Geographical distribution of hepatitis C virus genotypes in blood donors

JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1994, p. 884–892
0095-1137/94/$04.00+0

Vol. 32, No. 4

Geographical Distribution of Hepatitis C Virus Genotypes in Blood Donors: an International Collaborative Survey

F. McOMISH,[1] P. L. YAP,[1] B. C. DOW,[2] E. A. C. FOLLETT,[2] C. SEED,[3] A. J. KELLER,[3] T. J. COBAIN,[3] T. KRUSIUS,[4] E. KOLHO,[4] R. NAUKKARINEN,[4] C. LIN,[5] C. LAI,[5] S. LEONG,[5] G. A. MEDGYESI,[6] M. HÉJJAS,[6] H. KIYOKAWA,[7] K. FUKADA,[7] T. CUYPERS,[8] A. A. SAEED,[9] A. M. AL-RASHEED,[9] M. LIN,[10] AND P. SIMMONDS[11]*

Edinburgh and South East Scotland Blood Transfusion Service, Royal Infirmary of Edinburgh, Edinburgh EH3 9HB,[1] SNBTS Microbiology Reference Unit, Ruchill Hospital, Glasgow G20 9NB,[2] and Department of Medical Microbiology, Medical School, University of Edinburgh, Edinburgh EH8 9AG,[11] United Kingdom; Australian Red Cross Society, Blood Transfusion Service, Perth, Australia[3]; Finnish Red Cross Blood Transfusion Service, Helsinki, Finland[4]; Hong Kong Red Cross Blood Transfusion Service, Yaumatei, Hong Kong[5]; National Institute of Haematology, Blood Transfusion and Immunology, 1113 Budapest, Hungary[6]; Fukuoka Red Cross Blood Centre, Fukuoka 818, Japan[7]; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, 1066CX Amsterdam, The Netherlands[8]; Riyadh Armed Forces Hospital, Riyadh 11159, Saudi Arabia[9]; and Transfusion Medicine Laboratory, National Health Research Institute, Mackay Memorial Hospital, Taipei, Taiwan[10]

Received 13 July 1993/Returned for modification 23 September 1993/Accepted 20 December 1993

The frequency of infection with the six classified major genotypes of hepatitis C virus (HCV) was investigated in 447 infected volunteer blood donors from the following nine countries: Scotland, Finland, The Netherlands, Hungary, Australia, Egypt, Japan, Hong Kong, and Taiwan. Viral sequences in plasma from blood donors infected with HCV were amplified in the 5′-noncoding region and were typed by restriction fragment length polymorphism analysis. Electrophoresis of DNA fragments produced by cleavage with *Hae*III-*Rsa*I and *Scr*FI-*Hin*fI allowed HCV types 1 (or 5), 2, 3, 4, and 6 to be identified. Further analysis with *Mva*I-*Hin*fI allowed sequences of the type 5 genotype to be distinguished from sequences of the type 1 genotype. Types 1, 2, and 3 accounted for almost all infections in donors from Scotland, Finland, The Netherlands, and Australia. Types 2 and 3 were not found in the eastern European country (Hungary), where all but one of the donors were infected with type 1. Donors from Japan and Taiwan were infected only with type 1 or 2, while types 1, 2, and 6 were found in those from Hong Kong. HCV infection among Egyptians was almost always by type 4. Donors infected with HCV type 1 showed broad serological reactivity with all four antigens of the second generation Chiron RIBA-2 assay (Chiron Corporation, Emeryville, Calif.), while infection with divergent HCV genotypes elicited antibodies mainly reactive to c22-3 and c33c. Reactivities with antibodies 5-1-1 and c100-3 were infrequent and were generally weak, irrespective of the geographical origin of the donor. Because the envelope region of HCV is even more variable than the NS-4 region, it is likely that vaccines based on these proteins need to be multivalent and perhaps specifically adapted for different geographical regions.

The discovery of hepatitis C virus (HCV) (6) and the development of serological screening assays for HCV antibody in blood donors has provided a valuable method for the prevention of posttransfusion non-A, non-B hepatitis. The original anti-HCV enzyme immunoassay (EIA) used a recombinant protein, c100-3, derived from the NS-4 region of the viral genome as the solid-phase antigen in an indirect enzyme-linked immunosorbent assay (ELISA) and proved to be capable of detecting most, but not all, blood donations implicated in the transmission of HCV to recipients (12, 14). Second-generation EIAs include recombinant antigens from the core and NS-3 regions of HCV and are capable of detecting HCV antibody in blood donors and hemophiliacs at much higher rates (1). However, some HCV-infected blood donors may not be detected because of the delay between primary infection and seroconversion to positivity for antibody to HCV. Alternatively, infection with HCV isolates with sequences divergent from those of the prototype virus may elicit antibodies that do not cross-react with the antigens used in current screening assays.

Comparison of the complete genomic sequences of HCV reveals considerable heterogeneity between different isolates. For example, the prototype virus (HCV-1) (8) and HCV type HC-J6 (22) show a 33% nucleotide sequence divergence that is distributed throughout the genome in regions encoding both structural and nonstructural proteins (21). Divergence is most extreme for the envelope proteins and the part of the NS-4 gene that codes for the c100-3 antigen, with only 50% predicted amino acid sequence identity between different HCV genotypes.

HCV has been classified into a number of distinct groups or genotypes on the basis of the sequence similarities of complete genomic sequences (21) or fragments amplified by PCR (3, 4, 11, 19). Unfortunately, there are at least three different classification systems in the literature, and this has caused confusion and difficulty when results from different research groups are compared. However, several laboratories studying viral sequence variation have agreed upon a standardized system of HCV nomenclature (25) that reflects the two-titered nature of sequence variability. All known variants can be classified into a total of six major genotypes (types 1 to 6), of

* Corresponding author. Mailing address: Department of Medical Microbiology, Medical School, Teviot Place, Edinburgh EH8 9AG, United Kingdom. Phone: +44 31 650 3138. Fax: +44 31 650 6531.

which four (types 1 to 4) contain several more closely related subtypes (e.g., a, b, and c).

Variants previously described as belonging to types or groups I and II (3, 13, 19, 21) are designated types 1a and 1b. The more divergent HCV types, such as HC-J6 and HC-J8 (21, 22), are designated types 2a and 2b, respectively. Variants described as type 3 (4), type IV (3), or types V and VI (19) are grouped together as type 3, with two subtypes (subtypes a and b). HCV variants detected in the Middle East and Africa initially described as "type 4" (26) retain their designation as type 4a, as do the group V variants found in South Africa (type 5a) (2, 3). Finally, some blood donors and patients with non-A, non-B hepatitis from Hong Kong are infected with a variant of HCV characterized by a 2-base insertion in the 5′-noncoding region (5′-NCR) (2, 26); sequence analysis of NS-5 indicated that it represents another major genotype of HCV, now designated type 6a (25).

