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Short communication

Real-time polymerase chain reaction assays for leukocyte CD and cytokine mRNAs of the Eastern woodchuck (*Marmota monax*)

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Abstract

Real-time polymerase chain reaction (PCR) assays were developed for woodchuck leukocyte cluster of differentiation (CD) and cytokine mRNA expression. Plasmid DNA standards of each marker (CD3, CD4, CD8, IL-2, IFN-γ, TNF-α, IL-4, IL-10), and RNA standards from mitogen-stimulated woodchuck peripheral blood mononuclear cells (PBMCs) were used to validate and optimize the assays for TaqMan 7700® and iCycler® PCR instruments. The complementary DNAs (cDNAs) produced by reverse transcription (RT) of RNA were quantified by real-time PCR against the plasmid DNA standards (6–8 log range) with detection of as few as 10–50 copies of amplicon cDNA per reaction. Analysis of unstimulated and concanavalin A-stimulated woodchuck PBMC demonstrated increased CD and cytokine mRNA expression following mitogenic activation. A liver sample from a woodchuck hepatitis virus (WHV) infected woodchuck with histologically confirmed acute hepatitis had increased intrahepatic CD and cytokine mRNAs compared to liver from an uninfected control woodchuck. The real-time PCR assays were highly specific for the woodchuck markers in PBMC and liver samples and were equally applicable for use in alternate real-time PCR instrumentation. These assays will enable the high-throughput analyses of mRNA markers during WHV infection, and thereby facilitate continued modelling of the immunopathogenesis and immunotherapy of human hepatitis B virus (HBV) infection.

Keywords: Woodchucks; Woodchuck hepatitis virus; Leukocyte genes; Cytokine genes; RT-PCR; Hepatitis B virus

1. Introduction

The hepatitis B virus (HBV) is a major cause of severe liver disease throughout the world, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (reviewed in Chang and Chisari, 1999). The woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck (*Marmota monax*) have

Abbreviations: HBV, hepatitis B virus; CD, cluster of differentiation; cDNA, complementary DNA; C_T, threshold cycle; PRS, PBMC RNA standard; RT, reverse transcription; WHV, woodchuck hepatitis virus

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been used extensively as an animal model for HBV infection, disease, prevention, and therapy (reviewed in Cote and Gerin, 1996; Menne and Tennant, 1999; Tennant, 1999). The model is now being used to investigate the immunogenetics, immunopathogenesis, and immunotherapy of WHV infection (Cote and Gerin, 1995; Cote et al., 2000a,b; Garcia-Navarro et al., 2001; Guo et al., 2000; Hodgson and Michalak, 2001; Li et al., 2000; Menne et al., 1997, 1998, 2002a,b; Nakamura et al., 1997, 2001; Yang et al., 2000). Such studies have demanded a growing number of new cellular and molecular immunologic assays to investigate the immune response of woodchucks to infection and therapy. Accordingly, in this study, real-time polymerase chain reaction (PCR) technology was used to develop high-throughput PCR assays for several important leukocyte cluster of differentiation (CD) and cytokine mRNAs of the woodchuck.

2. Materials and methods

2.1. PBMC and liver RNA samples

The use of woodchucks for these studies was approved by the Cornell University Institutional Animal Care and Use Committee. PBMC RNAs were obtained from normal adult WHV-negative woodchucks following 48 h of in vitro mitogen stimulation (ConA and/or LPS) as described (Nakamura et al., 1997) using the Qiagen RNeasy kit (Qiagen, Valencia, CA). RNA was resuspended in 50 μl of RNase-free water and quantified by spectrophotometry (OD_{260}). Woodchuck liver tissues were obtained by surgical biopsy from one woodchuck (#5277) experimentally infected with WHV7P1 (Cote et al., 2000a) as a neonate, and from one age-matched uninfected control woodchuck (#4460). These two liver samples were characterized as described previously for viral markers and histology (Cote et al., 2000b), and for immunostaining of $CD3^+$ leukocytes and expression of type 1 cytokine mRNAs by first generation end-point reverse transcription (RT) PCR assays (Nakamura et al., 2001). For the present analysis, total RNA was isolated from 200 to 900 mg of liver tissue using the Qiagen RNA/DNA Maxi-kit (Qiagen) and quantified by spectrophotometry.

2.2. Real-time PCR instruments

The real-time PCR assays were developed and validated initially using the TaqMan 7700® instrument (Applied Biosystems [ABI], Foster City, CA). The assays then were adapted for use with the iCycler® instrument (BioRad Laboratories, Hercules, CA). Further details regarding the principles and nuances specific to each instrument are available via the respective manufacturers.

