T, Kaneko M, Matsumoto C, Sobata R, Igarashi M, Suzuki K, Matsubayashi K, Mio K, Uchida S, Satake M, Tadokoro K.	Establishment of culture systems for Genotypes 3 and 4 hepatitis E virus (HEV) obtained from human blood and application of HEV inactivation using a pathogen reduction technology system.	***Transfusion***	54	2820 - 2827	2014
Abe T, Shimada E, Takanashi M, Takamura T, Motoji K, Okazaki H, Satake M, Tadokoro K.	Antibody against immunoglobulin E contained in blood components as causative factor for anaphylactic transfusion reactions.	***Transfusion***	54	1953 - 1960	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, Hino S, Suzuki Y, Murokawa H, Uchida S, Tadokoro K.	Residual risk of transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan.	***Transfusion.***	53	1393 - 1404	2013
Luc S, Luis TC, Boukarabila H, Macaulay IC, Buza-Vidas N, Bouriez-Jones T, Lutteropp M,	The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential.	***Nat Immunol.***	13	412-419	2012

Woll PS, Loughran SJ, Mead AJ, Hultquist A, Brown J, **Mizukami T**, Matsuoka S, Ferry H, Anderson K, Duarte S, Atkinson D, Soneji S, Domanski A, Farley A, Sanjuan-Pla A, Carella C, Patient R, de Bruijn M, Enver T, Nerlov C, Blackburn C, Godin I, Jacobsen SE.					
Kuramitsu M, Sato-Otsubo A, Morio T, Takagi M, Toki T, Terui K, Wang R, Kanno H, Ohga S, Ohara A, Kojima S, Kitoh T, Goi K, Kudo K, Matsubayashi T, Mizue N, Ozeki M, Masumi A, Momose H, Takizawa K, **Mizukami T**, Yamaguchi K, Ogawa S, Ito E, Hamaguchi I.	Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia.	***Blood***	119	2376-2384	2012

III. 研究成果の刊行物・印刷

JCM
Journals.ASM.org

Identification of TL-Om1, an Adult T-Cell Leukemia (ATL) Cell Line, as Reference Material for Quantitative PCR for Human T-Lymphotropic Virus 1

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Quantitative PCR (qPCR) for human T-lymphotropic virus 1 (HTLV-1) is useful for measuring the amount of integrated HTLV-1 proviral DNA in peripheral blood mononuclear cells. Many laboratories in Japan have developed different HTLV-1 qPCR methods. However, when six independent laboratories analyzed the proviral load of the same samples, there was a 5-fold difference in their results. To standardize HTLV-1 qPCR, preparation of a well-defined reference material is needed. We analyzed the integrated HTLV-1 genome and the internal control (IC) genes of TL-Om1, a cell line derived from adult T-cell leukemia, to confirm its suitability as a reference material for HTLV-1 qPCR. Fluorescent *in situ* hybridization (FISH) showed that HTLV-1 provirus was monoclonally integrated in chromosome 1 at the site of 1p13 in the TL-Om1 genome. HTLV-1 proviral genome was not transferred from TL-Om1 to an uninfected T-cell line, suggesting that the HTLV-1 proviral copy number in TL-Om1 cells is stable. To determine the copy number of HTLV-1 provirus and IC genes in TL-Om1 cells, we used FISH, digital PCR, and qPCR. HTLV-1 copy numbers obtained by these three methods were similar, suggesting that their results were accurate. Also, the ratio of the copy number of HTLV-1 provirus to one of the IC genes, RNase P, was consistent for all three methods. These findings indicate that TL-Om1 cells are an appropriate reference material for HTLV-1 qPCR.

Human T-lymphotropic virus 1 (HTLV-1) was the first retrovirus to be found in humans (1, 2). HTLV-1 is a cause of adult T-cell leukemia (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1-associated uveitis (3). Areas where HTLV-1 is endemic are distributed across several different regions, including southern Japan, the Caribbean, South America, and tropical Africa (4, 5). A recent report has shown that the area affected by this infection has expanded from the southern part of Japan to the entire country, particularly the Tokyo metropolitan area (6). Diagnostic tests for HTLV-1 infection are performed mainly with serological assays, such as enzyme-linked immunoabsorbent assay, particle agglutination assay, and Western blotting. Recently, another diagnostic test has been developed. Quantitation of integrated proviral DNA in peripheral blood (proviral load [PVL]) can be performed by quantitative PCR (qPCR) as a risk assessment for ATL or HAM/TSP (7, 8).

A few studies reported that several samples were positive for viral DNA when tested by PCR even though those samples had been found seroindeterminate for HTLV-1 when tested by Western blotting (9, 10). Their results suggest that HTLV-1 qPCR could be used as an additional test to confirm infection in seroindeterminate samples.