The six major genotypes have distinct nucleotide sequences in the 5′-NCR (2, 26), and so viruses can be typed by restriction fragment length polymorphism (RFLP) analysis of DNA amplified from that region of the viral genome by reverse transcription and then PCR (18, 20). However, there are few, if any, consistent differences in the 5′-NCR sequences of subtypes within a single genotype (e.g., between types 1a and 1b), and so it is not generally possible to differentiate them reliably by the type of RFLP assay described here. In the study described here, we explored the distributions of the major genotypes without attempting to identify specific subtypes in particular countries.

The development of typing assays based on RFLP requires considerable amounts of comparative sequence data, because there is also some intratypic sequence variability that may lead each genotype to show more than one electropherotype (18, 20). Furthermore, existing assays may need to be modified as more HCV genotypes are discovered. For example, by using *Hae*III-*Rsa*I and *Scr*FI, it was possible to identify HCV types 1, 2, and 3 (18), but we have since found that HCV variants of type 4 (26) show the same electropherotype as type 1 with these restriction enzymes. We therefore modified the assay by using *Hin*fI in combination with *Scr*FI to differentiate the two genotypes. Type 6 can also be identified by using these restriction enzymes by virtue of insertions in the 5′-NCR that lead to detectable size variations in one of the cleaved fragments of DNA. Finally, we analyzed sequences showing type 1 electropherotypes with a new combination of restriction endonucleases (*Mva*I-*Hin*fI) to differentiate type 1 variants from type 5 variants.

In the current study, we investigated the distributions of the six recognized genotypes in 447 anti-HCV-positive volunteer blood donors from the following nine different countries: Scotland, Finland, The Netherlands, Australia, Egypt, Hungary, Japan, Hong Kong, and Taiwan. Serological reactivities to a series of structural and nonstructural HCV type 1 antigens in the Chiron recombinant immunoblot assay (RIBA-2) of samples from donors infected with different genotypes were assessed in parallel.

MATERIALS AND METHODS

Plasma samples. Samples from volunteer blood donors from Scotland, Egypt (expatriates working in Riyadh, Saudi Arabia), Finland, The Netherlands, Hungary, Australia, and Hong Kong were available from routine second-generation anti-HCV ELISA screening (Ortho Diagnostics Systems, Raritan, N.J., or Abbott (GmBH, Wiesbaden-Delkenheim, Germany). Samples from Japan were screened by anti-HCV passive hemagglutination assay by using erythrocytes coated with PHCV-34 and PHCV-31 antigens (Dainabot Co. Ltd., Abbott, Japan). Samples from Taiwan were screened by using a second-generation ELISA with HCV antigens equivalent to c22-3, c33c, and c100-3.

Donations from Finland, The Netherlands, Hungary, Australia, and Egypt that were repeatedly reactive on screening by the Abbott or the Ortho second-generation assays were tested further by the RIBA-2 assay (Chiron Corporation, Emeryville, Calif.) (see Table 1). Samples that were confirmed to be positive (significant reactivity with two or more recombinant antigens) or indeterminate (reactivity with one antigen) were shipped frozen to Edinburgh for further analysis by PCR. In Hong Kong, the Abbott Matrix assay was used for supplementary testing. Samples from Japanese donors that were reactive on screening and that showed titers of $>2^{12}$ in the HCV passive hemagglutination assay were referred to Edinburgh, while those from Taiwanese donors were referred without prior confirmatory testing. In interpreting the RIBA-2 assay results, we considered that reactivities with the 5-1-1 and c100-3 antigens do not represent true confirmation of positive results, since both antigens are derived from the same region of NS-4 and could be reacting with the same epitope in both proteins. In the present study, samples showing this type of dual reactivity were considered indeterminate (see Table 4).

HCV typing. RNA PCR was carried out as described previously (4) by using primers matching conserved regions in the 5′-NCR. The PCR assay has proved to be both sensitive and specific for the detection of HCV RNA sequences in plasma (9). To monitor possible contamination with exogenous sequences during extraction or setting up of the amplification reactions, RNAs from at least three negative control plasma samples were extracted, reverse transcribed, and amplified in each batch of donor samples examined by PCR. On no occasion were false-positive results obtained with these negative controls. The PCR product was radiolabelled by the addition of [^{35}S]dATP in the second PCR stage. Two aliquots of product DNA were cleaved with *Hae*III-*Rsa*I, *Scr*FI-*Hin*fI, or *Mva*I-*Hin*fI as described previously (18), and the DNA fragments were electrophoresed on a high-resolution (nondenaturing) polyacrylamide gel that allowed fragments of 30 to 500 bp to be resolved.

The nomenclature for the bands produced by *Hae*III-*Rsa*I and *Scr*FI-*Hin*fI broadly follows that described previously (18), but with additional patterns for genotypes 4 and 6. For simplicity, the two patterns with *Scr*FI (*Scr*FI-*Hin*fI) which were not distinguishable (patterns A and B) by electrophoresis are referred to here as pattern A. Similarly, a rare C→T change found in type 3 and type 4 sequences at position −220 (e.g., E-b1 [4] and EG-33 [26]) creates a new *Hin*fI site, but the position of cleavage is 3 bp downstream from a conserved *Scr*FI or *Mva*I site. Because these two sites could not be cleaved simultaneously, the polymorphism would not affect the overall band patterns. Since there are no other variable *Hin*fI or *Scr*FI sites in type 3 and 4 sequences, we assigned a single letter (G and H, respectively) to each sequence.

Nucleotide sequencing. To investigate samples showing unusual electropherotypes ($n = 2$) or to confirm the identities of samples showing unexpected distributions ($n = 3$), the 5′-NCR was amplified with unlabelled nucleotide triphosphates and directly sequenced by using both sense and antisense inner primers as described previously (4).

New sequences described in this report have been submitted to GenBank (accession numbers L28057 and L28058).

