Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma

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The role of humoral immunity in hepatitis C virus (HCV) infections is uncertain. Nevertheless, there is increasing evidence for neutralizing antibodies to HCV in the serum or plasma of chronically infected individuals. Immune globulins prepared by ethanol fractionation of plasma had long been considered safe until a commercial immune globulin product, Gammagard, prepared from plasma from which units containing anti-HCV had been excluded, transmitted HCV to recipients. Studies suggested that the exclusion might have removed neutralizing antibodies from the plasma and hence compromised the safety of the resulting immune globulins. In the present study, by using chimpanzees and a recently validated *in vitro* system based on neutralization of infectious HCV pseudoparticles, we found broadly reactive neutralizing and protective antibodies in experimental immune globulin preparations made from anti-HCV-positive donations. Neutralizing antibodies were also found in Gammagard lots made from unscreened plasma that did not transmit hepatitis C but not in Gammagard lots, which were prepared from anti-HCV-screened plasma, that did transmit hepatitis C. The results provide an explanation for the mechanism by which the safety of this product was compromised. Immune globulins made from anti-HCV-positive plasma and containing broadly reactive neutralizing antibodies may provide a method of preventing HCV infection.

Hepatitis C virus (HCV) is an enveloped virus containing a single-stranded, positive-sense RNA. It infects up to 170 million people worldwide. Although acute HCV infections are generally asymptomatic, the rate of persistence is remarkably high (80%), leading to chronic liver disease, cirrhosis, and hepatocellular carcinoma in some patients.

The role of the humoral immune response in preventing and/or controlling HCV infection has not been well defined, which may be due chiefly to the lack of a reliable cell culture system useful for *in vitro* neutralization assays, the genetically heterogeneous nature of HCV, and the limited resources for studying HCV infection in chimpanzees, the only species susceptible to HCV infection other than man. In addition, it is a general perception that humoral immunity is largely ineffective in resolving HCV infection or preventing reinfection, perhaps because of emergence of neutralization-resistant variants (1, 2) and/or the "masking" of HCV by serum lipoproteins (3).

Previously, Shimizu *et al.* (2, 4) and Farci *et al.* (1, 5) identified neutralizing antibodies (Nt Abs) to HCV by their ability to prevent replication of the virus in a lymphoid cell line and to prevent hepatitis C in chimpanzees, respectively. By using a recently established *in vitro* neutralization assay system based on the neutralization of infectious retroviral pseudoparticles bearing HCV envelope glycoproteins, Bartosch *et al.* (6) were able to confirm the existence of Nt Abs shown previously in both systems. Relatively high titers of Nt Abs were present in plasma or serum from chimpanzees and humans who were chronically infected with HCV (7).

Several lines of evidence also suggest the presence of Nt Abs in immune globulins. U.S.-licensed immune globulin products were historically considered safe with respect to hepatitis transmission until the "Gammagard incident," which began in late 1993 (8–12): one commercial i.v. immune globulin (IGIV) product prepared from pooled plasma from which anti-HCV-positive plasma donations were excluded transmitted HCV to recipients. Epidemiologic and laboratory studies suggested that such screening might have removed complexing and/or Nt Abs from plasma and hence compromised the safety of the immune globulins (9, 11–16).

In this study, we correlated the presence of Nt Abs in several experimental IGIV preparations (HCIGIV) made solely from anti-HCV-positive plasma donations with their ability to prevent HCV infection in chimpanzees. Preliminary data indicating that an experimental HCIGIV product could neutralize a low-dose HCV inoculum administered to a chimpanzee were reported (17). In addition, we measured Nt Abs in commercial Gammagard lots manufactured before or after the screening of plasma for anti-HCV was instituted. We demonstrated the presence of high-titer and broadly reactive Nt Abs to HCV in a pool of anti-HCV-positive plasma donations in three HCIGIV preparations made from anti-HCV-positive pools and in Gammagard lots prepared from unscreened plasma. In contrast, we did not find Nt Abs to HCV in a plasma pool from which anti-HCV-positive plasma donations had been excluded, in immune globulins prepared from such plasma pools, or in lots of Gammagard prepared from screened plasma. Thus, our data indicate that anti-HCV contributes to the historic safety of immune globulins and that anti-HCV screening of donors removes Nt Abs from plasma and could therefore compromise the safety of immune globulins unless their manufacturing procedures include one or more viral inactivation steps.

