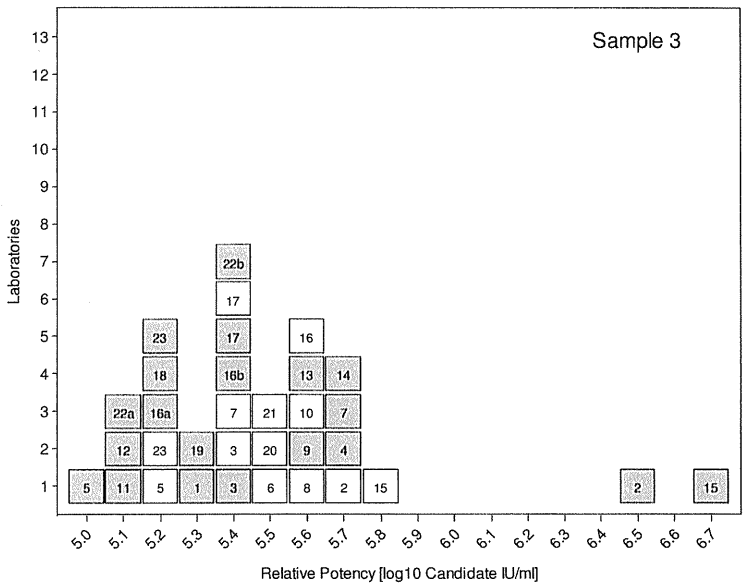
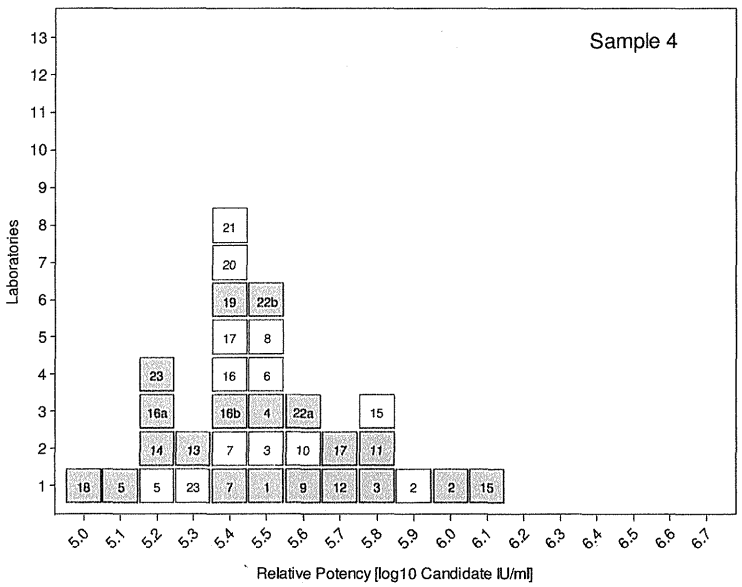
Figure 5 Potency of Sample 3 relative to Sample 1



Histogram of the potency of Sample 3 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). In the case of Laboratory 11, the data have been calculated relative to Sample 2.

Figure 6 Potency of Sample 4 relative to Sample 1



Histogram of the potency of Sample 4 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). In the case of Laboratory 11, the data have been calculated relative to Sample 2.

Table 1 Details of HEV strains lyophilized as candidate standards

Virus strain	HEV RNA (copies/ml)*	Genotype	Accession No.**	Anti-HEV IgM/IgG	ALT (IU/L)
HRC-HE104	1.6×10^7	3a	AB630970	-/-	36
JRC-HE3	2.5×10^7	3b	AB630971	+/-	398

*Concentrations determined by the Japanese Red Cross Hokkaido Blood Center
**Full length sequence

Table 2 Assay protocols used by participants

Laboratory code	Assay type (qualitative or quantitative)	Extraction method	NAT method	Assay target	Reference
1	Qual.	QIAamp MinElute Virus Spin kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
2	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2	Adlhoch *et al.* 2009
3	Qual./Quant.	High Pure Viral Nucleic Acid kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
4	Qual.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
5	Qual./Quant.	QIAamp DNA Mini Blood kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
6	Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
7	Qual./Quant.	QIAamp MinElute Virus Spin kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Matsubayashi *et al.* 2008
8	Quant.	SMI-TEST EX-R&D (Medical Biological Laboratories Co., Ltd.)	Real-time RT-PCR (TaqMan)	ORF2/3	Tanaka *et al.* 2007
9	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
10	Quant.	COBAS AmpliPrep Total Nucleic Acid Isolation kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
11	Qual.	COBAS AmpliScreen Multiprep Specimen Preparation and Control kit (Roche)	Conventional one step RT-PCR; analysis by agarose gel electrophoresis	ORF1	
12	Qual.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
13	Qual.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
14	Qual.	Viral DNA/RNA Isolation kit (GenMag Biotechnology)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	
15	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006 (modified)
16a	Qual./Quant.	MagNA Pure LC (Roche)	Real-time PCR (SYBR Green)	ORF2/3	Jothikumar *et al.* 2006

					(modified)
16b	Qual.	MagNA Pure LC (Roche)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	Meng *et al.* 2001
17	Qual./Quant.	QIAamp Virus BioRobot MDx kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Matsubayashi *et al.* 2008
18	Qual.	MagNA Pure LC Total Nucleic Acid Isolation kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
19	Qual.	easyMag (bioMérieux)	Real-time RT-PCR (TaqMan)	ORF2	
20	Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
21	Quant.	BioRobot Universal (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
22a	Qual.	QIAamp RNA Mini kit (Qiagen)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	Gyarmati *et al.* 2007
22b	Qual.	QIAamp RNA Mini kit	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al.* 2006
23	Qual./Quant.	QIAamp DNA Mini Blood kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Wenzel *et al.*, in press