A)

								HCV GENOTYPE					
								1	2	3	4	5	6
a	44	58	# 114/5	9	26			9	-	-	14	-	-
b	102		# 114/5	9	26			60	-	-	4	4	-
c	44	12	46	58	56	9	26	-	9	-	-	-	-
d	44	12	46	114		9	26	-	10	-	-	-	-
e	56		46	114		9	26	-	2	-	-	-	-
f	33	69		114		9	26	-	-	21	-	-	-
g	33	23	46	114		9	26	-	-	9	-	-	-
h	44	12	46	# 117		9	26	-	-	-	-	-	5

B)

							HCV GENOTYPE					
							1	2	3	4	5	6
A	53	15	48	9	32	94	68	-	-	-	4	-
C	53	15	57		32	94	1	-	-	-	-	-
D	53	15	48	41		94	-	11	-	-	-	-
E	53	15	183				-	9	-	-	-	-
F	53	15	48	135			-	1	-	-	-	-
G	53	15	41	16	126		-	-	30	-	-	-
H	53	15	41	7	9	# 32/3 94	-	-	-	18	-	-
I	53	15	48	9	# 35	94	-	-	-	-	-	5

C)

					HCV GENOTYPE					
					1	2	3	4	5	6
A	53	63	41	94	69	-	-	-	-	-
B	53	63	# 44	94	-	-	-	-	-	5
C	53	56	# 142/3		-	-	30	18	-	-
D	53	198			-	21	-	-	3	-

FIG. 1. Predicted association of different cleavage patterns of the 5′-NCR with sequences of HCV types 1 to 6 by cleavage with *Hae*III-*Rsa*I (A), *Scr*FI-*Hin*fI (B), and *Mva*I-*Hin*fI (C). The sources of type 1 (n = 69), 2 (n = 21), and 3 (n = 30) sequences were given previously (18), and some were published elsewhere (2, 28); the sequences of type 4 (n = 18) (26), type 5 (n = 4) (2), and type 6 (n = 5) (2, 26) have been described previously. The locations of cleavage sites are indicated by vertical lines in the box on the left; the positions of 1- or 2-bp insertions found in some 5′-NCR sequences are represented by #; the expected sizes of fragments are given in base pairs. The numbers of published sequences of each genotype associated with each pattern are given in the box on the right.

RESULTS

HCV typing method. The predicted cleavage patterns of sequences corresponding to the region in the 5′-NCR amplified by PCR varied considerably among the different major types of HCV (Fig. 1). By using the combination *Hae*III-*Rsa*I, patterns designated a and b were predicted for published type 1, type 4, and type 5 sequences, while patterns designated c, d, and e were uniquely associated with type 2 (Fig. 1A). Patterns f and g should be produced by all known type 3 sequences, and pattern h should be produced by all known type 6 sequences. The latter pattern differed from pattern d (type 2) by the two insertions (a total of 3 bp) in the 114-bp fragment. This size difference can readily be resolved by polyacrylamide gel electrophoresis of the cleaved DNA.

The second set of restriction enzymes (*Scr*FI-*Hin*fI) produced different bands for five of the six HCV genotypes (Fig. 1B). For type 6 sequences, a 3-bp insertion in the 35-bp fragment (pattern I) distinguished them from type 1 sequences (pattern A; 32 bp). With neither combination of enzymes was it possible to distinguish type 5 from type 1 sequences. However, these two genotypes can be reliably differentiated by using *Mva*I-*Hin*fI (Fig. 1C), in which of a total of four possible electropherotypes, all type 1 sequences produce pattern A and all type 5 sequences produce pattern D. These enzymes also distinguish many of the other genotypes, and in principle, it would be possible to identify all six by cleavage with *Hae*III-*Rsa*I in one reaction and by cleavage with *Mva*I-*Hin*fI in the other.

Identification of HCV genotypes in blood donors. Viral RNA sequences could be amplified from a total of 421 of the 526 RIBA-2 assay-positive samples from volunteer blood donors (80%) and from 26 of the 430 RIBA-2 assay-indeterminate samples (6%) (Table 1). The incidence of confirmed HCV infection (samples that were either RIBA-2 assay positive or PCR positive) varied from 0.01% (Finland, The Netherlands) to more than 8% (Egypt).

Amplified sequences from each of the 447 PCR-positive samples were typed by RFLP analysis by using the restriction endonucleases *Hae*III-*Rsa*I and *Scr*FI-*Hin*fI (Table 2). A wide range of electrophoretic patterns was found in the study samples, but almost all could be assigned to those predicted for published sequences (n = 443; Table 2). In no case were there incompatible combinations of RFLP patterns between the two sets of enzymes (Fig. 2A), and sequences from all 443 samples could be assigned to types 1 (type 5), 2, 3, 4, and 6. Because sequences of genotype 5 produce the same RFLP patterns as

TABLE 1. Supplementary testing of blood repeatedly reactive upon screening by second-generation EIAs

Country	No. of donors screened[a]	Supplementary test	No. of samples[b]	No. of RIBA-2 assay-positive (no. of PCR-positive) samples		No. (%) of PCR-positive samples[c]	% Infected donors[d]
				Confirmed[e]	Indetermined[f]		
Scotland	302,231	RIBA-2	510	203 (129)	307 (15)	144 (28)	0.07
Australia	20,000	Abbott (2 bead)[g]	48	27 (22)	21 (2)	24 (50)	0.15
Finland	137,000	RIBA-2	75	15 (10)	60 (2)	12 (16)	0.01
The Netherlands	321,400	RIBA-2	34	30 (27)	4 (4)	31 (91)	0.01
Hungary	9,707	RIBA-2	73	48 (44)	25 (3)	47 (64)	0.53
Egypt	264	RIBA-2	33	23 (19)	10 (0)	19 (58)	8.7
Taiwan	5,914	ND[h]	100	99 (93)	1 (0)	93 (93)	1.7
Japan	4,400	ND	44	44 (40)	0 (0)	40 (91)	1.0
Hong Kong	29,000	Abbott (Matrix)	39	37 (37)	2 (0)	37 (95)	0.13
Total			956	526 (421)	430 (26)	447 (47)	

[a] Donor samples from Scotland and Finland were part of a consecutive series; for The Netherlands, Hong Kong, and Australia, an estimate of the number of donations from which the samples were derived was made.
[b] Number of EIA-positive samples reactive with one or more antigens in the RIBA-2 assay.
[c] Samples either positive or indeterminate by the RIBA-2 assay were further tested by PCR; the HCV type was determined for all PCR-positive samples.
[d] Approximate incidence of infection obtained by dividing the number of confirmed infected donors (RIBA-2 assay positive or RIBA-2 assay indeterminate, PCR positive) by number of donations screened.
[e] Confirmed by the RIBA-2 assay (significant reactivity to two or more HCV recombinant antigens).
[f] Indeterminate by the RIBA-2 assay (reactivity to a single HCV antigen).
[g] Abbott (2 bead), two-bead supplementary assay (Abbott).
[h] ND, not done.

those of type 1 with both sets of restriction endonucleases (Fig. 1A and B), a third cleavage reaction with *Mva*I-*Hin*fI was carried out on the 252 samples showing the aA or bA electropherotypes (Fig. 2B). All but one sample showed the A electropherotype indicating type 1, while the exception showed the D pattern, which is consistent with a type 5 sequence.

