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Progress in antiretroviral drugs

Wataru Sugiura

AIDS Research Center, National Institue of Infectious Diseaseas
E-mail: wsugiura@nih.go.jp

HIV-1, causative agent of acquired immunodeficiency syndrome, was identified in the early 1980s . The plague quickly spread throughout the world and today 40 million people are living with HIV/AIDS. The first anti-HIV drug "zidovudine", was discovered in 1985, and many other inhibitory compounds have been developed successfully in the last decade. Today, three classes 17 antiretroviral drugs are available in Japan. This article overviews the history of anti-HIV drug discovery, present HIV-1 treatment, and on-going drug discovery.

総　　説

新規感染者における薬剤耐性HIV拡散の危機
Alert for Outbreak of Drug Resistance HIV-1 in Newly Infected Population

杉　浦　　亙
Wataru SUGIURA
国立感染症研究所エイズ研究センター
National Institute of Infectious Diseases

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はじめに

1985年に満屋裕明博士の手によりzidovudineが見いだされ[1]，HIV/AIDSの治療が実現してから今年で20年目にあたるが，この間の治療薬剤と治療法の開発に目覚しい進歩があったことはこの雑誌の読者には周知の事実であろう。平成17年現在，ヌクレオシド系逆転写酵素阻害剤7種類，非ヌクレオシド系逆転写酵素阻害剤3種類，そしてプロテアーゼ阻害剤7種類，合計17種類の薬剤が使用されている。改良された剤形や合剤などを含めると，その数はさらに増え23種類になる。現在もケモカインレセプター阻害剤，インテグラーゼ阻害剤などの新たなクラスの薬剤開発が活発に行われており，今後益々治療の選択肢が増えていくことが期待される。特定の病原体に対して，これほど多数の治療薬剤が作られてきた例は他には無く，一つ一つが研究者たちのHIV/AIDSを克服するために尽くしてきた努力の標石といえよう。

皮肉なことに治療薬剤の登場はHIV-1に薬剤耐性という新たな進化の扉を開き，優れた治療法の実現による死亡者の減少は，私たちからHIV-1感染症に対する恐れを忘れさせつつあるようである。今日，先進諸国では薬剤耐性HIV-1による新たな感染拡大が危惧されているが，これはこのようなウイルス学的そして意識の変化が根底にあると考えられる。あるいは，薬剤耐性株による感染の拡大はHIVに特有の問題ではなく，結核やマラリアなどの薬剤治療が行われている他の感染症においても解決すべき大きな課題となっていることを考えると，今の状況は抗HIV薬剤が登場した時から定められていた必然であるとも考えることができる。

著者連絡先：杉浦　亙（〒208-0011　武蔵村山市学園4-7-1
国立感染症研究所エイズ研究センター）
Fax：042-561-7446

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先進諸国における薬剤耐性HIV-1による新規感染者の問題

では，薬剤耐性HIV-1による新規感染はどのような状況になっているのであろうか。表1に日本と同等の治療環境をもつ先進各国で行われた新規感染者における薬剤耐性HIV-1の調査結果をまとめた[2-21]。この表を一瞥して判るのは，ほぼ同時期に調査が行われたにもかかわらず，薬剤耐性HIVの検出頻度が報告により大きく異なっていることである。一つの説明としては調査毎に薬剤耐性検査法，そして薬剤耐性の定義が異なっていたことが挙げられる。もう一つの説明として調査対象集団（地域，人種，感染経路）が耐性HIV-1の拡散を考える際の重要な因子であり，薬剤耐性による感染の拡大が特定の集団に限局していたことも考えられる。

このように薬剤耐性HIV-1の検出頻度がばらつく一方で，調査間で共通して見られている事象もある。それは，どの調査においても耐性の検出頻度がヌクレオシド系逆転写酵素阻害剤で最も高いことである。これは表に示す国々では，調査時点で既にヌクレオシド系逆転写酵素阻害剤は10年近く治療に用いられてきた薬剤となっており，HIV-1感染者集団内にヌクレオシド系逆転写酵素阻害剤耐性ウイルスが蔓延しつつあることを反映しているのであろう。これに対して非ヌクレオシド系逆転写酵素阻害剤とプロテアーゼ阻害剤に対する耐性検出頻度については調査によって順位が入れ替わっているが，概ね非ヌクレオシド系の耐性検出頻度のほうが高いようである。これら2クラスの薬剤はヌクレオシド系逆転写酵素阻害剤に比べるとまだ使用の歴史が浅い（調査時点で数年程度）ためであろう。

表に示した調査のほとんどが薬剤耐性遺伝子検査によって行われているが，薬剤耐性遺伝子検査と感受性検査を平

行して行った調査が2つある。#2のLittle等と#3のSimon等の調査である。この2つの調査結果における薬剤耐性遺伝子検査と感受性検査の結果を比較して気がつくのは，非ヌクレオシド系逆転写酵素阻害剤耐性とプロテアーゼ阻害剤耐性では遺伝子検査と感受性検査で行った調査結果がほぼ一致しているのに対して，ヌクレオシド系逆転写

表 1 諸外国における薬剤耐性による新規感染症例の報告

	Author and Group	country	year	test	N	prevalence				ref
						Total	NRTI	NNRTI	PI	
1	Novak RM Terry Berin Community Programs for clinical Research on AIDS_058 study team	US	1999-2001	Genotyping	n=491	11.6%	7.8%	3.0%	0.7%	10
2	Little SJ Richmann DD	USA	1995-1998	Genotyping	n=213	8.0%	8.5%	1.7%	0.9%	4
				Phenotyping	n=264	3.4%	2.3%	1.9%	0.4%	
			1999-2000	Genotyping	n=88	22.7%	15.9%	7.3%	10.2%	
				Phenotyping	n=114	12.4%	8.5%	7.1%	8.0%	
3	Simon V & Markowitz M	USA	1995-2001	Genotyping	n=76	13.2%	11.8%	2.6%	1.3%	9
				Phenotyping	n=60	10.0%	8.3%	3.0%	1.7%	
			1999-2001	Genotyping	n=78	19.7%	14.5%	6.6%	5.1%	
				Phenotyping	n=74	10.8%	2.7%	8.1%	5.4%	
4	Wegner S US military	USA	1997-1998	Genotyping	n=95	22.1%	4.0%	15.0%	10.0%	6
				Phenotyping	n=91	29.7%	8.0%	26.0%	1.0%	
5	Weinstock HS	USA	1997-2001	Genotyping	n=1082	8.3%	6.4%	1.7%	1.9%	12
6	Hanna GJ D'Aqila RT	USA	1999	Genotyping	n=88	18.0%	14.0%	5.0%	2.0%	11
7	Boden D Markowiz M	USA	1999	Genotyping	n=80	16.3%	12.5%	7.5%	3.8%	14
8	Alexander CS	Canada	1997-1998	Genotyping	n=479	6.3%	----- 3.4% -----		3.8%	2
9	Alexander CS	Canada Vancouver	1996-1998	Genotyping	n=57	19.2%	14.0%	7.0%	1.9%	3
10	Routy JP	Canada	1997-2000	Genotyping	n=127	13.0%	11.0%	2.4%	5.5%	15
		Montreal	2001-2003	Genotyping	n=53	4.0%	0.0%	1.9%	1.9%	
11	Salomon H Quebec Primary Infection Study	Canada	1997-1999	Genotyping	n=56	NR*	----- 22.0% -----		12.0%	18
				Genotyping	n=21**	NR	----- 24.0% -----		24.0%	
12	UK collaborative Group on Monitoring the transmission of HIV Drug Resistance	UK	1994-2000	Genotyping	n=69	14.5%	11.6%	4.3%	1.4%	5
13	Descamps D ANRS antiretroviral Resistance Study	France	1998	Genotyping	n=391	3.7% (51.1%)	3.3%	0.8%	1.9% (49.2%)	8
14	Chaix ML	France	1999-2000	Genotyping	n=249	10.0%	8.0%	4.0%	6.0%	7
15	Duwe	Germany	1996-1999	Genotyping		NR	----- 9.0% -----		5.0%	16
16	Romano L	Italy	1996-2000	Genotyping	n=116	12.9%	12.9%	0.0%	0.9%	13
17	Martinz-Picado	Spain	2004	Genotyping	n=182	3.8%	2.2%	1.1%	0.5%	20
18	Maljkovic I	Sweden	1998-2001	Genotyping	n=100	9.0%	7.0%	5.0%	1.0%	21
19	Jorgensen LB	Denmark	2000	Genotyping		NR	----- 2.0% -----		0.0%	19
20	Ammoranond P	Australia	1992-2001	Genotyping		NR	----- 9.0% -----		0.0%	17

* NR : Not reported
** : IVDU data

酵素阻害剤耐性では遺伝子検査での結果のほうがかなり高い頻度を示していることである。薬剤耐性HIV-1の調査は疫学的な視点から耐性ウイルスの拡散頻度と拡散様式を把握することが目的であるため，主要な耐性変異が1つでも見つかれば“耐性ウイルスが感染した”と判定する必要があるが，ヌクレオシド系逆転写酵素阻害剤耐性は耐性変異が複数集積しないと薬剤感受性自体は大きく変化しないためにこのような調査結果の開きが生じたのであろう。この2つの調査結果は，新規感染者における薬剤耐性HIV-1のサーベイランスには遺伝子検査が適しており，感受性検査では結果を低く見積もる恐れがあることを示している。

結　　語

HIV-1の感染成立には体液の物理的な接触が必要である。このような感染症では，教育による危険行為の回避と感染予防が効果を上げるはずである。事実，積極的な啓発活動は優れた成果をあげてきた。その一方で薬剤耐性ウイルスが新規感染者に広がっているという現実は教育・啓発活動に限界があることを示している。今年の初頭にNew York Timesで報道された多剤耐性且つ急速進行性の新型ウイルス騒ぎはニューヨーク市保健局によって，薬剤耐性HIV-1が広がりつつあるという現状に人々の関心を向けさせる絶好の話題として演出された。このような報道は新たな差別を助長しかねない最悪のやり方だという批判も出ているようだが，感染者の10人に1人に耐性ウイルスが検出される米国の現実をみると，このような荒療治も必要なのかも知れないと考えさせられる。なお，この症例にはウイルス学的な新事実は無く，多剤耐性を獲得した以外には特徴の無いウイルスであったことが明らかにされている。また症例自体も単発であり，急速進行性という特性は感染者の宿主因子に帰すると結論されている[22]。

わが国における新規感染者の薬剤耐性HIV-1については2003年に2つの研究グループの手により調査が行われた。国立国際医療センターエイズ治療・研究開発センターのグループによる調査では4%，国立名古屋医療センターのグループによる調査では17%という結果が示された[23]。このように2つの調査結果には大きな開きが認められているが，これは日本においても耐性HIV-1による感染が集団に限局していることを強く示唆している。この2つの調査結果を踏まえて平成16年度より厚生労働省エイズ対策事業の1つとして新規感染者における薬剤耐性HIV-1のサーヴェイランスが開始された。調査はHIV-1診療に携わる全国のブロック拠点，拠点病院の医師，薬剤耐性検査を担当する検査技師，研究者など多くの人々の協力を得て進められている。この調査を行うことにより，日本の新規感染者における薬剤耐性HIV-1の広がりが正確に把握でき，またどのような対策が薬剤耐性HIV-1による新たな感染を防ぐために有効か明らかにできると期待している。

文　　献

1) Mitsuya H, Weinhold KJ, Furman PA, St. Clair MH, Lehrman SN, Gallo RC, Bolognesi D, Barry DW, Broder S : 3′-Azido-3′-deoxythymidine (BW A509U) : an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc Natl Acad Sci U S A 82 : 7096-7100, 1985.
2) Alexander CS, Dong W, Chan K, Jahnke N, O'Shaughnessy MV, Mo T, Piaseczny MA, Montaner JS, Harrigan PR : HIV protease and reverse transcriptase variation and therapy outcome in antiretroviral-naive individuals from a large North American cohort. Aids 15 : 601-607, 2001.
3) Alexander CS, Dong W, Schechter MT, O'Shaughnessy MV, Strathdee SA, Mo T, Montaner JS, Harrigan PR : Prevalence of primary HIV drug resistance among seroconverters during an explosive outbreak of HIV infection among injecting drug users. Aids 13 : 981-985, 1999.
4) Little SJ, Holte S, Routy JP, Daar ES, Markowitz M, Collier AC, Koup RA, Mellors JW, Connick E, Conway B, Kilby M, Wang L, Whitcomb JM, Hellmann NS, Richman DD : Antiretroviral-drug resistance among patients recently infected with HIV. N Engl J Med 347 : 385-394, 2002.
5) UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance : Analysis of prevalence of HIV-1 drug reistance in primary infections in the United Kingdom. BMJ 322 : 1087-1088, 2001.
6) Wegner SA, Brodine SK, Mascola JR, Tasker SA, Shaffer RA, Starkey MJ, Barile A, Martin GJ, Aronson N, Emmons WW, Stephan K, Bloor S, Vingerhoets J, Hertogs K, Larder B : Prevalence of genotypic and phenotypic resistance to anti-retroviral drugs in a cohort of therapy-naive HIV-1 infected US military personnel. Aids 14 : 1009-1015, 2000.
7) Chaix ML, Descamps D, Harzic M, Schneider V, Deveau C, Tamalet C, Pellegrin I, Izopet J, Ruffault A, Masquelier B, Meyer L, Rouzioux C, Brun-Vezinet F, Costagliola D : Stable prevalence of genotypic drug resistance mutations but increase in non-B virus among patients with primary HIV-1 infection in France. Aids 17 : 2635-2643, 2003.

