表3　HBs抗体陽性の児童におけるHBc抗体陽性率の推移

出生年	HBs抗体陽性の児童数(人)	HBc抗体陽性数(%)
実施前 (1978-1980)		
1978	49	40 (81.6)
1979	72	64 (88.9)
1980	34	23 (76.7)
小計	155	127 (81.9)
治験による予防 (1981-1985)		
1981	30	23 (76.7)
1982	12	9 (75.0)
1983	14	6 (42.9)
1984	58	18 (31.0)
1985	43	12 (27.9)
小計	157	68 (43.3)
事業開始以降 (1986-1994)		
1986	41	10 (24.4)
1987	61	11 (18.0)
1988	58	9 (15.5)
1989	46	6 (13.0)
1990	67	6 (9.0)
1991	62	7 (11.3)
1992	72	2 (2.8)
1993	63	5 (7.9)
1994	66	3 (4.6)
小計	536	59 (11.0)

言える.

なお，静岡県においても，HBs抗原陽性率は，1980年までに出生した群における0.2％（7/3,446）から，1986年以降に出生した群における0.01％（2/23,792）にまで激減しており，同様にHBs抗体陽性率もまた0.96％（33/3,446）から0.21％（51/23,792）にまで大幅に減少していたことが確認されている.

2. 保険医療によるHBV母子感染の予防とその問題点—1995.4～

これまで述べてきたように，HBe抗原陽性のHBVキャリアの母親から出生する児に対象を絞り込んだHBV母子感染防止事業は他に類をみない合理的，かつ効率的なものであったことが明らかとなっている.

しかし，少子化の時代を迎え，事業の対象外とされていた児(HBe抗原陰性のHBVキャリアの母親から出生する児)であっても，自費により感染の予防を希望する母親が増えてきていたとの理由から，1995年4月1日からは，HBe抗原の有無にかかわらず，HBVキャリアの母親から生まれるすべての児を対象とした，保険医療による予防に切り換えられることとなった.

1) 変更後の問題点

保険医療による予防への変更が示された1994年末の時点で，対象の拡大などに伴って当初の標的(予防措置をせずに放置した場合にはキャリア化するリスク集団を対象とす

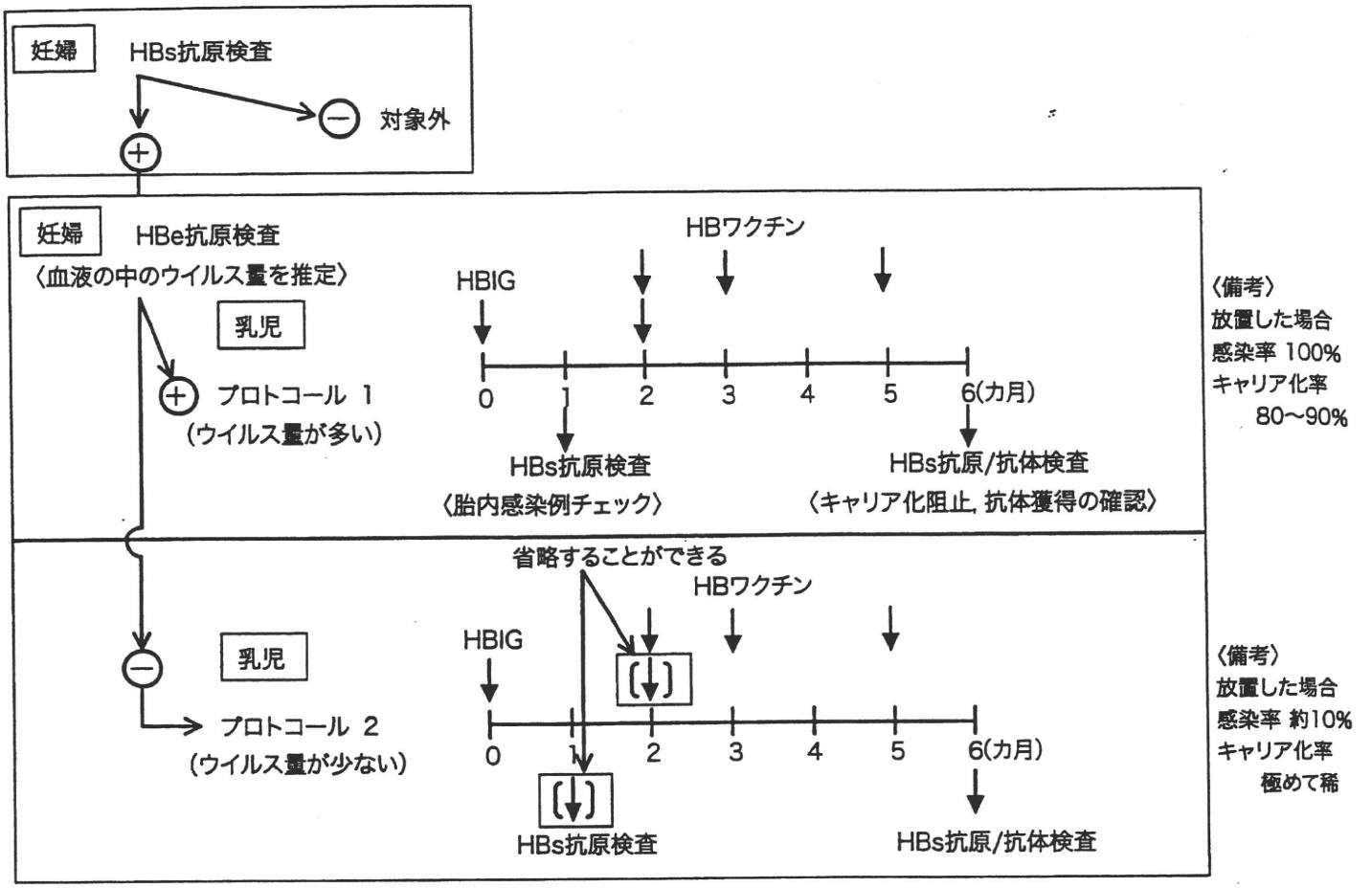

図2　保険医療による予防プロトコール　1995.4.1〜

ること)と，目的(リスク集団に対象を絞り込んでキャリア化を阻止すること)が不明確になり，治験による予防からHBV母子感染防止事業による予防を行ってきた過程で積み上げてきたことが失われてしまうことが懸念された．

このことに対処するために，筆者も加わって，図2に示すプロトコールを新たに作成し，特にHBe抗原陽性のHBVキャリアの母親から出生する児を主な標的として予防することの重要性を繰り返し強調してきた[4]．しかし，保険医療による予防は医師であればどこでも誰でも行うことができ，かつ，届出の必要もないことなどからHBV母子感染予防の実態がその後どのようになっているのかを捉える術もなくなっている現状にある．

本稿では，十全のプロトコールを作成して，講習会を通じて十分な説明と知識の伝達を行った上で臨んだ1986年のHBV母子感染防止事業による全面展開初年度におけるキャリア化阻止率が，それでも90％を割り込んだこと，また，定期的な症例検討会を開くことができなくなった1991年には，いったんは94〜97％にまで回復していたキャリア化阻止率が，再び90％を切ってしまったという表1に示したデータの重みを再度強調し，注意を換起しておきたい．

3　C型肝炎ウイルス（HCV）の母子感染について

はじめに述べたように，C型肝炎ウイルス(HCV)の母子感染については，(1) HCVキャリアの母親から出生する児への感染率は2.3％ (2/87) [7]〜8.3％ (7/84) [17]程度に止

まること，(2)仮に感染が起こった場合でも，早期(乳幼児期)に比較的高い頻度でキャリア状態からの離脱が起こること，(3)感染防止のためのワクチン，免疫グロブリンは未だ実用化の目処が立っていないこと，(4)万が一感染してキャリア化した場合でも，成人に達してからの抗ウイルス療法で十分に対処が可能であること，などの理由から，現時点においては分娩の方法，哺育などについて持別な対処は必要ない(自然分娩，母乳保育でも可)とされている．

4 おわりに

HBVの母子感染予防については，「HBVキャリアを次世代から消滅させる」という当初の目標を風化させないために，治験研究による予防，HBV母子感染防止事業による予防が行われていた時代に得られた知見，経験を再度総点検して，その結果を世代交代した産科，小児科の医師達に継承していく地道な努力が必要であることを改めて強調しておきたい．一方，HCVの母子感染については，現時点においては特に社会対応する術も，またその必要もないと言えよう．

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Incidence Rates of Hepatitis B and C Virus Infections among Blood Donors in Hiroshima, Japan, during 10 Years from 1994 to 2004

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Key Words
Hepatitis B virus · Hepatitis C virus · Blood donors · Incidence

Abstract
Objective: Although prevalence rates of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections have kept decreasing in blood donors, there is little information on incidence rates of these hepatitis viruses in Japan. ***Methods:*** During 10 years from June 1994 through April 2004, 418,269 inhabitants of Hiroshima, Japan, donated blood (1,409,465 units in total). They were screened for serum markers of HBV and HCV infections, and individuals who developed de novo infections were identified. ***Results:*** Infection with HBV occurred at a rate of 2.78 per 100,000 person-years (95% confidence interval: 1.78–4.14/100,000 person-years) and that with HCV at a rate of 1.86 per 100,000 person-years (95% confidence interval: 1.06–3.01/100,000 person-years). Residual risks of transmission by transfusions, based on the relationship risk [window period (estimated at 0.15 and 0.03 years in chimpanzees inoculated with minimum infectious doses for HBV and HCV, respectively) × incidence], were 1/243,000 for HBV and 1/1,960,000 for HCV infections. ***Conclusion:*** At present, incidence rates of HBV and HCV infections are extremely low in Japan.