2.3. Nucleotide sequences and recombinant plasmids

The panel of woodchuck-specific markers included leukocyte CD markers (CD3, CD4, CD8), type 1 cytokine markers (IL-2, IFN-γ, TNF-α), and type 2 cytokine markers (IL-4, IL-10) (Table 1). Several recombinant plasmids containing woodchuck-specific sequences were kindly provided by Dr. Christoph Seeger (Fox Chase Cancer Center) (Guo et al., 2000). A recombinant plasmid containing the CD8α sequence (Guo et al., 2000) was generated in our laboratory. Additional plasmids from our laboratory consisted of full-length complementary DNAs (cDNAs) for woodchuck IL-10, IFN-γ, and TNF-α, and partial-length CD4 and IL-2 (Cote et al., unpublished; Nakamura et al., 1997).

2.4. Primer and probe selection, external standards, and DNase treatment of RNA

Oligonucleotide primers and probes (Table 1) were purchased from ABI, except for one modified primer for CD8 (Invitrogen Life Technologies, Gaithersburg, MD) that was used in the iCycler® (Table 1). Primers and probes were designed from the respective gene sequences using the Primer Express® software (ABI). Probes contained the FAM-reporter dye (6-carboxyfluorescein) at the 5′-end and the TAMRA-quencher (6-caboxytetramethyl-rhodamine) at the 3′-end.

Recombinant DNA plasmids were purified (Nucleobond, Clontech Laboratories, Palo Alto, CA), quantified spectrophotometrically, and used as assay standards (i.e., plasmid DNA standard). Standard total RNAs were isolated from mitogen-stimulated PBMC, as described above, and served as positive controls for RT reactions, and as additional external PCR standards for each marker.

Table 1
Primers and probe sets used for real-time PCR assays of woodchuck leukocyte CD and cytokine mRNAs[a]

Marker	Function[b]	Sequence	Amplicon size (bp)
T cell			
CD3	F	CGG AGT TCG CCA GTC AAG A	118
	R	TTG GTG GTT TCC TTG AAG ACG	
	P[c]	CTT CAG ACA AGC AGA CTC TGT TGC CCA A	
CD4	F	AGG TCT CAA AGC CCG AGA AGA	66
	R	GTA GGC ACT GCC ACA TCC CT	
	P[c]	ATT CGG GTG CCA AAC CCC AAG G	
CD8	F	TGG ACT TCG CCT GTG ATA TCT ACA[d]	102
	R	GTT TCC GGT GGT GAC AGA TGA	
	P[c]	TGC GCG GTC CTT CTG TTG TCA CTG	
Type 1 cytokine			
IL-2	F	ATC CGC CAC GAC GTT CA	97
	R	CGA GAT GAT GCT TTG GCA AAA	
	P[e]	TCG CTG TCT CCT GGG CGT ACT CAC A	
IFN-γ	F	TCC TAA AGG TGT CTC AAG TTC AGG TA	106
	R	CTT AGG GTA GAG TGT GGT AAC AGA TCA T	
	P[e]	TCA CTG CTT TAC GCT GGA TCT TCA GGT CA	
TNF-α	F	CCT GCA AAC GGG CTA TAC CTT	83
	R	GTG TGG GTG AGG AGC ACG TA	
	P[c]	CAG CCT TGG CCC TTG AAG AGG ACC T	
Type 2 cytokine			
IL-4	F	GCA TGG AGG TGA TGG TAG CA	78
	R	TTG TAG CCG TGC AGA GGA TTT	
	P[c]	TTG CTG TCC CCA AGA ACA CAA CCG A	
IL-10	F	CCA AGC CTT GTC GGA GAT GA	128
	R	GCC GCA GTC TGA GGG TCT T	
	P[e]	TCA CGT GCT CCT TGA CAT CTG GGC T	

[a] Gene bank accession numbers: CD3γ (AF082493), CD4 (AF082497), CD8α (AF082499), IL-2 (AF082496), IFN-γ (AF081502), TNF-α (AF082491), IL-4 (AF082495), IL-10 (AF082492).
[b] F: forward primer (sense 5′ → 3′), R: reverse primer (antisense 5′ → 3′).
[c] Probe in 5′ → 3′ sense orientation relative to the sequence provided in the gene bank.
[d] CD8 forward primer was modified for use in the iCycler® instrument by deleting the terminal adenine.
[e] Probe in 5′ → 3′ antisense orientation relative to the sequence provided in the gene bank.

