Clearance of dengue virus in the plasma-derived therapeutic proteins

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BACKGROUND: Viral safety is of paramount importance for human plasma–derived therapeutic proteins. Recent reports of blood-associated transmission and continuous regional outbreaks of dengue fever have prompted a validation of clearance of dengue virus in the manufacture processes of the plasma-derived products.

STUDY DESIGN AND METHODS: A high titer of cultured dengue virus serotype 2 was spiked into process samples before individual steps of albumin and immunoglobulin manufacture processes, including cold ethanol precipitation, cation-exchange chromatography, pasteurization, solvent/detergent treatment, and virus filtration. Clearance of dengue virus was quantified with $TCID_{50}$ assays in the culture of Vero E6 cells and, when appropriate, real-time polymerase chain reaction (RT-PCR) assays.

RESULTS: The individual process steps were all effective in the inactivation and/or removal of dengue virus, and the data obtained clearly demonstrate that the risk of dengue virus transmission was reduced cumulatively by at least 10.12 and at least 14.24 log in the albumin and immunoglobulin manufacture processes, respectively.

CONCLUSION: The dedicated viral inactivation and/or removal approaches currently implemented in the manufacture of plasma-derived products provide a good safety margin with regard to the transmission of dengue virus.

Dengue virus infects 50 to 100 million people worldwide a year; of those infected, several hundred thousand develop the more severe and life-threatening diseases, dengue hemorrhagic fever and dengue shock syndrome. Dengue virus belongs to the family Flaviviridae, which in general is known to survive over long periods in fluids with high protein contents, for example, blood. Therefore, dengue viruses may be transmitted via transfusion of blood or blood components. Albeit rare, it has indeed been documented that blood-associated transmission of dengue virus occurs via routes including needle-stick injuries,[1] marrow transplantation,[2] intrapartum and vertical transmission,[2] and mucocutaneous transmission.[3] This can be a serious public health problem without proper control measures.

Dengue virus is a lipid-enveloped RNA virus, with a diameter of approximately 50 nm.[4] Reportedly, dengue virus has been effectively inactivated by photosensitizers[5,6] and is sensitive to high temperatures and acidic pH.[7] This study aims to demonstrate for the first time that the

ABBREVIATION: BVDV = bovine viral diarrhea virus.

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risk of dengue virus transmission in plasma derivatives is eliminated by specific virus removal and inactivation procedures. Log reduction of dengue virus is investigated at individual steps of the manufacture processes of plasma-derived albumin and immunoglobulins, which include cold ethanol precipitation, cation-exchange chromatography, pasteurization, solvent/detergent (S/D) treatment, and virus filtration. The evaluation of the manufacture processes provides a measure of confidence for eliminating dengue virus.

MATERIALS AND METHODS

Raw materials

Normal human plasma was obtained from the plasma fractionator Shenzhen Weiwu Guangming Biological Products Co. (Shenzhen, China). All chemicals used in this study were of either pharmaceutical grade or analytical grade. Virus filters (Planova 35N, 10 cm^2) were a gift from Asahi Kasei (Tokyo, Japan).

Virus culture and quantification

Dengue virus serotype 2 (S047/00 from Environmental Health Institute, Singapore) was propagated in C6/36 cells (CDC Guangdong, China) in minimal essential medium with 1 percent fetal bovine serum (Gibco, Grand Island, NY). Dengue virus was quantified with $TCID_{50}$ assays in the culture of Vero E6 cells (ATCC, Manassas, VA). Vero E6 cells (2.5×10^5 cells/mL) were seeded in 96-well plates in a volume of 100 µL per well. After 1 day of incubation, 50 µL of medium was added to each well. Each dilution of sample was added at 50 µL per well, and further incubation was carried out at 36 ± 2°C with 5 percent CO_2. Plates were assessed for $TCID_{50}$ endpoint as cytopathic effects developed on the fifth day. The $TCID_{50}$ endpoint was calculated according to the Spearman-Kärber method, and the Poisson distribution was used when no virus was detected in samples. Quantitative real-time polymerase chain reaction (RT-PCR) was used to determine virus titer in the chromatography and cold ethanol precipitation steps. RNA of dengue virus was extracted in duplicate from samples with a viral RNA mini kit (QIAamp, Qiagen, Hilden, Germany) according to the procedure provided by the manufacturer. Dengue virus cDNA was reverse transcribed with random hexamers with reverse transcriptase (Supercript III, Invitrogen, Carlsbad, CA). Quantitative RT-PCR utilizing TaqMan technology (Applied Biosystems, Foster City, CA) was performed on samples and proper controls with specific primers (GTCAACATAGAAGCAGAACCTCCA and CTCTATGATGATGTAGCTGTCTCCG) and SYBR Green fluorescent probes with conditions optimized to detect 4.67 copies of viral RNA for dengue virus. Duplicate PCR procedures were performed for each sample with a sequence detection system (ABI 7900 HT, Applied Biosystems), and the cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute, as well as a dissociation stage of 95°C for 15 minutes, 60°C for 15 minutes, and 95°C for 15 minutes.

