図３

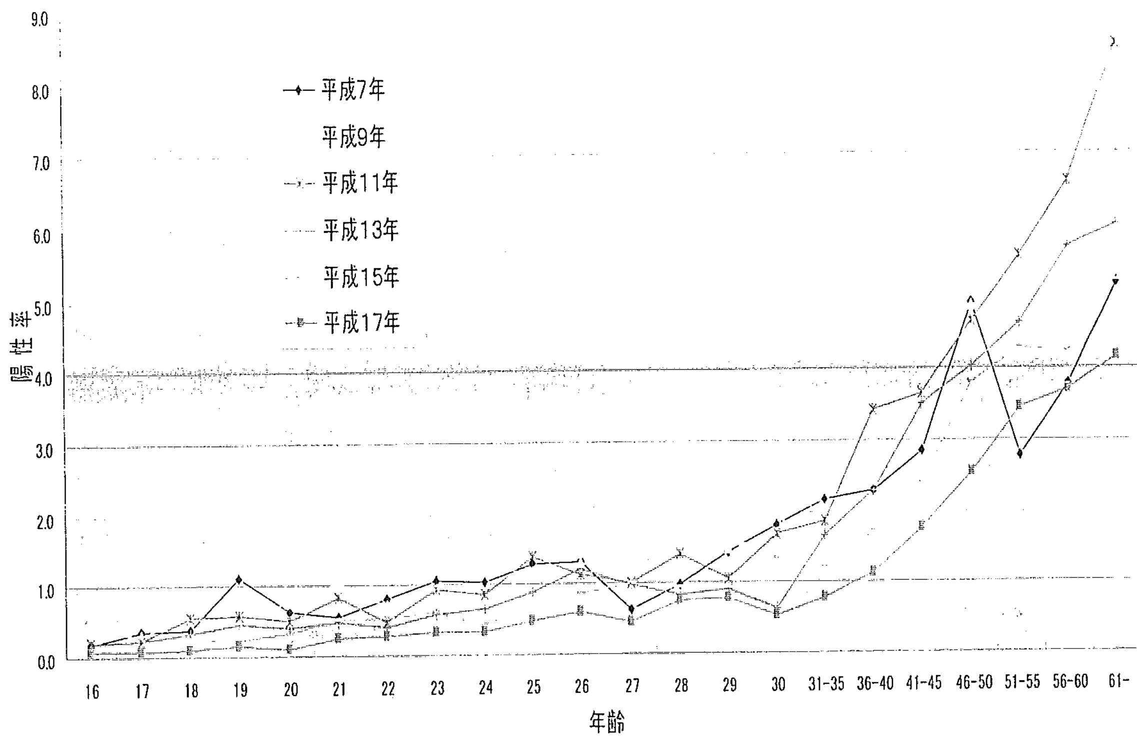



図４

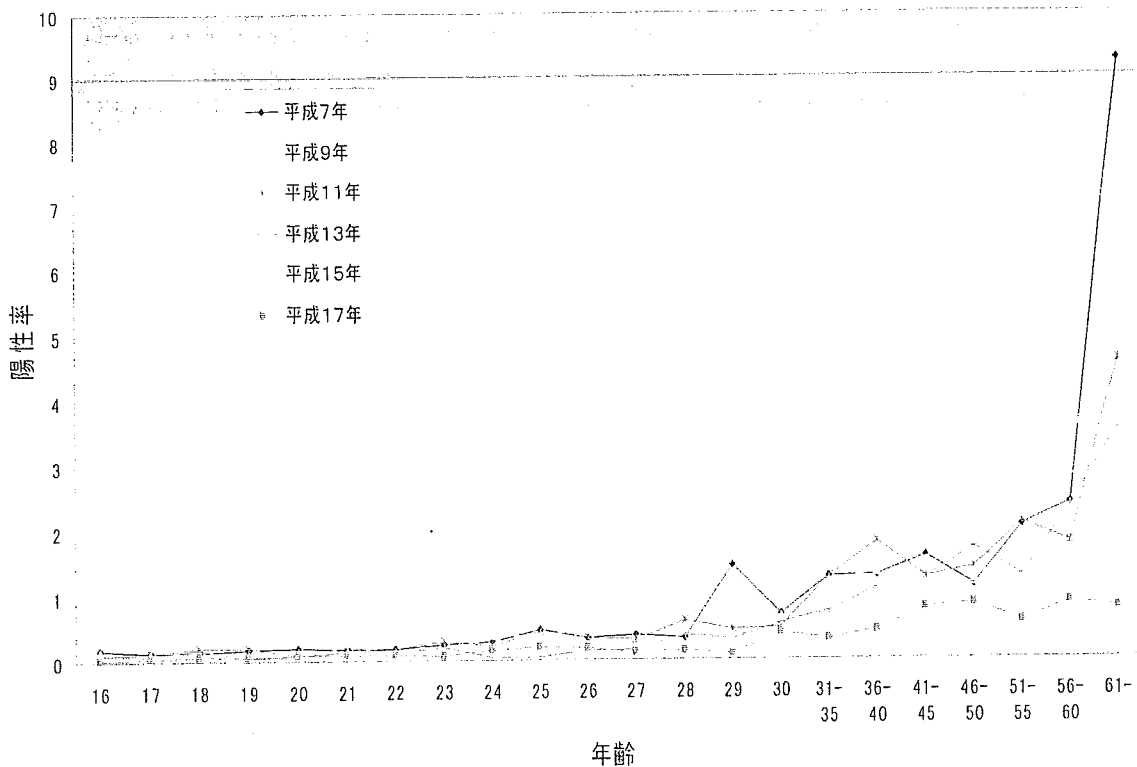


図5

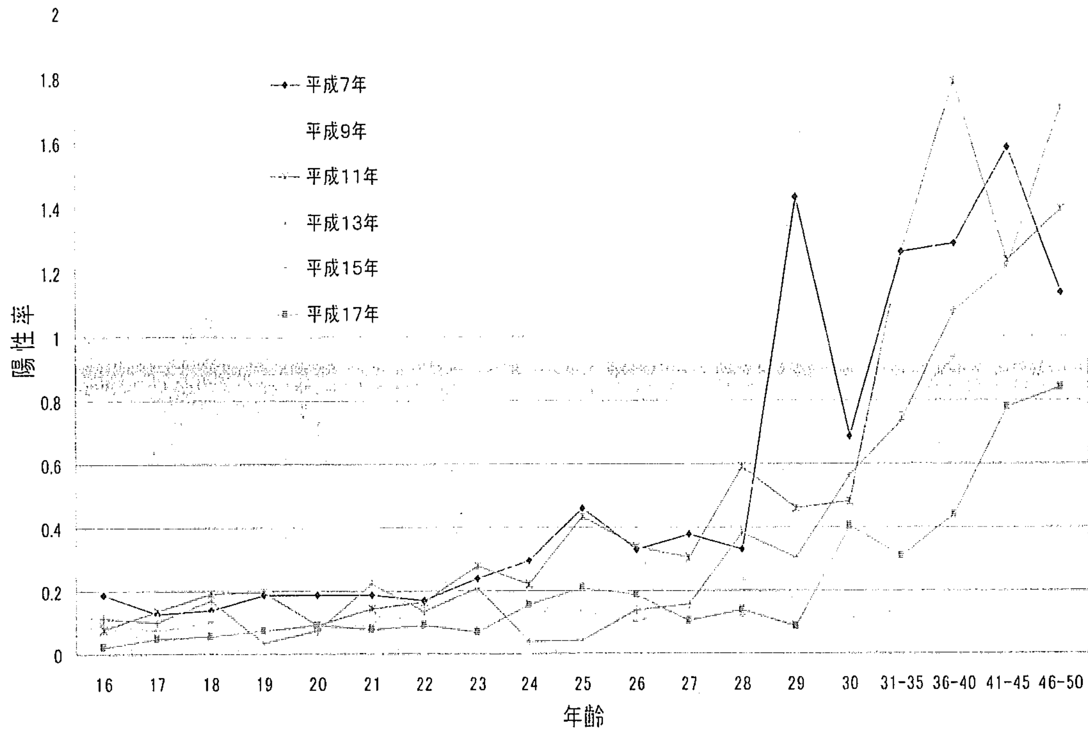



図6

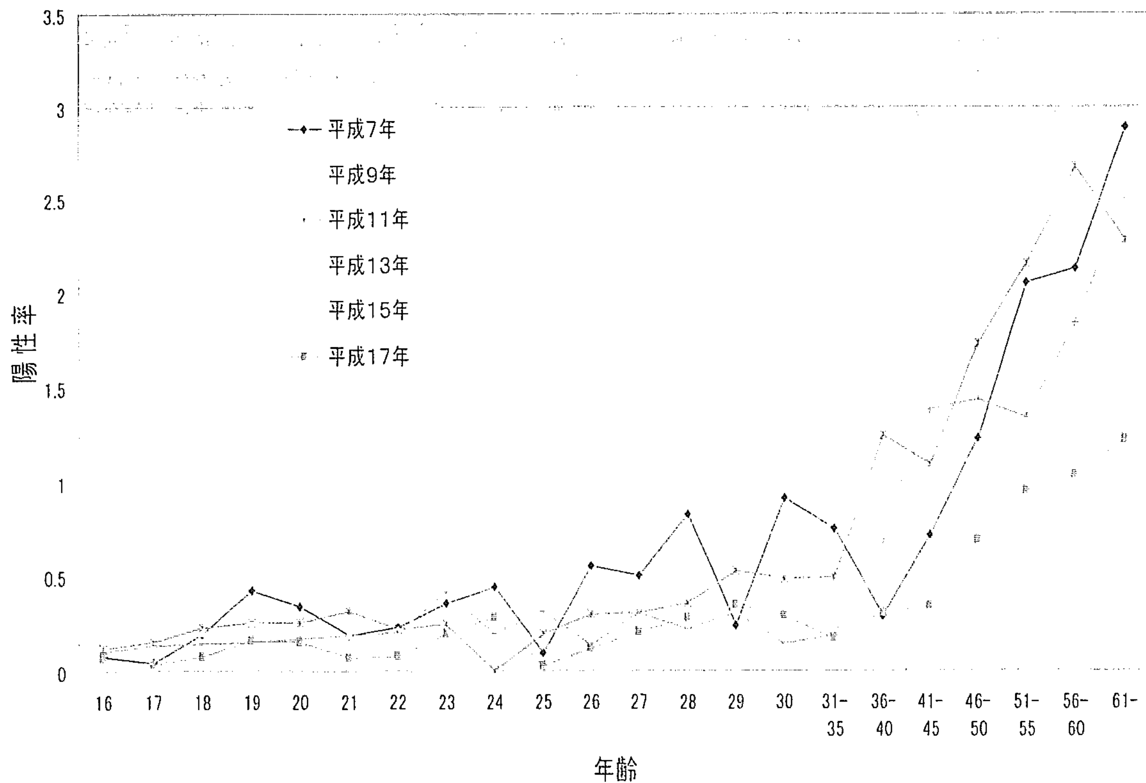


図7

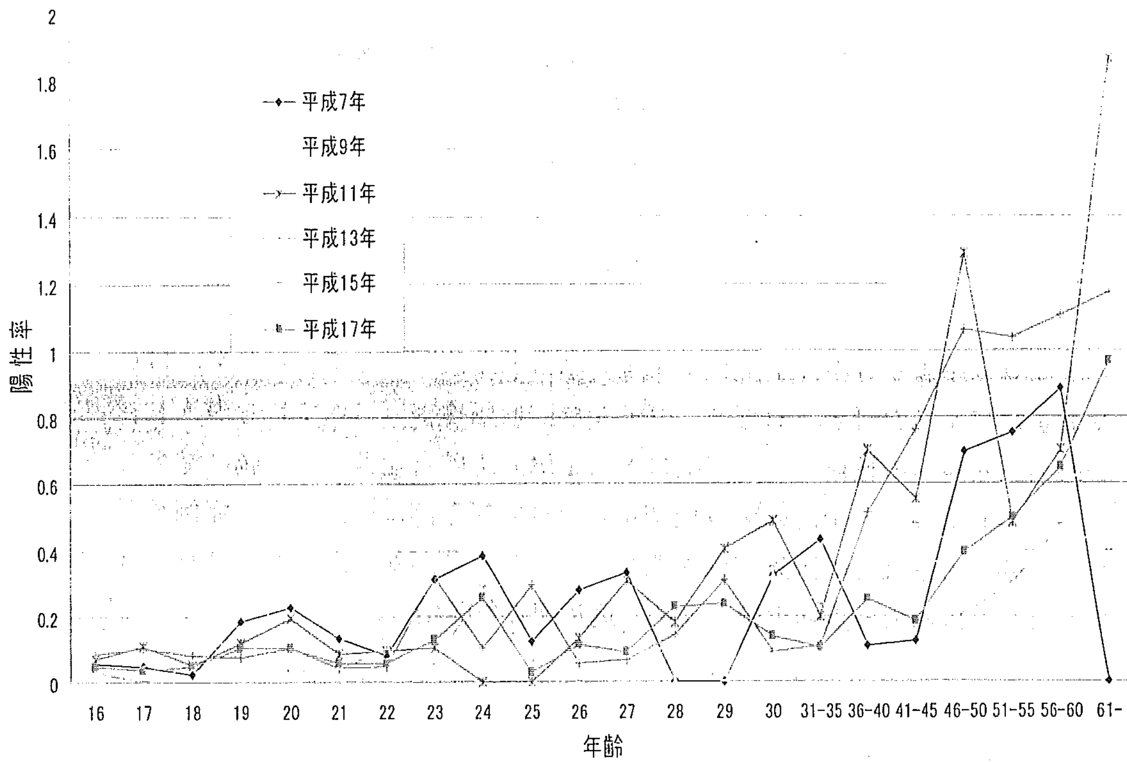
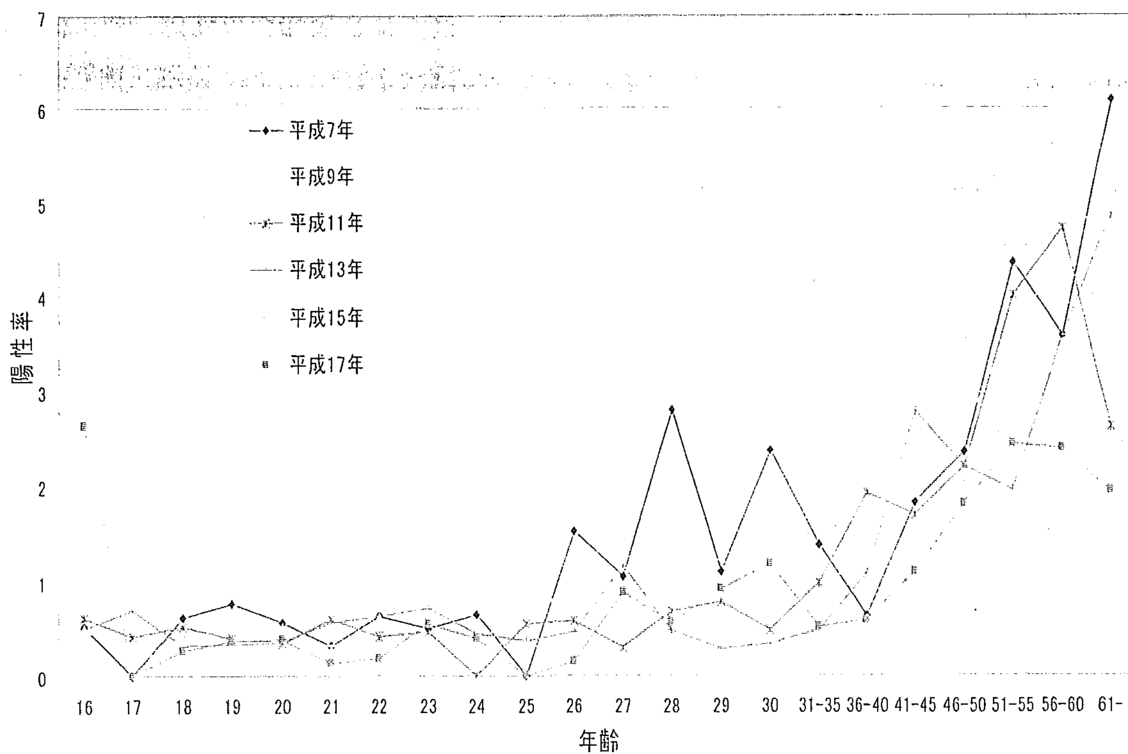



図8



厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

# ２００５年の輸血感染症報告

分担研究者：内田茂治（東京都西赤十字血液センター）

１９９９年より日本赤十字社は全国の献血者血液に対して、ＨＢＶ、ＨＣＶおよびＨＩＶを対象としてミニプール核酸増幅検査（ＮＡＴ）を導入し、これらウイルスの輸血による伝播はＮＡＴ導入以前と比較して大幅に減少した。しかしながら、全国の医療機関からの自発報告例は依然として症例数が多く、輸血と感染との因果関係を明らかにする必要がある。また、２００３年６月からは過去の陽転化例も含めた遡及調査を開始した。