Prior to RT reaction, 2–10 μg of RNA (20–400 ng RNA/μl) was treated with DNase I (0.4–1.0 U DNaseI/μg RNA, 21 °C, 15 min; Invitrogen, Rockville, MD). One-tenth volume of 2.5 mM EDTA was then added and the DNase was heat inactivated (65 °C, 10 min). When needed, DNase-treated RNA samples were vacuum centrifuged and resuspended in 6 μl of RNase-free water in order to concentrate the RNA for the RT reaction. If an RNA standard was diluted to less than 5 ng/μl prior to treatment with DNase, yeast tRNA (Invitrogen) was added as a carrier (5–10 ng/μl of DNase reaction) in order to minimize loss of target RNA during vacuum centrifugation.

2.5. RT of RNA into cDNA

RT reactions were performed in 50–200 μl volumes to enable replicate PCR reactions of multiple woodchuck markers using the (c)DNA produced from a single RT reaction. Sample RNA from woodchuck PBMC and liver were added to give final concentrations of 20–40 ng of total RNA per 1 μl of RT reaction.

Serial dilutions of the PBMC RNA standard were added to give final concentrations of 0.01–25 ng of total RNA per 1 μl of RT reaction. Two RT methods were used where indicated: (i) a generic RT method (Nakamura et al., 1997), and (ii) the TaqMan® Reverse Transcription Reagents kit (ABI). The respective RT reaction mixtures included: buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), dNTPs (500 μM each), $MgCl_2$ (3.0 mM (i) or 5.6 mM (ii)), RNase inhibitor (0.2–0.4 U/μl), random hexamers (i) or oligo-d$(T)_{16}$ (ii) (2.5 μM each), and DTT-activated (25 μM) murine leukaemia virus (MuLV) reverse transcriptase (5 U/μl (i) or 1.25 U/μl (ii)). RT reactions were annealed (37 °C (i) or 25 °C (ii); 10 min) followed by first strand cDNA synthesis (42 °C (i) or 48 °C (ii); 30 min) and heat inactivated (95 °C for 5 min). The resulting cDNA was stored frozen (−70 °C) until assayed by real-time PCR.

2.6. Real-time PCR assay

The real-time PCR reaction mixture contained 3 μl of the sample and 27 μl of a master mix containing probe (200 nM), forward and reverse primers (900 nM each), and the PCR core reagent (2X Core Reagent kit, ABI). The 3 μl volume of cDNA sample was added to the final reaction mixture either undiluted from the RT reaction, or as a 1:2 dilution of the RT reaction in DNase/RNase-free water. The 96-well real-time PCR format included six, 10-fold dilutions in duplicate of the plasmid DNA standards (i.e., starting at 5×10^7–5×10^8 copies per 3 μl), and six, threefold or fivefold dilutions in duplicate of PBMC RNA standards, each previously subjected to RT (i.e., starting at 12–75 ng of RNA per 3 μl of cDNA solution). Up to 22 test samples were assayed in triplicate on each plate, along with triplicate negative controls for the RT and PCR steps (i.e., water instead of sample). The wells of the plates were sealed with optical adhesive covers (ABI, BioRad) or caps (ABI), and were centrifuged at low speed (e.g., $300 \times g$, 5 min) to ensure complete mixing. Reactions were stored up to 18 h (4 °C, shielded from light), at which time the plates were centrifuged again, before placement into the real-time PCR instrument. The PCR protocols used involved an initial activation of AmpErase UNG®, in the PCR core reagent (50 °C, 2 min). This was followed by the inactivation of AmpErase UNG®, activation of AmpliTaq Gold® DNA polymerase in the PCR core reagent, and denaturation of DNA (all at 95 °C, 10 min), and then by 40–50 cycles of denaturation (95 °C, 15 s) and annealing with primer extension (60 °C, 1 min).

2.7. Analysis and normalization of real-time PCR data

Optical data obtained by real-time PCR was analysed by using the default and variable parameters available in the software provided with the respective instruments (TaqMan® Detector V1.6.3, ABI; iCycler® Optical System Interface V 2.3, BioRad). The PCR threshold cycle number (C_T) for each plasmid DNA standard, PBMC RNA standard, and test RNA sample was calculated at the point where the fluorescence exceeded the threshold limit. The threshold limit was fixed along the linear logarithmic phase of the fluorescence curves at 10–20 SDs above the average background fluorescence. Average C_T for duplicate standards and for triplicate samples were calculated. Standard curve equations were calculated by regression analysis of average C_T versus the $\log_{10}$ of the plasmid DNA standard copy number (i.e., the copy number of the plasmid DNA standard equals the initial amplicon DNA copy number). The plasmid DNA standard curve equations (R^2 usually greater than 0.96) were used to calculate the amplicon cDNA copy numbers for the PBMC RNA standards and test sample RNAs. Amplicon cDNA copy numbers were expressed relative to the amount of total RNA present (ng), as determined by the real-time PCR of 18S RNA in the sample using the TaqMan® Ribosomal RNA Control Reagents kit (ABI). Standards for 18S RNA consisted of either human RNA (supplied with the kit), or a woodchuck PBMC or liver RNA standard of known concentration.