8) Descamps D, Calvez V, Izopet J, Buffet-Janvresse C, Schmuck A, Colson P, Ruffault A, Maillard A, Masquelier B, Cottalorda J, Harzic M, Brun-Vezinet F, Costagliola D : Prevalence of resistance mutations in antiretroviral-naive chronically HIV-infected patients in 1998 : a French nationwide study. Aids 15 : 1777-1782, 2001.
9) Simon V, Vanderhoeven J, Hurley A, Ramratnam B, Louie M, Dawson K, Parkin N, Boden D, Markowitz M : Evolving patterns of HIV-1 resistance to antiretroviral agents in newly infected individuals. Aids 16 : 1511-1519, 2002.
10) Novak RM, Chen L, MacArthur RD, Baxter JD, Huppler Hullsiek K, Peng G, Xiang Y, Henely C, Schmetter B, Uy J, van den Berg-Wolf M, Kozal M : Prevalence of antiretroviral drug resistance mutations in chronically HIV-infected, treatment-naive patients : implications for routine resistance screening before initiation of antiretroviral therapy. Clin Infect Dis 40 : 468-74. Epub 2005 Jan 10, 2005.
11) Hanna GJ, Balaguera HU, Freedberg KA, Werner BG, Steger Craven KA, Craven DE, D'Aquila RT : Drug-selected resistance mutations and non-B subtypes in antiretroviral-naive adults with established human immunodeficiency virus infection. J Infect Dis 188 : 986-991. Epub 2003 Sep 10, 2003.
12) Weinstock HS, Zaidi I, Heneine W, Bennett D, Garcia-Lerma JG, Douglas JM, Jr., LaLota M, Dickinson G, Schwarcz S, Torian L, Wendell D, Paul S, Goza GA, Ruiz J, Boyett B, Kaplan JE : The epidemiology of antiretroviral drug resistance among drug-naive HIV-1-infected persons in 10 US cities. J Infect Dis 189 : 2174-2180. Epub 2004 May 21, 2004.
13) Romano L, Venturi G, Ferruzzi R, Riccio ML, Corsi P, Leoncini F, Vinattieri A, Incandela L, Valensin PE, Zazzi M : Detection of genotypically drug-resistant HIV-1 variants and non-B subtypes in recently infected antiretroviral-naive adults in Italy. Aids 14 : 2204-2206, 2000.
14) Boden D, Hurley A, Zhang L, Cao Y, Guo Y, Jones E, Tsay J, Ip J, Farthing C, Limoli K, Parkin N, Markowitz M : HIV-1 drug resistance in newly infected individuals. Jama 282 : 1135-1141, 1999.
15) Routy JP, Machouf N, Edwardes MD, Brenner BG, Thomas R, Trottier B, Rouleau D, Tremblay CL, Cote P, Baril JG, Remis RS, Sekaly RP, Wainberg. MA : Factors associated with a decrease in the prevalence of drug resistance in newly HIV-1 infected individuals in Montreal. Aids 18 : 2305-2312, 2004.
16) Duwe S, Brunn M, Altmann D, Hamouda O, Schmidt B, Walter H, Pauli G, Kucherer C : Frequency of genotypic and phenotypic drug-resistant HIV-1 among therapy-naive patients of the German Seroconverter Study. J Acquir Immune Defic Syndr 26 : 266-273, 2001.
17) Ammaranond P, Cunningham P, Oelrichs R, Suzuki K, Harris C, Leas L, Grulich A, Cooper DA, Kelleher AD : No increase in protease resistance and a decrease in reverse transcriptase resistance mutations in primary HIV-1 infection : 1992-2001. Aids 17 : 264-267, 2003.
18) Salomon H, Wainberg MA, Brenner B, Quan Y, Rouleau D, Cote P, LeBlanc R, Lefebvre E, Spira B, Tsoukas C, Sekaly RP, Conway B, Mayers D, Routy JP : Prevalence of HIV-1 resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or injecting drug use. Investigators of the Quebec Primary Infection Study. Aids 14 : F17-23, 2000.
19) Jorgensen LB, Christensen MB, Gerstoft J, Mathiesen LR, Obel N, Pedersen C, Nielsen H, Nielsen C : Prevalence of drug resistance mutations and non-B subtypes in newly diagnosed HIV-1 patients in Denmark. Scand J Infect Dis 35 : 800-807, 2003.
20) Martine-Picado J, Guiterrez C, deMendoza C, Erkicia I, Domingo P, Camino X, Galindo MJ, Blaonco JL, Leal M, Masabeu A, Guelar A, Baldovi JF, Pedereia JD, Gatell JM, Moreno S, Clotet B, Soriano V, Ruiz J : Presented at the 14th international HIV drug resistance workshop, Quebec City, Canada, June 7-11, 2005. 2005.
21) Maljkovic I, Wilbe K, Solver E, Alaeus A, Leitner T : Limited transmission of drug-resistant HIV type 1 in 100 Swedish newly detected and drug-naive patients infected with subtypes A, B, C, D, G, U, and CRF01_AE. AIDS Res Hum Retroviruses 19 : 989-997, 2003.
22) Volberding PA : The New York case : lessons being learned. Ann Intern Med 142 : 866-868. Epub 2005 Apr 21, 2005.
23) Ibe S, Hotta N, Takeo U, Tawada Y, Mamiya N, Yamanaka K, Utsumi M, Kaneda T : Prevalence of drug-resistant human immunodeficiency virus type 1 in therapy-naive patients and usefulness of genotype testing. Microbiol Immunol 47 : 499-505, 2003.

第18回日本エイズ学会シンポジウム記録

シンポジウム7.「薬剤耐性の新知見，基礎から臨床へ」を終えて

杉浦 亙[1]，潟永 博之[2]，田宮 貞宏[3]，松田 昌和[1]，松見信太郎[3,4]，
蜂谷 敦子[2]，John M. Coffin[5]，満屋 裕明[3,4]
[1] 国立感染症研究所，エイズ研究センター
[2] 国立国際医療センター，エイズ治療・研究開発センター
[3] 熊本大学医学部血液内科学・感染免疫診療部
[4] 米国国立癌研究所内科療法部門レトロウイルス感染症部
[5] HIV Drug Resistance Program, NCI-Frederick

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多剤併用療法が標準的なHIV感染症治療法として行われている今日，治療を適切に進めていく上で薬剤耐性変異の出現を如何に回避するかは重要な研究課題である。また，薬剤耐性HIVについて研究することは治療上の必要性だけでなく，治療薬剤と変異の対応関係の解析によるプロテアーゼおよび逆転写酵素の機能と構造，感染者体内における耐性変異の進化・選択のプロセス，そしてヒトにおけるレトロウイルス感染症の病態などの知見も得ることができる点でも意義の深いことである。第18回日本エイズ学会総会2日目に行われたシンポジウム「薬剤耐性の新知見，基礎から臨床へ」では，米国立癌研究所HIV Drug resistance Programの所長John Coffin博士を基調講演者として迎え，3人の日本人研究者とともに薬剤耐性HIV-1研究の最近の研究成果についての発表と活発な討論が行われた。以下各発表の概要を紹介する。

S7-1「非核酸系逆転写酵素阻害薬nevirapineに対する新規耐性変異パターンの解析」

潟永博之，蜂谷敦子
国立国際医療センター エイズ治療・研究開発センター

臨床的に広く使われている薬剤耐性検査はgenotype assayであるが，このassayは既知の耐性変異の有無でHIV-1の薬剤耐性を推測する。未知の耐性変異が存在した場合には，まったく無力となるため，genotype assayをより有効にするためには，新たな薬剤耐性変異の同定・解析が不可欠である。また，phenotype assayの結果がgenotype assayから予想されるものと大きく異なる場合，未知の耐性変異が存在している可能性があり注意を要する。

国立国際医療センターを受診した未治療のHIV-1感染患者44人から，HIV-1を分離培養し，MAGIC-5細胞によるphenotype assayを行ったところ，2株が非核酸系逆転写酵素阻害薬nevirapine（NVP）に対して高度耐性（69倍と310倍以上）であった。genotype assayでは，既知のNVP耐性変異は認められなかったが，2株とも，逆転写酵素の238番目のアミノ酸がlysine（K）からserine（S）に変異していた（K238S）。組換えHIV-1を作成して，増殖能・薬剤感受性を調べたところ，K238S単独でNVPに対し4.4倍耐性となり，既知のNVP耐性変異とK238Sが共存すると，V106A/K238Sでは530倍，V108I/K238Sでは56倍耐性となり，高度なNVP耐性を獲得することが明らかとなった[1)]。K238Sがgenotype assayで認められた場合には，NVPをkey drugとする治療法は不適切であると考えられる。近年，非核酸系逆転写酵素阻害薬耐性に関与する238番目付近のアミノ酸変異が相次いで報告されており，この領域のpolymorphismが治療結果に影響を与える可能性がある。

著者連絡先：杉浦 亙（〒208-0011 武蔵村山市学園4-7-1
国立感染症研究所エイズ研究センター）
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S7-2「プロテアーゼ阻害剤に対する薬剤耐性の発現」

松見信太郎[1,2]，田宮貞宏[2]，満屋裕明[1,2]
[1] 熊本大学医学部血液内科学・感染免疫診療部
[2] 米国国立癌研究所内科療法部門レトロウイルス感染症部

プロテアーゼ阻害剤（PIs）の標的であるHIVのプロテアーゼは対称的な二量体構造を有し，構造的に微生物のアスパラチルプロテアーゼに類似している。HIVプロテアーゼ阻害剤はこの複合蛋白の切断を阻害し，ウイルス粒子の成熟をブロックすることによって抗ウイルス活性を発揮する。PIsに対する耐性は，主要には，HIVがプロテアーゼの基質結合部位（あるいはその近傍）にアミノ酸置換を起こして，本来の基質（ウイルスの前駆体）との親和性を著しく低下させずに，阻害剤との親和性だけを下げることで起こる。HIVのプロテアーゼはかなりの可撓性（flexibility）

を有しており，99 個の構成アミノ酸のうち 60% 以上のアミノ酸に，プロテアーゼとしての酵素活性を保ちながら（減弱は見られるものの），置換を起こし得る[2]（図1)。