共同研究者
高橋雅彦（東京都赤十字血液センター）

## A．研究目的

輸血によるウイルス伝播の実態を調査することを目的として、２００５年に全国の医療機関から報告のあった自発報告例、ならびに遡及調査を含めた献血者の献血後情報による症例の解析を行った。

## B．対象と方法

２００５年１月から２００５年１２月までに、全国の医療機関から日本赤十字社中央血液センター医薬情報部に輸血後感染症として自発報告例、２００４年中に遡及調査の終了した症例、および試験的に行っている輸血実施例の全数調査症例を対象とした。自発報告例の内訳はＨＢＶ感染の疑いが９８例、ＨＣＶ感染の疑いが６１例であった。これらの症例における感染と輸血との関連性を調査するため、輸血に使用された血液の保管検体の精査（血清学的検査ならびに個別ＮＡＴ）を行った。

## C．結果

ＨＢＶ対象症例とその解析結果

自発報告９８例と、遡及調査ならびに全数調査症例の解析を行い、計１０例のＨＢＶ－ＤＮＡ陽性例が確認された(図１)。内訳は自発報告で５例、遡及調査で４例、および遡及調査陽性の同一採血血液の追跡調査で１例であった。図２に自発報告陽性例（同一採血血液の追跡調査を含む)、図３に遡及調査陽性例の概略を示す。

ＨＣＶ対象症例とその解析結果

６１例の解析を行い１例のＨＣＶ－ＲＮＡ陽性例が確認された(図４)。この例はＮＡＴスクリーニング検査開始後はじめての輸血感染例である。図５、６にこの輸血感染例の概略を示す。

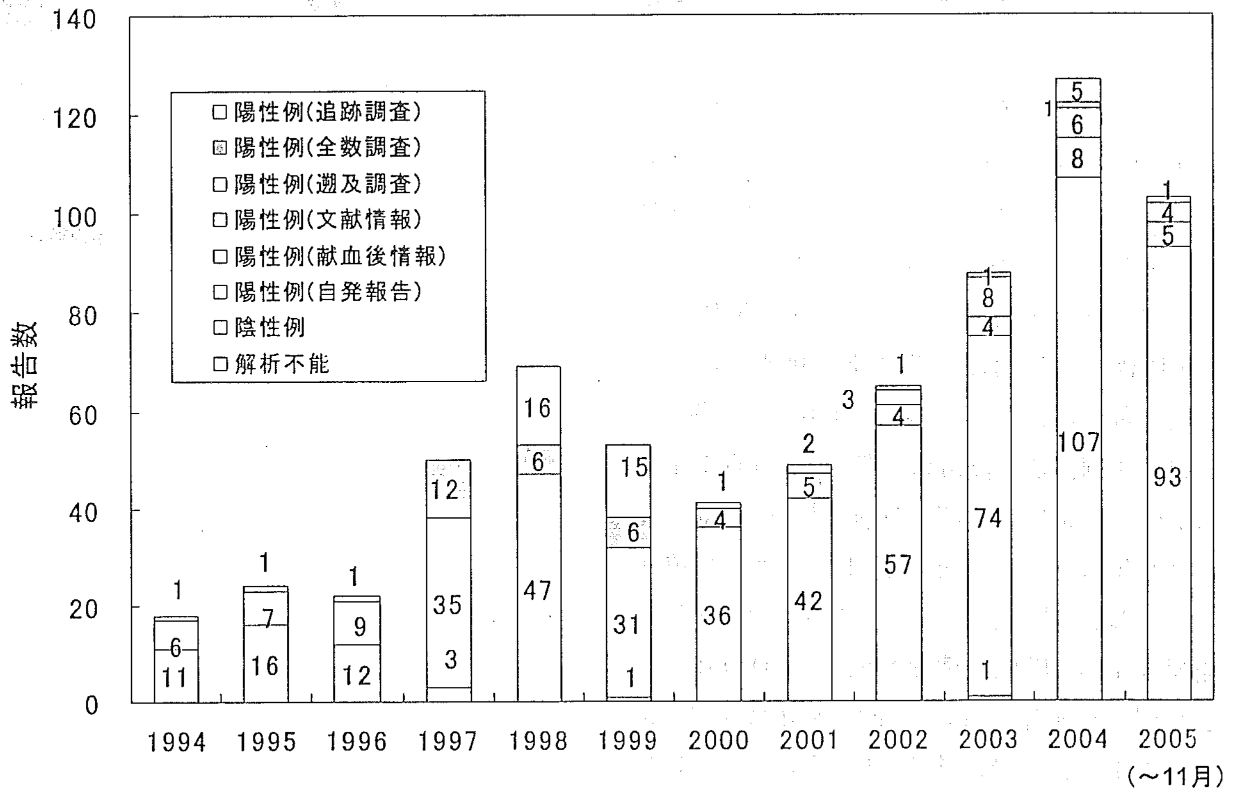
## D．考察

日本赤十字社中央血液センター医薬情報部

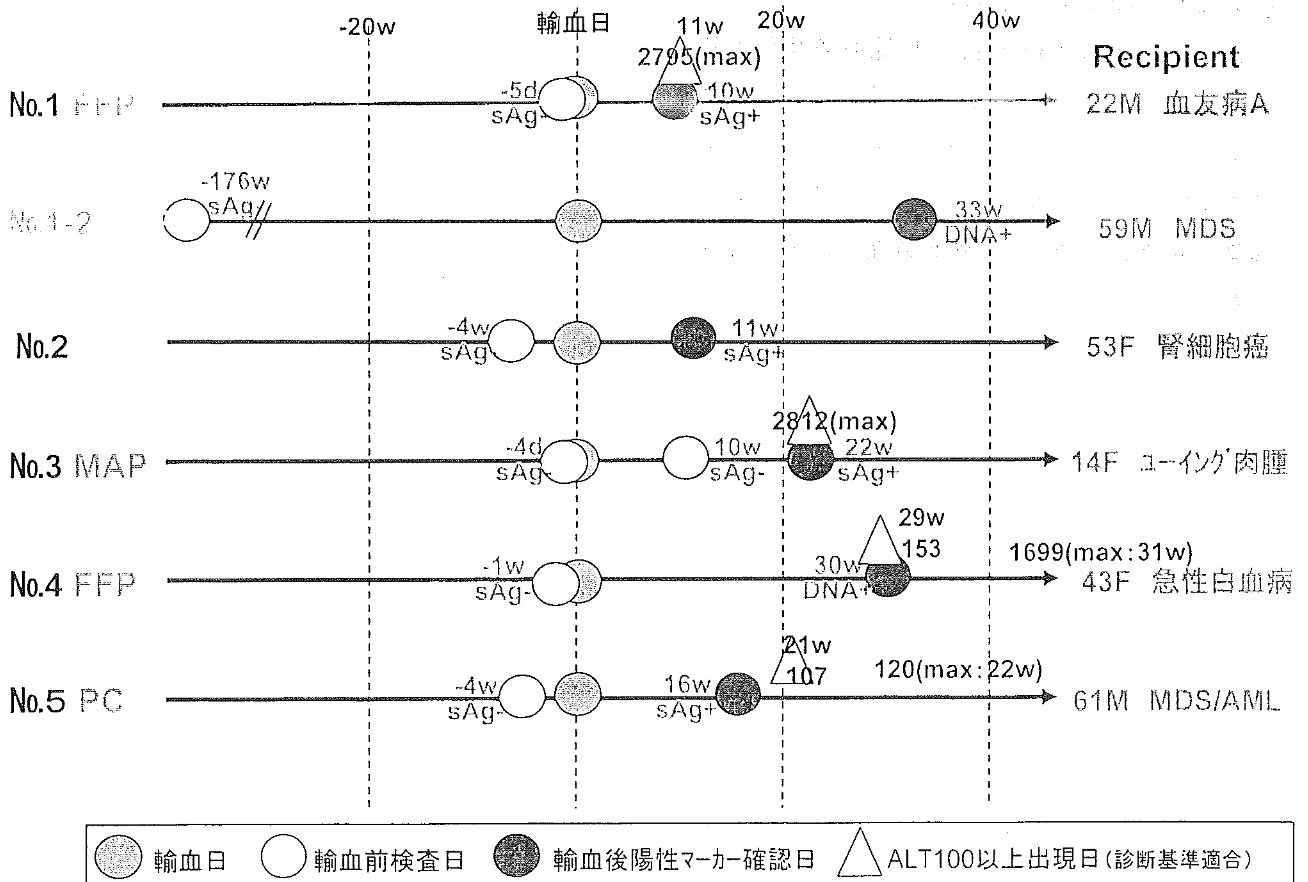
では、１９９４年から輸血によるウイルス感染疑い例の調査を行っているが、ＨＢＶ感染は１９９８年に２２例（自発報告６例、献血後情報１６）、１９９９年には２０例（自発報告５例、献血後情報１５例）が保管検体精査結果陽性で、輸血による感染の可能性が高い症例と考えられたが、２０００年には５例（自発報告４例、献血後情報１例）、２００１年には７例（自発報告５例、献血後情報２例）、２００２年は８例（自発報告４例、献血後情報３例、追跡調査１例）、２００３年は１２例（自発報告４例、献血後情報８例）、２００４年は２０例（自発報告８例、献血後情報６例、全数調査１例、追跡調査５例）であった。ＨＣＶ感染では１９９８年の７例、１９９９年の５例（いづれも献血後情報）の保管検体精査結果陽性の症例が認められたが、２０００年以降は２００４年まで１例も確認されていなかった。このように１９９９年のミニプールＮＡＴ導入後、ＨＣＶ、ＨＢＶの輸血による感染は大幅な減少が認められた。