Although many laboratories have developed qPCR methods for HTLV-1 detection in Japan, a wide variety of testing methods are used. For example, the target region, primers and probes, and internal control (IC) genes vary among the laboratories (8, 11–15). These variations lead to significant differences in HTLV-1 PVL when these laboratories measure the same samples (16). As a consequence of these differences, comparison of quantitative data between laboratories will continue to be difficult without standardization.

One possible solution is to establish a reference material, which is indispensable for standardizing multicenter test results. The target material for HTLV-1 qPCR is genomic DNA (gDNA) from peripheral blood mononuclear cells (PBMCs). Therefore, HTLV-1-infected cells would be an ideal source for a reference material. To date, many cell lines from ATL patients have been established, but few of them have been well characterized for the genomic features associated with reference materials for HTLV-1 qPCR.

In this study, we investigated the genomic structure of one of these ATL cell lines, TL-Om1, to establish it as a reference material for HTLV-1 nucleic acid amplification techniques (NATs), namely, HTLV-1 clonality, karyotyping, proviral sequencing, integration sites, and determination of gene copy number of HTLV-1 and cellular genes for IC.

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TABLE 1 Primers used for qPCR of HTLV-1 and IC genes

Target gene	Forward name	Forward sequence	Reverse name	Reverse sequence	Size (bp)	Primer correction factor	
						Plasmid	gDNA
HTLV-1 gene	LTR202F	ACAATGACCATGAGCCCCAAA	LTR202R	TTAGTCTGGGCCCTGACCT	101	0.9869	
	LTR215F	GCTCGCATCTCTCCTTCAC	LTR215R	AGTTCAGGAGGCACCACA	102	0.9942	
	LTR005F	CCTGACCCTGCTTGCTCAAC	LTR005R	TCAGTCGTGAATGAAAGGGAAAG	99	0.9917	
	056F	TAGTCCCACCCTGTTCGAAATG	056R	GCCAGGAGAATGTCATCCATGT	105	1.0013	
	084F	CCTGCCCCGCTTACTATCG	084R	GGCATCTGTGAGAGCGTTGA	102	0.9922	
	153F	TTGTCGCGCTACTCCTTCTTG	153R	AGGGATGACTCAGGGTTTATAAGAGA	118	0.9792	
	pX2-S[a]	CGGATACCCAGTCTACGTGTT	pX2-AS[a]	CAGTAGGGCGTGACGATGTA	100	0.9944	
RNaseP (RPPH1) gene	RPPH1-05F	TATGCACAATTATGTAATCCCCAAA	RPPH1-05R	CCAGCTCCCTATAACCTGCACTT	100	1.0025	1.0012
	RPPH1-08F	GCCGGAGCTTGGAACAGA	RPPH1-08R	AATGGGCGGAGGAGAGTAGTCT	109	0.9956	0.9937
	RPPH1-12F	AGGAAGCCCACGAAAATTCTAATT	RPPH1-12R	GTCCCCATACTCGGTGATTCTC	101	1.0019	1.0052
Albumin (ALB) gene	ALB-07F	TGCAATGAACACAGGAGAGCTACTA	ALB-07R	CCACCCAGGTAACAAAATTAGCAT	103	0.9971	0.9964
	ALB-19F	CCTGATGCTTCTCAGCCTGTT	ALB-19R	TCCATTTAAGAGTGTGTGTGGTAGGT	100	1.0019	1.0045
	ALB-26F	TGCATTGCCGAAGTGGAAA	ALB-26R	CCTCAGCATAGTTTTTGCAAACA	100	1.0038	1.0078
β-Actin (ACTB) gene	ACTB-06F	TCTGGTGTTTGTCTCTCTGACTAGGT	ACTB-06R	CCGCTTTACACCAGCCTCAT	100		0.9965
	ACTB-12F	TCCTGGGTGAGTGGAGACTGT	ACTB-12R	CCATGCCTGAGAGGGAAATG	107		1.0016
	ACTB-21F	AGCATCCCCCAAAGTTCACA	ACTB-21R	GGACTTCCTGTAACAACGCATCT	101		1.0106
CD81 gene	CD81-01F	GACACATCCCAAGGGTGCTT	CD81-01R	GGACTCAGTTCTCAATGCTTTGC	107		1.0015
	CD81-10F	ACCACGCCTTGCCCTTCT	CD81-10R	GAATCACGCCACTTCCATAACTG	111		1.0021
	CD81-21F	GGTGCACACAGCATGCATTT	CD81-21R	GTGCGCCTCTGGGTAATCAT	102		1.0009
β-Globin (HBB) gene	HBB-11F	TTGGACCCAGAGGTTCTTTGAG	HBB-11R	GGCACCGAGCACTTTCTTG	103		1.0021
	HBB-15F	AGCAGCTACAATCCAGCTACCAT	HBB-15R	GAGGTATGAACATGATTAGCAAAAGG	105		1.0033
	HBB-24F	CCCACCCCAAATGGAAGTC	HBB-24R	AGCACCATAAGGGACATGATAAGG	104		1.0111
RAG-1 gene	RAG1-03F	GCAATCCCATTTGTCCACTTTT	RAG1-03R	TCCCACTGGCCTGCATTACTA	100		1.0045
	RAG1-27F	GAAGTTTAGCAGTGCCCCATGT	RAG1-27R	ACGGGCAGTGTTGCAGATG	100		1.0006
	RAG1-32F	TCAAAGTCATGGGCAGCTATTGT	RAG1-32R	AGGGAATTCAAGACGCTCAGAA	100		0.9993

