JOURNAL OF CLINICAL MICROBIOLOGY, June 1993, p. 1493–1503
0095-1137/93/061493-11$02.00/0

Vol. 31, No. 6

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 Types 1, 2, and 3

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Received 14 December 1992/Accepted 16 March 1993

The effect of sequence variability between different types of hepatitis C virus (HCV) on the antigenicity of the NS-4 protein was investigated by epitope mapping and by enzyme-linked immunosorbent assay with branched oligopeptides. Epitope mapping of the region between amino acid residues 1679 and 1768 in the HCV polyprotein revealed two major antigenic regions (1691 to 1708 and 1710 to 1728) that were recognized by antibody elicited upon natural infection of HCV. The antigenic regions were highly variable between variants of HCV, with only 50 to 60% amino acid sequence similarity between types 1, 2, and 3. Although limited serological cross-reactivity between HCV types was detected between peptides, particularly in the first antigenic region of NS-4, type-specific reactivity formed the principal component of the natural humoral immune response to NS-4. Type-specific antibody to particular HCV types was detected in 89% of the samples from anti-HCV-positive blood donors and correlated almost exactly with genotypic analysis of HCV sequences amplified from the samples by polymerase chain reaction. Whereas almost all blood donors appeared to be infected with a single virus type (97%), a higher proportion of samples (40%) from hemophiliacs infected from transfusion of non-heat-inactivated clotting factor contained antibody to two or even all three HCV types, providing evidence that long-term exposure may lead to multiple infection with different variants of HCV.

The identification of hepatitis C virus (HCV) (9) and the development of serological assays to detect infection in blood donors (24) provide an important opportunity for prevention of posttransfusion non-A, non-B hepatitis (NANBH). Early serological assays used the NS-4-derived recombinant protein, c100-3, as an antigen on the solid phase in an indirect enzyme-linked immunosorbent assay (ELISA). This test was of major value in early surveys of the incidence of HCV infection in different risk groups for infection (11), such as patients with posttransfusion NANBH and implicated donors (2, 16, 54) and hemophiliacs (4, 15, 27, 29). However, the test yielded frequent false-positive reactions (44), particularly with samples containing autoantibodies (21, 31, 49). In the absence of a satisfactory confirmatory assay, particularly misleading results were obtained when the assay was used to screen populations with a low incidence of infection, such as volunteer blood donors (12, 34).

However, a more serious problem was the frequent occurrence of false-negative results. An indication of the relative insensitivity of the test was provided by the finding that not all hemophiliacs treated with non-heat-inactivated factor VIII or IX were anti-C100 positive despite suffering from NANBH (4, 15, 27, 29). That HCV was responsible was demonstrated by the frequent detection of circulating RNA in plasma by the polymerase chain reaction (PCR) in seronegative hemophiliacs (43), particularly those with concurrent infection with human immunodeficiency virus. In retrospective studies of posttransfusion NANBH patients, a considerable delay was found between transfusion and the onset of hepatitis with the development of antibody to c100-3 (6 weeks to 6 months) (2, 16, 26, 51, 53, 55, 57). Transfusion of blood collected during this period may account for many of the continued transmissions of HCV to blood recipients (16, 22) after anti-C100 assays were adopted for blood donation screening.

Another possible cause for the absence of reactivity to c100-3 in infected individuals could be the sequence variability of different HCV isolates in the NS-4 region. Hepatitis C viruses are a heterogeneous group of viruses, which make up several major types (7, 14, 35, 50) showing approximately 70% homology overall, similar to that which exists between different serotypes of other flaviviruses (6). Currently, three major types have been characterized in detail and will be referred to here as HCV type 1 (10, 23, 46), type 2 (37, 38) and type 3 (7, 35), as recently proposed (7). Each major type can in turn be further differentiated into a number of subtypes, termed 1a, 1b, and 2a, etc. When complete sequence data are available, it has been found that the subtypes show 77 to 79% similarity, consistently greater than that found between the major types of HCV. We have evidence for at least four subtypes of type 1 and three subtypes of type 2 (19a), while comparative sequence data of HCV type 3 variants in the NS-5 region reveal two subtypes (35).

The NS-4 region encoding the c100-3 antigen is variable between different isolates of HCV, showing only 75 to 77% sequence similarity between HCV types 1 and 2. That this

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TABLE 1. Sequences of primers used for amplification of the NS-4 region of HCV types 1, 2, and 3

Primer	HCV type[a]	Position[b]	Orientation[c]	Sequence 5′ to 3′
007	All	5293	−	AACTCGAGTATCCCACTGATGAAGTTCCACAT
220	All	5278	−	CACATGTGCTTCGCCCAGAA
253	1	4858	+	GGGCCAACACCCCTGCTATA
196	1	4878	+	CAGACTGGGCGCCGTTCAGAATG
281	2	4858	+	GGTCCCACCCCTCTCCTGTA
509	2	4878	+	CCGCTTGGGTTCCGTTACCAACG
221	3	4858	+	GGACCTACGCCCCTTCTATA
008	3	4878	+	TCGGTTGGGGCCTGTCCAAAATG

[a] Separate sense primers required to enable amplification of each HCV type.
[b] Position of the 5′ base relative to the HCV genomic sequence given in reference 10.
[c] Orientation of primer sequence (+, sense; −, antisense).

affects serological recognition is shown by the relatively infrequent serological reactivity of sera from blood donors infected with HCV types 2 and 3 with NS-4-derived antigens in immunoblot assays (7, 33) and frequent negative results in the original first generation blood donor screening assays. In the current study, we have investigated the distribution of epitopes in the NS-4 region encoding c100-3 for HCV types 1, 2, and 3. The frequency of serological reactivity to synthetic peptides corresponding to two antigenic regions of the sequenced region of the three HCV types was investigated with samples from blood donors infected with known HCV types. This allowed an investigation of the frequency of type-specific and cross-reactive antibody specificities to be determined in naturally infected individuals. Finally, an assay for type-specific antibody provided evidence for infection with different HCV types in anti-HCV-positive blood donors and a higher incidence of mixed infection in hemophiliacs multiply exposed to HCV.

MATERIALS AND METHODS

Samples. Plasma samples were obtained from 137 HCV-infected blood donors identified by screening with the Abbott or Ortho second generation ELISA (33). Plasma samples were also obtained from 27 human immunodeficiency virus-uninfected hemophiliacs who had been treated with non-heat-inactivated factor VIII and IX concentrates and who had been infected with HCV for several years. All samples were anti-HCV positive by several second generation screening and confirmatory assays (13a, 56). Control samples were obtained from anti-HCV-negative blood donors. All samples from anti-HCV-positive blood donors were screened for antibody to the recombinant c100-3 recombinant protein using the Abbott first generation enzyme immunoassay according to the manufacturer's instructions.

Sequence analysis of NS-4. RNA was extracted from a series of HCV-infected blood donors and reverse transcribed with a primer complementary in sequence to a relatively well conserved segment within the region of the HCV genome encoding NS-4. Complementary DNA was amplified by using this primer in combination with a series of sense orientation primers for each of the major types of HCV (Table 1). The sequences of these primers were determined by comparison of those obtained in the NS-3 region in a previous study and other published sequences (7, 10, 23, 38, 46). The primary PCR product was amplified further by a second PCR with a conserved antisense inner primer and one of three type-specific sense primers for amplification of HCV types 1, 2, and 3. Amplified DNA was directly sequenced as previously described (7).

HCV genotyping. HCV RNA sequences were reverse transcribed and amplified from plasma samples of infected blood donor samples as previously described (7). The HCV type was determined either by direct nucleotide sequencing of the PCR product and phylogenetic analysis (7) or by restriction fragment length polymorphism analysis of radiolabelled amplified DNA with *Hae*III-*Rsa*I and *Scr*FI (33).

