感染例から分離したウイルスからの感染性分子クローンを新たに加え、また抗原濃度を各社新規開発診断キットの公称感度近傍の低濃度域に振り現在認可承認され市販されている第４世代 HIV 抗原・抗体同時測定診断キットの各種 HIV-1 subtype/CRF 抗原検出感度比較を行ない、樹立した感染性分子クローンが標準品として有用か否か更に検討した。

B. 研究方法

HIV-1 ウイルスのクローニングおよび Long PCR と全長ゲノム Plasmid 構築は先に報告した HIV 感染価測定系 Indicator 細胞 MAGIC-5/HeLa4.5 nEGFP 細胞株を用いた HIV ウイルスクローニングと感染性クローンの構築系(HIV Trapping System；HIV 捕捉実験系)により行った。対象としたウイルスは、米国 NIH, NIAID, AIDS Research and Reference Reagent Program から分与を受けた subtype C, A, CRF01_AE ウイルス、ガーナ国野口記念医学研究所との共同研究で分離した CRF02_AG, subtype G ウイルス及び国立感染症研究所エイズ研究センター第２グループにより国内感染者から分離されたウイルスである。本年度は特に昨年度の成績において各種キット間で検出感度の差異が認められた subtype A と国内感染例から流行している subtype B, CRF01_AE ウイルスと、近年南米で流行している BF 組換え体ウイルス由来の感染性分子クローン 12 クローンを加え総計 32 クローンの感染性分子クローン p24 gag 抗原パネルを作成した。

まず末梢血リンパ球の共培養で分離したウイルスを直接感染させた MAGIC-5A 細胞からゲノム DNA を抽出し，それを鋳型として Long PCR を行い 5'-および 3'-側のプロウイルスゲノムを増幅，それらを連結することによって完全長の DNA クローンを得た。完全長 DNA クローン作製の戦略は患者ウイルスの RT-PCR 法によって得られていた pol 領域における Rare cutter の制限酵素を選定し、その塩基配列を含む Primer で HIV-1 genome pol 下流領域を増幅し pMT1 に組込み、pol 上流領域を増幅した断片を酵素処理後組込む「Half & Half 戦略」を用いて全長クローンを得た。得られたクローンは HeLa4.5nEGFP 細胞に transfection しその培養上澄を MAGIC-5A 細胞にかけ感染性を確認した。

現在まで樹立した感染性分子クローンのうち国内で感染例の多い subtype/CRF を含む多くの種類を選定し FuGENE 6 (Roche) にて 293T 細胞へ transfect し培養２日後に培養上澄を回収し、その感染価を HIV 感染価測定細胞株 MAGIC-5A で測定した。感染性ウイルス液を Triton X-100 処理して感染性を失活させ、その HIV-1 p24 CA gag 抗原量を「ルミパルス HIV-1 p24」キット（富士レビオ）で測定し、各感染性分子クローン由来ウイルス p24 抗原量を標準化した。米国 BBI 社の HIV/HBV/HCV 陰性ヒト・プール血漿 BaseMatrix を希釈液として、各ウ

イルス液の５もしくは１０倍希釈系列を作製した。国内で HIV-1 診断キットを販売している各社の協力を得て、この抗原液を配付し測定を依頼した。データを回収後、各キットの抗原検出感度を比較し、本ウイルス抗原液が HIV-1 抗原検出感度測定における標準品となり得るかについて検討した。

本研究では、血液などヒト臨床材料が使用される場合には，材料提供者の個人情報が漏出しないよう厳格なプライバシー保護に努める。このためヒト材料を用いた研究は連結不可能匿名化（unlinked anonymous）の手法を行いて個人情報の漏洩を防ぎ，患者の非特定性を保つ。また、研究方法および研究により生じうる研究対象者に対する不利益、危険性の排除について充分な説明を加え，守秘義務を守る。以上を遵守することで倫理面の問題は無いと判断した。

C. 研究結果

本年度は既に当室で樹立した種々の subtype/CRF HIV-1 感染性分子クローンから世界での流行状況と国内感染者での subtype 分布を踏まえて subtype A, B, C, D 及び G と、流行組換体 CRF01_AE、CRF02_AG 及び BF 組換え体から合計 32 クローンを選定した。各感染性分子クローンを transfection し２日間培養して回収し、TritonX-100 で可溶化した HIV-1 virion 全体の抗原液中における p24 gag 抗原量を「ルミパルス HIV-1 p24」キット（富士レビオ）で測定し抗原原液とした。現在各社新規開発診断キットの公称感度近傍の低濃度域の検出感度を測定する目的で、各クローンの p24 gag 抗原量を 100, 10 及び 1 pg/mL に HIV 抗原・抗体陰性の BaseMatrix で調整し、現在国内で市販されている HIV 抗原・抗体同時測定キットのメーカーの協力を得て配布の上測定を依頼した。得られた測定結果を集計し、比較を容易にするため、全ての数値を C.O.I (cut-off index)に換算し 1.0 以上を陽性と判定した（表)。又新たに欧州で事実上 HIV-1 p24 gag 抗原測定のデフォルトアッセイとされる HIV-1 p24 INNOTEST Assay による測定もドイツ Dade-Bhering 社研究所の協力を得て、同じパネルを送付して測定した。

その結果、現在市販されているキット、今後申請予定のキット、及び国内では市販されていないが欧米では既に認可を受け市販されている HIV 感染診断キット間でそれぞれの subtype/CRF の p24 gag 抗原に対する検出感度が比較可能であった。各診断キットは感染の有無を判定する定性的なもので必ずしも定量的なものではないが、感度の良いキットは高い C.O.I 値を示していることが判明した。また subtype/CRF に対する陽性・陰性判定は、いわゆるグレイゾーン（判定保留）となる場合も多いが、現在市販されている各社キットが公称する感度であるところの 100 pg/mL に沿うものであった。又新規開発の第４世代抗原・抗体同時測定キットでは公称する 10 pg/mL を検出感度とする

ものが多かった。しかしながら一部の会社の新規開発第４世代キットにおいては現在市販されているものを含めて、一部の subtype A, subtype C 及び AG 組換え体クローンにおいては 100 pg/mL の高濃度域でも検出できなかった。一方欧州で hIV-1 p24 gag 抗原測定計のデフォルトとされる INNOTEST Assay の結果は、今回測定した最も感度に優れた測定キットと同等な成績を示した。

D. 考察

本研究では、極めて多様性に富む病原体である HIV-1 p24 gag 抗原の各種 subtype/CRF の標準となりうるか、HIV-1 感染性分子クローンを用いての HIV-1 抗原・抗体同時測定診断キットの抗原検出感度比較を行った。現在の公的な HIV-1 抗原・抗体検出感度比較試験は、BBI 社などから購入した、同一感染者から採取した Seroconversion Panel を供試して行われている。しかしながら同一感染者から継時的に採取した一連の血清は量的に限られており、さらに感染した HIV-1 の subtype/CRF まで特定して整備することは不可能である。また Seroconversion 前の抗体陰性・抗原陽性期に当たる比較的短い期間の感染者を特定することも実際上困難であることから、抗原検出感度を適確に判断するために適当なパネルを整備することは至難な状況である。以上の観点から、今回検討した感染性分子クローンによる抗原パネルは、HIV-1 抗原診断キットの品質管理において貴重な標準品となり得る可能性が高いと考える。今回供試したクローンは様々な年代に分離された様々な subtype/CRF ウイルスを含んでいるが、ここ数年に国内で分離されたウイルスクローンも含んでいる。診断キットの検出標的抗原である HIV-1 gag p24 抗原のアミノ酸配列の系統樹から判断すると、各クローンはそれぞれの subtype/CRF の Reference Clone のクラスターに属している。なにより感染性分子クローンを主体とした標準パネルは原理上、量的な制限はなく、全ての標的抗原である p24 gag 抗原のアミノ酸配列が明らかである事からより標準としての資格がある。以上の検討結果から本感染性分子クローンパネルにより、各社診断キットの抗原検出感度を明確に比較できることから、本パネルは HIV-1 抗原検出キットの品質管理に貴重な標準品となり得ることが示唆された。

今回の検討で判明した重要な点は、この抗原パネルが市販されている（申請予定も含む）抗原・抗体同時測定系の診断キットにおける相対的な抗原検出感度を明確に判定できることである。ある一社の診断キットが subtype A, subtype C のみならず AG 組換え体のクローンで、高い抗原濃度でも検出できないことが明らかになった（表を参照）。欧州で抗原測定のデフォルトとされる抗原測定キットではそのようなクローンによる検出出来ない例がないことから、これらの検出不可能で