3. Results and discussion

3.1. Real-time PCR assay of plasmid DNA standards

The primers and probes used for real-time PCR assays of woodchuck markers are shown in Table 1. One primer (CD8 forward) was modified slightly by

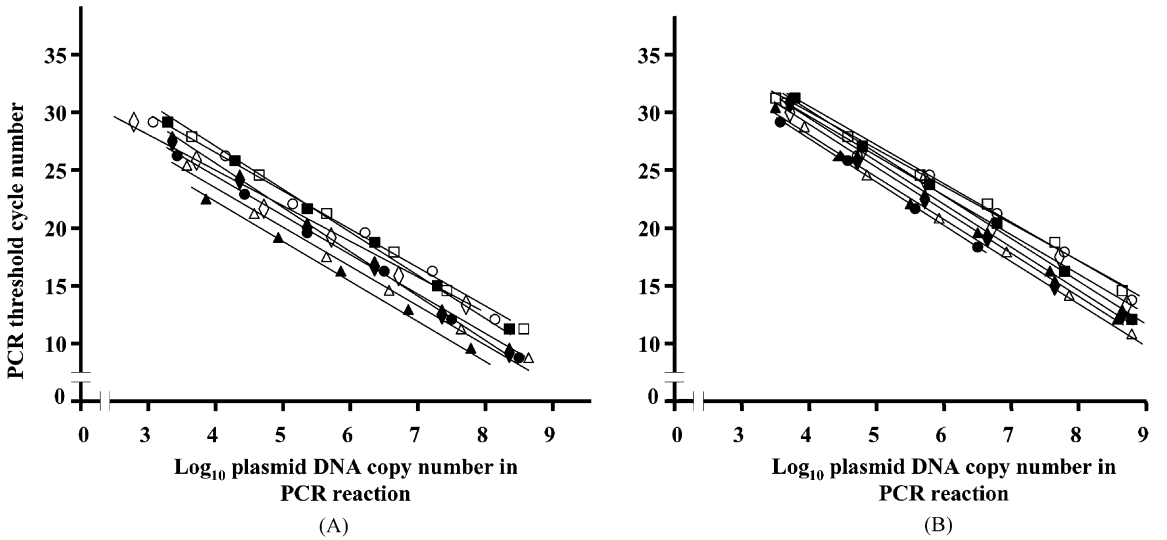

Fig. 1. Representative plasmid DNA standard curves for woodchuck leukocyte CD markers and cytokines assayed by real-time PCR using the TaqMan 7700® (A) and iCycler® (B) instruments. (◆) CD3, (●) CD4, (◇) CD8, (○) IL-2, (■) IFN-γ, (□) TNF-α, (△) IL-4 and (▲) IL-10. The primer and probe sets are listed in Table 1. The CD3 and IL-4 plasmids assayed with both instruments were the same except that the individual DNA plasmid stocks were prepared separately. The CD8 plasmid assayed with both instruments was the same plasmid preparation. The CD4, IFN-γ, TNF-α, IL-2, and IL-10 plasmids assayed on each instruments were different DNA plasmids with stocks prepared separately as described in methods.

deleting the 3′-terminal adenine, which appeared to enhance its function in the iCycler® instrument. In representative assays using the TaqMan 7700®, plasmid DNA standard slopes over 6 logs of concentration ranged from −2.98 to −3.40 and the intercepts ranged from 34.3 to 41.1 (Fig. 1A). In representative assays using the iCycler®, plasmid DNA standard slopes ranged from −3.25 to −3.67 and intercepts ranged from 38.5 to 44.9 (Fig. 1B). The results indicated that the primer and probe sets functioned comparably in either instrument to detect plasmid DNA standards.