Epitope mapping. Four sets of overlapping nonapeptides corresponding to the consensus amino acid sequences of HCV subtypes 1a, 1b, 2b, and 3a were synthesized as previously described (32) and modified for Fmoc chemistry by using kits from Cambridge Research Biochemical (Norwich, United Kingdom) according to the manufacturer's instructions. Each series comprised a set of 82 oligopeptides from positions 1679 to 1768 in the NS-4 region (10), each of which overlapped the adjacent peptide by eight residues. The peptides were synthesized in covalent linkage to polypropylene rods having the format and spacing of the wells in a microtiter plate.

Antibody reactivity to individual peptides was assayed with incubation of each pin overnight at 4°C in 175 μl of a 1/40 dilution of anti-HCV-positive or -negative plasma in phosphate-buffered saline (PBS) supplemented with 1% ovalbumin, 1% bovine serum albumin, and 0.1% Tween 20 overnight. Pins were washed in four changes of PBS–0.1% Tween 20 (PBST), and bound antibody was detected by a second incubation with a 1/20,000 dilution of affinity-isolated horseradish peroxidase-conjugated anti-human immunoglobulin G (Sigma Chemical Company, Poole, United Kingdom) for 2 h at 20°C. After washing, pins were immersed in 100 μl of a freshly prepared solution of 0.5 μg of 2,2′-azino-bis(3-ethylbenzthiazoline 6-sulphonic acid) (Sigma) per ml in 0.1 M sodium phosphate-sodium citrate buffer (pH 4.0) containing 0.03% hydrogen peroxide (30 volumes) for 30 min at 20°C. Optical densities (ODs) were measured at 410 nm, with a reference wavelength of 650 nm by using a dual-wavelength spectrophotometer; values ranged from −100 to 750 mU. Pins were reused by stripping off antibody by sonication (30 min) in 1% sodium dodecyl sulfate–0.1% 2-mercaptoethanol–0.1 M sodium dihydrogen orthophosphate, followed by reblocking in 1% ovalbumin–1% bovine serum albumin–0.1% Tween 20 in PBS for 1 h at room temperature.

Synthesis of branched peptides. Branched peptides (40, 47) corresponding to antigenic regions identified by epitope mapping were synthesized by continuous-flow Fmoc chemistry as previously described (3, 32). Some peptides were made to contain an equimolar mixture of two amino acids at one position (Table 2). To do this, the peptide was assembled on a single batch of resin up to the relevant amino acid, at which point the resin was split into two aliquots. One of the

TABLE 2. Sequences of NS-4 peptides used for type-specific serological assay

HCV type	Region[a]	Peptide	Sequence
1	1	370-C	KPA(I/V)IPDREVLYREFDEM[b]
	1	370-B	RPAV(I/V)PDREVLYQEFDEM[b]
	2	373-C	ECSQHLPYIEG(M/A)LAEQF
2	1	370-A	RVVVTPDKEILYEAFDEM
	2	373-A	ECASRAALIEEGQRIAEML
	2	373-B	ECASKAALIEEGQRMAEML
3	1	368-A	KPALVPDKEVLYQQYDEM
	2	368-B	ECSQAAPYIEQAQVIAHQF

[a] Amino acid positions 1691 to 1708 (region 1) and 1710 to 1728 (region 2), numbered as described in reference 10.
[b] Alternative peptides, where there is variability of an HCV type.

two amino acids was added to one aliquot, and the other was added the second aliquot. The two portions of resin were then combined, and the synthesis was continued. For cases in which alternative variants showed two amino acid differences, peptides were produced separately to avoid synthesis of hybrid sequences not present in the original samples (e.g., peptides 373-A and 373-B of HCV type 2). The amino acid compositions of the branched peptides were determined by amino acid analysis (Cambridge Research Biochemicals, Cambridge, United Kingdom) and were in agreement with expectations.

Indirect ELISA. Approximately 1 mg of peptide was reconstituted in distilled water (dH_2O) to give a final concentration of 1 mg/ml immediately before use. Peptides 368A and 368B (type 3, regions 1 and 2) required acidification with a small volume of acetic acid to ensure complete solubilization, while the remainder was dissolved only after alkalinization by bubbling 1 to 2 ml of ammonia vapor through the water. One hundred-microliter volumes of peptides at concentrations ranging from 20 ng/ml to 20 μg/ml in PBS were coated onto polypropylene microtiter wells (Immunoplate-GIBCO BRL, Paisley, United Kingdom) overnight at 4°C. After washing, the wells were blocked in 150-μl volumes of blocking solution (PBST–1% bovine serum albumin, 1% ovalbumin) for 1 h at room temperature. Serological reactivity to an irrelevant branched peptide (HIV V3 loop [42]) was measured for each serum, which served as a control for possible nonspecific antibody binding to the polylysine spacer residues.

Plasma from HCV-infected blood donors was diluted in PBST–0.5% bovine serum albumin (PBSAT), and 100 μl was added to antigen-coated and blocked wells. The first incubation was carried out at room temperature for 1 h. Plates were washed four times in PBST, followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G (1/20,000 in PBSAT; 1 h, room temperature). The plates were finally washed four times in PBST and incubated with substrate (50 μg of *o*-phenylenediamine per ml, 0.1% H_2O_2 [30 volumes]; 30 min at room temperature). ODs were read at 490 nm, with values ranging from −100 to 2,000 mU.

Blocking assays were carried out as described above, except that blocking peptide (in a 100:1 excess over those used for coating microtiter wells) was added to the first antibody diluent.

RESULTS

Derivation of NS-4 consensus sequences for HCV types 1 to 3. Part of the 5′ noncoding region of HCV was amplified by PCR from circulating viral RNA in plasma samples from 14 anti-HCV-positive Scottish blood donors. Direct sequence analysis of the region allowed each sample to be assigned to type 1 ($n = 5$), 2 ($n = 4$), or 3 ($n = 5$) variants by comparison with previously published sequences (7). To amplify the NS-4 region, cDNA was synthesized with primer 007 from plasma viral RNA, amplified with this primer and the appropriate type-specific sense primer (Table 1), and then subjected to a second reaction with primer 220 and primer 008, 509, or 196, depending on the HCV type. Direct sequence analysis of the amplified DNA yielded nucleotide sequences corresponding to positions 4911 to 5271 in the HCV type 1 prototype genome (10).

The inferred amino acid sequences (Fig. 1A) of variants from the five blood donors infected with HCV type 1 were similar to those of the prototype HCV type 1 variant (subtype 1a [7]) and differed at several sites from sequences of subtypes 1b, 2a, and 2b (Fig. 1). One sequence from a type 2-infected donor was similar to that of type 2a, while the remaining three were type 2b. The five donors with type 3 infection yielded NS-4 sequences distinct from any of those published to date, further confirming its status as a separate major type of HCV (7).

Within each subtype, remarkably little sequence variation was apparent; only 8 polymorphic amino acids of 90 were found among the type 1 sequences, and only 2 were found in the five type 3 sequences. Furthermore, most of the substitutions were between similar amino acids, such as substitutions of arginine for lysine, valine for alanine, and valine for isoleucine. From the type 1a, 2b, and 3 sequences, it was possible to derive a consensus sequence that was representative of each subtype of HCV (Fig. 1B). However, these consensus sequences differed considerably from each other and from those of the two subtype 1b and two subtype 2a sequences, with only 36 amino acid residues of 90 being conserved between all five subtypes.

