TABLE 2. Comparison of demographic, clinical, and laboratory findings in patients infected with the four dengue serotypes*

	PCR† type 1 (n=27)	PCR type 2 (n=13)	PCR type 3 (n=9)	PCR type 4 (n=7)	Overall (n=56)	P value
Gender (M:F)	13:14	6:7	5:4	3:4	27:29	1.0000
Age, median (IQR)	36.0 (24.0-52.0)	54.0 (33.0-66.0)	28.0 (23.5-61.0)	35.0 (21.0-63.0)	36.0 (26.3-57.8)	0.3559
Duration of hospitalisation, median (IQR) [days]	5.0 (4.0-7.0)	6.0 (3.0-7.5)	7.0 (4.5-8.5)	5.0 (4.0-6.0)	5.0 (4.0-7.0)	0.4589
Retro-orbital pain	9/18 (50)	3/10 (30)	0/4 (0)	1/6 (17)	13/38 (34)	0.2297
Rash—symptom	15/25 (60)	8/13 (62)	2/9 (22)	2/7 (29)	27/54 (50)	0.1332
Rash—sign	17/26 (65)	10/13 (77)	6/9 (67)	1/7 (14)	34/55 (62)	0.0509
Abdominal pain	3/25 (12)	2/12 (17)	1/8 (13)	1/7 (14)	7/52 (13)	1.0000
Diarrhoea	11/25 (44)	5/12 (42)	2/8 (25)	1/6 (17)	19/51 (37)	0.5956
Bleeding manifestation (epistaxis, gum bleeding, petechiae, haematuria)	13/26 (50)	8/12 (67)	5/8 (63)	2/6 (33)	28/52 (54)	0.5775
Hepatomegaly	2/26 (8)	2/13 (15)	0/9 (0)	1/7 (14)	5/55 (9)	0.5883
Leukopenia	25/26 (96)	10/13 (77)	9/9 (100)	5/7 (71)	49/55 (89)	0.0529
Lymphopenia	20/22 (91)	9/13 (69)	8/9 (89)	4/6 (67)	41/50 (82)	0.2550
Atypical lymphocyte	18/26 (69)	10/13 (77)	7/9 (78)	6/7 (86)	41/55 (75)	0.8848
Thrombocytopenia	26/26 (100)	11/13 (85)	8/9 (89)	6/7 (86)	51/55 (93)	0.0931
Elevated aspartate aminotransferase	8/9 (89)	3/4 (75)	4/4 (100)	2/2 (100)	17/19 (89)	1.0000
Elevated alanine aminotransferase	23/26 (88)	11/13 (85)	7/9 (78)	6/7 (86)	47/55 (85)	0.8954
Hypoalbuminaemia	10/26 (38)	5/13 (38)	5/9 (56)	4/7 (57)	24/55 (44)	0.6658
Highest temperature, mean (SD)	38.6 (1.0)	38.2 (1.1)	38.6 (1.3)	38.7 (0.6)	38.5 (1.0)	0.6893
Transfusion	4/23 (17)	2/12 (17)	1/8 (13)	2/6 (33)	9/49 (18)	0.8548

* Data are shown in No. (%), except otherwise stated
† PCR denotes polymerase chain reaction

TABLE 3. Demographic, clinical, and laboratory findings in patients with dengue haemorrhagic fever

Sex/age (years)	Ethnicity	Fever	Haemorrhagic manifestations	Lowest platelet count (x 10^9/L)	Plasma leakage	Laboratory findings	
						Serotype	Serology titer
M/38	Thai	37.2°C	Petechiae, bloody diarrhoea	9	Pleural effusion	Not done	Immunoglobulin M +ve
M/46	Chinese	38.4°C	Petechiae, bruises	9	Ascites	DEN 2	Immunoglobulin M +ve
F/49	Thai	38°C	Coffee ground vomitus, petechiae	8	Hypoalbuminaemia, haemoconcentration	DEN 1	4-fold increase*

* 1st titre: 640 (DEN-1), 5120 (DEN-2), 1280 (DEN-3), 1280 (DEN-4); 2nd titre: 5120 (DEN-1), 10 240 (DEN-2), 10 240 (DEN-3), 10 240 (DEN-4)

macrocytic anaemia and pancytopenia. She was diagnosed to have vitamin B12 deficiency anaemia, which was treated by vitamin B12 replacement and received a blood transfusion on 24 August 2002. On day 2 post-transfusion, she developed low-grade fever, but no skin rash, headache, myalgia, arthralgia, or retro-orbital pain. The patient was treated with antibiotics as for a urinary tract infection, based on the microbiological findings. The fever subsided 3 days later and the patient recovered uneventfully. The blood product she received was donated by a 17-year-old asymptomatic patient living in Ma Wan, during his viremic phase on 17 July 2002. On 24 July 2002, he developed generalised skin rash and attended the Accident and Emergency Department of Yan Chai Hospital. In October, he was subsequently picked up as one of the dengue cases based on serology results during the active case finding exercise in Ma Wan. Molecular testing performed on the donated blood product was positive for dengue virus type 1. The woman who had received the blood transfusion was recalled for blood testing on 7 October 2002, and was found to be positive for corresponding IgM antibodies and had a haemagglutination-inhibition titre of 1:2560. This incident was the first documented cases of such transmission in the literature, and since October

2002, the Hong Kong Red Cross Blood Transfusion Service (BTS) has intensified its donor deferral systems to counter this possibility. Specifically, it now asks about symptoms of dengue fever in the Blood Donor Registration Form (Supplement) by reminding all prospective donors to inform the BTS staff of all instances for flu, fever, headache, eye pain, muscle/joint pain, vomiting, and skin rash experienced 2 weeks before or after blood donation.

In our study, dengue fever was far more common than DHF and dengue shock syndrome, which were rare events. Our patients only manifested mild bleeding with good clinical outcomes and no fatalities. The clinical presentations of dengue fever, such as fever, myalgia, headache, and arthralgia, were comparable to findings reported in other studies.[10-12] Our patients (35%) presented with fewer gastroenteritis symptoms compared to those of others (50-98%).[11,12] Lymphadenopathy was documented in only 16% of our patients, which is much lower than the figure of 50% reported elsewhere.[13] This difference may be accounted for by less-than-adequate physical examination. Gum bleeding and epistaxis were reported in 12% and 10% of our patients respectively, which was also much lower than that reported previously.[11,12] Such differences could be due to the populations studied; patients recruited in endemic countries were mainly encountered during outbreaks in which both dengue fever and DHF were common. Previous studies showed dengue disease severity correlated with high viremia titres, secondary infection, and DEN-2 serotype infection.[14,15] Our findings showed that the haemorrhagic tendencies and duration of hospitalisation were not related to specific serotypes. Although some of our patients did receive platelet transfusions, the efficacy of such treatment in speeding recovery remains controversial. According to Thai experts, platelets are almost immediately destroyed by immune lysis after administration.[16]

Our study had several limitations. First, the target patients were limited to those with laboratory-confirmed dengue admitted to public hospitals. During 1998 to 2005, DH received notification of 203 dengue cases, including 77 who were admitted to private hospitals or consulted general practitioners only. The disease burden might also be underestimated, because some patients might have recovered, without seeking medical attention, while others might not have undergone serological testing. Second, statistical analysis could not be carried out to compare clinical and laboratory parameters in patients with dengue fever and DHF, as there were too few of the latter. Third, laboratory results before 2002 were not available in the Public Health Laboratory Information System. Fourth, not all clinical symptoms and signs listed in Table 2 could be retrieved from the medical records, as some may not have been specifically asked for or looked for.

In conclusion, dengue fever should be considered in the differential diagnosis of febrile patients with or without a travel history. Health care providers should therefore have an understanding of the infection, the spectrum of its clinical features, and methods of diagnosis and appropriate treatment. Until the *Aedes* mosquito can be effectively controlled or a cost-effective vaccine is developed, dengue fever will remain a public health concern, especially in South-East Asia. Control at source is one of the keys to combating dengue fever and requires active participation from all sectors of the community.

Acknowledgements

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References

1. Dengue/dengue haemorrhagic fever. Wkly Epidemiol Rec 2000;75:193-6.
2. General considerations. In: Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva: World Health Organization; 1997:1-11.
3. Auyeung TW, Que TL, Lam KS, Ng HL, Szeto ML. The first patient with locally acquired dengue fever in Hong Kong. Hong Kong Med J 2003;9:127-9.
4. Nimmannitya S. Clinical spectrum and management of dengue haemorrhagic fever. Southeast Asian J Trop Med Public Health 1987;18:392-7.
5. Nimmanitya S, Cardosa J. Dengue haemorrhagic fever. In: Weatherall DJ, Ledingham JGG, Warrell DA, editors. Oxford textbook of medicine. 3rd ed. Oxford, UK: Oxford Medical Publications; 1995.
6. Situation of dengue fever/dengue haemorrhagic fever in the South-East Asia Region. WHO Regional Office for South East Asia website: http://www.searo.who.int/EN/Section10/Section332_1100.htm. Accessed 23 Aug 2007.
7. Weekly Infectious Disease Bulletin [serial online] 2005;31(1). Singapore, Ministry of Health, Singapore: http://www.moh.gov.sg/mohcorp/statisticsweeklybulletins.aspx. Accessed 23 Aug 2007.
8. Limkittikul K, Yingsakmongkon S, Jittmittraphap A, et al. Clinical differences among PCR-proven dengue serotype infections. Southeast Asian J Trop Med Public Health

2005;36:1432-8.
9. Average Monthly Ovitrap Index, 2000-2006. HKSAR Food and Environmental Hygiene Department website: http://www.fehd.gov.hk/safefood/dengue_fever/index.html. Accessed 23 Aug 2007.
10. Kalayanarooj S, Vaughn DW, Nimmannitya S, et al. Early clinical and laboratory indicators of acute dengue illness. J Infect Dis 1997;176:313-21.
11. Singh NP, Jhamb R, Agarwal SK, et al. The 2003 outbreak of Dengue fever in Delhi, India. Southeast Asian J Trop Med Public Health 2005;36:1174-8.
12. Hussin N, Jaafar J, Naing NN, Mat HA, Muhamad AH, Mamat MN. A review of dengue fever incidence in Kota Bharu, Kelantan, Malaysia during the years 1998-2003. Southeast Asian J Trop Med Public Health 2005;36:1179-86.
13. Ryan ET, Wilson ME, Kain KC. Illness after international travel. N Engl J Med 2002;347:505-16.
14. Vaughn DW, Green S, Kalayanarooj S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000;181:2-9.
15. Thein S, Aung MM, Shwe TN, et al. Risk factors in dengue shock syndrome. Am J Trop Med Hyg 1997;56:566-72.
16. Alex C, Djatnika S, Ridad A, et al. Thrombocytopenia and platelet transfusions in Dengue Haemorrhagic Fever and Dengue Shock Syndrome. Dengue Bulletin 2003;27:138-43.

別紙様式第2

別紙(3)-3

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 2008年8月11日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	フィブリノゲン加第 XIII 因子		研究報告の公表状況	Clearance of dengue virus in the plasma-derived therapeutic proteins. Transfusion. 2008 Jul;48(7):1342-7. 2008 Feb 22.	公表国 中国	
販売名（企業名）	ベリプラスト P コンビセット （CSL ベーリング株式会社）					

研究報告の概要

問題点（血漿分画製剤でのデングウイルスの不活化・除去）

デングウイルスは年間に世界で 5 千万から 1 億人が感染し、感染者の数十万人がより重篤で生命を脅かすデング出血熱やデングショック症候群に進展する。

デングウイルスはフラビウイルス科に属し、直径 50nm のエンベロープを有する RNA ウイルスである。一般に血液などの高蛋白な体液で長期間生存するので、輸血により感染する可能性がある。針刺し事故や骨髄移植、分娩での血液に関連するデングウイルス感染が報告されている。

本研究は血漿分画製剤でのデングウイルス伝播の危険性が、特定のウイルス除去・不活化工程で除去されることを初めて証明するため実施された。

低温エタノール分画、陰イオン交換クロマトグラフィー、パスツリゼーション、S/D 処理とウイルスろ過を含むアルブミンやグロブリンの各製造工程前に、高力価の培養デングウイルスセロタイプ 2 を正常人血漿にスパイクし、各製造工程でのデングウイルスのクリアランスを $TCID_{50}$ アッセイ、RT-PCR で測定した。

デングウイルスの不活化・除去に対して、各製造工程前は全てで有効であった。

また、アルブミンの全製造工程（低温エタノール分画、パスツリゼーション）で少なくとも 10.12 log 減少すること、グロブリンの全製造工程（低温エタノール分画、ウイルスろ過、S/D 処理、クロマトグラフィー）では少なくとも 14.24 log 減少することが証明された。

現在実施されている血漿分画製剤の製造方法は、デングウイルス伝播に関して安全である。

使用上の注意記載状況・その他参考事項等

報告企業の意見	今後の対応
本剤の製造工程（低温エタノール分画、パスツリゼーション、イオン交換樹脂等）でデングウイルスが不活化・除去できると考えられる。	今後とも新しい感染症に関する情報収集に努める所存である。

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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

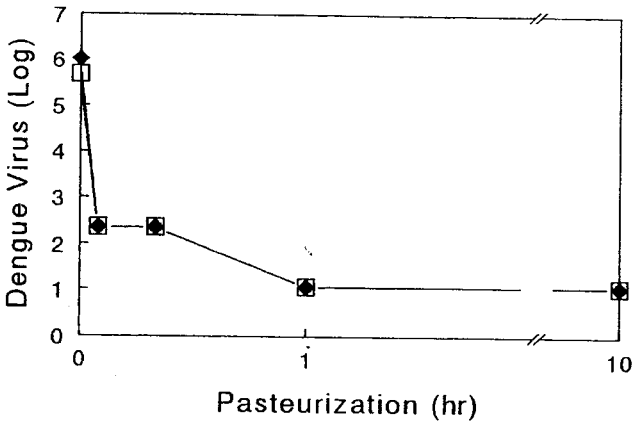

Fig. 1. Inactivation of dengue virus in albumin by pasteurization over time. (□) Bulk pasteurization; (◆) terminal pasteurization. Stock dengue viruses (6.67 or 7.50 log/mL) were spiked at a ratio (vol/vol) of 1:20 and 1:25, respectively, in the bulk pasteurization and terminal pasteurization.

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.

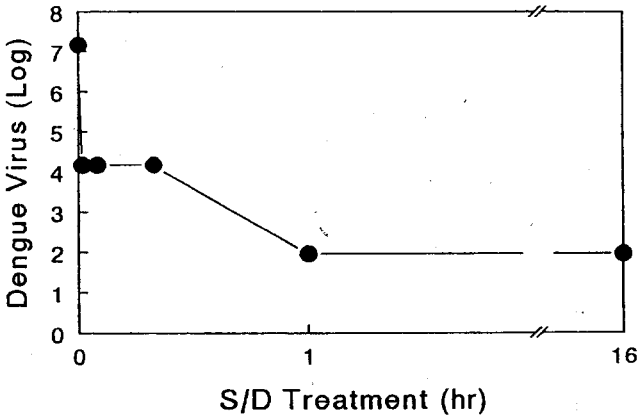

Fig. 2. Inactivation of dengue virus in immunoglobulins by S/D treatment over time. Stock dengue viruses (7.16 log/mL) was spiked at a ratio (vol/vol) of 1:9 in the S/D treatment step; when it was spiked to the immunoglobulin sample neutralized to pH 7.0 a viral titer of 7.00 log was obtained.

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.

REFERENCES

1. de Wazieres B, Gil H, Vuitton DA, Dupond JL. Nosocomial transmission of dengue from a needlestick injury. Lancet 1998;351:498.
2. Rigau-Perez JG, Vorndam AV, Clark GG. The dengue and dengue hemorrhagic fever epidemic in Puerto Rico, 1994-1995. Am J Trop Med Hyg 2001;64:67-74.
3. Chen LH, Wilson ME. Transmission of dengue virus without a mosquito vector: nosocomial mucocutaneous transmission and other routes of transmission. Clin Infect Dis 2004;39:e56-60.
4. Kuhn RJ, Zhang W, Rossmann MG, Plentnev SV, Corver J, Lenches E, Jones CT. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 2002;108:717-25.
5. Lin YL, Lei HY, Wen YY, Luh TY, Chou CK, Liu HS. Light-independent inactivation of dengue-2 virus by carboxyfullerene C3 isomer. Virology 2000;275:258-62.
6. Huang Q, Fu WL, Chen B, Huang JF, Zhang X, Xue Q. Inactivation of dengue virus by methylene blue/narrow bandwidth light system. J Photochem Photobiol B 2004;77:39-43.
7. Manning JS, Collins JK. Effects of cell culture and laboratory conditions on type 2 dengue virus infectivity. J Clin Microbiol 1979;10:235-9.
8. Cohn EJ, Strong LE, Hugles WL, Mulford DJ, Ashworth JN, Melin M, Taylor HL. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J Am Chem Soc 1946;68:459-75.
9. Lundblad JL, Seng RL. Inactivation of lipid-enveloped viruses in proteins by caprylate. Vox Sang 1991;60:75-81.
10. Korneyeva M, Hotta J, Lebing W, Rosenthal RS, Franks L, Petteway SR Jr. Enveloped virus inactivation by caprylate: a robust alternative to solvent-detergent treatment in plasma derived intermediates. Biologicals 2002;30:153-62.
11. Lebing W, Remington KM, Schreiner C, Paul HI. Properties of a new intravenous immunoglobulin (IGIV-C, 10%) produced by virus inactivation with caprylate and column chromatography. Vox Sang 2003;84:193-201.
12. Johnston A, Uren E, Johnstone D, Wu J. Low pH, caprylate incubation as a second viral inactivation step in the manufacture of albumin. Parametric and validation studies. Biologicals 2003;31:213-21.
13. Kreil TR, Berting A, Kistner O, Kindermann J. West Nile virus and the safety of plasma derivatives: verification of high safety margins, and the validity of predictions based on model virus data. Transfusion 2003;43:1023-8.
14. Remington KM, Trejo SR, Buczynski G, Li H, Osheroff WP, Brown JP, Renfrow H, Reynolds R, Pifat DY. Inactivation of West Nile virus, vaccinia virus and viral surrogates for relevant and emergent viral pathogens in plasma-derived products. Vox Sang 2004;87:10-8.

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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。

Dengue virus in blood donations, Puerto Rico, 2005

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BACKGROUND: A single instance of transfusion-transmitted dengue infection has been reported. The high incidence of dengue in endemic countries, the high proportion of asymptomatic infection, and the median 5-day viremia, however, suggest that transfusion-associated dengue transmission may be more widespread than documented.

STUDY DESIGN AND METHODS: The prevalence of dengue virus (DENV) RNA was determined in all blood donations to the American Red Cross in Puerto Rico from September 20 to December 4, 2005, using a specific type of nucleic acid amplification test called transcription-mediated amplification (TMA). TMA-positive donations were defined as those having two repeatedly reactive TMA results. TMA-positive donations were tested by enzyme-linked immunosorbent assay for immunoglobulin M (IgM) antibodies, by reverse transcription–polymerase chain reaction (RT-PCR), and by viral culture.