Qualitative (Qual.) and quantitative (Quant.) assays

Table 3 Mean estimates from quantitative assays (log_{10} copies/ml)

Laboratory code	Sample			
	1	2	3	4
2	4.69	4.82	5.09	5.08
3	5.69	5.62	5.43	5.65
5	6.51	6.48	6.24	6.20
6	5.75	5.80	5.77	5.83
7	5.50	5.46	5.45	5.44
8	5.07	4.97	5.14	5.06
9	5.43	5.52	5.62	5.61
10	5.18	5.22	5.30	5.39
15	5.66	5.73	6.02	5.93
16a	5.59	5.62	5.64	5.51
17	5.40	5.34	5.35	5.41
20	5.70	5.65	5.74	5.65
21	5.25	5.23	5.25	5.23
23	6.54	6.53	6.31	6.41

Table 4 Mean estimates from qualitative assays (log_{10} NAT detectable units/ml)

Laboratory code	Sample			
	1	2	3	4
1	5.76	6.05	5.62	5.91
2	4.42	4.85	5.49	5.02
3	5.35	5.40	5.35	5.76
4	6.20	6.37	6.47	6.33
5	4.70	4.84	4.27	4.42
7	5.34	5.62	5.62	5.34
9	5.02	5.03	5.18	5.26
11		4.00	3.72	4.42
12	4.91	5.48	4.61	5.18
13	5.51	5.66	5.71	5.44
14	4.71	4.43	5.00	4.57
15	6.11	6.36	7.42	6.87
16a	5.32	5.17	5.17	5.17
16b	4.74	4.74	4.74	4.74
17	5.39	5.52	5.42	5.67
18	5.13	5.13	4.98	4.76
19	5.68	5.42	5.56	5.71
22a	5.21	4.92	4.91	5.44
22b	4.53	4.53	4.52	4.68
23	5.76	5.76	5.60	5.60

Laboratory 11, sample 1, omitted due to 2 log_{10} higher cut-off

Table 5a Overall mean estimates from quantitative assays (log_{10} copies/ml)

Sample	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
1	123	5.58	0.29	5.32	5.85	5.46	4.36	6.85	98%
2	125	5.60	0.28	5.33	5.87	5.46	4.43	6.69	94%
3	124	5.66	0.20	5.40	5.93	5.50	4.49	6.63	77%
4	125	5.66	0.20	5.40	5.93	5.48	4.64	6.77	76%

n – number of dilutions analysed (in linear range), sd – standard deviation, lowercl/uppercl – 95% confidence limits for the mean, cv_geo – geometric coefficient of variation [%]

Table 5b Combined mean estimates from quantitative assays (log_{10} copies/ml)

Candidate	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
WHO	248	5.59	0.30	5.33	5.86	5.46	4.36	6.85	99%
NIID	249	5.66	0.20	5.40	5.93	5.48	4.49	6.77	76%

Combined data for Samples 1 and 2, replicate samples of the candidate IS (WHO); combined data for Samples 3 and 4, replicate samples of the candidate Japanese National Standard (NIID)

Table 6a Overall means of estimates from qualitative assays (log_{10} NAT detectable units/ml)

Sample	n	mean	sd	Lower cl	Upper cl	median	min	max	cv_geo
1	19	5.25	0.51	5.01	5.50	5.32	4.42	6.20	150%
2	20	5.26	0.62	4.97	5.56	5.29	4.00	6.37	179%
3	20	5.27	0.79	4.90	5.64	5.27	3.72	7.42	226%
4	20	5.31	0.64	5.02	5.61	5.30	4.42	6.87	183%

n – number of tests, lowercl/uppercl – 95% confidence limits for the mean, cv_geo – geometric coefficient of variation [%]

Table 6b Combined means of estimates from qualitative assays (log_{10} NAT detectable units/ml)

Candidate	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
WHO	39	5.26	0.56	5.08	5.44	5.32	4.00	6.37	163%
NIID	40	5.29	0.71	5.07	5.52	5.30	3.72	7.42	202%

Combined data for Samples 1 and 2, replicate samples of the candidate IS (WHO); combined data for Samples 3 and 4, replicate samples of the candidate Japanese National Standard (NIID)

Table 7 Potency relative to Sample 1 (quantitative assays)

Sample	Laboratory code	Relative potency (log_{10} copies/ml)	95% Confidence Interval	
2	2	5.54	5.29	5.78
	3	5.45	5.15	5.74
	5	5.39	5.15	5.63
	6	5.45	5.20	5.71
	7	5.38	5.28	5.47
	8	5.31	5.17	5.45
	9			
	10	5.47	5.34	5.59
	15	5.53	5.46	5.60
	16a	5.40	5.22	5.59
	17	5.36	5.29	5.43
	20	5.36	5.26	5.46
	21	5.39	5.35	5.44
	23	5.41	5.29	5.53
3	2	5.74	5.50	5.97
	3	5.36	5.07	5.65
	5	5.21	4.97	5.46
	6	5.48	5.21	5.75
	7	5.38	5.29	5.47
	8	5.55	5.41	5.69
	9			
	10	5.55	5.43	5.68
	15	5.83	5.76	5.90
	16a	5.55	5.36	5.73
	17	5.39	5.31	5.46
	20	5.52	5.42	5.62
	21	5.46	5.41	5.50
	23	5.20	5.09	5.32
4	2	5.90	5.66	6.15
	3	5.45	5.17	5.74
	5	5.17	4.93	5.42
	6	5.54	5.29	5.80
	7	5.37	5.28	5.46
	8	5.46	5.32	5.60
	9			
	10	5.63	5.50	5.76
	15	5.75	5.68	5.83
	16a	5.35	5.17	5.53
	17	5.44	5.37	5.52
	20	5.43	5.33	5.52
	21	5.44	5.39	5.48
	23	5.27	5.16	5.39