The consensus sequences from types 1a, 1b, 2b, and 3 were used to specify four sets of 82 overlapping peptides, each of which was nine residues in length and overlapped by eight residues. For example, the sequences of the peptides of the subtype 1a series were CVVIVGRIV (first), VVI VGRIVL (second), and VIVGRIVLS (third), etc., to FWA KHMWNF, the last (82nd) peptide. The distribution of changes between the amino acid sequences of the types 1a, 1b, 2b, and 3 resulted in almost all of corresponding peptides in the four series having different sequences (Fig. 1B).

Serological reactivity between the subtype 1a peptides and a plasma sample from a type 1-infected blood donor was confined to three or four relatively well defined sequential epitopes in the NS-4 region (Fig. 2A), spanning peptides 12 to 22 (region 1), 32 to 38 (region 2), and 52 to 55 (region 3). Reactivity to peptides 12 to 20 and 32 to 38 was also found in the three additional anti-HCV type 1 samples tested (data not shown). In contrast, a sample from an anti-HCV-negative blood donor showed no significant reactivity with any of the peptides in the subtype 1a series (Fig. 2E). Reactivity of these sera to the subtype 1b series of peptides was similar both quantitatively and in antibody specificity to that of subtype 1a (Fig. 2A and B). Reactivity of a type 2-infected donor samples with the type 2b peptide series (Fig. 2C) and of a type 3-infected donor with type 3 peptides (Fig. 2D) was to regions corresponding almost exactly to those of regions 1

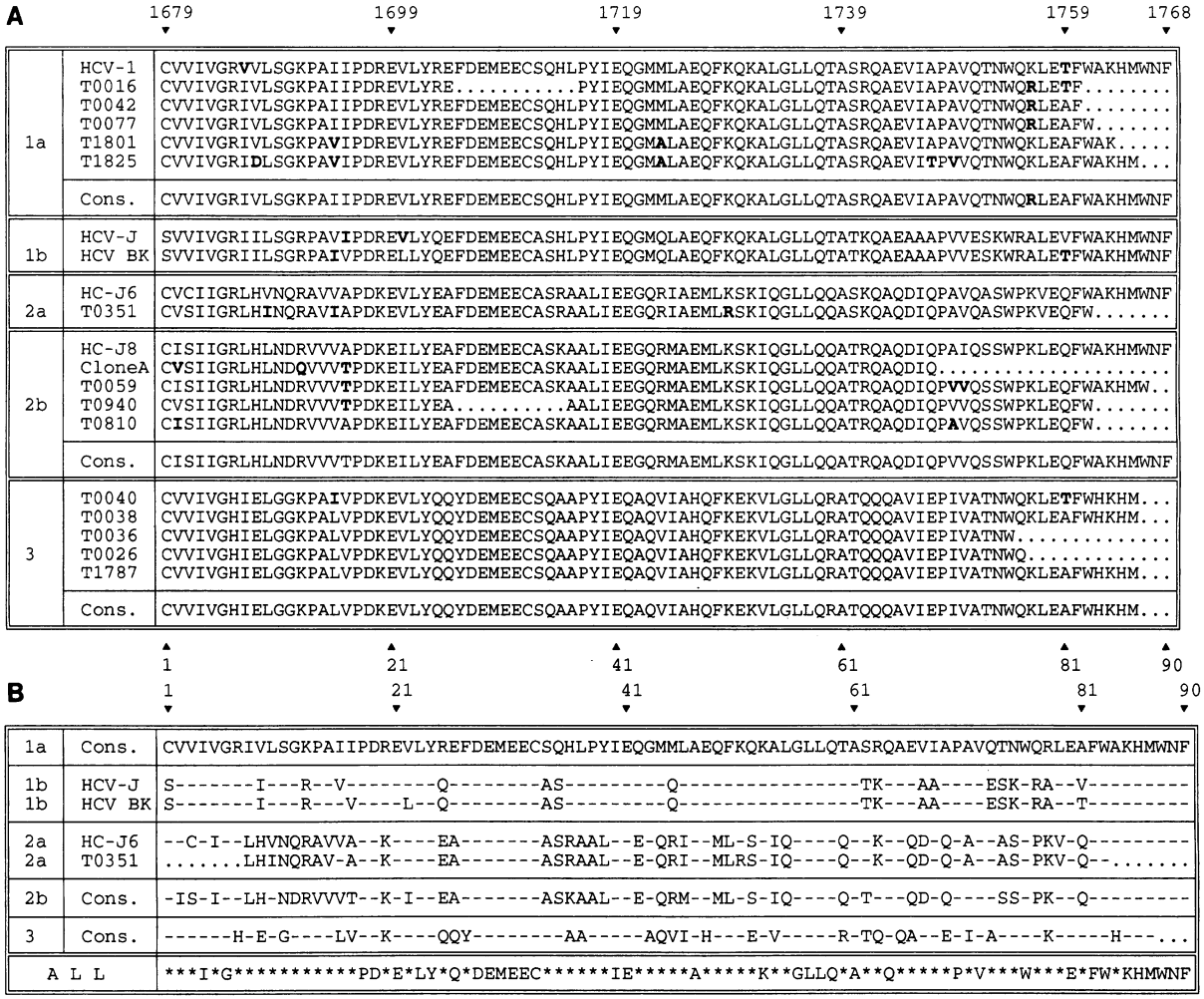

FIG. 1. Comparison of amino acid sequences of HCV types 1 to 3 in NS-4 region. (A) Intratypic variation. (B) Comparison of consensus sequence of HCV types in NS-4 region. Only two samples from subtypes 1b and 2a were obtained, and therefore both sequences are shown. Residues are numbered according to those of HCV type 1 (10). The sources of the published sequences for the following are given in parentheses: HCV type 1 (10), HCV-J (23), HCV-BK (46), HC-J6 (38), HC-J8 (37), clone A (50). Sequences T0016 to T1825 were derived from Scottish blood donors. The HCV type is indicated in the left column. Variable amino acid residues are indicated in bold. . . . , sequence has not been determined; —, residue identical to that of HCV type 1 (10); *, polymorphic amino acid residues between HCV types; cons., consensus.

and 2 in type 1 and to further antigenic regions between 58 and 63, downstream from region 3 of type 1 (Fig. 2A and B). Again, homologous type reactivity to regions 1 and 2 was found in other type 2- and 3-infected donors (data not shown). Despite the substantial sequence differences between the major types of HCV, the antigenic regions are located in similar places in the NS-4 protein of all three HCV types.

To investigate whether antibody elicited by infection with one HCV type cross-reacted with epitopes of the other types, serum samples from HCV-infected donors were incubated with each of the three sets of heterologous type peptides, and the reactivity was compared with that of the homologous type antigens (Fig. 3). The sample from the type 1-infected donor showed weak reactivity to the first antigenic region of the type 2 and 3 peptides (Fig. 3D and G) but showed equivalent reactivity with the subtype 1b series of peptides (Fig. 2B). The type 2 plasma sample again cross-reacted with heterologous peptides in only the first antigenic region (Fig. 3B and H), while the type 3 sample showed weak reactivity with both the first and second regions of the type 1 series and with region 1 of type 2 (Fig. 3C and F). Further experiments with other sera confirmed that cross-reactivity between types was variable and generally weaker than that to homologous type peptides (data not shown).