あったことは、この診断キットに特異的なものと判断される。HIV-1 gag p24 抗原検出感度は、第一義的には p24 抗原を捕捉するために用いる抗体の各種 subtype/CRF p24 抗原に対する affinity/avidity の差に起因するものと推測される。現在 HIV 診断キットの申請には抗原捕捉に用いる抗体の特異性の明示は求められていないが、今後は必要な事項になるかもしれない。このキットが、これら subtype A, subtype C 及び AG 組換え体クローンに対してのみ反応性が劣るのか、他の subtype A HIV-1 ウイルスに対しても感度が劣るのか、今後さらに subtype A などの感染性分子クローンの数を増やして再検討する必要がある価も知れない。また今回の感度比較は、各社新規開発キットの公称感度が p24 gag 抗原量で 10 pg/mL という算定から低濃度域を設定したが、感度の良い診断キットは最低濃度 10 pg/mL を全ての subtype/CRF で検出したことから、この成績はある種のキットが p24 gag 抗原検出感度が明らかに劣る事を示しているものと判断された。

現在 HIV 診断の第 1 次スクリーニングにおいて、抗体測定系よりウインドウ期を短縮することから推奨されている抗原・抗体同時測定系において種々の診断キットが、実際にその抗原検出感度に、これほどの相違があることは想定外であった。しかしながら HIV 感染症例を除外することを厳格に守らなければならない輸血行政と比較して、一般の HIV 感染診断における抗原・抗体同時測定系の優位性が抗原検出感度にあるならば、市販キットの性能検定を目的に改めて Control Survey を行い、その性能が明らかに劣るものは適切な行政指導が望まれる。一方、薬事法改正による申請方式の変更により、明らかに性能が向上した改良型感染診断キットの市場への導入が遅滞する状況に陥る事態にならないように迅速な審査体制構築が望まれる。周知のように HIV 感染拡大は本邦でも徐々に進行しており厚生行政上の観点から時期を失せず、この状況に適確な判断を下さなければならないものと考えられる。それには以前に増して科学的データに基づいた HIV 抗原標準パネルの整備が求められると考える。

E. 結論

種々の subtype/CRF HIV-1 ウイルスから樹立した感染性分子クローン由来の HIV-1 p24 抗原を市販キットの抗原検出感度比較に用いたところ、各キットの検出感度の測定に有用な標準品として使用できる可能性が示された。

健康危険情報

該当する事項はない。

F. 研究発表

該当無し。

Sensitivity Comparison of HIV-1 Combination Assays with A Panel of Various HIV-1 Subtype/CRF Infectious Molecular Clones

No	Subtype/ CRF	Infectious Clone	p24(pg/ml)	Manufacture	A	B	A	C	D	E	B	D	HIV p24 gag Standard in EU
					Kit 2	Kit 2	Kit 1	Kit 1	Kit 2	Kit 1.1	Kit 1	Kit 1	Innotest
					Approved	In prep	Approved	Approved	In prep	In prep	Approved	Approved	EU Approved
					CLIA	EIA	EIA	CLIA	EIA	ECLIA	EIA	EIA	EIA
					C.O.I.	C.O.I.	C.O.I.	C.O.I.	C.O.I.	C.O.I.	C.O.I.	C.O.I.	C.O.I.
1	A	p92UG031A2	100		51.20	8.25	18.89	19.45	0.14	3.78	1.71	0.12	6.49
2			10		6.17	1.68	2.40	2.20	0.19	0.66	0.27	0.10	1.38
3			1		0.62	0.63	0.61	0.45	0.12	0.33	0.11	0.07	0.11
4		p92UG029A4	100		61.74	9.34	18.50	22.87	2.82	9.74	6.13	1.88	60.40
5			10		6.76	2.51	2.48	2.79	0.40	1.26	0.86	0.29	12.73
6			1		0.72	0.72	0.71	0.52	0.15	0.40	0.17	0.10	0.97
7		p92RW008A4	100		116.12	10.07	32.69	37.48	0.21	20.91	3.37	2.85	65.55
8			10		12.17	3.38	4.49	4.66	0.14	2.37	0.50	0.51	24.45
9			1		1.43	0.80	0.87	0.71	0.12	0.50	0.13	0.11	2.39
10		p92UG037A35	100		104.48	10.62	29.67	36.89	9.73	14.43	8.25	2.73	63.96
11			10		11.41	3.53	4.01	4.31	2.05	1.71	1.26	0.43	18.69
12			1		1.30	0.84	0.79	0.68	0.33	0.44	0.22	0.11	1.95
13	B	pJPDR6073B68	100		52.76	9.03	14.56	20.81	4.07	7.52	1.60	1.76	54.03
14			10		6.56	2.08	1.87	2.51	0.61	1.02	0.25	0.28	9.22
15			1		0.68	0.67	0.60	0.49	0.17	0.37	0.11	0.09	0.63
16		pJPDR6075B50	100		39.73	8.06	12.77	21.04	9.23	6.61	3.39	2.40	47.53
17			10		4.25	1.76	1.74	2.43	1.45	0.94	0.48	0.37	7.82
18			1		0.50	0.64	0.58	0.48	0.25	0.36	0.14	0.10	0.55
19		pBa-L	100		31.28	7.60	12.88	20.18	9.20	6.35	1.38	2.18	47.50
20			10		3.15	1.53	1.70	1.96	1.83	0.87	0.21	0.34	7.87
21			1		0.43	0.61	0.55	0.48	0.30	0.35	0.11	0.10	0.51
22		pARES1	100		30.37	8.01	11.30	19.13	9.14	5.66	3.14	2.04	43.92
23			10		3.05	1.65	1.63	2.19	1.52	0.82	0.44	0.30	6.61
24			1		0.40	0.64	0.56	0.45	0.29	0.36	0.14	0.09	0.38
25		pLAI	100		31.92	9.54	11.79	24.87	11.02	6.15	3.41	2.08	43.95
26			10		3.20	2.17	1.56	2.75	2.04	0.86	0.46	0.31	6.51
27			1		0.42	0.72	0.56	0.48	0.34	0.36	0.14	0.10	0.56
28	C	pIndie-C1	100		33.14	8.42	9.74	6.81	9.99	7.08	4.07	2.08	49.39
29			10		3.27	1.81	1.41	0.88	1.84	0.94	0.49	0.30	7.62
30			1		0.41	0.65	0.50	0.33	0.32	0.38	0.12	0.09	0.39
31		p02ZMDBC33	100		34.11	7.95	12.98	14.92	9.47	6.31	2.13	1.95	43.44
32			10		3.44	1.64	1.78	1.69	1.84	0.88	0.29	0.30	6.12
33			1		0.48	0.61	0.56	0.43	0.29	0.36	0.10	0.08	0.35
34		p02ZM108C12	100		68.30	9.42	15.62	17.36	1.24	8.20	3.11	1.74	52.76
35			10		6.77	2.23	2.11	2.00	0.24	1.05	0.40	0.27	10.34
36			1		0.77	0.66	0.59	0.43	0.13	0.37	0.12	0.09	1.00
37		p02ZM110C7	100		26.88	5.93	9.02	10.01	0.66	3.26	0.74	1.00	49.48
38			10		2.54	1.20	1.22	1.21	0.18	0.59	0.15	0.16	7.26
39			1		0.36	0.56	0.52	0.37	0.13	0.33	0.09	0.08	0.52
40		p02ZM114C27	100		32.10	7.71	12.28	14.17	9.30	6.15	2.04	1.94	41.08
41			10		3.23	1.59	1.55	1.60	1.44	0.85	0.28	0.25	5.46
42			1		0.43	0.62	0.53	0.40	0.27	0.37	0.11	0.09	0.39
43	D	pNLD-T	100		33.70	8.17	12.88	12.86	8.56	6.71	2.27	1.71	42.00
44			10		3.35	1.73	1.71	1.47	1.22	0.91	0.31	0.25	6.73
45			1		0.48	0.64	0.56	0.40	0.22	0.36	0.11	0.09	0.53
46	G	p03GH175G12	100		53.32	8.03	13.08	11.32	0.73	8.54	1.34	1.05	39.90
47			10		6.29	1.69	1.63	1.32	0.16	1.07	0.20	0.17	5.24
48			1		0.61	0.64	0.53	0.37	0.12	0.38	0.11	0.08	0.22
49	AE	pJPDR5032AE2	100		59.38	8.59	15.19	19.92	11.30	8.54	3.12	2.31	50.16
50			10		5.76	1.73	1.97	2.19	2.45	1.08	0.40	0.34	8.19
51			1		0.72	0.55	0.57	0.47	0.38	0.38	0.12	0.10	0.64
52		pJPDR3730AE21	100		47.01	8.76	14.61	16.84	9.40	7.98	0.60	2.18	50.76
53			10		6.08	1.29	1.85	1.87	1.86	1.03	0.14	0.33	8.35
54			1		0.63	0.51	0.55	0.44	0.29	0.37	0.09	0.10	0.57
55		p93TH051AE13	100		48.45	7.69	13.79	17.63	9.28	7.56	3.26	2.30	48.27
56			10		4.87	1.49	1.81	1.97	2.01	0.98	0.42	0.35	7.08
57			1		0.62	0.53	0.56	0.44	0.31	0.37	0.12	0.10	0.40
58		p93TH054AE30	100		67.49	9.53	19.76	29.08	9.52	10.16	3.68	1.96	53.46
59			10		6.40	2.11	2.39	3.21	1.77	1.22	0.47	0.29	10.12
60			1		0.74	0.59	0.64	0.56	0.29	0.41	0.13	0.10	1.12
61		p93TH060AE62	100		52.59	8.49	13.95	22.39	8.96	7.77	3.37	2.30	49.79
62			10		5.10	1.66	1.92	2.44	2.09	1.03	0.45	0.35	7.99
63			1		0.62	0.56	0.57	0.49	0.35	0.39	0.12	0.10	0.48
64		p93TH062AE39	100		62.18	8.32	14.36	22.63	9.61	7.88	3.27	2.41	48.14
65			10		5.14	1.58	1.73	2.51	2.14	1.00	0.43	0.32	7.17
66			1		0.64	0.55	0.56	0.51	0.33	0.38	0.12	0.10	0.45
67		p93TH065AE25	100		49.18	8.18	13.35	21.66	9.30	7.58	3.21	2.12	48.47
68			10		4.79	1.56	1.73	2.36	1.98	0.99	0.43	0.34	7.87
69			1		0.59	0.55	0.56	0.47	0.32	0.37	0.13	0.10	0.70
70		pJPDR6824AE96	100		76.44	8.87	21.91	27.93	9.39	13.02	2.02	3.12	57.26
17			10		7.73	1.77	2.77	3.17	2.49	1.50	0.27	0.47	13.51
72			1		0.91	0.58	0.66	0.55	0.40	0.43	0.12	0.11	0.99
73		p93TH057AE18	100		78.52	5.66	17.62	48.39	11.14	11.37	3.37	3.28	57.88
74			10		7.96	1.00	2.24	5.64	4.11	1.33	0.44	0.53	13.95
75			1		0.89	0.49	0.61	0.97	0.56	0.41	0.13	0.12	1.11
76	AG	p97GHAG1	100		49.19	7.86	11.80	21.45	7.11	7.36	1.13	1.79	48.17
77			10		5.00	1.72	1.56	2.46	0.96	1.02	0.20	0.26	7.42
78			1		0.62	0.63	0.52	0.64	0.23	0.38	0.10	0.09	0.40
79		p97GHAG2	100		105.48	10.06	27.44	46.29	9.50	7.47	6.03	3.13	62.80
80			10		10.75	3.45	3.38	5.47	4.15	0.98	0.76	0.49	17.46
81			1		1.26	0.86	0.71	0.97	0.60	0.40	0.15	0.11	1.49
82		p03GH185AG10	100		13.50	4.76	4.80	10.56	6.16	2.81	1.35	0.84	22.75
83			10		1.38	1.02	0.90	1.40	0.78	0.54	0.21	0.14	2.36
84			1		0.22	0.56	0.43	0.52	0.20	0.34	0.10	0.08	0.53
85		p03GH188AG7	100		43.10	6.34	9.40	18.64	9.24	6.76	2.60	1.85	45.57
86			10		4.40	1.88	1.34	2.21	1.74	0.92	0.34	0.28	6.05
87			1		0.55	0.67	0.50	0.63	0.32	0.39	0.12	0.09	0.56
88		p03GH193AG15	100		23.29	6.48	7.84	14.80	5.71	3.37	1.66	0.90	29.84
89			10		2.37	1.33	1.20	1.88	0.74	0.62	0.25	0.15	3.04
90			1		0.33	0.61	0.49	0.57	0.19	0.35	0.11	0.08	0.31
91		p03GH196AG4	100		44.04	8.66	8.93	14.29	9.28	7.26	2.86	1.82	43.25
92			10		4.32	2.04	1.33	1.72	1.96	0.98	0.39	0.29	5.28
93			1		0.59	0.69	0.50	0.59	0.34	0.41	0.13	0.09	0.19
94	BF	pJPDR0769BF3	100		64.30	10.12	23.15	35.71	9.51	9.54	4.68	2.69	51.40
95			10		6.82	3.10	2.79	4.12	2.50	1.22	0.67	0.37	8.67
96			1		0.84	0.84	0.65	0.81	0.42	0.41	0.16	0.10	0.41
		No. of Positive			67	64	63	63	50	47	31	28	71