Introduction

The prevalence of hepatitis B virus (HBV) infection has kept decreasing in Japan during the past 4 decades, which is reflected in the sex- and age-specific frequency of hepatitis B surface antigen (HBsAg) among the first-time blood donors [1]. The prevalence of HBsAg is the lowest in blood donors born after 1981 at 0.23%, in marked contrast to 1.50% in those born between 1941 and 1950. The decrease would be due to improved sanitary conditions after the end of the World War II (1939–1945), and immunoprophylaxis on babies born to carrier mothers with hepatitis B e antigen (HBeAg) in serum implemented nationally since 1986 [2].

The decrease in the sex- and age-specific prevalence of infection with hepatitis C virus (HCV) is even more remarkable. The frequency of antibody to HCV (anti-HCV) is the highest in blood donors born before 1940 at 3.38%, in outstanding contrast to 0.13% in those born after 1984 [1]. At present, it is said that the safety of the blood supply became top level also in the world [3]. It would be attributable, not only to various screening tests, but also to the exclusion of remunerated blood donors heavily contaminated with HCV. In Japan, anti-HCV screening has been performed since November 1989, and nucleic acid technology (NAT) for screening HCV RNA was initiated in October 1999 [4].

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On the basis of decreasing prevalence of HBV and HCV infections, incidence of these hepatitis viruses is reasonably expected to be low in Japan. It has been pointed out, however, that prevalence and incidence do not necessarily behave in parallel [5]. Therefore, incidence rates of HBV and HCV infections were examined in blood donors at the Hiroshima Red Cross Blood Center during 10 years from 1994 to 2004.

Materials and Methods

Blood Donors

During 10 years from June 1, 1994, through April 30, 2004, 418,269 individuals donated 1,409,465 blood units at the regional Japanese Red Cross Blood Center in Hiroshima. Of them, 219,292 (52.4%) individuals were negative for HBsAg at the first donation and gave 1,209,788 units at two or more occasions during that period, and the incidence of HBV infection was determined in them. Likewise, the incidence of HCV infection was calculated in 218,797 (52.3%) individuals who were negative for anti-HCV at the first donation and submitted 1,207,773 units thereafter. They were followed for serum markers of HBV and HCV infections.

In Japan, all donors were asked to fill in the questionnaire at regional blood centers before giving blood units in accordance with the universal standard. It included their current health status and history of liver disease, travels abroad, minor and major surgeries, skin-breaking maneuvers such as tattooing, piercing and acupuncture, high-risk behaviors like homosexual and promiscuous sexual contacts as well as illicit intravenous drugs. Their wish, if any, to be tested for infections with blood-borne viruses through recent risk-taking actions, was rigorously denied under a signed declaration.

Observation periods in person-years were compiled in these blood donors, stratified by sex and age at the donation, and incidence rates of HBV and HCV infections were estimated in each group.

Markers of HBV and HCV Infections

HBsAg was determined by reversed passive hemagglutination, antibody to anti-HBc by hemagglutination inhibition and antibody to HBsAg (anti-HBs) by passive hemagglutination (PHA); reagents for hemagglutination tests were prepared by the Japanese Red Cross. In brief, serial twofold dilutions (2^N) of the serum were prepared in microtiter plates and tested for HBV markers, and wells exhibiting hemagglutination or inhibition thereof were detected in an automated system (PK7200; Olympus Co., Ltd, Tokyo, Japan). Positive results were recorded for wells showing hemagglutination or its inhibition in $\geq 2^3$ dilutions. Donors with solitary anti-HBc with hemagglutination inhibition titers $\geq 2^6$ had been screened out since March 1996, and those with $\geq 2^5$ after April 1996 [6]. HBeAg was determined by enzyme-linked immunosorbent assay with commercial kits (HBeAg ELISA, Institute of Immunology, Tokyo, Japan). Anti-HCV was determined by PHA of the second generation with commercial assay kits (HCV PHA; Abbott Laboratories, Abbott Park, Ill., USA) with a cut-off limit set at 2^5. HCV RNA was determined in blood units testing positive for anti-HCV by polymerase chain reaction with nested primers deduced from well-conserved areas in the 5'-noncoding region of the viral genome [7].

Incident Cases of HBV and HCV Infections

New infections with HBV were diagnosed in donors who turned positive for HBsAg between any two donations during the observation period of 10 years, and those with HCV in donors who seroconverted to anti-HCV with or without HCV RNA. At the Japanese Red Cross Blood Center in Hiroshima, blood donations negative for all serological markers of HBV or HCV infection were examined by NAT, in a 500-pool since October 10, 1999 and in a 50-minipool after February 1, 2000; the pool size was reduced to 20 as of August 1, 2004. Donors who had turned positive for NAT were also counted as incident cases.

Relationship Risk for HBV and HCV Infections by Blood Transfusion

The relationship risk of blood transfusion, defined by (window period in years) × (incidence/100,000 person-years) [8, 9], was calculated for both HBV and HCV.

Statistical Analysis

Incidence rates of HBV and HCV infections were calculated in the total as well as subgroups of donors stratified by sex and age at the blood donation, and their 95% confidence intervals (CIs) were computed by the Poisson distribution.

Results

Incidence of HBV Infection

HBV infection developed in 24 of the 219,292 donors who gave two or more blood units during 10 years from 1994 to 2004 in Hiroshima (fig. 1). The occurrence of HBV infection is tabulated in table 1 stratified by sex and age at the blood donation. The incidence of HBV infection was calculated to be 2.78/100,000 person-years (95% CI: 1.78–4.14). Two examples of typical HBV seroconversion are presented in figure 2.

Caution was exercised to identify and exclude low-level carriers who had resolved HBV infections a long time ago, and in whom anti-HBc persisted along with waxing and waning of HBsAg in low titers. Figure 3 demonstrates two such examples.

Incidence of HCV Infection

HCV infection developed in 16 of the 218,797 donors who gave two or more blood units during 10 years in Hiroshima (fig. 1). The occurrence of HCV infection is tabulated in table 2 stratified by sex and age at the donation. The incidence of HCV infection was calculated to be 1.86/100,000 person-years (95% CI: 1.06–3.01/100,000 person-years). Examples of HCV seroconversion are ex-

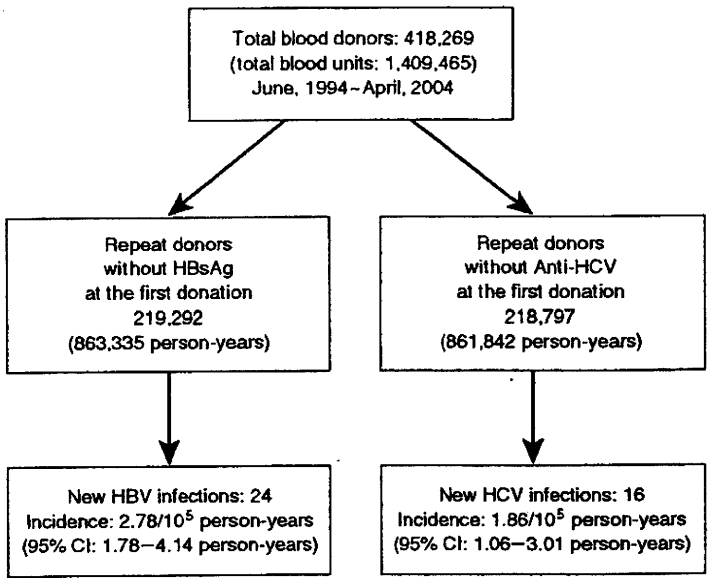

Fig. 1. Blood donors in whom incidence rates of HBV and HCV infections were determined.