It was not possible to estimate the relative potency for laboratory 9 since there were only two assay runs performed, each at a different dilution

Table 8 Potency relative to Sample 1 (qualitative assays)

Sample	Laboratory code	Relative potency (log_{10} NAT detectable units/ml)	95% Confidence Interval	
2	1	5.68	5.10	6.27
	2	5.82	5.26	6.38
	3	5.44	4.81	6.08
	4	5.56	4.90	6.22
	5	5.53	5.09	5.97
	7	5.68	5.16	6.23
	9	5.40	5.15	5.66
	12	5.96	5.35	6.51
	13	5.54	5.14	5.91
	14	5.11	4.71	5.50
	15	5.65	4.90	6.40
	16a	5.24	4.85	5.64
	16b	5.39	4.77	6.01
	17	5.52	4.96	6.08
	18	5.39	4.88	5.90
	19	5.13	4.71	5.56
	22a	5.10	4.57	5.63
	22b	5.39	4.79	5.99
	23	5.39	4.74	6.04
3	1	5.25	4.67	5.81
	2	6.46	5.90	7.14
	3	5.39	4.76	6.02
	4	5.66	5.00	6.32
	5	4.96	4.53	5.39
	7	5.68	5.16	6.23
	9	5.55	5.30	5.80
	11	5.11	4.52	5.69
	12	5.09	4.51	5.64
	13	5.59	5.19	5.96
	14	5.67	5.27	6.08
	15	6.67	5.90	7.44
	16a	5.24	4.85	5.64
	16b	5.39	4.77	6.01
	17	5.43	4.87	5.98
	18	5.24	4.73	5.75
	19	5.28	4.85	5.70
	22a	5.10	4.56	5.63
	22b	5.38	4.78	5.97
	23	5.24	4.59	5.89
4	1	5.54	4.96	6.12
	2	5.99	5.43	6.55
	3	5.80	5.15	6.48
	4	5.52	4.86	6.18
	5	5.11	4.70	5.51
	7	5.39	4.87	5.92
	9	5.64	5.38	5.90

	11	5.81	5.23	6.40
	12	5.65	5.07	6.20
	13	5.32	4.93	5.71
	14	5.24	4.85	5.64
	15	6.13	5.39	6.88
	16a	5.24	4.85	5.64
	16b	5.39	4.77	6.01
	17	5.68	5.12	6.23
	18	5.02	4.51	5.52
	19	5.43	5.00	5.87
	22a	5.62	5.08	6.18
	22b	5.54	4.94	6.17
	23	5.24	4.59	5.89

N.B. The relative potency for laboratory 11 was estimated relative to Sample 2 (Sample 1 had a cut-off 2 $\log_{10}$ dilutions higher)

Table 9 Stability testing

Incubation time	Incubation temperature				
	-20°C	+4°C	+20°C	+37°C	+45°C
1 month	ND	ND	ND	ND	5.03
2 months	ND	ND	ND	4.98	4.55*
4 months	5.56	5.52	5.33	ND	ND

ND Not determined
*Material could not be completely reconstituted
Titres expressed as $\log_{10}$ candidate International Units/ml

Appendix 1 List of participants

Scientist	Affiliation
Akihiro Akaishi	Nihon Pharmaceuticals Co., Ltd. Chiba, Japan
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Silvia Dorn	Mikrogen GmbH Neuried, Germany
Thomas Gärtner	Octapharma Frankfurt am Main, Germany
Samreen Ijaz/Renata Szypulska	Health Protection Agency London, UK
Jacques Izopet	Institut Fédératif de Biologie Purpan Toulouse, France
Shintaro Kamei/Katsuro Shimose	Chemo-Sero-Therapeutic Research Institute Kumamoto, Japan
Li Ma/Mei-ying Yu	Center for Biologics Evaluation and Research/Food and Drug Administration Bethesda, USA
Thomas Laue	Astra Diagnostics Hamburg, Germany
Keiji Matsubayashi/Hidekatsu Sakata	Japanese Red Cross Hokkaido Blood Center Sapporo, Japan
Birgit Meldal/Daniel Candotti	Cambridge University and NHS Blood and Transplant Cambridge, UK
Takao Minagi	Benesis Corporation Kyoto, Japan
Saeko Mizusawa/Yoshiaki Okada	National Institute of Infectious Diseases Tokyo, Japan
Elisa Moretti/Francesca Bonci	BioSC-Kedrion S.p.A. Bolognana-Lucca, Italy
Tonya Mixson/Saleem Kamili	Centers for Disease Control and Prevention Atlanta, USA
Andreas Nitsche/Marco Kaiser	Robert Koch-Institut Berlin, Germany
Mats Olsson/Anders Olofsson	Octapharma Stockholm, Sweden
Giulio Pisani/Francesco Marino	CRIVIB, Istituto Superiore di Sanità Rome, Italy
James Wai Kuo Shih	Xiamen University Fujian, China
Ko Suzuki	Central Blood Institute, Japanese Red Cross Society Tokyo, Japan
Isabelle Thomas	Scientific Institute of Public Health,Brussels, Belgium
Youchun Wang/Yansheng Geng	National Institutes for Food and Drug Control Beijing, China
Jürgen Wenzel/Wolfgang Jilg	University of Regensburg Regensburg, Germany