耐性株で最初に出現する一群のアミノ酸置換は，投与された阻害剤に特異的なものが多い。そうしたアミノ酸置換は「一次変異」と呼ばれる。「一次変異」はウイルス酵素の構造を変えて阻害剤と酵素の結合が起こらないようにするなどしてウイルスに耐性を付与すると思われるが，その構造変化のために酵素本来の活性が低下して増殖能などが損なわれることが多い。この構造変化を修復，補正するために続いて起こってくる一連のアミノ酸置換が「二次変異」と呼ばれる。複数の「二次変異」が加わってくると，HIV は増殖能を取り戻し，また高度の交差耐性を獲得するようになる[2]。PIs に対する薬剤耐性の発現には複数のメカニズムが関与している。プロテアーゼと PI の結合という観点から最も重要な機序と考えられるものは，酵素の活性部位にアミノ酸の置換が起こって PI との結合性が失われて（あるいは減弱して）耐性が発現するというものである[2]。プロテアーゼは構造的に活性中心部位付近に PI の側鎖が入り込んで強固な結合を形成する幾つもの「ポケット（又はサブサイト)」を有している。この酵素はウイルスの基質とも同様な水素結合パターンを形成すると考えられており，それぞれの PI で観察される基質特異性は PI とサブサイトのアミノ酸側鎖との間で形成される結合パターンに依存していると考えられる。このような特異性を規定するサブサイトのアミノ酸に置換が起これば当然その PI に特異的な耐性が発現することになる[2]。プロテアーゼの活性中心部位でない領域にもアミノ酸の置換が起こって，それが耐性発現に関与することがある。HIV はアミノ酸置換によって PI に対する耐性を獲得するが，その度に本来のプロテアーゼ酵素の活性がしばしば減弱する。これを補うために HIV は，基質である複合蛋白（例えば Gag 蛋白の前駆体部分）の切断部位に突然変異を起こして切断されやすくする等して酵素活性の減弱を代償させ，結果的に酵素活性の損なわれたプロテアーゼによるウイルス蛋白の前駆体（基質）の切断・成熟を進める[3]。切断部位とは異なった Gag 領域にもアミノ酸置換を起こして viral fitness を維持するメカニズムも確認されている[4]。極く最近我々は複数のプロテアーゼ阻害剤に対する耐性変異株（PIR-HIV）で HIV の Gag 部分の p17/p24 と p1/p6 の切断部位の近傍に「繰返し配列」が集積することを発見した[5]（図2)。このような一連の付加アミノ酸（例えば図2の HIV-1B 変異株における TGNS）は何れも近傍のアミノ酸配列の繰返し配列で，この「繰返し配列」を野生 HIV 株（HIVWT）に導入すると増殖能が低下したが，full-size の感染性 PIR-HIV クローンをそれぞれ作成してこのクローンから「繰返し配列」を除くと PIR-HIV クローンの増殖能が低下したことから，この「繰返し配列」は PI に対する耐性を付与する複数のアミノ酸置換のために損なわれたプロテアーゼ活性を代償して酵素による p17/p24 と p1/p6 の切断部位での切断が起こりやすいようにするものと考えられた。ウェスタンブロットで検討すると HIVWT に「繰り返し配列」を導入すると成熟 p24Gag の産生量が著減した。PIR-HIV の増殖速度は HIVWT に比べて遅く，また PIR-HIV 内の成熟 p24Gag 量も HIVWT に比べると少ないが，PIR-HIV から「繰り返し配列」を除くと PIR-HIV 内の成熟 p24Gag 量は更に減少した（図3)。これらのデータは HIVWT の増殖にとっては「繰り返し配列」は不要かつ有害であるが，多剤耐性能を獲得した PIR-HIV にとっては損なわれた変異プロテアーゼの酵素活性を回復するのに必須であることを示すものと思われた[5]。

S7-3 「本邦における薬剤耐性 HIV-1 の現状と今後の課題」

松田昌和，杉浦　亙
国立感染症研究所エイズ研究センター

多剤併用療法が HIV-1 感染症の標準的な治療法として 1997 年に開始されてから今日までの約 7 年間，多数の新薬が登場して HIV-1 感染症を取り巻く治療環境は大きく進歩した。我々は多剤併用療法の導入が HIV-1 の進化・選択にどのような影響を及ぼしてきたのか理解するために（i）既治療感染症例および（ii）新規感染・慢性未治療感染症例という 2 つの異なる集団について解析を行った。解析に用いた HIV-1 の遺伝子領域は治療薬剤の標的である protease および逆転写酵素遺伝子あわせて 1.3Kb である。HIV-1 サブタイプはプロテアーゼ領域あるいは *env* C2V3 領域 350 bps の系統樹解析により判定をした。既治療症例については国立感染症研究所エイズ研究センターで薬剤耐性遺伝子検査を実施した症例の集団における薬剤耐性出現頻度の年次推移について解析を行った。薬剤耐性変異の判定は IAS-USA の耐性変異リストに準じた。新規感染・慢性未治療症例については，図4に示すような遺伝子進化学的な視点から，1997 年と 2003 年の新規・慢性未治療症例各集団内における遺伝子多様性の解析と，1997 年と 2003 年の集団間の多様性のネット変化の算出を行い，抗 HIV-1 治療薬剤の導入がこの 7 年間に HIV-1 に直接的，間接的にどのような影響を及ぼしてきたかについて考察をした。

まず既治療症例における薬剤耐性変異の頻度と推移であるが，ヌクレオシド系およびプロテアーゼ阻害剤耐性変異の頻度は多剤併用療法の導入とともに 1999 年から 2000 年ころまで増加をたどっており，興味深いことにこの 2 クラスの耐性検出頻度はその後横ばいからやや減少に転じてい

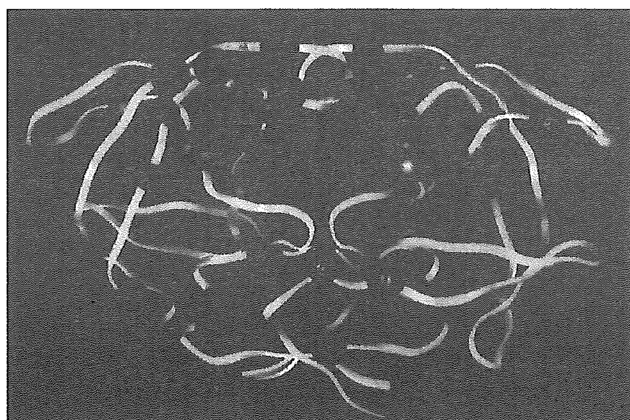


図 1 プロテアーゼ阻害剤に対する薬剤耐性を付与するアミノ酸置換 HIV のプロテアーゼは極めて柔軟性に富んでおり，99 個の構成アミノ酸のうち 60% 以上のアミノ酸に置換を起こして「偽の基質」であるプロテアーゼ阻害剤との結合を回避，正常の基質（ウイルスのポリ蛋白）を認識するようになるとウイルスの複製が再び始まる。これが HIV のプロテアーゼ阻害剤に対する耐性獲得の主要な機序である[2)]。

```
                                         p17 | p24
HIV-1NL4-3:    SKKKAQQA----AADTGNN----SQVSQNYPIVQ
HIV-1B:        SKKKAQQA----AADTGNSTGNSSQVSQNYPIVQ
HIV-1G:        S-KKAQQA----AADTGNS----SKVSQNYPIVQ
HIV-1EV:       SKKKAQQA--AAAADTGNSSQVNSQVSQNYPIVQ
HIV-1ES:       SKKKAQQAAQQAAADTGSN----SQVSQNYPIVQ

                          p1|p6
HIV-1NL4-3:    HKGRPGNFLQSRPE------PTAPP---EESFR
HIV-1B:        HKGRPGNFLQSRPE------PTAPPAPPEESFR
HIV-1G:        HKGRPGNFLQSRPESRPE--PTAPP---EESFR
HIV-1EV:       QKGRPGNFFQSRPE------PTAPP---EESFR
HIV-1ES:       HKGRPGNLLQSRLEPTAPPAPTAPP---AESFR
```

図 2 複数のプロテアーゼ阻害剤に対する耐性変異株(PIR-HIV)で見られた HIV の Gag 部分での「繰返し配列」PIR-HIV の p17/p24 と p1/p6 の切断部位の近傍で集積して見られたアミノ酸置換（例えば HIV-1B 変異株における TGNS）は何れも近傍のアミノ酸配列の繰返し配列であった。この「繰返し配列」を野生 HIV 株に導入すると増殖能が低下したが，full-size の感染性 PIR-HIV クローンをそれぞれ作成してこのクローンから「繰返し配列」を除くと PIR-HIV クローンの増殖能が低下したことから，この「繰返し配列」は PI に対する耐性を付与する複数のアミノ酸置換のために損なわれたプロテアーゼ活性を代償して酵素による p17/p24 と p1/p6 の切断部位での切断が起こりやすいようにするものと考えられた。野生株（HIVWT）のアミノ酸配列は紫，置換アミノ酸は青，「繰返し配列」は赤，鋳型となった元の配列は下線で示す[5)]。

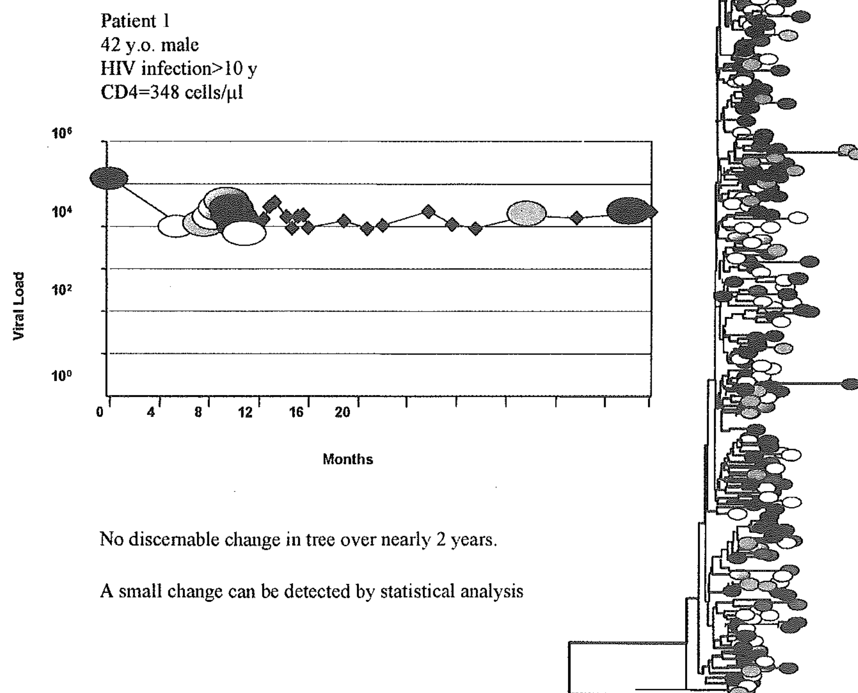


図 8

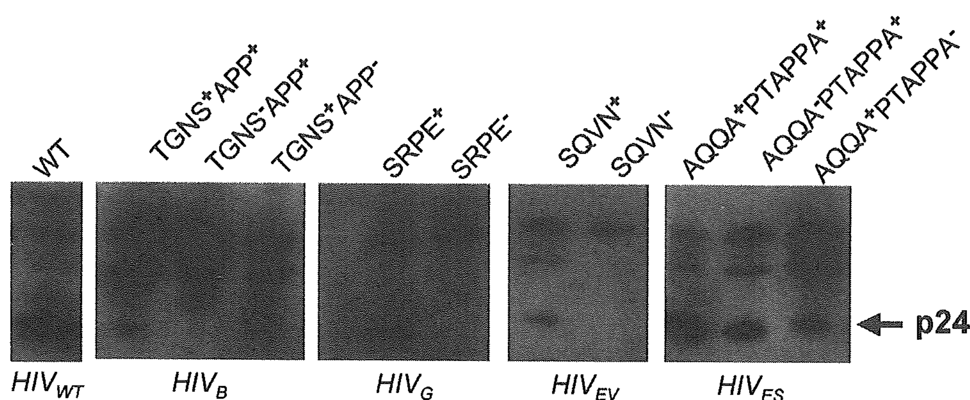


図 3 Gag 部分での「繰り返し配列」は PIR-HIV の増殖能維持に必須である。PIR-HIV の増殖速度は HIVWT に比べて遅く，また PIR-HIV 内の成熟 p24Gag 量も HIVWT に比べると少ないが，PIR-HIV から「繰り返し配列」を除くと PIR-HIV 内の成熟 p24Gag 量は更に減少した[5]。

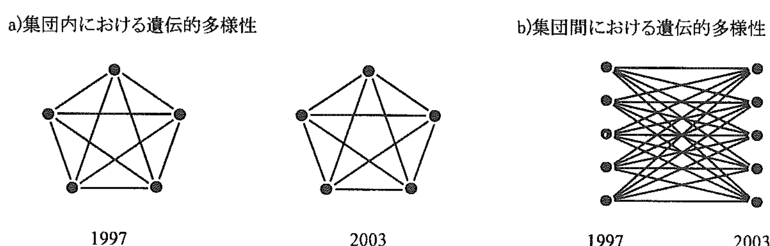


図 4

る。一方，非ヌクレオシド系逆転写酵素阻害剤耐性変異の検出頻度は 2004 年まで緩やかに増加している。個々の耐性変異毎にその検出頻度を見ると，出現頻度により大まかに高度（20% 以上），中等度（5-20%）そして低度（5% 未満）の 3 群に分類された。このような耐性変異の出現頻度を左右する因子としては，その時々の処方薬剤のトレンド，変異が耐性に及ぼす影響，変異間の相互作用，そして変異がウイルスの増殖能力に及ぼす酵素化学的および構造学的な因子の関与が示唆された。

新規感染・慢性未治療症例における遺伝的多様性の変化は図 5 に示したように，逆転写酵素遺伝子では $D_{1997}=0.032\pm0.003$ に対し $D_{2003}=0.048\pm0.004$ と集団における多様性が有意に拡大している。プロテアーゼ遺伝子についても $D_{1997}=0.042\pm0.005$ に対し $D_{2003}=0.055\pm0.007$ と多様性の拡大が観察された。この集団内における多様性の拡大は図 5 の無根系統樹の広がりから視覚的にも捕らえることができる。しかしながら 1997 年の症例群と 2004 年症例群の集団間のネット変化については逆転写酵素では 0.002，プロテアーゼでは 0.003±0.001 にとどまっており，7 年という時間では遺伝子の大きな進化・淘汰は起きてはいないと考えられた。集団間の多様性の拡大については，これが治療薬剤の出現による選択。淘汰の変化なのか，それとも自然界における通常の変化の範疇に収まっているのか，更なる解析と検討が必要であろう。

S7-基 "The HIV-Host Interaction : New Insights from New Tools."