Table 1. Incidence of HBV infection stratified by age and sex

Age at donation years	Donors	Person-years	Infection	Incidence/10^5 person-years (95% CI)
Total	309,252	863,335	24	2.78 (1.78–4.14)
≤19	33,705	40,354	0	0.00 (0.00–9.14)
20–29	85,947	234,737	13	5.54 (2.95–9.47)
30–39	71,407	216,993	6	2.77 (1.01–6.02)
40–49	61,593	196,628	3	1.53 (0.31–4.46)
50–59	44,137	139,833	2	1.43 (0.17–5.17)
≥60	12,463	34,789	0	0.00 (0.00–10.60)
Men	161,830	464,943	16	3.44 (1.97–5.59)
≤19	13,388	15,870	0	0.00 (0.00–23.24)
20–29	40,658	110,115	7	6.36 (2.56–13.10)
30–39	41,831	130,452	5	3.83 (1.24–8.94)
40–49	36,833	119,144	2	1.68 (0.20–6.06)
50–59	23,270	73,012	2	2.74 (0.33–9.90)
≥60	5,850	16,349	0	0.00 (0.00–22.56)
Women	147,422	398,392	8	2.01 (0.87–3.96)
≤19	20,317	24,484	0	0.00 (0.00–15.07)
20–29	45,289	124,623	6	4.81 (1.77–10.48)
30–39	29,576	86,541	1	1.16 (0.03–6.44)
40–49	24,760	77,483	1	1.29 (0.03–7.19)
50–59	20,867	66,821	0	0.00 (0.00–5.52)
≥60	6,613	18,440	0	0.00 (0.00–20.01)

Calculated from a database of 219,292 repeat donors.

Table 2. Incidence of HCV infection stratified by age and sex

Age at donation years	Donors	Person-years	Infection	Incidence/10^5 person-years (95% CI)
Total	308,791	861,842	16	1.86 (1.06–3.01)
≤19	33,737	40,380	0	0.00 (0.00–9.14)
20–29	85,983	234,827	5	2.13 (0.69–4.97)
30–39	71,388	216,885	2	0.92 (0.11–3.33)
40–49	61,485	196,277	2	1.02 (0.12–3.68)
50–59	43,903	139,170	5	3.59 (1.17–8.38)
≥60	12,295	34,303	2	5.83 (0.71–21.06)
Men	161,691	464,382	5	1.08 (0.35–2.51)
≤19	13,404	15,881	0	0.00 (0.00–23.23)
20–29	40,692	110,181	1	0.91 (0.02–5.06)
30–39	41,827	130,403	0	0.00 (0.00–2.83)
40–49	36,802	118,983	1	0.84 (0.02–4.68)
50–59	23,176	72,766	1	1.37 (0.03–7.66)
≥60	5,790	16,169	2	12.37 (1.50–44.69)
Women	147,100	397,459	11	2.77 (1.38–4.95)
≤19	20,333	24,499	0	0.00 (0.00–15.06)
20–29	45,291	124,647	4	3.21 (0.87–8.22)
30–39	29,561	86,482	2	2.31 (0.28–8.35)
40–49	24,683	77,293	1	1.29 (0.03–7.21)
50–59	20,727	66,404	4	6.02 (1.64–15.42)
≥60	6,505	18,134	0	0.00 (0.00–20.34)

Calculated from a database of 218,797 repeat donors.

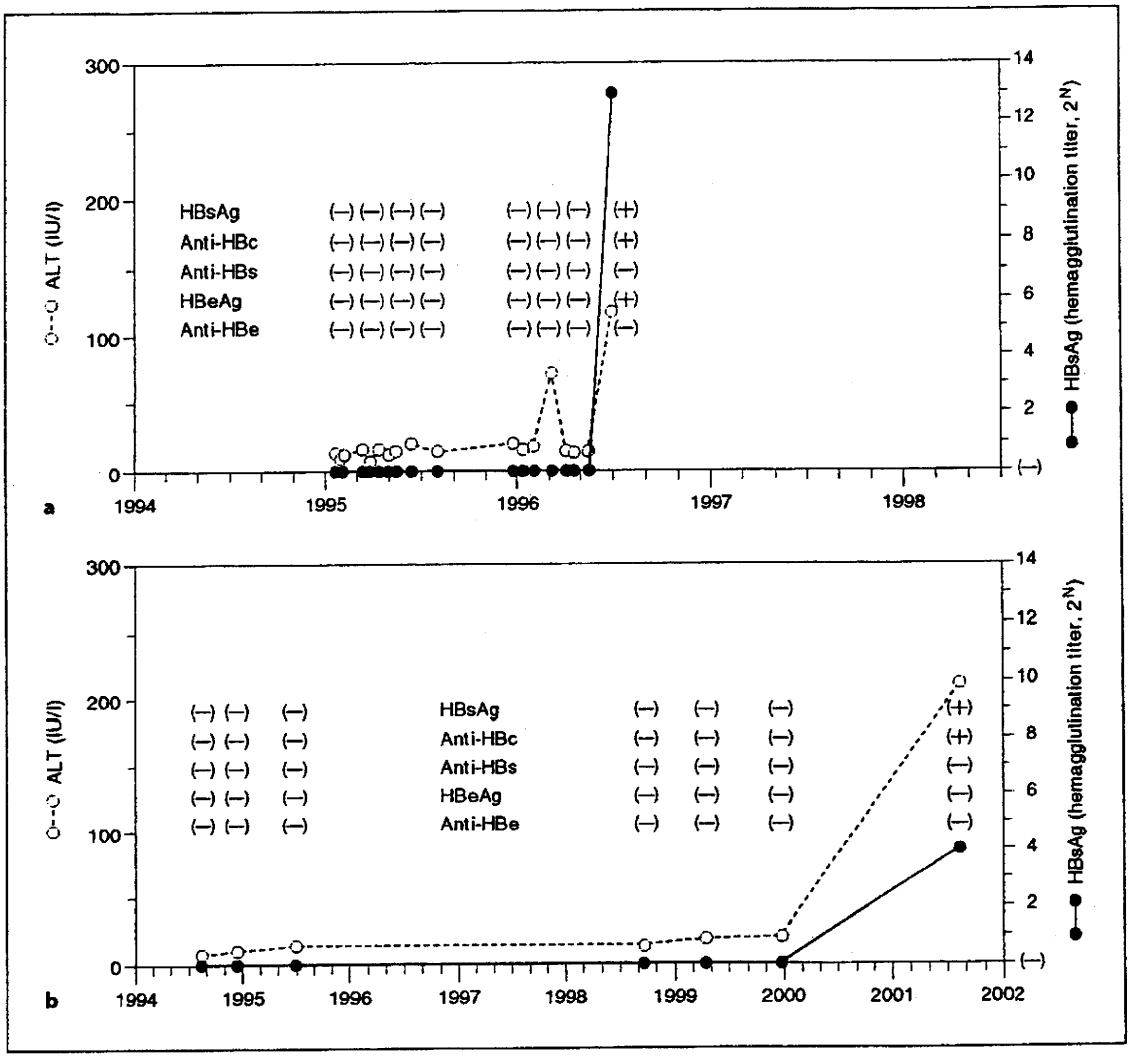

Fig. 2. Blood donors who were infected with HBV during repeat donations. Two representatives are shown. **a** A male 24-year-old blood donor had been seronegative through the initial 18 donations, but at the 19th donation 6 weeks after the previous one, HBsAg in a high hemagglutination titer (2^{13}) was detected in his serum along with anti-HBc and HBeAg. **b** Another male donor, 38 years old, had been seronegative through 6 donations, but at the 7th donation 20 months from the previous one, he seroconverted to HBsAg along with the development of anti-HBc.

hibited in figure 4. Coinfection with HBV and HCV did not develop in any donor.

Sex- and Age-Specific Incidence Rates of HBV and HCV Infections

Figure 5 illustrates incidence rates of HBV and HCV infections in donors stratified by sex and age. HBV infections tended to occur more frequently in men than women (3.44 vs. 2.01/100,000 person-years), but the difference fell short of being significant due to small numbers of de novo HBV infections (fig. 5a). Both in men and women, HBV infection occurred most frequently in male and female donors aged 20–29 years [7/16 (44%) and 6/8 (75%), respectively], and it decreased gradually with age.