Fraction IV precipitation

The supernatant II + III was prepared from frozen human plasma through two consecutive steps of cold ethanol precipitation with 8 percent ethanol at pH 7.1 followed by 19 percent ethanol at pH 5.85.[8] Duplicates of 20 mL of supernatant II + III were spiked with 7.00 log per mL each of dengue virus at a ratio (vol/vol) of 1:10. Ethanol (95%) was added drop by drop into the supernatant II + III to a final ethanol concentration of 40 percent, which was further mixed at –5 to 5.5°C for 1 hour, before being centrifuged at 2300 × *g* to separate the fraction (F)IV from the supernatant IV. The supernatant II+III, the FIV, and the supernatant IV were titrated for quantity of viruses by $TCID_{50}$ assay or RT-PCR assay.

Pasteurization

The purified albumin solution was diafiltrated with 8 volumes of water and then concentrated to a concentration of 22 percent with a 30-kDa cutoff cassette (Millipore, Bedford, MA). Sodium caprylate was added to the concentrated albumin solution to a final concentration of 32 mmol per L, before adjustment of pH to 6.8 to make 20 percent albumin bulk. Two-hundred milliliters of albumin bulk and duplicates of the sterile-filtered 20 percent albumin in a 50-mL bottle was heated to 59°C in a water bath, followed by spiking with dengue virus (6.67, 7.50, or 7.67 log/mL) at a ratio (vol/vol) of 1:20 and 1:25, respectively. Gentle mixing with a mechanical stirrer (stainless steel) was applied to the bulk pasteurization. Samples were taken out for virus titration during the time course of a 10-hour treatment at 59 to 60°C.

FIII precipitation

The FII + III separated from the supernatant II + III above was redissolved, and NaAc-HAc buffer (0.8 mol/L-4 mol/L, pH 3.9) was added dropwise to adjust pH to 5.1. Dengue virus (7.17 or 7.67 log/mL) was spiked at a ratio (vol/vol) of 1:10 into duplicates of 20 mL of the pH-adjusted FII + III. Ethanol (95%) was added drop by drop into the FII + III to a final ethanol concentration of 15 percent, which was further mixed at –5 to 5.5°C for 1.5 hour, before being centrifuged at 2300 × *g* to separate the FIII from the supernatant III. The FII + III, the supernatant III, and the FIII were titrated for quantity of viruses by $TCID_{50}$ assay.

Virus filtration

A quantity of 196 mL of partially purified immunoglobulin was spiked with 7.67 log per mL dengue virus at a ratio (vol/vol) of 1:49, followed by filtration with a 0.22-μm filter (Steritop, Millipore) to remove viral aggregates. The filtered immunoglobulin was subject to virus filtration with the 35N filter in a normal-flow manner, under constant pressure of 80 kPa. Samples were titrated for quantity of viruses by $TCID_{50}$ assay or RT-PCR assay.

S/D treatment

Duplicates of 27 mL of the immunoglobulin purified through virus filtration were heated to 28°C in a water bath, followed by spiking with 7.16 or 7.83 log per mL dengue virus at a ratio (vol/vol) of 1:9. Triton X-100 and tri-*n*-butyl phosphate were added drop by drop into the immunoglobulin to a final concentration of 1 and 0.3 percent, respectively. Gentle mixing was achieved with a mechanical stirrer (stainless steel) for the time course of 16-hour treatment at 28 to 30°C, during which samples were removed for virus titration by $TCID_{50}$ assay.