２００５年は１例のＨＣＶ輸血感染例が確認されたが、チンパンジーの感染実験では１０コピーのＨＣＶで感染するといわれており、ＮＡＴ感度以下の低コピー数の血液でも輸血感染の可能性はあり、遡及調査の徹底が重要と考えられた。

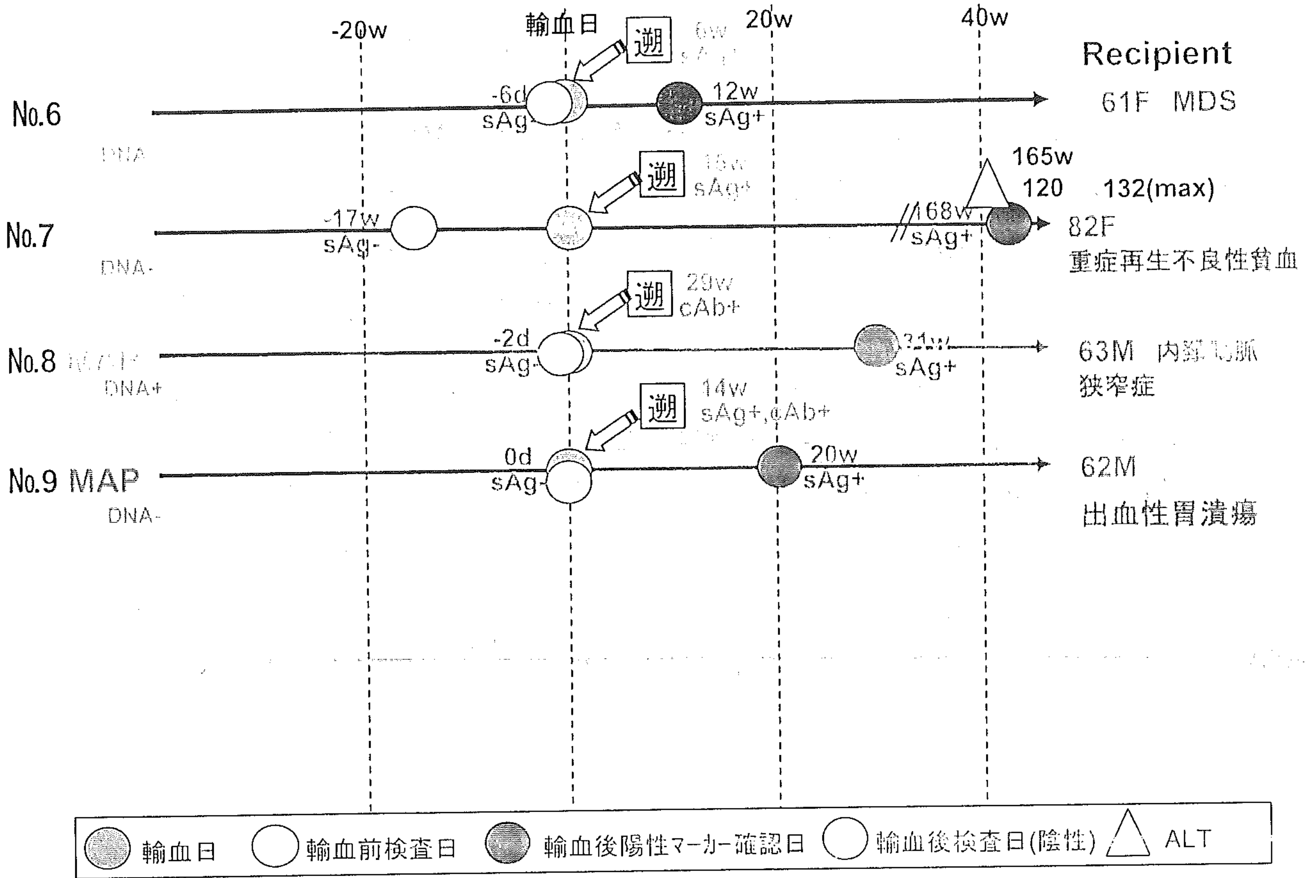
# 図1. HBV症例と解析結果



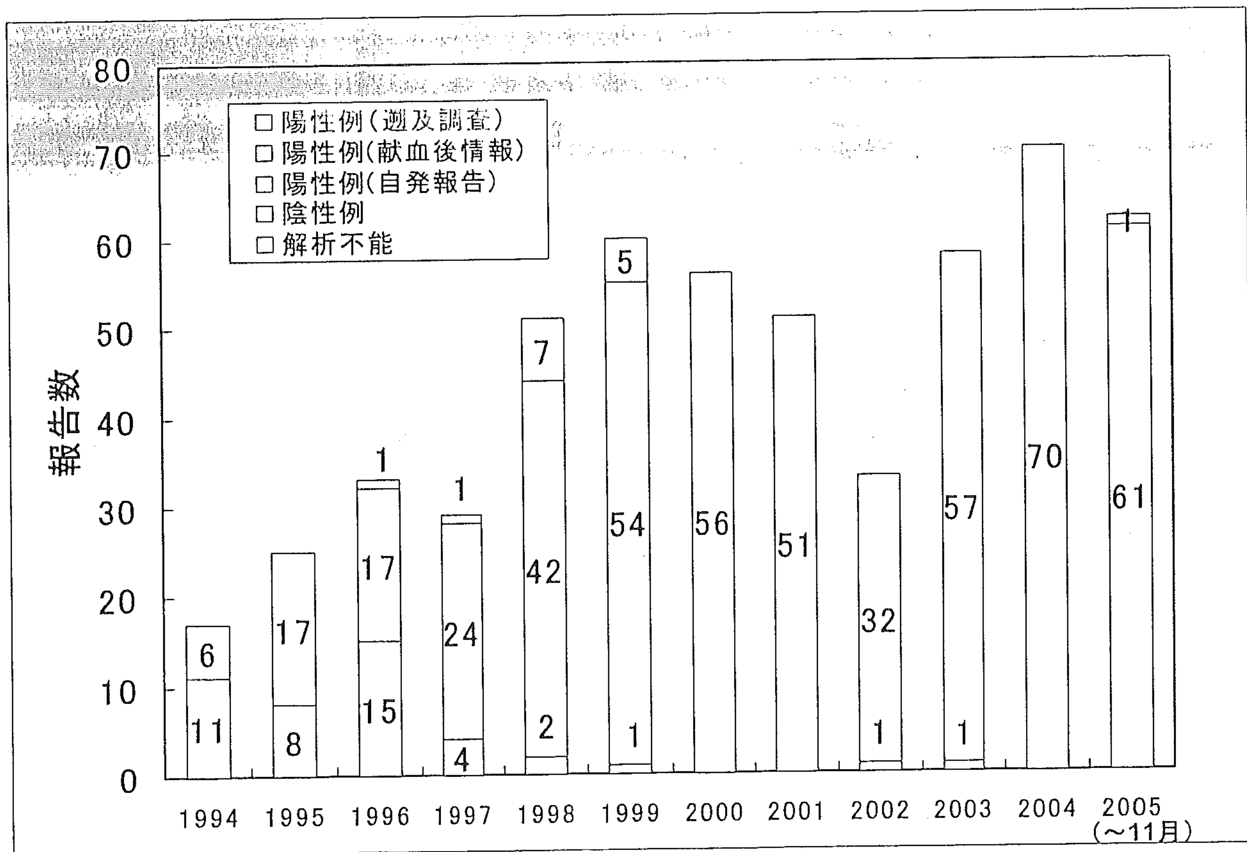

＊2003年までの献血後情報を2004年以降遡及調査、追跡調査等に分類した。



## HBV陽性症例の経過概要－遡及調査－




## 図4．HCV症例数と解析結果

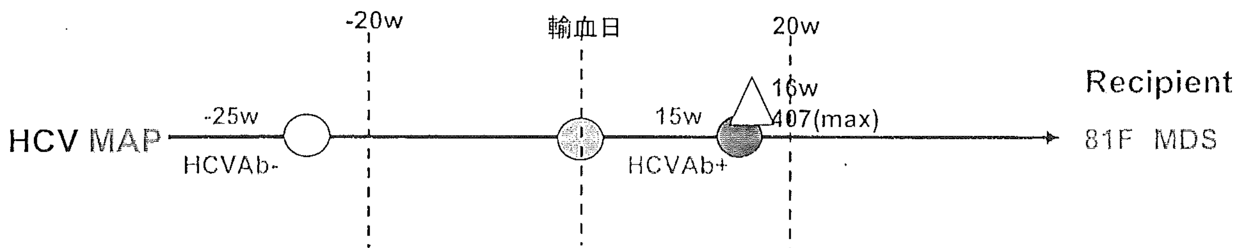


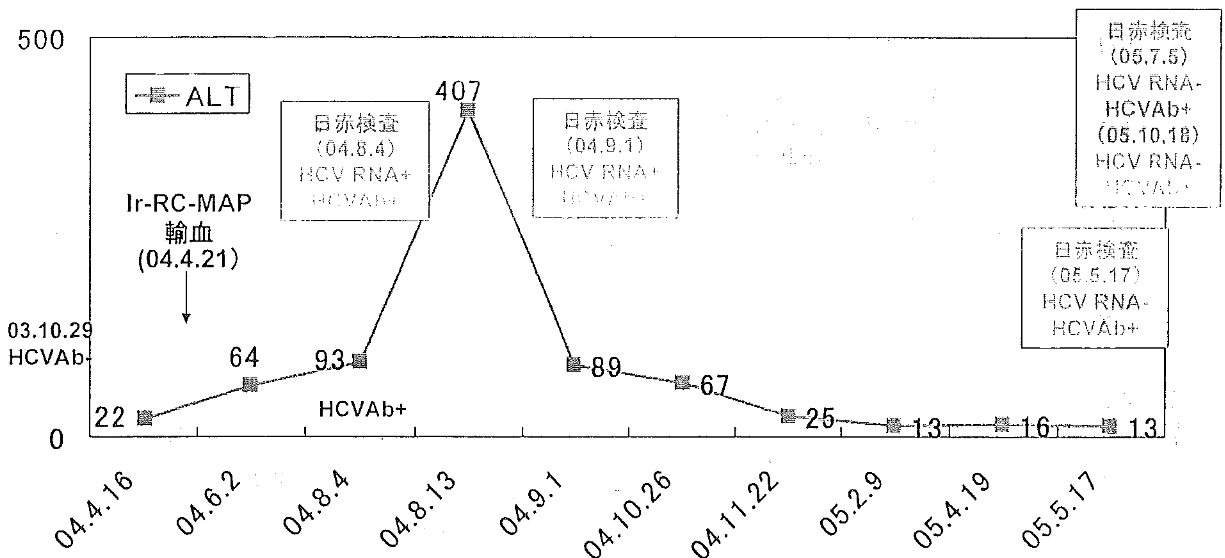
# 献血者の遡及調査により輸血後HCV感染が明らかとなった事例（2005年5月）

平成17年度第2回薬事・食品衛生審議会血液事業部会運営委員会報告（平成17年5月30日開催）

| | | |
|---|---|---|
| 発端：今回献血 | '05.02.26 | HCVAb（＋）、個別NAT（－） |
| **前回献血** | **'04.04.07** | HCVAb（－）、個別NAT（＋）【遡及調査】 |
| 前々回 | '03.12.10 | HCVAb（－）、個別NAT（－）【遡及調査】 |

50プールNAT導入後のすり抜け例としては、平成15年の1例に続き本事例は2例目。ただし、平成15年の事例は受血者が原疾患にて死亡のため、受血者情報が得られ感染が確認できたのは今回が初めての事例である。




献血者；44F

原因輸血製剤；Ir-RC-MAP2（2004/4/21 輸血）、採血日2004/04/07　ALT19

個別NAT(+)　HCV-RNA; genotype 1b（Ⅱ），$8.2 \times 10^2$ copies/mL

解析結果：Core領域前半部（nt.-2～363）の塩基配列について、すべて一致。

研究成果の刊行に関する一覧表

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# Human Papillomavirus 16 Minor Capsid Protein L2 Helps Capsomeres Assemble Independently of Intercapsomeric Disulfide Bonding

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**Abstract.** The human papillomavirus (HPV) capsomeres (pentamers of major capsid protein L1), which constitute along with L2 the virion capsid, can assemble themselves alone into the L1-capsid particles *in vivo* and *in vitro*, depending on intercapsomeric disulfide bonds. To study a possible role of L2 in capsid assembly, we examined the interaction between HPV16 L2 and capsomeres under the conditions that inhibit the formation of disulfide bonds *in vitro* and *in vivo*. The purified L2 bound to free capsomeres prepared by disassembling L1-capsids but not to the L1-capsids *in vitro*. And the L2 was found to help capsomeres assemble into smaller capsid-like particles independently of intercapsomeric disulfide bonding. Similar particles were obtained from the Sf9 cells co-infected with baculoviruses expressing L2 and an L1 mutant that lacks a C-terminal cysteine (C428S) and can form capsomeres but no capsids when expressed alone. These findings suggest that L2, which is known to bind both viral DNA and L1, may contribute to the formation of the virion by linking viral DNA and capsomeres and by helping capsomeres assemble before the virion capsid structure is completed by dintercapsomeric disulfide bonding.

**Key words:** capsid-like particles, capsomere, HPV, minor capsid protein L2

## Introduction

Human papillomavirus (HPV) is a non-enveloped virus having an 8-kb double-stranded circular genomic DNA encapsidated in the icosahedral capsid. Since cell cultures supporting efficient replication of HPV are not available, surrogate systems capable of expressing major (L1) and minor (L2) structure proteins have been developed to obtain the HPV capsid for structural analysis. Expression of L1 either alone or together with L2 from recombinant vaccinia viruses or recombinant baculoviruses results in production of self-assembled the L1-capsid [1-3] or L1/L2-capsid [2,4,5]. The L1-capsid is composed of 360 L1 molecules arranged as 72 pentameric capsomeres [6], and the L1/L2-capsid contains additional 12 L2 molecules [5,6]. These particles without viral DNA are morphologically indistinguishable, under an electron microscope, from HPV virions extracted from the lesions [1].