RESULTS: Twelve (0.07%) of 16,521 blood donations tested were TMA-positive. Four were positive by RT-PCR (DENV serotypes 2 and 3). Virus was cultured from 3 of 4 RT-PCR–positive donations. One of the 12 TMA-positive donations was IgM-positive. Only 5 donations remained TMA-positive when diluted 1:16, as is done for routine minipool screening for other transfusion-transmissible viral infections (hepatitis C, human immunodeficiency, West Nile viruses [WNVs]).

CONCLUSION: Nearly 1 in 1000 blood donations contained DENV RNA, and virus could be cultured from TMA-positive donations, suggesting a transfusion transmission risk similar to that which existed in the United States for WNV before universal donation screening. Similar to WNV, IgM antibody screening is likely to be ineffective, and some potentially infectious donations will be missed by minipool screening. Transfusion transmission should be considered in patients with dengue after blood transfusion.

Dengue virus (DENV) is a mosquito-borne flavivirus transmitted by the bite of an infected *Aedes* spp. mosquito. Infection by each of the antigenically distinct serotypes (DENV-1, -2, -3, and -4) confers lifelong serotype-specific immunity. Subsequent infection with another serotype is possible because immunity to heterologous serotypes is short-lived. Most (53%-87%) dengue infections are asymptomatic or mildly symptomatic.[1-3] Dengue infection is characterized by a median 5-day viremia, and in clinically apparent infections, symptom onset occurs 1 day after onset of viremia.[4,5] The clinical spectrum of dengue infection ranges from dengue fever to dengue hemorrhagic fever, dengue shock syndrome, and death. Primary dengue infections often present with features of classic dengue fever including acute onset of fever, arthralgia, myalgia, retroorbital pain, headache, and rash. Subsequent infection with a second dengue serotype increases the risk of developing dengue hemorrhagic fever, which is characterized by fever, thrombocytopenia (platelet count $\leq 100 \times 10^9$/L), hemorrhagic manifestations, and evidence

ABBREVIATIONS: ARC = American Red Cross; DENV = dengue virus; IC = internal control; IR = initially reactive; S/CO ratio = signal-to-cutoff ratio; TMA = transcription-mediated amplification; WNV = West Nile virus.

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Conflict of interest: JML and ASB are employees of Gen-Probe, Inc. and are owners of equity stock options in Gen-Probe.

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of increased vascular permeability and plasma leakage.[6,7] With timely supportive care, dengue hemorrhagic fever case-fatality rates can be reduced to less than 1 percent.[8,9]

The principal dengue vector is *Aedes aegypti*. It is found throughout the tropics and subtropics and in limited areas of some states in the southeastern United States. *Aedes albopictus* is also a competent vector for dengue and has been implicated previously in dengue outbreaks.[10] Although it has not been detected in Puerto Rico, *A. albopictus* exists in some parts of the Americas including more than 20 states in the eastern half of the United States.[11] Autochthonous dengue transmission does sporadically occur in southern Texas along the United States-Mexico border, with the most recent outbreak occurring in the contiguous border towns of Brownsville, Texas, and Matamoros, Tamaulipas (Mexico).[12,13] This suggests the endemicity of dengue in South Texas and the risk of reemergence of dengue in states that border Mexico as well as in southeastern states with competent vector(s) and subtropical climates. Research, however, has found that differences in housing (e.g., use of air conditioning and screens) and lifestyle may prevent this from happening.[12,13]

Although few reports document DENV transmission through receipt of infected blood,[14] tissues,[15] or organs,[16] transfusion-associated dengue transmission may be more common than previously recognized. The high proportion of asymptomatic infections, the median 5-day period of detectable viremia, and the high incidence, especially during outbreaks, suggest that a substantial number of donors could be viremic at the time of donation. In addition, nosocomial transmission of DENV via needle-stick injury[17-21] further indicates the transmissibility of DENV by infected blood. Viremic individuals may unknowingly donate blood before symptom onset or if they remain asymptomatic. West Nile virus (WNV), a related mosquito-borne flavivirus, may provide a useful model for assessing transfusion-associated DENV transmission. Transfusion transmission of WNV is well documented, and all blood donations in the United States are screened using WNV-specific nucleic acid amplification tests (NATs).[22,23]

Dengue was first identified in Puerto Rico in 1963 and is now endemic year-round with occasional islandwide outbreaks. A mean of 5446 (range, 2416-10,048) suspected cases were reported annually during the nonoutbreak years from 1990 to 2004, whereas 6039 cases were reported in 2005 (incidences of 151 versus 159 per 100,000 population/year). Approximately 77,000 blood donations are collected annually by the American Red Cross (ARC) collection centers and blood donation drive sites in Puerto Rico. These donations are used in the continental United States, Puerto Rico, and elsewhere in the Caribbean. To assess the potential for transfusion-associated dengue infection in Puerto Rico, we tested all blood donations to the ARC for dengue viral nucleic acid using a recently developed dengue-specific NAT during an 11-week period of seasonally heightened dengue activity in 2005.

MATERIALS AND METHODS

We analyzed demographic data collected from blood donors and plasma specimens from all blood donations to ARC blood collection centers and blood drives in Puerto Rico from September 20 to December 4, 2005. This study period commenced 2 weeks after the peak of seasonally heightened dengue activity in Puerto Rico (Fig. 1). Plasma specimens containing ethylenediaminetetraacetate as an anticoagulant (BD Vacutainer PPT plasma preparation tubes, BD, Franklin Lakes, NJ) from all blood donations during this study period were retained in a repository at the ARC facility in Gaithersburg, Maryland.

All specimens were first screened for the presence of DENV RNA using a DENV-specific NAT developed by Gen-Probe, Inc. (San Diego, CA) that uses transcription-mediated amplification (TMA). Specimens were tested by TMA at Gen-Probe by trained ARC staff. All initially reactive (IR) specimens were retested and TMA-positive

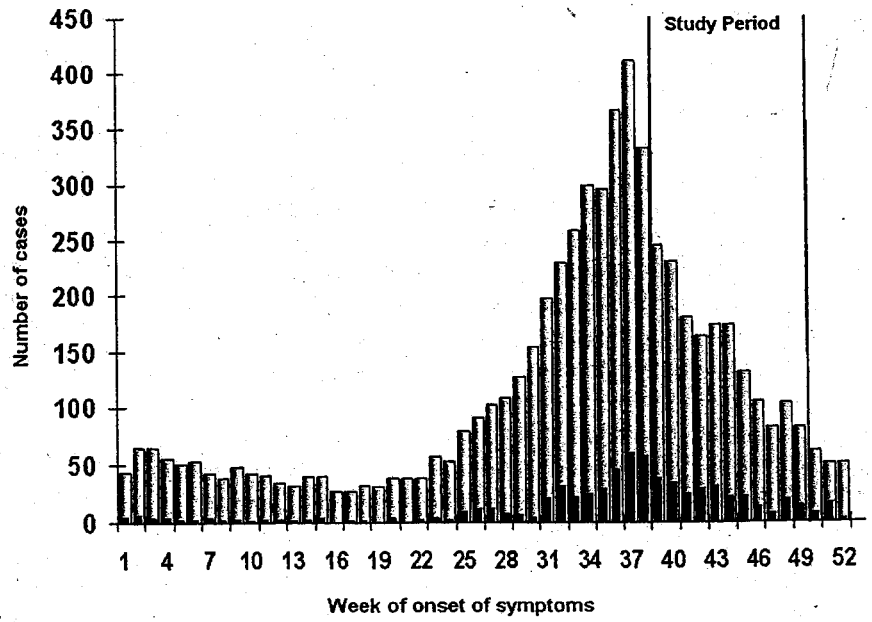

Fig. 1. Number of suspected* (□) and confirmed† (■) dengue cases by week of symptom onset, Puerto Rico, 2005. *Suspected = reported case of dengue with a clinical suspicion of dengue. †Confirmed = laboratory-confirmed (by serology or virology) case of dengue.

specimens were those that were repeatedly reactive; all others were considered to be TMA-negative. Both initial and repeat TMA screening were performed using individual specimens. All IR specimens were sent to the CDC's Dengue Branch Laboratory in San Juan, Puerto Rico, for supplemental testing by reverse transcription–polymerase chain reaction (RT-PCR).[24] Testing of donations was unlinked to donor personal identifiers; thus, subsequent contact with donors or recipients was not possible. Deidentified data from blood donation records were used in the statistical analysis (described below). The data were stored on a single password-protected terminal at the CDC, and no attempt was made to trace the donors. The study protocol was approved by the Institutional Review Board of the ARC.

TMA

Testing was performed using a prototype dengue TMA assay on a fully automated system for NAT blood screen (Procleix Tigris system, Chiron Corp., Emeryville, CA). The assay uses the same chemistry as other human immunodeficiency virus-1/hepatitis C virus and WNV assays (Procleix and Ultrio, respectively, Chiron Corp.)[25-27] and targets sequences that are conserved across all four serotypes. Thus the assay used is capable of detecting all four dengue serotypes. TMA is an isothermal RNA transcription amplification system using bacteriophage T7 RNA polymerase and Moloney murine leukemia virus reverse transcriptase (MMLV RT) to produce RNA amplicons via DNA intermediates. Viral lysis and magnetic-based target capture of viral RNA are followed by amplification and detection with the use of chemiluminescent probes.[26] This technique is able to detect 3.4 West Nile viral copies per mL at a 50 percent detection rate.[28] The analytical sensitivity of the DENV TMA assay used in this study is very similar, with 50 percent detection at 3.5 viral RNA copies per mL and a sample volume of 0.50 mL.[29] Assay results were reported in relative light units, which were used to derive signal-to-cutoff (S/CO) ratios. Cutoff values for the Dengue TMA assay internal control (IC) and analyte signals were calculated using the same formulae used for the Procleix WNV Assay.[30] A sample was considered reactive if the analyte S/CO ratio was at least 1.0, nonreactive if the analyte S/CO ratio was less than 1.0 and the IC signal was above the IC cutoff, and invalid if the analyte S/CO ratio value was less than 1.0 and the IC signal was below the IC cutoff.

Supplemental testing

All TMA-positive specimens were retested at a 1:16 dilution in plasma screened negative for all infectious disease markers including dengue RNA at Gen-Probe to determine the efficacy of testing blood donations by minipooled methods. The TMA-positive and IR specimens were tested using a real-time RT-PCR assay for the detection of NS5 gene sequence (TaqMan, Applied Biosystems, Foster City, CA).[24] This RT-PCR test is multiplexed and detects the four dengue serotypes in one reaction. It can also be used to quantitatively measure viral RNA in blood specimens with a sensitivity of approximately 1×10^3 to 5×10^3 viral RNA copies per mL. The sample volume is 20 μL derived from a 100-μL RNA extract obtained from a 0.24-mL serum specimen. All TMA-positive and IR specimens were also tested for the presence of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody using IgM MAC–enzyme-linked immunosorbent assay (ELISA) and IgG ELISA, respectively.[31,32] Virus was isolated on C6/36 cells and by mosquito isolation.[33,34]

Statistical analysis

A trend analysis using simple linear regression was performed to determine if there was a change in the number of blood donations collected during the study period. The prevalence of DENV RNA was determined by dividing the number of TMA-positive donations by the number of blood donations collected during the study period. Information about donor characteristics (see Table 1) was obtained from the ARC's electronic donor database and included date of collection, gender, date of birth, zip code of residence, zip code of donation site, donation status (first-time donor or repeat donor), phlebotomy procedure (whole blood, plateletpheresis, or leukapheresis), and donation type (allogeneic, directed, or autologous). Both donor residence and donation site were recoded into the three regions of Puerto Rico set by the United States Postal Service (San Juan Metropolitan Area, west, and east) and then treated as binary variables in the analyses (i.e., San Juan metropolitan area versus other). Age was stratified by its median and considered as a binary categorical variable in analyses.

Differences in TMA positivity by donor characteristics were assessed by the Fisher's exact test and exact logistic regression. Potential covariates identified for inclusion in the final multivariable model included covariates with a p value less than 0.20 on bivariate analysis. Age was added to the model a priori given its association with dengue infection.[2] All comparisons were made with the use of a two-tailed test, and a Type I error rate of 0.05 was used to assess significance.

RESULTS

A total of 16,521 blood donations were collected during the 11-week study period (mean, 1502 donations per week [range, 281-1864] without a significant trend in donation frequency). Twelve donations (0.73 per 1000 donations) were TMA-positive, with two or less identified per week. Eleven of these 12 donations were whole

blood-collections, while the other was a plateletpheresis from an O-donor. Donor and donation characteristics were similar among the TMA-positive and -negative donations ($p > 0.05$ for all variables, Fisher's exact test). In a multivariable model adjusted for age, donors with residence in the San Juan metropolitan area were approximately three times more likely than donors residing outside of the metropolitan area to be TMA-positive (adjusted odds ratio, 3.0; 95% confidence interval, 0.9-10.1).

The five blood donations that had the highest S/CO ratios on initial TMA testing were the only specimens to be TMA-reactive at a dilution of 1:16 (Table 2). Four of these five specimens were positive by RT-PCR and had quantifiable viral loads ranging from 2×10^3 to 8×10^7 viral RNA copies per mL. Three were identified as DENV-2 and the other as DENV-3. DENV was cultured from three of the four specimens, two by mosquito inoculation and one in cell culture. DENV-2 and DENV-3 were the predominant serotypes in circulation in Puerto Rico in 2005.

Serologic testing of the 12 TMA-positive blood donations revealed that only 1 was IgM-positive and 9 were IgG-positive by ELISA (Table 2). The lack of IgG antibody titers in Specimens 1, 4, and 8 indicates no previous dengue infections in these patients. The presence of IgG antibodies in the absence of IgM antibodies could reflect evidence of previous infections in Specimens 2, 3, 5, 7, 9, 10, 11, and 12, and IgG titers equal or greater than 1:163,840 in Specimens 3, 7, 9, and 10 indicate a recent or current secondary infection in those patients.[32] The presence of IgG in the sole donor with IgM antibodies (Specimen 6) could similarly be reflective of recent or current infection.

Other than the 12 TMA-positive specimens, there were an additional three IR specimens with S/CO ratios on initial testing of 1.00, 1.03, and 11.58 and on repeat testing of 0.92, 0.40, and 0.07, respectively. All were negative on PCR, IgM MAC-ELISA, and virus recovery. They were, however, positive on IgG ELISA. In the WNV TMA assay, an S/CO ratio of greater than or equal to 17 has a positive predictive value for confirmation of 95 percent (ARC data, unpublished); it is likely that this relationship is the same for DENV TMA.

TABLE 1. Characteristics of all and TMA-positive blood donors in Puerto Rico, September 20 to December 5, 2005*

Characteristic	All donors (n = 16,521)	TMA-positive donors (n = 12)
Age (years)	37.0 (13-85)	36.5 (16-65)
Male	10,654 (64.5)	8 (67)
Donation status		
First-time donor	5,056 (30.6)	5 (42)
Repeat donor	11,465 (69.4)	7 (58)
Region of residence		
San Juan Metropolitan Area	6,631 (40.1)	8 (67)
East	5,182 (31.4)	3 (25)
West	4,706 (28.5)	1 (8)
Phlebotomy procedure		
Whole blood	15,838 (95.9)	11 (92)
Plateletpheresis	627 (3.8)	1 (8)
Plateletpheresis/RBC pheresis	48 (0.3)	0 (0)
Double RBC pheresis	7 (0.0)	0 (0)
Leukapheresis	1 (0.0)	0 (0)
Donation type		
Allogeneic	16,400 (99.3)	12 (100)
Directed	67 (0.4)	0 (0)
Autologous	54 (0.3)	0 (0)
Region of donation site		
San Juan Metropolitan Area	8,984 (54.4)	8 (67)
East	3,870 (23.4)	4 (33)
West	3,667 (22.2)	0 (0)

* Data are reported as median (range) or number (%).

TABLE 2. Results of supplementary testing of TMA IR specimens (n = 12)

TMA test Gen-Probe (S/CO ratio)*				Supplementary testing CDC dengue branch					
Specimen	Initial test	Second test	1:16	PCR†	Number viral RNA/mL	IgM‡	IgG	Cell culture	Mosquito inoculation
1	31.96	26.99	27.73	D2	7.14×10^3	0.229	Negative	Negative	D2
2	30.31	31.28	28.78	D3	8.12×10^7	0.337	1:10,240	Negative	D3
3	29.22	27.86	27.12	D2	7.74×10^5	0.409	1:163,840	D2	Negative
4	29.17	24.84	22.92	D2	2.0×10^3	0.229	Negative	Negative	Negative
5	23.89	20.59	8.54	Negative	Undetected	0.469	1:2,560	Negative	Negative
6	21.22	5.28	0.21	Negative	Undetected	8.870	1:160	Negative	Negative
7	17.78	23.10	0.15	Negative	Undetected	0.409	1:655,360	Negative	Negative
8	17.41	18.44	0.31	Negative	Undetected	0.198	Negative	Negative	Negative
9	17.24	21.05	0.33	Negative	Undetected	1.540	1:163,840	Negative	Negative
10	5.97	7.73	0.15	Negative	Undetected	0.440	1:655,360	Negative	Negative
11	4.08	4.15	0.13	Negative	Undetected	0.368	1:10,240	Negative	Negative
12	1.53	5.56	0.60	Negative	Undetected	0.270	1:2,560	Negative	Negative

* S/CO ≥ 1 considered to be reactive.
† D2 = DENV-2, D3 = DENV-3.
‡ >2.000 considered positive.

Nine of the 12 repeat-reactive samples had S/CO values in one or both tests of 17 or greater.

DISCUSSION

This study, and a similar one recently conducted using donations in Honduras, Brazil, and Australia,[29] are the first to document the presence of dengue viral nucleic acid in blood donations. In Puerto Rico, nearly 1 in 1000 donations was positive for the presence of dengue viral nucleic acid by TMA. Furthermore, live virus was recovered from three of the 12 TMA-positive donations, indicating that at least these 3 were capable of transmitting infection to recipients. The prevalence of dengue viral nucleic acid in blood donations in this study was similar to that estimated for WNV in the areas experiencing outbreaks in the continental United States in 2002[35] before universal screening using minipool NAT was implemented in July 2003.[22] Assuming an annual prevalence rate of 0.73 per 1000 (as found in this study) and that each donation is made into a mean of 1.45 transfusable components,[36] there may be as many as 56 potentially viremic donations and 81 components generated from the approximately 77,000 blood donations collected annually by the ARC in Puerto Rico. Dengue incidence is highly seasonal and varies considerably from year to year,[37,38] however, so the prevalence of potentially viremic donors could be considerably higher or lower than this figure at any given time. Furthermore, the three IR specimens lacking reproducible results in repeat TMA testing may have been true-positive specimens but with lower viral loads. If the case, this would underestimate the true prevalence of TMA positivity.

The unlinked study design did not permit contact with the recipients of the TMA-positive donations to assess whether transmission occurred. Nevertheless, virus was cultured from three donations and the viral loads of the four RT-PCR–positive donations indicate that their transfusion would have resulted in inocula orders of magnitude greater than the amount of virus secreted in the saliva of *Aedes* mosquitoes, documented to be as low as 10^2 viral particles per secretion.[39] The RT-PCR assay used in this study had lower sensitivity than the TMA assay, and it was not possible to assess the viral load of the RT-PCR–negative specimens.