Cation-exchange chromatography

A chromatography column of 10-mm diameter was packed to a bed height of 11 cm with either new CM Sepharose Fast Flow resin (Pharmacia Biotech, Uppsala, Sweden) or the used resin that had previously been recycled 476 times with the immunoglobulin purification process. The column was equilibrated with 20 mmol per L NaAc buffer, pH 4.0. Adjusted to a pH of 4.0 with 1 M HCl and an ionic strength of 1.4 mS per cm with purified water, duplicates of 75 mL of the S/D-treated immunoglobulin solution were spiked with 7.67 or 7.83 dengue virus at a ratio (vol/vol) of 1:20. The virus-spiked immunoglobulin solution was applied to the column at a linear flow rate of 40 cm/hr at ambient temperature. After washing of the column with 10 column volumes of 10 mmol per L glycine, pH 7.0, immunoglobulins were eluted with 100 mmol per L glycine together with 150 mmol per L sodium chloride, pH 9.0. The column load, the flow-through fraction, and the eluate fraction containing immunoglobulins were titrated for quantity of viruses by $TCID_{50}$ assay or RT-PCR assay.

RESULTS

FIV precipitation

After the 40 percent ethanol precipitation of the supernatant II + III, no dengue virus was detected with the $TCID_{50}$ assay in both the supernatant IV and the FIV (Table 1). Despite its direct cytotoxicity to the virus detector Vero E6 cells, when diluted 500-fold, 40 percent ethanol did not affect the determination of virus titer. Results of quantitative RT-PCR clearly showed that genetic materials of dengue virus were concentrated in the FIV (Table 1), which is discarded during the albumin manufacture. Because chemical inactivation by high concentrations of ethanol is mechanistically different from the physical partitioning effects between fractions, this FIV precipitation step provides an extra safety margin in the effective clearance of dengue viruses.

Pasteurization

The kinetics of inactivation of dengue virus in the 20 percent albumin during the 10-hour pasteurization at 59 to 60°C are shown in Fig. 1. The pasteurization was carried out at 0.5°C below what is normally used in the manufacture, representing a worst-case scenario. Dengue

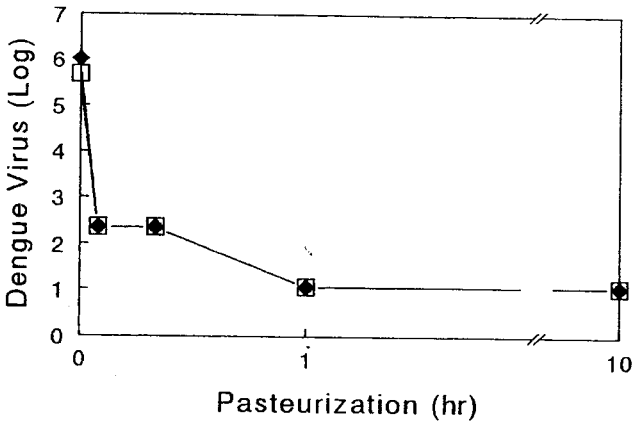

Fig. 1. Inactivation of dengue virus in albumin by pasteurization over time. (□) Bulk pasteurization; (◆) terminal pasteurization. Stock dengue viruses (6.67 or 7.50 log/mL) were spiked at a ratio (vol/vol) of 1:20 and 1:25, respectively, in the bulk pasteurization and terminal pasteurization.

TABLE 1. Clearance of dengue virus in the precipitation of FIV*

Assay (log)	Supernate II + III	FIV	Supernate IV	Log reduction, II + III → supernate IV
$TCID_{50}$	6.83/7.00	2.06†/2.06†	1.65†/1.65†	≥5.18/≥5.35
Quantitative RT-PCR	7.15/8.33	7.40/7.56	3.30/4.98	3.85/3.35

* Data shown are total viral titers (log number multiplied by volume) from duplicate experiments, where stock virus had a titer each of 7.00 log per mL spiked at a ratio of 1:10.
† No dengue virus was detected, and the number was calculated according to the Poisson distribution.

virus was quickly inactivated by the heat treatment, and infectious virus became undetectable within 5 minutes (Fig. 1). Total viral reduction for both the bulk pasteurization and the terminal pasteurization is shown in Table 2.