Intercapsomeric disulfide bonding appears to contribute greatly to the formation of the capsid from capsomeres (pentamers of L1 protein). The capsomeres, produced from L1-capsids under the reducing conditions that disrupt disulfide bonds, can re-assemble themselves into the capsid structure following the removal of the reducing agent by dialysis [4,7]. Mutational analyses further support the relevance of the intercapsomeric bonding; three cysteine residues at amino acid (aa) 175, 185, and 428 of HPV16 L1 (comprised of 505 aa) are important for the structural integrity of the L1-capsid [8]. An L1 mutant with the substitution of serine for cysteine at aa 428 (C428S) can form capsomeres but no capsids [8].

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Although the papillomavirus capsid can be assembled from L1 alone, the virion capsid always contains L2 as a minor component. In fact L2 appears to play an important role in encapsidating the viral genome into the virion during the viral morphogenesis [9–13]. But it is as yet to be investigated how L2 and L1 interact each other in the formation of particles.

In this study, to investigate a possible role of L2 in capsid assembly *in vitro* and *in vivo*, we examined the interaction between HPV16 L2 and capsomeres under the conditions that inhibit the formation of disulfide bonds, using the reducing agent or L1 substitution mutant C428S. From analyses of the L2/capsomere complexes by sucrose density gradient centrifugation and electron microscopy, we found that L2 helps wild-type capsomeres in reducing conditions and C428 capsomeres assemble into smaller capsid-like particles without disulfide bonding.

## Materials and Methods

### *Purification of L1-Capsids (wild-type), L1 Mutant Capsomeres (C428S), and L2*

Recombinant baculoviruses capable of expressing HPV16L1 and C428S, which has a substitution of serine for cysteine at aa 428 of HPV16 L1, were described previously [8]. A DNA fragment encoding HPV16 L2 codon mutant was generated as described [14] and subcloned into pFastBac-1 plasmid (Bac System, Invitrogen Co., Ltd., Carlsbad, USA). The resultant plasmid was used to produce a recombinant baculovirus capable of expressing L2. Sf9 cells were grown in SFM medium (Invitrogen Co., Ltd.) supplemented with 3% fetal calf serum at 27°C. The cells were inoculated with the recombinant baculoviruses at multiplicity of infection of 10 and cultured for 3 days. The cells were harvested and incubated in phosphate buffered saline (PBS) containing 0.5% NP-40 for 15 min at room temperature, and then the nuclei were precipitated by a centrifugation at 10,000*g* for 15 min at 4°C.