Materials and Methods

Anti-HCV Testing. Antibodies to HCV core and nonstructural proteins (anti-HCV) in immune globulins and chimpanzee sera were determined by a second-generation enzyme immunoassay (EIA)-2 or a third-generation EIA-3 kit (both from Ortho Diagnostics) according to the manufacturer's instructions. Immune globulin preparations were serially 2-fold-diluted with a specimen diluent provided, and the reported titer represented the highest dilution that gave a reading above the cutoff value specified for the kit. The presence of anti-HCV in immune globulins was confirmed by a second-generation strip recombinant immunoblot assay (RIBA-II.

Abbreviations: HCV, hepatitis C virus; Nt Abs, neutralizing antibodies; IGIV, intravenous immune globulin; HCIGIV, experimental IGIV made from anti-HCV-positive plasma; CID, chimpanzee infectious dose; CID_{50}, CID at 50%; EIA, enzyme immunoassay; ALT, alanine aminotransferase.

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Table 1. Samples tested under code

Code	Sample	Anti-HCV plasma screening	Anti-HCV RIBA II	Anti-E1*	Anti-E2*	HCV RNA	Virus inactivation	IgG, %
1	Control IGIV	EIA-2 neg	Neg	Neg	Neg	Neg	Yes	5
2	HCIGIV	EIA-2 pos	Pos	Pos	Pos	Neg	Yes	5
3	HCIGIV	EIA-1 pos	Pos	Pos	Pos	Pos	No	5
4	HCIGIV	EIA-1 pos	Pos	ND	ND	Neg	Yes	5
5	Human albumin	EIA-2 neg	N/A	N/A	N/A	Neg	Yes	(5% prot)
6	Plasma pool	EIA-1 pos	Pos	Pos	Pos	Pos	No	1†
7	Plasma pool	EIA-2 neg	Neg	Neg	Neg	Neg	No	1†
8	Gammagard	EIA-2 neg	Neg	Neg	Neg	Neg	No	10
9	Gammagard	EIA-2 neg	Neg	Neg	Neg	Pos	No	10
10	Gammagard	EIA-2 neg	Neg	Neg	Neg	Pos	No	10
11	Gammagard	EIA-2 neg	Neg	Neg	Neg	Pos	No	10
12	Gammagard	EIA-2 neg	Neg	Neg	Neg	Pos	No	10
13	Gammagard	Unscreened	Pos	Pos	Pos	Neg	No	10
14	Gammagard	Unscreened	Pos	Pos	Pos	Neg	No	10
15	Gammagard	Unscreened	Pos	ND	ND	Neg	No	10
16	Gammagard	Unscreened	Pos	ND	ND	Neg	No	10
17	IGIV	EIA-3 neg	ND	ND	ND	Neg	Yes	10
18	Gammagard	Unscreened	Pos	ND	ND	Neg	No	10
19	Gammagard	Unscreened	Pos	ND	ND	Neg	No	10

pos, positive; neg, negative; ND, not determined; N/A, not applicable.
*In-house EIA procedures for anti-E1 and anti-E2 (see *Materials and Methods* for details).
†Assumed value.

Chiron). Determination of antibodies to HCV envelope glycoprotein E1 and E2 has been described (14). Briefly, the antibodies were determined by in-house EIA methods by using partially purified fusion proteins expressed in baculovirus. Immune globulins were first diluted to a 5% IgG solution and then diluted with PBS, pH 7.4, containing 0.05% Tween 20 before being incubated in wells coated with either the expressed E1 (7.2 μg/ml) or expressed E2 antigen (38 μg/ml) at 4°C overnight. Subsequently, standard EIA procedures were followed. The absorbance at 490 nm was measured, and cutoff values for anti-E1 and anti-E2 were set at 0.5 and 0.6, respectively.