[a] Primer sequences were previously reported in reference 11.

MATERIALS AND METHODS

Cells and gDNA preparation. Jurkat clone E6-1 cells were obtained from the American Type Culture Collection. HUT102 and SLB-1 cells, which are HTLV-1-infected cell lines, were a kind gift from Masahiro Fujii (Division of Virology, Niigata University Graduate School of Medical and Dental Sciences). PBMCs were kindly provided by the Japanese Red Cross or purchased from AllCells (Alameda, CA, USA). TL-Om1 cells, an ATL-derived cell line established by Sugamura et al. (17), were maintained in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 2 mmol/liter L-glutamine, and 10 ng/ml interleukin-2 (PeproTech, London, United Kingdom). Jurkat, HUT102, and SLB-1 cells were maintained in RPMI 1640 containing 10% FBS supplemented with 100 U/ml penicillin-streptomycin and 2 mmol/liter L-glutamine. DNA was extracted using a QIAamp DNA blood mini or maxi kit (Qiagen, Valencia, CA, USA).

Southern blotting. Southern blotting was performed by SRL Inc. (Tokyo, Japan). DNA was digested with EcoRI and PstI and separated on a 0.8% agarose gel as previously reported (18, 19). DNA was transferred onto nylon membranes (Roche, Mannheim, Germany). The membrane was hybridized with digoxigenin (DIG)-labeled HTLV-1 probe at 42°C overnight. DNA fragments for HTLV-1 probes were obtained from Oncor Inc. (Gaithersburg, MD, USA). Sense and antisense HTLV-1 DNA probes were prepared by random primed labeling using a DIG-High Prime kit (Roche). After the membrane was washed, HTLV-1 probe signals were obtained using a DIG luminescent detection kit (Roche).

FISH analysis. To stop the cell cycle at M phase, Colcemid (Sigma) was added to the cell culture medium at a concentration of 0.02 μg/ml and incubated for 1 h. Cells were harvested and washed with phosphate-buffered saline (PBS). After treatment with 0.075 M KCl hypotonic solution at 37°C for 1 h, cells were fixed with a solution containing acetic acid and methanol (3:1). Cells were fixed to a glass slide and dried. The complete HTLV-1 genome inserted in pUC18 (15) was used as a probe for provirus, bacterial artificial chromosome (BAC) clone RP11-919G18 was used as a probe for the albumin (ALB) gene, and BAC clones CTD-2326H15 and RP11-203M5 were used as probes for the RNase P (RPPH1) gene. BAC clones were selected from NCBI (http://www.ncbi.nlm.nih.gov/clone/) and were purchased from Advanced Geno Techs Co. (Tsukuba, Japan). The probe for 1q44 was commercially prepared by Chromosome Science Labo Inc. (Sapporo, Japan). For the detection of ALB and RPPH1 genes, the BAC clones were labeled with cyanine 3 (Cy3) and Cy5, respectively. For the detection of provirus, the DIG-labeled probe was prepared by the nick translation method. The probe was hybridized to the sample at 70°C for 5 min, followed by incubation at 37°C overnight. The probe was stained with anti-DIG-Cy3 antibody. Signals were detected by a Leica DMRA2 system and analyzed with Leica CW4000 fluorescent *in situ* hybridization (FISH) software (Wetzlar, Germany).

Splinkerette PCR analysis. Splinkerette PCR was performed as previously reported (20). The first-round PCR was performed as indicated in reference 20. The second-round, nested PCR was performed using the HTLV-1 long-terminal-repeat (LTR)-specific primer. The nested PCR product was loaded onto 3% Tris-acetate-EDTA buffer (TAE) agarose gels. Two distinct DNA bands were cut from the agarose gel and purified using a QIAquick gel extraction kit (Qiagen). After thymine and adenine (TA) cloning, each band was sequenced by the Sanger method (21).