For purification of L1-capsids or capsomeres, the nuclei were resuspended in 10 ml of PBS containing protease inhibitor cocktail (Roche Diagnostics Co., Ltd., Mannheim, Germany) and lysed by a brief sonication. Then the lysate was mixed with CsCl (final density:1.27 g/ml) and centrifuged at 100,000*g* for 20 h at 20°C. Fractions containing L1 were collected and dialyzed against 10 mM phosphate buffer (pH 7.5) containing 0.5 M NaCl. The solution was loaded on the top of 5 and 60% discontinuous sucrose gradient in 10 mM phosphate buffer (pH 7.5) containing 0.5 M NaCl and centrifuged at 120,000*g* for 2.5 h at 4°C. The L1-rich fractions below the interface between 5 and 60% sucrose were collected, dialyzed against buffer A (50 mM Tris–HCl, pH 7.5, 0.1% CHAPS, and 10% glycerol) containing 150 mM NaCl. The solution was applied to a heparin-sulfate column chromatography. L1-capsids or capsomeres were eluted by buffer A containing 2 M NaCl. Purified materials were stored at −80°C.

For purification of L2, the nuclei were resuspended in 10 ml PBS containing protease inhibitor cocktail (Roche Diagnostics Co., Ltd.) and lysed by a brief sonication. The lysate was centrifuged at 100,000*g* for 30 min at 4°C. The pellet was suspended in 10 ml 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 1% Triton-X 100, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, and 0.5 mM EGTA. Then the sample was centrifuged again at 100,000*g* for 1 h at 4°C. The pellet was suspended in 50 mM Tris–HCl (pH 7.5) containing 8 M urea and 5 mM DTT. After a low speed centrifugation to remove debris, the supernatant was collected and adjusted to L2 concentration of 1 μg/μl. The purified L2 was stored at 4°C.

### *SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting*

Samples were suspended in the standard SDS sample buffer (153 mM Tris–HCl, pH 7.5, 4.9% SDS, 6.13% 2-mercaptoethanol (2ME), 24.5% glycerol, and 0.0025% bromophenol blue), heated for 5 min at 98°C, and electrophoresed in SDS-polyacrylamide gel (10%). Proteins separated in the gel were stained by coomassie brilliant blue (CBB) or transferred to a polyvinylidene difluoride (PVDF) membrane, Hybond-P (Amersham Biosciences Inc., Uppsala, Sweden) After blocking with 5% skim milk, L1 and L2 on the membrane were probed by anti-HPV 16 L1 mouse monoclonal antibody (#554171, Becton Dickinson Co.,

Ltd., San Diego, USA) and mouse anti-HPV16L2 antibody, which was newly generated by immunizing mice with the N-terminal region of HPV16 L2, respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (SC-2031, Santa Cruz Biotechnology Inc., Santa Cruz, USA) was allowed to bind to mouse IgG on the membrane. Peroxidase activity was detected with BCL Western Blotting Detection System (Amersham Biosciences Inc.) and an image analyzer (Storm, Amersham Bioscience Inc.).

*Complex Formation of L2 with L1-Capsids or Capsomeres In Vitro*

The purified L1-capsids or capsomeres were dialyzed against 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% CHAPS (Buffer B). Then, 12.5 μg of the L1-capsids were incubated in 50 μl Buffer B with or without 5% 2ME for 30 min at 37°C. The L1-sample was reacted with 2.5 μg of the purified L2 in 100 μl buffer B containing $CaCl_2$ (final concentration: 0.5 mM) with or without 5% 2ME by incubation for 1 h at room temperature.

*Co-Immunoprecipitation Assay*

Ten μl of the mixtures were diluted with 90 μl buffer B containing 0.5 mM $CaCl_2$ and mixed with rabbit anti-L2-antibody that had bound with protein A sepharose. The anti-L2 antiserum was produced by immunizing rabbits with a synthetic peptide with amino acid sequence of HPV16 L2 aa 107–122. After incubation for 1 h at 4°C the resins were collected and washed. The proteins bound to the resins were analyzed by SDS-PAGE and immunoblotting.

*Sucrose Density Gradient Centrifugation*

Samples (the complex of L2 and capsormeres, the L1-capsid, capsomeres, and purified L2 protein) were layered each on the top of a 5 to 45% liner sucrose-density gradient in 50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl and 5 mM DTT (for reducing condition only) and centrifuged at 120,000g for 2.5 h at °C. Aliquots (400 μl each) were collected from the tube bottom. L1 and L2 in each fraction were analyzed by SDS-PAGE and immunoblotting.

*Electron Microscopy*

Purified particle or capsomere fractions were allowed to settle on carbon-coated copper grids and stained with 4% uranylacetate. The grids were examined in a HITACHI model H-7600 transmission electron microscope (Hitachi Co., Ltd., Tokyo, Japan) and photographed at an instrumental magnification of 200,000×.

## Results and Discussion

*Formation of Complex of HPV16 L2 and Capsomeres In Vitro*

HPV16 L2 was found to bind to capsomeres but not to L1-capsids *in vitro*. Purified L2 protein and L1-capsids (Fig. 1a) were mixed at a protein molar ratio of 1:5 and incubated in a buffer with or without reducing agent 2ME (5%) for 1 h at room temperature. This concentration of 2ME is known to disassemble the L1-capsids into capsomeres [4,8]. After the incubation, the sample was diluted at 1 to 10 to lower the concentration of 2ME and mixed with an anti-HPV16 L2 rabbit serum. The dilution did not induce re-assembly of capsomeres into the L1-capsid (data not presented). Co-precipitation of L1 and L2 proteins was examined by immunoprecipitation with the rabbit anti-L2 antibody followed by electrophoresis and immunoblotting with an anti-L1 mouse monoclonal antibody and the anti-L2 antibody (Fig. 1b). L1 was not precipitated together with L2 from the mixture of L2 and L1-capsids but was precipitated together with L2 from the mixture of L2 and capsomeres. The results indicate that L2 do not bind to the L1 in the L1-capsid but can complex with free capsomeres *in vitro*, being consisted with the previous data that L2 of bovine papillomavirus type 1 (BPV1) binds to capsomeres but not to L1-capsids of BPV1 [13].

The sedimentation pattern of the complex of L2 and capsomeres was compared with those of the L1-capsid, capsomeres, and purified L2 protein (Fig. 1c). Samples were loaded each on a sucrose density gradient (5–45%), centrifuged, and fractionated. Proteins L1 and L2 in each fraction were