John M. Coffin[1], Frank Maldarelli[1], Sarah Palmer[1], Valerie Boltz[1], Mary Kearney[1], Ann Weigand[1], and John W. Mellors[2]
[1] HIV Drug Resistance Program, NCI-Frederick,
[2] Division of Infectious Disease, University of Pittsburgh.

To obtain more detailed information about the dynamics and evolution of HIV in infected individuals, we have developed 3 assays to detect and quantitate virus and analyze its genetic makeup (図 6). The first of these, the single copy

1997年と2003年の2つの集団の系統樹と多様性

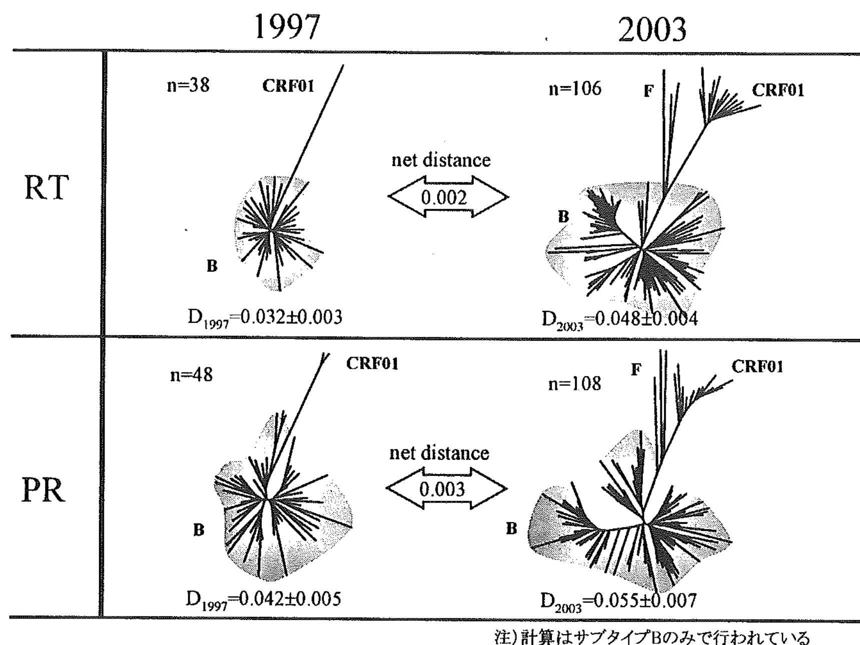


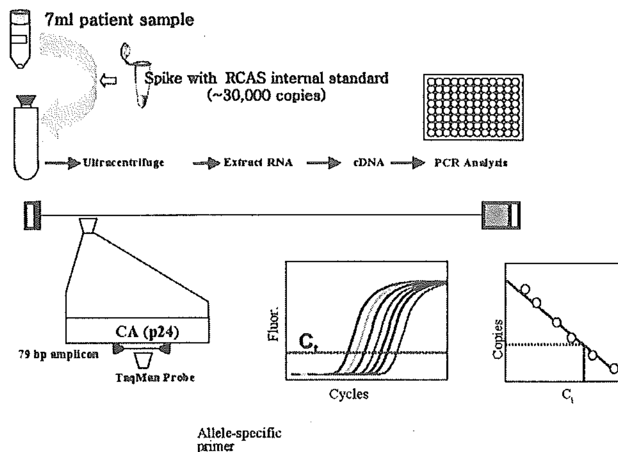
図 5

assay (SCA) allows us to detect and accurately quantitate 1 copy of HIV RNA. In routine use, we can measure as little as 0.3 copies of HIV RNA (or 0.15 virions) per ml of patient plasma. The second assay is use of allele-specific PCR (ASP) to detect specific point mutations such as K103N in HIV RT, which confers resistance to NNRTIs. Using ASP, we can detect and quantitate mutations at this codon (AAA to AAT or AAC) comprising less than 0.1% of the total virus population. The third assay is single-genome sequencing (SGS), in which multiple single cDNA molecules derived from reverse transcription of plasma virus are amplified over a region extending from the p6 region of *gag* through most of RT, and sequenced in bulk. This approach allows us to obtain a snapshot of the genetic diversity within the virus population in a single patient at any point in time, with minimal assay based error, and essentially no artifacts due to resampling or assay-based recombination. We have used these assays to study the virus in both naïve and drug-treated patients, with the following results.

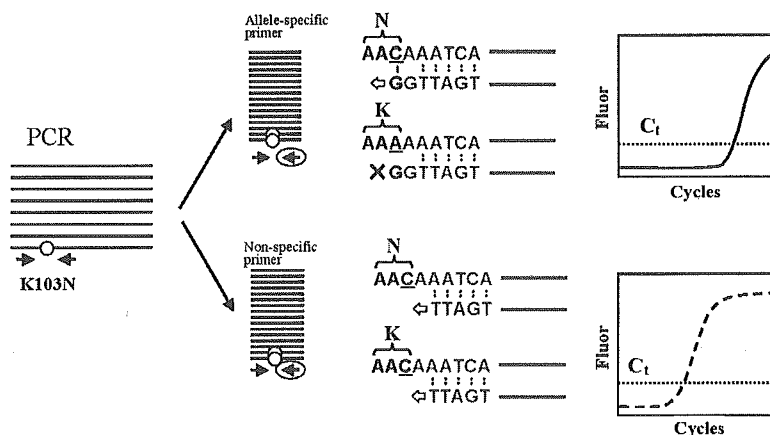
1. In a large set of patients with levels of plasma virus that are "undetectable" by standard assays, we find that about 2/3 of them have viremia in the range of 1–20 copies of RNA per ml, with an average around 5 copies/ml. These levels are stable over periods of a year or more（図 7）, and are likely to be the source of rebound viremia observed in all patients following interruption of therapy. We do not yet know whether this virus is the result of ongoing low-level replication or is derived from cells infected before initiation of therapy, although preliminary results are consistent with the latter possibility.

2. In individuals who have been infected for long periods of time and remained untreated, the virus has diversified to about 1–2% in the *gag-pol* region, as determined by SGS（図 8）. This diversity is remarkably stable so that samples taken years apart can not be distinguished by phylogenetic analysis. Similarly, virus populations retain their diversity through a 100-fold decline in viremia following initiation of therapy. Samples taken soon after infection, by contrast, are usually almost perfectly monomorphic, exhibiting levels of diversity indistinguishable from background up to 70 days after infection. Thus, we can conclude that infection is usually effectively clonal, and the virus population is large and subject to strong purifying selection leading to gradual diversification up to a point where the population is both highly diverse and stably so, although later populations gradually become distinguishable from earlier ones (by subtle statistical tests), there are no significant bottlenecks or episodes of obvious selective sweeps over long periods of time.

1) Single Copy Assay



2) 103N/K Allele-Specific PCR



3) Limiting Dilution PCR Sequencing
AKA: single genome sequencing (SGS)

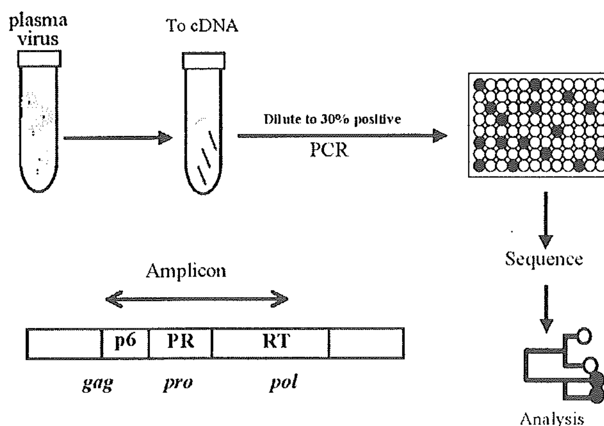


図 6

Viremia Persists after Suppression by Antiretroviral Therapy

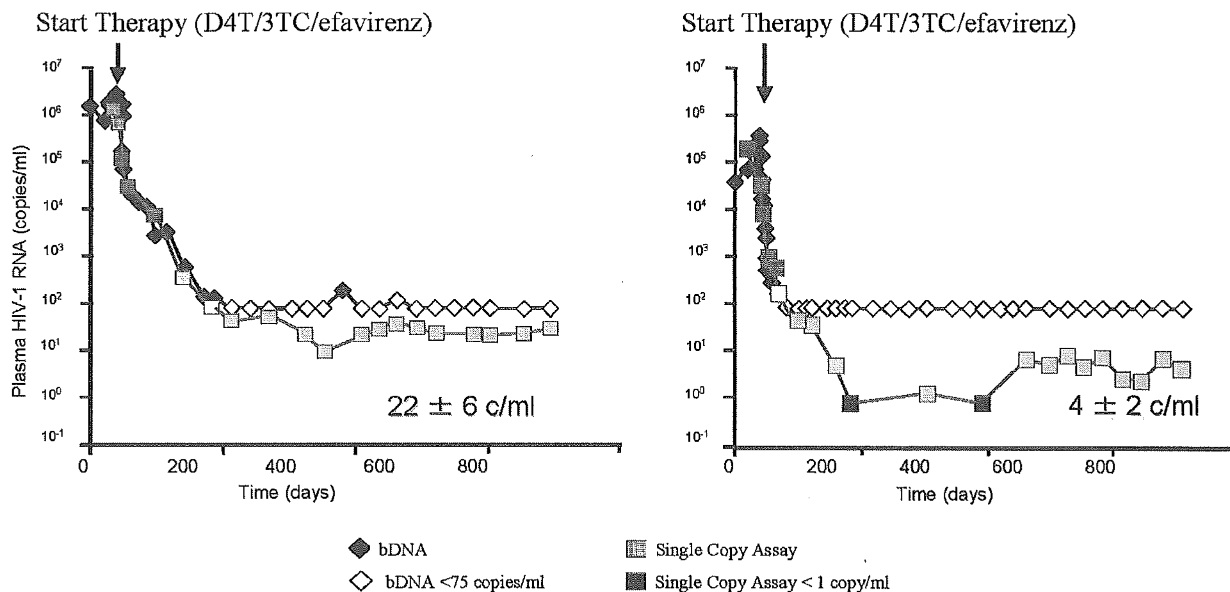


図 7

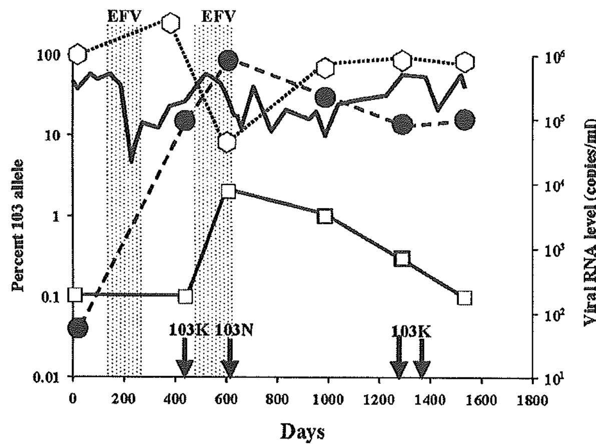
3. Both theory and experience imply that the virus in individuals who have been infected with HIV for more than a short time should have a low level of preexisting drug resistance mutations. We have therefore used allele-specific PCR to analyze levels of K103N in infected, untreated individuals. The background in the assay was about 0.02%, and the large majority of patient samples gave results were very close to this value, implying that the assay is not yet sufficiently sensitive to detect the true values in most patients. A few patients had values significantly larger than background, however, suggesting the possibility of stochastic fluctuations in frequency — such fluctuations — although rare — could have significant implications for the success of subsequent therapy.