Types 1, 2, and 3 were found among samples from the three western European countries surveyed and Australia, with types 1 and 3 being similar in prevalence and type 2 forming approximately 20% of the total (Table 3). One Dutch donor was infected with a variant that showed an RFLP pattern with *Scr*FI-*Hin*fI that is usually associated with type 4; the DNA in that sample was sequenced and found to be similar to those obtained from the Middle East and Egypt (26), Zaire (2), and Burundi (28) and which were previously assigned to the type 4 genotype (26, 28). Upon checking of the donor's records, this individual was found to have originated from Indonesia and may have acquired the HCV infection there. The one sample identified by RFLP as type 5 (bA, D with *Mva*I-*Hin*fI) originated from Australia; direct nucleotide sequence analysis confirmed its identity as type 5, because it was identical to the 5′-NCR sequences of the previously identified type 5 variants SA1, SA3, SA7, and SA11 (2, 25). This donor was of French and Polish ancestry. Her only admitted risk factor for HCV infection was a blood transfusion that she received in France. No history of travel to South Africa was reported. The distribution of HCV genotypes was markedly different in Hungary, where all but one of the donors were infected with type 1. The one exception produced an RFLP pattern consistent with type 4 (aH), an identification also confirmed by direct sequence analysis.

Type 1 was also the most frequently found genotype in countries in the Far East, although samples from those countries differed from many of those from Europe by the complete absence of HCV type 3. Approximately a quarter of donors from Hong Kong were infected with a novel HCV type, designated type 6 (2, 25, 26). A completely different distribution of HCV infection was found in Egypt, where all donors whose HCV strains could be typed (17 of 19) were infected with HCV type 4 (25, 26).

Only two of the donors showed evidence of infection with more than one genotype. Both of these were from Hong Kong

TABLE 2. Distributions of electropherotypes among donors from different countries

Country	No. of donor samples of electropherotype[a]:											
	aA	bA	cD	dD	eD	dE	eE	fG	gG	aH	bH	hI
Scotland	4	63	10	2	3	4	2	48	8			
Australia	1	12	1		1	1		8				
Finland		3				5		4				
The Netherlands		18	2	3		2		5			1	
Hungary		45								1		
Egypt										12	5	
Japan		31	6	2		1						
Taiwan	1	52	16	5	7	6	6					
Hong Kong	1	21				1						12

[a] The first letter (lowercase a to h) indicates the cleavage pattern observed upon digestion with *Hae*III-*Rsa*I (Fig. 1A). The second letter (capital A to I) refers to the cleavage pattern observed upon digestion with *Scr*FI-*Hin*fI (Fig. 1B).

A)

HaeIII/RsaI	ScrFI/HinfI A	C	D	E	F	G	H	I	
a	8	-	-	-	-	-	13	-	1,
b	244	-	-	-	-	-	6	-	4,5
c	-	-	35	-	-	-	-	-	
d	-	-	12	20	-	-	-	-	2
e	-	-	11	8	-	-	-	-	
f	-	-	-	-	-	65	-	-	3
g	-	-	-	-	-	8	-	-	
h	-	-	-	-	-	-	-	12	6
	1,	5	2			3	4	6	

B)

HaeIII/RsaI	MvaI/HinfI A	B	C	D	
a	8	-	-	-	1,
b	244	-	-	1	4,5
c	-	-	-	-	
d	-	-	-	-	2
e	-	-	-	-	
f	-	-	-	-	3
g	-	-	-	-	
h	-	-	-	-	6
	1	6	3,4	2,5	

FIG. 2. Observed combinations of cleavage patterns with *Hae*III-*Rsa*I (a to h; left margin), *Scr*FI-*Hin*fI (A to I; top margin), and *Mva*I-*Hin*fI (A to D; top margin) for identification of genotypes 1 to 6 in blood from 443 donors. The specific genotypes associated with cleavage patterns are shown on the right and lower margins (see Fig. 1). For *Mva*I-*Hin*fI, only those samples showing RFLP pattern a or b with *Hae*III-*Rsa*I were tested further with *Mva*I-*Hin*fI.

and showed a mixed pattern of type 1 and type 6 electropherotypes. Coinfection with two genotypes was confirmed by limiting dilution of cDNA amplification of single molecules by nested PCR (24). RFLP analysis of DNAs amplified from five single molecules yielded three type 1 and two type 6 sequences from one of the two donors who showed this type of mixed pattern.

Two of the 19 samples from Egypt produced anomalous RFLP patterns, producing bands with *Hae*III-*Rsa*I larger than any of those predicted in Fig. 1A. Sequence analysis confirmed that an A→U or an A→C change at position −176 eliminated the usually well conserved *Rsa*I site to produce bands of 44, 173 (i.e., 58 plus 115 bp [see pattern a]), 9, and 26 bp or 217 (i.e., 102 plus 115 bp [see pattern b]), 9, and 26 bp. The first sequence showed the greatest similarity to those of type 4; they were both left unassigned until we obtain sequence data for the NS-5 region to allow formal classification (25) (see Discussion).

Serological reactivity. Irrespective of the geographical origins of the donors, sera from those infected with HCV type 1 showed broad reactivities with all four antigens in the second-generation RIBA-2 assay, with a high mean score in all cases (Fig. 3; Table 4). The antibody response elicited by infection with HCV types 2, 3, 4, and 6 showed reactivity that was largely restricted to c33c and c22-3 antigens (Fig. 3). Reactivity to the 5-1-1 antigen was found in 79, 17, 7, 42, and 33% of donors infected with types 1, 2, 3, 4, and 6, respectively, and reactivity to the c100-3 antigen was found in 78, 34, 33, 58, and 58%, respectively (Table 4). There was a significant difference between the proportion of type 1 samples that were reactive with antigen 5-1-1 compared with the proportion of samples of the other four genotypes that were reactive with antigen 5-1-1, either individually (type 2, $P < 0.0001$; type 3, $P < 0.0001$; type 4, $P < 0.001$; type 6, $P < 0.001$) or collectively ($\chi^2 = 176$; 4 degrees of freedom on a 2-by-2 contingency table; $P < 0.0001$). There were also significant differences between type 1 and types 2, 3, 4, and 6 in the frequency of reactivity to antigen c100-3 ($P < 0.0001$, $P < 0.0001$, $P < 0.05$, and $P \approx 0.1$ for types 2, 3, 4, and 6, respectively). Between type 1 and the four genotypes together, there was a highly significant difference ($\chi^2 = 82$; 4 degrees of freedom; $P < 0.0001$).