Appendix 2 Draft Instructions For Use for 6329/10

Paul-Ehrlich-Institut → → A WHO Collaborating Centre
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel → for Quality Assurance of Blood Products and
Federal Institute for Vaccines and Biomedicines → → in vitro Diagnostic Devices → →

1st World Health Organization International Standard for Hepatitis E Virus RNA Nucleic Acid Amplification Techniques (NAT)-Based Assays

PEI code 6329/10

(Version 1.0, 7th July 2011)

1. INTENDED USE

The 1st World Health Organization International Standard for hepatitis E virus (HEV) is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for HEV. The need to develop a standard was demonstrated in an initial study investigating performance of HEV NAT assays (Baylis *et al.*, J. Clin. Microbiol., 2011). The standard has been prepared using a genotype 3a strain of HEV, derived from the plasma of a blood donor and further diluted in human plasma. The material has been lyophilized in 0.5 ml aliquots and stored at -20°C. The material has been evaluated in an international collaborative study involving 23 laboratories performing a wide range of HEV NAT assays. Further details of the collaborative study are available in the report WHO/BS/11.XXXX.

2. UNITAGE

This reagent has been assigned a unitage of 250,000 International Units/ml.

3. CONTENTS

Each vial contains 0.5 ml of lyophilized plasma containing infectious HEV.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

The preparation contains material of human origin, and contains infectious HEV. The reference materials has been diluted in human plasma negative for HIV-1 RNA, HCV RNA, HBV DNA, HBsAg, anti-HBs, anti-HBc, anti-HIV-1/2, anti-HCV and anti-HEV (IgM and IgG).

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.

The material is supplied lyophilized and should be stored at or below -20°C. Each vial should be reconstituted in 0.5 ml of sterile nuclease-free water. The product should be reconstituted just prior to use, once reconstituted, freeze-thawing of the product is not recommended.

6. STABILITY

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

The reference materials are held at PEI within assured, temperature-controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. Once, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact PEI.

7. REFERENCES

Baylis, S.A., K.M. Hanschmann, J. Blümel, and C.M. Nübling; on behalf of the HEV Collaborative Study Group. 2011. Standardization of hepatitis E virus (HEV) nucleic acid amplification technique (NAT)-based assays: an initial study to evaluate a panel of HEV strains and investigate laboratory performance. J. Clin Microbiol. 49:1234-1239.

S. A. Baylis, K. M. Hanschmann. Collaborative Study to Establish a World Health Organization International Standard for Hepatitis E Virus RNA for Nucleic Acid Amplification Technology (NAT)–Based Assays. WHO Report 2011, WHO/BS/YY.XXXX.

8. ACKNOWLEDGEMENTS

We are grateful to the Japanese Red Cross Hokkaido Blood Center for supplying the candidate materials, the National Institute of Infectious Diseases, Japan for their collaboration and to the study participants.

9. FURTHER INFORMATION

This material: whoccivd@pei.de
WHO Biological Reference Preparations:
http://www.who.int/biologicals/en/

10. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to whoccivd@pei.de

11. CITATION

In any circumstance where the recipient publishes a reference to PEI materials, it is important that the title of the preparation and the PEI code number, and the name and address of PEI are cited correctly.

12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)	
Physical appearance → →	Lyophilized powder
Fire hazard → → →	None
Chemical properties	
Stable → → →	Yes — Corrosive: No
Hygroscopic → →	No — Oxidising: No
Flammable → →	No — Irritant: No
Other (specify) → CONTAINS HUMAN PLASMA & INFECTIOUS HEPATITIS E VIRUS (HEV)	
Handling: → ..... See caution, section 4	
Toxicological properties	

Paul-Ehrlich-Institut → → Tel: +49 (0)6103 77 0
Paul-Ehrlich-Str. 51-59 → → Fax: +49 (0)6103 77 123
63225 Langen, Germany → → Email: whoccivd@pei.de
→ → Web: http://www.pei.de

Effects of inhalation: → → → Avoid – *contains infectious HEV*	
Effects of ingestion: → → → Avoid – *contains infectious HEV*	
Effects of skin absorption: → Avoid – *contains infectious HEV*	
Suggested First Aid	
Inhalation → ..... Seek medical advice – *contains infectious HEV*	
Ingestion → ..... Seek medical advice – *contains infectious HEV*	
Contact with eyes ..... Wash thoroughly with water. Seek medical advice – *contains infectious HEV*	
Contact with skin ..... Wash thoroughly with water. Seek medical advice – *contains infectious HEV*	
Action on Spillage and Method of Disposal	
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. ¶ Absorbent materials used to treat spillage should be treated as biological waste.	

¶

13. LIABILITY AND LOSS ¶

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use. ¶

¶

It is the responsibility of the Recipient to determine the appropriateness of the materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute. ¶

¶

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party. ¶

¶

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement. ¶

¶

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods. ¶

¶

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition. ¶

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Toll-like receptor (TLR) 3 as a surrogate sensor of retroviral infection in human cells

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Interferon gamma-induced protein 10 (IP-10)/CXCL10

ABSTRACT

The toll-like receptor (TLR)-7 has been shown to sense the retroviral infection. However, a surrogate sensor has been implicated. We examined whether retrovirus serves as a TLR3 ligand in human cells by utilizing cell lines LNCaP and PC-3 lacking TLR7, and the xenotropic murine leukemia virus-relamoted virus (XMRV) insensitive to human tripartite motif-containing (TRIM) 5, a newly characterized pattern recognition receptor (PRR). A dominant-negative TLR3 or a chemical inhibitor of TLR3 attenuated the XMRV-induced IP-10/CXCL10 expression, a marker of TLR3 response. These data clearly indicated that retroviral infection exemplified by XMRV activates the TLR3 signal in human cells.