As serological reactivity to antigenic regions 1 and 2 was the most frequently found between the HCV types, the consensus sequences for these regions were used to specify a series of branched oligopeptides for the type-specific assay (Table 2). The peptides from region 1 spanned peptides 13 to 22 in the mapping series (HCV residues 1691 to 1708), while those of region 2 corresponded to the sequences of peptides 32 to 42 (residues 1720 to 1738). Sequence variation within each type (Fig. 1A) was allowed for by synthesizing two peptides, each bearing the alternative amino acid residues (see Materials and Methods). For example, peptide 370-C (Table 2) contains an equimolar mixture of two peptides, one with isoleucine and the other with valine at position 4

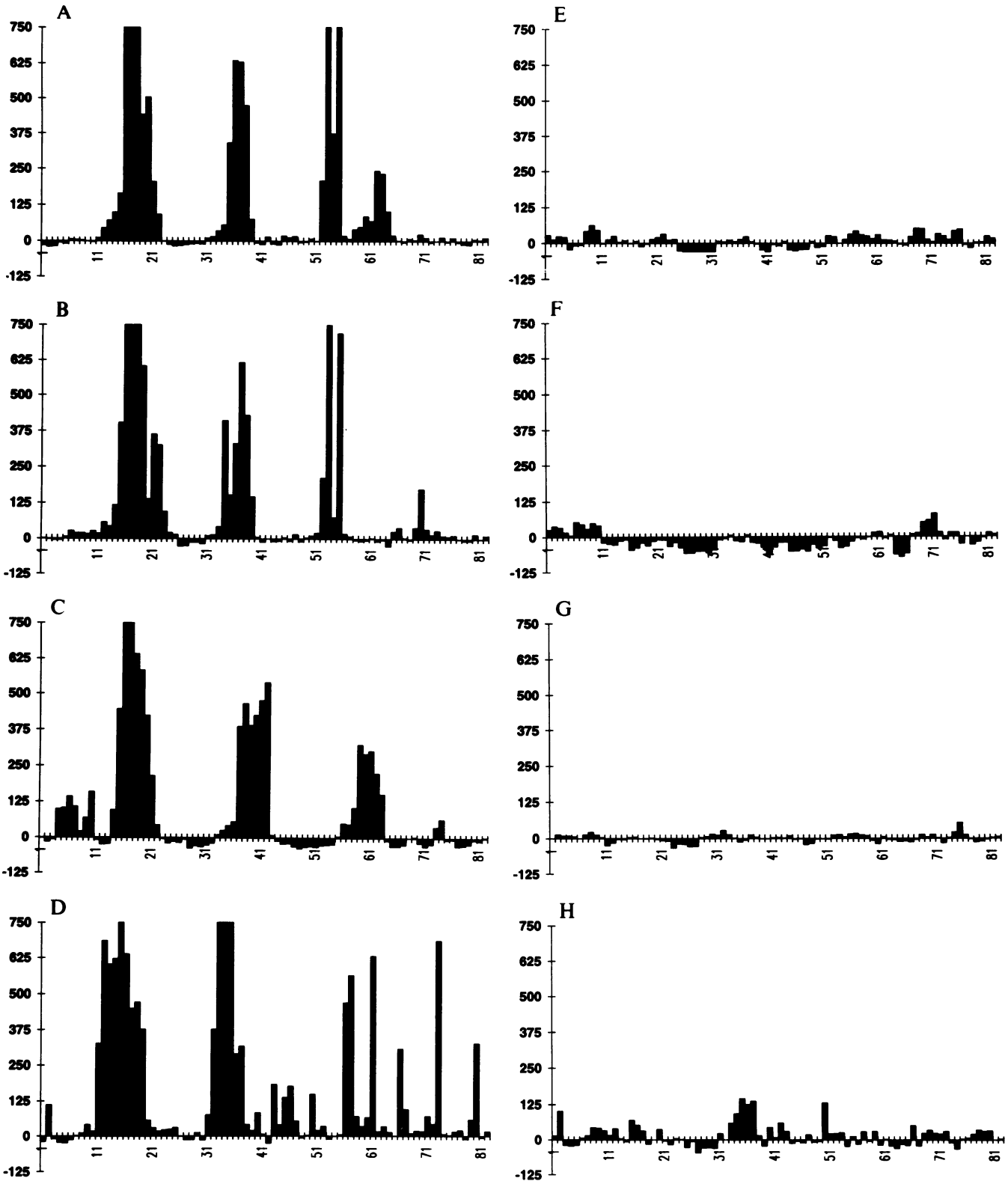

FIG. 2. Reactivity of representative anti-HCV-positive plasma samples (A to D) and a negative plasma sample (E to H) with overlapping peptides (numbered 1 to 82) corresponding to sequences within the NS-4 region of HCV. Reactivity of samples from an HCV type 1-infected donor (A and B) and an anti-HCV-negative donor (E and F) with peptides of subtype 1a (A and E) or 1b (B and F) peptides; reactivity of a type 2-infected donor (C) and anti-HCV negative donor (G) with type 2b peptides; reactivity of a type 3-infected donor (D) and anti-HCV negative donor (H) with type 3 peptides. ODs are expressed in milliunits in the range of 125 to 750 mU.

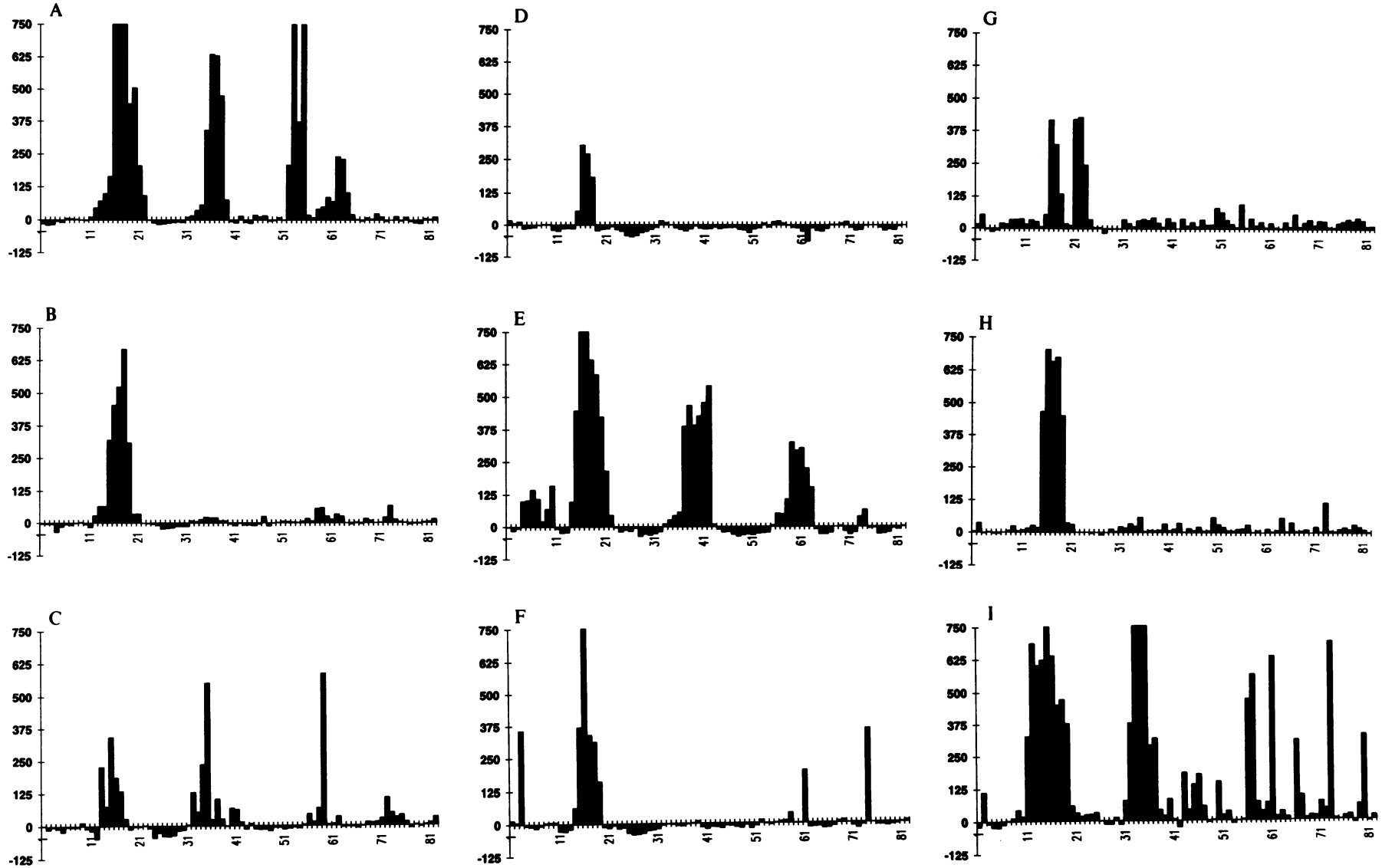

FIG. 3. Comparison of reactivity to type homologous and heterologous peptides. Reactivity of sera from donors infected with HCV type 1, 2, or 3 with type 1a (A, B, and C, respectively), type 2b (D, E, and F, respectively), type 3 (G, H, and I, respectively) peptides.