3.2. Real-time PCR assay of PBMC RNA standards

Two separate woodchuck PBMC RNA standards (PRS1 and PRS2) prepared from different woodchucks and under slightly different in vitro conditions were assayed by real-time PCR. PRS1 was serially diluted threefold, converted to cDNA by the generic RT method, and assayed using the TaqMan 7700® instrument. PRS2 was serially diluted fivefold, converted to cDNA by the ABI RT method, and assayed using the iCycler® instrument. An amplicon cDNA copy number for each marker was calculated from the observed C_T at each dilution using the respective DNA standard curve. These were multiplied by the respective dilutions and averaged over the best fit points to determine a mean amplicon cDNA copy number per 3 μl of cDNA solution (Table 2). Second generation curves and equations then were developed for each marker by plotting the observed PRS standard C_T against a dilution range beginning with the calculated mean amplicon cDNA copy number (Table 2). This enabled a direct comparison of the linear ranges and endpoints of detection (i.e., intercepts) relative to the respective DNA plasmid standard.

The slopes and intercepts for the dilution curves for PRS1 and PRS2 (Table 2) were always similar to those measured for the homologous DNA plasmid standards (e.g., Fig. 1). The slopes and intercepts obtained using the TaqMan 7700® instrument were usually slightly lower as a group for both DNA plasmid and PBMC RNA standards. However, the linear range of endpoint detection for cDNA amplicons was similar for these experiments involving the different PBMC RNA standards, dilution series, RT methods, and PCR instruments (Table 2). PRS2 had a higher specific marker

Table 2
Representative CD and cytokine amplicon cDNA copy numbers and slope–intercept parameters in two woodchuck PBMC RNA standards using alternate RT conditions and real-time PCR instruments[a]

Standard and RNA marker	Amplicon cDNAs after RT			Slope–intercept parameters	
	Copy number		Copies/ng RNA	Slope (*95% CI*)	Intercept (*95% CI*)
	Input	Limit			
PRS1					
CD3	12454	51	166	3.22 (*2.69–3.74*)	37.0 (*35.5–38.4*)
CD4	69268	285	924	3.08 (*2.73–3.42*)	35.6 (*34.4–36.8*)
CD8	44707	184	596	3.36 (*3.20–3.53*)	38.1 (*37.5–38.6*)
IL-2	6764	28	90	3.33 (*3.14–3.51*)	38.3 (*37.8–38.9*)
IFN-γ	202155	831	2695	3.32 (*3.02–3.62*)	38.8 (*37.7–40.1*)
TNF-α	51482	211	686	3.07 (*2.58–3.55*)	38.7 (*37.0–40.4*)
IL-4	1335	6	18	3.26 (*2.70–3.81*)	36.5 (*35.3–37.6*)
IL-10	16982	70	226	3.10 (*2.63–3.56*)	34.4 (*32.9–35.9*)
PRS2					
CD3	nt[b]				
CD4	42526	68	3571	3.31 (*2.73–3.89*)	40.2 (*38.3–42.2*)
CD8	15419	25	1295	3.13 (*2.70–3.56*)	39.5 (*38.3–40.8*)
IL-2	17808	28	1495	3.42 (*3.03–3.81*)	41.9 (*40.8–43.0*)
IFN-γ	589607	189	49505	3.66 (*3.01–4.32*)	42.7 (*39.8–45.7*)
TNF-α	123871	40	10401	3.25 (*2.80–3.70*)	43.9 (*42.4–45.5*)
IL-4	6308	10	530	3.48 (*2.92–4.04*)	43.2 (*41.8–44.7*)
IL-10	50888	81	4273	3.69 (*3.45–3.94*)	43.8 (*43.1–44.6*)

[a] PRS1 and PRS2 represent two different PBMC RNA standards produced from two different WHV-negative normal woodchucks. Briefly, PBMC were isolated from 30 to 40 ml of whole blood. Approximately, 50–75% of the PBMC (10^6/ml) were stimulated in culture with ConA (10–20 μg/ml, 48 h) and 25–50% were stimulated with LPS (25–50 μg/ml, 48 h). The PBMC were harvested and lysed as described in methods. The ConA and LPS lysates from a given woodchuck were pooled for subsequent isolation of total RNA. PRS1 was serially diluted threefold and the cDNA was generated for each dilution of RNA by using the generic RT method (i). PRS1 cDNA was left undiluted and a 3 μl volume was assayed using the TaqMan 7700® instrument (starting at 75 ng RNA/3 μl of cDNA solution down to 1:243 dilution). PRS2 was serially diluted fivefold and the cDNA was generated for each dilution of RNA by using the ABI RT method (ii). PRS2 cDNA was then diluted 1:2 and a 3 μl volume of each dilution was assayed using the iCycler® instrument (starting at 11.91 ng RNA/3 μl of cDNA solution at 1:2 dilution down to 1:3125 dilution). Input: calculated mean amplicon cDNA copy number for the starting amount of RNA per 3 μl of cDNA solution; coefficients of variation for means were 8–25%. Limit: minimum detection of cDNA amplicon copy number in the linear range of C_T at or near the lowest amount of cDNA assayed for the experiment; these values may not necessarily represent the lower limit of detection. The extrapolated intercept represents the C_T for theoretical detection of one copy of the amplicon cDNA. The slope (minus sign omitted) represents the number of PCR cycles per 10-fold dilution of target amplicon. The 95% confidence intervals (CIs) are given in italics.