1. Introduction

The pattern recognition receptor (PRR) plays a key role in the innate immune response to microbial infection [1,2]. Viral RNA can serve as a ligand for the PRR system. Such RNA sensors are present in both endosomes (for example, toll-like receptor (TLR)-3 and -7, double and single-stranded RNA sensors, respectively) and cytoplasm (for example, retinoic acid-inducible gene-I, RIG-I; melanoma differentiation associated gene-5, MDA5). TLR3 is also known to be expressed on the cell surface of epithelial origin [3]. The use of RNA sensors in host defense against retroviral infection remains controversial.

Retroviruses are enveloped viruses with single-stranded RNA genomes. Retroviruses replicate using a unique strategy to protect the viral genomic RNA from being recognized by host RNA sensors. In the production phase of the retroviral life cycle, transcription from proviral DNA integrated into the host chromosome occurs via a mechanism that is essentially identical to that of transcription of cellular genes. The accumulation levels of viral transcript in the infected cells are modest relative to other RNA viruses encoding their own RNA polymerases to amplify viral RNA. In this sense, the mRNA of proviral DNA is barely distinguishable from mRNA transcribed from cellular genes unless the viral RNA has some sequences that evoke anti-viral responses [4,5], which does not apply to all the retroviral species. No evidence has been reported whether the TLR3/7-mediated signal is activated by retroviral RNA transcribed from the provirus. The retroviral genome is not exposed to the surface of virion. Thus, the recognition of retroviral genomic RNA at the cell surface appears unlikely. In the entry phase, the viral genome released into the cytoplasm after the virus-cell membrane fusion is packed in the core. The reverse transcription of viral genome takes place in the cytoplasm and the reverse-transcribed viral DNA is mostly covered with proteins forming the preintegration complex (PIC) [6]. Thus, the exposure of viral genome to the cytoplasmic viral RNA sensor appears limited. Thus, the reverse-transcribed viral DNA may not serve as an efficient ligand for cytoplasmic DNA sensors, such as the DNA-dependent activator of interferon regulatory factor (DAI).

A fraction of retroviral particles, either infectious or non-infectious, are actively endocytosed and degraded in the endosome/lysosome, providing the viral genome as a ligand for RNA sensors, namely a single-stranded RNA sensor TLR7 [7–11]. This model has been tested directly in dendritic cell/human immunodeficiency

Abbreviations: AZT, azidothymidine; dnTLR, dominant negative TLR; PIC, preintegration complex; TRIM, tripartite motif-containing.

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virus type 1 (HIV-1) systems [7]. Human T-cell luekemia virus type 1 (HTLV-1) has been also shown to activate TLR7-mediated signal [12]. Not only TLR7, TLR8 and TLR9 have been involved in the recognition of retroviruses [7,9]. Interestingly, the retroviral infection still evokes some host immune response in the absence of TLR7, suggesting a surrogate sensor of retroviral infection [7,9,10]. It has been reported that HIV-based lentiviral vectors evoke signals from TLR3 as well as TLR7 in mouse cells [1]. However, the involvement of TLR3 in the recognition of infecting retroviral genomes remains to be clarified in human cells.

The retroviral genome is predicted to dimerize and form an extensive secondary structure [13–15]. In these processes, it is likely that double-stranded RNA, a ligand of TLR3, is formed. We hypothesized that retroviruses can potentially activate TLR3 during the viral entry phase. In this study, we demonstrated definitively that TLR3 is a sensor of retroviral genome in human cells through a genetic and a chemical biology approaches.

2. Materials and methods

2.1. Tissue culture

Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Tokyo, Japan) at 37 °C in a humidified 5% CO_2 atmosphere. 22RV-1 and LNCaP (clone FGC) were obtained from Dainippon Sumitomo Pharma Biomedical (Osaka, Japan), while PC-3 and DU145 were obtained from the National Institute of Radiological Sciences. AZT was obtained from the NIH AIDS Research and Reference Reagent Program. The TLR3 ligand, poly(I:C12U), was used at a concentration of 25 μg/ml (Hemispherx Biopharma, Philadelphia, PA). Imiquimod (Sigma) was used at a concentration of 40 μM. The TLR3 inhibitor 4a has been described previously [16].

2.2. Cytokine measurement

The levels of IP-10/CXCL10 were measured using Quantikine IP-10 ELISA kit for the most of the data (R&D Systems, Minneapolis, MN), except 23-Plex panel of the Bioplex cytokine assay system was used for the experiment in Fig. 1D (Bio-Rad Laboratories, Hercules, CA).