Immune Globulins and Plasma Pools. Nineteen coded samples were tested in this study (Table 1). The code was not broken until after testing was completed. Samples 1, 2, and 5 were previously described in a preliminary report (17). Briefly, sample 1 was a commercial 5% IGIV solution (Venoglobulin-S, Alpha Therapeutic, Los Angeles) prepared from >43,000 plasma donations screened for anti-HCV (EIA-2) and virally inactivated by a solvent–detergent treatment (18). Sample 1 had no detectable antibodies to HCV, including antibodies to envelope glycoproteins E1 and E2, and, hence, was used as a control IGIV. Sample 2 (HCIGIV) was an experimental 5% IGIV that had been solvent–detergent treated (kindly provided by Nabi Biopharmaceuticals, Boca Raton, FL). Sample 2 was made from pooled plasma of 198 anti-HCV (EIA-2)-positive donors who otherwise met the requirements for normal plasma donor, i.e., negative for both anti-HIV and hepatitis B surface antigen and without elevated levels of alanine aminotransferase (ALT). This HCIGIV contained the highest levels of anti-E1 and anti-E2 among all experimental and commercial IGIVs examined (14) and had an anti-HCV (EIA-3) titer of 1:2048. Sample 5 (albumin) was derived from commercial 25% albumin (human) that had been virally inactivated by heating at 60°C for 10–11 h and was diluted to 5% with PBS before its use as a control. Sample 3 (HCIGIV), a 5% IgG solution prepared by the Food and Drug Administration from 4 liters of pooled plasma from 186 anti-HCV (EIA-1)-positive plasma donations as described (19), was not virally inactivated and contained HCV RNA. This HCIGIV also had high levels of anti-E1 and anti-E2 (14). Sample 4 (HCIGIV), also prepared by the Food and Drug Administration, was similar to sample 3, except that the former was prepared from portions of 23 plasma donations selected from among the 186 donations for sample 3, was virally inactivated at low pH, and was used previously in a chimpanzee study by Krawczynski *et al.* (20). In that study, a chimpanzee was inoculated with 30 chimpanzee infectious doses (CID) of HCV, strain HCV-1 (closely related to the strain H77 used herein), and infused with the HCIGIV 1 h later.

Sample 6 was a plasma pool consisting of the 186 donations (19) that formed the starting pool for the HCIGIV designated sample 3. Sample 7 was a plasma pool from anti-HCV (EIA-2)-screened donors provided by a manufacturer. Samples 8–12, i.e., Gammagard lots implicated in transmission of HCV to recipients, were made solely from anti-HCV (EIA-2)-screened plasma donations in 1993, whereas samples 13–16 and 18–19 were different Gammagard lots from plasma unscreened for anti-HCV and manufactured between 1988 and 1990. Sample 17 was a current, virally inactivated IGIV product made by another manufacturer from anti-HCV (EIA-3)-negative plasma donations. Many of the lots of IGIV and the human albumin were from product lots submitted by manufacturers for release by the Food and Drug Administration. Lyophilized Gammagard lots were reconstituted to 10% IgG solutions with PBS before use.

HCV Inoculum, Chimpanzee Inoculation, and Monitoring. An inoculum diluted to contain ≈64 CID_{50} (50% chimpanzee infectious doses) of HCV was prepared from the acute phase plasma of patient H as described (1, 5). By limiting dilution, the inoculum was found to contain 630 copies of HCV RNA per milliliter based on our sample extraction and subsequent RT-PCR procedures. This ratio of infectious doses to genome equivalents was similar to that found for the H strain inoculum described (ref. 21 and unpublished data). One-milliliter portions of the inoculum were incubated with 50 ml of the test substance overnight at 4°C before administering to chimpanzees. Sample 3, HCIGIV that had not been virally inactivated, was infused into a chimpanzee at a dose of 1.0 g of IgG per kg of body weight, divided into two daily doses. Serum samples were obtained weekly from the chimpanzees and tested for anti-HCV by EIA-2 or EIA-3. Serum ALT levels were measured weekly by standard methods (Antech Diagnostics, Baltimore, MD). ALT was considered elevated when it reached levels twice the upper limit of normal. Sera were also tested weekly for HCV RNA. The housing,

maintenance, and care of the chimpanzees met or exceeded all relevant guidelines and requirements.

Detection and Quantitation of HCV RNA by RT-PCR. Procedures were performed as described previously with some modifications (11, 15). To detect low levels of HCV RNA in some immune globulin samples, 4-ml aliquots were used for RNA extraction. For plasma pools, chimpanzee sera, and HCV inocula, sample aliquots ranging from 0.05 to 0.2 ml were used. The extracted RNA was dissolved in 100 μl, and 25-μl serially diluted (0.5 or 1 log) aliquots were assayed by RT-PCR in a total reaction mixture of 50 μl that contained 5 units of rTth DNA polymerase, 1× EZ buffer, 2.5 mM $Mn(OAc)_2$, 300 mM each of the 4 deoxyribonucleotides, and 0.4 mM each of the outer primer pair [nucleotides 39–59, 5′-ACTCCCCTGTGAGGAACTACT and nucleotides 329–310, 5′-ACGAGACCTCCCGGGGCACT]. All RT-PCR reagents were from Applied Biosystems except the primers, which were prepared by GIBCO/BRL. The RT reaction was carried out for 30 min at 60°C followed by heating for 1 min at 94°C to denature the RNA:DNA hybrid. The first PCR step was performed in the same tube for 30 cycles, each cycle consisting of 15 s at 94°C and 30 s at 60°C, followed by one cycle of 7 min at 60°C. The second PCR step was performed by adding 5-μl aliquots of the first PCR product to the same ingredients and incubating them under the same conditions as described for the first PCR step but with the inner primer pair (nucleotides 57–76, 5′-ACTGTCTTCACGCAGAAAGC, and nucleotides 312–293, 5′-ACTCGCAAGCACCCTATCAG). The amount of HCV RNA, expressed as copies per ml, was determined by limiting dilution analysis as described (13). In the RT-PCR assays, two to four copies of HCV RNA were equivalent to 1 international unit when compared to the established international standard for HCV RNA (22).