Only 26 of 430 (6%) RIBA-2 assay-indeterminate samples were PCR positive, whereas 421 of 526 (80%) samples confirmed to be positive by the RIBA-2 assay were PCR positive

TABLE 3. Prevalence of HCV types in different donor populations

Country	No. of samples[a]	No. (%) of donor samples of HCV genotype:					
		1	2	3	4	5	6
Scotland	144	67 (47)	21 (14)	56 (39)	0	0	0
Australia	24	12 (50)	3 (13)	8 (33)	0	1 (4)	0
Finland	12	3 (25)	5 (42)	4 (33)	0	0	0
The Netherlands	31	18 (58)	7 (23)	5 (16)	1 (3)	0	0
Hungary	47	46 (98)	0	0	1 (2)	0	0
Egypt	19[b]	0	0	0	17 (90)	0	0
Taiwan	93	53 (57)	40 (43)	0	0	0	0
Japan	40	31 (77)	9 (23)	0	0	0	0
Hong Kong	37[c]	22 (59)	1 (3)	0	0	0	12 (32)
Total	447	252	86	73	19	1	12

[a] Number of PCR-positive samples typed by RFLP.
[b] Two donors were infected with variants that could not be classified as type 1 to 6.
[c] Two donors showed evidence of mixed infection with types 1 and 6.

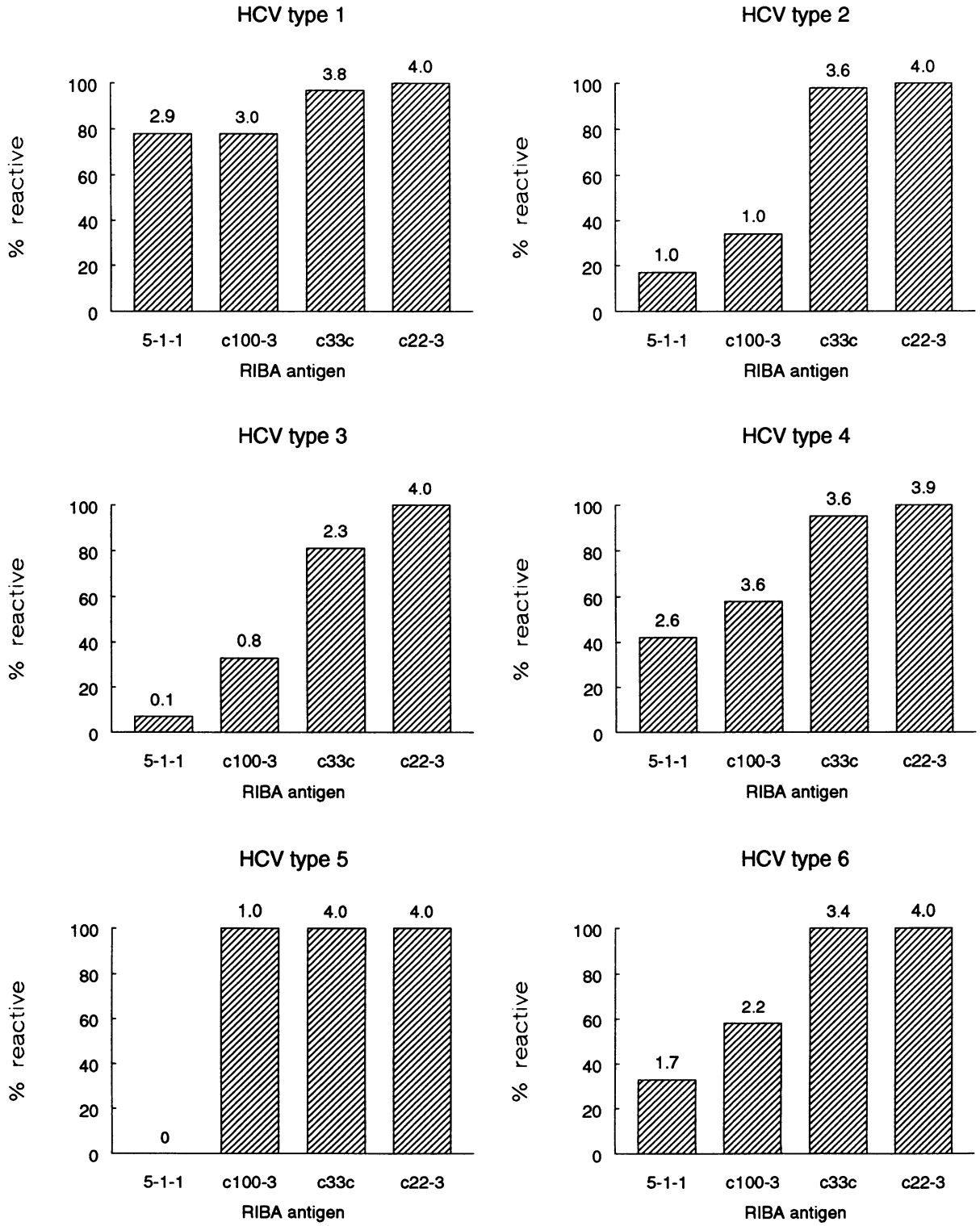

FIG. 3. Frequency of reactivities to antigens in the RIBA-2 assay by samples from blood donors infected with different genotypes of HCV. The values above the histogram indicate the mean score for each type with each antigen, from 0 (nonreactive) to 4+. Samples from 252 type 1-, 86 type 2-, 73 type 3-, 19 type 4-, 1 type 5-, and 12 type 6-infected donors were used for the comparison.