Unlike HBV infection, the infection with HCV tended to be less frequent in men than women (1.08 vs. 2.77/100,000 person-years); the difference was not significant

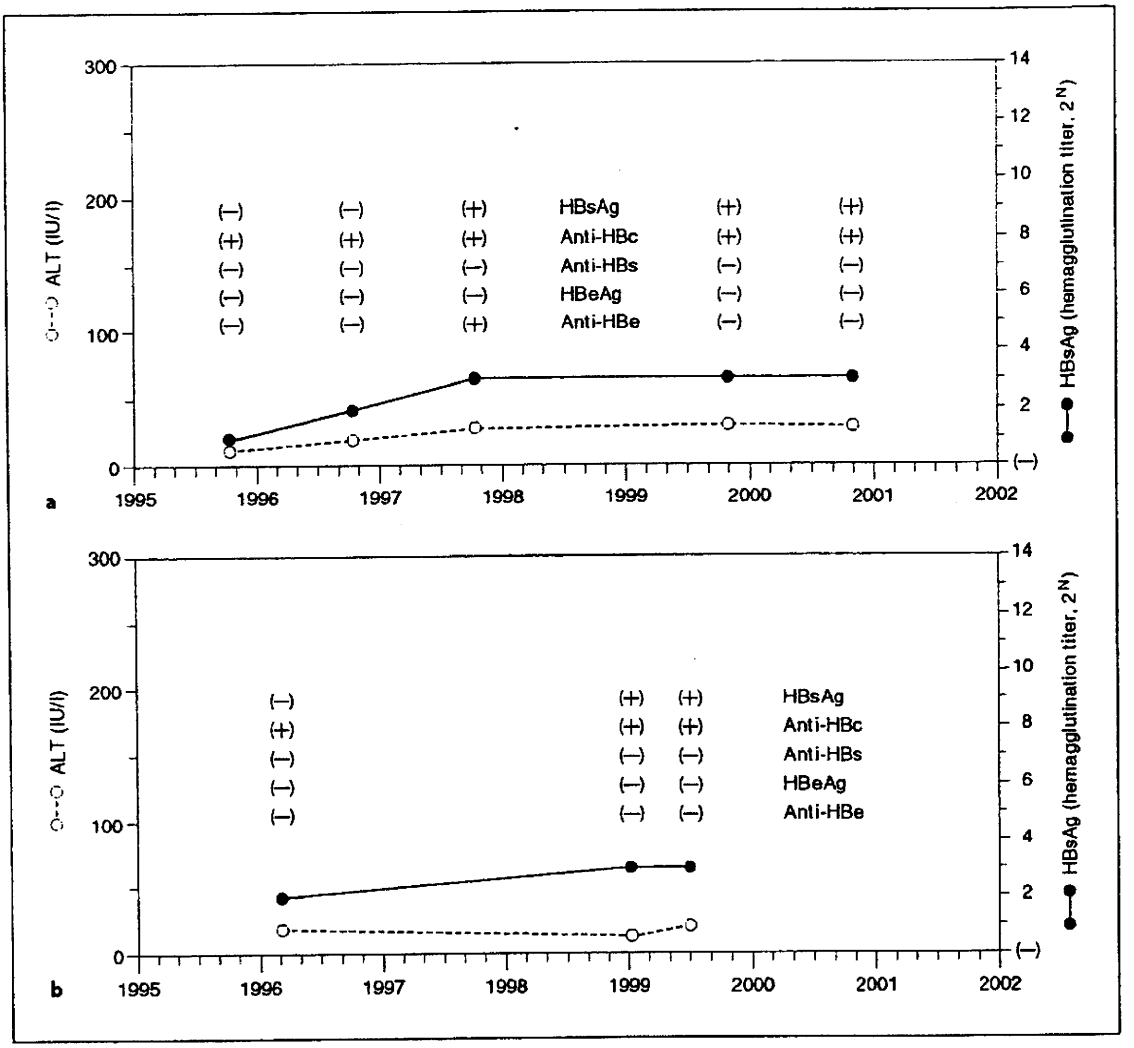

Fig. 3. Blood donors in whom HBV infection was reactivated. Two representatives are shown. **a** A male blood donor 35 years of age tested positive for HBsAg in low hemagglutination titers just above the negative range on his 3rd to 5th donations. He had possessed anti-HBc through his 5 donations, in titers $<2^4$ and kept donating blood units. On these grounds, he was diagnosed with the reactivation of past HBV infection. **b** Likewise, another female blood donor, 41 years old, had her past HBV infection reactivated between the 1st and 2nd donations.

owing to small numbers of new HCV infections, however (fig. 5b). In contrast to HBV infection, the infection with HCV was inclined to increase with age.

Estimates of the Relationship Risk for HBV and HCV Infections by Transfusion

The mean window periods in chimpanzees who had received the minimal infectious dose (~10^1 copies: digit of ten) of HBV or HCV were 54 days (0.15 years) [10] and 10 days (0.03 years) [11]; respectively; HBV DNA and HCV RNA levels rose to 10^3/ml within these windows, which is required for testing positive by NAT in a 20-minipool [12, 13]. Multiplying these figures by incidence rates of HBV and HCV infections (2.78 and 1.86/100,000 person-years, respectively), the relationship risk of HBV infection was estimated at 1/243,000 and that of HCV infection at 1/1,960,000 blood donations.

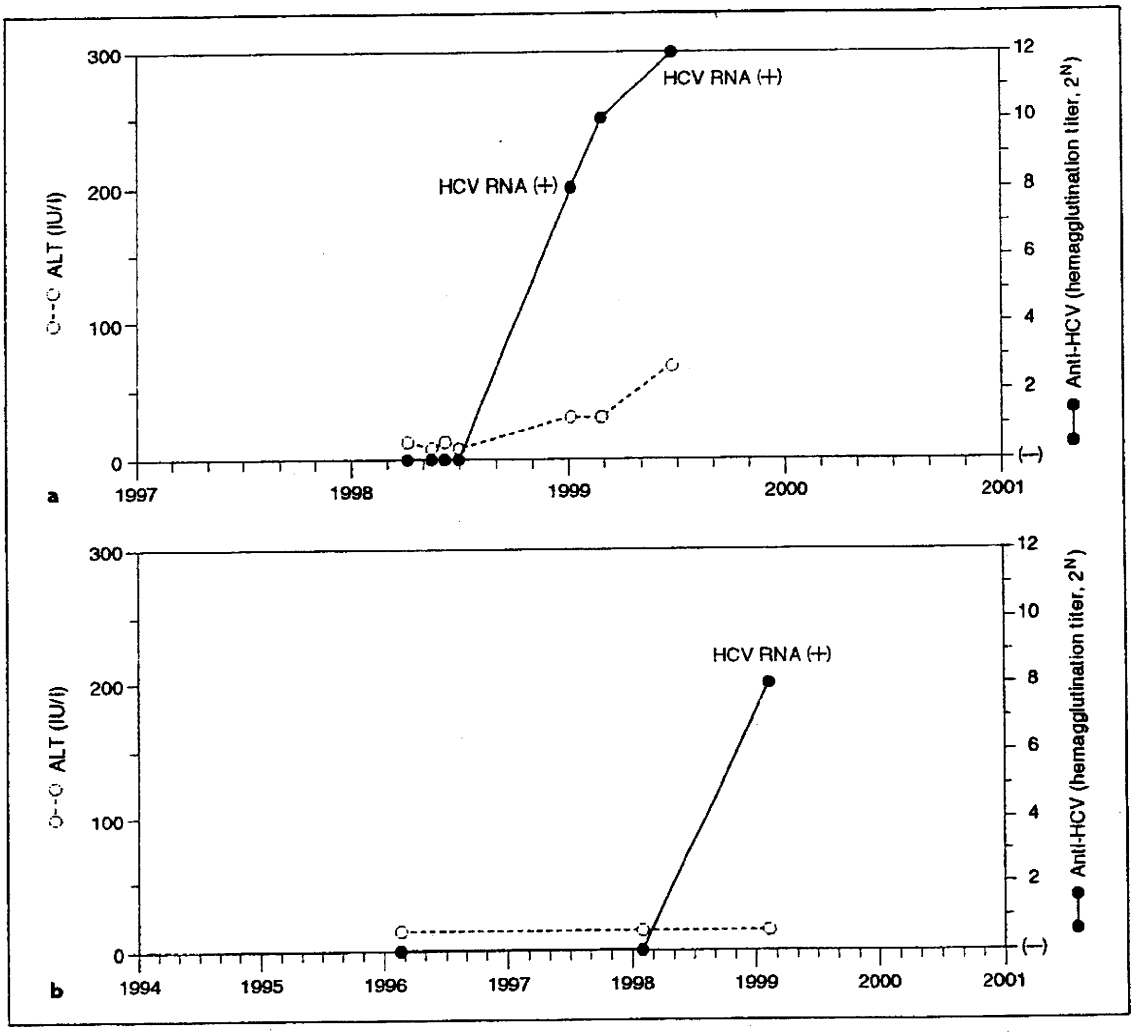

Fig. 4. Blood donors who contracted HCV infection during repeat donations. Two representatives are shown. **a** A female blood donor aged 24 years turned positive for anti-HCV on her 5th donation along with the development of HCV RNA in the serum. **b** Another female blood donor, 53 years old, developed anti-HCV at her 3rd donation accompanied by HCV RNA in the serum.