Production and Neutralization of Pseudoparticles. Pseudoparticles were generated as described (6). Briefly, 293T cells were transfected with expression vectors encoding HCV envelope glycoproteins, a retroviral core/packaging component, and the GFP gene with integration signal. Expression plasmids encoding both E1 and E2 glycoproteins of either HCV genotype 1a strain H77 or genotype 2a were used. The medium was replaced 16 h after transfection. Supernatants containing the pseudoparticles were harvested 24 h later, filtered through 0.45-μm-pore membranes, and used to infect Huh-7 cells that had been seeded the day before at a density of 8×10^4 cells per well in 12-well plates. Coded samples were diluted and tested for neutralization of HCV pseudoparticles of both genotypes by preincubating the mixture at room temperature for 1 h before adding it to the target cells. After 3 h, the supernatants were removed and the cells were incubated in regular medium for 96 h at 37°C. Input pseudoparticles infected 2–4% of the cells in the absence of antibody. The infectivity of pseudoparticles exposed to sera from two healthy seronegative human donors from France was standardized to 100% for comparison with the two plasma pool samples (samples 6 and 7), whereas that exposed to albumin (sample 5) was used as a negative control for all immune globulin samples. All dilutions of test samples were compared with the same dilutions of these negative control samples. A diminished number of infected cells that was ≤50% of the negative controls was considered to be evidence of significant neutralization. The positive control sample was from a French patient (Vu) who was chronically infected with HCV, genotype 1b. Control neutralization experiments were also performed with an irrelevant target by using pseudoparticles bearing glycoproteins derived from the feline endogenous retrovirus RD114 as described because antibodies to this virus are not found in human sera (6).

Results

Prevention of HCV Infection in Chimpanzees by Experimental IGIV Preparations Made from Anti-HCV-Positive Plasma. To investigate whether withholding anti-HCV-positive plasma units from a plasma pool and, hence, the resulting IGIV product, depleted Nt Abs, we assessed the capabilities of two IGIV preparations to neutralize 64 CID_{50} of HCV, strain H77. One IGIV was an experimental globulin preparation (HCIGIV) made solely from plasma of anti-HCV-positive donors. The preparation had high levels of both anti-E1 and anti-E2 (14). The other was a commercial globulin preparation made from anti-HCV (EIA-2)-negative plasma donations; it was devoid of detectable anti-E1 and anti-E2 (14). To confirm the viability of the inoculum, a 5% human albumin solution was used as a control. All three preparations had been virally inactivated, and each was incubated with the inoculum as described above before infusing the globulin–virus (or albumin–virus) mixtures i.v. into individual chimpanzees.

The control chimpanzee, CH 1560, infused with an incubation mixture of the inoculum and a 5% human albumin solution (sample 5), was infected and HCV RNA was detected in the serum within one week (Fig. 1*A*, which is published as supporting information on the PNAS web site). The other control chimpanzee, CH 1587, which was infused with a mixture of the same inoculum and the control IGIV (sample 1), was similarly infected (Fig. 1*B*). Both CH 1560 and CH 1587 had serum ALT elevations, and both seroconverted (EIA-3) at weeks 9 and 11, respectively. In contrast, the HCIGIV (sample 2) neutralized the HCV inoculum: CH 1588 exhibited neither elevated ALT nor detectable HCV RNA over a period of 58 weeks (Fig. 1*C*). Passively acquired anti-HCV (EIA-3) from the HCIGIV was present in CH 1588 from week 1 through week 22.

To determine whether CH 1588 had remained fully susceptible to infection with HCV, it was challenged at week 59, after all passively acquired anti-HCV had disappeared, with 1 ml of the same inoculum containing 64 CID_{50} of HCV. As expected, HCV RNA was detected within 1 week after challenge, and serum ALT levels became elevated 11 weeks after challenge, thereby demonstrating susceptibility of the chimpanzee to HCV infection. Interestingly, anti-HCV (EIA-3) was again found 1 week after challenge, at week 60. Given that the challenge inoculum did not have anti-HCV, such a rapid appearance of HCV antibodies in the chimpanzee suggests priming of the immune system by HCV-specific antigens associated with the first challenge, as described (23). Our data strongly suggest that the HCIGIV designated sample 2, prepared from anti-HCV-positive donor units, contained specific Nt Abs to HCV.