2.3. Virus

The xenotropic murine leukemia virus-relamoted virus (XMRV) was prepared from the tissue culture supernatant of 22RV-1 cells. Tissue culture supernatants of 22RV-1 cells were passed through nitrocellulose filters (0.45 μm) and the virions were collected by centrifugation over 20% (w/w) sucrose/PBS (Optima™ L-70 k, SW 55 Ti rotor, 11 k × *g* for 2 h; Beckman Coulter, Miami, FL). The pellet was resuspended in tissue culture medium to 1/10–20 the original volume. Approximately 5% of LNCaP cells were infected with XMRV at 2 days-postinfection as determined by immunofluorescent assay using anti-R-MuLV p30 (Gag) polyclonal serum (NCI BCB repository, 81S263). The MuLV vector was produced as described previously [17]. Replication of XMRV was measured by assessing RT acitivity using the EnzChek Reverse Transcriptase Assay kit (Invitrogen).

2.4. Western blotting

Western blotting was performed as described previously [18]. The following probes were used: an anti-hemagglutinin (HA) monoclonal antibody 6E2 (Cell Signaling Technology, Beverly, MA); an anti-actin monoclonal antibody 1501R (Millipore); and a biotin conjugated secondary antibody and streptavidin conjugated with horseradish peroxidase (HRP, GE Healthcare, Tokyo, Japan).

2.5. RT-PCR

Total RNA from LNCaP cells was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). RT-PCR was carried out using the OneStep RT-PCR Kit (Qiagen, Valencia, CA) using the following primers: TLR3, 5′-TGG TTG GGC CAC CTA GAA GTA-3′ and 5′-TCT CCA TTC CTG GCC TGT G-3′; TLR7, 5′-TTT ACC TGG ATG GAA ACC AGC TA-3′ and 5′-TCA AGG CTG AGA AGC TGT AAG CTA-3′; IP-10/CXCL10, 5′-TTC AAG GAG TAC CTC TCT CTA G-3′ and 5′-CTG GAT TCA GAC ATC TCT TCT C-3′; GAPDH for Fig. 1A, 5′-GTG GAA GGA CTC ATG ACC ACA GTC-3′ and 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′; and GAPDH for Fig. 2B, 5′-GTC GGA GTC AAG GAT TTG-3′ and 5′-TGG TGG AAT CAT ATT GGA A-3′.

2.6. Cloning

The cDNA library of human PBMCs was used as a template (Takara, Otsu, Japan). The following primers were used: forward, 5′-AGC GGC CGC ACC ATG AGA CAG ACT TTG CCT TGT ATC TAC TTT TGG-3′ and reverse, 5′-AAC CGG TTA GGC GTA GTC TGG CAC ATC ATA GGG GTA AAA CTG TTC TGT CTG TCT GTC TAT TTC TTT G-3′. The reverse primer contained the HA tag sequence. The PCR fragment spanning the ectodomain and transmembrane domain of tlr3 was cloned into NotI-AgeI sites of pQcXIP (Clontech, Palo Alto, CA). The pQcXIP without an insert was used as a control for pQcdnTLR3. The LNCaP cells were infected with the MuLV vector and selected with 1.0 μg/ml puromycin.

3. Results and discussion

To clearly differentiate the TLR3 signal from TLR7 in human cells, we carefully chose the experimental system. The prostate cancer cell lines LNCaP and PC-3 have been chosen for cells because they expressed TLR3 endogenously but not TLR7 [19]. We verified the lack of TLR7 expression by RT-PCR in these cell lines in agreement with the previous report (Fig. 1A) [19]. We chose XMRV because this retrovirus is not restricted by a newly-identified PRR protein human tripartite motif-containing (TRIM) 5 [20–22]. The exposure of LNCaP cells to bacteria-derived plasmid DNA induced the robust production of a PRR-responsive cytokine, namely interferon gamma-induced protein 10 (IP-10)/CXCL10. Thus, the viral vectors were not suitable for this study since the complete removal of plasmids or bacteria-derived contaminants from the preparation of viral vectors was difficult [23]. The advantage of XMRV is that the plasmid-free viral preparation is achievable using 22RV-1 cells latently infected with XMRV [24]. The mouse mammary tumor virus (MMTV) activates the signal from TLR4 that targets non-nucleic acid components [25]. The signals from TLR7, 8, and 9 have been activated by retroviruses [7–12]. Another advantage of using the LNCaP-XMRV system is that LNCaP cells do not express TLR4, 8 and 9, in addition to TLR7 [19]. Furthermore, RNase L and JAK in LNCaP cells are defective, both are involved in the interferon (IFN)-mediated anti-viral responses [26–29]. Thus, the signal we detected was not due to any retroviral sensors identified thus far that target non-nucleic acid components. We chose IP-10/CXCL10, one of the PRR-inducible cytokines, as a marker to monitor the cellular response toward TLR3 ligand according to Galli et al. [19] for the induction of IP-10/CXCL10 by TLR3 ligand was reproducible (Fig. 1B). The replication kinetics of XMRV were measured in relation to the production of IP-10/CXCL10. The IP-10/CXCL10 production profile was almost identical