Our results indicate the feasibility of NAT as a screening strategy for DENV, as has been successfully used for WNV. Of concern, we found that simulated minipool NAT (dilution 1:16) would not have detected the majority (7 of 12, or 58%) of the TMA-positive specimens; however, the experience with WNV suggests that not all of these donations may be infectious. Approximately 30 percent of WNV NAT–positive donations have viral loads below the limits of detection by minipool NAT and can only be detected by screening of individual donations.[23,40] Although WNV has been transmitted from transfusions detectable only by individual unit screening and with an estimated level of viremia as low as 0.06 plaque-forming units (PFUs) per mL (1 PFU is approximately 400 viral copies),[41] most donations only detectable by individual unit screening had IgM and IgG antibodies and were likely not infectious given the fact that nearly all WNV transfusion transmissions have occurred from antibody-negative donations.[22,25,42] Unfortunately, this same marker of infectivity is not applicable to dengue because of the high prevalence of preexisting, cross-reactive dengue antibodies in the population and the complex and variable serologic response after secondary dengue infection.[32,43]

The global incidence of dengue has risen more than 30-fold in the past 50 years. In areas where dengue is endemic, however, transfusion transmission of the agent is rarely investigated for many reasons, including the fact that this mode of transmission is difficult to prove against a background of endemic dengue. In such cases, the distinction between a recipient infection via mosquito-borne transmission as opposed to transfusion transmission may be too complex to distinguish. Furthermore, many dengue-endemic countries lack hemovigilance systems with sufficient resources to investigate cases of recipient infection that are potentially related to transfusion of blood components. Finally, sophisticated laboratory testing may not be readily available in many dengue-endemic countries and such testing is required to distinguish dengue from other arboviral infections as well as distinguishing current dengue infection from prior infections.

In contrast, when WNV entered the United States, it was against a background of a naïve population. This permitted the laboratory linkage of multiple transfusion recipients with WNV infection to a single infected donor within several clusters of WNV cases. Infectious virus and/or viral RNA could also be recovered from retrieved cocomponent plasma units; in these cases, WNV was readily identified in the absence of competing arboviral infections. The transmissibility of WNV via blood transfusion has been established, and our findings documenting the presence of DENV RNA in the Puerto Rican blood supply, at a level comparable to that which triggered screening of the US blood supply for WNV in 2003, highlight the risks to transfusion safety posed by emerging diseases such as the vector-borne flaviviruses. Further evaluation is required to assess the risk of dengue transmission by TMA-positive donations and the cost and benefit of routine dengue screening in endemic regions.

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REFERENCES

1. Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, Rothman AL, Vaughn AL, Ennis FA. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng phet, Thailand. Am J Epidemiol 2002;156:40-51.
2. Rodriguez-Figueroa L, Rigau-Perez JG, Suarez EL, Reiter P. Risk factors for dengue infection during an outbreak in Yanes, Puerto Rico in 1991. Am J Trop Med Hyg 1995;52: 496-502.
3. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg 1988;38:172-80.
4. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000;181:2-9.
5. Nishiura H, Halstead SB. Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. J Infect Dis 2007;195:1007-13.
6. Halstead S. Dengue hemorrhagic fever—a public health problem and a field for research. Bull World Health Organ 1980;58:1-21.
7. Haemorrhagic D. Fever: diagnosis, treatment, prevention and control, 2nd ed. Geneva, Switzerland: World Health Organization; 1997.
8. Thang Hung N, Trong Lan N. Improvement of case management—a key factor to reduce case-fatality rate of dengue hemorrhagic fever in Southern Viet Nam. Dengue Bull 2003;27:144-8.
9. Chareonsook O, Foy H, Teeratkul A, Silarug N. Changing epidemiology of dengue hemorrhagic fever in Thailand. Epidemiol Infect 1999;122:161-6.
10. Effler PV, Pang L, Kitsutani P, Vorndam V, Nakata M, Ayers T, Elm J, Tom T, Reiter P, Rigau-Perez JG, Hayes JM, Mills K, Napier M, Clark GG, Gubler DJ; Hawaii Dengue Outbreak Investigation Team. Dengue fever, Hawaii, 2001-2002. Emerg Infect Dis 2005;11:742-9.
11. Moore C. Aedes albopictus in the United States: current status and prospects for further spread. J Am Mosq Control Assoc 1999;15:221-7.
12. Ramos M, Mohammed H, Zielinski-Gutierrez E, Hayden MH, Lopez JL, Fournier M, Trujillo AR, Burton R, Brunkard JM, Anaya-Lopez L, Banicki AA, Morales PK, Smith B, Munoz JL, Waterman SH, The Dengue Serosurvey Working Group. Epidemic dengue and dengue hemorrhagic fever at the Texas-Mexico Border: results of a household-based seroepidemiological survey, December 2005. Am J Trop Med Hyg 2008 Mar;78(3):364-9.
13. Reiter P, Lathrop S, Bunning M, Biggerstaff B, Singer D, Tiwari T, Baber L, Amador M, Thirion J, Hayes J, Seca C, Mendez J, Ramirez B, Robinson J, Rawlings J, Vorndam V, Waterman S, Gubler D, Clark G, Hayes E. Texas lifestyle limits transmission of dengue virus. Emerg Infect Dis 2003; 9:86-9.
14. Tsang C. Local dengue fever cases in 2002. Hong Kong, China: Public Health and Epidemiology Bulletin—Department of Health; 2002.
15. Rigau-Perez JG, Vorndam AV, Clark GG. The dengue and dengue hemorrhagic fever epidemic in Puerto Rico, 1994-1995. Am J Trop Med Hyg 2001;64:67-74.
16. Tan FL-S, Loh DL, Prabhakaran K. Dengue haemorrhagic fever after living donor renal transplantation. Nephrol Dial Transplant 2005;20:447-8.
17. Chen LN, Wilson ME. Nosocomial mucutaneous transmission and other routes of transmission. Emerg Infect Dis 2005;11:775.
18. De Wazieres B, Gil H, Vuitton D, Dupond J. Nosocomial transmission of dengue from a needle stick injury. Lancet 1998;351:498.
19. Nemes Z, Kiss G, Madarassi EP, Peterfi Z, Ferenczi E, Bakonyi T, Ternak G. Nosocomial transmission of dengue [letter]. Emerg Infect Dis 2004;10:1880-1.
20. Wagner D, de With K, Huzly D, Hufert F, Weidmann M, Breisinger S, Eppinger S, Kern WV, Bauer TM. Nosocomial acquisition of dengue. Emerg Infect Dis 2004;10:1872-3.
21. Hirsch J, Deschampes C, Lhuillier M. Metropolitan transmission of dengue by accidental inoculation at a hospital. Ann Intern Med 1990;141:629.
22. Pealer LN, Marfin AA, Petersen LR, Lanciotti RS, Page PL, Stramer SL, Stobierski MG, Signs K, Newman B, Kapoor H, Goodman JL, Chamberland ME; West Nile Virus Transmission Investigation Team. Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med 2003;349:1236-45.
23. Stramer SL, Fang CT, Foster GA, Wagner AG, Brodsky JP, Dodd RY. West Nile virus among blood donors in the United States, 2003 and 2004. N Engl J Med 2005;353:451-9.
24. Chien LJ, Liao TL, Shu PY, Huang JH, Gubler DJ, Chang GJ. Development of real-time reverse transcriptase PCR assays to detect and serotype dengue viruses. J Clin Microbiol 2006;44:1295-304.
25. Busch MP, Tobler LH, Saldanha J, Caglioti S, Shyamala V, Linnen JM, Gallarda J, Phelps B, Smith RI, Drebot M, Kleinman SH. Analytical and clinical sensitivity of West Nile virus RNA screening and supplemental assays available in 2003. Transfusion 2005;45:492-9.
26. Giachetti C, Linnen JM, Kolk DP, Dockter J, Gillotte-Taylor K, Park M, Ho-Sing-Loy M, McCormick MK, Mimms LT,

McDonough SH. Highly sensitive multiplex assay for detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol 2002;40:2408-19.
27. McCormick MK, Dockter J, Linnen JM, Kolk D, Wu Y, Giachetti C. Evaluation of a new molecular assay for detection of human immunodeficiency virus type 1 RNA, hepatitis C virus RNA, and hepatitis B virus DNA. J Clin Virol 2006;36:166-76.
28. Linnen JM, Deras ML, Cline J, Wu W, Broulik AS, Cory RE, Knight JL, Cass MM, Collins CS, Giachetti C. Performance evaluation of the PROCLEIX West Nile virus assay on semi-automated and automated systems. J Med Virol 2007;79: 1422-30.
29. Linnen JM, Vinelli E, Sabino EC, Tobler LH, Hyland C, Lee TH, Kolk DP, Broulik AS, Collins CS, Lanciotti RS, Busch MP. Dengue viremia in blood donors from Honduras, Brazil and Australia. Transfusion DOI: 10.1111/j.1537-2995.2008.01772.x
30. Linnen JM, Deras ML, Cline J, Wu W, Broulik AS, Cory RE, Knight JL, Cass MM, Collins CS, Giachetti C. Performance evaluation of the PROCLEIX West Nile virus assay on semi-automated and automated systems. J Med Virol 2007;79: 1422-30.
31. Burke DS, Nisalak A, Ussery MA. Antibody capture immunoassay detection of Japanese encephalitis virus immunoglobulin M and G antibodies in cerebrospinal fluid. J Clin Microbiol 1982;16:1034-42.
32. Miagostovich MP, Nogueira RM, dos Santos FB, Schatzmayr HG, Araujo ES, Vorndam V. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. J Clin Microbiol 1999;14:183-9.
33. Rosen L, Gubler D. The use of mosquitoes to detect and propagate dengue viruses. Am J Trop Med Hyg 1974;23: 1153-60.
34. Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am J Trop Med Hyg 1984;33:158-65.
35. Biggerstaff BJ, Petersen LR. Estimated risk of transmission of the West Nile virus through blood transfusion in the US, 2002. Transfusion 2003;43:1007-17.
36. Marshall DA, Kleinman SH, Wong JB, AuBuchon JP, Grima DT, Kulin NA, Weinstein MC. Cost-effectiveness of nucleic acid test screening of volunteer blood donations for hepatitis B, hepatitis C and human immunodeficiency virus in the United States. Vox Sang 2004;86:28-40.
37. Rigau-Perez JG. Dengue activity in Puerto Rico during an interepidemic period (1995-1997). Am J Trop Med Hyg 2001;64:75-83.
38. Rigau-Perez JG. The reappearance of dengue-3 and a subsequent dengue-4 and dengue-1 epidemic in Puerto Rico in 1998. Am J Trop Med Hyg 2002;67:355-62.
39. Kraiselburd E, Gubler DJ, Kessler MJ. Quantity of dengue virus required to infect rhesus monkeys. Trans R Soc Trop Med Hyg 1985;79:248-51.
40. Busch MP, Caglioti S, Robertson EF, McAuley JD, Tobler LH, Kamel H, Linnen JM, Shyamala V, Tomasulo P, Kleinman SH. Screening the blood supply for West Nile virus RNA by nucleic acid amplification testing. N Engl J Med 2005;353:460-7.
41. Montgomery SP, Brown JA, Kuehnert M, Smith TL, Crall N, Lanciotti RS, Macedo de Oliveira A, Boo T, Marfin AA; 2003 West Nile Virus Transfusion-Associated Transmission Investigation Team. Transfusion-associated transmission of West Nile virus, United States 2003 through 2005. Transfusion 2006;46:2038-46.
42. Petersen LR, Epstein JS. Problem solved? West Nile virus and transfusion safety. N Engl J Med 2005;353:516-7.
43. Gubler DJ, Sather GE. Laboratory diagnosis of dengue and dengue hemorrhagic fever. In: Homma A, Cunha JF, editors. Proceedings of the international symposium on yellow fever and dengue. Rio de Janeiro, Brazil: Rio de Janeiro; 1988. p. 291-322.

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別紙 3-7

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008 年 6 月 13 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. Ehrlich, H.J. et al, New Engl. J. Med., 358, 2573-2584 (2008)	公表国 オーストリア	
販売名（企業名）						

研究報告の概要

本稿では，抗 H5N1 型鳥インフルエンザワクチンの安全性及び有効性を検討する無作為化用量漸増第 1・2 相試験の試験成績について記載した。Baxter Bioscience 社が同ワクチンを開発し，試験を実施した。当該ワクチンの主な特徴は，サルの腎臓細胞の培養株（ベロ細胞）で作成された自然発生するウイルス株 A/Vietnam/1203/2004 を利用していることである。ミョウバンアジュバントによる作用も検討し，ウイルス全体をワクチンとして使用した。有効性エンドポイントとして，ワクチンの（i）ヘマグルチニン阻害を生じさせる能力，（ii）中和抗体を誘発する能力，（iii）注射 21 日後にセロコンバージョンを生じさせる能力を検討した。各被験者に対し，それぞれ 3.75，7.5，15 又は 30 ug のヘマグルチニン抗原を含有するワクチンをアジュバントとともに，もしくは 7.5 又は 15 ug の抗原を含有するワクチンをアジュバントなしで 21 日の間隔をおいて 2 回投与した。免疫寛容は非常に良好に成立した。いずれのワクチン処方でも，注射部位の軽度疼痛（被験者の 9～27%）及び頭痛（被験者の 6～31%）が最も高頻度に報告された有害事象であった。有効性に関する限り，免疫応答はアジュバントなしの処方を投与した被験者において最も高い割合で認められたが，いずれの処方でも第 21 日目から第 42 日目で中和抗体の抗体価は同程度に増加した。さらに，ウイルス株 A/Indonesia/05/2005 及び A/Hong Kong/156/1997 に対する交差中和が認められた。再度，アジュバントなしの処方が最も高い免疫原性を示した。本試験では重要な用量反応性の関連が示されなかったことから，今後の開発にあたってアジュバントなしの 7.5 ug の処方が選択された。

使用上の注意記載状況・その他参考事項等

BYL-2008-0338

報告企業の意見	今後の対応
抗 H5N1 型鳥インフルエンザワクチンが利用可能となれば、パンデミックの発生及び拡大を防ぐために有効であろう。血漿由来製剤工程におけるウイルス除去は、インフルエンザウイルス除去に対しても有効と考えられる。弊社製品の製造工程に使用されている血漿分画成分は，製造工程中のウイルスバリデーションにおいて，インフルエンザウイルスと同様のエンベロープ RNA ウイルスである HIV（レトロウイルス）の不活化・除去能が確認されている。各成分の製造工程における不活化・除去能は以下のとおり。 • アルブミン・カッター及びコージネイト FS の製造工程培地に使用されているヒト血清アルブミン：17.8 log 以上 • プラスマネート・カッター，コージネイト FS 及びコージネイト FS バイオセットの製造工程培地に使用されている加熱ヒト血漿タンパク：15 log 以上 • コージネイト FS の製造工程に使用されているトランスフェリン：9.1 log 以上 • ベタフェロン皮下注，ゼヴァリン イットリウム (^{90}Y) 静注用セット及びゼヴァリン インジウム (^{111}I) 静注用セットの製造工程に使用されているヒト血清アルブミン：9.98 log 以上	現時点で新たな安全対策上の措置を講じる必要はないと考える。

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

A Clinical Trial of a Whole-Virus H5N1 Vaccine Derived from Cell Culture

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ABSTRACT

BACKGROUND

Widespread infections of avian species with avian influenza H5N1 virus and its limited spread to humans suggest that the virus has the potential to cause a human influenza pandemic. An urgent need exists for an H5N1 vaccine that is effective against divergent strains of H5N1 virus.

METHODS

In a randomized, dose-escalation, phase 1 and 2 study involving six subgroups, we investigated the safety of an H5N1 whole-virus vaccine produced on Vero cell cultures and determined its ability to induce antibodies capable of neutralizing various H5N1 strains. In two visits 21 days apart, 275 volunteers between the ages of 18 and 45 years received two doses of vaccine that each contained 3.75 μg, 7.5 μg, 15 μg, or 30 μg of hemagglutinin antigen with alum adjuvant or 7.5 μg or 15 μg of hemagglutinin antigen without adjuvant. Serologic analysis was performed at baseline and on days 21 and 42.

RESULTS

The vaccine induced a neutralizing immune response not only against the clade 1 (A/Vietnam/1203/2004) virus strain but also against the clade 2 and 3 strains. The use of adjuvants did not improve the antibody response. Maximum responses to the vaccine strain were obtained with formulations containing 7.5 μg and 15 μg of hemagglutinin antigen without adjuvant. Mild pain at the injection site (in 9 to 27% of subjects) and headache (in 6 to 31% of subjects) were the most common adverse events identified for all vaccine formulations.

CONCLUSIONS

A two-dose vaccine regimen of either 7.5 μg or 15 μg of hemagglutinin antigen without adjuvant induced neutralizing antibodies against diverse H5N1 virus strains in a high percentage of subjects, suggesting that this may be a useful H5N1 vaccine. (ClinicalTrials.gov number, NCT00349141.)

From the Department of Global Research and Development, Baxter BioScience (H.J.E., G.B., S.F., A.L.-B., N.V., R.B., B.G.P., E.M.P., O.K., P.N.B.), and the Department of Clinical Pharmacology, Medical University of Vienna, Vienna General Hospital (M.M., C.J.) — both in Vienna; Changi General Hospital (H.M.L.O.) and the National University of Singapore and National University Hospital (P.A.T., D.F.) — all in Singapore; and the University of Siena, Siena, Italy (E.M.). Address reprint requests to Dr. Müller at the Department of Clinical Pharmacology, Medical University of Vienna, Vienna General Hospital (AKH), Währinger Gürtel 18-20, 1090 Vienna, Austria, or at markus.mueller@meduniwien.ac.at.

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THE EMERGENCE OF A NEW HUMAN influenza pandemic caused by an avian virus strain is possible. Vaccination against pandemic influenza is considered to be the most effective option to limit its spread. However, the conventional approaches to the manufacture of influenza vaccines have a number of disadvantages and raise concern about whether sufficient quantities of an effective vaccine can be made available early enough at the onset of a pandemic to have a major effect on public health.[1] In addition, clinical studies of conventional split-vaccine formulations without adjuvant have shown poor immunogenicity.[2,3] It has been suggested that whole-virus vaccines have the potential to be more immunogenic than split-virus or subunit vaccines in previously unvaccinated populations.[4,5] The first clinical study of a whole-virus vaccine against avian influenza H5N1 virus showed that a substantially reduced antigen dosage (10 μg) with an alum formulation induced seroconversion in nearly 100% of subjects.[6]

All these studies were carried out with vaccines manufactured by conventional methods (i.e., with the use of embryonated chicken eggs and modified, attenuated reassortant viruses produced by reverse genetics).[7] We have devised a strategy for the development of an H5N1 vaccine that involves the use of a wild-type virus (i.e., the strain circulating in nature) grown in a Vero cell culture. This strategy has the advantage that the lead time for pandemic vaccine production can be reduced, since the generation of attenuated reassortants is not required, although the requirement for the use of enhanced biosafety level 3 (BSL-3) facilities for such a strategy is a relative drawback. In addition, cell culture provides a robust manufacturing platform that eliminates dependence on embryonated chicken eggs, which would be an advantage in the event of limited availability of such eggs during a pandemic caused by a highly pathogenic avian virus. This technique was used to develop a whole-virus vaccine that was highly immunogenic in animal models.[8] We report on the safety and immunogenicity of this vaccine, using formulations with and without alum adjuvant.