To determine whether the presence of anti-HCV correlated with the apparent safety of immune globulins (which often contained HCV RNA) (13), we infused CH 1430 with sample 3, which contained not only high levels of anti-HCV, including both anti-E1 and anti-E2 (14), but also HCV RNA (Fig. 1*D*). Furthermore, this HCIGIV had not been subjected to viral inactivation. The chimpanzee was infused intravenously with 20 g of sample 3 and hence received a total of 3×10^4 copies of HCV RNA. After the infusion, the chimpanzee had neither elevated ALT nor detectable HCV RNA in serum samples examined weekly for a period of 50 weeks. Passively acquired anti-HCV was detected until week 17. The chimpanzee was proven to be fully susceptible to HCV by subsequently rechallenging it with 1 ml of plasma from patient H, diluted to contain $10^{3.5}$ CID_{50} of HCV, strain H-77, at week 51. The chimpanzee became infected and developed ALT elevation at week 56 and anti-HCV at week 60. Thus, the lack of infectivity for HCV of an immune globulin positive both for anti-HCV and for HCV RNA suggested that the antibody in the globulin neutralized the large dose of potentially infectious virus that was also present.

As noted, the two chimpanzees that were protected from HCV infection by HCIGIV were rechallenged to ascertain their susceptibility to infection. Both animals not only became infected with HCV but also developed hepatitis. Although reinfection of previously infected chimpanzees with HCV is widely recognized, such infections seldom result in hepatitis (24). Thus, the demonstration of hepatitis in these two rechallenged chimpanzees is strong evidence that they were not infected previously.

Table 2. Comparison of neutralizing activities detected in chimpanzee and pseudoparticle assays

Coded sample	Composition	Anti-HCV status of plasma	Chimpanzee 1a*	Pseudoparticle†	
				1a	2a
5	Albumin	Neg	Not protected	<1:20	<1:20
1	Control IGIV	Neg	Not protected	<1:20	<1:20
2	HCIGIV	Pos	Protected	≥1:320	1:160
3	HCIGIV	Pos	Protected	≥1:320	≥1:320
4	HCIGIV	Pos	Modified infection‡	≥1:320	1:80

*Genotype of HCV inoculum.
†Highest dilution that neutralized indicated genotype of pseudoparticle. Some values are extrapolated, based on values of consecutive 4-fold dilutions.
‡Postexposure passive immunoprophylaxis: prolonged incubation period relative to negative control globulin (see text).

***In Vitro* Assay for Nt Abs to HCV in Selected Immune Globulins and Plasma Pools.** We measured the Nt Abs of those preparations tested in chimpanzees along with the other selected IGIV lots and plasma pools listed in Table 1 by using the recently established *in vitro* neutralization assay (7), based on neutralization of infectious HCV pseudoparticles. The coded samples were diluted 1:20, 1:80, and 1:320 and tested against pseudoparticles bearing HCV envelope glycoproteins of genotype 1a or 2a. The Nt Ab titer of the globulin samples was calculated as described in *Materials and Methods*.

All immune globulins prepared from known or presumed anti-HCV-positive plasma donations (coded samples 2–4, 13–16, and 18–19) and sample 6 (plasma pool for globulin sample 3) neutralized the pseudoparticles of HCV genotype 1a, i.e., exhibited ≥50% reduction in percent of GFP-positive cells. (Tables 2 and 3 and Fig. 2*A*, which is published as supporting information on the PNAS web site). The titers of Nt Abs in all these samples were very high, ≥1:320, when assayed with HCV genotype 1a pseudoparticles. To estimate the breadth of neutralization against different genotypes, the samples were also tested against pseudoparticles of genotype 2a, the genotype most divergent from genotype 1a. All three HCIGIV preparations (samples 2–4, Table 2) and sample 6 (anti-HCV-positive plasma pool, Fig. 2*B*) also exhibited significant but less potent neutralization than that against genotype 1a pseudoparticles. Samples 13–16 and 18–19, Gammagard lots manufactured from unscreened plasma, were more variable in their ability to neutralize the genotype 2a pseudoparticles; only samples 16 and 19 were positive for such Nt Abs and only at a dilution of 1:20. However, all diminished the titer of pseudoparticles when compared with the control, but with the two exceptions, by <50% (Table 3 and Fig. 2*B*). The EIA-2-screened plasma pool (coded sample 7) and immune globulins prepared from anti-HCV-screened plasma (coded samples 1, 8–12, and 17) did not demonstrate neutralization against HCV pseudoparticles of either genotype (Tables 2 and 3 and Fig. 2). Neutralization observed with the plasma pool and the various globulins prepared from unscreened plasma was specific because none of the samples neutralized the pseudoparticles with an irrelevant envelope glycoprotein derived from the feline endogenous virus RD 114 (data not shown).