FIII precipitation

After the 15 percent ethanol precipitation, dengue virus was detectable with the $TCID_{50}$ assay in both the supernatant III and the FIII, with a majority of infectious virus in the FIII. Viral reduction from the FII + III to the supernatant III was calculated and is shown in Table 3.

Virus filtration

The immunoglobulin spiked with dengue virus was processed at 24 to 25°C through the 35N virus filter within 7 hours. No infectious virus was detectable by the $TCID_{50}$ assay in the immunoglobulin filtrate, the sample obtained when the virus filter was reversely flushed with purified water, or the virus-spiked immunoglobulin control standing along the whole virus filtration process. To differentiate physical separation from the chemical inactivation by the low pH, the samples were further quantified for dengue virus with the quantitative RT-PCR assay. The RT-PCR data show that dengue virus was much more concentrated in the back-flush fraction than in the immunoglobulin filtrate. Viral reduction by the virus filtration was calculated and shown in Tables 3 and 4. These results indicate that dengue virus is effectively removed by the 35N virus filtration.

TABLE 2. Clearance of dengue virus in the albumin process*

Process step	Reduction of virus (log)
FIV precipitation	≥5.18†/≥5.35†
Bulk pasteurization	≥4.61
Terminal pasteurization	≥4.94/≥5.44
Cumulative	≥10.12‡/≥10.79‡

* Data are shown from duplicate experiments, except bulk pasteurization, which was conducted once.
† The data from the $TCID_{50}$ assay, but not the RT-PCR assay, are included.
‡ "Bulk pasteurization" is not included in the "cumulative," because it is similar mechanistically to "terminal pasteurization."

TABLE 3. Clearance of dengue virus in the immunoglobulin process*

Process step	Reduction of virus (log)
FIII precipitation	2.16/2.65
Virus filtration	3.37†
S/D treatment	≥5.05/≥5.38
Chromatography	3.66‡/4.18‡
Cumulative	≥14.24/≥15.58

* Data of single virus filtration experiment and duplicate experiments of other processing steps are shown.
† Only RT-PCR data are included.
‡ Virus reduction caused by the presence of S/D is not included.

S/D treatment

The presence of S/D was cytotoxic to the virus detector Vero E6 cells; when diluted 1000-fold, S/D did not affect the determination of virus titer. The kinetics of inactivation of dengue virus in the immunoglobulin during the 16-hour S/D treatment at 28 to 30°C is shown in Fig. 2. Dengue virus was quickly inactivated by the S/D treatment, and infectious virus became undetectable within 1 minute. Total viral reduction for the S/D treatment was shown in Table 3.

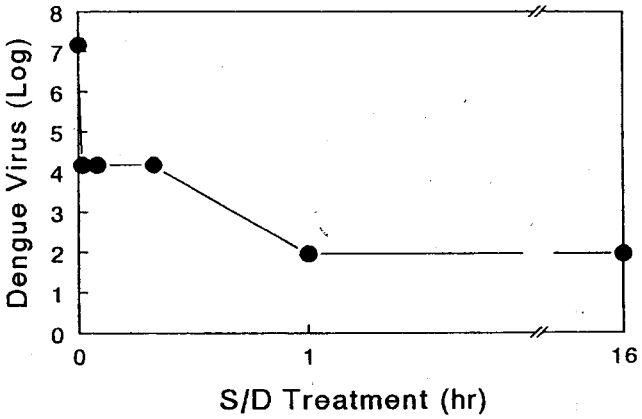

Fig. 2. Inactivation of dengue virus in immunoglobulins by S/D treatment over time. Stock dengue viruses (7.16 log/mL) was spiked at a ratio (vol/vol) of 1:9 in the S/D treatment step; when it was spiked to the immunoglobulin sample neutralized to pH 7.0 a viral titer of 7.00 log was obtained.