4. In individuals who have failed complex combinations of therapy, including the NNRTI efavirenz (EFV), the K103 N resistance mutation shows a wide variety of behaviors, including persistence at a level of nearly 100% for 5 years after the end of EFV treatment ; rapid reduction to about 10% of the virus population and persistence at that level, and a complete switch in the relevant codon in the virus from AAC to AAT and back again (図 9). Persistence is not due to linkage to other resistance mutations, but the codon switching is the result of linkage to the M184V mutation selected by treatment with 3TC during part of the EFV therapy.

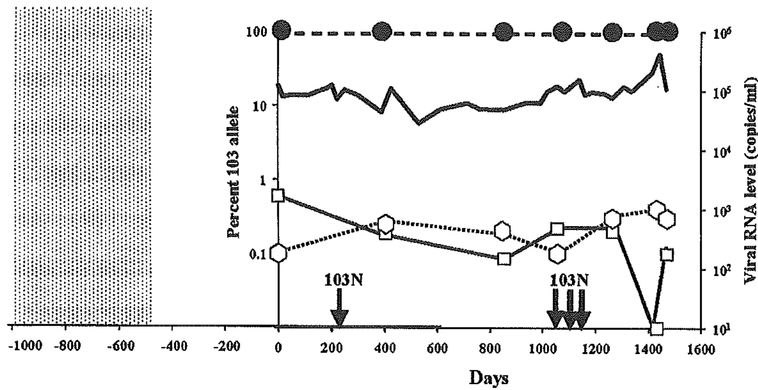
5. In patients who have failed combination antiretroviral therapy, it is standard to test for potentially active drugs by bulk sequence analysis of RT-PCR products derived from plasma virus. To test the ability of standard analysis to detect important resistance mutations, we compared bulk sequences with those of SGS products from plasma virus obtained from highly drug-experienced patients. SGS revealed the presence of resistance mutations that were not detected by standard bulk genotype analysis in 3-20% of genomes analyzed. In some cases, furthermore, the undetected mutations are linked on the same genomes. Since even minor populations of resistant virus are likely to cause rapid failure, bulk sequencing approaches, while useful for predicting resistance to specific drugs, are unlikely to be reliable in predicting sensitivity to them.

Conclusions. The tests we have developed are bringing new insights to the analysis of HIV in infected patients. We have uncovered a new therapeutic steady state viremia in most or all patients that explains our inability to cure the infection. We have found that the genetically highly diverse population of HIV in long-term infected patients is very stable in its diversity. We have learned that standard sequencing ap-

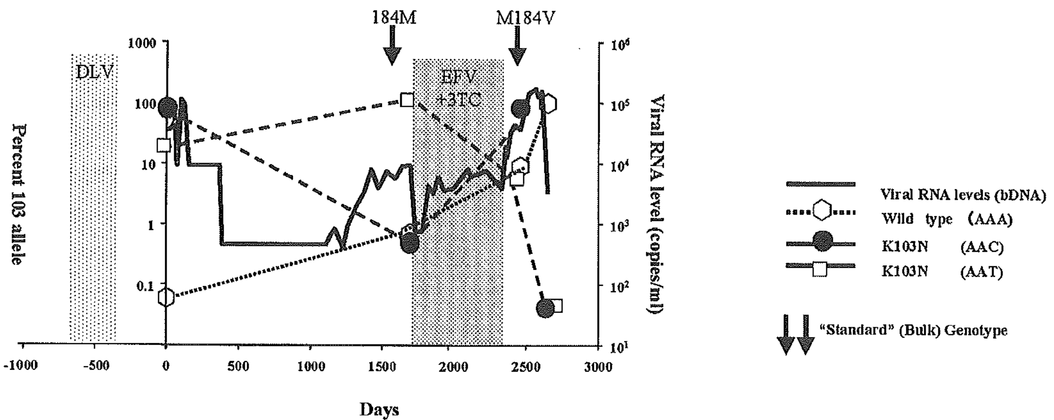
Patient 1 : Persistent K103N mutation



Patient 3 : Persistent K103N mutation:



Patient 5 : 103N codon switch



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proaches miss a large fraction of resistance mutations in highly experienced patients, and we have uncovered a puzzling diversity of patterns of loss of drug resistant mutations long after treatment has ceased. We expect further studies of these phenomena to be equally rewarding in their ability to uncover new aspects of the host-virus relationship.

文　献

1) Hachiya A, Gatanaga H, Kodama E, Ikeuchi M, Matsuoka M, Harada S, Mitsuya H, Kimura S, Oka S : Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naive patients. Virology 327 : 215-224, 2004.

2) Mitsuya H, Erickson J : Discovery and development of antiretroviral therapeutics for HIV infection, *In* Merigan TC, Bartlet JG, Bolognesi D eds, Textbook of AIDS Medicine, Williams & Wilkins, Baltimore, p 751-p 780, 1999.

3) Doyon L, Croteau G, Thibeault D, Poulin F, Pilote L, Lamarre D : Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. J Virol 70 : 3763-3769, 1996.

4) Gatanaga, H, Suzuki Y, Tsang H, Yoshimura K, Kavlick MF, Nagashima K, Gorelick RJ, Mardy S, Tang C, Summers MF, Mitsuya H : Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. J Biol Chem 277 : 5952-5961, 2002.

5) Tamiya S, Mardy S, Kavlick MF, Yoshimura K, Mistuya H : Amino acid insertions near Gag cleavage sites restore the otherwise compromised replication of human immunodeficiency virus type 1 variants resistant to protease inhibitors. J Virol 78 : 12030-12040, 2004.

Both Regulatory T Cells and Antitumor Effector T Cells Are Primed in the Same Draining Lymph Nodes during Tumor Progression[1]

Toru Hiura,[2]* Hiroshi Kagamu,[2,3]* Satoru Miura,* Akira Ishida,* Hiroshi Tanaka,* Junta Tanaka,* Fumitake Gejyo,* and Hirohisa Yoshizawa†

The peripheral tolerance mechanism prevents effective antitumor immunity, even though tumor cells possess recognizable tumor-associated Ags. Recently, it has been elucidated that regulatory T cells (Treg) play a critical role in maintaining not only self-tolerance, but also tolerance of tumor cells. However, because the Treg that maintain self-tolerance arise naturally in the thymus and are thought to be anergic in peripheral, it is still unclear where and when Treg for tumor cells are generated. In this study we analyze tumor-draining lymph nodes (LNs) and demonstrate that both antitumor effector T cells and Treg capable of abrogating the antitumor reactivity of the effector T cells are primed in the same LNs during tumor progression. The regulatory activity generated in tumor-draining LNs exclusively belonged to the CD4$^+$ T cell subpopulation that expresses both CD25 and a high level of CD62L. Forkhead/winged helix transcription factor gene expression was detected only in the CD62L^{high}CD4$^+$CD25$^+$ T cells. CD62L^{high}CD4$^+$CD25$^+$ Treg and CD62L^{low}CD4$^+$CD25$^+$ T cells, which possess effector T cell functions, had comparable expression of LFA-1, VLA-4, CTLA-4, lymphocyte activation gene-3, and glucocorticoid-induced TNFR. Thus, only CD62L expression could distinguish regulatory CD4$^+$CD25$^+$ cells from effector CD4$^+$CD25$^+$ cells in draining LNs as a surface marker. The Treg generated in tumor-draining LNs possess the same functional properties as the Treg that arise naturally in the thymus but recognize tumor-associated Ag. CD62L^{high}CD4$^+$CD25$^+$ Treg contained a subpopulation that expressed CD86. Blocking experiments revealed that ligation of CTLA-4 on effector T cells by CD86 on Treg plays a pivotal role in regulating CD4$^+$ effector T cells. *The Journal of Immunology,* **2005, 175: 5058–5066.**

The purpose of the immune system is to discriminate and eliminate invading nonself. To do this, it possesses not only an effector system to eliminate nonself, but also a regulatory system that abrogates the attack of effector cells against self-somatic cells. Thus, the balance between immunity and tolerance determines the outcome of an immune reaction. Although tumor cells have Ags altered by mutation, a lack of danger signals and antigenic similarity to self-somatic cells, from which tumor cells are derived, engage the peripheral tolerance mechanism (1, 2). This tolerance makes it difficult to obtain effective antitumor immunity.

Recent studies revealed CD4$^+$ T cells that constitutively express CD25 to play a critical role in maintaining peripheral tolerance during infection, transplantation, autoimmunity, and tumor immunity (2–7). CD4$^+$CD25$^+$ regulatory T cells (Treg)[4] that arise naturally in the thymus to maintain self-tolerance are considered anergic in peripheral (8). However, it has been demonstrated that Treg proliferate in peripheral tissues in response to antigenic stimulation and can be converted from naive CD4$^+$ T cells (9, 10). Thus, it is still unclear where and when Treg are generated for tumor cells.

APCs that acquire Ags migrate into secondary lymphoid organs, where the Ag information is converted to adaptive immune responses. Although CD4$^+$CD25$^+$ is the best surrogate marker, it is difficult to distinguish Treg based on CD25 expression, especially in lymph nodes (LNs) where T cell priming is going on, because the expression of CD25 is also up-regulated on effector T cells upon TCR engagement before clonal expansion. This problem makes it impossible to analyze how regulatory T cells are primed in LNs. We reported that effector T cells, which are capable of mediating antitumor reactivity, are primed in LNs draining growing tumors and that these T cells exclusively belong to the CD62L^{low} subpopulation (11). Additional CD40 stimulation as help signals for APC resulted in increased numbers of CD62L^{low} T cells in draining LNs (12, 13). CD62L^{high} T cells have been considered naive cells that have never been encountered by cognate Ag. However, our findings suggested that CD62L^{high} tumor-draining LN T cells contained a regulatory subpopulation, because the elimination of CD62L^{high} cells promotes the generation of highly potent antitumor T cells upon stimulation with CD3 (14).

*Graduate School of Medical and Dental Sciences, Course for Biological Functions and Medical Control, Department of Homeostatic Regulation and Development, Division of Respiratory Medicine, Niigata University, Niigata, Japan; and †Bioscience Medical Center, Niigata University Medical and Dental Hospital, Niigata, Japan

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[2] T.H. and H.K. equally contributed to this work.

[3] Address correspondence and reprint requests to Dr. Hiroshi Kagamu, Division of Respiratory Medicine, Department of Homeostatic Regulation and Development, Course for Biological Functions and Medical Control, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510, Japan. E-mail address: hkagamu@med.niigata-u.ac.jp

[4] Abbreviations used in this paper: Treg, regulatory T cell; CM, complete medium; DC, dendritic cell; Foxp3, forkhead/winged helix transcription factor gene; GITR, glucocorticoid-induced TNFR; LAG3, lymphocyte activation gene-3; LN, lymph node; m, murine.

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Recent studies have demonstrated that CD62L^{high} CD4$^+$CD25$^+$ T cells possess superior suppressive activity (15–18).

In this study we demonstrate that the expression of CD62L distinguishes regulatory CD4$^+$CD25$^+$ cells from effector CD4$^+$CD25$^+$ T cells and that both antitumor effector T cells and regulatory T cells, which are capable of abrogating the therapeutic efficacy of the antitumor effector T cells in vivo, are primed in the same tumor-draining LNs with different kinetics. CTLA-4 ligation by CD86 exclusively expressed on regulatory CD62L^{high}CD4$^+$ CD25$^+$ LN T cells plays a pivotal role in regulating effector CD4$^+$ T cell functions via direct T-T interaction.

Materials and Methods

Mice

Female C57BL/6J (B6) mice were purchased from CLEA Laboratory. They were maintained in a specific pathogen-free environment and used for experiments at the age of 8–10 wk. All animal experiments were conducted with the permission of the Niigata University ethics committee for animal experiments.