Inverse PCR analysis. TL-Om1 gDNA was digested with BamHI or XbaI. Digested DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Briefly, 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol were added to the sample. After centrifugation at $2 \times 10^4 \times g$ for 15 min, the DNA pellet was washed with 70% ethanol and then air dried. Purified DNA was self-ligated using a Ligation-Convenience kit (Nippon Gene, Tokyo, Japan). Ligated DNA was purified again by phenol-chloroform extraction followed by ethanol precipitation. PCR was performed with KOD FX (Toyobo, Osaka, Japan). The PCR mixture contained 20 ng gDNA, 0.4 mM forward and reverse primers, 1 mM deoxynucleoside triphosphate (dNTP), 1× KOD FX buffer, and 0.5 U KOD FX in a total volume of 25 μl, in duplicate. The forward primer sequence was 5′-ACAAATACACCTTGCAATCCTATG G-3′, and the reverse primer sequence was 5′-CGCTTGGGAGACTTCT TGCT-3′. PCR mixtures were denatured at 94°C for 2 min, followed by 34 cycles of 98°C for 10 s and 68°C for 10 min. PCR products were loaded onto 0.8% agarose gels and detected by LAS-3000 (Fujifilm, Tokyo, Japan).

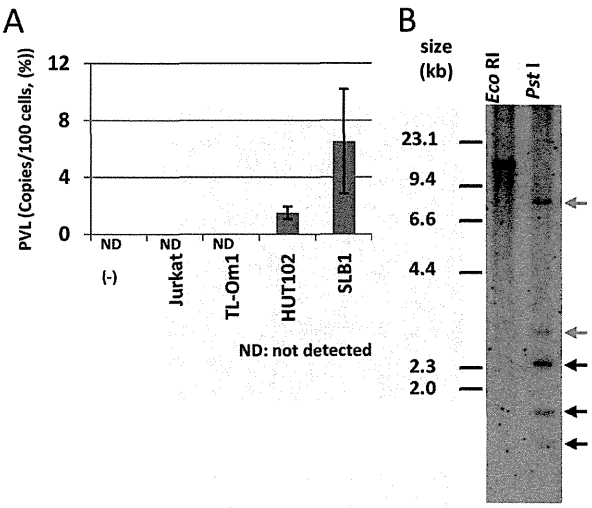

FIG 1 Infectivity and clonality of HTLV-1 provirus in TL-Om1 cells. (A) Mitomycin C-treated Jurkat, TL-Om1, HUT102, and SLB1 cells were cocultured with Jurkat cells. PVL (%) was measured 2 weeks later by qPCR. (B) gDNA from TL-Om1 cells digested with EcoRI or PstI was subjected to Southern blotting probed by the full HTLV-1 genome. Three black arrows show bands for typical HTLV-1 genomic sequences; two gray arrows show bands for host genomic sequences ligated to the HTLV-1 genome. Because the EcoRI site is not included in the HTLV-1 sequence, the number of bands indicates the number of clones in the cells. Detection of two gray bands indicates that there is a pair of 5′ and 3′ HTLV-1 genomes conjugated with the host genome, signifying that the HTLV-1 provirus is monoclonal. On the other hand, detection of more than two gray bands indicates that it is multiclonal.

Genomic long PCR. Genomic long PCRs were performed using KOD FX (Toyobo). Primers are listed in Table S1 in the supplemental material. The conditions for the PCR mixture and thermal cycling program were the same as those for the inverse PCR analysis.

DNA sequencing analysis. The genomic long PCR and inverse PCR products were purified by a GenElute PCR Clean Up kit (Sigma). Direct sequencing was performed using a BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence primers are listed in Table S2 in the supplemental material. Sequences were read and analyzed using a 3120× genetic analyzer (Applied Biosystems).

Synchronized qPCR analysis. The primers used for the synchronized qPCR amplification are listed in Table 1. The PCR mixture was prepared with SYBR premix *Ex Taq* II (TaKaRa, Tokyo, Japan) containing 100 ng gDNA and 0.4 mM forward and reverse primers in a total volume of 15 μl, in triplicate. PCR was performed according to the manufacturer's protocol. The ΔC_T(RPPH1) value (where C_T is threshold cycle) was calculated by the following equation: ΔC_T(RPPH1) = average C_T of target gene primer results − average C_T of RPPH1. The gene copy number was calculated by the following equation: target gene copy number (N) = copy number determined by FISH $\times 2^{-\Delta CT(\mathrm{RPPH1})}$. Using normal PBMCs or plasmids, the primer correction factor, which can compensate for small differences in amplification efficiency among different primers, was calculated. The correction factor was determined by the difference of each C_T