別紙５

研究成果の刊行に関する一覧表

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研究

臨床検査 52：111-115，2008

国内で販売されている抗HBs抗体定量用体外診断用医薬品の評価：国内標準品を用いた検討*

水落利明[1)]/小高千加子[1)]/山口一成[1)]

〔SUMMARY〕 国内で販売されている8種類の抗HBs抗体測定キットを用いて，抗HBsヒト免疫グロブリン(以下，抗HBs抗体)国内標準品の希釈系列検体を測定した．6種類のキットにおいてはほぼ期待される測定値を示した．しかしながら，期待値に比較して約0.5倍と1.5倍の測定値を示すキットが各1種類あった．このような乖離の原因を調査しその修正が行われた結果，1種類のキットは新規申請に向けて開発中であり，もう一種類のキットでは一部変更の手続きが行われている．これにより今後はHBV感染防御の基準と考えられる抗体価の"10 mIU/ml"という数値について，いずれのキットを用いてもほぼ均一な定量が可能になることが期待される．

〔KEYWORDS〕 HBV，抗HBs抗体測定キット，抗HBs抗体国内標準品

緒言

HBV(hepatitis B virus)感染者数は全国民の約2%にも及ぶと推定されており，B型肝炎は「国民病」とも言われている．HBV感染防御については，安全で副作用の少ないワクチンが既に開発され，わが国ではハイリスクグループおよびHBV感染者から出産した児に投与され効果をあげている．HBV感染防御に有効な抗HBs抗体価については，WHO(世界保健機関)やCDC(米国疾病予防管理センター)では10 mIU/mlという数値を提唱している[1~3]．日本国内においても同様な基準を採用するかについては議論の余地があるが，それ以前の問題として，現在国内で使用されている様々な抗HBs抗体測定キット(定量キット)が，お互いに乖離がない測定値を提供できるかについての検証がなされていない．本研究では，抗HBs抗体国内標準品(WHO国際標準品を用いて値付けしたもの)の希釈系列検体を，国内で販売されている8種類の測定キットを用いて測定し，その値を比較検討した．

材料および方法

1. 抗HBs抗体国内標準品

原料はグラクソスミスクライン社製ENGERIX-B®(HBs抗原のサブタイプはadwと考えられる)の接種により抗体価を高めた国外血由来の血漿である．内容物は白色粉末凍結乾燥品で，1バイアル(含湿度0.5%)を1.0 ml注射用水で溶解したとき320 IU/mlとなる．またpH＝6.94，浸透圧＝1.65，蛋白質含量＝29.2 mg/ml，免疫グロブリンG含量＝99.6%であり，HBsAg，HAV-RNA，HBV-DNA，HCV-RNA，HIV-1-RNAはすべて陰性である．なお本標準品の力価は国際標準品(WHO 1st，1977)を基にして値付けされ，2001年4月より国立感染症研究所から供与され

* Evaluation of anti-HBs antibody assay kits commercially available in Japan by utilizing the national standard

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表　使用した抗 HBs 抗体測定キットの名称，測定方法，および抗体検出に用いた HBs 抗原のサブタイプ

Kit No.	メーカー名	製品名	原理/方法
1	アボット・ジャパン	アキシム オーサブ・ダイナパック	EIA
2	アボット・ジャパン	アーキテクト・オーサブ	CLIA
3	アボット・ジャパン	IMx オーサブ・ダイナパック	EIA
4	オーソ・クリニカル・ダイアグノスティックス	ビトロス HBs 抗体	CLEIA
5	東ソー	E テスト「TOSOH」II (HBsAb)	EIA
6	デイドベーリング	エンザイグノスト Anti-HBs II	EIA
7	富士レビオ	ルミパルス II HBsAb	CLEIA
8	和光純薬	スフィアライト HBs 抗体	CLEIA

CLIA：chemiluminescent immunoassay(蛍光免疫法).
EIA：enzyme immunoassay(酵素免疫法).
CLEIA：chemiluminescent enzyme immunoassay(蛍光酵素免疫法).

ている.

2. 希釈系列作成手順

(1) 抗 HBs 抗体国内標準品(320 IU/vial)1 バイアルを 1 ml の精製水で溶解した.

(2) 標準品溶液 0.1 ml に検体希釈液 9.9 ml を加え均一な溶液にした(3,200 mIU/ml).

(3) 3,200 mIU/ml の溶液を 5 倍希釈(溶液 1 ml + 検体希釈液 4 ml)して 640 mIU/ml の抗体液(5 ml)を作成した.

(4) 以下，順次 2 倍希釈系列を作成した. (2.5 ml + 2.5 ml) 系列：0，10，20，40，80，160，320，640(mIU/ml).

3. 抗 HBs 抗体測定キット

抗 HBs 抗体測定に使用したキットは，表に示した 8 種類である．測定は各検体につき 3 重測定で行った.