TABLE 4. Patterns of serological reactivity in Chiron RIBA-2 assay associated with different genotypes of HCV

Sample	RIBA-2 pattern				No. of samples	No. PCR positive	No. of donor samples of HCV genotype:							
	5-1-1	c100-3	c33c	c22-3			1	2	3	4	5	6	U[a]	M[b]
Confirmed by RIBA-2 assay	+	+	+	+	271	222	187	15	5	8	0	4	1	2
	+	−	+	+	19	12	12	0	0	0	0	0	0	0
	−	+	+	+	70	50	10	14	19	3	1	3	0	0
	−	−	+	+	166	137	33	56	35	7	0	5	1	0
Subtotal					526	421	242	85	59	18	1	12	2	2
Indeterminate by RIBA-2 assay	+	−	−	−	8	0	0	0	0	0	0	0	0	0
	−	+	−	−	51	0	0	0	0	0	0	0	0	0
	−	−	+	−	24	2	2	0	0	0	0	0	0	0
	−	−	−	+	339	24	8	1	14	1	0	0	0	0
	+[c]	+	−	−	8	0	0	0	0	0	0	0	0	0
Subtotal					430	26	10	1	14	1	0	0	0	0

[a] U, two samples from Egyptian donors could not be classified by RFLP analysis (see text).
[b] M, two samples showed evidence of mixed infection with types 1 and 6.
[c] Samples showing reactivities to proteins 5-1-1 and c100-3 were not considered confirmed for the reasons described in the text.

(Table 4). All samples that were indeterminate and that were reactive with the NS-4-derived proteins c100-3 or 5-1-1 were PCR negative, while a significant proportion of those showing an indeterminate reaction with the c33c or c22-3 antigen were PCR positive (2 of 24 and 23 of 339 samples, respectively). Approximately 20% of samples from donors infected with type 3 were reactive with c22-3 only, whereas 3% of samples from those infected with type 1 were reactive with c22-3 only ($\chi^2 = 23$; $P < 0.0001$). However, sequence variability was not the only cause of restricted serological reactivity, because many of the samples that failed to react with antigens c100-3 and 5-1-1 were obtained from donors infected with type 1 (10 of 430 RIBA-2 assay-indeterminate samples and 33 of the 166 samples that were reactive with antigens c22-3 and c33c only). We are analyzing whether this restricted pattern of reactivity is associated with proximity to seroconversion by testing previous donations and follow-up samples from the donors. From retrospective testing, we found that the type 1-infected donor whose sera reacted with antigen c33c only (Table 4) showed a similar serological profile for at least 2 years (data not shown).

DISCUSSION

In the study described here, we modified an existing typing assay so that all six known major genotypes of HCV could be recognized. By the addition of *Hin*fI to *Scr*FI, a novel site present in all type 4 (and type 3) variants resulting from a change from U to C at position −166 could be recognized. New cleavage patterns resulting from the 3-base insertion in the 5′-NCR of type 6 variants were also classified in the study as electropherotype h with *Hae*III-*Rsa*I and electropherotype I with *Scr*FI-*Hin*fI. The type 5 sequences could be distinguished from type 1, replacing *Scr*FI (GG*n*CC) with *Mva*I (GG[A/T]CC) to identify the A/G polymorphism at position −162 and the U/C polymorphism at position −121. We are in the process of exploring whether *Mva*I-*Hin*fI can be used to entirely replace the original *Scr*FI-*Hin*fI combination for the identification of all six genotypes, as suggested by analysis of the predicted fragment patterns of the published sequences (Fig. 1A and C).

The main problem with any HCV typing assay is the need for continual modifications as new HCV genotypes are discovered. Many of the assays that use type-specific primers could be predicted to either fail to differentiate genotypes or fail to amplify sequences unrecognized when the primers were originally designed. For example, none of the primers used for the identification of genotypes I, II, III, and IV (types 1a, 1b, 2a, and 2b, respectively) described by Okamoto et al. (23) would be likely to amplify either type 3 or type 4 sequences. Similarly, type-specific probes for amplified NS-5 or 5′-NCR based on these genotypes would fail to hybridize or would hybridize inappropriately to many of the newly recognized genotypes (11, 28, 31).

There is now a considerable amount of comparative sequence data on the 5′-NCRs of HCV types 1, 2, and 3 from several geographical regions. It is therefore possible to predict a comprehensive range of possible electropherotypes with the restriction enzymes used in the present study and to reliably differentiate these genotypes from each other. However, the designation of specific electropherotypes to HCV types 4, 5, and 6 is preliminary at this stage because so few sequences in the 5′-NCR have been described. For example, the existence of types 4, 5, and 6 is based on phylogenetic analysis of 3, 4, and 1 sequences, respectively, in the NS-5 region (25). Each of these new genotypes contains sequences that are distinct in the 5′-NCR, and to date, a total of 18, 6, and 6 5′-NCR sequences similar to those of types 4, 5, and 6 that have been definitively genotyped have been published (2, 26). It is likely that a wider range of electropherotypes will be found for these genotypes, and continuous modification of Fig. 1 will be required as new data accumulate. Indeed, some of the sequences closely resembling type 4 (26), but which were obtained from West Africa rather than the Middle East (2, 28), would produce patterns with *Hae*III-*Rsa*I other than a and b, although all would produce the C electropherotype with *Mva*I-*Hin*fI.

Finally, the method described here detects only the majority circulating sequence of HCV; coinfection with two or more genotypes is apparent only when approximately equimolar concentrations of different sequences are present within a sample. Our finding of only two coinfections (both from Hong Kong donors infected with type 1 and type 6) could clearly be

an underestimate of the true rate of mixed infections. Assays based on type-specific amplification of different genotypes would be expected to be more sensitive indicators of multiple infections (16, 23), provided that separate amplification reactions were carried out for each pair of type-specific primers.

Accepting the above qualifications, it is clear from this first large-scale survey of blood donors that major differences in the geographical distributions of HCV variants exist. Donors in western European countries (Scotland, Holland, and Finland) were almost exclusively infected with types 1, 2, and 3, while those in the Far East (Japan, Taiwan, and Hong Kong) were mainly infected with type 1, with lower frequencies of infection with type 2 (and type 6 in Hong Kong). The situation is highly complex and difficult to interpret because there are genotypes with worldwide distributions (e.g., types 1 and 2) that coexist with those which are apparently confined to specific geographical areas (types 4 and 6).