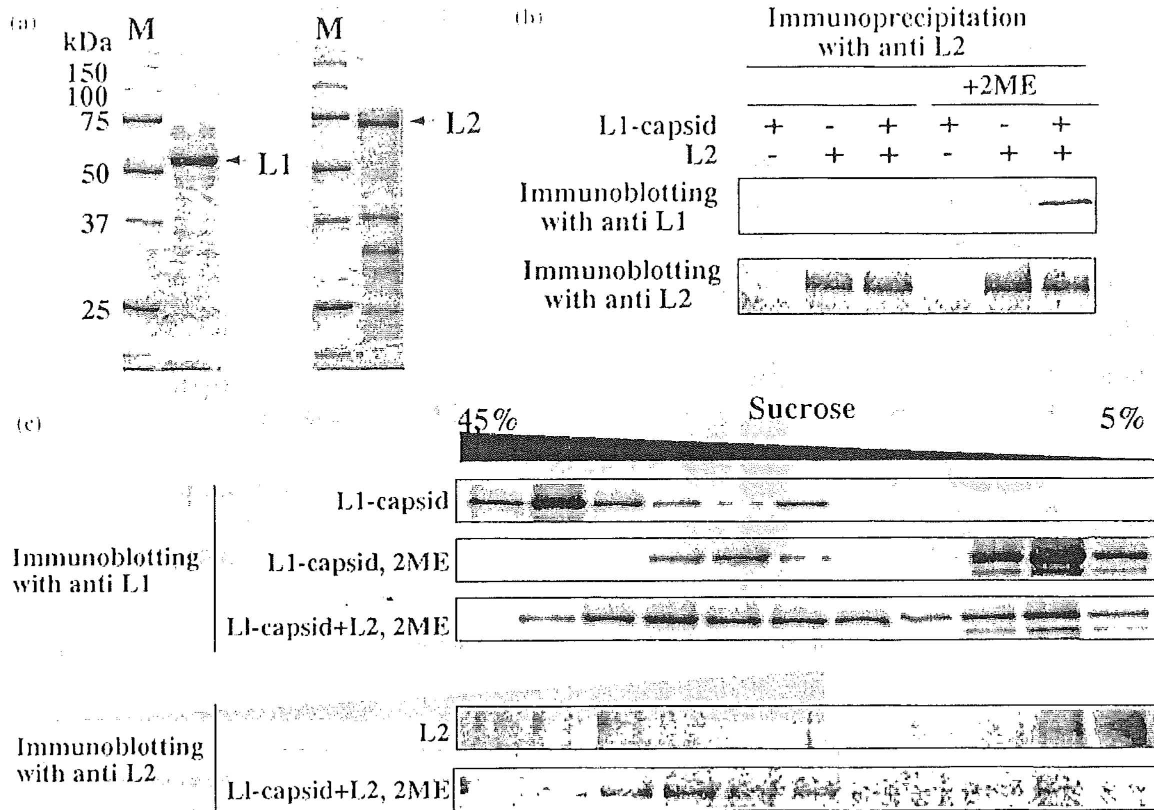


*Fig. 1.* Analysis of the complex of L2 and capsomeres obtained by disassembly of L1-capsids by incubation with 2ME *in vitro*. (a): Purified L2 and L1-capsids, which were used in the assays shown (b) and (c), were electrophoresed in SDS-polyacrylamide gel and stained with coomassie brilliant blue. Lane M is for protein size markers. (b): Co-precipitation assay of L1 and L2 immunoprecipitated by anti-L2-antibody. L2 was incubated with L1-capsids in a buffer with or without 2-ME, and then L2 was immunoprecipitated with anti-L2-antibody. The precipitate was analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using anti-L1- or anti-L2-antibody. (c): Sedimentation analyses of L1-capsids, capsomeres (obtained by disassembly of the L1-capsid by incubation with 2ME), and the complex of L2 and capsomeres. Samples were loaded each on a 5–45% linear sucrose-density gradient and centrifuged. After fractionation, each aliquot was electrophoresed in SDS-polyacrylamide gel (10%) and was subjected to immunoblotting for detection of L1 and L2.

detected by immunoblotting using anti-L1 and anti-L2 antibodies, respectively. The L1-capsids and capsomeres peaked near the bottom and near the top of the centrifuge tube, respectively, as reported previously [8]. L2 stayed near the top of the centrifuge tube. The complex of L2 and capsomeres was analyzed in a sucrose density gradient containing DTT (5 mM) to avoid disulfide bond-mediated assembly of capsomeres into the L1-capsid. L1 and L2 were detected in the same fractions with 30–35% sucrose, indicating that the L2/capsomere complex was sedimented more slowly than the L1-capsid. Extensive pretreatment of the L2 preparation and the capsomeres with NDase I did not affect the sedimentation pattern, indicating that the complex formation was not mediated by DNA in the materials. The data suggest that the complex is smaller than the L1-capsid. The molar ratio of L2 and L1 proteins in the complex was estimated as approximately 1 to 10–30.

### *Formation of Complex of HPV16 L2 and C428S Capsomeres In Vivo*

HPV16 L2 was found to bind to the capsomeres of L1 mutant C428S (substitution of serine for cysteine at aa 428) *in vivo*, which forms capsomeres but no capsid structure when expressed in insect Sf9 cells [8]. The extract from the cells expressing

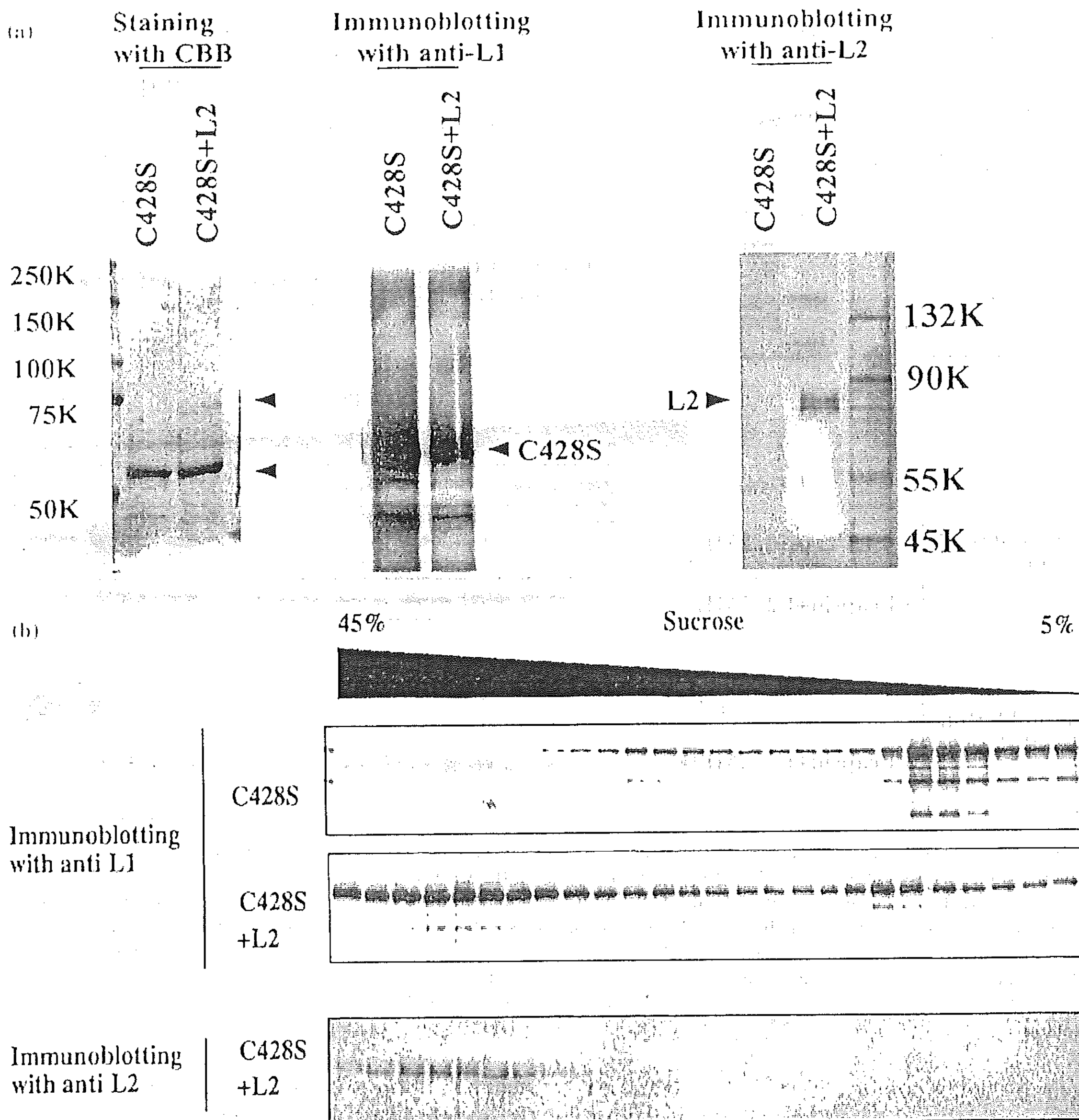


*Fig. 2.* Analysis of the complex of L2 and L1 mutant C428S capsomeres. Co-purification of L2 and L1 of C428S capsomeres. (a): Co-purification of L2 and L1 of C428S capsomeres. Sf9 cells were infected with either recombinant baculovirus capable of expressing C428S alone or together with another recombinant baculovirus capable of expressing L2. Purified samples from cell lysates were electrophoresed in SDS-polyacrylamide gel (10%) and stained with coomassie brilliant blue (left panel), and were subjected to immunoblotting for detection of L1 (central panel) and L2 (right panel). (b): Sedimentation analysis of C428S sample and L2/C428S sample. Purified samples were loaded each on a 5–45% linear sucrose-density gradient and centrifuged. After fractionation, each aliquot was electrophoresed in SDS-polyacrylamide gel (10%) and was subjected to immunoblotting for detection of C428S.