[b] Not tested.

content (i.e., cDNA amplicon copies/ng RNA assayed). This did not relate to the sensitivities of the instruments, as indicated above, but could relate instead to the woodchuck donor, the slight variation in culture conditions for the respective PBMC stimulations (Table 2), or the RT method used to generate cDNA. The overall results indicate and predict that the linear range of endpoint detection for cDNA in the assays approaches less than 50–100 amplicon copies per reaction for most markers, and ranges as low as 5–10 amplicon copies per reaction in some cases (e.g., IL-4, Table 2).

3.3. Real-time PCR assay of paired PBMC and unpaired liver RNAs

Total RNA isolated from as few as 1×10^6–2×10^6 cultured woodchuck PBMC was sufficient to perform assays for the entire panel of markers. Comparison of paired unstimulated and ConA-stimulated PBMC from the same woodchuck demonstrated at least four-fold increases in the expression of the various markers following stimulation (Table 3). The most remarkable increase in mRNA expression were noted for IL-10 (25-fold) and IFN-γ (176-fold). In representative

Table 3
Measurement of woodchuck leukocyte CD mRNAs and of type 1 and type 2 cytokine mRNAs in paired and unpaired samples

RNA marker	Amplicon cDNA copy number/mass total RNA			
	Paired PBMC samples[a]		Unpaired liver samples[b]	
	Unstimulated	ConA-stimulated	Uninfected	WHV-infected
Leukocyte CD				
CD3	1236	4847 (4.0)	7835	66630 [8.5]
CD4	406	2561 (6.8)	25560	256981 [10.1]
CD8	81	334 (4.2)	57835	556198 [9.6]
Type 1 cytokine				
IL-2	11	44 (4.0)	25	63 [2.5]
IFN-γ	20	3915 (176.0)	242	59129 [244.3]
TNF-α	359	2185 (5.9)	3884	69774 [17.7]
Type 2 cytokine				
IL-4	2	10 (5.0)	8	42 [5.3]
IL-10	3	84 (25.4)	150	399 [2.7]

[a] Paired PBMC samples: PBMC from a normal WHV-negative woodchuck were isolated, cultured, and either stimulated with ConA (8 μg/ml) or left unstimulated. Values are the amplicon cDNA copy number of CD and cytokine mRNA markers per nanogram of RNA assayed, as determined by a separate real-time PCR assay for 18S RNA. The fold-changes in the markers (in parentheses) are representative for PBMC cultures from five different normal woodchucks.

[b] Unpaired liver samples: one woodchuck (#5277) was sampled for liver during the mid-acute stage of neonatal WHV infection at the time of acute hepatitis (i.e., week 14) (see Cote et al., 2000b; Nakamura et al., 2001). The other woodchuck (#4620) provided age-matched uninfected control liver. Values are the amplicon cDNA copy number of CD and cytokine mRNA markers per microgram of RNA assayed, as determined by a separate real-time PCR assay of 18S RNA. The fold differences in the markers [in brackets] were representative for acute hepatitis followed by resolution of the infection (Nakamura et al., 2001).

unpaired liver samples, one from an uninfected woodchuck and one from a WHV-infected woodchuck at the time of acute hepatitis (Cote et al., 2000b; Nakamura et al., 2001), the intrahepatic expression of most of the markers were increased above normal in the WHV-infected liver (Table 3). The most dramatic increase in intrahepatic cytokine mRNAs were for TNF-α (17-fold) and IFN-γ (244-fold). Increases in the intrahepatic mRNAs for IL-2, IL-4, and IL-10 were detected in the infected woodchuck by real-time PCR (e.g., 2–5-fold), but were less remarkable overall in view of the generally low levels of expression. Moderate increases in the intrahepatic expression of each leukocyte CD marker in the WHV-infected liver (e.g., 8–10-fold) were consistent with an ongoing acute inflammatory response.

3.4. Real-time PCR assays for leukocyte CD and cytokine mRNAs of the woodchuck

Molecular immunologic assays involving the RT of host RNA followed by conventional PCR (Holland et al., 1991) are inherently sensitive and usually require only small amounts of tissue RNA. PCR methodology has now been improved immensely with the development of high-throughput instruments capable of measuring amplified PCR products quantitatively in real-time (reviewed in Bustin, 2000). The TaqMan 7700® and iCycler® real-time PCR instruments detect an ideal interval in real-time approximating 3.32 cycles (i.e., C_T) for each 10-fold dilution of standard. This enables the sensitive discrimination of target concentrations in samples that differ by as little as twofold (i.e., corresponding to one C_T unit).