結果

図 1 は各キットでの抗 HBs 抗体国内標準品希釈系列検体の測定結果である．いずれのキットも測定範囲内(0～640 mIU/ml)で期待値と測定値の関係において非常に良好な直線性が得られた(r^2 = 0.98～1.00)．しかし，キット #7 と #8 では直線の傾きがそれぞれ 1.7，および 0.56 と理想である 1.0 からの明らかな乖離がみられた．そこで，対表示値を算定し，それらの結果を図 2 にまとめた．ここでの対表示値とは，各検体の抗体濃度(期待値)と測定値の比を，各濃度ごとに計算しそれらを平均した値のことである．したがって理想的な対表示値は 100 となる.

図 2 に示したように，#1～#6 のキットではおおよそ 120 前後の対表示値を示しているが，図 1 の結果から予想されたように，キット #7，#8 の対表示値はそれぞれ 169.2 および 50.1 であり，100 からの乖離が大きかった．なお WHO や CDC が提唱している，HBV 感染防御に有効な免疫獲得の基準と考えられる 10 mIU/ml の低濃度検体を用いた測定値を比較したところ，図 2 に示した「対表示値」と良く相関していることが確認された．このような測定値乖離の原因について，それぞれのメーカーと協議した結果，以下の結論に達した．#7 においてはキットを製造する際の検討に，WHO 国際標準品の抗 HBs 抗体を基準にしていなかったことが原因であると結論された．そこで現在新たなキットが開発され，それを用いた測定では対表示値が 117.2 となった(未発表データ)．また #8 においては，乖離の原因は不明であるが，WHO 国際標準品を用いて見直しを行い，再測定の結果，対表示値が 88.4 となった(未発表データ，なおこのキットは現在一部変更申請中である).

考察

現在国内における抗 HBs 抗体価の表示には，凝集法による「管数」表示と，EIA (enzyme immunoassay)，CLIA (chemiluminescent immuno-

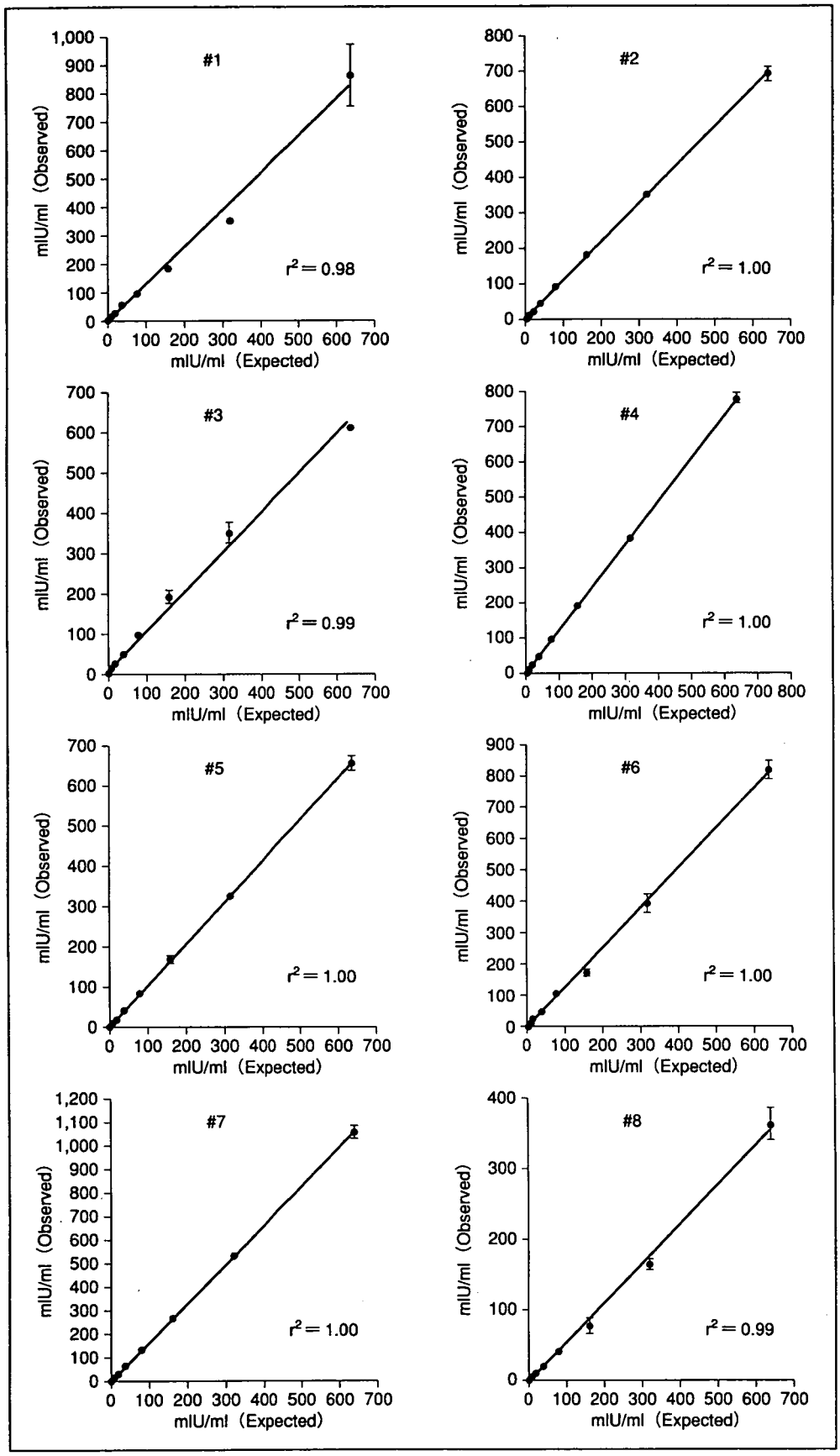

図1　各キットを用いた抗 HBs 抗体希釈系列検体の測定結果
表に示した8種類のキットを用いて抗 HBs 抗体国内標準品の希釈系列検体(0～640 mIU/ml)を測定した結果を表す．なお，図におけるキットの番号は，表におけるキットの番号に対応する．

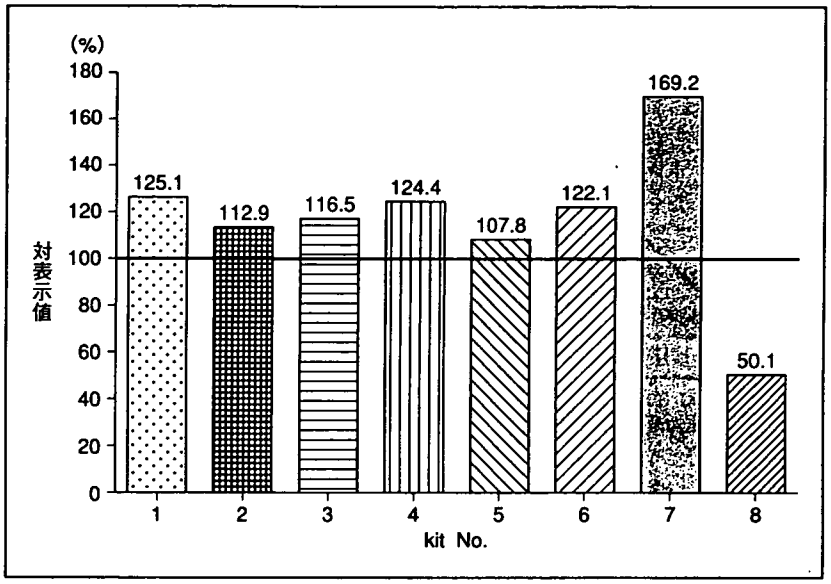

図2　各キットの対表示値
対表示値(%)とは，各検体の抗体濃度(期待値)と測定値の比を各濃度ごとに計算し，それらを平均した値のことである．

assay)，CLEIA 法による「mIU/ml」表示の両者が混在して用いられている．しかし今後は国際的調和の観点からも，WHO の国際標準品を基にした「mIU/ml」に統一されるべきである．また“緒言”でも述べたように，HBV 感染防御に有効な抗 HBs 抗体価の基準について，WHO や CDC が提唱する 10 mIU/ml という基準についてわが国でも詳細に検討するべき時期がきている．以上の観点から，現在国内で承認を受け販売/使用されている抗 HBs 抗体測定キットにおいて，同一検体系列を測定しその結果を比較した場合，使用するキットによる測定結果に乖離がないことが今後の基準作成の前提条件となるだろうと考える．

本研究の結果から，現在国内で使用されている 8 種類のキットのなかで，6 種類のキットにおいてはほぼ同様の測定値を示したが，2 種類のキットではそれぞれ約 1.5 倍，約 0.5 倍の測定値を示した(図 2)．この結果は Ogata によってすでに報告されているアーキテクト(#2)とルミパルス(#7)における測定値の乖離と良く一致する[4,5]．そこでこれらのキットについて製造メーカーと検討した結果，その原因が判明し，それに従いキットが改善されることとなった．そして，それぞれのキットにおいて現在申請に向けて開発中，および一部変更申請が行われている．したがって，これらの申請が認められた段階では，8 種類すべてのキットで同一検体に対してほぼ同一の測定値を示すことになるだろう．