Discussion

Here, we report incidence rates of HBV and HCV infections in Japanese blood donors. There are obvious limitations in the present study. First, the incidence was examined in blood donors in Hiroshima; it is not certain how they represent the general population in Japan. Nevertheless, we would have to face the reality that blood donors are the only cohort in whom prevalence and incidence rates of blood-borne viruses are to be estimated. In fact, the great majority of epidemiological surveys of HBV and HCV have been performed on blood donors worldwide. Secondly, the time period of our survey was rather long, spanning 10 years from 1994 to 2004. This has made it difficult to estimate the accurate incidence of HBV or HCV infection in Hiroshima blood donors. These constraints notwithstanding, some facts and trends have surfaced in this study.

First, incidence rates of HBV and HCV infections are low at 2.78/100,000 person-years (95% CI: 1.78–4.14/100,000 person-years) and 1.86/100,000 person-years (95% CI: 1.06–3.01/100,000 person-years), respec-

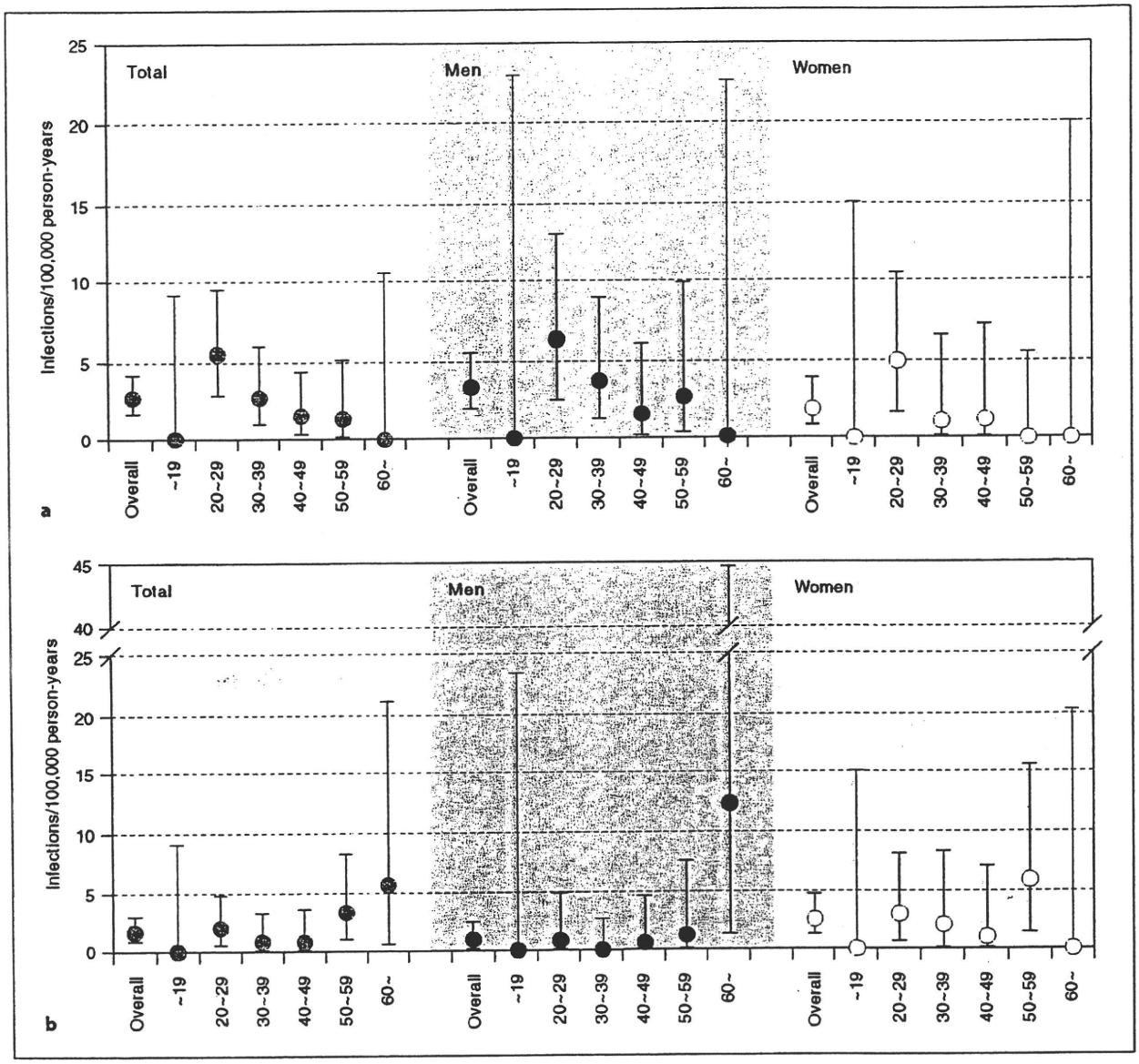

Fig. 5. Sex and age-specific incidence rates of HBV (**a**) and HCV (**b**) infections. Bars indicate 95% CIs.

tively. They are less than 12.4 (per 100,000) and 16.83 (per 100,000), respectively, during 11 years from 1990 through 2000 in Canada [14], and comparable with 1.27 and 1.89/100,000 person-years, respectively, during 1995 through 2001 in the United States [8]. Observed low incidence rates of HBV and HCV infections in Japan would be attributed, at least in part, to rare intravenous drug users as well as less sexual behavior including homosexual in Japan than in Western countries up to the present. In addition, the medical treatment hygiene environment, such as disposable medical supplies, has improved and successful passive and active immunoprophylaxis of babies born to carrier mothers with HBeAg in serum, implemented on the national scale since 1986, would have had some direct and indirect effects on suppressing reproduction of HBV carriers in Japan [2]. Moreover, there

might be a possibility that questionnaires before donation in Japan are more effective for excluding high risk donors than those in Western countries.

Secondly, there would be an age-specific risk for HBV infection in both men and women. HBV infection clustered in male and female donors aged 20–29 years [7/16 (44%) and 6/8 (75%), respectively]. As acute HBV infection is transmitted mostly by sexual contacts in young men in Japan [15], young female blood donors may have been infected with HBV in a similar manner. Since they are infected with HBV of foreign genotypes represented by genotype A [15, 16], genotyping HBV DNA in donors with de novo infection would be able to trace the route of transmission in them. There are serious ramifications for the HBV infection of genotype A in that it tends to become chronic even in the adulthood and persists in >10% [17], and that it elicits HBV mutants resistant to lamivudine more early and frequently than infections with the other genotypes [18]. HCV infection tended to occur more frequently in women than men (2.77 vs. 1.08/100,000 person-years), in opposite to HBV infection (2.01 vs. 3.44/100,000 person-years). Unlike HBV infection that inclined to decrease with age, HCV infection had the tendency to increase with age (fig. 5).

Thirdly, in view of the prevalence of HCV infection in Japan that decreases sharply from the southwest to the northeast along its axis [19], the incidence of HCV infection in Hiroshima may not be generalized elsewhere. An HCV incidence of 5.38/100,000 person-years (95% CI: 4.10–6.95/100,000 person-years) was reported in 448,020 blood donors during 6 years from 1992 to 1997 in Osaka where HCV is prevalent [20]. Of note, the incidence of HCV infection there is higher in donors aged 16–24 years than in those 35–49 years of age (8.89/100,000 person-years, 95% CI: 6.04–12.61/100,000 person-years, vs. 1.81/100,000 person-years, 95% CI: 0.67–3.95/100,000 person-years) [20]. Such a wide difference between Hiroshima and Osaka blood donors (1.86 vs. 5.38/100,000 person-years) in the incidence of HCV infection is hardly accounted for. The incidence during 10 years from 1994 to 2004 in Hiroshima at 1.86/100,000 person-years (95% CI: 1.06–3.01/100,000 person-years) is essentially the same as 1.78/100,000 person-years (96% CI: 0.37–5.19/100,000 person-years) in 114,266 donors during the previous 3 years from 1992 to 1994 in the identical prefecture [21]; both are much lower than 5.38/100,000 person-years in Osaka during 1992 through 1997. Although the incidence was calculated according to the change to anti-HCV positive in Osaka, that is, confirmation of infection by NAT had not been done, the incidence in Osaka might be almost 30% reduction. On the other hand, there might be some regional risks for HCV infection in Osaka that are not shared by Hiroshima. There is a possibility that the background of routes of blood-borne infection, for instance intravenous injection of methamphetamine with shared syringes and needles, might be more common among a specific group in Osaka than in Hiroshima. Therefore, the source of infection, that is HCV carriers, is greater in Osaka than in Hiroshima.