Comparison of Neutralizing Activities Detected with Chimpanzees and Pseudoparticles. As seen in Table 2, neutralizing activity detected with pseudoparticles bearing HCV glycoproteins of genotype 1a agreed well with that observed with chimpanzees (Fig. 1). The results thus provided a possible explanation for the lack of infectivity as shown in a chimpanzee infused with sample 3, the HCIGIV that was positive for both HCV RNA and anti-HCV. Sample 4, which was prepared from a subset of plasma donations used to prepare sample 3, also contained a high titer of Nt Ab. This preparation, administered to a chimpanzee after exposure to HCV, had been shown previously to prolong the incubation period to hepatitis (120 days versus 30 days) but not to viremia (20).

Correlation Between the Number of Reported Human Cases of Hepatitis C Associated with Gammagard Lots and Titers of Nt Abs. We assessed the presence of Nt Abs in Gammagard lots prepared before and after anti-HCV screening of plasma. As shown in Table 3, none of the five tested Gammagard lots prepared from screened plasma had detectable Nt Abs, and four of the lots were associated with reported hepatitis cases (the fifth did not contain detectable HCV RNA). In contrast, all six Gammagard lots made from unscreened plasma had Nt Ab titers of ≥1:320 against pseudopar-

Table 3. Correlation between number of reported human cases of hepatitis C associated with Gammagard lots and the titer of Nt Abs detected with pseudoparticles

Sample number	Date of manufacture	Plasma screening by anti-HCV EIA-2	HCV RNA	Hepatitis cases*	Pseudoparticle†	
					1a	2a
8	1993	Screened	Neg	0	<1:20	<1:20
9	1993	Screened	Pos	4	<1:20	<1:20
10	1993	Screened	Pos	2	<1:20	<1:20
11	1993	Screened	Pos	60	<1:20	<1:20
12	1993	Screened	Pos	18	<1:20	<1:20
13	1990	Unscreened	Neg	0	≥1:320	<1:20
14	1990	Unscreened	Neg	0	≥1:320	<1:20
15	1989	Unscreened	Neg	0	≥1:320	<1:20
16	1989	Unscreened	Neg	0	≥1:320	1:20
18	1989	Unscreened	Neg	0	≥1:320	<1:20
19	1989	Unscreened	Neg	0	≥1:320	1:20

*From case reports received by the manufacturer; patients often received either multiple or unidentified lots.
†Same as described in Table 2.

ticles of genotype 1a. Among these six lots, two were found to have Nt Abs against the 2a particles, albeit at a low titer of 1:20.

Discussion

Evidence that normal immune globulin could protect against hepatitis C (previously designated non-A non-B hepatitis) dates back at least to 1967, when U.S. soldiers stationed in Korea participated in a randomized, double-blind clinical trial of the protective effect of globulin against viral hepatitis (25). Other clinical trials of immune globulin for the prevention of transfusion-associated non-A/non-B hepatitis (virtually all of which was hepatitis C) revealed that such globulin was protective if administered before exposure to the virus (26, 27) but not after (28, 29).

When chimpanzees were vaccinated with recombinant HCV glycoproteins E1 and E2, they were partially protected against a subsequent low-lose homologous HCV challenge (30). This finding suggests that antibodies to E1 and/or E2 may have protected against HCV. The existence of Nt Abs against HCV was first directly demonstrated by *in vitro* and *in vivo* test systems in a patient who was chronically infected with HCV (1, 2). However, such antibodies appeared to be isolate-specific and not protective over time, possibly because of the emergence of neutralization-resistant variants of HCV or limitations in the tests. Even though the principal neutralization epitopes were not identified in those earlier studies, antibodies to the putative envelope proteins of HCV, especially to the hypervariable region of the E2 envelope protein, were suspected. With the newly available *in vitro* neutralization system, based on neutralizing the infectivity of pseudoparticles bearing HCV envelope glycoproteins (6), the presence of Nt Abs in plasma or serum of chronically infected hepatitis C patients was confirmed (7). Furthermore, in addition to the neutralization epitope(s) previously found in the hypervariable region of the E2 envelope protein, one or more additional epitopes have been tentatively localized to other regions of E2 (31).