今後は HB ワクチン投与の有効性(感染防御能の獲得)を検証するためにも，抗 HBs 抗体価の正確な測定が重要である．そのためには測定キット間での測定結果に乖離がないことが必須であり，今回の報告でそれが実現されつつあることが示された．

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本研究における抗 HBs 抗体価の測定にあたり，各キットの製造/販売会社の方々に多大な御協力をいただきましたことを深謝いたします．また本研究の遂行にあたり，数多くの御助言をいただきました燕労災病院の小方則夫博士に深く御礼申し上げます．

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Review

Molecular biology of hepatitis C virus

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Infection with hepatitis C virus (HCV), which is distributed worldwide, often becomes persistent, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma. For many years, the characterization of the HCV genome and its products has been done by heterologous expression systems because of the lack of a productive cell culture system. The development of the HCV replicon system is a highlight of HCV research and has allowed examination of the viral RNA replication in cell culture. Recently, a robust system for production of recombinant infectious HCV has been established, and classical virological techniques are now able to be applied to HCV. This development of reverse genetics-based experimental tools in HCV research can bring a greater understanding of the viral life cycle and pathogenesis of HCV-induced diseases. This review summarizes the current knowledge of cell culture systems for HCV research and recent advances in the investigation of the molecular virology of HCV.

Key words: hepatitis C virus, translation, polyprotein processing, RNA replication, viral assembly, ubiquitin

Introduction

Hepatitis C virus (HCV), discovered in 1989, is a major etiologic agent of posttransfusion- and sporadic non-A, non-B hepatitis[1] and at present infects approximately 200 million people worldwide.[2,3] Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma.[3,4–8] HCV is a small, enveloped RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family.[9,10] Its genome consists of a single-strand of positive-sense RNA of approximately 9.6kb, which contains an open reading frame (ORF) coding for a polyprotein precursor of approximately 3000 residues.[11] The precursor is cleaved into at least ten different proteins: the structural proteins core, E1, E2, and p7, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 1).

To date, six major genotypes of HCV have been identified that differ by 31%–34% in their nucleotide sequence and by about 30% in their amino acid sequence. It has been shown that HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies.[12] This quasispecies model of mixed virus populations may confer a significant survival advantage, because the simultaneous presence of multiple variant genomes and the high rate of generation of new variants allows rapid selection of mutants better suited to new environmental conditions.[13]

Specific anti-HCV drugs that efficiently block virus production are not yet available. The current standard care is combination therapy with interferon (IFN)-α and the nucleoside analog ribavirin, which cures about 40% of hepatitis C patients infected by HCV genotype 1, the most prevalent genotype in industrialized countries, and about 80% of those infected by genotype 2 or 3.[14,15] Since many patients still do not benefit from the treatment and IFN therapy is associated with undesirable side effects such as headache, fever, severe depression, myalgia, arthralgia, and hemolytic anemia, development of innovative treatment alternatives for hepatitis C patients is immediately needed. Studies of HCV life cycle in cell cultures have been greatly facilitated by the development of genetically engineered viral genomes that are capable of self-amplifying to high levels (replicon system), and by recent establishment of a production system for recombinant infectious HCV. Such progress will aid in the development of significantly improved HCV antiviral agents.

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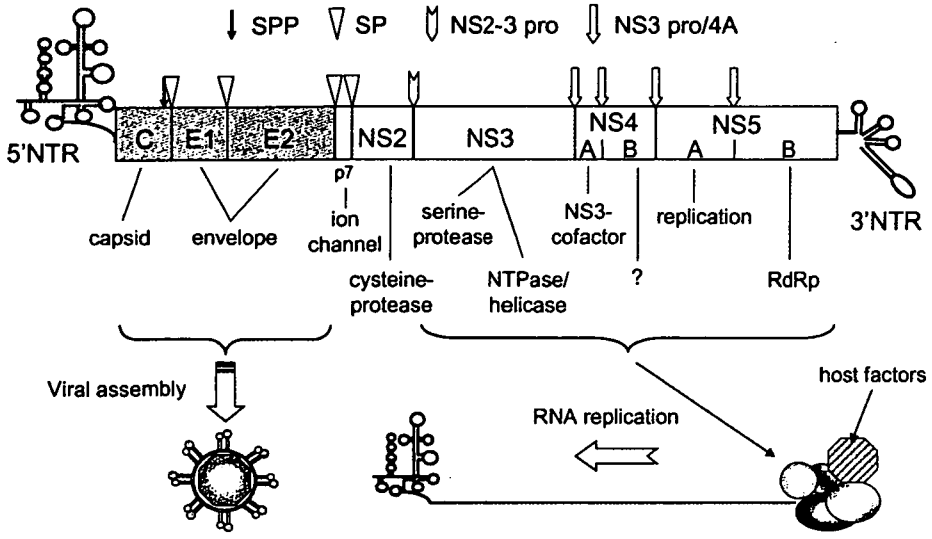

Fig. 1. Hepatitis C virus (HCV) genome organization and polyprotein processing. Posttranslational cleavages by signal peptide peptidase (*SPP*), signal peptidase (*SP*), NS2–NS3 protease (*NS2–3 pro*), and NS3 protease and NS4A complex (*NS3 pro/4A*) lead to the production of functional HCV proteins. *NTR*, nontranslated region

Cell culture systems for HCV research

Although substantial information on HCV protein structure and function has been obtained from the use of a variety of cell culture and in vitro expression systems, for many years, HCV research has been hampered by the restricted host range and the inefficiency of cell culture models for viral infection and propagation. The development of the HCV replicon system, therefore, is a milestone in HCV research and has allowed examination of viral RNA replication in cell culture.[16] Expression systems of heterologous virus genes based on RNA replicons have been established in a variety of positive-strand RNA viruses such as polio virus,[17–20] the alphavirus Semliki Forest virus,[21] Sindbis virus,[22–25] Kunjin virus,[26] human rhinovirus 14,[27] and bovine viral diarrhea virus.[28] In general, advantages of replicon systems are (1) a high level of gene expression and RNA replication, (2) easy construction of recombinants, and (3) a wide permissible host range.

The HCV replicons are typically composed of selectable, bicistronic RNA, with the first cistron containing the HCV 5′ nontranslated region (NTR), which directs translation of the gene encoding the neomycin phosphotransferase, and the second cistron containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus, which directs translation of HCV NS3 through NS5B region, and the 3′ NTR. The prototype subgenomic replicon utilized a particular HCV genotype 1b clone termed Con1. Following transfection of RNA generated by in vitro transcription of the cloned replicon sequences into a human hepatoma cell line Huh-7, antibiotic G418-resistant cells could be obtained in which the subgenomic RNA replicated autonomously. RNA replication was first detected at relatively low frequency, followed by the identification of replicons harboring cell culture-adaptive mutations, which increased the efficiency of replication initiation by several orders of magnitude.[29–31]

Adaptive mutations were found primarily at the N-terminus of the NS3 helicase, in NS4B, and in the center of NS5A, which is upstream of the region putatively involved in IFN sensitivity. Most of the mutations in NS5A are located at highly conserved serine residues and lead to change in the phosphorylation state of NS5A.[32,33] A combination of adaptive mutation in NS3 and NS5A resulted in the highest level of replication of a particular HCV genotype 1b isolate.[31] Later work, however, has indicated that adaptive mutations can arise in most of the viral nonstructural proteins.[34,35] The mechanisms by which adaptive mutations increase RNA replication efficiency are not well understood.

In the last 7 years, a variety of different replicons have been generated, including replicons with reporters or markers such as luciferase and green fluorescent protein, replicons from genotype 1a and 2a, and genome-length dicistronic HCV RNAs (genomic HCV replicons). HCV replicons with reporter genes allow us to execute fast and reproducible screening of large series of compounds for antivirals.[36–38] Huh-7 cells are the most permissive for HCV replicons. However, variability in the permissiveness for replicons has been observed for a given Huh-7 cell pool, and the cells that are able to support efficient replication of the viral genome are enriched during selection such as G418 treatment. A so-called "cured" cell clone, which can be prepared by removing the replicons by treatment with IFN, supports viral replication to a much higher level in many cases and is useful for introducing genome-length HCV RNAs.[39,40]

An HCV genotype 2a replicon with the JFH-1 strain, which was first isolated from the serum of a Japanese patient with fulminant hepatitis C by our group,[41] replicates efficiently in not only Huh-7 cells but also other

hepatocyte-derived cell lines, HepG2 and IMY-N9, and nonhepatocyte-derived cell lines, HeLa and 293.[42–44] Interestingly, the JFH-1 replicon does not require adaptive mutations for replicating in these cell lines, and enormously efficient RNA replication is detected by transient replication assay as well as by colony formation assay with G418 selection,[42] suggesting that the JFH-1 genome can replicate autonomously without the help of drug selection or the requirement of adaptive mutations. This observation laid the basis for a breakthrough in HCV research.