In this report, real-time PCR assays for woodchuck leukocyte CD and type 1 and type 2 cytokine mRNA expression were validated using DNA plasmid standards and the cDNA produced by RT of standard PBMC RNAs. Regression slopes for plasmid DNA and for amplicon cDNA from the PBMC RNA standards were all within the range of the ideal constant (i.e., 3.32) on both real-time PCR instruments (Fig. 1, Table 2). The similarity of regression equations for plasmid DNA and amplicon cDNA from standard

RNAs suggested an approximation of first-order reaction kinetics when the RT was performed after serial dilution of the RNA standards.

The quantification of an average cDNA amplicon copy number in PBMC RNA standards provided additional external standards for the sample RNAs. The absolute copy number for each mRNA transcript in the PBMC RNA standards and samples could not be determined by the present method. This requires specific in vitro-transcribed RNAs of known molecular weight and concentration that should also closely resemble the native mRNA and be diluted in a manner similar to the samples. Such RNA standards can be used to determine the efficiency of amplicon cDNA production in a given RT reaction (i.e., the number of amplicon cDNAs produced per RNA transcript), and hence, for determining the absolute copy number for target RNA transcripts in the sample. This is often useful and convenient when applied for a single marker (e.g., Garcia-Navarro et al., 2001; Matsumura et al., 2001). However, large numbers of such quantitative RNA standards can be unusually difficult and laborious to prepare, quality-control, and maintain (Bustin, 2000). This is relevant to the present situation where a large panel of different markers is tested. The PBMC RNA standards used in this study were stable and convenient for application to the real-time PCR assays for multiple markers.

The real-time PCR assays detected the expected fold changes in marker expression between paired unstimulated and ConA-stimulated PBMC cultures. They also demonstrated several expected changes in marker expression of unpaired, uninfected and WHV-infected woodchuck liver with histologically confirmed acute hepatitis (Cote et al., 2000b). This liver specimen had increased expression of CD3 mRNA (Table 3), consistent with the moderate to marked infiltration by $CD3^+$ leukocytes shown previously (Nakamura et al., 2001). The increased expression of IFN-γ and TNF-α mRNAs, and the nominal change in IL-2 mRNA expression (Table 3), confirmed previous results using end-point PCR (Nakamura et al., 2001). The role of type 2 cytokines (IL-4, IL-10) in acute hepatitis appeared to be minimal based on the present analysis. However, the increased intrahepatic accumulation of CD4 and CD8 mRNAs suggested that the acute hepatitis leading to recovery from neonatal WHV infection is characterized by increased T helper and cytolytic T cell infiltration, as also shown for self-limited WHV infection of adult woodchucks (Guo et al., 2000).

The woodchuck is becoming an important model for studying host immune responses during experimental WHV infections. Applications of high-throughput real-time PCR assays to WHV-induced host immune responses in the peripheral blood and hepatic compartments should facilitate the characterization of molecular events and their kinetics during acute and chronic WHV infections in adult and neonatal woodchucks. Accordingly, such assays will facilitate the identification of markers that are predictive for the onset and maintenance of chronic WHV infection, the progression of acute to chronic liver disease, and successful responses to antiviral and immunotherapy.

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References

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Chang, K.M., Chisari, F.V., 1999. Immunopathogenesis of hepatitis B virus infection. Clin. Liver Dis. 3, 221–239.

Cote, P.J., Gerin, J.L., 1995. In vitro activation of woodchuck lymphocytes measured by radiopurine incorporation and interleukin-2 production: implications for modeling immunity and therapy in hepatitis B virus infection. Hepatology 22, 687–699.

Cote, P.J., Gerin, J.L., 1996. The woodchuck as a model of hepadnavirus infection, pathogenesis and therapy. Forum Trends Exp. Clin. Med. 6, 131–159.

Cote, P.J., Korba, B.E., Miller, R.H., Jacob, J.R., Baldwin, B.H., Hornbuckle, W.E., Purcell, R.H., Tennant, B.C., Gerin, J.L., 2000a. Effects of age and viral determinants on chronicity as an outcome of experimental woodchuck hepatitis virus infection. Hepatology 31, 190–200.