Tumors

MCA 205 is a fibrosarcoma of B6 origin induced by i.m. injection of 3-methylcholanthrene (19). Single-cell suspensions were prepared from solid tumors by enzymatic digestion as described previously (20). An MCA 205 tumor cell line was established and maintained in vitro.

mAbs and flow cytometry

Hybridomas producing mAbs against murine CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), CD3 (2C11), and murine CD62L (MEL14) were obtained from American Type Culture Collection. Anti-CD4 mAb, anti-CD8 mAb, and anti-CD62L mAb were produced as ascites fluid from sublethally irradiated (500 cGy) DBA/2 mice. PE-conjugated anti-CD80 (16-10A), anti-CD86 (GL1), anti-CD62L (MEL14), anti-CTLA-4 (UC10-4F10-11), anti-lymphocyte activation gene-3 (anti-LAG3; C9B7W), anti-CD8 (2.43), and anti-CD25 (PC61) mAbs and FITC-conjugated anti-Thy1.2 (30-H12), and anti-CD4 (GK1.5) mAbs were purchased from BD Pharmingen. PE-conjugated anti-glucocorticoid-induced TNFR (anti-GITR; 108619) mAb was purchased from R&D Systems. Analyses of cell surface phenotypes were conducted by direct immunofluorescent staining of 0.5–1 × 10^6 cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan flow microfluorometer (BD Biosciences). PE-conjugated subclass-matched Abs used as isotype controls were also purchased from BD Pharmingen.

Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Wako Pure Chemical Industries). To yield highly purified (>90%) cells with down-regulated CD62L expression (CD62L^{low}), LN T cells were further isolated by a panning technique using T-25 flasks precoated with goat anti-rat Ig Ab (Jackson ImmunoResearch Laboratories)/anti-CD62L mAb (MEL14) and sheep anti-rat-Ig Ab/anti-CD62L mAb-coated Dynabeads M-450 (Dynal Biotech). T cells with high CD62L expression (CD62L^{high}) were obtained as cells attached to flasks coated with goat anti-rat Ig Ab/anti-CD62L mAb. In some experiments cells were also separated into CD4$^+$ and CD8$^+$ cells by depletion using magnetic beads as described previously (14). For in vitro experiments, highly purified CD4$^+$ cells were obtained using anti-CD4 mAb-coated Dynabeads and Detachabeads (Dynal Biotech) according to the manufacturer's instructions. CD25$^+$ cells were isolated using PE-conjugated anti-CD25 mAb and anti-PE microbeads (Miltenyi Biotec) according to the manufacturer's directions. Cell purity was >90%.

Bone marrow-derived dendritic cells (DCs)

DCs were generated from bone marrow cells as described previously. In brief, bone marrow cells obtained from femurs and tibias of naive mice were placed in T-75 flasks for 2 h at 37°C in complete medium (CM) containing 10 ng/ml rmGM-CSF (a gift from KIRIN). Nonadherent cells were collected by aspirating the medium and were transferred into fresh flasks. On day 6, nonadherent cells were harvested by gentle pipetting. CM consists of RPMI 1640 medium supplemented with 10% heat-inactivated LPS-qualified FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (all from Invitrogen Life Technologies), and 5 × 10^{-5} M 2-ME (Sigma-Aldrich).

Tumor-draining LN cells

B6 mice were inoculated s.c. with 2 × 10^6 MCA 205 tumor cells in both flanks. Inguinal LN draining tumors were harvested. Single-cell suspensions were prepared mechanically as described previously (20).

Adoptive immunotherapy

B6 mice were injected s.c. with 1.5 × 10^6 MCA 205 tumor cells in 100 μl of HBSS to establish s.c. tumors. Three days after inoculation, mice were sublethally irradiated (500 cGy) and then infused i.v. with T cells isolated from tumor-draining LNs. Perpendicular diameters of s.c. tumors were measured with calipers. The significance of differences in the diameters between groups was analyzed by Student t test. A value of $p < 0.05$ was considered significant.

Cytokine ELISA

T cells were stimulated with immobilized anti-CD3 mAb or tumor Ag-pulsed bone marrow-derived DCs in CM. Supernatants were harvested and assayed for mouse IFN-γ content by a quantitative sandwich enzyme immunoassay using a mouse IFN-γ ELISA kit (Genzyme) according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated from T cells using Isogen (Nippon Gene) and used for cDNA synthesis. The cDNAs were used as templates for PCR (94°C for 2 min, 58°C for 30 s, and 72°C for 1.5 min), and 35 cycles were performed using primers specific for forkhead/winged helix transcription factor gene (*foxp3*; forward, 5′-GGCCCTTCTCCAGGACAGA-3′; 5′-GCTGATCAT GGCTGGGTTGT-3′). To ensure the quality of the product, RT-PCR was also performed using primers specific for β_2-microglobulin.

Proliferation assay

T cells isolated from tumor-draining LNs were stimulated with immobilized anti-CD3 mAb for 48 h in 2 ml of CM on 24-well plates at 2 × 10^6/ml. CD62L^{low} T cells were labeled with 5 μM CFSE (Molecular Probes) in HBSS at 37°C for 15 min and washed twice before CD3 stimulation. The ratio of CD62L^{low} T cells to CD62L^{high} CD4$^+$CD25$^+$ T cells was 2:1. After a 48-h stimulation, cells were counted and washed twice with HBSS. Then, T cells were cultured in CM supplemented with 10 U/ml human rIL-2 (gift from Shionogi) at 1 × 10^5/ml. Three wells were analyzed for each condition.

Results

CD62L^{high} T cells derived from tumor-draining LNs, but not from naive spleens, abrogated antitumor reactivity of CD62L^{low} LN T cells

It was believed that CD62L^{high} T cells are naive T cells; however, we reported that the elimination of CD62L^{high} T cells promotes the generation of highly potent antitumor CD4$^+$ T cells upon stimulation with CD3. To determine CD62L^{high} LN T cells possess activity to abolish the antitumor reactivity of effector T cells primed in tumor-draining LNs, mice with established s.c. tumors were infused with 2 × 10^6 CD62L^{low} LN T cells in the presence or the absence of 10 × 10^6 CD62L^{high} T cells. T cells were isolated from LNs draining growing MCA 205 tumors for 12 days or from spleens of naive mice. The ratio of CD62L^{low} to CD62L^{high} T cells was approximately the same as that in LNs, because 15–25% of all T cells were CD62L^{low} in 12-day tumor-draining LNs. As shown in Fig. 1*b*, 2 × 10^6 CD62L^{low} T cells alone successfully mediated the antitumor efficacy to regress s.c. tumor growth. In contrast, the s.c. tumor growth curve of the mice infused with 2 × 10^6 CD62L^{low} T cells in the presence of 10 × 10^6 CD62L^{high} T cells derived from tumor-draining LNs was identical with that of the no treatment group. CD62L^{high} T cells derived from naive splenocytes did not affect the antitumor reactivity of CD62L^{low} tumor-draining LN T cells. Thus, CD62L^{high} T cells of tumor-draining LNs contain a subpopulation that is capable of abrogating the antitumor reactivity of effector T cells primed in the same tumor-draining LNs.

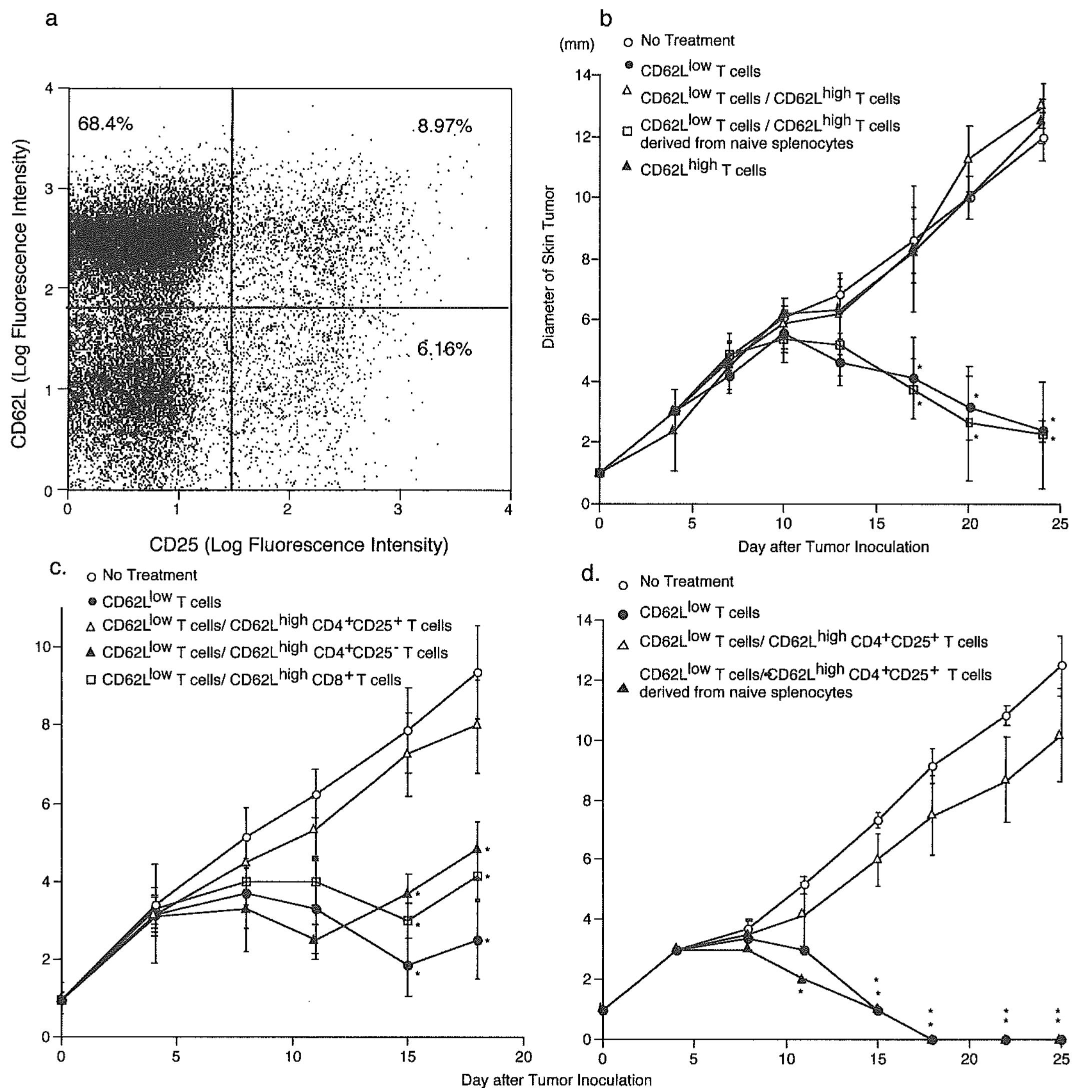

FIGURE 1. *a*, CD62L and CD25 expression of CD4^{+} T cells in 12-day tumor-draining LNs. LN cells were stained with FITC-conjugated anti-CD4 mAb, PE-conjugated anti-CD62L mAb, and PerCP-conjugated anti-CD25 mAb. Gated CD4^{+} cells were analyzed with a microfluorometer. *b–d*, Subcutaneous tumor growth of mice infused with CD62L^{low} T cells in the presence or the absence of CD62L^{high} T cells. Two million MCA 205 tumor cells were injected s.c. along the midline of the abdomen to establish s.c. tumors. Three days later, mice were adoptively infused i.v. with 2×10^6 CD62L^{low} T cells alone or with 10×10^6 CD62L^{high} T cells after sublethal whole body irradiation (500 cGy; *b*). CD62L^{low} T cells were isolated from LNs draining growing MCA 205 s.c. tumors for 12 days. CD62L^{high} T cells were isolated from tumor-draining LNs or naive splenocytes. *c* and *d*, CD62L^{high} T cells were further fractionated according to CD4, CD8, and CD25 expression using magnetic beads. One million fractionated CD62L^{high} T cells were infused into mice bearing established 3-day s.c. tumors with 2×10^6 (*c*) or 4×10^6 (*d*) CD62L^{low} LN T cells. Diameters of s.c. tumors were measured twice weekly with calipers, and size was recorded as the average of two perpendicular diameters. Statistical analyses were performed with Student's *t* test. *, $p < 0.01$ compared with the no treatment group. Each group contained five mice.