C428S alone or both together (co-infected with two recombinant baculoviruses capable of expressing L2 and C428S at a high multiplicity of infection of ten for each virus) was subjected to the purification procedure for L1-capsids and capsomeres, and resulting purified samples were electrophoresed in SDS-polyacrylamide gel (Fig. 2a). Coomassie brilliant blue (CBB) staining showed that the purified material contained proteins of 57 kDa and 70 kDa. Immunostaining with

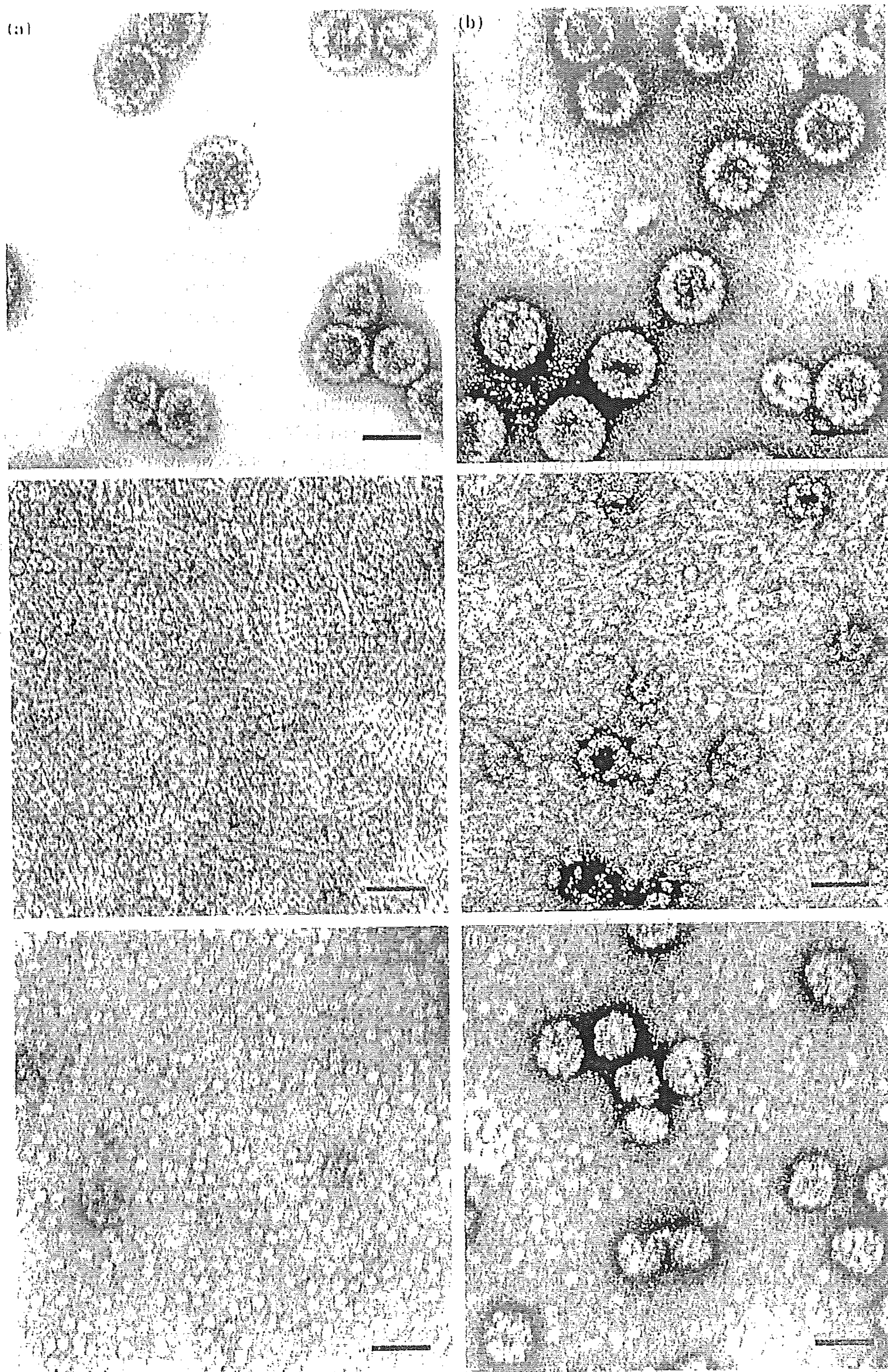


*Fig. 3.* Electron micrographs of purified samples. (a): L1-capsids. (b): L1/L2-capsids. (c): Capsomeres obtained by disassembly of L1-capsids by incubation with 2-ME. (d): Complex of L2 and capsomeres. (e): C428S capsomeres. (f): Complex of L2 and C428S capsomeres. Scale bar represents 50 nm.

anti-L1 and anti-L2 antibodies confirmed that the major 57-kDa protein and the minor 70 kDa protein were L1 and L2, respectively. The presence of L2 in the purified sample provides part of evidence that L2 can complex with C428S protein or more likely capsomeres as shown with wild-type capsomeres *in vitro* (Fig. 1). An approximate protein molar ratio of L2 to C428S was estimated as 1 to 10.

The two purified samples were analyzed by sedimentation through a sucrose density gradient (5–45%) (Fig. 2b). The sample containing only C428S showed the pattern characteristic of capsomeres, as expected from the previous report [8]. The sample containing both L2 and C428S showed a pattern resembling that of the wild-type L2/capsomere complex formed *in vitro*. The putative L2/C428S capsomere complex was sedimented more slowly than the L1-capsid (Fig. 2b), suggesting the complex is smaller.

### *Electron Microscopy of the HPV16 L2/Capsomere and L2/C428S Capsomere Complexes*

The L1-capsid and the L1/L2-capsid are particles with a diameter of 55 nm (Fig. 3a, b). The L1-capsid was disassembled to capsomeres after incubation in a buffer containing 2ME (5%) (Fig. 3c), as previously described [4,8]. The L2/capsomere complex formed *in vitro* under the reducing conditions (Fig. 1) was smaller capsid-like particles (Fig. 3d), of which sizes were about 35–40 nm in diameter. Whereas the purified sample containing only C428S appeared to be comprised of capsomeres (Fig. 3e), the putative L2/C428S capsomere complex (Fig. 2) was capsid-like particles somewhat heterogeneous in size, with a diameter of 30–50 nm (Fig. 3f). Unlike the others, the C428S particles appeared not to have an empty structure filled with uranylacetate under an electron microscope.

In summary, the *in vitro* study showed that under reducing conditions, L2 can bind to capsomeres but not to L1-capsids, and that L2 helps capsomeres assemble into smaller capsid-like particles independently of intercapsomeric disulfide bonding. These findings were further supported by the study using an L1 mutant incapable of regular intercapsomeric disulfide bonding.

The observed interaction between L1 and L2 proteins would reflect the intrinsic properties of the proteins and was revealed only under the conditions used in this study. Probably the intercapsomeric disulfide bonds are so dominant in capsid formation that possible interaction of the two proteins would be difficult to detect when permissive for the formation of the bonds. And it may be that the lack of disulfide bonding has made the particles smaller and somewhat heterogeneous in size and shape. It remains to be investigated how these properties contribute to the morphogenesis of virions.

Although the roles of L2 in the morphogenesis of the papillomavirus virion is not fully elucidated at present, L2 is believed to mediate the DNA incorporation into the virion because it binds to both viral DNA [15] and L1 [13,16,17]. The lack of L2 binding to L1-capsids shown in this study suggests that the binding of L2 (possibly L2-viral DNA complex) to capsomeres or L1 occurs before the formation of the capsid structure. It may be that in productive infection, L2 links viral DNA and capsomeres and helps capsomeres assemble before the vision capsid structure is completed by intercapsomeric disulfide bonding.

**Acknowledgments**

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# IgG antibodies to HPV16, 52, 58 and 6 L1-capsids and spontaneous regression of cervical intraepithelial neoplasia

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**Abstract**

To identify the predictive markers for spontaneous regression of cervical intraepithelial neoplasia (CIN), we examined whether IgG antibody responses to common human papillomavirus (HPV) L1-capsids correlate with CIN regression. In a cohort study, a total of 116 Japanese women with CIN grade I/II were tested for cervical HPV DNA and serum IgG antibodies to HPV16/52/58/6 L1-capsids. Our data suggest that baseline IgG reactivities to HPV L1-capsids do not serve as a predictive marker of CIN regression, in contrast to histological CIN grades and HPV DNA status.
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*Keywords:* HPV; L1-capsids; VLP; CIN; Regression

## 1. Introduction

Cervical cancer remains the second most common cancer in women worldwide, with nearly 500,000 women developing the disease every year [1]. The marked decrease in mortality rates in Western countries is attributed to the development of cytology screening programs that detect women with precursor lesions (cervical intraepithelial neoplasia, CIN). Although many CIN lesions are known to regress without treatment [2], women with CIN are usually treated because we cannot predict which CIN lesions will regress spontaneously. Thus, the establishment of predictive markers for spontaneous regression would

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