METHODS

STUDY DESIGN AND OBJECTIVE

From June 2006 through September 2006, we enrolled a total of 284 men and women between the ages of 18 and 45 years in a randomized, partially blinded (between groups) clinical trial at three sites: one in Austria and two in Singapore. The study was designed by its sponsor, Baxter. Data were collected by the investigators and were held and analyzed by Baxter. The manuscript was written by a subgroup of industry and academic authors; all authors contributed to the content, had full access to the data, and vouch for the completeness and accuracy of the data and data analysis.

The appropriate local review boards and ethics committees approved the protocol for the study, which was conducted in compliance with Good Clinical Practice guidelines and the provisions of the Declaration of Helsinki. The study investigators were unaware of assignments to study groups. (For details of the study design, see the Supplementary Appendix, available with the full text of this article at www.nejm.org.)

The objective was to identify the immunogenicity and safety of various doses of inactivated H5N1 whole-virus vaccine in formulations with and without adjuvant. The primary immunogenicity outcome was the number of subjects with hemagglutination-inhibition and neutralizing antibodies to the vaccine strain (A/Vietnam/1203/2004) 21 days after the first and second doses of vaccine. The primary safety outcome was any systemic reaction after the first and second doses.

VACCINE

The monovalent avian influenza H5N1 whole-virus vaccine (Baxter) was produced with the wild-type strain A/Vietnam/1203/2004, which was obtained from the Centers for Disease Control and Prevention and was inactivated with formalin and ultraviolet light. The vaccine was manufactured in Vero cell culture in an enhanced BSL-3 facility (as required for wild-type H5N1 virus), as described previously.[9]

RANDOMIZATION AND FOLLOW-UP

Subjects were eligible to participate if they were clinically healthy, understood the study procedures, provided written informed consent, and agreed to keep a daily record of symptoms. Women were required to have a negative pregnancy test at screening and before each vaccination.

Subjects were recruited in three study cohorts in a dose-escalating manner and were randomly assigned to receive two 0.5-ml injections into the deltoid muscle at an interval of 21 days (range,

19 to 23) with an H5N1 whole-virus formulation containing 3.75 μg, 7.5 μg, 15 μg, or 30 μg of hemagglutinin antigen with a 0.2% alum adjuvant or 7.5 μg or 15 μg of hemagglutinin antigen without adjuvant. There was no placebo group. Subjects and investigators were unaware of the dose of vaccine administered within the subgroups (Fig. 1, and the Supplementary Appendix). Blood samples were taken for serologic testing before the first dose of vaccine and on day 21 after the first and second doses.

Using a diary provided by the investigators, subjects were asked to record daily oral body temperature (using study-issued digital thermometers), local reactions, and systemic adverse events for 7 days after each vaccination. On days 7 and 21 after each vaccination, subjects were asked to return for a review of the diary and assessment for any adverse events.

ASSAYS

We evaluated all immunogenicity outcomes against the influenza-virus strain used in the vaccine (A/Vietnam/1203/2004) according to hemagglutination-inhibition and virus-neutralization assays. To assess cross-reactivity of antibodies, all assays were also conducted with known related influenza strains — for example, an original prototype clade 3 strain (A/Hong Kong/156/1997) and a clade 2 strain (A/Indonesia/05/2005).

Using a hemagglutination-inhibition or virus-neutralization assay, we investigated secondary

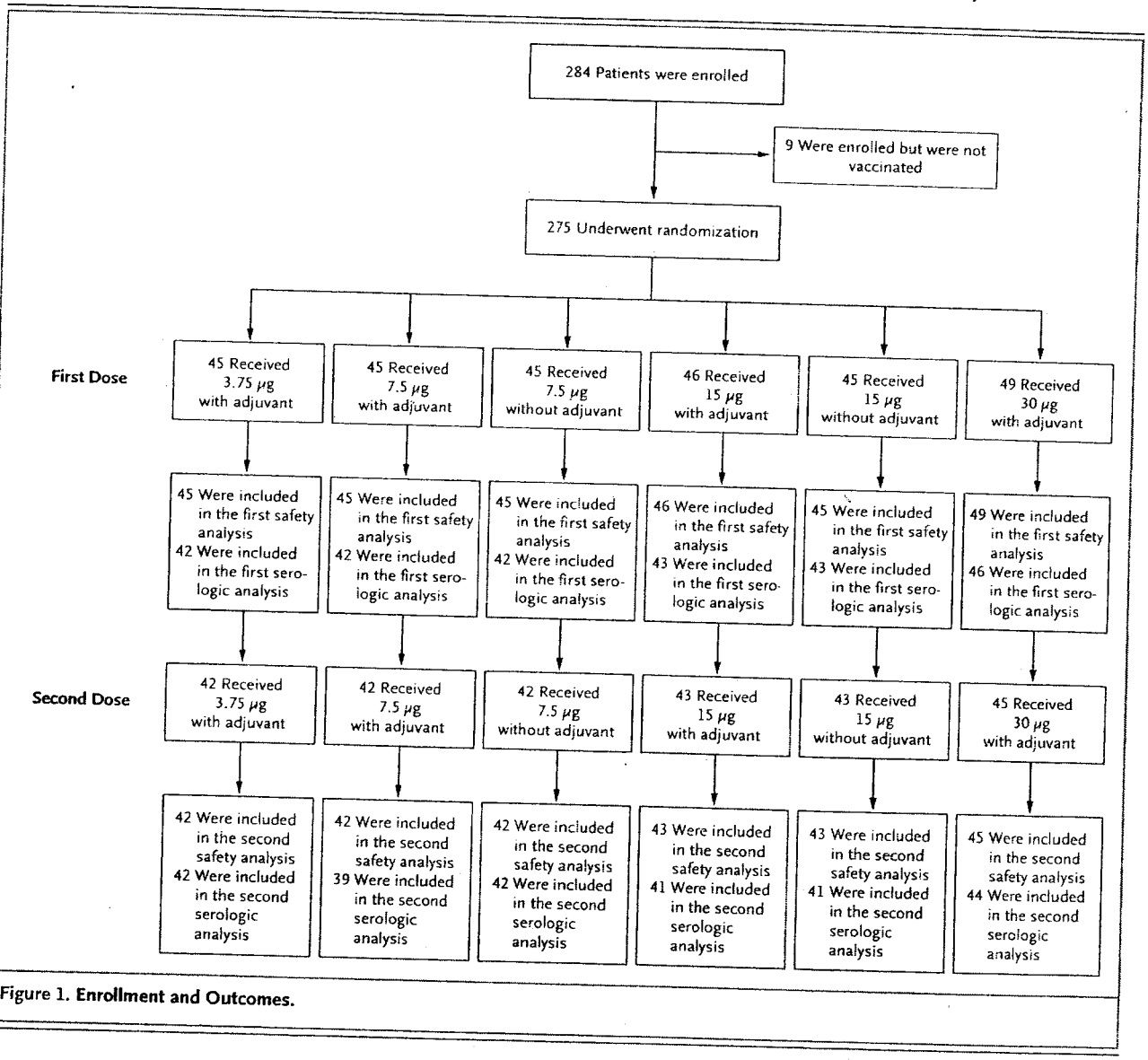

Figure 1. Enrollment and Outcomes.

Table 1. **Proportion of Subjects with Injection-Site and Systemic Reactions within 7 Days after the First and Second Doses of Vaccine.**

Variable	3.75 μg with Adjuvant	7.5 μg with Adjuvant	7.5 μg without Adjuvant	15 μg with Adjuvant	15 μg without Adjuvant	30 μg with Adjuvant
First dose						
No. of subjects	45	45	45	46	45	49
			percent (95% confidence interval)			
Injection-site reaction						
Any	29 (16–44)	22 (11–37)	11 (4–24)	28 (16–43)	20 (10–35)	24 (13–39)
Pain	27 (15–42)	20 (10–35)	9 (2–21)	26 (14–41)	18 (8–32)	24 (13–39)
Erythema*	0 (0–8)	2 (0–12)	2 (0–12)	4 (1–15)	0 (0–8)	0 (0–7)
Swelling*	0 (0–8)	0 (0–8)	0 (0–8)	2 (0–12)	0 (0–8)	2 (0–11)
Induration*	0 (0–8)	2 (0–12)	0 (0–8)	0 (0–8)	4 (1–15)	2 (0–11)
Ecchymosis*	0 (0–8)	0 (0–8)	0 (0–8)	0 (0–8)	2 (0–12)	2 (0–11)
Systemic reaction						
Any	51 (36–66)	31 (18–47)	38 (24–53)	30 (18–46)	47 (32–62)	18 (9–32)
Fever†	2 (0–12)	4 (1–15)	0 (0–8)	4 (1–15)	2 (0–12)	2 (0–11)
Headache	31 (18–47)	18 (8–32)	20 (10–35)	13 (5–26)	24 (13–40)	6 (1–17)
Malaise	13 (5–27)	11 (4–24)	4 (1–15)	13 (5–26)	9 (2–21)	6 (1–17)
Myalgia	9 (2–21)	16 (6–29)	4 (1–15)	9 (2–21)	9 (2–21)	2 (0–11)
Shivering	0 (0–8)	9 (2–21)	7 (1–18)	9 (2–21)	2 (0–12)	0 (0–7)
Second dose						
No. of subjects	42	42	42	43	43	45
			percent (95% confidence interval)			
Injection-site reaction						
Any	17 (7–31)	12 (4–26)	14 (5–29)	19 (8–33)	16 (7–31)	13 (5–27)
Pain	14 (5–29)	10 (3–23)	12 (4–26)	19 (8–33)	16 (7–31)	11 (4–24)
Erythema*	0 (0–8)	2 (0–13)	2 (0–13)	0 (0–8)	0 (0–8)	0 (0–8)
Swelling*	0 (0–8)	2 (0–13)	0 (0–8)	2 (0–12)	0 (0–8)	0 (0–8)
Induration*	5 (1–16)	0 (0–8)	0 (0–8)	2 (0–12)	0 (0–8)	0 (0–8)
Ecchymosis*	0 (0–8)	2 (0–13)	0 (0–8)	0 (0–8)	2 (0–12)	2 (0–12)
Systemic reaction						
Any	31 (18–47)	24 (12–39)	26 (14–42)	28 (15–44)	44 (29–60)	18 (8–32)
Fever†	0 (0–8)	2 (0–13)	5 (1–16)	0 (0–8)	7 (1–19)	2 (0–12)
Headache	19 (9–34)	10 (3–23)	5 (1–16)	9 (3–22)	12 (4–25)	13 (5–27)
Malaise	5 (1–16)	7 (1–19)	5 (1–16)	2 (0–12)	12 (4–25)	9 (2–21)
Myalgia	12 (4–26)	2 (0–13)	2 (0–13)	2 (0–12)	7 (1–19)	0 (0–8)
Shivering	0 (0–8)	2 (0–13)	5 (1–16)	2 (0–12)	7 (1–19)	0 (0–8)

* Listed are injection-site reactions with a diameter of more than 1 cm.
† Fever was defined as an oral temperature of 38°C (100.4°F) or more.

immunogenicity outcomes by analyzing the antibody response 21 days after the first and second doses of vaccine; the increase in the antibody response 21 days after the first and second doses, as compared with baseline; and the number of subjects with seroconversion (which we defined as a minimum increase by a factor of 4 in the titer) 21 days after the first and second doses, as compared with baseline.

The hemagglutination-inhibition assay is the standard test for detection of antibodies against influenza after infection or vaccination. However,

Table 2. **Proportion of Subjects with a Virus-Neutralization Antibody Titer of 1:20 or More.**

Virus Strain and Day	3.75 μg with Adjuvant	7.5 μg with Adjuvant	7.5 μg without Adjuvant	15 μg with Adjuvant	15 μg without Adjuvant	30 μg with Adjuvant
A/Vietnam/1203/2004 (clade 1)						
Day 0						
No./total no. (%)	0/42	3/42 (7.1)	0/42	1/43 (2.3)	0/43	0/46
95% CI	0.0–8.4	1.5–19.5	0.0–8.4	0.1–12.3	0.0–8.2	0.0–7.7
Day 21						
No./total no. (%)	9/42 (21.4)	11/42 (26.2)	17/42 (40.5)	7/43 (16.3)	17/43 (39.5)	5/46 (10.9)
95% CI	10.3–36.8	13.9–42.0	25.6–56.7	6.8–30.7	25.0–55.6	3.6–23.6
Day 42						
No./total no. (%)	29/42 (69.0)	25/39 (64.1)	32/42 (76.2)	25/41 (61.0)	29/41 (70.7)	29/44 (65.9)
95% CI	52.9–82.4	47.2–78.8	60.5–87.9	44.5–75.8	54.5–83.9	50.1–79.5
A/Indonesia/05/2005 (clade 2)						
Day 0						
No./total no. (%)	1/42 (2.4)	1/42 (2.4)	0/42	1/43 (2.3)	0/43	0/46
95% CI	0.1–12.6	0.1–12.6	0.0–8.4	0.1–12.3	0.0–8.2	0.0–7.7
Day 21						
No./total no. (%)	5/42 (11.9)	5/42 (11.9)	10/42 (23.8)	1/43 (2.3)	7/43 (16.3)	3/46 (6.5)
95% CI	4.0–25.6	4.0–25.6	12.1–39.5	0.1–12.3	6.8–30.7	1.4–17.9
Day 42						
No./total no. (%)	12/42 (28.6)	14/39 (35.9)	19/42 (45.2)	3/41 (7.3)	15/41 (36.6)	13/44 (29.5)
95% CI	15.7–44.6	21.2–52.8	29.8–61.3	1.5–19.9	22.1–53.1	16.8–45.2
A/Hong Kong/156/1997 (clade 3)						
Day 0						
No./total no. (%)	0/42	4/42 (9.5)	2/42 (4.8)	2/43 (4.7)	1/43 (2.3)	1/46 (2.2)
95% CI	0.0–8.4	2.7–22.6	0.6–16.2	0.6–15.8	0.1–12.3	0.1–11.5
Day 21						
No./total no. (%)	9/42 (21.4)	13/42 (31.0)	20/42 (47.6)	9/43 (20.9)	18/43 (41.9)	7/46 (15.2)
95% CI	10.3–36.8	17.6–47.1	32.0–63.6	10.0–36.0	27.0–57.9	6.3–28.9
Day 42						
No./total no. (%)	28/42 (66.7)	25/39 (64.1)	32/42 (76.2)	26/41 (63.4)	32/41 (78.0)	34/44 (77.3)
95% CI	50.5–80.4	47.2–78.8	60.5–87.9	46.9–77.9	62.4–89.4	62.2–88.5

this assay may be insensitive for the detection of anti-H5 antibodies.[10,11] For this reason, immunogenicity analyses focused on a determination of functional neutralizing-antibody responses. Since most licensing authorities typically request data regarding hemagglutination-inhibition assays or single radial hemolysis, these determinations are also reported but only for the vaccine virus strain A/Vietnam/1203/2004. (For details on hemagglutination-inhibition and virus-neutralization assays and single radial hemolysis,[12-14] see the Supplementary Appendix.)

STATISTICAL ANALYSIS

The protocol called for the recruitment of 45 subjects per study group. With this number of subjects, the 95% confidence interval for the percentage of subjects with an antibody response that was associated with protection did not extend more than 15% from the observed rate, assuming a seroprotection rate of approximately 80%.

We used the likelihood-ratio chi-square test to compare the number of subjects with local or systemic reactions within 7 days after vaccination among the various vaccine formulations. For bi-

Table 3. **Geometric Mean of the Increase from Baseline (GMI) and Proportion of Subjects with Seroconversion.***

Virus Strain and Day	3.75 μg with Adjuvant		7.5 μg with Adjuvant		7.5 μg without Adjuvant	
	GMI	Seroconversion	GMI	Seroconversion	GMI	Seroconversion
	value (95% CI)	*% (95% CI)*	*value (95% CI)*	*% (95% CI)*	*value (95% CI)*	*% (95% CI)*
A/Vietnam/1203/2004 (clade 1)						
Day 21	2.0 (1.6–2.4)	11.9 (4.0–25.6)	2.0 (1.6–2.5)	9.5 (2.7–22.6)	3.2 (2.4–4.2)	35.7 (21.6–52.0)
Day 42	4.4 (3.5–5.6)	54.8 (38.7–70.2)	4.0 (3.1–5.2)	51.3 (34.8–67.6)	5.3 (4.1–6.9)	69.0 (52.9–82.4)
A/Indonesia/05/2005 (clade 2)						
Day 21	1.7 (1.4–1.9)	4.8 (0.6–16.2)	1.6 (1.3–1.9)	7.1 (1.5–19.5)	2.2 (1.8–2.8)	19.0 (8.6–34.1)
Day 42	2.8 (2.3–3.4)	19.0 (8.6–34.1)	2.7 (2.1–3.4)	28.2 (15.0–44.9)	3.2 (2.5–4.0)	31.0 (17.6–47.1)
A/Hong Kong/156/1997 (clade 3)						
Day 21	2.3 (1.8–2.9)	16.7 (7.0–31.4)	2.3 (1.8–2.8)	14.3 (5.4–28.5)	3.4 (2.5–4.7)	38.1 (23.6–54.4)
Day 42	5.8 (4.4–7.7)	69.0 (52.9–82.4)	5.2 (3.8–7.1)	51.3 (34.8–67.6)	5.9 (4.3–8.1)	66.7 (50.5–80.4)

* Seroconversion was defined as an increase in the virus-neutralization titer by a factor of 4 or more.

nary variables (i.e., seroprotection and seroconversion), response rates and 95% confidence intervals were computed for each strain and time point. The confidence intervals were interpreted in a descriptive manner, and no adjustment for multiplicity was made.[15]

In addition, for the log-transformed values of virus-neutralization titers and single radial hemolysis, a longitudinal analysis was performed within a repeated mixed-model framework of analysis of covariance. Changes from baseline were analyzed, accounting for the fixed effects of vaccine formulation, day, sex, age, baseline titer, interaction between the vaccine formulation and day, and random effects for subjects. Vaccine formulations without adjuvant were compared with formulations with adjuvant within this model. Comparisons were also made between groups receiving 7.5 μg and 15 μg of hemagglutinin antigen without adjuvant. We calculated the proportion of subjects with a virus-neutralization titer of 1:20 or more and that of subjects with results of 25 mm^2 or more on single radial hemolysis, using a generalized linear model with repeated measurements and the general-estimating-equations method (see the Supplementary Appendix).