CD4^{+}CD25^{+} subpopulation of CD62L^{high} tumor-draining LN T cells mediated regulatory functions

Because CD4^{+}CD25^{+} is the best surrogate marker for regulatory T cells identified to date, CD4^{+}CD25^{+}, CD4^{+}CD25^{-}, or CD8^{+} CD62L^{high} T cells isolated from tumor-draining LNs were infused with CD62L^{low} LN T cells into mice bearing established s.c. tumors to determine which subpopulation of CD62L^{high} T cells mediates regulatory functions. In Fig. 1*c*, the antitumor efficacy of 2×10^6 CD62L^{low} T cells was not enough to cure the s.c. tumor, which, after starting to regress, eventually grew in the mice. Neither CD62L^{high}CD4^{+}CD25^{-} nor CD62L^{high}CD8^{+} T cells showed any additive antitumor or regulatory activity. The s.c. tumor growth curves showed no significant differences from the curve for the mice who received CD62L^{low} T cells alone. In contrast, CD62L^{high}CD4^{+}CD25^{+} T cells abolished the antitumor efficacy of effector T cells, resulting in a growth curve identical with that of the no treatment group. Thus, the CD4^{+}CD25^{+} subpopulation of CD62L^{high} LN T cells is probably made up of Treg cells, whereas CD62L^{high}CD4^{+}CD25^{-} and CD8^{+} cells are functionally irrelevant naive T cells. Moreover, Fig. 1*d* shows that 1×10^6 CD62L^{high}CD4^{+}CD25^{+} tumor-draining LN T cells were capable of abrogating the antitumor reactivity of 4×10^6 CD62L^{low} LN T cells, which was sufficient to cure established 3-day s.c. tumors. In contrast, the same number of CD62L^{high}CD4^{+}CD25^{+} T cells derived from naive spleens, which are considered resident Treg (21), did not influence the therapeutic efficacy of antitumor effector T cells in vivo.

In 12-day MCA 205 s.c. tumor-draining LNs, ~20% of T cells are CD62L^{low}, and 5–7% are CD62L^{high}CD4^{+}CD25^{+} (Fig. 1*a*). The ratio of CD62L^{low} to CD62L^{high} CD4^{+}CD25^{+} T cells is

3–4:1. Thus, it seems that Treg cells sufficient to abolish the antitumor reactivity of primed effector T cells are generated in tumor-bearing hosts.

CD62L^{high}CD4$^+$CD25$^+$ inhibited IFN-γ production by either CD4$^+$ or CD8$^+$ CD62L^{low} T cells via direct T-T interaction

To test whether CD62L^{high}CD4$^+$CD25$^+$ T cells generated in tumor-draining LNs influence cytokine production, we measured the amount of IFN-γ produced by 1×10^5 CD62L^{low} T cells in the presence or the absence of 5×10^4 CD62L^{high}CD4$^+$CD25$^+$ T cells in 200 μl of CM on 96-well plates. As shown in Fig. 2*a*, CD62L^{high}CD4$^+$CD25$^+$ T cells in tumor-draining LNs abolished the Ag-specific production of IFN-γ by tumor-draining LN effector T cells stimulated with 5×10^4 tumor Ag-loaded DCs. As shown in Fig. 2*b*, CD62L^{high}CD4$^+$CD25$^+$ T cells derived from naive splenocytes did not affect the production of IFN-γ stimulated by tumor-associated Ag, although they inhibited IFN-γ production in the presence of nonspecific stimulation with immobilized anti-CD3 mAb (data not shown).

Next, we examined whether this suppression of cytokine production can be reproduced without APC and tested whether it is cell-cell contact dependent, because the Treg naturally arise in the thymus to maintain self-tolerance. One million CD62L^{low} LN T cells on the bottom of 24-well plates were cocultured with 0.5×10^6 CD62L^{high}CD4$^+$CD25$^+$ T cells on either 0.4-μm pore size Transwell inserts (Costar) or the bottom of plates in 0.5 ml of CM. Both 24-well plates and Transwell inserts were coated with anti-CD3 mAb. As shown in Fig. 2, *c* and *d*, CD62L^{high}CD4$^+$CD25$^+$ Treg cells abrogated IFN-γ production by either CD8$^+$ or CD4$^+$ effector T cells in the absence of APC upon stimulation with CD3, and the suppression was dependent on cell-cell contact.

CD62L^{high}CD4$^+$CD25$^+$ T cells abrogated proliferation of both CD4$^+$ and CD8$^+$CD62L^{low} T cells

To elucidate whether CD62L^{high}CD4$^+$CD25$^+$ T cells generated in tumor-draining LNs inhibit cell proliferation, a T cell proliferation assay was performed as described in *Materials and Methods*. CFSE-labeled CD62L^{low} T cells stimulated with immobilized anti-CD3 mAb increased the total number of cells by 7-fold during a 3-day culture period accompanied by a reduction in the intensity of CFSE (Fig. 3, *a* and *b*). CD62L^{high}CD4$^+$CD25$^-$ T cells did not affect CD62L^{low} T cell proliferation, because the total number of cells increased and the reduction in intensity of CFSE intensity during the 3-day culture was identical with that of CD62L^{low} T cells alone. In contrast, CD62L^{low} T cells stimulated in the presence of CD62L^{high}CD4$^+$CD25$^+$ T cells did not proliferate at all. CFSE intensity did not change during the 3-day culture. Fig. 3*c* demonstrates the relative number of CD8$^+$ or CD4$^+$ cells according to phenotypic analysis. Thus, CD62L^{high}CD4$^+$CD25$^+$ T cells generated in tumor-draining LNs have the ability to abrogate the proliferation of both CD4$^+$ and CD8$^+$ T cells.

CD62L^{low}CD4$^+$CD25$^+$ LN T cells had effector, but not regulatory, functions

To examine the properties of CD62L^{low}CD4$^+$CD25$^+$ T cells, which comprise 20–30% of the CD62L^{low} T cell population in 12-day tumor-draining LNs, we tested whether CD62L^{low} CD4$^+$CD25$^+$ LN T cells affect IFN-γ production by CD62L^{low}CD4$^+$CD25$^-$ T

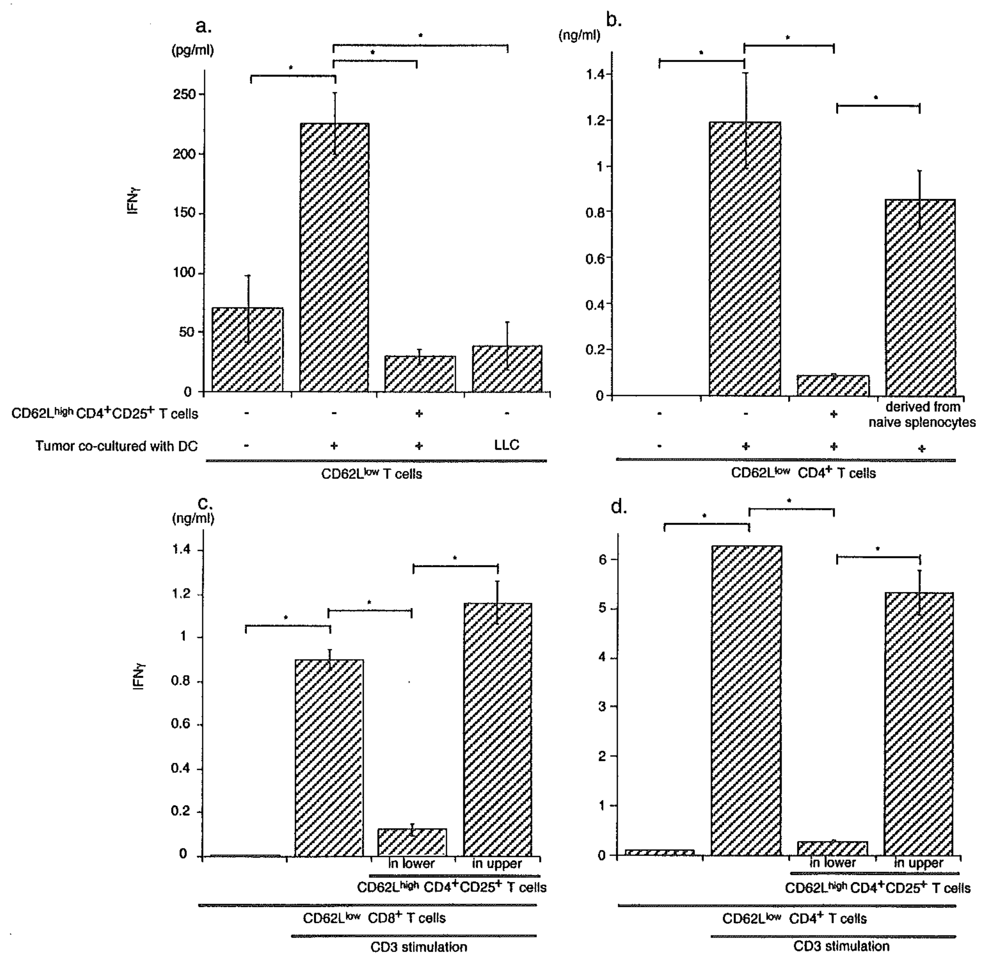

FIGURE 2. Measurement by ELISA of IFN-γ secreted in the medium. CD62L^{low} T cells 1×10^5 were cocultured with 0.5×10^5 DCs in the presence or the absence of 0.5×10^5 CD62L^{high}CD4$^+$CD25$^+$ T cells for 72 h in 200 μl of CM in 96-well plates (*a* and *b*). Before coculture with T cells, DCs were incubated with 5000 cGy-irradiated MCA 205 or LLC tumor cells overnight. The amount of IFN-γ produced by 1×10^6 CD62L^{low}CD8$^+$ or CD4$^+$ T cells cultured with 0.5×10^6 CD62L^{high}CD4$^+$CD25$^+$ T cells upon CD3 stimulation is shown in *c* and *d*. CD62L^{low} T cells on the bottom of 24-well plates were cultured with CD62L^{high}CD4$^+$CD25$^+$ T cells either on 0.4-μm pore size Transwell inserts or on the bottom. Both the Transwell inserts and 24-well plates were coated with anti-CD3 mAb. T cells were isolated from LNs draining growing MCA 205 s.c. tumors for 12 days. Three wells were analyzed for each condition. ELISA was performed in duplicate. Statistical analyses were performed by Student's *t* test. *, $p < 0.01$.

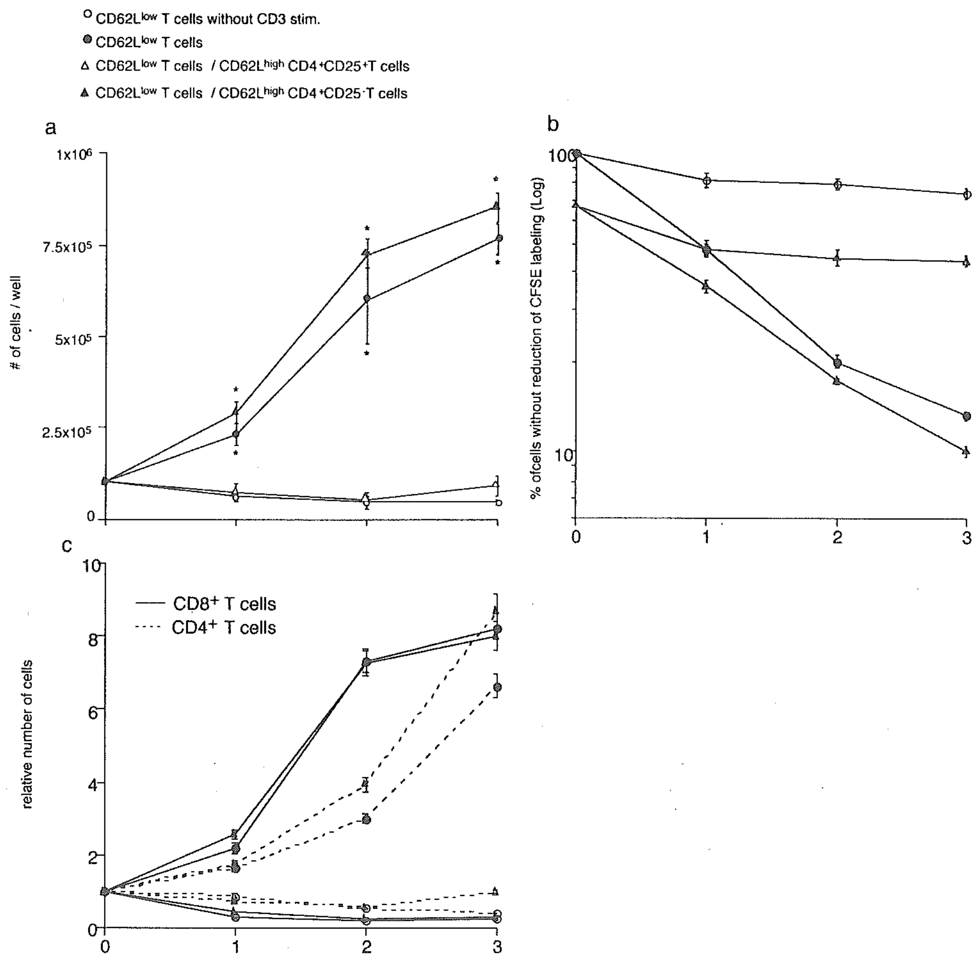

FIGURE 3. T cell proliferation was evaluated in the presence of 10 U/ml IL-2 after stimulation with CD3. CD62L^{low} T cells isolated from tumor-draining LNs were labeled with CFSE and cultured alone or with CD62L^{high}CD4^{+} LN T cells at 2×10^6/ml in 2 ml of CM on 24-well plates coated with anti-CD3 mAb for 48 h. The ratio of CD62L^{low} to CD62L^{high} cells was 2:1. After CD3 stimulation, T cells were harvested and counted, then recultured at 0.1×10^6/ml in 2 ml of CM supplemented with 10 U/ml IL-2 on new 24-well plates. T cells were counted and analyzed with a microfluorometer every 24 h. *a*, Total number of cells per well. *b*, Percentage of T cells that still possess high levels of CFSE labeling and represent cells without division. *c*, Relative number of CD8^{+} or CD4^{+} T cells. Three wells were analyzed for each condition. *, $p < 0.01$ compared with the no stimulation group.