TABLE 3. Reactivity of anti-HCV-positive and -negative samples to peptides corresponding to two antigenic regions in NS-4

HCV type(s)	Antigenic region	Peptide	No. of reactive samples[a]			Total no.
			1	2	3	
1	1	370-C	**39**[b]	3	24	66
	1	370-B	**40**	3	18	61
	2	373-C	**46**	3	10	59
2	1	370-A	35	**6**	17	58
	2	373-A	24	**7**	2	33
	2	373-B	0	**7**	2	9
3	1	368-A	39	6	**25**	70
	2	368-B	39	1	**30**	70
1+2+3	1+2	ALL	**48**	**7**	**35**	90
1	1+2	c100-3[c]	**43**	3	15	61
Total no. tested			49	9	37	95

[a] Number of reactive samples from blood donors subdivided according to infection with types 1, 2, or 3.
[b] Type-homologous reactions are indicated in boldtype.
[c] Reactivity with commercially available recombinant c100-3-type antigen.

(residue 1694). The amount of branched peptide used for the ELISA was determined by a series of checkerboard titrations of peptide with anti-HCV-positive and -negative sera. Virtually no nonspecific binding to peptides was found, even at the highest concentration of peptides tested (10 μg per well) and the highest plasma concentration (1/10) (data not shown). For subsequent assays, we used a coating antigen concentration of 1 μg of antigen per ml and a test dilution of serum or plasma of 1/40.

For the ELISA, the cutoff value for each peptide was calculated as the mean of four negative-control values (reactivity to HCV peptide minus reactivity to irrelevant [non-HCV] peptides) + 2 standard deviations and ranged from 13 to 105 mU. We found that all 26 samples from anti-HCV-negative blood donors yielded net OD values (that of HCV peptide minus that of irrelevant peptides) below this cutoff value, while samples from HCV-infected blood donors showed frequent reactivity with each of the NS-4 peptides (Table 3). OD values ranged continuously from just above the cutoff to >2,000 mU. In general, reactivity to type homologous peptides was more frequent than that to other HCV types; in particular, very few sera from type 1- and type 3-infected donors reacted with peptides corresponding to the second antigenic region of type 2 (peptides 373-A and 373-B). In contrast, high frequencies of cross-reactivity were observed between HCV types 1 and 3 in both antigenic regions; 39 of the 49 type 1 samples reacted with type 3 antigens, while 30 of the 37 type 3 samples reacted with type 1 peptides. A total of 95% of anti-HCV-positive samples showed significant reactivity in an ELISA for which wells were coated with all eight peptides, substantially higher than that achieved with the recombinant c100-3 antigen (61 of 95) and higher than the frequency of reactivity with any one NS-4 peptide used alone (maximum, 70 of 95, peptides 368-A and 368-B; Table 3).

The ELISA was modified in order to detect type-specific antibody by the addition of a blocking concentration of peptides to plasma or serum samples to absorb cross-reactive antibody. In the HCV type-specific antibody assay (HCV-TSAA), wells were coated with 100 ng of peptides corresponding to each of the three HCV types per ml. The first well was coated with the three type 1 peptides, the second contained the three type 2 peptides, and the third well was coated with the two type 3 peptides. Test samples at a 1/40 dilution were incubated in each of the three wells in the presence of a 100:1 molar excess of type heterologous peptides in solution. Thus, for the well coated with type 1 peptides, the test sample was diluted in buffer containing 10 μg of each of the three type 2 peptides and the two type 3 peptides per ml. The net effect of this blocking is to ensure that the only antibody that could bind to the antigens on the solid phase was one that reacted with epitopes not shared with other HCV types. Type-specific reactivity was considered significant when it was greater than twice the residual OD obtained from incubating the test serum in a well coated with all eight peptides and blocked with all eight.

To illustrate how the method may be used for serological typing of HCV infection, the OD values of a series of representative samples in the three type-specific wells and the blocked and unblocked controls are shown graphically in Fig. 4. Four samples from blood donors infected with HCV type 1 (Fig. 4A to D) show variable reactivity with the unblocked well coated with all eight peptides (first bar [labelled C] in each histogram). Blocking with all eight reduced binding in all cases to background levels (<100 mU [second bar]). Reactivity to the type 2 and type 3 peptides on their own could also be completely blocked (fourth and fifth bars, respectively), while type 2 and 3 peptides in solution failed to block reactivity to the type 1 antigens (third bar). From this data, we can infer that the four samples illustrated contain antibody solely to HCV type 1. Equivalent results were obtained with the four type 2 and four type 3 serum samples (Fig. 4). Comparison of the OD of unblocked control with that of the blocked homologous type antigen showed that the type-specific antibody formed a substantial proportion and usually the majority of the total anti-NS-4 peptide antibody within each sample.

A total of 137 samples from donors typed by restriction fragment length polymorphism analysis of sequences amplified by PCR in the 5′ noncoding region (33) were assayed blind for the presence of type-specific antibodies by using the HCV-TSAA (Table 4). Upon decoding the results, we observed a remarkable correlation between the results of the two assays. Almost all samples that could be serologically typed contained antibody of a single type specificity corresponding to the HCV type detected by PCR (118 of 122). Antibody to one of the HCV types found in the four samples with dual specificity also corresponded to the circulating HCV type identified by PCR. Only 15 samples could not be typed; 7 were nonreactive with the NS-4 antigens at either 1/40 or upon retesting at a 1/10 test dilution, while the remaining 8 contained no detectable type-specific antibody. These samples showed (usually weak) reactivity with the unblocked peptides, that was completely blocked out not only by all eight peptides together but also in each of the wells blocked with heterologous peptides, indicating that these samples contain insufficient type-specific antibody.

To investigate whether the HCV-TSAA could reliably detect infection with multiple HCV types, we screened samples from 27 HCV-infected hemophiliacs treated for several years with non-heat-treated factor VIII or IX and who had been potentially exposed to all three types of HCV over the years of treatment (18, 27, 56). We found that a higher proportion of samples showed type-specific antibody to multiple HCV types. Whereas in blood donors, only 4 (3%) of 122 samples showed evidence of multiple infection, and dual or even triple antibody specificities were found in 8