TABLE 4. Clearance of dengue virus in the virus filtration*

Assay	Load	Immunoglobulin filtrate	Back-flush	Log reduction, load → filtrate
$TCID_{50}$	8.37	≤2.58†	≤2.51†	≥5.79
Quantitative RT-PCR	8.47	5.10	7.08	3.37

* The numbers shown are total viral titers (log number multiplied by volume), and the stock virus spiked at a ratio of 1:49 had a titer of 7.67 log per mL.
† No dengue virus was detected, and the number was calculated according to the Poisson distribution.

TABLE 5. Clearance of dengue virus in the chromatography*

Assay	Resin	Load	Flow through	Eluate	Log reduction, load → eluate
$TCID_{50}$	New	8.20	≤6.19†	≤1.16†	≥7.04
	Used	8.04	≤6.19†	≤1.11†	≥6.93
Quantitative RT-PCR	New	9.24	8.31	5.58	3.66
	Used	8.99	8.14	4.81	4.18

* The numbers shown are total viral titers (log number multiplied by volume), and the stock virus spiked at a ratio of 1:20 had a titer of 7.67 and 7.83 log per mL for the new and used resins, respectively.
† No dengue virus was detected, and the number was calculated according to the Poisson distribution.

Cation-exchange chromatography

After the chromatography step of the immunoglobulin process, no infectious virus was detectable by the $TCID_{50}$ assay in the column load, the flow-through fraction, the eluate fraction, or the virus-spiked load control standing along the whole chromatography process. Total viral reduction from the column load to the eluate fraction was at least 7.04 and at least 6.93 log for the new resin and the 476-cycled used resin, respectively (Table 5). Because S/D was present in the starting material, the elimination of dengue virus could be a result of inactivation by the chemicals. To differentiate physical removal from chemical inactivation, the samples were further quantified for dengue virus with the quantitative RT-PCR assay. The RT-PCR data show that a majority of dengue virus was observed in the flow-through fraction. Total viral removal by the chromatography process was calculated to be 3.66 and 4.18 log for the new resin and the 476-cycled used resin, respectively (Tables 3 and 5).

DISCUSSION

Viral safety is of paramount importance for human plasma–derived therapeutic proteins such as albumin, α1-proteinase inhibitor, clotting factors, and immunoglobulins. Recent documentation of blood-associated transmission[1-3] and continuous regional outbreaks of dengue fever have prompted a validation of clearance of dengue virus in the manufacture processes of the plasma-derived products. It was the intention of this study to investigate clearance of dengue virus in individual steps of manufacture processes of plasma-derived albumin and immunoglobulins. The results shown in Tables 2 and 3 clearly demonstrate for the first time that specific virus removal and inactivation procedures reduce the risk of dengue virus transmission by more than 10 log cumulatively in plasma-derived albumin and immunoglobulins.

In this study, cold ethanol precipitation is very effective in inactivating dengue virus in the albumin process, but mildly effective in removing dengue virus in the immunoglobulin process. This difference in effectiveness is probably due to the fact that higher concentrations of ethanol were used in the albumin process. It is fairly reasonable to speculate that other therapeutic proteins prepared from plasma by similarly high concentrations of ethanol, for instance, α1-proteinase inhibitor and transferrin purified from the Cohn FIV, would have a good safety margin with regard to transmission of dengue virus.

Pasteurization inactivated dengue virus very quickly and effectively in the albumin process. The presence of a high concentration of albumin or the albumin stabilizing agent sodium caprylate did not seem to protect dengue virus from the heat inactivation. Caprylate has been shown to be an effective virus-inactivating agent at millimolar concentrations under acidic conditions;[9-12] however, caprylate appears unlikely to contribute much to the viral inactivation capacity of the pasteurization step as in the albumin formulation it is used under neutral pH, which do not favor the formation of the active component—the nonionized form of caprylate. As shown by albumin's long history of viral safety in clinical applications, the dedicated viral inactivation step in albumin manufacture processes has been very robust in the inactivation of many different viruses including West Nile virus and bovine viral diarrhea virus (BVDV), both from the same Flaviviridae family as dengue virus.[13,14]

Virus filtration was very effective in separating dengue virus from the immunoglobulin filtrate (Table 4). The data suggest that chemical inactivation by the low pH condition can probably contribute to the viral clearance capacity of this process step. In a separate study with BVDV, which is of similar size but not sensitive to low pH treatment, a majority of the spiked BVDV was trapped in the Planova 35N filter, which was recovered in the back-flush sample (unpublished observation).