Cote, P.J., Toshkov, I.A., Bellezza, C., Ascenzi, M., Roneker, C.A., Graham, L.A., Baldwin, B.H., Gaye, K., Nakamura, I., Korba, B.E., Tennant, B.C., Gerin, J.L., 2000b. Temporal pathogenesis of experimental neonatal woodchuck hepatitis virus infection: increased initial viral load and decreased severity of acute hepatitis during the development of chronic viral infection. Hepatology 32, 807–817.

Garcia-Navarro, R., Blanco-Urgoiti, B., Berraondo, P., Sanchez De La Rosa, R., Vales, A., Hervas-Stubbs, S., Lasarte, J.J., Borras, F., Ruiz, J., Prieto, J., 2001. Protection against woodchuck hepatitis virus (WHV) infection by gene gun coimmunization with WHV core and interleukin-12. J. Virol. 75, 9068–9076.

Guo, J.T., Zhou, H., Liu, C., Aldrich, C., Saputelli, J., Whitaker, T., Barrasa, M.I., Mason, W.S., Seeger, C., 2000. Apoptosis and regeneration of hepatocytes during recovery from transient hepadnavirus infections. J. Virol. 74, 1495–1505.

Hodgson, P.D., Michalak, T.I., 2001. Augmented hepatic interferon gamma expression and T-cell influx characterize acute hepatitis progressing to recovery and residual lifelong virus persistence in experimental adult woodchuck hepatitis virus infection. Hepatology 34, 1049–1059.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA 88, 7276–7280.

Li, D.H., Havell, E.A., Brown, C.L., Cullen, J.M., 2000. Woodchuck lymphotoxin-alpha, -beta, and tumor necrosis factor genes: structure, characterization and biologic activity. Gene 242, 295–305.

Matsumura, M., Ijichi, M., Shiratori, Y., Togo, G., Hikiba, Y., Inoued, K., Kohara, M., Omata, M., 2001. Simple quantitative assay of alpha-fetoportein mRNA in liver tissue using real-time detection polymerase chain reaction assay—its application for clinical use. Hepatology Res. 20, 84–96.

Menne, S., Tennant, B.C., 1999. Unraveling hepatitis B virus infection of mice and men (and woodchucks and ducks). Nat. Med. 10, 1125–1126.

Menne, S., Maschke, J., Tolle, T.K., Lu, M., Roggendorf, M., 1997. Characterization of T-cell response to woodchuck hepatitis virus core protein and protection of woodchucks from infection by immunization with peptides containing a T-cell epitope. J. Virol. 71, 65–74.

Menne, S., Maschke, J., Lu, M., Grosse-Wilde, H., Roggendorf, M., 1998. T-cell response to woodchuck hepatitis virus (WHV) antigens during acute self-limited WHV infection and convalescence and after viral challenge. J. Virol. 72, 6083–6091.

Menne, S., Roneker, C.A., Gerin, J.L., Cote, P.J., Tennant, B.C., 2002a. Deficiencies in the acute phase cell-mediated immune response to viral antigens are associated with development of chronic woodchuck hepatitis virus infection following neonatal inoculation. J. Virol. 76, 1769–1780.

Menne, S., Roneker, C.A., Korba, B.E., Gerin, J.L., Tennant, B.C., Cote, P.J., 2002b. Immunization with surface antigen vaccine alone and after treatment with l-(Z-fluoro-5-methyl-β-L-arabinofuranosyl)-uracil (L-FMAU) breaks humoral and cell-mediated immune tolerance in chronic woodchuck hepatitis virus infection. J. Virol. 76, 5305–5314.

Nakamura, I., Nupp, J.T., Rao, B.S., Buckler-White, A., Casey, J.L., Gerin, J.L., Cote, P.J., 1997. Cloning and characterization of partial cDNAs for woodchuck cytokines and CD3ε with applications for the detection of RNA expression in tissues by RT-PCR assay. J. Med. Virol. 53, 85–95.

Nakamura, I., Nupp, J.T., Cowlen, M., Hall, W.C., Tennant, B.C., Casey, J.L., Gerin, J.L., Cote, P.J., 2001. Pathogenesis of neonatal woodchuck hepatitis virus infection: chronicity as an outcome of infection is associated with a diminished acute hepatitis that is temporally deficient for the expression of interferon-gamma and tumor necrosis factor-alpha messenger RNAs. Hepatology 33, 439–447.

Tennant, B.C., 1999. Animal models of hepatitis B virus infection. Clin. Liver Dis. 3, 241–266.

Yang, D.L., Lu, M., Hao, L.J., Roggendorf, M., 2000. Molecular cloning and characterization of major histocompatibility complex class I cDNAs from woodchuck (*Marmota monax*). Tissue Antigens 55, 548–557.