Historically, U.S.-licensed immune globulins prepared by ethanol fractionation of plasma had a remarkable safety record until one commercial IGIV product, Gammagard, prepared from anti-HCV (EIA-2)-screened plasma was withdrawn worldwide in February 1994 because of HCV transmission to recipients (8–12). Before 1993, globulin-related transmission of hepatitis C by licensed products had not been reported in the U.S. The mechanism of this apparent safety of immune globulins before the Gammagard incident was unknown, although the possible neutralization of HCV by antibodies in products not screened for such antibodies had long been suspected (13, 32). Epidemiological and laboratory follow-up studies of the Gammagard incident revealed the following:

(*i*) Anti-HCV screening with the EIA-2 test resulted in the presence of HCV RNA in lots prepared from such screened plasma. In contrast, HCV RNA was rarely detected in Gammagard lots made from either unscreened plasma or plasma screened with the relatively insensitive EIA-1 test (9, 15). The lots derived from plasma screened with the more sensitive EIA-2 test were associated with many reported cases of hepatitis C transmission.

(*ii*) Gammagard was the only IGIV product implicated (8–12,15). All other IGIV products were not similarly affected because of deliberately incorporated viral inactivation steps or other manufacturing (e.g., purification) procedures that fortuitously inactivated or removed virus.

(*iii*) Anti-HCV screening greatly reduced levels of antibodies to HCV E1 and E2 envelope proteins in plasma and the resulting immune globulin products (14).

(*iv*) The presence of anti-HCV in plasma had a substantial effect on the partitioning of HCV away from the resultant immune globulin. Overall, ethanol fractionation of anti-HCV-negative plasma resulted in a 3.5 log reduction in HCV RNA (16), whereas a 4.7 log reduction was achieved with anti-HCV-positive plasma (19).

(*v*) The lot of Gammagard associated with the most reported cases of hepatitis C (sample 11 in Table 3) (9, 12, 15) had the highest level of HCV RNA found among the commercial lots tested, ≈7,000 copies of HCV RNA per g of IgG. This anti-HCV-negative lot exhibited poor ultracentrifugal recovery of HCV RNA: only 10% was recovered in the pellet when compared with that from anti-HCV-positive immune globulins (15). Furthermore, the HCV in this lot had a low buoyant density, 1.08 g/ml (15) (determined by sucrose gradient ultracentrifugation), suggesting that the virus was not complexed with antibody (3, 21). This finding was in contrast to the buoyant density of HCV recovered from anti-HCV-positive immune globulin, 1.16 g/ml, as found in intramuscular globulin derived from plasma that was not screened for anti-HCV (13) or found in plasma from patients chronically infected with HCV (21).

In line with these findings, a retrospective cohort study conducted by Feray *et al.* (33) clearly showed that the prevalence of HCV viremia was lower in liver transplant patients who received hepatitis B immune globulin made before the time when anti-HCV screening of plasma began, suggesting that the hepatitis B immune globulin also contained Nt Abs against HCV. All these studies, therefore, provided evidence that globulins prepared from anti-HCV-positive plasma contained antibodies that complexed with HCV and that these antibodies neutralized the virus.

In the present study we found that HCIGIV (sample 2), prepared from healthy anti-HCV-positive donors, contained specific Nt Abs to HCV, as evidenced by protection of a chimpanzee and by neutralization of HCV pseudoparticles bearing HCV envelope glycoproteins. In contrast, IGIV prepared from anti-HCV-screened plasma did not have any detectable Nt Abs as measured by either system.

Similarly, we showed that HCIGIV (sample 3) prepared from anti-HCV-positive donors and containing high levels of both anti-HCV and HCV did not transmit HCV to a chimpanzee, although the preparation was not virally inactivated. This preparation also contained high levels of Nt Abs, as measured by neutralization of HCV pseudoparticles. Thus, our data confirm that globulins made from anti-HCV-positive plasma contain Nt Abs to HCV and that these antibodies are capable of neutralizing both endogenous and exogenous HCV.

The presence of HCV RNA in plasma or its derived product does not necessarily equate with infectivity, especially when anti-HCV antibodies are present as immune complexes (13, 15). It has been suggested that when HCV is present as circulating immune complexes, its infectivity is lower (21). In addition, density heterogeneities of HCV measured in human sera have been attributed to binding with IgG (3, 21). An early finding that mutations within the hypervariable region of E2 led to the accumulation of IgG-free virus particles in the sera of patients with chronic HCV infection strongly implied that anti-E2 was involved (34). When 180 of the 186 EIA-1 reactive plasma units making up sample 6 were tested, 73% of them were positive for anti-E2, 63% contained anti-E1, and 58% had both antibodies (14). Neutralizing antibodies would be directed against E1, E2, or both envelope glycoproteins. In contrast, the antibodies measured by the EIA tests (EIA-1, EIA-2, and EIA-3) are directed against the nonstructural proteins and core protein of HCV and would not be neutralizing.