Finally, incidence rates of HBV and HCV infections documented herein have implications for blood safety in Japan. The relationship risk (window period × incidence) has been proposed to estimate residual risks of the exposure to blood-borne viruses by transfusions [8, 9]. Window periods while HBV DNA and HCV RNA are not detectable by NAT were determined in chimpanzees who had received the minimum infectious doses (~10^1 copies: digit of ten) of these hepatitis viruses [10, 11]. It took 54 and 10 days, respectively, before HBV DNA and HCV RNA reached 10^3 copies/ml; they translate into 0.15 and 0.03 years, respectively. Multiplying these figures by 2.78 and 1.86/100,000 person-years for incidence rates of HBV and HCV, respectively, the residual risks for the exposure to HBV and HCV by transfusion in Japan are calculated to be 1/243,000 and 1/1,960,000, respectively. They are close to 1/488,000 for HBV and 1/1,935,000 for HCV in the United States during 1955–2001 [8], and would guarantee the safety of blood transfusion in Japan at present.

In conclusion, incidence rates of HBV and HCV, as well as residual risks of the exposure to these hepatitis viruses by transfusion, were found to be low in Japan during the past decade. This would be attributable to successful suppressing of hepatitis virus infections and of reproduction of carriers by the national program to interrupt mother-to-baby transmission of HBV [2] and ever improving screening methods for preventing transfusion-transmitted infections with HBV and HCV [6, 19]. There seem to be risk behaviors for getting HBV infection that would be age dependent. Hence, low incidence rates of hepatitis viruses should not be deemed as auspicious readily, and further efforts are warranted to identify and interrupt insidious transmission routes for containing infections with HBV and HCV in various age groups.

Acknowledgments

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Journal of Medical Virology 80:2064–2068 (2008)

Titration of Hepatitis B Virus Infectivity in the Sera of Pre-Acute and Late Acute Phases of HBV Infection: Transmission Experiments to Chimeric Mice With Human Liver Repopulated Hepatocytes

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Studies of hepatitis B virus (HBV) infection in non-human primates such as chimpanzees are no longer possible due to ethical considerations and the endangered status of chimpanzees since April 2007 in Japan. A human hepatocyte transplanted chimeric mouse was used to characterize HBV infectivity in serial stages of acute infection. Chimeric mice were inoculated intravenously with serum samples obtained from an experimentally infected chimpanzee with HBV. Sera from the pre-acute phases (i.e., rump-up viremia prior to anti-HBc) and late acute phases (i.e., declining phase of HBsAg and anti-HBcAb positive) were collected from the chimpanzees 57 and 244 days after inoculation. These sera contained 2.6×10^6 and 2.8×10^6 copies/ml of HBV DNA, respectively. Three chimeric mice inoculated intravenously with 100 μl of pre-acute serum (equivalent to 10^0 copy of HBV DNA) developed an HBV infection. The three chimeric mice that received 100 μl of pre-acute serum (equivalent to 10^1 copies of HBV DNA), developed high levels of serum HBV DNA. None of the three chimeric mice inoculated with 100 μl of $1:10^4$ dilution (equivalent to 10^1 copies of HBV DNA) of late-acute serum was infected, while only one of three chimeric mice inoculated with 100 μl of $1:10^3$ dilution (equivalent to 10^2 copies of HBV DNA) of late-acute serum developed an HBV infection. Based on these results, chimeric mice can be used as animal models for the study of HBV infectivity, pathogenesis and control. The results show that pre-acute phase HBV serum is about 100-times more infectious than late acute phase serum. ***J. Med. Virol. 80:2064–2068, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus (HBV); acute HBV infection; HBV serum markers; HBV DNA; chimeric mice; minimum infectious HBV dose; experimental transmission of HBV

INTRODUCTION

In the past, non-human primates such as chimpanzees, have been utilized successfully as human surrogates for the experimental transmission of human hepatitis viruses [Rizzetto et al., 1981; Dienstag, 1983; Prince and Brotman, 2001; Murray et al., 2005]. At present, however, the use of chimpanzees in such experiments is prohibited in Japan since April 2007 and many other countries. As an alternative animal model mice with severe combined immunodeficiency disease (SCID) transgenic for the urokinase-type plasminogen activator gene under the control of albumin

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promotor (uPA/SCID mice) can be transplanted with human hepatocytes [Heckel et al., 1990; Rhim et al., 1994; Tateno et al., 2004] and utilized for studies of viral transmission, replication and pathogenesis of human hepatitis in vivo [Dandri et al., 2001; Mercer et al., 2001; Meuleman et al., 2005; Tsuge et al., 2005; Sugiyama et al., 2007].

An understanding of the minimal infectious dose of hepatitis B virus (HBV) required for parenteral transmission from blood collected from donors in progressive stages of HBV infection is important for assessing the safety of transfusions and guiding decisions on implementation of nucleic acid amplification tests to interdict infectious units [Yugi et al., 2006; Komiya et al., 2008]. Such studies would also be useful to understand mechanisms of viral–host interactions controlling replication and disease pathogenesis. The present study using these chimeric mice offers a rare opportunity in determining the minimal infectious dose for human hepatitis viruses without resorting to the use of chimpanzees. This study shows that chimeric mice can be used as models in the study of the pathogeneses and control of HBV infection and in determining the infectious status of implicated human sera in the transmission of an HBV infection.

MATERIALS AND METHODS

Sera in Pre-Acute and Late Acute Phases of HBV Infection

A chimpanzee (13 years old, male, 60.7 kg) designated as chimp-246, was injected intravenously with 1 ml of fresh frozen plasma obtained from a blood donor in the pre-acute phase of HBV infection [Komiya et al., 2008]. The donor was identified by a nucleic acid amplification test (NAT) for HBV DNA at a Japanese Red Cross Blood Center. The plasma contained 6.9×10^4 copies/ml of HBV DNA, genotype A, and was reactive for hepatitis B surface antigen (HBsAg) and negative for antibody to hepatitis B core antigen (anti-HBc). Chimpanzee serum samples were collected serially and immediately aliquoted in 1 ml volume, snap-frozen in liquid nitrogen and stored immediately at −80°C for future studies. Individual tubes were thawed gently by immersing in a 37°C water bath before inoculation of chimeric mice. Experiments using chimpanzees were done before 2006 and ethical approval for each experiment was obtained and applied according to the available facilities.

Inoculation of Chimeric Mice With the Liver Repopulated for Human Hepatocytes

The human hepatocytes were transplanted into the urokinase-type plasminogen activator transgenic SCID mice ($uPA^{+/+}/SCID^{+/+}$ mice) and these chimeric mice using the same human hepatocytes lot; Chimeric mice's lot was BD 61.

These chimeric mice (ChiM); lot BD61 were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). The chimeric mice were kept in a clean room and supplied with sterilized laboratory chaw and water. They were inoculated with 100 μl of diluted chimpanzee sera containing known copy numbers of HBV DNA via the tail vein. Blood samples were taken from orbital venous plexus of the chimeric mice, and sera were separated.

LABORATORY TESTS

HBsAg, antibody to HBsAg (anti-HBs) and antibody to core antigen (anti-HBc) were determined by microparticle enzyme immunoassay (MEIA) with AxSYM® kits (Abbott Japan, Co., Ltd, Tokyo, Japan) according to the manufactures instructions. The results were expressed in S/N ratio for HBsAg, mIU/ml for anti-HBs and per cent inhibition for anti-HBc. HBV DNA in serum samples was quantitated by TaqMan PCR (Roche Diagnostics KK, Tokyo, Japan) with an established sensitivity of 10^2 HBV DNA copies/ml. Human serum albumin in the sera of chimeric mice was determined by the latex turbidimetric immunoassay (Eiken Chemical Co., Ltd, Tokyo, Japan).