cells. CD62L^{high}CD4^{+}CD25^{+} T cells completely inhibited the production of IFN-γ by CD62L^{low}CD4^{+}CD25^{-} T cells (Fig. 4), whereas the addition of CD62L^{low}CD4^{+}CD25^{+} T cells increased production. Moreover, CD62L^{low}CD4^{+}CD25^{+} T cells alone produced the same amount of IFN-γ as CD62L^{low}CD4^{+}CD25^{-} T cells. In contrast, CD62L^{high}CD4^{+}CD25^{+} T cells produced no IFN-γ. Thus, it is likely that CD62L^{low}CD4^{+}CD25^{+} cells possess effector T cell function, but not regulatory activity.

Foxp3 *mRNA expression was specific to CD62L^{high}CD4^{+}CD25^{+} T cells*

It has been reported that mutation of Foxp3 is responsible for immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance, a syndrome of systemic autoimmunity in humans (22, 23). It is now believed that Foxp3 is a master switch of regulatory functions (9, 24, 25). Thus, we tested whether fractionated T cells derived from tumor-draining LNs express mRNA for Foxp3. As depicted in Fig. 5*A*, only CD62L^{high}CD4^{+}CD25^{+} T cells expressed *foxp3* mRNA.

Functionally distinct CD62L^{high} and CD62L^{low} CD4^{+}CD25^{+} T cells expressed comparable levels of GITR, CTLA-4, LAG3, VLA-4, and LFA-1

Next, we analyzed the phenotype of fractionated T cells derived from tumor-draining LNs. Because it was demonstrated that CD4^{+}CD25^{+} Treg cells express GITR, CTLA-4, and LAG3 (26–29), we tested for these molecules and adhesion molecules that were important for T cell migration. Although CD62L^{high}CD4^{+}CD25^{+} regulatory LN T cells have an up-regulated expression of

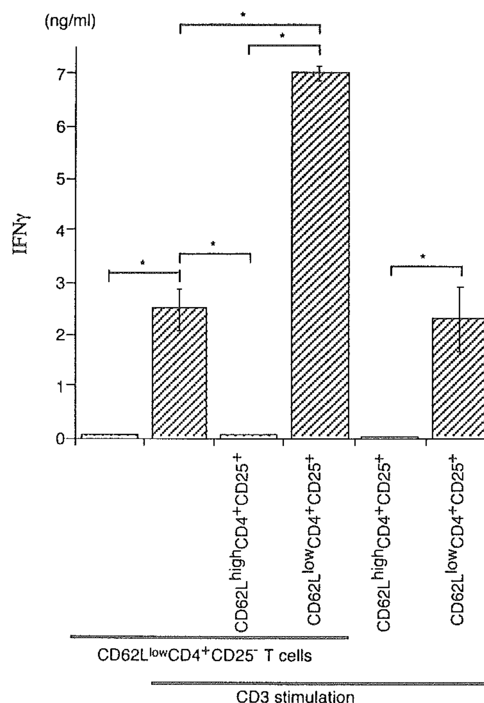

FIGURE 4. Measurement by ELISA of IFN-γ secreted in the medium. CD62L^{low}CD4^{+}CD25^{-} T cells alone (1×10^5) or with 0.5×10^5 T cells fractionated according to the expression of CD62L and CD25 were stimulated with immobilized anti-CD3 mAb in 200 μl of CM on 96-well plates for 48 h. *, $p < 0.01$.

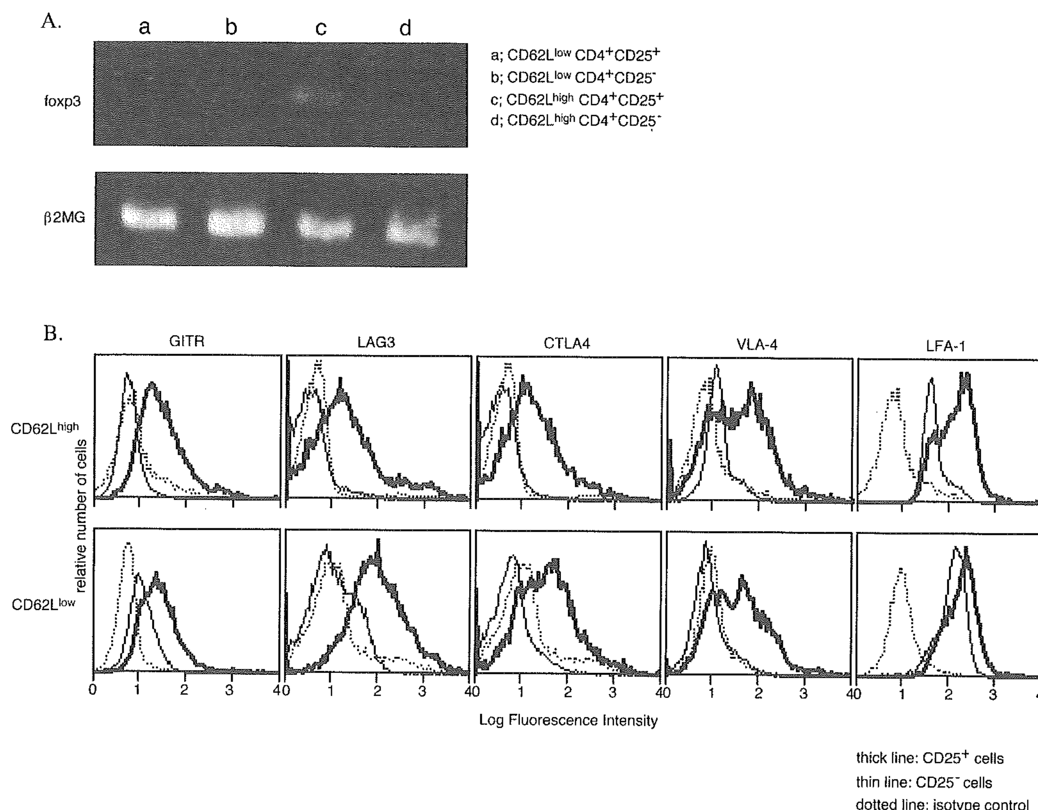

FIGURE 5. *A*, *Foxp3* mRNA expression in isolated CD62L^{low}CD4$^+$CD25$^+$, CD62L^{low}CD4$^+$CD25$^-$, CD62L^{high}CD4$^+$CD25$^+$, or CD62L^{high}CD4$^+$CD25$^-$ T cells derived from LNs draining MCA 205 s.c. tumors for 12 days. Total RNA was isolated from T cells and analyzed by RT-PCR for *foxp3*. β_2-Microglobulin gene expression is shown to confirm that equal amounts of RNA were used in each RT-PCR. Results shown are representative of three separate experiments. *B*, GITR, LAG3, CTLA-4, VLA-4, and LFA-1 expression on isolated T cells derived from MCA 205 tumor-draining LNs. Immediately after fractionation, T cells were double stained with PE-labeled anti-CD25 and FITC-conjugated anti-GITR, anti-LAG3, anti-CTLA-4, anti-VLA-4, anti-LFA-1, or isotype control Ab. Either CD25$^+$ or CD25$^-$ cells were gated for analyses. A total of 10^6 cells were analyzed for each sample. Each frame consists of 10,000 cells. Dotted lines indicate the isotype control.

GITR, CTLA-4, and LAG3, it is difficult to distinguish CD62L^{high}CD4$^+$CD25$^+$ T cells from CD62L^{low}CD4$^+$CD25$^+$ T cells, which possess effector T cell properties, from these molecules (Fig. 5*B*). Furthermore, CD62L^{high}CD4$^+$CD25$^+$ Treg cells and CD62L^{low}CD4$^+$CD25$^+$ effector T cells had a comparable upregulated expression of VLA-4 and LFA-1. In contrast, CD62L^{high}CD4$^+$CD25$^-$ T cells possessed the naive cell phenotype, such as no VLA-4, GITR, or CTLA-4, and a low level of LFA-1.

Different kinetics of CD62L^{high}CD4$^+$CD25$^+$ and CD62L^{low}CD4$^+$ T cell priming in LNs draining growing s.c. tumors

To address the priming of CD62L^{high}CD4$^+$CD25$^+$ and CD62L^{low}CD4$^+$ T cells in LNs draining growing MCA 205 s.c. tumors, we examined the number and phenotype of LN cells. Kinetic analysis revealed that the proportion of both CD62L^{low}CD4$^+$ CD25$^+$ and CD4$^+$CD25$^-$ T cells started to increase 7 days after s.c. tumor inoculation and peaked on the 11th day (Fig. 6). The percentage of CD62L^{low} T cells rapidly decreased, reaching the starting level by the 14th day. Although the proportion of CD62L^{high}CD4$^+$CD25$^+$ T cells started to increase 7 days after s.c. tumor inoculation, like that of CD62L^{low} cells, it kept increasing until the total number of LN cells started to decrease. The increase in CD62L^{high}CD4$^+$CD25$^+$ T cells was not caused by a nonspecific accumulation of CD62L^{high} cells, because the proportion of CD62L^{high}CD4$^+$CD25$^-$ naive T cells decreased in tumor-draining LNs. Hence, it is likely that CD62L^{high}CD4$^+$CD25$^+$ Treg cells underwent clonal expansion in tumor-draining LNs during tumor progression.

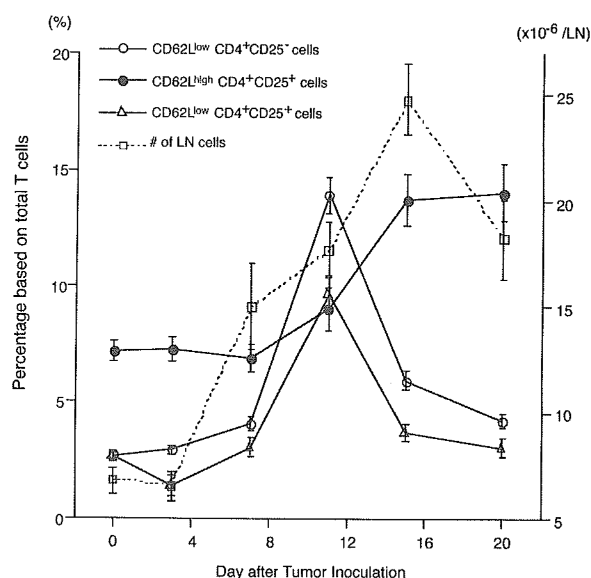

FIGURE 6. Kinetics of cellularity and the ratio of the indicated T cell subpopulations based on total T cells in LNs draining growing MCA 205 s.c. tumors. MCA 205 tumor cells (1.5×10^6) were inoculated s.c. into both flanks of mice. Inguinal LNs were harvested from three mice serially 0, 3, 7, 11, 15, and 20 days after s.c. injection and analyzed.