The data from blood donors presented here are consistent with those obtained from smaller surveys of a variety of different patient groups with non-A, non-B hepatitis or hepatocellular carcinoma (16, 29–31). However, these latter studies are potentially biased toward those genotypes that are more likely to cause more severe liver disease. Current evidence suggests that genotype 1 is associated with a higher rate of chronic active hepatitis or cirrhosis than is genotype 2, 3, or 4 (10) and is associated with a much poorer response to treatment with alpha interferon than are type 2 (15, 29, 33) and type 3 (10). It is perhaps significant that among the asymptomatic blood donors in the present study, we found a much higher rate of non-type 1 variants (43%) than in previous surveys (20, 23, 30, 31).

Any attempt to interpret these geographical differences is hampered by current ignorance surrounding the method of virus transmission. Needle sharing, transfusion of blood and blood products, tattooing, and other forms of parenteral exposure have been implicated in the transmission of HCV, while current evidence suggests that the intrafamilial, mother-to-child, sexual, and intrafamilial forms of transmission are relatively inefficient modes of virus spread. There is therefore little understanding of how or whether HCV might spread in societies where blood transfusion and parenteral drug abuse are uncommon. As an example of the difficulty in interpreting the current data, the similarities in the distributions of HCV genotypes in Australia and western Europe (and their difference from the genotypes in other Far East countries) could be explained by assuming an ancient origin for HCV and relating the distributions of genotypes of the majority of Australian blood donors to their original European ancestry. On the other hand, it might be that HCV was introduced relatively recently by needle sharing among HCV-infected drug abusers from Europe or the United States.

The existence of variants of HCV that show up to 30% sequence diversity could potentially lead to significant antigenic variations among homologous proteins encoded by the different genotypes of HCV. The effects that this sequence variation would have on the effectiveness of antibody screening for HCV remains a relatively unexplored area. All currently used assays use antigens derived from HCV type 1 sequences and rely on cross-reacting antibodies to detect infection with other HCV genotypes. This is illustrated by the first-generation assays that used the c100-3 recombinant protein derived from NS-4. The antigenic region was found to be particularly variable in sequence between HCV types 1, 2, and 3 (27) and led to a low rate of detection of infection with variants other than type 1 (5, 18). In the present study, we showed that the majority of individuals infected with genotypes 2 to 6 showed weak or absent reactivity to c100-3, confirming our previous observations for types 2 and 3 on a relatively smaller number of individuals (18). This lack of cross-reactivity provides at least one explanation for the continuing transmission of HCV by blood screened by anti-C100 assays alone (12, 14).

All donors infected with HCV types 2 to 6 reacted with the recombinant core protein c22-3, probably reflecting the protein's greater degree of amino acid sequence conservation (90%) than that among the nonstructural proteins (75 to 80%). However, this observation is misleading, because c22-3 is a component of screening and confirmatory assays for HCV infection. In interpreting these results, the preselection by the screening assays precluded an analysis of the proportion of donors with infections with divergent HCV types that elicited an even more restricted serological response so as to be undetectable by current blood donor screening assays. The actual frequency with which samples from individuals infected with variants other than type 1 are missed by current second-generation screening assays (which contain c33c and c22-3 as well as NS-4 proteins) is unknown, but it is probably low. However, it is recognized that the serological response to HCV-encoded antigens is often narrow in specificity and is generally of low titer. Acute infections with HCV following transfusion of infectious blood or blood products may fail to elicit antibodies for several months (32). Furthermore, individuals who are only marginally immunosuppressed (such as patients undergoing renal dialysis, elderly people, hemophiliacs, and neonates) generally show extremely restricted and idiosyncratic patterns of serological reactivity, often to only one (or possibly none) of the four antigens used in current screening assays (17). In all of these cases, it is clearly desirable to have a test that is optimally sensitive for all variants of HCV. Evaluation of assays that incorporate additional peptide antigens corresponding to epitopes of other HCV types not shared with type 1 may represent a first step in improving current assays.

A recent report described the immunization of several chimpanzees with E1 and E2 proteins produced in mammalian cells and their protection from infection with homologous type virus (type 1) (7). However, these viral envelope glycoproteins have highly variable sequences among different genotypes of HCV. For example, amino acid sequence similarities of 55 and 74% were found upon comparison of the E1 and E2 proteins, respectively, of a type 1 and a type 2 variant (HCV-1 and HC-J8) (21). By analogy with other enveloped viruses, it is likely that the antibodies elicited by one genotype will not protect an individual from reinfection with heterologous variants. Indirect evidence for this possibility is shown by the frequent detection of infection with multiple genotypes in a variety of different patient groups (23, 27). These data suggest that a fully protective vaccine may have to contain a range of different envelope proteins corresponding to the common genotypes in particular geographical regions. For example, those for use in western Europe might include the E1 and E2 proteins from HCV types 1, 2, and 3, while those for use in the Middle East might be based on type 4 sequences.

ACKNOWLEDGMENTS

We are indebted to the following for assistance in carrying out the donor survey: Debbie Rankin, SMLSO, Riyadh Armed Forces Hospital; W.-T. Liu, School of Medical Technology, National Yang-Ming Medical College, Taipei, and W. S. Lin, Taichung Blood Donation Centre, Taichung, Taiwan; J. Andreis, V. Shannahan, and D. Kay, Blood Transfusion Service, Perth, Australia; and I. Coote and H. Munro, Ruchill Hospital, Glasgow, United Kingdom. We thank Donald Smith for careful reading of the manuscript. Finally, we are grateful to J. D. Cash for continued support and encouragement.

The work in Scotland was funded by the Scottish National Blood Transfusion Service and the Medical Research Council (grant G9020615CA).