RESULTS

Chronology of HBV Markers After Experimental HBV Infection in Chimpanzee-246

In chimpanzee-246, infected experimentally with HBV, four HBV makers were shown to appear sequentially during the course of disease. These markers appeared in the following sequential order: HBV DNA, HBsAg, anti-HBc and anti-HBs. The titers and time course of these markers are shown graphically in Figure 1. HBV DNA became detectable at 17 days after inoculation and increased exponentially to 10^9 copies/ml until day 83 and then started to decrease at day 118, although it stayed detectable until 335 days after inoculation. HBsAg became detectable 35 days after inoculation and increased to an S/N ratio of greater than 400 until day 71 followed by a gradual decrease and became undetectable 286 days after inoculation. Anti-HBc appeared 79 days after inoculation and increased sharply to high levels until 441 days after inoculation. Anti-HBs appeared 441 days after inoculation at a level 18.1 mIU/ml. Elevated ALT levels were observed at day 97 and remained elevated until 307 days after inoculation. A window of approximately 61 days was observed between the loss of HBsAg and the emergence of anti-HBs. The only markers of any previous HBV infection in this window period were HBV DNA and anti-HBs.

Dilution of Sera From Chimp-246 in Pre-Acute and Late Acute Phases of Infection

Chimeric mice were inoculated intravenously with 100 μl of serially diluted sera in the pre-acute phase (day 57) and late acute phase (day 244) of the acute HBV infection. Six chimeric mice each were inoculated with dilutions of sera in pre-acute and late acute phases of resolving HBV infection. Their sex, body weight and rate index of human hepatocytes repopulation that was

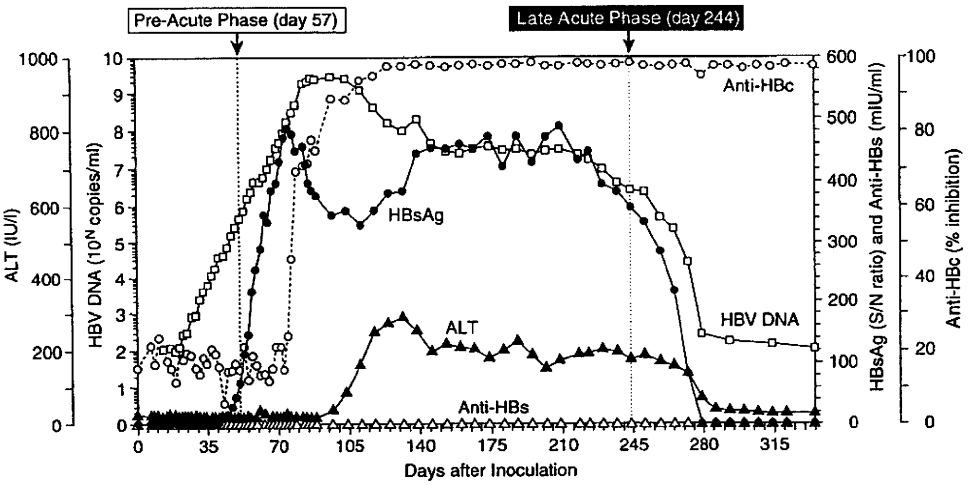

Fig. 1. Markers of HBV infection and transaminase levels of a chimpanzee (chimp-246) inoculated with fresh frozen plasma (FFP) from a donor in the pre-acute phase of infection with HBV of genotype A. Sera from chimp-246 obtained at pre-acute (day 57) and late acute phase (day 244) phases were titrated for HBV DNA, and infectious activity of them were determined in chimeric mice with the liver repopulated for human hepatocytes.

estimated by serum levels of human albumin, are shown in Table I.

Sera of chimp-246 collected either at day 57 in the pre-acute phase when HBV DNA was on the exponential rise or in a late acute phase at day 244 were serially diluted tenfold in pooled SCID mouse sera, and quantitated for HBV DNA by TaqMan PCR (Table II). HBV DNA (26–46 copies/ml) were estimated to be contained in $1:10^6$ dilutions of serum in the pre-acute phase (day 57), and 20–35 copies/ml of HBV DNA in $1:10^5$ dilutions of serum in a late acute phase (day 244). Every tenfold dilution of sera was aliquoted into tubes, snap frozen in liquid nitrogen and kept frozen at −80°C until used for inoculation into chimeric mice.

Infectious Activity of Serum in the Pre-Acute Phase of HBV Infection

Three chimeric mice (ChiM-1,-2,-3) were inoculated with 100 μl of $1:10^5$ dilution inocula of pre-acute phase taken 57 days after inoculation from chimp-246, containing approximately a 10^0 (min–max: 2.6–4.6) copy of HBV DNA. HBV DNA was detected in their serum 4 weeks after the inoculation (Fig. 2A). Similarly in the sera of three chimeric mice (ChiM-5,-6,-7) that were inoculated with 100 μl of $1:10^4$ dilution of sera containing approximately 10^1 (min–max: 26–46) copies of HBV DNA, HBV DNA was detected (Fig. 2B).

Infectious Activity of Serum in a Late Acute Phase of HBV Infection

None of the three chimeric mice inoculated with 100 μl of a $1:10^4$ dilution, containing approximately 10^1 (20–35) copies of HBV DNA, developed HBV DNA in serum (Fig. 3A). In contrast, one (ChiM-10) of the three chimeric mice inoculated with 100 μl of a $1:10^3$ dilution, containing approximately 10^2 (200–350) copies of HBV DNA, exhibited HBV DNA in serum (Fig. 3B). Hence, the dose of HBV in serum in a late acute phase, required

TABLE I. Chimeric Mice Used for Experimental HBV Infection

Mouse no.	Sex	Body weight (g)	Human hepatocyte lot. no.	Serum level of human-albumin (mg/ml)	Rate of hepatocyte repopulation (%)
Mice inoculated with pre-acute serum of chimp 246 (57 days after inoculation)					
1	Female	15.3	BD61	13.0	86.0
2	Female	15.8	BD61	9.4	78.1
3	Male	16.6	BD61	6.9	70.7
4	Female	16.0	BD61	10.0	79.6
5	Female	15.5	BD61	7.5	72.7
6	Male	17.0	BD61	9.1	77.4
Mice inoculated with late-acute serum of chimp 246 (244 days after inoculation)					
7	Female	14.0	BD61	6.7	70.0
8	Male	15.2	BD61	5.3	64.3
9	Male	14.1	BD61	6.6	69.6
10	Male	15.8	BD61	6.5	69.2
11	Male	15.6	BD61	5.5	65.2
12	Male	16.8	BD61	5.7	66.1

TABLE II. Titration of HBV DNA in Sera From Chimpanzee Serially Diluted in Mouse Sera

	Dilution of chimpanzee serum					
	Original	$1:10^1$	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$
Serum of chimp-246 in pre-acute phase (57 days after inoculation)						
HBV DNA (copies/ml)	2.6×10^6	NT	4.6×10^4	2.7×10^3	3.4×10^2	NT
Serum of chimp-246 in late acute phase (244 days after inoculation)						
HBV DNA (copies/ml)	2.8×10^6	3.5×10^5	2.6×10^4	2.7×10^3	2.0×10^2	NT

NT, not tested.

for transmitting infection to one third of chimeric mice, was estimated to be 10^2 (200–350) copies. This is about 100 times higher than serum in pre-acute phase at a single (2.6–4.6) copy.