RESULTS

STUDY POPULATION

A total of 275 subjects between the ages of 18 and 45 years received the first dose of vaccine, and 257 received the second dose. All vaccinated subjects were included in the safety analysis. Two subjects who initially gave their consent withdrew from the study because of nonserious adverse events, including four events in one subject (chills, fatigue, malaise, and insomnia) and one event in the second subject (papular rash); the majority of these symptoms abated within 24 hours. Immunogenicity data were available for 258 subjects for the first dose of vaccine and for 249 subjects for the second dose of vaccine.

SAFETY

The rates of occurrence of injection-site and systemic reactions during the first 7 days after each dose of vaccine are presented in Table 1. No serious, vaccine-related adverse events were recorded. There were two serious adverse events recorded in two subjects: hospitalization due to a contusion of the left foot and hospitalization for an elective abortion.

The most commonly occurring injection-site reaction after vaccination was pain, which occurred in 9 to 27% of subjects; the most frequently reported systemic reaction was headache, which occurred in 6 to 31% of subjects.

There were no significant differences between the vaccine formulations with respect to local reactions after the first dose and the second dose of vaccine (P=0.32 and P=0.97, respectively, for all comparisons). With respect to systemic reactions, a slight difference was observed between the vaccine formulations after the first dose of vaccine (P=0.01), a finding that was largely due

15 μg with Adjuvant		15 μg without Adjuvant		30 μg with Adjuvant	
GMI *value (95% CI)*	Seroconversion *% (95% CI)*	GMI *value (95% CI)*	Seroconversion *% (95% CI)*	GMI *value (95% CI)*	Seroconversion *% (95% CI)*
1.9 (1.5–2.4)	11.6 (3.9–25.1)	3.1 (2.5–4.0)	34.9 (21.0–50.9)	2.1 (1.8–2.5)	13.0 (4.9–26.3)
3.9 (3.0–5.0)	46.3 (30.7–62.6)	5.7 (4.3–7.5)	68.3 (51.9–81.9)	4.6 (4.0–5.4)	61.4 (45.5–75.6)
1.4 (1.2–1.7)	2.3 (0.1–12.3)	2.3 (1.8–2.9)	16.3 (6.8–30.7)	1.7 (1.5–2.0)	2.2 (0.1–11.5)
2.5 (2.1–2.9)	9.8 (2.7–23.1)	3.6 (2.9–4.5)	43.9 (28.5–60.3)	2.9 (2.5–3.5)	29.5 (16.8–45.2)
2.0 (1.5–2.7)	11.6 (3.9–25.1)	3.3 (2.5–4.3)	30.2 (17.2–46.1)	1.9 (1.6–2.3)	15.2 (6.3–28.9)
4.9 (3.7–6.5)	53.7 (37.4–69.3)	7.8 (5.7–10.6)	75.6 (59.7–87.6)	5.7 (4.6–7.0)	63.6 (47.8–77.6)

to an unexpectedly low rate of headache observed in the group receiving the 30-μg formulation with adjuvant. No difference was shown regarding systemic reactions after the second dose of vaccine (P=0.15).

IMMUNE RESPONSE

At 21 days after the first and second doses, functional neutralizing antibodies against strain A/Vietnam/1203/2004 were detected in patients receiving any of the six formulations. Table 2 shows the rates of response in subjects with a virus-neutralization titer of 1:20 or more, and Table 3 shows the geometric mean increase (GMI) of the titer from baseline and the percentage of seroconversion. Numerically, the formulations without adjuvant induced the highest rates of a virus-neutralization titer of 1:20 or more after the first dose (40.5% and 39.5% for 7.5 μg and 15 μg without adjuvant, respectively) and the second dose (76.2% and 70.7% for 7.5 μg and 15 μg without adjuvant, respectively) (Table 2). Similar results were obtained with respect to GMI (Table 3), since the highest GMIs were obtained for the formulations without adjuvant (5.3 and 5.7 for 7.5 μg and 15 μg without adjuvant, respectively) (Table 3). Among subjects with seroconversion (an increase in the titer by a factor of at least 4 after immunization), the highest rates of response were again seen in subjects who received a 7.5-μg or 15-μg formulation without adjuvant (69.0% and 68.3%, respectively) (Table 3).

Statistical analysis with the use of a mixed model on log-transformed virus-neutralization values confirmed that the formulations without adjuvant induced significantly higher immune responses than did the formulations with adjuvant (P<0.001). There were no significant differences between the two formulations without adjuvant or among the four formulations with adjuvant. All vaccine formulations showed a similar ratio of increase in antibody titer between day 21 and day 42, as shown by the nonsignificant interaction between vaccine formulation and day (Table 4, and Table 4 in the Supplementary Appendix).

Table 5 compares the presumed rates of seroprotection, as measured by hemagglutination-inhibition assay (i.e., the proportion of subjects with a titer ≥40) and single radial hemolysis (i.e., the proportion of subjects with an area of ≥25 mm^2 on single radial hemolysis). Numerically, the formulations without adjuvant again were more immunogenic than those with adjuvant. On single radial hemolysis, the percentage of seroprotection 21 days after the second dose of vaccine without adjuvant was 78.6% for the 7.5-μg dose and 61.0% for the 15-μg dose. Single radial hemolysis for H5N1 antibodies appeared to be more sensitive than hemagglutination-inhibition assay, since the equivalent values for hemagglutination-inhibition assay were 47.6% and 26.8%, respectively.

We also analyzed changes from baseline in results on single radial hemolysis using a mixed-model analysis of covariance for the log-transformed values, and the results were similar to those obtained for the virus-neutralization titers. Again, we observed a significant effect of the

Table 4. Mixed-Model Analysis of Log-Transformed Values of Virus-Neutralization Titer.

Effects and Comparison	A/Vietnam/1203/2004 (Clade 1)	A/Indonesia/05/2005 (Clade 2)	A/Hong Kong/156/1997 (Clade 3)
		P Value	
Effect			
Vaccine formulation	0.004	0.001	0.01
Day 21 vs. day 42	<0.001	<0.001	<0.001
Baseline	<0.001	<0.001	<0.001
Sex	0.009	0.08	0.01
Age	0.41	0.18	0.03
Vaccine formulation–day interaction	0.06	0.36	0.01
Comparison			
With adjuvant vs. without adjuvant	<0.001	<0.001	<0.001
Without adjuvant, 7.5 μg vs. 15 μg	0.80	0.97	0.70

vaccine formulations, with formulations without adjuvant showing higher response rates than those with adjuvant. There was no significant difference between the two formulations without adjuvant or among the formulations with adjuvant (Table 4, and Table 5 in the Supplementary Appendix).

CROSS-NEUTRALIZATION

The 7.5-μg and 15-μg formulations without adjuvant showed high levels of cross-reactivity against the A/Hong Kong strain (76.2% and 78.0%, respectively, with a neutralizing titer of ≥1:20) (Table 2). The responses against the clade 2 strain were somewhat lower (with rates of a virus-neutralization titer of ≥1:20 of 45.2% and 36.6% for the 7.5-μg and 15-μg formulations without adjuvant, respectively) (Table 2).

We also analyzed the virus-neutralization response to the heterologous strains using the mixed model. Results were similar to those for the homologous strain. Formulations without adjuvant elicited significantly higher immune responses than those with adjuvant. Antibody titers increased significantly from baseline, independently of the vaccine dose (Table 4, and Tables 3 and 4 in the Supplementary Appendix).

The reverse cumulative distribution curves for antibody titers after the first and second doses of vaccine against all three strains support the finding of higher immunogenicity from the formulations without adjuvant (Fig. 2). Analysis of rates of seroprotection with homologous and heterologous immune responses showed results that were consistent with those obtained by direct analysis of values of virus-neutralization titers and single radial hemolysis (Tables 6 and 7 in the Supplementary Appendix).

DISCUSSION

It has been reported that whole-virus trivalent influenza vaccines are more immunogenic than subvirion vaccines but are also more prone to cause adverse reactions.[5] In our study, a monovalent whole-virus H5N1 vaccine had a side-effect profile similar to that of subvirion H5N1 formulations described previously.[2,3,16] Most important, the low rate of fever among subjects in our study (2 to 7%) compares favorably with that reported both for subvirion H5N1 vaccines and for an egg-derived whole-virus H5N1 vaccine with adjuvant.[2,3,6,16] However, it should be noted that reporting systems and characteristics of the subjects differ among the various studies.

With respect to immunogenicity, the highest neutralizing-antibody response after the second dose of vaccine (76.2%) was obtained with the 7.5-μg formulation without adjuvant, which was equivalent to a rate of seroconversion of 69.0% and represented an increase by a factor of 4 or more in the neutralization titer after two doses of vaccine (Tables 2 and 3). These data are also similar to the levels of immunogenicity reported in a study of an egg-derived whole-virus H5N1 vaccine, in which 96% of subjects who received

Table 5. **Antibody Response to the Homologous Virus Strain after the First and Second Doses of Vaccine.***

Dose with or without Adjuvant	Assay	Seroprotection			Seroconversion		GMI	
		Day 0	Day 21	Day 42	Day 21	Day 42	Day 21	Day 42
		percent (95% CI)					*value (95% CI)*	
3.75 μg with adjuvant	HI	2.4 (0.1–12.6)	33.3 (19.6–49.5)	40.5 (25.6–56.7)	33.3 (19.6–49.5)	38.1 (23.6–54.4)	2.7 (1.7–4.4)	4.5 (2.4–8.4)
	SRH	4.8 (0.6–16.2)	26.2 (13.9–42.0)	50.0 (34.2–65.8)	21.4 (10.3–36.8)	47.6 (32.0–63.6)	1.7 (1.2–2.3)	2.9 (2.0–4.2)
7.5 μg with adjuvant	HI	4.8 (0.6–16.2)	35.7 (21.6–52.0)	38.5 (23.4–55.4)	35.7 (21.6–52.0)	35.9 (21.2–52.8)	3.2 (1.9–5.4)	3.6 (1.9–6.8)
	SRH	4.8 (0.6–16.2)	26.2 (13.9–42.0)	35.9 (21.2–52.8)	21.4 (10.3–36.8)	33.3 (19.1–50.2)	1.7 (1.2–2.3)	2.3 (1.5–3.4)
7.5 μg without adjuvant	HI	0.0 (0.0–8.4)	47.6 (32.0–63.6)	47.6 (32.0–63.6)	47.6 (32.0–63.6)	47.6 (32.0–63.6)	4.5 (2.7–7.6)	5.3 (3.0–9.5)
	SRH	7.1 (1.5–19.5)	69.0 (52.9–82.4)	78.6 (63.2–89.7)	61.9 (45.6–76.4)	73.8 (58.0–86.1)	4.8 (3.2–7.2)	6.3 (4.3–9.1)
15 μg with adjuvant	HI	0 (0.0–8.2)	14.0 (5.3–27.9)	14.6 (5.6–29.2)	14.0 (5.3–27.9)	14.6 (5.6–29.2)	1.5 (1.1–2.2)	1.7 (1.1–2.7)
	SRH	4.7 (0.6–15.8)	16.3 (6.8–30.7)	39.0 (24.2–55.5)	11.6 (3.9–25.1)	36.6 (22.1–53.1)	1.4 (1.1–1.8)	2.2 (1.6–3.2)
15 μg without adjuvant	HI	0 (0.0–8.2)	25.6 (13.5–41.2)	26.8 (14.2–42.9)	25.6 (13.5–41.2)	26.8 (14.2–42.9)	2.8 (1.6–4.9)	3.2 (1.7–6.0)
	SRH	2.3 (0.1–12.3)	41.9 (27.0–57.9)	61.0 (44.5–75.8)	39.5 (25.0–55.6)	58.5 (42.1–73.3)	2.8 (1.9–4.2)	4.7 (3.1–7.1)
30 μg with adjuvant	HI	0 (0.0–7.7)	34.8 (21.4–50.2)	36.4 (22.4–52.2)	34.8 (21.4–50.2)	36.4 (22.4–52.2)	3.4 (2.0–5.7)	4.5 (2.4–8.6)
	SRH	2.2 (0.1–11.5)	21.7 (10.9–36.4)	58.1 (42.1–73.0)	19.6 (9.4–33.9)	58.1 (42.1–73.0)	1.5 (1.2–2.0)	3.6 (2.5–5.2)

* GMI denotes geometric mean of the increase, HI hemagglutination-inhibition assay, and SRH single radial hemolysis.

two doses of 5-μg or 10-μg formulations had a neutralization titer of 1:20 or more,[6] although differences in assay systems must be taken into account in making such direct comparisons.

Lower rates of seroprotection and seroconversion (as defined in the guidelines of the Committee for Proprietary Medicinal Products[17]) were obtained with the hemagglutination-inhibition assay than with the virus-neutralization assay, which supports the finding that the hemagglutination-inhibition assay is less sensitive for detection of anti-H5 antibodies, as reported previously.[10,11] In our study, single radial hemolysis, which is considered to have a sensitivity equivalent to that of the hemagglutination-inhibition assay for seasonal influenza strains,[18] was shown to be more sensitive than the hemagglutination-inhibition assay for H5N1.

The lack of enhancement of vaccine immunogenicity by the use of alum adjuvant at the doses studied here was consistent with data from a previous study, which showed that no effect of alum adjuvant was seen with a 15-μg dose of subvirion vaccine, and a 7.5-μg formulation without alum was more immunogenic than the formulation with adjuvant.[3] In the previous study, an enhanced immune response with the use of alum was seen only with the 30-μg formulation. We did not investigate this dose without alum in our study.

However, other studies have described substantial positive effects of other adjuvants on H5N1 immunogenicity. The use of an oil-in-water–based emulsion in a 3.8-μg dose of split-virus vaccine resulted in 82% seroconversion, as compared with 4% seroconversion without adjuvant.[16] The addition of another oil-in-water–based adjuvant (MF-59) to an H5N3 vaccine was also associated with a substantial increase in antibody response.[19]

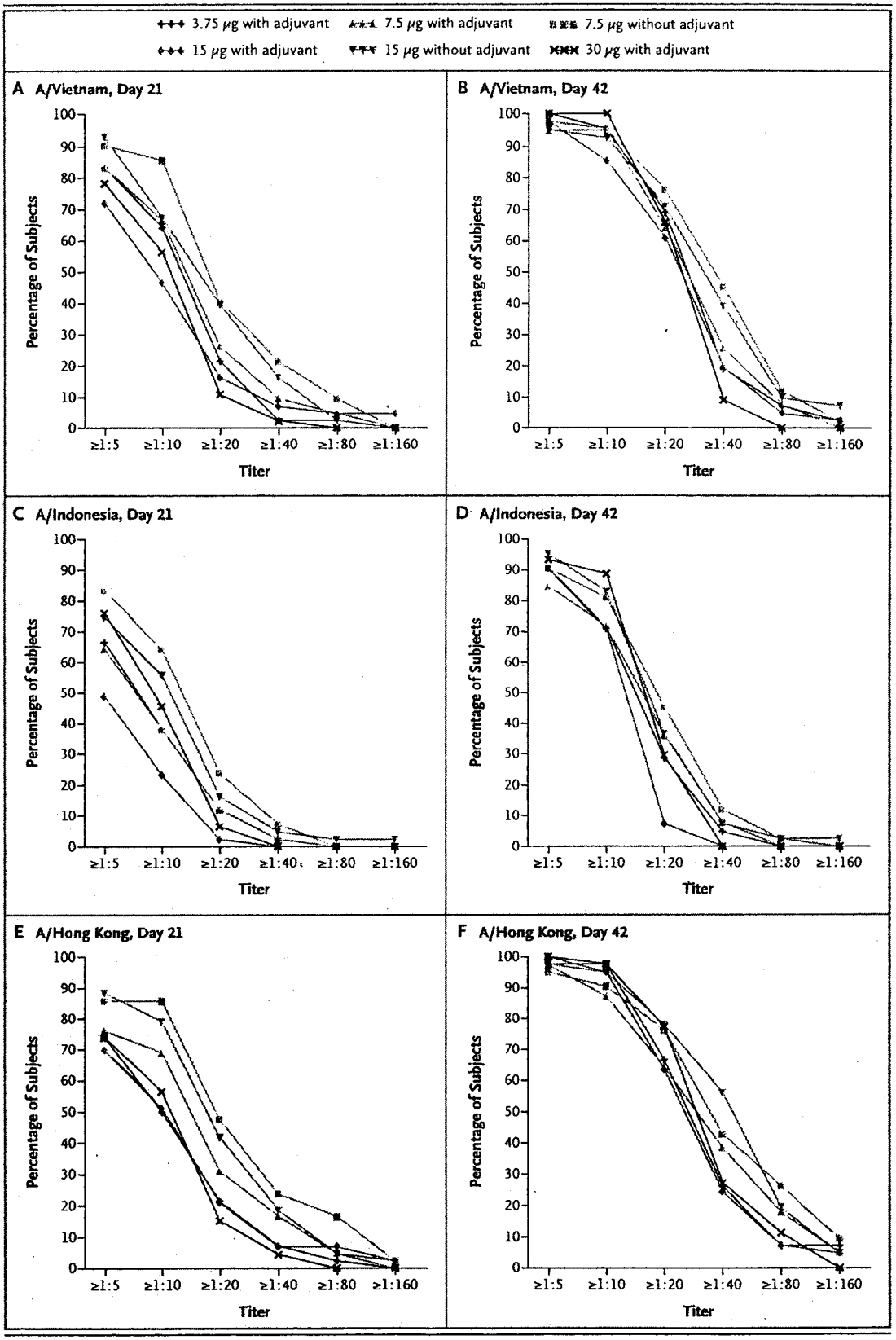

Figure 2 (facing page). **Reverse Cumulative Distribution Curves for Titers of Neutralizing Antibodies in Six Study Groups after the First and Second Doses of Vaccine against Three Strains of Avian Influenza.**

Shown are the percentages of subjects with specific virus-neutralization titers after the first dose (day 21) and second dose (day 42) of vaccine against A/Vietnam/1203/2004 (clade 1) (Panels A and B, respectively), A/Indonesia/05/2005 (clade 2) (Panels C and D, respectively), and A/Hong Kong/156/1997 (clade 3) (Panels E and F, respectively).

Our data also showed that the whole-virus clade 1–based vaccine can induce a substantial cross-neutralizing response against clade 2 and clade 3 strains. The results described in Table 2 are encouraging: after two doses of 7.5-μg of the formulation without adjuvant, the proportions of subjects with neutralizing titers of 1:20 or more were 45% of those immunized against the clade 2 Indonesia strain and 76% of those immunized against the clade 3 Hong Kong strain. However, there is no available evidence to indicate which neutralizing titer is sufficient to confer protection. Most studies of H5N1 split-virus and whole-virus vaccines have not described attempts to determine the cross-reactivity of antibodies to other H5N1 virus strains. However, a recent study of a novel split-virus vaccine with adjuvant also showed high levels of cross-neutralization against a clade 2 strain.[16] In addition, in a study involving 15 subjects, two doses of an H5N3 vaccine with MF-59 as adjuvant induced intermediate levels of cross-reactivity to antigenically distinct H5N1 strains, and three doses induced high levels of cross-reactivity.[20]

The apparent absence of a dose–response relationship in our study may be surprising. However, it is in agreement with a number of studies of vaccine for pandemic influenza. Leroux-Roels et al. reported no relationship between the dose of antigen and the neutralizing-antibody response for H5N1 formulations with adjuvant,[16] and there appeared to be an inverse dose–response relationship with respect to responses to the clade 2 strain. A number of other studies involving other pandemic-strain vaccines — H9N2,[21] H5N3,[19] and H2N2[22] — have shown no dose–response relationship or even a reduced response at higher doses. The reasons for these findings are unclear, but at least with respect to vaccines with adjuvant, it has been speculated that the ratio of adjuvant to antigen may be critical in determining the immune-enhancing effect rather than the antigen concentration alone.[19] For other viral vaccines, particularly those with soluble proteins, it has been reported that there are distinct dose–response relationships for induction of various cytokines. In many studies, responses similar to those mediated by type 2 helper T cells have been elicited at low doses of vaccine, and responses similar to those mediated by type 1 helper T cells have been elicited at higher doses.[23] Further studies focusing on T-cell responses will be required to investigate this phenomenon. In addition, these studies will be extended by the use of antigen doses lower than 3.75 μg to confirm and extend the results obtained in our study.