REFERENCES

1. **Bresters, D., H. T. M. Cuypers, H. W. Reesink, W. P. Schaasberg, C. L. van der Poel, E. P. Mauserbunschoten, M. Houghton, Q. L. Choo, G. Kuo, R. Lesniewski, H. Troonen, and P. N. Lelie.** 1992. Enhanced sensitivity of a 2nd generation ELISA for antibody to hepatitis C virus. Vox Sang. **62:**213–217.
2. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1992. Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proc. Natl. Acad. Sci. USA **89:**4942–4946.
3. **Cha, T. A., E. Beall, B. Irvine, J. Kolberg, D. Chien, G. Kuo, and M. S. Urdea.** 1992. At least five related, but distinct, hepatitis C viral genotypes exist. Proc. Natl. Acad. Sci. USA **89:**7144–7148.
4. **Chan, S.-W., F. McOmish, E. C. Holmes, B. Dow, J. F. Peutherer, E. Follett, P. L. Yap, and P. Simmonds.** 1992. Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. J. Gen. Virol. **73:**1131–1141.
5. **Chan, S.-W., P. Simmonds, F. McOmish, P.-L. Yap, R. Mitchell, B. Dow, and E. Follett.** 1991. Serological reactivity of blood donors infected with three different types of hepatitis C virus. Lancet **338:**1391.
6. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA derived from a blood-borne non-A, non-B hepatitis genome. Science **244:**359–362.
7. **Choo, Q. L., R. Ralston, G. Kuo, A. Weiner, D. Chien, G. Van Nest, J. Han, E. Muchmore, and M. Houghton.** 1993. Vaccine approaches to the hepatitis C viruses. ISVHLD **8:**61. (Abstract B17.)
8. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
9. **Dow, B. C., I. Coote, H. Munro, F. McOmish, P. L. Yap, P. Simmonds, and E. A. C. Follett.** 1993. Confirmation of hepatitis C virus antibody in blood donors. J. Med. Virol. **41:**215–220.
10. **Dusheiko, G., H. Schmilovitz-Weiss, D. Brown, F. McOmish, P. L. Yap, S. Sherlock, N. McIntyre, and P. Simmonds.** Hepatitis C virus genotypes. An investigation of type-specific differences in geographic origin and disease. Hepatology, in press.
11. **Enomoto, N., A. Takada, T. Nakao, and T. Date.** 1990. There are two major types of hepatitis C virus in Japan. Biochem. Biophys. Res. Commun. **170:**1021–1025.
12. **Esteban, J. I., A. Gonzalez, J. M. Hernandez, L. Viladomiu, C. Sanchez, J. C. Lopez Talavera, D. Lucea, C. Martin Vega, X. Vidal, R. Esteban, and J. Guardia.** 1990. Evaluation of antibodies to hepatitis C virus in a study of transfusion-associated hepatitis. N. Engl. J. Med. **323:**1107–1112.
13. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q. L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14:**381–388.
14. **Japanese Red Cross Non-a, Non-b Hepatitis Research Group.** 1991. Effect of screening for hepatitis C virus antibody and hepatitis B virus core antibody on the incidence of post-transfusion hepatitis. Lancet **338:**1040–1041.
15. **Kanai, K., M. Kako, and H. Okamoto.** 1992. HCV genotypes in chronic hepatitis C and response to interferon. Lancet **339:**1543.
16. **Kato, N., Y. Ootsuyama, S. Ohkoshi, T. Nakazawa, S. Mori, M. Hijikata, and K. Shimotohno.** 1991. Distribution of plural HCV types in Japan. Biochem. Biophys. Res. Commun. **181:**279–285.
17. **Lelie, P. N., H. T. Cuypers, H. W. Reesink, C. L. van der Poel, I. Winkel, E. Bakker, P. J. Exel Oehlers, D. Vallari, J. P. Allain, and L. Mimms.** 1992. Patterns of serological markers in transfusion-transmitted hepatitis C virus infection using second generation HCV assays. J. Med. Virol. **37:**203–209.
18. **McOmish, F., S.-W. Chan, B. C. Dow, J. Gillon, W. D. Frame, R. J. Crawford, P. L. Yap, E. A. C. Follett, and P. Simmonds.** 1993. Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serological reactivity and rate of alanine aminotransferase abnormalities. Transfusion **33:**7–13.
19. **Mori, S., N. Kato, A. Yagyu, T. Tanaka, Y. Ikeda, B. Petchclai, P. Chiewsilp, T. Kurimura, and K. Shimotohno.** 1992. A new type of hepatitis C virus in patients in Thailand. Biochem. Biophys. Res. Commun. **183:**334–342.
20. **Nakao, T., N. Enomoto, N. Takada, A. Takada, and T. Date.** 1991. Typing of hepatitis C virus (HCV) genomes by restriction fragment length polymorphisms. J. Gen. Virol. **72:**2105–2112.
21. **Okamoto, H., K. Kurai, S. Okada, K. Yamamoto, H. Lizuka, T. Tanaka, S. Fukuda, F. Tsuda, and S. Mishiro.** 1992. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology **188:**331–341.
22. **Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyakawa, and M. Mayumi.** 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J. Gen. Virol. **72:**2697–2704.
23. **Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. **73:**673–679.
24. **Simmonds, P., P. Balfe, J. F. Peutherer, C. A. Ludlam, J. O. Bishop, and A. J. Brown.** 1990. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. **64:**864–872.
25. **Simmonds, P., E. C. Holmes, T. A. Cha, S.-W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea.** 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol. **74:**2391–2399.
26. **Simmonds, P., F. McOmish, P. L. Yap, S. W. Chan, C. K. Lin, G. Dusheiko, A. A. Saeed, and E. C. Holmes.** 1993. Sequence variability in the 5′ non coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. J. Gen. Virol. **74:**661–668.
27. **Simmonds, P., K. A. Rose, S. Graham, S. W. Chan, F. McOmish, B. C. Dow, E. A. C. Follett, P. L. Yap, and H. Marsden.** 1993. Mapping of serotype-specific, immunodominant epitopes in the NS-4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infections with HCV type 1, type 2, and type 3. J. Clin. Microbiol. **31:**1493–1503.
28. **Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Heuverswyn, and G. Maertens.** 1993. Typing of hepatitis C virus isolates and characterisation of new subtypes using a line probe assay. J. Gen. Virol. **74:**1093–1102.
29. **Takada, N., S. Takase, N. Enomoto, A. Takada, and T. Date.** 1992. Clinical backgrounds of the patients having different types of hepatitis C virus genomes. J. Hepatol. **14:**35–40.
30. **Takada, N., S. Takase, A. Takada, and T. Date.** 1992. HCV genotypes in different countries. Lancet **339:**808.
31. **Takada, N., S. Takase, A. Takada, and T. Date.** 1993. Differences in the hepatitis C virus genotypes in different countries. J. Hepatol. **17:**277–283.
32. **van der Poel, C. L., D. Bresters, H. W. Reesink, A. A. D. Plaisier, W. Schaasberg, A. Leentvaar Kuypers, Q. L. Choo, S. Quan, A. Polito, M. Houghton, G. Kuo, P. N. Lelie, and H. T. M. Cuypers.** 1992. Early anti-hepatitis C virus response with 2nd generation C200/C22 ELISA. Vox Sang. **62:**208–212.
33. **Yoshioka, K., S. Kakumu, T. Wakita, T. Ishikawa, Y. Itoh, M. Takayanagi, Y. Higashi, M. Shibata, and T. Morishima.** 1992. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus. Hepatology **16:**293–299.