Transfection of the full-length JFH-1 genome into Huh-7 cells leads to the production of HCV particles that are infectious both for naïve cells and for animal models.[45] As a first attempt, an in vitro transcribed full-length JFH-1 RNA was introduced into naïve Huh-7 cells, which is the original cell line used for subgenomic replicon studies. Efficient RNA replication in the transfected cells was detectable by Northern blot analysis, and the viral-enveloped particles, which are spherical structures with an outer diameter of approximately 55 nm, were secreted to the culture medium.[45] Secreted virus was found to be infectious, although at low efficiency, for naïve Huh-7 cells, and its infectivity can be neutralized by anti-CD81 antibody and hepatitis C patients' sera.[45] Subsequently, to increase the infection efficiency, "cured" Huh-7 cell lines such as Huh7.5, Huh7.5.1, and Huh7-Lunet were used. Infectivity of these cured cell lines with JFH-1 became more intense compared with standard Huh-7 cells, and the virus titers released from cells freshly transfected with the JFH-1 genome were markedly increased by continuous passage of the cells carrying persistent replicating viral RNA.[46] Further, chimeric constructs with the core to NS2 region of another genotype 2a clone, J6, improved the infectivity.[47] Thus, this recombinant infectious HCV cell culture system opens avenues of biochemical and genetic studies of the HCV life cycle.

Besides isolating functional molecular clones of HCV that replicate to high levels, to generate a cell culture model that mimics natural host cell environments may be advantageous for improving HCV production systems suitable for studying the virus–host interaction. It is likely that HCV morphogenesis occurs in a complex cellular environment in which host factors may either enhance or reduce the assembly and budding process. Generally, the interaction of viruses with polarized epithelia in the host is one of the key steps in the viral life cycle. A variety of enveloped viruses mature and bud from distinct membrane domains of the host cells.[48–51] We found that a dicistronic HCV genome of genotype 1b supports the production and secretion of infectious HCV particles in two independent three-dimensional (3D) culture systems, the radial-flow bioreactor (RFB) and the thermoreversible gelation polymer (TGP), but not in monolayer cultures, although its productivity is much lower than that observed in the JFH-1 system[52] (Fig. 2). The RFB system was initially aimed at the

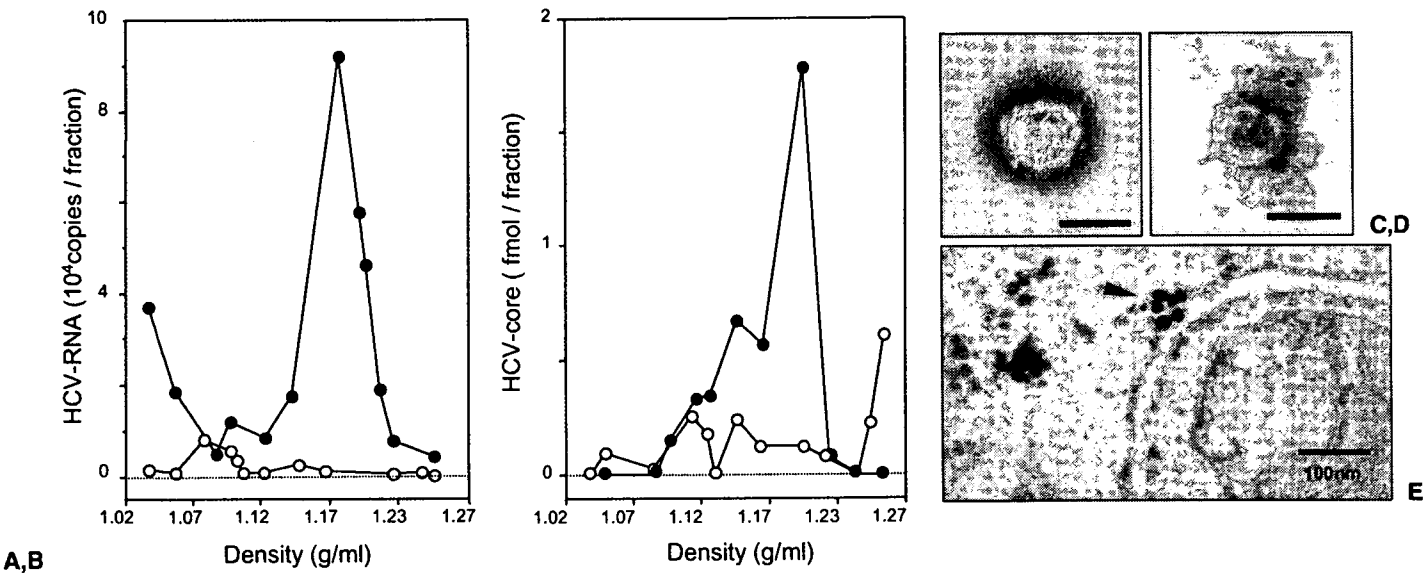

Fig. 2A–E. Production of HCV particles in the three-dimensional, thermoreversible gelatin polymer (TGP) culture of the Huh-7 cell line (RCYM1) carrying genome-length dicistronic HCV RNA of genotype 1b. **A, B** Sucrose density gradient analysis of culture supernatants of RCYM1 cells. The culture supernatants were fractionated and then HCV RNA (**A**) and core protein (**B**) in each fraction were determined by enzyme-linked immunosorbent assay and real-time reverse transcription polymerase chain reaction, respectively. *Closed circles*, TGP culture; *open circles*, monolayer culture. **C, D** Electron microscopy of HCV particles in the supernatants of TGP-cultured RCYM1 cells. **C** Negative staining. **D** Immunogold labeling with an anti-E2 antibody. Gold particles, 5 nm; bars, 50 nm. **E** Silver-intensified immunogold staining with anti-E1 antibody. The *arrowhead* indicates virus-like particles reacting with anti-E1 antibody

development of artificial liver tissue, and the bioreactor column consists of a vertically extended cylindrical matrix through which liquid medium flows continuously from the periphery toward the center of the reactor.[53] In RFB culture, human hepatocyte-derived cells can grow spherically or cubically, and they retain liver functions such as albumin synthesis[53-55] and drug-metabolizing activity mediated by cytochrome P450 3A4.[56] TGP is a chemically synthesized biocompatible polymer which has a sol–gel transition temperature, thus enabling us to culture cells three-dimensionally in the gel phase at 37°C and to harvest them in the sol phase at 4°C, without enzyme digestion.[57] In contrast to other matrix gels made from conventional natural polymers, TGP has several advantages that allow us to investigate the functional characteristics of epithelial cells, their tissue-like morphology, and their potential clinical applications. For example, the use of 3D culture materials other than TGP requires treatment with appropriate digestive enzymes or heating to collect cells grown as spheroids from the culture media, and the matrices may damage the cultured cells to some extent. A 3D culture system based on RFB and TGP, in which human hepatoma cells can assemble into spheroids with potentially polarized morphology, is a valuable tool in studies of HCV morphogenesis.

Translation

The approximately 341-nucleotide (nt)-long 5′ NTR is one of the most conserved regions of the HCV genome, and the secondary structural model, which is also largely conserved, reveals four distinct RNA domains in the region, reflecting its importance in both viral translation and replication.[58-61] The 5′ NTR forms four highly structured domains (domains I–IV), which may be conserved among HCV and related flaviviruses and pestiviruses,[59,60] and it is functionally characterized as an IRES to direct cap-independent translation of the genome.[62,63] To determine the minimal sequence required for HCV IRES-dependent translation, as in the earlier studies of picornaviruses, the bicistronic RNAs in which two reporter protein-coding sequences are separated by an IRES sequence were analyzed. Translation of the upstream reading frame occurs in a 5′ end-dependent fashion, while translation of the downstream reading frame is driven by the IRES element. The IRES comprises nearly the entire 5′ UTR of the genome. There is evidence to suggest that the first 12 to 30 nt of the coding sequence are also important for IRES activity.[64-66] The first 40 nt of the 5′ NTR, which includes a single stem–loop (domain I), is not essential for the translation; the 5′ border of the IRES was mapped between nt 38 and 46.[61,67,68] Domains II and III are relatively more complex and contain multiple stems and loops.[60,69] Domain IV consists of a small stem–loop containing the polyprotein start codon at nt 342 and forms a pseudoknot via base-pairing with a loop in domain III.

Recruitment of the 43S ribosomal complex, containing a small 40S ribosomal subunit, eukaryotic initiation factor (eIF) 3, and a tRNA–eIF2–GTP ternary complex, to mRNA molecules is critical for initiation of eukaryotic protein synthesis. The 40S subunit and eIF3 can bind independently to the HCV IRES.[64,70-72] However, it appears that interaction between IRES RNA and the 40S subunit drives formation of an IRES–40S subunit–eIF3 complex, since HCV IRES RNA demonstrates similar affinity to both the 40S subunit and the 40S–eIF complex.[71] Other cellular factors such as La autoantigen,[73-75] heterogeneous ribonucleoprotein L,[76] poly-C binding protein,[77,78] and pyrimidine tract-binding protein,[79,80] also bind to the IRES element and modulate translation.