DISCUSSION

A chimpanzee (chimp-246) was infected experimentally with HBV genotype A obtained from a blood donor during the early acute phase of infection [Komiya et al., 2008]. The patterns of appearance, disappearance, and persistence of hepatitis B markers in this chimpanzee with acute resolving HBV infection were basically similar to experiments carried out previously [Ling et al., 1979]. Sera were collected from chimp-246 during the pre-acute phase (57 days after inoculation) before the appearance of anti-HBc in circulation, and also during the late acute phase (244 days) when both serum HBV DNA and HBsAg started to decline. A marked difference was observed in HBV infectivity of the two sera in vivo, based on the determination of serum HBV DNA concentration. All the three chimeric mice that were inoculated with pre-acute phase chimpanzee serum containing 2.4–4.6 copies of HBV DNA were infected

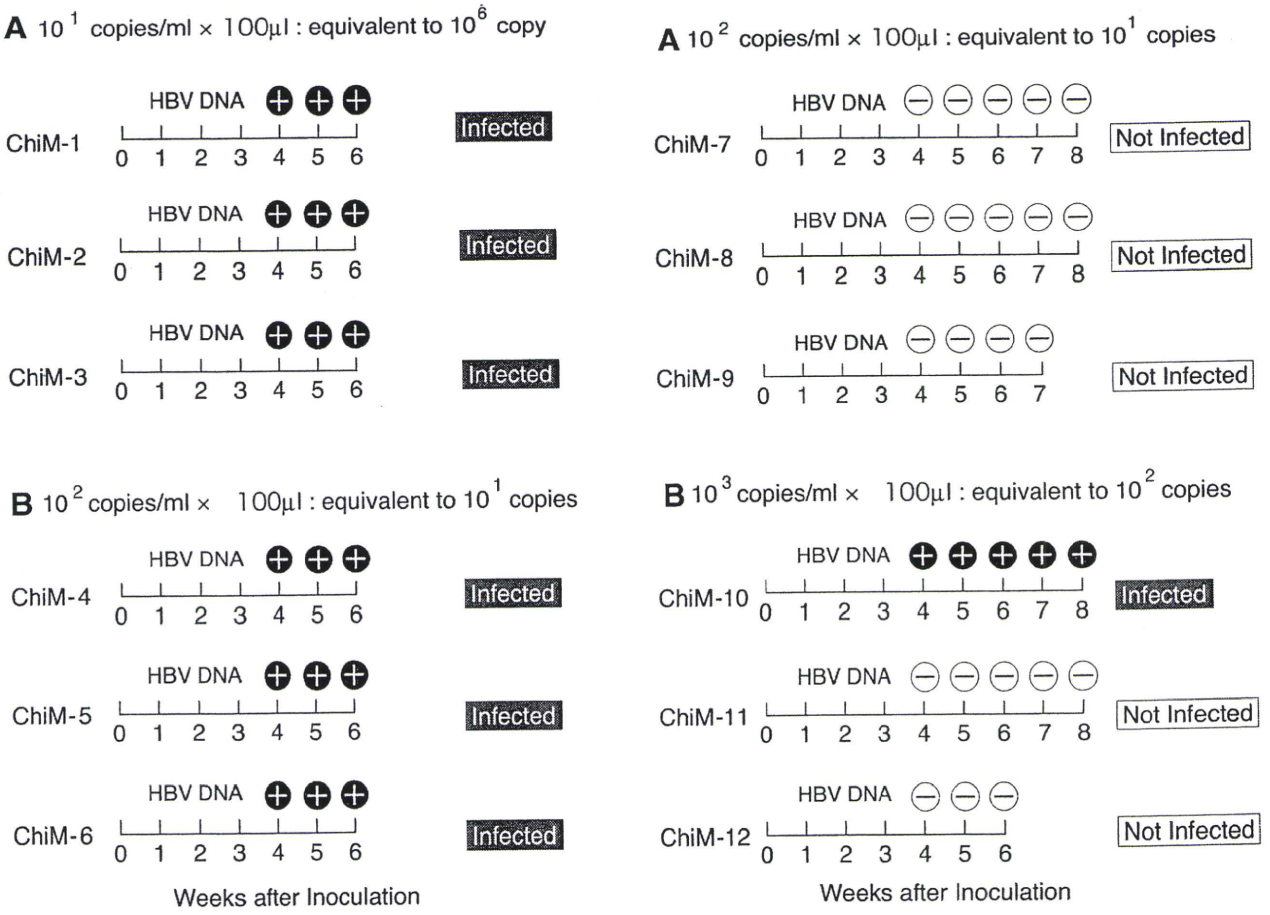

Fig. 2. Courses of six chimeric mice inoculated with serum from chimp-246 in the pre-acute phase 57 days after the inoculation. Three each mice were inoculated with 10^0 copy (**A**) and 10^1 copies (**B**) of HBV DNA.

Fig. 3. Courses of six chimeric mice inoculated with serum from chimp-246 in the late acute phase 244 days after the inoculation. Three each mice were inoculated with 10^1 copies (**A**) and 10^2 copies (**B**) of HBV DNA.

with HBV (Fig. 2). In contrast, only one chimeric mouse out of three developed an HBV infection upon inoculation with late acute phase serum containing 200–350 copies of HBV DNA (Fig. 3A). Based on these limited studies with the absence of a human immune system precludes study of the impact of the innate and adaptive immune responses on viral replication and pathogenesis, the minimum infectious dose of a pre-acute and late acute HBV serum that is required for HBV transmission to chimeric mice with livers repopulated with human hepatocytes is about 10^0 and 10^2 copies, respectively. This difference may be an indirect indication of higher replication rate of HBV in pre-acute HBV sera. It is also reasonable to assume that the lower potency of HBV in late acute sera may be due to the formation of immune complexes of HBsAg and anti-HBs, 244 days after inoculation. These complexes as expected would be less infectious in chimeric mice than the free (uncomplexed) HBV in pre-acute sera as already has been demonstrated previously [Prince et al., 2001].

Non-human primates such as chimpanzees, have been used for many years to study the natural history, pathogenesis and treatment of several human hepatitis viruses. Substantial progress has been made in the last four decades in understanding the molecular virology, immune pathogenesis, diagnosis and treatment of various forms of hepatitis by the use of chimpanzees for such purposes. However, due to the scarcity of these animals and ethical considerations, the use of chimpanzees in such studies is prohibited in Japan and many other countries. Instead, chimeric mice, with severe combined immunodeficiency disease (SCID) transgenic for urokinase-type plasminogen activator gene under the control of albumin promoter (uPA/SCID mice) can be transplanted with human hepatocytes and used successfully for such studies. In this study, chimeric mice are as practical and chimpanzees for estimating the minimum infectious dose of HBV with a sensitivity 10-times higher than in chimpanzees. Furthermore, chimeric mice have already been used instead of chimpanzees for transmission experiments not only with HBV [Dandri et al., 2001; Meuleman et al., 2005; Tsuge et al., 2005; Sugiyama et al., 2007], but also for HCV transmission [Mercer et al., 2001; Meuleman et al., 2005].

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List of Errata

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Modified Part	Wrong	Correct
P2066 left line 8	$1{:}10^6$	$1{:}10^5$
P2067 Fig.2. A Title	equivalent to 10^6 copy	equivalent to 10^0 copy

Minimum infectious dose of hepatitis B virus in chimpanzees and difference in the dynamics of viremia between genotype A and genotype C

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BACKGROUND: In planning optimal hepatitis B virus (HBV) blood screening strategies, the minimum infectious dose and early dynamics of HBV need to be determined for defining the window period for HBV DNA as well as for hepatitis B surface antigen (HBsAg).

STUDY DESIGN AND METHODS: Pairs of chimpanzees were inoculated with preacute-phase inocula containing HBV of genotype A or genotype C to determine the minimum infectious dose, and two pairs of chimps infected with the lowest infectious dose of genotypes A and C were followed for HBV markers.

RESULTS: The minimum 50 percent chimpanzee infectious dose (CID_{50}) was estimated to be approximately 10 copies for genotype A and for genotype C. In the two chimps inoculated with the lowest infectious dose, the HBV DNA window was 55 to 76 days for genotype A and 35 to 50 days for genotype C, respectively. The HBsAg window was 69 to 97 days for genotype A and 50 to 64 days for genotype C, respectively. The doubling times of HBV DNA were 3.4 days (95% confidence interval [CI], 2.6-4.9 days) for genotype A and 1.9 days (95% CI, 1.6-2.3 days) for genotype C. When comparing the replication velocity of HBV DNA between the two genotypes, the doubling time of genotype C was significantly shorter than that of HBV genotype A ($p < 0.01$).

CONCLUSION: Although the CID_{50} of approximately 10 copies was similar for the two HBV genotypes, the doubling time and pre-HBV nucleic acid amplification technology (<100 copies/mL) window period in chimps infected with the lowest infectious dose seemed to be shorter for genotype C than for genotype A.

Posttransfusion infection with hepatitis B virus (HBV) has decreased dramatically since screening for hepatitis B surface antigen (HBsAg) was introduced in the early 1970s. The number of reported posttransfusion hepatitis B cases has been further reduced after screening for antibody to HBV core (anti-HBc) was implemented in the late 1980s in the United States and Japan.[1,2] Japan introduced HBV DNA screening by nucleic acid amplification technology (NAT) in minipools (MPs) in 1999. Since introduction of MP-NAT, more than 500 seronegative donations with detectable HBV DNA have been interdicted, although there are still units of blood in an early or late phase of HBV infection

ABBREVIATIONS: CID_{50} = 50 percent chimpanzee infectious dose; CLIA = chemiluminescent immunoassay; JRC = Japanese Red Cross; MP(s) = minipool(s).

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