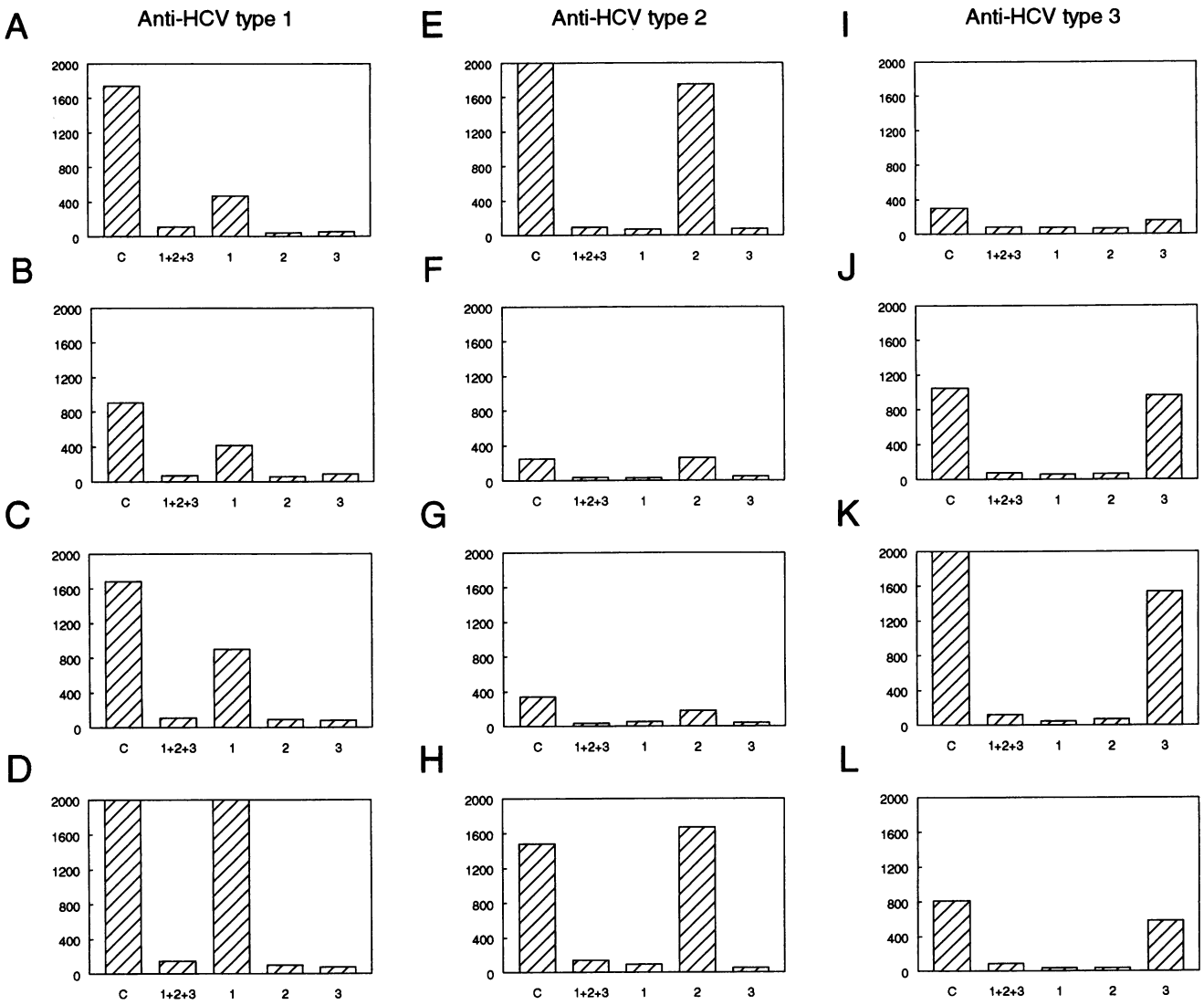

FIG. 4. Reactivity of representative plasma samples from donors of known HCV type in HCV-TSAA. Samples from HCV type 1 (A to D)-, type 2 (E to H)-, or type 3 (I to L)-infected donors. The histograms show reactivity to all eight peptides, with no blocking (total anti-NS-4 reactivity bar labelled (C); all eight peptides blocked with all eight (blocked control) (bar labelled 1+2+3): type 1 peptides blocked with type 2 and 3 peptides (type 1-specific antibody) (bar labelled 1); type 2 peptides blocked with type 1 and 3 peptides (type 2-specific antibody) (bar labelled 2); and type 3 peptides blocked with type 1 and 2 peptides (type 3-specific antibody) (bar labelled 3).

(40%) of the 20 hemophiliac samples typed by HCV-TSAA (Table 4). Antibodies to types 1 and 3 were found most frequently in the hemophiliacs, most of whom had been treated with only factor VIII or IX concentrates derived from local plasma donations. This frequency of antibody types is consistent with previous observations these two types are the predominant HCV variants detected in Scottish blood donors (33).

DISCUSSION

Epitope mapping of NS-4. In this study, we have investigated the antigenicity of the NS-4 protein of HCV. Two major sequential antigenic determinants occurred at similar positions within the NS-4 sequences of HCV types 1, 2, and 3 and coincided with substantial sequence differences between the HCV types. For example, the consensus subtype 1a sequence of the first antigenic region (residues 1691 to 1708) differed from subtype 2b at 9 of the 18 amino acid residues and from type 3 by 6 residues. In the second antigenic region (1710 to 1728), sequences of types 1a, 2b, and 3 differed from each other by 7 to 12 of 19 residues. This amino acid sequence diversity implies substantial type-specific differences in antigenicity between peptides corresponding to the two regions, which is consistent with the absence of serological reactivity of most sera from HCV type 2- and 3-infected individuals to the recombinant protein 5-1-1 (8, 33), which extends from residues 1694 to 1736 and therefore includes part of the first antigenic region and all of the second (20). Similar type-specific reactivity has been also observed with the longer c100-3 protein, which includes all of these residues, those upstream in NS-3, and those downstream towards the end of NS-4; residues between 1679 and 1768 probably comprise the major antigenic region. The

5-1-1 region has been previously shown to be the target of the human monoclonal antibodies derived from an HCV-infected individual (5). Reactivity of sera from HCV-infected individuals to a series of overlapping peptides in the 5-1-1 region was normally restricted to peptides containing sequences corresponding to the first antigenic region described in this paper. However, as the HCV genotype of the study patients was not determined, it is possible that the infrequent reactivity to the second antigenic region resulted from a lack of cross-reactivity (Table 3) rather than an absence of antibody (5).

Serological reactivity to several branched peptides corresponding to the two antigenic regions was more frequent than that of the HCV type 1 c100-3 protein used in the original assays for anti-HCV (Table 3). Of the 95 donations screened, 61 showed reactivity with c100-3, compared with between 66 and 70 for individual HCV peptides from the first antigenic region of HCV type 1 and regions 1 and 2 of HCV type 3. Two factors undoubtedly contribute to this increased rate of detection. Firstly, oligopeptides sometimes act as antigens better than do recombinant proteins. This is illustrated by the increased rate of detection of anti-NS-4 antibody in recently developed third generation assays that use a series of NS-4-encoded peptides in place of c100-3 (13a, 25). Secondly, the peptides used in this study were synthesized on a branched polylysine core (40, 47). The presentation of a polyvalent target allows smaller amounts of antibodies to be detected than can be with the equivalent monomeric peptide (48). The increased sensitivity may arise from higher-affinity (i.e., multisite) binding of antibody to branched peptide antigen (30), reduced physical constraints, and greater binding of the branched peptides to the solid phase (48). When sera were assayed with all eight peptides from all three HCV types, antibodies were detected in 90 of the 95 samples tested. This combination of peptides is therefore as effective as the recombinant c22-3 (core region) protein for detecting antibody to HCV (33, 56). In combination with other proteins, the NS-4 peptides described in this paper are likely to be of major value in screening assays for anti-HCV.

The serological response to HCV infection is very variable, with often a delayed appearance of antibody to the core protein in cases of posttransfusion NANBH (52). Therefore, in many cases, antibody to the more variable nonstructural proteins, such as c33c, c100-3, and 5-1-1, may be the only evidence of infection, particularly in individuals who are marginally (human immunodeficiency virus-negative hemophiliacs and renal dialysis patients) or severely immunosuppressed (human immunodeficiency virus-infected individuals [56] and transplant recipients). The absence of serological cross-reactivity between antibody elicited by infection with divergent HCV types with the nonstructural proteins in current anti-HCV assays may therefore be one explanation for the continued finding of NANBH patients and blood donors who are antibody negative but positive by PCR and who have been implicated in transmission of HCV by transfusion (1, 19, 45).