Regulation of IRES-dependent translation of HCV is also likely to involve viral factors. We found that the core protein specifically inhibits HCV translation, possibly by binding to a stem–loop IIId domain, particularly a GGG triplet within the hairpin loop structure of the domain, within the IRES (Fig. 3).[79-81] Although a conflicting report has suggested that inhibition of HCV translation is due to an RNA–RNA interaction, rather than to an interaction between RNA and the core protein,[82] later studies support the role of a core protein sequence spanning amino acids (aa) 34–44 in inhibition of viral translation through its interaction with the IRES.[83] Furthermore, the N-terminal 20 residues of the core protein have been shown to selectively inhibit translation mediated by HCV IRES in a cell type-specific manner.[84] We propose a model in which competitive binding of the core protein to the IRES and 40S ribosomal subunit regulates HCV translation.

By analogy with other RNA viruses with IRES-mediated expression, the HCV 5′ NTR has been expected to contain not only determinants for translation but also *cis*-acting elements for RNA replication. Recent studies demonstrated that (1) the sequence upstream of the IRES is essential for viral RNA replication, (2) sequences within the IRES are required for high-level HCV replication, and (3) the stem–loop domain II of the IRES is crucial for the replication.[85]

Polyprotein processing

IRES-mediated translation of the HCV ORF yields a polyprotein precursor that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins (Fig. 1). As deduced from the hydrophobicity profile and the dependence on micro-

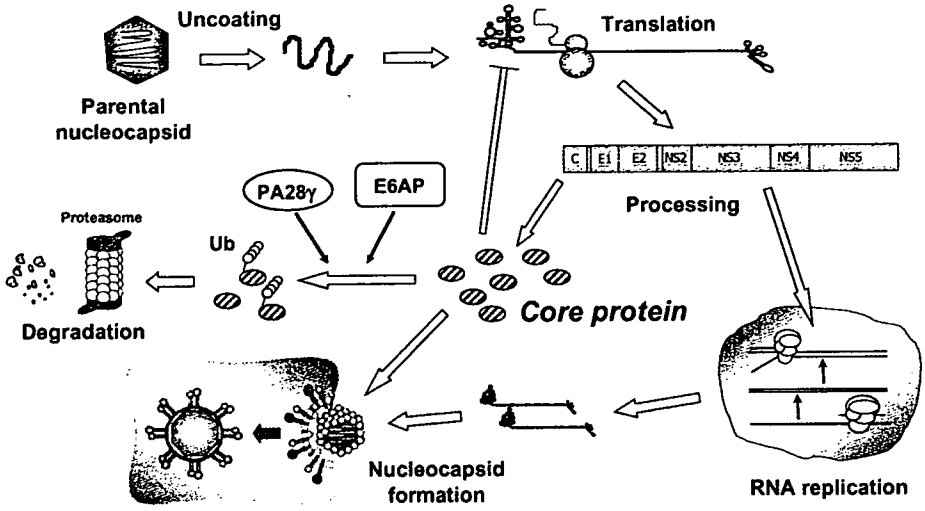

Fig. 3. The role and fate of HCV core protein in the postulated HCV life cycle. See text for further explanation and details

somal membranes, junctions at core/E1, E1/E2, E2/p7, and p7/NS2 are processed by host signal peptidases. For instance, secondary structure analysis of the core protein reveals that all major alpha helices are located in the C-terminal half of the protein. A predicted alpha helix encoded by aa 174–191 is extremely hydrophobic and resembles typical signal peptide sequences. Further posttranslational cleavage close to the C terminus of the core protein takes place, removing the E1 signal sequence by the signal peptide peptidase.[86–89] This peptidase has recently been identified[90] and exhibits protease activity within cellular membranes, resulting in cleavage of peptide bonds in the plane of lipid bilayers.

The viral nonstructural proteins are processed by two viral proteases: processing between NS2 and NS3 is a rapid intramolecular reaction that is accomplished by the NS2–3 protease, which spans NS2 and the N-terminal domain of NS3, whereas the remaining four junctions are cleaved by the serine protease located at the N-terminal 180 residues of NS3 protein. Efficient cleavage at the NS2/3 site requires the 130 C-terminal residues and the first 180 aa of NS3. Recombinant proteins lacking the N-terminal membrane domain of NS2 were found to be enzymatically active, allowing further characterization of this activity.[91,92] Deletion of NS2 from the nonstructural polyprotein did not abolish the replication of HCV RNA in cell cultures, indicating that NS2 is not essential for vial RNA replication.[16,29]

The NS3–NS5B region is processed presumably with the following preferred order of cleavage: NS3/4A→NS5A/5B→NS4A/4B→NS4B/5A.[93–96] Processing at the NS3/4A site is an intramolecular reaction, whereas cleavage at the other sites can be mediated intermolecularly. NS3 is a multifunctional molecule. Besides its N-terminal protease activity, the helicase and nucleotide triphosphatase (NTPase) activities reside in the C-terminal 500 residues of the NS3 protein.[97–101] NS4A functions as a cofactor of the NS3 serine protease and is required for efficient polyprotein processing. There are significant differences in the stability and activity of the NS3 protease in the presence or absence of NS4A. NS3 protein is relatively unstable when expressed in cells in the absence of NS4A.[102] Structural studies by nuclear magnetic resonance and X-ray methods show that the NS3–4A complex has a more highly ordered N-terminal domain and NS4A binding leads the NS3 protease to a rearrangement of the active site triad to a canonical conformation.[103] It has been predicted that the N-terminus of NS4A forms a transmembrane helix, which presumably anchors the NS3–4A complex to the cellular membrane.[104]

RNA replication

HCV is assumed to replicate its genome through the synthesis of a full-length negative-strand RNA. Positive-strand RNA is then produced from the negative-strand template; it is several-fold more abundant than the negative-stranded RNA and is utilized for translation, replication, and packaging into progeny viruses. RNA replication of most RNA viruses involves certain intracellular membrane structures, including the endoplasmic reticulum (ER),[105–107] Golgi,[108] endosomes, and lysosomes.[109] HCV RNA replication is also believed to occur in the cytoplasm of the virus-infected cells.

Although NS5B protein has RNA-dependent RNA polymerase (RdRp) activity in vitro, its recombinant product alone is presumably short of strict template specificity and fidelity, which are essential for viral RNA synthesis. It is highly likely that other viral or host factors are important for conferring proper RNA replication and that the replication complexes (RCs), which are composed of NS5B and additional components re-

quired for modulating polymerase activity, are involved in catalyzing HCV RNA synthesis during the replication process. NS3 is directly involved in RNA synthesis, possibly through its helicase/NTPase activities. The helicase activity is presumed to be involved in unwinding a putative double-stranded replication intermediate or to remove regions of secondary structure so that MS5B RdRp can copy both strands of the viral RNA. It is likely that the NTPase activity is coupled with the helicase function, supplying the energy required for disrupting RNA duplexes. Although little is known about the function of NS4B in the HCV life cycle to date, NS4B protein can induce a membranous web, consisting of small vesicles embedded in a membranous matrix,[110] and it has been reported that the newly synthesized HCV RNA and most of the viral nonstructural proteins occur in these membrane webs or speckle-like structures.[111–113] NS4B may play an important role in the formation of the HCV RNA replication complex.[114] Evidence indicating an involvement of NS5A in viral RNA replication is now accumulating. As described above, a hot spot of the cell culture-adaptive mutations that increase replication efficiency of HCV RNA is located in the central region of NS5A.[29–31] The membrane association of NS5A through its amino-terminal transmembrane domain[115] and the interaction between NS5A and 5B[116] are essential for RNA replication. Several cellular proteins interacting with NS5A have been identified, and human vesicle-associated membrane protein-associated proteins (hVAP-A and -B) are likely to play a key role in RNA replication through the interaction with NS5A.[114,117] The 3′ NTR also contains a significant predicted RNA structure with three distinct domains: a variable region of about 40 nt, a variable length poly(U/UC) tract, and a highly conserved, 98-nt 3′ terminal segment (3′X) that putatively forms three stem–loop structures.[118–120] Viral RNA replication was not detected when any of the three putative stem–loop structures within the 3′X region or the entire poly(U/UC) was deleted.[121] The variable region segment also contributes to efficient RNA replication.[122]