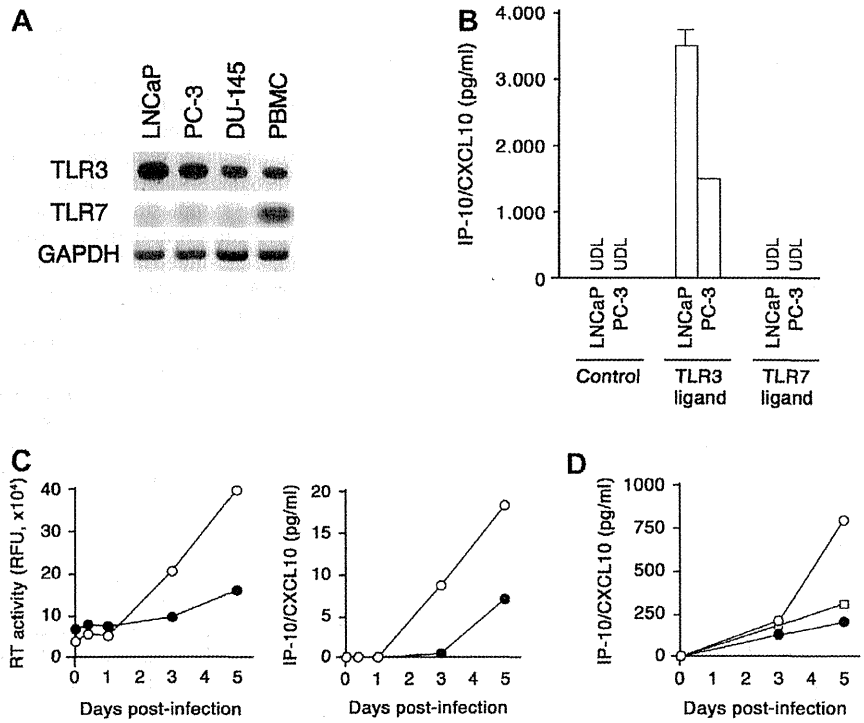

Fig. 1. Expression profiling of TLR3/7 and IP-10/CXCL10 in prostate cancer cell lines. (A) Verification of TLR3 expression in LNCaP, PC-3, and DU-145 cell but not TLR7 by RT-PCR. Total RNA isolated from peripheral blood mononucler cells (PBMC) was used as a positive control. (B) Production of IP-10/CXCL10 in response to TLR3 and TLR7 ligands. LNCaP and PC-3 cells were exposed to TLR3 ligand or TLR7 ligand for 5 days, and the tissue culture supernatants were examined by ELISA. The control is solvent only (PBS and DMSO for TLR3 and TLR7 ligands, respectively). The error bar represents the SD of triplicated wells. Representative data from three independent experiments are shown. UDL, under the detection limit. (C) Correlation of XMRV replication and IP-10/CXCL10 production kinetics in LNCaP cells. The culture supernatant was subjected to RT assay (left) and IP10/CXCL10 ELISA (right). For the control, the replication of XMRV was inhibited by 5 μM AZT (filled). Representative data from three independent experiments are shown. RFU, relative fluorescent units. (D) Induction of IP-10/CXCL10 by XMRV infection in PC-3 cells. Cells were infected with XMRV and maintained in the absence (open circle) or presence (filled circle) of AZT. The MOCK control was also shown (open rectangle).

to the replication profile of XMRV (Fig. 1C). The production of IP-10/CXCL10 from LNCaP cells was reduced when XMRV replication was inhibited by azidothymidine (AZT). Similar results were obtained in PC-3 cells (Fig. 1D). The upregulation of IP-10/CXCL10 was at the transcriptional levels as demonstrated below. These data indicate that XMRV replication induces the expression of IP-10/CXCL10 under the TLR7-null conditions. Note that the IP-10/CXCL10 levels in TLR3 ligand-exposed LNCaP cells were higher than those in XMRV-infected cells (Fig. 1B v.s. Fig. 1C). This is likely because almost all the cells were fully activated by the TLR3 ligand, whereas XMRV infection was limited to a portion of cells.

We then asked whether TLR3 is responsible for these responses. The specific involvement of TLR3 in the upregulation of IP-10/CXCL10 by XMRV infection was investigated by both genetic and chemical approaches. The RNA silencing approach was not employed because siRNA/shRNA potentially serves as a TLR3 ligand [3,30]. First, a dominant-negative derivative of TLR3 (dnTLR3) [31], devoid of cytoplasmic Toll/IL-1 receptor (TIR) domain required for TLR3 signaling, was transduced into LNCaP cells by a murine leukemia virus (MuLV) vector. We verified dnTLR3 expression in LNCaP cells by Western blot analysis where the dnTLR3 was tagged with a HA epitope tag (Fig. 2A). The baseline of the IP-10/CXCL10 production levels was increased in puromycin-selected LNCaP cells. It was assumed that puromycin triggers production of IP-10/CXCL10 since the removal of puromycin from the culture medium reduced the IP-10/CXCL10 levels (data not shown). The control cells responded to both TLR3 ligand and XMRV infection to produce IP-10/CXCL10 (Fig. 2A). In contrast, both XMRV infection and the TLR3 ligand did not upregulate the expression of IP-10/CXCL10 in LNCaP/dnTLR3 cells (Fig. 2A). Second, we treated LNCaP cells with a TLR3 inhibitor and infected cells with XMRV [16]. RT-PCR was employed to examine whether the induction of IP-10/CXCL10 by XMRV infection was at the transcriptional level. Under the conditions whereby the solvent control did not affect the induction of IP-10/CXCL10 by XMRV infection, TLR3 inhibitor was shown to limit the induction of IP-10/CXCL10 by XMRV infection from LNCaP cells (Fig. 2B). The inhibition of IP-10/CXCL10 induction by XMRV was dose-dependent (Fig. 2B). These data suggest that the induction of IP-10/CXCL10 by XMRV infection occurs at the transcriptional level. Taken together, it is suggested that TLR3 is responsible for the recognition of XMRV to induce production of IP-10/CXCL10.