Direct assessment of neutralizing antibodies in various Gammagard lots by tests in chimpanzees would not be feasible. However, by means of the *in vitro* assay system, we demonstrated that all six tested Gammagard lots prepared from unscreened plasma contained high titers (≥1:320) of Nt Abs against the HCV pseudoparticles of genotype 1a but only 2 lots had titers as high as 1:20 of Nt Abs against genotype 2a pseudoparticles. We did not perform end

point titrations of Nt Abs in the Gammagard lots and HCIGIV preparations. However, because levels of both anti-E1 and anti-E2 were previously found to be lower in all unscreened Gammagard lots when compared with those obtained with HCIGIV (14), levels of Nt Abs in Gammagard lots from unscreened plasma would be expected to be lower than in HCIGIV. By contrast, implicated Gammagard lots derived from anti-HCV-screened plasma had neither detectable antibodies to E1 or E2 glycoproteins (14) nor Nt Abs to HCV pseudoparticles. The results agreed well with the results of retrospective studies of the safety of Gammagard summarized in Table 3. Therefore, our data clearly demonstrate that the apparent safety of immune globulins before the Gammagard incident can be attributed to the presence of Nt Abs to HCV. The lack of Nt Abs in Gammagard lots manufactured after the implementation of anti-HCV screening of plasma by EIA-2 provides a mechanism by which the safety of this noninactivated product was compromised. This conclusion is consistent with the concept that infectivity depends on the balance between the virus and the Nt Abs. In addition, anti-HCV screening would not result in the exclusion of units with high levels of HCV RNA associated with the window period of HCV infection, i.e., before anti-HCV seroconversion. Similarly, we observed in chimpanzees (Fig. 1) that the levels of HCV RNA before anti-HCV seroconversion were always higher than after seroconversion. Plasma units obtained from donors during the incubation period of hepatitis C, when HCV titers are the highest, would be exactly the units that would escape screening for anti-HCV in the absence of a nucleic acid screening test for HCV RNA.

It is desirable to have HCIGIV containing Nt Abs against all genotypes of HCV. In the present study, all three HCIGIV preparations could neutralize HCV pseudoparticles bearing HCV envelopes derived from two different genotypes, 1a and 2a, the two most genetically divergent genotypes of HCV. It is not known whether Nt Abs against other genotypes are present in these samples. However, genotype analysis of HCV in sample 6, the 186-donor plasma pool, revealed that all genotypes (i.e., 1–6) were present (data not shown). All six Gammagard lots derived from unscreened plasma had high levels of Nt Abs to genotype 1a but less Nt Abs against genotype 2a. Genotype analysis of HCV RNA-positive Gammagard lots made from screened plasma revealed that some lots contained only one HCV genotype, e.g., genotype 1a for sample 9 (12) and genotype 1b for sample 12 (15). Genotypes 1a and 1b are the most common genotypes in the U.S. (35). However, some Gammagard lots (not included in this study) contained mixed genotypes, e.g., 1a/2b, 1a/2a–c, 1a/3b (data not shown). Such a finding is not surprising given that every lot of Gammagard and of other IGIV products is prepared from plasma of at least 10,000 donors. Whether the extended profiles of HCV genotypes in the starting plasma pools can correlate with the broad neutralizing capabilities in the resulting immune globulins is currently unknown. Nevertheless, in view of the genetic diversity of HCV, the neutralizing capabilities and, hence, the therapeutic utility of HCIGIV may be enhanced by incorporating plasma from a greater number of anti-HCV-positive plasma donors or by selecting donors who are infected with diverse HCV genotypes. Further studies are required to address this issue.

In conclusion, we have confirmed and extended the previous observation by Bartosch *et al.* (7) that neutralization of pseudoparticles bearing HCV envelope glycoproteins has biological significance and have evaluated the role of Nt Abs in the passive immunoprophylaxis of hepatitis C. We have also confirmed that such Nt Abs are not strain-specific as previously thought but may be broadly protective. In addition, we have demonstrated that Nt Abs to HCV were present in commercial immune globulins derived from plasma unscreened for anti-HCV and that they contributed to the historic safety of these products. Finally, screening tests for anti-HCV can be used to identify plasma units that contain Nt Abs. These units may be useful for preparing immunoprophylactic products to prevent hepatitis C infection, if protective levels of antibody and schedules of administration can be developed.

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