Detection of type-specific antibody. The amino acid sequences of the antigenic regions of the three HCV types differed considerably from each other, and it was originally anticipated that serological reactivity to them would be type specific. However, substantial degrees of cross-reactivity were observed upon epitope mapping (Fig. 2) and ELISA with branched peptides (Table 3). To detect type-specific antibody, it was necessary to absorb cross-reactive antibody with a molar excess of heterologous peptides in solution, on the basis that the only antibody that could bind to the solid phase was that which reacted with epitopes not shared with other HCV types. In general, the circulating HCV "geno"-type detected by PCR in samples from HCV-infected blood donors corresponded to the detection of type-specific antibody to the corresponding HCV "sero"-type (Table 4). The detection of dual antibody specificity in samples from four donors suggested that they had been exposed and infected with more than one HCV type; in such cases, the PCR type corresponded to one of the HCV types for which type-specific antibody was detected. A total of 122 (89%) of 137 blood donor samples were successfully typed, of which all but one were consistent with HCV genotyping by PCR. Although the serological assay for type-specific antibody to different HCV types does not necessarily reflect actively replicating virus in cases of recent infection, it is obviously a practical alternative to current PCR-based typing assays (14, 33, 36, 39) in the majority of circumstances and the only possible assay in those who are anti-HCV positive but negative by PCR. At a practical level, it costs virtually nothing to carry out, uses standard laboratory equipment, requires small volumes of sera, and could be used on old or incorrectly stored samples in which the RNA has degraded (13).

TABLE 4. Comparison of serological typing by HCV-TSAA with PCR

HCV type by PCR[a]	No. of samples tested	No. of samples with the indicated type-specific antibody:								
		1	2	3	1+2	1+3	2+3	1+2+3	NTS[b]	NR[c]
1	68	61	0	0	0	1	0	0	3	3
2	13	0	11	0	0	0	1	0	1	0
3	56	1	0	45	0	2	0	0	4	4
Hem[d]	27	10	0	2	1	6	0	1	2	5

[a] Genotype of HCV sequences amplified by PCR and typed by restriction fragment length polymorphism (33); none, untypeable samples from HCV-infected hemophiliacs.
[b] NTS, no type-specific antibody detected.
[c] NR, nonreactive with NS-4 peptides.
[d] Samples from HCV-infected hemophiliacs, untyped by PCR.

Type-specific antibody to a short peptide antigen of the core protein has also been described, allowing those samples that were reactive to be differentiated into anti-HCV type 1 and type 2 (28). However, this assay could serologically type a smaller proportion of samples (36 of 56) than the anti-NS-4-based assay described here, and the assay would be unlikely to function correctly with samples from individuals infected with other HCV types, such as type 3. The type-specific core peptide of type 1 differed by only 2 residues from the corresponding peptide sequence of type 3, both of which were conservative replacements of uncharged amino acids for other uncharged amino acids, comparing unfavorably with the six differences between type 1 and type 2 core peptide sequences (28). Furthermore, an essential element of the HCV-TSAA described herein is the blocking step that serves to absorb cross-reactive antibody. It is therefore capable of detecting a minority component of type-specific antibody against a background of cross-reactive antibody, providing a stringent assay for dual or even triple antibody specificities.

In this study, we found that almost all blood donors had antibody to only a single type. In some ways, this is surprising in view of previous observations that the major risk factor for HCV infection in this particular group of blood

donors is past intravenous drug abuse by needlesharing and that both HCV types 1 and 3 are approximately equally prevalent in this particular risk group in Scotland (33). A higher rate of multiple infection was detected in hemophiliacs treated with noninactivated factor VIII or IX (40%), higher than that detected by PCR-based typing of circulating RNA sequences (39), but which still does not reflect the full extent of their exposure to HCV over the years of treatment with factor VIII. It is possible that past infection with HCV completely or partially protects the individual from further infection or attenuates the secondary immune response to the extent that type-specific antibody to the reinfecting virus type remains undetectable. Evidence that previous exposure modifies the course of disease upon reinfection is provided by experimental cross-challenge experiments in chimpanzees (17, 41). In many cases, challenge of previously infected chimpanzees to HCV appeared to lead to reinfection, but this was accompanied by only a slight elevation of alanine aminotransferase levels and infrequent clinical hepatitis. Furthermore, it is not clear whether the material used for the challenge experiments reflects that of normal human exposure. The infectious dose of HCV in clotting factor and in shared needles from intravenous drug abusers is likely to be relatively low, and reinfection in these risk groups may therefore be correspondingly rarer than that in animal models.

ACKNOWLEDGMENTS

We thank staff at the Glasgow and West of Scotland and the Edinburgh and South East Scotland Blood Transfusion Service Regional Transfusion Centres for blood donor samples. We are grateful to C. A. Ludlam, H. G. Watson, and staff at the Haemophilia Centre, Royal Infirmary of Edinburgh, for samples from hemophiliacs.

The work was supported in part by a grant from the Scottish National Blood Transfusion Service (K.R. and F.M.) and by grant no. G9020615CA from the Medical Research Council (S.-W.C.).

REFERENCES

1. **Aach, R. D., C. E. Stevens, F. B. Hollinger, J. W. Mosley, D. A. Peterson, P. E. Taylor, R. G. Johnson, L. H. Barbosa, and G. J. Nemo.** 1991. Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. N. Engl. J. Med. **325**:1325–1329.
2. **Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q. L. Choo, and G. Kuo.** 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. **321**:1494–1500.
3. **Atherton, E., and R. C. Sheppard.** 1989. Solid phase peptide synthesis: a practical approach. IRL Press, Oxford.
4. **Brettler, D. B., H. J. Alter, J. L. Dienstag, A. D. Forsberg, and P. H. Levine.** 1990. Prevalence of hepatitis C virus antibody in a cohort of hemophilia patients. Blood **76**:254–256.
5. **Cerino, A., and M. U. Mondelli.** 1991. Identification of an immunodominant B cell epitope on the hepatitis C virus nonstructural region defined by human monoclonal antibodies. J. Immunol. **147**:2692–2696.
6. **Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice.** 1990. Flavivirus genome organisation, expression and replication. Annu. Rev. Microbiol. **44**:649–688.
7. **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.
8. **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.
9. **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.
10. **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.
11. **Choo, Q. L., A. J. Weiner, L. R. Overby, G. Kuo, M. Houghton, and D. W. Bradley.** 1990. Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis. Br. Med. Bull. **46**:423–441.
12. **Contreras, M., and J. A. J. Barbara.** 1989. Screening for hepatitis C virus antibody. Lancet **334**:505.
13. **Cuypers, H. T. M., D. Bresters, I. N. Winkel, H. W. Reesink, A. J. Weiner, M. Houghton, C. L. van der Poel, and P. N. Lelie.** 1992. Storage conditions of blood samples and primer selection affect the yield of cDNA polymerase chain reaction products of hepatitis C virus. J. Clin. Microbiol. **30**:3220–3224.

13a. **Dow, B. C., et al.** Unpublished data.