Several groups have succeeded in demonstrating the in vitro replication activities of HCV RCs in crude membrane fractions of cells harboring the subgenomic replicons.[123–126] These cell-free systems provide a valuable complement to the in vitro RdRp assays for biochemical dissection of HCV RNA replication and are a useful source for isolation of viral RCs. From the in vitro replication studies, it appears that RNA synthesis can be initiated in the absence of added negative-strand template RNA, suggesting that preinitiated template RNA copurifies with the RC.[124,125,127] Although the newly synthesized single-strand RNA can be used as a template for a further round of double-strand RNA synthesis, no exogenous RNA serves as a template for HCV RC preparation.[125] Added RNA templates might not access the active site of the HCV RCs owing to sequestration by membranes. The HCV RCs contain both positive- and negative-strand RNAs.[124,127] It has also been reported that cell-free replication activity increases at temperatures ranging from 25° to 40°C, and divalent cations (Mn^{2+} and Mg^{2+}) can be used in the reaction.[125,127]

Membrane flotation analysis and a replication assay have shown that viral RNA and proteins are present in detergent-resistant membrane structures, most likely a lipid-raft structure, and RNA replication activity was detected even after treatment with detergent.[123,128] Lipid rafts are cholesterol- and sphingolipid–rich microdomains characterized by detergent insolubility.[129–131] These structures are known to play a critical role in a number of biological processes, such as as regulators and organizing centers of signal transduction and membrane traffic pathways, including virus entry and assembly of, for example, influenza virus,[132–134] human immunodeficiency virus type-1,[27,135,136] Ebola virus, Marburg virus,[137] enterovirus,[138] avian sarcoma and leukosis virus,[139] Coxsackie B virus, adenovirus,[140] measles virus,[16] and respiratory syncytial virus.[141] However, HCV may be the first example of the association of a lipid raft with viral RNA replication.

On the other hand, it has been widely believed that most of the HCV life cycle, including protein processing and genome replication, takes place in the ER, where cholesterol–sphingolipid rafts are not assembled.[110,142–144] Several studies using the replicon system have indicated that the nonstructural proteins are associated with the ER.[143,145] Nevertheless, it is still possible that HCV nonstructural proteins synthesized at the ER relocalize to lipid-raft membranes when they are actively engaged in RNA replication. It has been shown by membrane separation analysis that HCV nonstructural proteins are present both in the ER and the Golgi, but the activity of viral RNA replication was detected mainly in the Golgi fraction.[123,146] Further studies to elucidate where and how the HCV genome replicates in infected cells are needed.

Viral assembly

The assembly of HCV and the virion structure remains largely unknown. By analogy with related viruses, the mature HCV virion presumably possesses a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. HCV virions are thought to have a diameter of 40–70 nm.[147,148] These observations were recently confirmed by immunoelectron microscopy of infectious HCV particles produced in cell cultures.[45,52] It has been reported that HCV circulates in various forms

in the sera of infected hosts, for example, as (1) free mature virions, (2) virions bound to low-density lipoproteins and very low density lipoproteins, (3) virions bound to immunoglobulins, and (4) nonenveloped nucleocapsids, which exhibit physicochemical and antigenic properties.[147-150]

The HCV structural proteins (core, E1, and E2) are located in the N-terminal one-third of the precursor polyprotein (Fig. 1). A crucial function of the core protein, which is derived from the N-terminus of the viral polyprotein, is assembly of the viral nucleocapsid. The aa sequence of this protein is well conserved among different HCV strains, compared with other HCV proteins. The N-terminal domain of the core protein is highly basic, while its C-terminus is hydrophobic. When expressed in mammalian cells and transgenic mice, the core protein is found on membranes of the ER, on the surface of lipid droplets, on the mitochondrial outer membrane, and, to some extent, in the nucleus.[151-156] The core protein is likely multifunctional and is not only involved in formation of the HCV virion but also has a number of regulatory functions, including modulation of lipid metabolism and hepatocarcinogenesis.[153,157-159] The envelope proteins E1 and E2 are extensively glycosylated and have an apparent molecular weight of 30–35 and 70–75 kDa, respectively. Predictive algorithms and genetic analyses of deletion mutants and glycosylation-site variants of the E1 protein suggest that E1 can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop.[160] E1 and E2 proteins form a noncovalent complex, which is believed to be the building block of the viral envelope.

Several expression systems have been used to investigate HCV capsid assembly using mammalian, insect, yeast, bacteria, and reticulocyte lysates, as well as purified recombinant proteins.[148,161-170] The results suggest that immunogenic nucleocapsid-like particles are heterologous in size and range from 30 to 80 nm in diameter. The N-terminal half of the core protein is important for nucleocapsid formation.[163,169,170] HCV capsid formation occurs in the presence or absence of ER-derived membrane, which supports cleavage of the signal peptide at the C-terminus.[170]

Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. In fact, study of a recombinant mature core protein has shown it to exist as a large multimer in solution under physiological conditions, within which stable secondary structures have been observed.[171] Studies using yeast two-hybrid systems have identified a potential homotypic interaction domain within the N-terminal region of the core protein (aa 1–115 or –122), with particular emphasis on the region encompassing aa 82–102.[172,173] However, other studies have identified two C-terminal regions, extending from aa 123 to 191 and from 125 to 179, as responsible for self-interaction. Furthermore, Pro substitution within these C-terminal regions has been observed to abolish core protein self-interaction.[171,174] Circular dichroism spectroscopy has further shown that a Trp-rich region spanning aa 76–113 is largely solvent-exposed and unlikely to play a role in multimerization.[171] Recently, our group demonstrated that self-oligomerization of the core protein is promoted by aa 72–91 in the core.[160]

Once a HCV nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through intracellular membranes. Interactions between the core and E1/E2 proteins are considered to determine viral morphology. Expression of HCV structural proteins using recombinant virus vectors has led to successful generation of virus-like particles with similar ultrastructural properties to HCV virions. Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the ER has been noted.[161,175,176] Mapping studies to determine the nature of interaction between core and E1 proteins have demonstrated the importance of C-terminal regions in this interaction.[177,178] Since corresponding sequences are not well conserved among various HCV isolates, interactions between core and E1 proteins might depend more on hydrophobicity than on specific sequences. By contrast, it has been shown that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 protein.[160]

Implication of the ubiquitin–proteasome pathway in core protein maturation

The ubiquitin–proteasome pathway is the major route by which selective protein degradation occurs in eukaryotic cells and is now emerging as an essential mechanism of cellular regulation.[179,180] This pathway is also involved in the posttranslational regulation of the core protein.[158,181-183] We have reported that processing at the carboxyl-terminal hydrophobic domain of the core protein leads to its efficient polyubiquitylation and proteasomal degradation.[181] Recently, our group identified the ubiquitin ligase E6AP as an HCV core-binding protein and showed that E6AP enhances ubiquitylation and degradation of the mature as well as the carboxyl-terminally truncated core proteins, and that the core protein produced from infectious HCV is degraded via an E6AP-dependent pathway (Fig. 3).[183] E6AP, the prototype of HECT domain ubiquitin ligases,[184] was initially identified as the cellular factor that stimulates ubiquitin-dependent degradation of the tumor suppres-

sor p53 in conjunction with E6 protein of cancer-associated human papillomavirus types 16 and 18.[185,186] Exogenous expression of E6AP reduces intracellular core protein levels and supernatant viral infectivity in infected cell cultures. Knockdown of exogenous E6AP by siRNA increases intracellular core protein levels and virus titers in the culture supernatants. The core protein interacts with E6AP through the aa 58–71 region of the core, which is highly conserved among all HCV genotypes, suggesting that E6AP-dependent degradation of the core protein is common to a variety of HCV isolates and plays a critical role in the HCV life cycle or viral pathogenesis.

A role for the proteasome activator PA28γ core-binding protein in degradation of the core protein has also been demonstrated (Fig. 3).[158,182] Overexpression of PA28γ promotes proteolysis of the core protein. PA28γ predominates in the nucleus and forms a homopolymer, which associates with the 20S proteasome,[187] thereby enhancing proteasomal activity.[188] Both nuclear retention and core protein stability are regulated via a PA28γ-dependent pathway.

In eukaryotic cells, targeted protein degradation is increasingly understood to be an important mechanism by which cells regulate levels of specific proteins, and thereby regulate their function. The core protein is believed to play a key role in viral replication and pathogenesis since it forms the viral particle and regulates a number of host cell functions. Although the biological significance of ubiquitylation and proteasomal degradation of the core protein is not fully understood, E6AP possibly affects the production of HCV particles through controlling the amount of core protein (Fig. 3). This mechanism may contribute to virus persistence by maintaining a (moderately) low level of the viral nucleocapsid. The E6AP binding domain within the core protein resides in the region that is considered to be important for binding to the viral RNA and several host factors.[189] These factors may affect the interaction between the core and E6AP, resulting in control of E6AP-dependent core degradation. A recent study demonstrated that a knockdown of the *PA28γ* gene induces the accumulation of the core protein in the nucleus of hepatocytes of HCV core gene-transgenic mice and disrupts development of both hepatic steatosis and hepatocellular carcinoma.[158] Upregulation of several genes related to fatty acid biosynthesis and lipid homeostasis by the core protein was observed in the cells and the mouse liver in the PA28γ-dependent manner. Thus, it is likely that PA28γ plays an important role in the development of liver pathology induced by HCV infection.

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