Like pasteurization, S/D treatment very quickly and effectively inactivated dengue virus in the immunoglobulin process. This dedicated viral inactivation step in the immunoglobulin manufacture processes has been very robust in the inactivation of many different viruses including West Nile virus and BVDV.[13,14]

The cation-exchange chromatography was originally intended to remove S/D from the immunoglobulin process; however, it was also observed in this study to effectively remove dengue virus by affinity adsorption. In addition, this purification step was mildly effective in the physical removal of BVDV (unpublished observation). Although the chromatographic process may not be a

robust viral removal step in general, it is indeed effective in the clearance of dengue virus.

In summary, this study has shown that effective clearance of dengue virus is achieved in the manufacture processes of albumin and immunoglobulins, providing additional evidence supporting the viral safety of plasma-derived products.

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一般的名称	①乾燥抗HBs人免疫グロブリン ②ポリエチレングリコール処理抗HBs人免疫グロブリン		研究報告の公表状況	TRANSFUSION 2008; 48: 1348-1354	公表国 プエルトリコ	
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研究報告の概要

＜背景＞
輸血を介したデングウイルス感染が1例報告されている。しかし、流行地域でのデング熱の発生率が高いこと、無症候感染の割合が高いこと、およびウイルス血症の中央値が5日であることから、輸血に関連したデング感染は、報告されているよりもさらに広範囲に拡がっている可能性がある。
＜研究デザインおよび方法＞
2005年9月20日から12月4日までにプエルトリコにある米国赤十字へ提供された全ての血液ドネーションのデングウイルス(DENA) RNAの陽性率を、transcription mediated amplification (TMA) 法と呼ばれる特異タイプの核酸増幅検査を使って測定した。TMAの測定結果が2回くり返して陽性であったものを、TMA法陽性のドネーションと定めた。TMA陽性のドネーションについて、IgM抗体のELISA、RT-PCRおよびウイルス培養による検査を行った。
＜結果＞
検査を行った血液ドネーション16,521検体のうちの12検体（0.07%）が、TMA陽性であった。4検体がRT-PCR陽性（DENVセロタイプ2、3）であった。4つのRT-PCR陽性のうちの3つでウイルスが培養できた。TMA陽性であった12のドネーションのうちの1つがIgM陽性であった。他の輸血ウイルス感染（C型肝炎、WNV）のルーチンのミニプールスクリーニングでされているのと同じように1:16に希釈すると、5つのドネーションだけがTMA陽性であった。
＜結論＞
1,000のドネーション中のほぼ1つがDENV RNAを含み、TMA陽性ドネーションからウイルスが培養できたが、このことは輸血感染のリスクが、WNVについて供血者全員へのスクリーニングがされる前の米国に存在していたリスクに類似していることを示している。WNVと同じく、IgM抗体のスクリーニングは有効でない可能性があり、感染性を有しているドネーションのいくつかはミニプールによって見逃されるであろう。輸血後のデング感染の患者において、輸血感染を考慮すべきである。

報告企業の意見	今後の対応
デングウイルス感染症の流行地域における献血中からデングウイルスが約0.1%の確率で検出されたとの報告である。 血漿分画製剤からのデングウイルス伝播の事例は報告されていない。万一、原料血漿にデングウイルスが混入しても、BVDをモデルウイルスとしたウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

使用上の注意記載状況・その他参考事項等

代表として静注用ヘブスブリンーIHの記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体陰性で、かつALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗HBs抗体を含有する血漿を原料として、Cohnの低温エタノール分画で得た画分からポリエチレングリコール4000処理、DEAEセファデックス処理等により抗HBs人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びろ過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。