Our study provides initial safety and immunogenicity data for a whole-virus H5N1 vaccine produced on Vero cell culture. It also shows that a broadly reactive immune response to clade 2 and clade 3 of H5N1 virus can be obtained with the use of a low-dose clade 1 vaccine without adjuvant. Since we observed no significant dose–response relationship, the 7.5-μg formulation without adjuvant has been chosen for further development.

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REFERENCES

1. Wood JM. Selection of influenza vaccine strains and developing pandemic vaccines. Vaccine 2002;20:Suppl 5:40-4.
2. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med 2006;354:1343-51.
3. Bresson JL, Perronne C, Launay O, et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 2006;367:1657-64.
4. Wood JM. Developing vaccines against pandemic influenza. Philos Trans R Soc Lond B Biol Sci 2001;356:1953-60.
5. Nicholson KG, Tyrrell DAJ, Harrison P, et al. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. J Biol Stand 1979;7:123-36.
6. Lin J, Zhang J, Dong X, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: phase 1 randomised controlled trial. Lancet 2006;368:991-7.
7. Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005;23:2943-52.
8. Kistner O, Howard K, Spruth M, et al. Cell culture (Vero) derived whole-virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses. Vaccine 2007;25:6028-36.
9. Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F. Development of a mammalian cell (Vero)-derived candidate influenza virus vaccine. Vaccine 1998;16:960-8.
10. Rowe T, Abernathy RA, Hu-Primmer J, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol 1999;37:937-43.
11. Lu BL, Webster RG, Hinshaw VS. Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect Immun 1982;38:530-5.
12. Stephenson I, Wood JM, Nicholson KG, Charlett A, Zambon MC. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. Virus Res 2004;103:91-5.
13. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493-7.
14. Schild GC, Pereira MS, Chakraverty P. Single-radial-hemolysis: a new method for the assay of antibody to influenza haemagglutinin: applications for diagnosis and seroepidemiologic surveillance of influenza. Bull World Health Organ 1975;52:43-50.
15. Fitting a model that contains a random effect. In: Collett D. Modeling binary data. Boca Raton, FL: Chapman & Hall/CRC Press, 1991:202-12.
16. Leroux-Roels I, Borkowski A, Vanwolleghem T, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 2007;370:580-9.
17. Committee for Proprietary Medicinal Products. Note for guidance on harmonisation of requirements for influenza vaccines. London: European Agency for the Evaluation of Medicinal Products, 1997. (Publication no. CPMP/BWP/214/96.)
18. Wood JM, Gaines-Das RE, Taylor J, Charkraverty P. Comparison of influenza serological techniques by international collaborative study. Vaccine 1994;12:167-74.
19. Nicholson KG, Colegate AE, Podda A, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001;357:1937-43.
20. Stephenson I, Bugarini R, Nicholson KG, et al. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J Infect Dis 2005;191:1210-5.
21. Stephenson I, Nicholson KG, Glück R, et al. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. Lancet 2003;362:1959-66.
22. Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. Virus Res 2004;103:163-71.
23. Constant SL, Bottomly K. Induction of TH1 and TH2 CD4+ T cell responses: the alternative approaches. Annu Rev Immunol 1997;15:297-322.

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識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008 年 6 月 4 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Identification of trimeric peptides that bind porcine parvovirus from mixtures containing human blood plasma. Heldt, C.L. et al, Biotechnol. Prog., 24, 554-560 (2008).	公表国 カナダ	
販売名（企業名）						

研究報告の概要

本稿では，ヒト血漿溶液から従来の除去法で十分に取り除けなかったパルボウイルス（粒径が小さい非エンベロープウイルス）を除去する新たな手法について記載する。固相法で三アミノ酸残基よりなるペプチドライブラリーを樹脂上に合成し，種々の溶液に添加したブタパルボウイルス（PPV）への選択的な結合能を有するペプチドをスクリーニングした。カラムから溶出した PPV は，その後の感染性検査で感染能を測定した。その結果，16 種のペプチドを単離し，配列決定したところ，塩基性アミノ酸の存在が PPV 結合に非常に重要であることが判明した。PPV をリン酸緩衝生理食塩水（PBS）に混ぜた場合，すべてのペプチドが最初の 9 カラム体積でウイルスを完全に除去した。著者らは，この方法が浄水への適用に有用と示唆している。それに対してウイルスを 7.5%のヒト血漿（蛋白質 5 mg/mL）を含有する溶液に添加した場合には，アミノ酸配列がトリプトファン－アルギニン－トリプトファン（WRW）の場合のみ，最初の 3 カラム体積で検出可能な PPV を除去することができた。しかし、その後血漿はペプチド樹脂へのウイルスの結合を阻害し始めた。本手法は，改良の余地があるものの，短いペプチドを特定のウイルスの除去プロセスに利用できることを示している。

使用上の注意記載状況・その他参考事項等

BYL-2008-0333

報告企業の意見	今後の対応
本研究は，まだ対処されていない非エンベロープウイルスに対する効果的な病原体除去法に対応し得る新規の手法が述べられている。	現時点で新たな安全対策上の措置を講じる必要はないと考えるが，今後も利用可能な本手法に関する情報収集に努める。

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Identification of Trimeric Peptides That Bind Porcine Parvovirus from Mixtures Containing Human Blood Plasma

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Virus contamination in human therapeutics is of growing concern as more therapeutic products from animal or human sources come into the market. All biopharmaceutical processes are required to have at least two distinct viral clearance steps to remove viruses. Most of these steps work well for enveloped viruses and large viruses, whether enveloped or not. That leaves a class of small non-enveloped viruses, like parvoviruses and hepatitis A, which are not easily removed by these typical steps. In this study, we report the identification of trimeric peptides that bind specifically to porcine parvovirus (PPV) and their potential use to remove this virus from process solutions. All of the trimeric peptides isolated completely removed all detectable PPV from buffer in the first nine column volumes, corresponding to a clearance of 4.5−5.5 log of infectious virus. When the virus was spiked into a more complex matrix consisting of 7.5% human blood plasma, one of the trimers, WRW, was able to remove all detectable PPV in the first three column volumes, after which human blood plasma began to interfere with the binding of the virus to the peptide resin. These trimer resins removed considerably more virus than weak ion exchange resins. The results of this work indicate that small peptide ligand resins have the potential to be used in virus removal processes where removal of contaminating virus is necessary to ensure product safety.

1. Introduction

The removal of viruses, pathogenic microorganisms, and toxins is an important problem in the growing area of human therapeutics. Every year, more therapeutic products are produced from animal, human, or cell culture sources (*1*), and these sources contain an inherent risk of viral contamination. Therapeutic products from human blood plasma, antibodies, albumin, and factor VIII, just to name a few, could be infected with human immunodeficiency virus (HIV), hepatitis B, B19 virus (formally known as parvovirus B19), SARS coronavirus, or one or more emerging viruses that have yet to be identified (*2*). Cell cultures are often contaminated with retrovirus particles, belonging to the family of viruses that include HIV (*3*). Cell culture lines often used in the production of human antibodies may contain viruses such as murine parvovirus (MVM) or cytomegalovirus (*4*). While this contamination has been greatly reduced since the requirement of strict characterization of cell culture lines and the careful screening of human plasma donors (*3*), the risk of low levels of contamination still exists.

The FDA requires that any process that uses materials from living sources must have two viral clearance steps to lower the risk of contamination (*5*). These steps must demonstrate a distinct mechanism of virus clearance and achieve a minimum of 4 log removal, or 99.99%. There are two broad categories for viral clearance, inactivation and removal (*6*). Inactivation is often performed toward the beginning of the purification of a therapeutic and could involve a lowering of pH or heating of the product. Both of these processes work well against enveloped viruses, but caution must be taken to not harm the desired protein product. Virus removal is often done at the end of a process and most commonly involves nanofiltration of the final product directly before formulation. Nanofiltration works well for viruses of large size. However, small viruses like parvoviruses are often of approximately the same size as the protein product, making it difficult to separate them by filtration (*2*). Complete removal of small non-enveloped viruses with 20 nm pore size filters has been accomplished, but there is significant fouling of these small-pored membranes that can lead to reductions in production rates (*7*, *8*). Filtration of parvoviruses has been improved by flocculation of the virus particles through addition of cationic polymers (*9*) or amino acids (*10*), which allows the use of larger pore membranes that do not foul as quickly. Virus removal can also be accomplished using functionalized membrane surfaces. Quaternary amine groups have been attached to membrane surfaces to facilitate the removal of viruses through an ion exchange mechanism (*11*−*13*). Viral clearance validation may be achieved by conducting spiking experiments on normal process steps used in the purification of a therapeutic (i.e., chromatography columns, precipitation) (*14*), but care should be taken if a chromatography step is to be used concurrently as a viral clearance step and a protein purification step. If both the virus and the protein bind to the resin, it is possible for viruses to accumulate in the column. Without proper cleaning, the virus may elute from the column in subsequent batches and contaminate the therapeutic product (*14*).

Affinity adsorption is rarely used to remove viruses from process streams because the most common affinity ligands for viruses are antibodies. Antibodies are expensive to produce, often cannot withstand the harsh conditions required for the

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cleaning of process equipment, and carry an inherent risk of being contaminated with viruses in their own right (*3*). However, affinity adsorption has been applied to the reduction of infectious prions from blood (*15*, *16*), and a small peptide ligand has been found to remove staphylococcal enterotoxin B, a small toxin, from *E. coli* lysate (*17*). Small peptides are more robust than antibodies, and they are also less expensive and can be chemically synthesized, eliminating the risk of virus contamination. Peptides can handle the cycling of production and cleaning much better than antibodies, and by using small peptides, there is no three-dimensional structure that may be destroyed during processing. In this work, several trimeric peptides have been discovered that remove PPV from phosphate buffered saline containing as high as 7.5% human blood plasma. The peptides were found when a synthetic, solid-phase combinatorial library was screened for ligands that bind to porcine parvovirus (PPV). Solid-phase libraries allow screening directly on the chromatographic support that will be used as the separation media and have been successful in the discovery of many affinity peptide ligands (*18*−*21*). The discovered peptides can completely remove any detectable PPV from PBS and completely remove any detectable PPV from the first 3 column volumes when 7.5% human plasma is present. This work demonstrates that small peptides may offer a novel and effective method for removing viruses from complex mixtures.

2. Materials and Methods

2.1. Materials. Phosphate buffered saline (PBS) containing 0.01 M phosphate, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4 was purchased from Sigma (St. Louis, MO), and human blood plasma was a donation from the American Red Cross (Rockville, MD). Amino acids, phenol red, sodium carbonate, sodium phosphate, glucose, calcium chloride, sodium chloride, potassium chloride, and magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA). Eagles Minimum Essential Media (EMEM) was purchased from Quality Biologicals (Gaithersburg, MD). MEM vitamins, sterile PBS, trypsin, gentamicin, and glutamine, all for cell culture, were purchased from Invitrogen (Carlsbad, CA).

2.2. Virus Propagation and Titration. The porcine parvovirus (PPV) NADL-2 strain was titrated and propagated on porcine kidney (PK-13) cells, which were a gift from the American Red Cross (Rockville, MD). The PK-13 cells were maintained and the PPV propagated as described in Heldt et al. (*22*) using complete media, which consisted of EMEM supplemented with 2 mM glutamine, 1x gentamicin, and 10% non-heat-inactivated fetal calf serum (Hyclone, Logan, UT). Upon propagation of the virus, the cell culture flasks were frozen at −20 °C and thawed at room temperature. The cells were then scraped from the flask, and the solution was clarified by centrifugation at 3000 rpm for 10 min in an IEC Centra CL2 centrifuge (Thermo Electron, Waltham, MA). This solution was then stored at −80 °C until further use.

Radioactive PPV was prepared by metabolically incorporating a radiolabel during propagation by addition of ^{35}S methionine and cysteine to the cell culture media. This was done by seeding the cells at 6×10^5 cells per 75 cm^2 flask. The next day, the flask was infected with 10^3 MTT units of PPV in 1 mL of PBS. An MTT unit was defined as the concentration of virus where 50% of the cells were considered viable, as determined by the metabolic cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) as previously described (*22*). The MTT concentration can be observed optically and quantified by spectrophotometry. The flasks were placed in the incubator at 5% CO_2, 37 °C, and 100% humidity for 1 h. At this time, 5 mL of complete media was added to the flask, which was returned to the incubator. The next day, the media was removed from the flask and starvation media was added, which contained the same amino acids and essential nutrients as the EMEM, except for methionine. The cells were exposed to 5 mL of this starvation media for 1.5 h, and then EasyTag Protein Labeling mixture (Perkin-Elmer, Waltham, MA) was added to a final concentration of 50 μCi/mL. The cells were frozen at −20 °C when approximately 90% cytopathic effect was observed, usually after 4−5 days. Virus purification was done by CsCl gradient centrifugation, as described elsewhere (*22*), after which solutions were dialyzed against PBS for 3 days at 4 °C, stored at 4 °C, and used within 2 weeks.

All infectivity measurements were made using the MTT assay, which previously has been correlated to a $TCID_{50}$ (50% tissue culture infectious dose), a common method for the titration of infectious viruses (*22*).

2.3. Primary Screening of Library. A solid-phase combinatorial trimer library was made by Peptides International (Louisville, KY) using the divide-couple-recombine technique (*23*) on Toyopearl Amino 650 EC (Tosoh Bioscience, Montgomeryville, PA). The library had an alanine and 2 mini-PEG spacer arms [Toyopearl resin-Ala-$(COCH_2$-$(OCH_2CH_2)_2$-$NH)_2$-X-X-X], where X is any naturally occurring amino acid except cysteine or methionine. The library was swelled in 20% methanol in DI water overnight and then buffer exchanged with PBS three times, with the last buffer exchange being overnight. Ten milligrams of dry library was taken and mixed with 50% human blood plasma in PBS for 1 h. ^{35}S-Labeled PPV was added to the library at 10,000 CPM (about 1×10^{-3} μCi) and allowed to equilibrate for about 1.5 h. The library was then placed in a disposable 10 mL fritted column (Bio-Rad Laboratories, Hercules, CA). The beads were washed with PBS followed by PBS containing an additional 1 M NaCl or KCl until no radioactivity could be detected from the wash. The beads were washed again in PBS to remove excess salt and then put into 20 mL of 1% low melt agarose (Bio-Rad Laboratories, Hercules, CA). This was poured onto a 160 mm × 180 mm GelBond (BioWhittaker Inc, Walkersville, MD) and allowed to dry for 3 days. Kodak BioMax MR Film (Kodak, Rochester, NY) was placed onto the dried gel for 10 days and developed with a Konica Medical Film Processor (Tokyo, Japan). A proprietary ligand found by the American Red Cross that binds to PPV (positive control) and a negative control of Amino 650M were used as markers to line up the film and the gel for visualization of radioactive beads. Positive beads were excised from the gel, boiled in water for 10 min each, and vortexed and the water changed for a total of three repeats to remove the agarose and the bound PPV from the beads. The beads then were sent to the Texas A&M Protein Laboratory (College Station, TX) for sequencing by Edman degradation.

2.4. Chromatography to Verify Screening Results. Peptide resins were synthesized on Toyopearl Amino 650M resin (Tosoh Bioscience, Montgomeryville, PA) by Peptides International (Louisville, KY) and were packed into disposable PIKSI columns (ProMetic Biosciences Ltd, Cambridge, England) with a total of 0.5 mL of settled resin in PBS per column. A Rainin (Oakland, CA) 8-channel peristaltic pump was used to add a solution of PPV supernatant in either PBS or 7.5% human blood plasma in PBS, at a rate of 0.1 mL/min. Ten 0.5 mL fractions were collected and tested for infectivity using the MTT assay and compared to the titer of the starting material before addition to the column.

2.5. Acetylated Control. The acetylated control was made by the acetylation of Toyopearl Amino 650M resin. About 50 mL of settled resin was added to a sintered glass funnel and allowed to drain. The resin was washed three times with 100 mL of 0.1 M NaOH. The resin was then washed with deionized water until the pH was below 8. The resin was placed into three separate 50 mL conical tubes and 30 mL of 0.5 M sodium acetate was added to each tube followed by end-over-end rotation for 10 min. A 100% excess of acetic anhydride (Riedel-de Haen, Germany), which amounted to a total of 755 μL, was dissolved into 3 mL of acetone, and 1.2 mL of the solution was added to each conical tube. The tubes were mixed for 2 h. The resin was then returned to the sintered glass funnel and washed three times with 100 mL of DI water, four times with 100 mL of 0.5 M NaOH, and finally at least 10 times with 100 mL of DI water, until the pH of the rinse was below 8. The acetylation was confirmed by taking 50 μL of acetylated resin, 50 μL of Toyopearl Amino 650M resin, and 50 μL of DI water and adding two drops of ninhydrin reagent, 2% solution (Sigma, St. Louis, MO). After 1−2 min the resins were observed for color change; the acetylated resin and the DI water remained yellow, whereas the amino resin turned purple.

3. Results and Discussion

3.1. Library Design. Many hexameric peptide ligands have been found that can purify proteins (*19*, *24*, *25*) and toxins (*17*). Each of these peptide ligands were selected from a hexamer library, which contains over 34 million different combinations, when 18 of the 20 naturally occurring amino acids are used for library production. It would take a tremendous amount of effort to screen all of these sequences, and it is not necessary when purification is the intended use of the ligand. In general, a purification ligand is useful if it can bind over 90% of the target protein and is specific enough to produce an eluted protein that is 80−90% pure, but for virus removal, the goal is reduction of ≥99.99% of a virus, which is at femtomolar to picomolar concentrations. To improve the possibility of finding a ligand that can accomplish this, a trimeric library was designed and screened. This library contained only 5832 different sequences and could be screened many times over to compare different screening conditions. Further, by screening the entire library, there was a greater probability that one or more strongly binding ligands would be found, which would not necessarily occur with a hexamer library.