It has been reported that HIV-1 activates TLR7-mediated signals but the contribution of TLR3, a double-stranded RNA sensor in the endosome and on the cell surface, in the anti-retroviral response has remained elusive [7–11]. We demonstrated that not only TLR7 but also TLR3 is able to recognize the retroviral genome and evokes an anti-viral response using XMRV as a model. Retrovirus has a single-stranded RNA as a genome. The retroviral RNA is predicted to form a higher order structure by base-pairing [13–15]. Thus, a portion of retroviral RNA should be able to serve as a TLR3 ligand. Although XMRV was employed in this experiment, it is likely that the recognition of retroviral genome via TLR3 applies to retroviruses in general, including HIV-1. We favor the model that XMRV activates TLR3 signaling at the endosomes given that a similar mechanism has been implicated in the recognition of HIV-1 and HTLV-1 by TLR7 [8,9,11,12]. XMRV has been shown to enter cells via endocytosis [32]. However, some viruses may fail

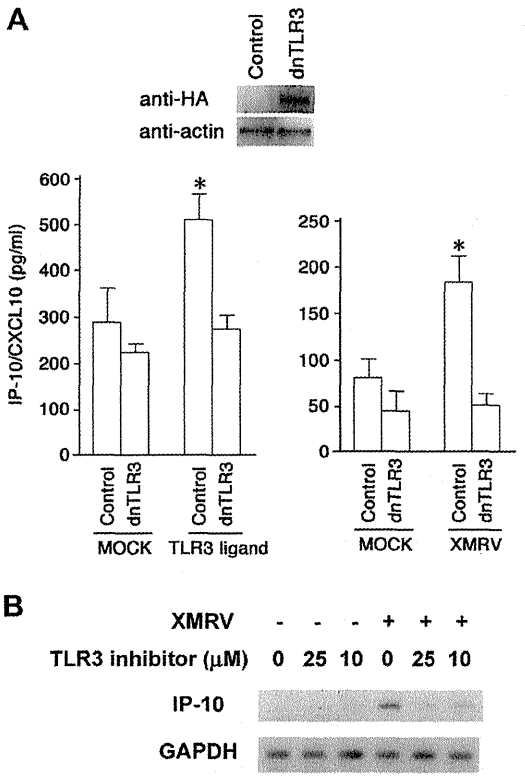

Fig. 2. Specific contribution of TLR3 to XMRV-induced IP-10/CXCL10. (A) Inhibition of IP-10/CXCL10 production by a dominant-negative derivative of TLR3 (dnTLR3). The dnTLR3 was transduced into LNCaP cells by MuLV vector and the cells were selected with puromycin. The constitutive expression of dnTLR3 in LNCaP cells was verified by Western blot analysis (upper panel). Actin was used as the internal control. The TLR3 ligand and XMRV infection failed to upregulate production of IP-10/CXCL10 by cells expressing dnTLR3 (lower panel). The error bar represents the SD of triplicated wells. Representative data from three independent experiments are shown. Statistical significance was detected between ligand-exposed or XMRV-infected control cells and each of the other groups by two-tailed Student's *t*-test (asterisk, $P < 0.01$). (B) Inhibition of XMRV-induced IP-10/CXCL10 production by a TLR3 inhibitor. The RNA isolated from LNCaP cells infected with XMRV in the presence or absence of the TLR3 inhibitor 4a (10 and 25 μM) was subjected to RT-PCR designed to detect IP-10/CXCL10 mRNA. GAPDH was used as the internal control. Representative data from three independent experiments are shown.

to infect cells because retroviruses are intrinsically unstable due to the loss of Env function [33]. Such defective viruses should be degraded in the endosome where the genomic RNA from these viral particles could be exposed to the host RNA sensors, including TLR3. The "endosome model" can be tested by using endosomal acidification inhibitors as reported by Beignon et al. [1]. However, this experimental approach was not possible in our experimental setting because IP-10/CXCL10 production was attenuated by such inhibitors, including Chloroquine or Bafilomycin A1. Retrovirus is an enveloped virus. Thus, the genomic RNA of XMRV should not be exposed to the surface of the virion. It appears unlikely, therefore, that the recognition of viral genomic RNA by TLR3 takes place at the cell surface unless TLR3 recognizes non-nucleic acid component on the surface of XMRV particle.

The historical studies on recognition of retroviruses by TLRs did not assess the involvement of a newly-identified PRR, TRIM5 [22]. In our experimental system, this potential caveat is clarified because human TRIM5 does not restrict XMRV entry [21,22]. The contribution of TLR3 to the recognition of retroviruses could have been difficult to detect partly because the expression levels of TLR7 might be higher than those of TLR3 in that experimental system, or that the TLR3-induced signal might not have been robust enough to detect. Thus, the failure of TLR3 signal detection does not necessarily mean that retroviruses do not activate the TLR3 signal. In the study by Breckpot et al. [34], activation of the TLR signal depended on reverse transcription of the viral genome using a replication-defective HIV-1-based lentiviral vector in mouse-derived dendritic cells. The reverse transcription of retroviral genome is considered to take place in the cell cytoplasm. Thus, the molecular sensor that recognizes the reverse-transcribed nucleic acid should be present in the cytoplasm, not in the endosomal compartment. The activation of anti-viral signal reported by Breckpot et al. should be, therefore, TLR3-independent. More recently, Kane et al. reported that MMTV and MuLV activate humoral responses via the TLR7 signal in mouse [10]. This, again, does not necessarily prove that TLR3 is not activated by these retroviruses, for it was noted that some immune responses were evoked by retroviral infection in a TLR7-independent manner. Although TLR7 serves as a front line, the TLR3 may be the second line PRR-mediated host defense against retroviruses. The *in vivo* relevance of TLR3 activation by retroviruses remains to be clarified in future studies.

Acknowledgments

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