14. **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.
15. **Esteban, J. I., R. Esteban, L. Viladomiu, J. C. Lopez Talavera, A. Gonzalez, J. M. Hernandez, M. Roget, V. Vargas, J. Genesca, M. Buti, et al.** 1989. Hepatitis C virus antibodies among risk groups in Spain. Lancet **ii**:294–297.
16. **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.
17. **Farci, P., H. J. Alter, S. Govindarajan, D. C. Wong, R. Engle, R. R. Lesniewski, I. K. Mushahwar, S. M. Desai, R. H. Miller, N. Ogata, and R. H. Purcell.** 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science **258**:135–140.
18. **Fletcher, M. L., J. M. Trowell, J. Craske, K. Pavier, and C. R. Rizza.** 1983. Non-A, non-B hepatitis after transfusion of factor VIII in infrequently treated patients. Br. Med. J. **287**:1754–1757.
19. **Gretch, D., W. Lee, and L. Corey.** 1992. Use of aminotransferase, hepatitis C antibody, and hepatitis C polymerase chain reaction RNA assays to establish the diagnosis of hepatitis C virus infection in a diagnostic virology laboratory. J. Clin. Microbiol. **30**:2145–2149.

19a. **Holmes, E. C., et al.** Unpublished data.

20. **Houghton, M., Q. L. Choo, and G. Kuo.** 1989. European patent application 88,310,992.5 and Publ. 318,216.
21. **Ikeda, Y., G. Toda, N. Hashimoto, and K. Kurokawa.** 1990. Antibody to superoxide dismutase, autoimmune hepatitis, and antibody tests for hepatitis C virus [letter; comment]. Lancet **335**:1345–1346.
22. **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.
23. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87**:9524–9528.
24. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, F. Tegtmeier, F. Bonino, M. Columbo, W.-S. Lee, C. Kuo, K. Berger, J. R. Schuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244**:362–364.
25. **Lam, J. P. H., F. McOmish, S. M. Burns, P. L. Yap, J. Y. Q. Mok, and P. Simmonds.** Infrequent vertical transmission of hepatitis C virus. J. Infect. Dis., in press.
26. **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.

27. **Ludlam, C. A., D. Chapman, H. Cohen, and P. A. Litton.** 1989. Antibodies to hepatitis C virus in haemophilia. Lancet **ii:**560.
28. **Machida, A., H. Ohnuma, F. Tsuda, E. Munekata, T. Tanaka, Y. Akahane, H. Okamoto, and S. Mishiro.** 1992. Two distinct subtypes of hepatitis C virus defined by antibodies directed to the putative core protein. Hepatology **16:**886–891.
29. **Makris, M., F. E. Preston, D. R. Triger, J. C. Underwood, Q. L. Choo, G. Kuo, and M. Houghton.** 1990. Hepatitis C antibody and chronic liver disease in haemophilia. Lancet **335:**1117–1119.
30. **Marsden, H. S., A. M. Owsianka, S. Graham, C. A. Robertson, G. W. McLean, and J. H. Subak-Sharpe.** 1992. Advantages of branched peptides in serodiagnosis: detection of HIV-specific antibodies and the use of glycine spacers to increase sensitivity. J. Immunol. Methods **147:**65–72.
31. **McFarlane, I. G., H. M. Smith, P. J. Johnson, G. P. Bray, D. Vergani, and R. Williams.** 1990. Hepatitis C virus antibodies in chronic active hepatitis: pathogenetic factor or false-positive result? Lancet **335:**754–757.
32. **McLean, G. W., A. M. Owsianka, J. H. Subak-Sharpe, and H. S. Marsden.** 1991. Generation of antipeptide and antiprotein sera: effect of presentation on immunogenicity. J. Immunol. Methods **137:**149–157.
33. **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.
34. **Mimms, L., D. Vallari, L. Ducharme, P. Holland, I. K. Kuramoto, and J. Zeldis.** 1990. Specificity of anti-HCV ELISA assessed by reactivity to three immunodominant HCV regions. Lancet **336:**1590–1591.
35. **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.
36. **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.
37. **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.
38. **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.
39. **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.
40. **Posnett, D. N., H. McGrath, and J. P. Tam.** 1988. A novel method for producing anti-peptide antibodies. J. Biol. Chem. **265:**1719–1725.
41. **Prince, A. M., B. Brotman, T. Huima, D. Pascual, M. Jaffery, and G. Inchauspe.** 1992. Immunity in hepatitis C infection. J. Infect. Dis. **165:**438–443.
42. **Robertson, C. A., J. Y. Q. Mok, K. S. Froebel, P. Simmonds, S. M. Burns, H. S. Marsden, and S. Graham.** 1992. Maternal antibodies to gp120 V3 sequence do not correlate with protection against vertical transmission of human immunodeficiency virus. J. Infect. Dis. **166:**704–709.
43. **Simmonds, P., L. Q. Zhang, H. G. Watson, S. Rebus, E. D. Ferguson, P. Balfe, G. H. Leadbetter, P. L. Yap, J. F. Peutherer, and C. A. Ludlam.** 1990. Hepatitis C quantification and sequencing in blood products, haemophiliacs, and drug users. Lancet **336:**1469–1472.
44. **Skidmore, S.** 1990. Recombinant immunoblot assay for hepatitis C antibody. Lancet **335:**1346.
45. **Sugitani, M., G. Inchauspe, M. Shindo, and A. M. Prince.** 1992. Sensitivity of serological assays to identify blood donors with hepatitis C viraemia. Lancet **339:**1018–1019.
46. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. **65:**1105–1113.
47. **Tam, J. P.** 1988. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple peptide system. Proc. Natl. Acad. Sci. USA **85:**5409–5413.
48. **Tam, J. P., and F. Zavala.** 1989. Multiple antigen peptide. A novel approach to increase detection sensitivity of synthetic peptides in solid phase immunoassays. J. Immunol. Methods **124:**53–61.
49. **Theilman, L., M. Blasek, T. Goeser, K. Gmelin, B. Kommerell, and W. Fiehn.** 1990. False-positive anti-HCV tests in rheumatoid arthritis. Lancet **335:**1346.
50. **Tsukiyama Kohara, K., M. Kohara, K. Yamaguchi, N. Maki, A. Toyoshima, K. Miki, S. Tanaka, N. Hattori, and A. Nomoto.** 1991. A second group of hepatitis C viruses. Virus Genes **5:**243–254.
51. **Vallari, D. S., B. W. Jett, H. J. Alter, L. T. Mimms, R. Holzman, and J. W.-K. Shih.** 1992. Serological markers of posttransfusion hepatitis C viral infection. J. Clin. Microbiol. **30:**552–556.
52. **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.
53. **van der Poel, C. L., H. W. Reesink, P. N. Lelie, A. Leentvaar Kuypers, Q. L. Choo, G. Kuo, and M. Houghton.** 1989. Anti-hepatitis C antibodies and non-A, non-B post-transfusion hepatitis in The Netherlands. Lancet **334:**297–298.
54. **van der Poel, C. L., H. W. Reesink, W. Schaasberg, A. Leentvaar Kuypers, E. Bakker, P. J. Exel Oehlers, and P. N. Lelie.** 1990. Infectivity of blood seropositive for hepatitis C virus antibodies. Lancet **335:**558–560.
55. **Wang, J. T., T. H. Wang, J. T. Lin, J. C. Sheu, J. L. Sung, and D. S. Chen.** 1990. Hepatitis C virus in a prospective study of posttransfusion non-A, non-B hepatitis in Taiwan. J. Med. Virol. **32:**83–86.
56. **Watson, H. G., C. A. Ludlam, S. Rebus, L. Q. Zhang, J. F. Peutherer, and P. Simmonds.** 1992. Use of several second generation assays to determine the true prevalence of hepatitis C infection in haemophiliacs treated with non-virus inactivated factor VIII and IX concentrates. Br. J. Haematol. **80:**514–518.
57. **Widell, A., G. Sundstrom, B. G. Hansson, T. Moestrup, and E. Nordenfelt.** 1991. Antibody to hepatitis-C-virus-related proteins in sera from alanine-aminotransferase-screened blood donors and prospectively studied recipients. Vox Sang. **60:**28–33.