A spacer arm of two sets of two ethylene glycol units separated by a peptide bond (*26*) (designated AEEA-AEEA by Peptides International) was added to the library to increase the chances of finding a peptide ligand that bound to a conserved area on the virus surface. This spacer arm separated the peptide approximately 15 Å from the undisclosed spacer on the Toyopearl resin. It has also been shown that hydrophilic ethylene glycol does not bind proteins and makes a flexible yet inert spacer arm that allows movement of the ligand, improving binding (*27*−*29*). A surface map of PPV shows that there are canyons on the surface of the virus that are approximately 15 Å in depth (*30*), and so the spacer arm was designed to allow the peptide to reach into the depths of the canyons. For most non-enveloped viruses, it is accepted that the conserved amino acid sequences are located in the depth of these canyons because these are often the location of the receptor binding sites.

3.2. Primary Screening. The library beads were originally blocked with 50% human blood plasma before the virus was added. This blocked any of the peptides that had a high affinity for plasma proteins before the addition of PPV to the library.

Table 1. Peptide Sequences Found from Primary Screening

wash	sequence	
1 M NaCl	KNY	AKL
	WRW	KTF
	KKK	VWR
	KGK	RAA
	KYY	KRR
	FVV	
1 M KCl	FRH	KHR
	KAA	RTG
	RQQ	

After incubation with PPV, the beads were washed to remove any nonspecifically bound virus. One screening run was washed in 1 M NaCl and yielded a total of 24 positive beads from about 10,000; another screening run was washed in 1 M KCl and gave a total of 9 positive beads. Only those positive beads that had a large signal to size ratio (i.e., a small bead that gave a large signal), as determined by visual inspection, were chosen for sequencing. The results of the returned sequences are shown in Table 1. To better determine the significance of the different chemical groups, the amino acids were counted and compared to their probability of random occurrence (Table 2). A random occurrence was determined as the number of amino acids in the chemical group divided by the number of different amino acids in the library and then multiplied by the total number of amino acids found from the sequencing. For example, there are five different aliphatic amino acids, so the random number of aliphatic amino acids is calculated by dividing 5 amino acids the by the 18 different amino acids used in this study, and the result multiplied by the 48 total amino acids in the 16 trimers found by screening. This gives the random occurrence of aliphatic amino acids of 13.3 indicated in Table 2. If the number of amino acids from a certain chemical group was close to the random occurrence number, then it was suspected that the chemical group was just randomly found and may have little to do with the binding of the virus. However, if the number was much higher than that expected to occur randomly, then that group was considered to be significant in the binding of the virus.

Table 2 shows the importance of basic amino acids in the binding of PPV and, to complement this, the lack of acidic groups associated with ligands found to bind to PPV. The results show that positive charges are important for the binding of virus. This is an expected result because canine parvovirus, a related parvovirus, has an isoelectric point of 5.3 (*31*), leaving it negatively charged at physiological pH, which corresponded to the conditions used in the screening studies. There was a random distribution of aromatic and aliphatic groups with seven sequences that contained aromatics and seven sequences that contained aliphatics. The sequences were then categorized into the following: those containing an aromatic amino acid, those containing an aliphatic amino acid, and those containing neither. Since all but one sequence contained a basic group, all sequences chosen for further screening contained a basic amino acid. From these categories, five sequences were chosen for additional screening using column chromatography: WRW and KYY, which contain aromatics; RAA, which contains an aliphatic; and KHR, which contains a histidine. Also, KKK and KRR were combined to form KRK, which contains basic residues.

3.3. Column Chromatography. The resins were packed into disposable columns and tested for breakthrough of PPV in the eluent using infectivity as the enumeration method. First, cell culture supernatant containing PPV was diluted with PBS to a final titer of about 6−7 log (MTT/mL) (approximately a 1:100

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Table 2. Chemical Characterization of Sequences

amino acid type	random[a]	actual[b]
aliphatic	13.3	11
cyclic imino	2.7	0
imidazole	2.7	2
basic	5.3	21
aromatic	8.0	9
hydroxy	5.3	2
amide	5.3	3
acidic	5.3	0

[a] The number of amino acids expected from a random distribution of amino acids in 16 trimeric peptides. [b] The number of each type of amino acid that was present in the 16 trimeric peptides.

to 1:10 dilution) and was filtered through a 0.22 μm filter. Virus spiking studies should not be carried out at a dilution more concentrated than 1:10, as the virus solution, which contains contaminants from the cell culture from which it was created, may start to interfere with the virus clearance mechanism (*3*). However, the larger the initial viral load, the better the opportunity to validate a high degree of virus clearance. Virus clearance was calculated in accordance with the expression

$$\log \text{clearance} = -\log\left(\frac{\text{virus detected after clearance step}}{\text{total virus load}}\right) \quad (1)$$

The PPV breakthrough curves were determined by pumping virus-spiked solutions onto the peptide columns at 0.1 mL/min. Fractions equivalent to 1 column volume were collected, for a total of 10 column volumes, and the amount of PPV in the flowthrough fractions was determined. The results are plotted in Figure 1 as the percentage of the detectable PPV as a function of column volume. Presenting the results in terms of a percentage of the detectable clearance automatically accounts for the different initial virus titers of the various batches analyzed in these experiments.

In PBS, all of the resins were able to clear completely the detectable virus available in the solutions, as shown in Figure 1. This is in contrast to the amino resin control (with no peptides, which is considered a weak ion-exchange resin) that was not able to remove any significant amounts of PPV from PBS. Clearly, the peptides were responsible for the binding of the virus, and nonspecific binding to the resin surface was ruled out.

The small peptide resins have the ability to remove viruses from simple solutions such as water, suggesting potential application to water treatment. In fact, microfiltration is being considered as an alternative to chlorine treatment of water supplies (*32*), but the method suffers from many of the same difficulties as nanofiltration for therapeutic processes. Small viruses, like hepatitis A virus, which has a diameter of 27−32 nm (*33*), and norovirus, with a diameter of 30−40 nm (*34*), are able to pass through many nanofiltration and all microfiltration membranes. These viruses are shed in the feces of infected humans and are common contaminants of water supplies. Small peptide ligands theoretically could be placed on microfiltration membranes to improve virus removal without the need to use membranes of small pore size which often cause fouling (*8*) and may require high back pressures.

To challenge the peptides for their ability to remove PPV under therapeutic processing conditions, virus-spiked 7.5% human blood plasma was used. A 7.5% human blood plasma solution contains about 5 mg/mL of protein, which is approximately the amount of protein that can be found in a therapeutic protein product. There are two general viral clearance steps in a monoclonal antibody production process, i.e., a low

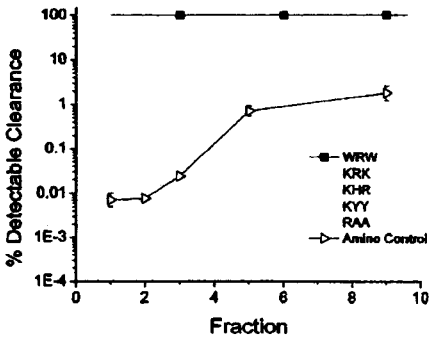

Figure 1. Binding of trimeric peptides to PPV in PBS. All ligands were able to bind 100% of the detectable PPV. The amino control, which is a weak ion-exchange resin, was able to clear less than 1% of the detectable PPV. Columns were run in duplicate, and the error bars represent the detectable clearance of each column.

pH step after cell harvest and a nanofiltration step before or during formulation (*35*). If effective, small peptides theoretically could be used on membranes on the nanofiltration step at the end of the process. All of the peptides were challenged with protein loads similar to those that would be found at this phase of the purification process.

Data on the removal of PPV from this complex mixture using the peptides are presented in Figure 2. All of the resins tested, except for KHR, were able to remove all of the detectable PPV in the first column volume. All of the resins had a breakthrough of PPV before the fifth column volume. It is believed that the proteins found in human blood plasma also began to nonspecifically bind to the peptides at this point, leaving less peptide available for specific binding to PPV. There may be a way to improve the selectivity of the virus over the plasma proteins by optimization of the chromatography process through changes in peptide density or buffer ionic strength.

The amino control resin had the ability to bind to some of the PPV and remove it from solutions. In the first two column volumes in PBS and 7.5% human blood plasma, the amino control achieved about 1 log clearance. This is not surprising, as anion exchange columns are often tested for their ability to clear viruses (*35*). It has been shown that a Q-Sepharose column was able to clear 3 log of PPV when loaded at pH 6.5 (*36*) and as high as 5 log of MVM when loaded in Tris buffer at pH 8.0 (*14*). This follows the trend that increasingly basic solutions will make the virus surface more negatively charged, which would cause increasingly stronger binding of the virus to an anion exchange column. The control in our experiments was a weak ion-exchange resin at pH 7.4, which showed clearance lower than that seen with the Q-Sepharose columns, as would be expected.

An increase in binding of virus over time was found with the amino control resin both in the presence and absence of human blood plasma (Figures 1 and 2). The PPV solution used in the experiments was cell culture supernatant that had been clarified by low-speed centrifugation and filtered through a 0.22 μm filter, and so the solution contained cellular debris. Since the amino control is a weak ion-exchange resin, it is possible that the resin was binding the cellular debris, and the virus then bound to the debris on the resin, as many proteins in host cell proteins are known to be negatively charged and removed by anion exchange (*35*). Since the amino resin has a high positive charge density, it is also possible that these cellular proteins may denature onto the surface, as has been suspected in ion exchange purification (*37*, *38*) and is known to happen when proteins adsorb to surfaces (*39*). Protein denaturation was also

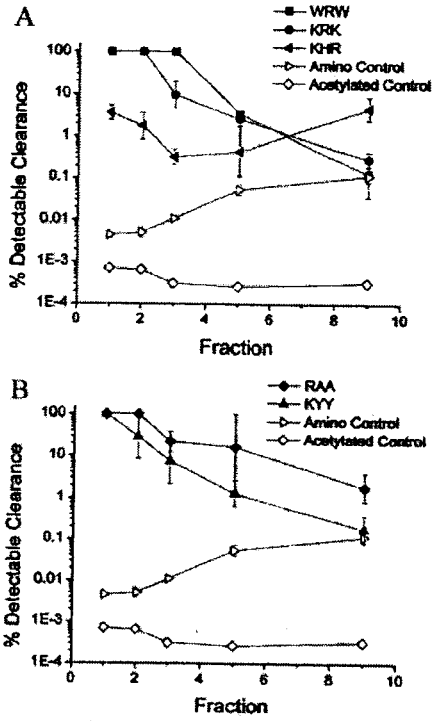

Figure 2. Binding of trimeric peptides to PPV in 7.5% human blood plasma. WRW was able to bind 100% of the detectable PPV in the first 3 column volumes. After 9 column volumes, WRW was still able to bind as much as the amino control. The amino control and acetylated control are the same in each figure as a reference point for comparison of the different peptide resins. Columns were run in duplicate, and the error bars represent the detectable clearance of each column.

a possibility, as this was observed in the purification of tumor necrosis factor-α using an ion exchange column (*37*). The longer these host cell proteins are retained on the column, the greater the potential for protein denaturation, and this may provide different binding sites for the virus. Virus binding to host cell proteins is confirmed by the fact that when highly purified virus suspensions containing less than 100 μg/mL of total protein are used in resin challenge, the amino control resin binds no more than 1 log of PPV even after 10 column volumes (data not shown). Viruses are also known to easily aggregate (*40*), and so the presence of denatured protein could become a new binding surface for the virus.

The trimer KHR was able to achieve 4 logs clearance in the first column volume but still left in solution 3 log (MTT/mL) of virus. This peptide column showed the same decrease in viral clearance in the first three column volumes seen with the other peptide resins, but in subsequent column volumes, it exhibited an increase in detectable clearance. This latter behavior was only seen in the amino control resin and not the other peptide resins. It is suspected that this resin may be causing denaturation of proteins in solution as discussed above, but this issue was not examined further. This resin was just discarded as one of the lead candidates, as its performance as a viral clearance ligand was unacceptable.

The trimers WRW, KRK, RAA, and KYY all exhibited breakthrough of PPV in the presence of plasma proteins after the first three column volumes (Figure 2). There was no detectable cooperative binding observed for these resins in the flowthrough fractions tested. Of these resins, only WRW was able to completely clear all detectable PPV in the first three column volumes from 7.5% human blood plasma. With optimization of the peptide density and spacer length, this resin may be able to clear PPV in all nine column volumes of challenge solution containing 7.5% human blood plasma. Human blood plasma also contains many different proteins, and only one or two may be interfering with the binding of PPV. For example, if albumin is the predominant protein binding to the resin, then the peptide may be able to clear PPV very well from a solution that contains other proteins but not albumin. In this case, WRW has the potential to be used effectively for final purification of a pure protein with excellent removal efficiency.

Chromatographic beads are not the most efficient way to remove large particles from process streams. The viruses, having a diameter on the same order of magnitude as the pore diameter, have small diffusion coefficients in the pores of the beads, and viruses quickly clog the pores. Consequently, the accessible surface area of the beads is mainly associated with the outside surface of the bead, and the inner pore surface is not available for binding. Membranes have a better geometry for binding of particles such as viruses, as there are not any diffusional limitations. However, the screening of a combinatorial library of peptides is difficult to do on a membrane surface. The SPOT method, developed by Ronald Frank (*41*), is used to produce peptide libraries on a cellulose membrane surface, but if done manually, only several hundreds of peptides can be created in 2−3 days (*42*). This is a small library compared to the thousands of peptides that can be screened on chromatographic beads. In addition, the binding to a peptide on cellulose fibers may be quite different from that observed on other membrane materials. There are currently no large ligand libraries on any membrane surface that is likely to be used for large-scale virus removal. This study provides proof-of-concept that peptides have the ability to remove viruses specifically. In the future, it may be beneficial to change the geometry of the support for improved access of all of the ligands to the viral particle, but currently the bead geometry offers a better screening platform.

4. Conclusions

Small trimeric ligands that specifically bind to porcine parvovirus were isolated from a solid-phase peptide library. In PBS, 100% of detectable infectious virus was removed from solution for every fraction that was tested, up to nine column volumes. This demonstrates the potential of these peptides for use in virus removal from samples of relatively simple composition, such as for water purification applications. In more complex mixtures, such as 7.5% human blood plasma, peptide WRW was able to remove all detectable infectious viruses in the first three column volumes. This is impressive for a ligand that contains only three amino acids, as most peptide ligands are a minimum of six amino acids in length. Enhanced specificity and binding affinity may be found using an increased number of amino acids in the ligand, and this is currently being examined. Tethering one or more of the ligands to a membrane with more suitable geometry may improve virus removal efficiency from complex mixtures. The ligands could also be optimized for application to specific process streams, so that a single ligand must only compete with one therapeutic protein, thereby overcoming competitive binding and facilitating use as an efficient virus absorbent.

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References and Notes

(1) Huang, P. Y.; Peterson, J. Scaleup and virus clearance studies on virus filtration in monoclonal antibody manufacture. In *Membrane Separations in Biotechnology*; Wang, W., Ed.; Marcel Dekker: New York, 2001; pp 327−350.

(2) Kempf, C.; Stucki, M.; Boschetti, N. Pathogen inactivation and removal procedures used in the production of intravenous immunoglobulins. *Biologicals* **2007**, *35* (1), 35−42.

(3) Darling, A. Validation of biopharmaceutical purification processes for virus clearance evaluation. *Mol. Biotechnol.* **2002**, *21* (1), 57−83.

(4) Valdes, R.; Ibarra, N.; Ruibal, I.; Beldarrain, A.; Noa, E.; Herrera, N.; Aleman, R.; Padilla, S.; Garcia, J.; Perez, M. Chromatographic removal combined with heat, acid and chaotropic inactivation of four model viruses. *J. Biotechnol.* **2002**, *96* (3), 251.

(5) Food and Drug Administration. *Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use;6* U.S. Department of Health and Human Services: Rockville, MD, 1997.

(6) Cai, K.; Gierman, T. M.; Hotta, J.; Stenland, C. J.; Lee, D. C.; Pifat, D. Y.; Petteway, S. R., Jr. Ensuring the biological safety of plasma-derived therapeutic proteins. *Biodrugs* **2005**, *19* (2), 79−96.

(7) Furuya, K.; Murai, K.; Yokoyama, T.; Maeno, H.; Takeda, Y.; Murozuka, T.; Wakisaka, A.; Tanifuji, M.; Tomono, T. Implementation of a 20-nm pore-size filter in the plasma-derived factor VIII manufacturing process. *Vox Sang.* **2006**, *91* (2), 119−125.

(8) Lute, S.; Bailey, M.; Combs, J.; Sukumar, M.; Brorson, K. Phage passage after extended processing in small-virus-retentive filters. *Biotechnol. Appl. Biochem.* **2007**, *47*, 141−151.

(9) Wickramasinghe, S. R.; Han, B.; Carlson, J. O.; Powers, S. M. Clearance of minute virus of mice by flocculation and microfiltration. *Biotechnol. Bioeng.* **2004**, *86* (6), 612−621.

(10) Yokoyama, T.; Murai, K.; Murozuka, T.; Wakisaka, A.; Tanifuji, M.; Fujii, N.; Tomono, T. Removal of small non-enveloped viruses by nanofiltration. *Vox Sang.* **2004**, *86* (4), 225−229.

(11) Specht, R.; Han, B.; Wickramasinghe, S. R.; Carlson, J. O.; Czernak, P.; Wolf, A.; Reif, O.-W. Densonucleosis virus purification by ion exchange membranes. *Biotechnol. Bioeng.* **2004**, *88* (4), 465−473.

(12) Zhou, J. X.; Tressel, T.; Gottschalk, U.; Solamo, F.; Pastor, A.; Dermawan, S.; Hong, T.; Reif, O.; Mora, J.; Hutchison, F.; Murphy, M. New Q membrane scale-down model for process-scale antibody purification. *J. Chromatogr. A* **2006**, *1134* (1−2), 66.

(13) Phillips, M.; Cormier, J.; Ferrence, J.; Dowd, C.; Kiss, R.; Lutz, H.; Carter, J. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing. *J. Chromatogr. A* **2005**, *1078* (1−2), 74.

(14) Norling, L.; Lute, S.; Emery, R.; Khuu, W.; Voisard, M.; Xu, Y.; Chen, Q.; Blank, G.; Brorson, K. Impact of multiple re-use of anion-exchange chromatography media on virus removal. *J. Chromatogr. A* **2005**, *1069* (1), 79.

(15) Gregori, L.; Lambert, B. C.; Gurgel, P. V.; Gheorghiu, L.; Edwardson, P.; Lathrop, J. T.; MacAuley, C.; Carbonell, R. G.; Burton, S. J.; Hammond, D.; Rohwer, R. G. Reduction of transmissible spongiform encephalopathy infectivity from human red blood cells with prion protein affinity ligands. *Transfusion* **2006**, *46* (7), 1152−1161.

(16) Gregori, L.; Gurgel, P. V.; Lathrop, J. T.; Edwardson, P.; Lambert, B. C.; Carbonell, R. G.; Burton, S. J.; Hammond, D. J.; Rohwer, R. G. Reduction in infectivity of endogenous transmissible spongiform encephalopathies present in blood by adsorption to selective affinity resins. *Lancet* **2006**, *368* (9554), 2226−2230.

(17) Wang, G.; Carbonell, R. G. Characterization of a peptide affinity support that binds selectively to staphylococcal enterotoxin B. *J. Chromatogr. A* **2005**, *1078*, 98−112.

(18) Mondorf, K.; Kaufman, D. B.; Carbonell, R. G. Screening of combinatorial peptide libraries: Identification of ligands for affinity purification of proteins using a radiological approach. *J. Pept. Res.* **1998**, *52*, 526−536.

(19) Yang, H.; Gurgel, P. V.; Carbonell, R. G. Hexamer peptide affinity resins that bind the Fc region of human immunoglobulin G. *J. Pept. Res.* **2005**, *66*, 120−137.

(20) Wang, G.; De, J.; Schoeniger, J. S.; Roe, D. C.; Carbonell, R. G. A hexamer peptide ligand that binds selectively to staphylococcal enterotoxin B: isolation from a solid phase combinatorial library. *J. Pept. Res.* **2004**, *64* (2), 51−64.

(21) Gurgel, P. V.; Carbonell, R. G.; Swaisgood, H. E. Identification of peptide ligands generated by combinatorial chemistry that bind alpha-lactalbumin. *Sep. Sci. Technol.* **2001**, *36* (11), 2411−2431.

(22) Heldt, C. L.; Hernandez, R.; Mudiganti, U.; Gurgel, P. V.; Brown, D. T.; Carbonell, R. G. A colorimetric assay for viral agents that produce cytopathic effects. *J. Virol. Methods* **2006**, *135* (1), 56.

(23) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. General-method for rapid synthesis of multicomponent peptide mixtures. *Int. J. Pept. Protein Res.* **1991**, *37* (6), 487−493.

(24) Kaufman, D. B.; Hentsch, M. E.; Baumbach, G. A.; Buettner, J. A.; Dadd, C. A.; Huang, P. Y.; Hammond, D. H.; Carbonell, R. G. Affinity purification of fibrinogen using a ligand from a peptide library. *Biotechnol. Bioeng.* **2002**, *77* (3), 278−289.

(25) Gurgel, P. V.; Carbonell, R. G.; Swaisgood, H. E. Fractionation of whey proteins with a hexapeptide ligand affinity resin. *Biosepa-rations* **2001**, *9*, 385−392.

(26) Ohtsuka, K.; Uemura, K.; Nojima, T.; Waki, M.; Takenaka, S. Immobiliation of RNase S-Peptide on a single-stranded DNA-fixed gold surface and effective masking of its surface by an acridinyl poly(ethylene glycol). *Analyst* **2006**, *131* (1), 55−61.

(27) Leckband, D. E.; Kuhl, T.; Wang, H. K.; Herron, J.; Muller, W.; Ringsdorf, H. 4-4-20 anti-fluorescyl IgG Fab′ recognition of membrane bound hapten: Direct evidence for the role of protein and interfacial structure. *Biochemistry* **1995**, *34*, 11467−11478.

(28) Soltys, P. J.; Etzel, M. R. Equilibrium adsorption of LDL and gold immunoconjugates to affinity membranes containing PEG spacers. *Biomaterials* **2000**, *21*, 37−48.

(29) Weimer, B. C.; Walsh, M. K.; Wang, X. Influence of a polyethylene glycol spacer on antigen capture by immobilized antibodies. *J. Biochem. Biophys. Methods* **2000**, *45* (2), 211.

(30) Simpson, A. A.; Herbert, B.; Sullivan, G. M.; Parrish, C. R.; Zadori, Z.; Tijssen, P.; Rossmann, M. G. The structure of porcine parvovirus: Comparison with related viruses. *J. Mol. Biol.* **2002**, *315*, 1189−1198.

(31) Weichert, W. S.; Parker, J. S. L.; Wahid, A. T. M.; Chang, S.-F.; Meier, E.; Parrish, C. R. Assaying for structural variation in the parvovirus capsid and its role in infection. *Virology* **1998**, *250* (1), 106.

(32) Ali, N. A.; Hasan, A. R.; Wong, L. Y. Development of a novel aymmetric ultra low pressure membranes and a preliminary study for bacteria and pathogen removal applications. *Desalination* **2007**, *206*, 474−484.

(33) Vaidya, S. R.; Kharul, U. K.; Chitambar, S. D.; Wanjale, S. D.; Bhole, Y. S. Removal of hepatitis A virus from water by polyacrylonitrile-based ultrafiltration membranes. *J. Virol. Methods* **2004**, *119* (1), 7−9.

(34) Sano, D.; Ueki, Y.; Watanabe, T.; Omura, T. Membrane separation of indigenous noroviruses from sewage sludge and treated wastewater. *Water Sci. Technol.* **2006**, *54* (3), 77−82.

(35) Shukla, A. A.; Hubbard, B.; Tressel, T.; Guhan, S.; Low, D. Downstream processing of monoclonal antibodies−Application of platform approaches. *J. Chromatogr. B* **2007**, *848* (1), 28.

(36) Kim, I. S.; Choi, Y. W.; Lee, S. R.; Woo, H. S.; Lee, S. Removal and inactivation of viruses during manufacture of a high purity antihemophilic factor VIII concentrate from human plasma. *J. Microbiol. Biotechnol.* **2001**, *11* (3), 497−503.

(37) Li, M.; Wang, Y. S.; Li, W. J.; Su, Z. G. Stability of tumor necrosis factor-alpha during ion exchange chromatography. *Biotechnol. Lett.* **2003**, *25* (11), 905−908.

(38) Sugihara, A.; Senoo, T.; Enoki, A.; Shimada, Y.; Nagao, T.; Tominaga, Y. Purification and characterization of a lipase from Pichia burtonii. *Appl. Microbiol. Biotechnol.* **1995**, *43* (2), 277−281.

(39) Oscarsson, S. Factors affecting protein interaction at sorbent interfaces. *J. Chromatogr., B: Biomed. Sci. Appl.* **1997**, *699* (1−2), 117.

(40) Berkowitz, S. A.; Philo, J. S. Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation. *Anal. Biochem.* **2007**, *362* (1), 16–37.

(41) Frank, R. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* **1992**, *48* (42), 9217.

(42) Reichl, U. Antibody epitope mapping using arrays of synthetic peptides. In *Antibody Engineering: Methods and Protocols*; Lo, B. K. C., Ed.; Humana Press Inc: Totowa, NJ, 2004; Vol. 248.

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研究報告の概要

パルボウイルス B19 DNA を検出するための実施手順に関するガイダンス案が示された。
・全ての血漿由来製剤について、製造プール中のパルボウイルス B19 DNA のウイルス負荷を確実に 10^4IU/mL 以下とするため、製造過程の品質管理検査としてパルボウイルス B19 の NAT を実施すべきである。
・ミニプールサンプルのパルボウイルス B19 の NAT 検査は 10^6IU/mL 以上の個別ユニットを検出できる感度とすべきである。
・個別ユニットのパルボウイルス B19 DNA の力価が 10^6IU/mL 以上であることがわかった場合、又は、製造用プール血漿で 10^4IU/mL のパルボウイルス B19 DNA を上回る可能性がある場合、その後の製造に使用すべきではない。

原料血漿及び回収血漿において、パルボウイルス B19 DNA を検出するため、ならびに製造プールにおけるパルボウイルス B19 DNA のウイルス負荷が 10^4IU/mL 以下であることを示すために用いるパルボウイルス B19 の NAT 検査の精度、感度、特異度、再現性及びその他の性能特性を示すバリデーションデータを維持管理すべきである。

報告企業の意見	今後の対応
血漿由来製剤製造用原料血漿に関するパルボウイルス B19 の NAT 検査に関するガイダンス案の情報である。 当社血漿分画製剤は最終製品において NAT 検査を行い、パルボウイルス B19DNA 陰性であることを確認している。	今後ともパルボウイルス B19 に関する血漿分画製剤の安全性に関する情報に留意していく。

使用上の注意記載状況・その他参考事項等

慎重投与(次の患者には慎重に投与すること)
・溶血性・失血性貧血の患者［ヒトパルボウイルスＢ19の感染を起こす可能性を否定できない。感染した場合には、発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。］
・免疫不全患者・免疫抑制状態の患者［ヒトパルボウイルスＢ19の感染を起こす可能性を否定できない。感染した場合には、持続性の貧血を起こすことがある。］

重要な基本的注意
(1)本剤の原材料となる‥［スクリーニング項目、不活化・除去工程］‥投与に際しては、次の点に十分注意すること。
1)血漿分画製剤の現在の製造工程では、ヒトパルボウイルスB19等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。

妊婦、産婦、授乳婦等への投与
妊婦又は妊娠している可能性のある婦人には治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。［妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルスB19の感染の可能性を否定できない。感染した場合には胎児への障害（流産、胎児水腫、胎児死亡）が起こる可能性がある。］

Guidance for Industry

Nucleic Acid Testing (NAT) to Reduce the Possible Risk of Parvovirus B19 Transmission by Plasma-Derived Products

DRAFT GUIDANCE

This guidance document is for comment purposes only.

Submit comments on this draft guidance by the date provided in the *Federal Register* notice announcing the availability of the draft guidance. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. Submit electronic comments to either http://www.fda.gov/dockets/ecomments or http://www.regulations.gov. You should identify all comments with the docket number listed in the notice of availability that publishes in the *Federal Register*.

Additional copies of this draft guidance are available from the Office of Communication, Training and Manufacturers Assistance (HFM-40), 1401 Rockville Pike, Suite 200N, Rockville, MD 20852-1448, or by calling 1-800-835-4709 or 301-827-1800, or from the Internet at http://www.fda.gov/cber/guidelines.htm.

For questions on the content of this guidance, contact Mahmood Farshid, Ph.D., at 301-496-0952, or by Fax at 301-402-2780.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
July 2008

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Contains Nonbinding Recommendations

Draft – Not for Implementation

Guidance for Industry

Nucleic Acid Testing (NAT) to Reduce the Possible Risk of Parvovirus B19 Transmission by Plasma-Derived Products

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

We, FDA, are issuing this guidance to provide you, manufacturers of plasma-derived products, with recommendations for performing parvovirus B19 nucleic acid testing (NAT) as an in-process test for Source Plasma and recovered plasma used in the further manufacturing of plasma-derived products. Such testing will identify and help to prevent the use of plasma units containing high levels of parvovirus B19. This guidance also recommends how to report to the FDA implementation of parvovirus B19 NAT.

We recognize that in the current business practice for parvovirus B19 NAT in-process testing, several weeks can elapse between collection of the units of Source Plasma or recovered plasma and identification of B19 NAT-positive pools or units. We encourage manufacturers of plasma-derived products to employ practices that will reduce the time between product collection and in-process testing to allow for the meaningful notification of blood and plasma collection establishments of positive test results within the dating period of components.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

Parvovirus B19 is a small, non-enveloped single strand DNA virus. This virus is highly resistant to all commonly used inactivation methods, including heat and solvent/detergent (S/D) treatment, and is also difficult to remove because of its small size. The parvovirus B19 can be

transmitted by blood components and certain plasma derivatives, and may cause morbidity to susceptible recipients such as pregnant women (and their fetuses exposed in utero), persons with underlying hemolytic disorders, and immune compromised individuals (Refs. 1 and 2). The disease transmission by transfusion of blood components is rare; however, extremely high levels of parvovirus B19, up to 10^{12} IU/mL, in plasma of acutely infected but asymptomatic donors may present a greater risk in plasma derivatives due to pooling of large numbers of plasma units in the manufacture of these products. The virus can be detected by NAT in plasma pools when there are high levels of parvovirus B19 DNA in viremic donations. For example, the parvovirus B19 DNA can be detected in various plasma-derived products, particularly in coagulation factors (Refs. 3 and 4). There have been a few reports of parvovirus B19 infection associated with the administration of coagulation factors (Refs. 5 and 6) and S/D Treated Pooled Plasma (Refs. 1 and 7). Parvovirus B19 DNA is less frequently detected in albumin and immunoglobulin products and, when detected, the levels are usually low. There are no confirmed reports that albumin and immunoglobulin products have transmitted parvovirus B19 infection.

We have held or participated in several meetings to discuss the potential risk of parvovirus B19 infection by plasma-derived products, and the strategy for reducing such risk. The meetings included FDA-sponsored NAT workshops in 1999 and 2001 (Refs. 8 and 9), Blood Products Advisory Committee (BPAC) meetings in 1999, and 2002 (Refs. 10, 11, and 12), the National Heart, Lung, and Blood Institute-sponsored Parvovirus B19 workshop in 1999 (Ref. 1), and an ad hoc Public Health Service (PHS) panel in 2002 (discussed at the 2002 BPAC meeting (Ref. 12)). In these meetings, it was recognized that the scientific data indicate that parvovirus B19 is highly resistant to the available viral inactivation methodologies, and is difficult to remove because of its small size. The viral inactivation/removal steps routinely used in the manufacturing process of plasma-derived products do not alone appear to be sufficient to completely clear the virus if high viral load is present in the starting material. Therefore, in these meetings, a common recommendation for mitigating the risk of parvovirus B19 transmission by plasma derivatives has been to limit the virus load in the manufacturing plasma pool by testing the plasma donations for high titer parvovirus B19 DNA, using a minipool format. This viral load reduction strategy combined with the ability of the manufacturing process to clear the residual virus could greatly reduce the risk of parvovirus B19 infection by plasma-derived products.

The recommended limit in this guidance for viral load of parvovirus B19 DNA in the manufacturing plasma pool (i.e., not to exceed 10^4 IU/mL) was primarily derived from studies that were conducted on the transmission of parvovirus B19 associated with S/D Treated Pooled Plasma (Refs. 1, 7, and 10). In principle, testing in a minipool format to measure the viral load for parvovirus B19 DNA in a manufacturing plasma pool is acceptable in order to exclude only the high-titer plasma donations, thereby avoiding too great a loss of plasma for further manufacturing. Furthermore, during the viremic period for parvovirus B19 infected donors, which can be very lengthy, low levels of parvovirus B19 coexist with parvovirus B19 antibodies (potentially complexing with and neutralizing the virus). Therefore, it is undesirable to remove plasma units with low levels of B19 DNA, because it would diminish the parvovirus B19 antibody levels in plasma pools and in some of the resulting plasma-derived products (Refs. 13 and 14).

III. RECOMMENDATIONS

We recommend that you implement the following procedures to detect the presence of parvovirus B19 DNA:

- For all plasma-derived products, you should perform parvovirus B19 NAT as an in-process quality control test to ensure that the viral load of parvovirus B19 DNA in the manufacturing pools does not exceed 10^4 IU/mL.
- Use parvovirus B19 NAT on minipool samples to screen plasma units intended for further manufacturing into plasma-derived products. The sensitivity of the NAT assay, in any size minipool, should be at least 10^6 IU/mL for detection of any single donation when tested in the minipool (i.e., if the titer of an individual unit is 10^6 IU/mL or higher, the test result on the minipool will be positive). Primers and probes selected for parvovirus B19 NAT should detect all known genotypes of the virus (Ref. 15).
- When identified, you should not use individual plasma units intended for further manufacturing into plasma-derived products, when such units are found to have a titer of parvovirus B19 DNA at or above 10^6 IU/mL, or when use of a positive unit might result in plasma manufacturing pools exceeding a parvovirus B19 DNA titer of 10^4 IU/mL.

You should maintain validation data demonstrating the accuracy, sensitivity, specificity, reproducibility, and other performance characteristics of the parvovirus B19 NAT assay used for the detection of parvovirus B19 DNA in the Source Plasma and recovered plasma, and for demonstrating that the viral load of parvovirus B19 DNA in the manufacturing pool does not exceed 10^4 IU/mL.

If the recommendations are implemented, you must notify FDA of the changes to an approved application under 21 CFR 601.12(c)(5) ("Supplement-Changes Being Effected"), and submit the information required in 21 CFR 601.12(b)(3)(i) through (vii).

IV. REFERENCES

1. Brown, K.E., Young N.S., Alving, B.M., Luiz, H., and Barbosa, L.H. Parvovirus B19 implications for transfusion medicine. Transfusion (2001) 41:130-135.

2. Young, N.S., and Brown, K.E. Parvovirus B19. The New England Journal of Medicine (2004) 350:586-597.

3. Saldanha, J., and Minor, P. Detection of human parvovirus B19 DNA in plasma pools and blood products derived from these pools: implications for efficiency and consistency of removal of B19 DNA during manufacture. British Journal of Haematology (1996) 93:714-719.

4. Schmidt, I., Blumel, J., Seitz, H., Willkommen, H., and Lower, J. Parvovirus B19 DNA in plasma pools and plasma derivatives. Vox Sanguinis (2001) 81:228-235.

5. Blumel, J., et al. Parvovirus B19 transmission by heat-treated clotting factor concentrates. Transfusion (2002) 42:1473-1481.

6. Wu, C.G., et al. Parvovirus B19 transmission by a high-purity factor VIII concentrate. Transfusion (2005) 45:1003-1010.

7. Koenigbauer, U.F., Eastlund, T., and Day, J.W. Clinical illness due to parvovirus B19 infection after infusion of solvent/detergent-treated pooled plasma. Transfusion (2000) 40:1203-1206.

8. Transcript of Workshop on Implementation of Nucleic Acid Testing, December 14, 1999. http://www.fda.gov/cber/minutes/nucleic121499.pdf.

9. Transcript of Application of Nucleic Acid Testing To Blood Borne Pathogens and Emerging Technologies Workshop, December 4, 2001. http://www.fda.gov/cber/minutes/nuclacd1204p1.pdf. http://www.fda.gov/cber/minutes/nuclacd1204p2.pdf.

10. Transcript of FDA's Blood Products Advisory Committee (BPAC) Meeting, September 1999. http://www.fda.gov/cber/advisory/bp/bpmain.htm.

11. Transcript of FDA's Blood Products Advisory Committee (BPAC) Meeting, December 2002. http://www.fda.gov/cber/advisory/bp/bpmain.htm.

12. Transcript of FDA's Blood Products Advisory Committee (BPAC) Meeting, March 14, 2002. http://www.fda.gov/cber/advisory/bp/bpmain.htm.

13. Lefrere, Jean-Jacques, et al. Persistent B19 infection in immunocompetent individuals: implications for transfusion safety. Blood (2005) 106(8):2890-2895.

14. Cassinotti, P., Siegl, G. Quantitative evidence for persistence of human parvovirus B19 DNA in an immunocompetent individual. Eur J Clin Microbiol Infect Dis (2000) 19:886-895.

15. Servant, A., et al. Genetic diversity within human erythroviruses: identification of three genotypes. J Virol (2002) 76:9124-34.

別紙様式第2-1

No. 6

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2008. 7. 17	該当なし	

一般的名称	(製造販売承認書に記載なし)	研究報告の公表状況		公表国
販売名(企業名)	合成血-LR「日赤」(日本赤十字社) 照射合成血-LR「日赤」(日本赤十字社)		Vrioni G, Pappas G, Priavali E, Gartzonika C, Levidiotou S. Clin Infect Dis. 2008 Jun 15;46(12):e131-6.	ギリシャ

研究報告の概要

○永続的な微生物:ブルセラDNAは臨床的治癒後何年間も存続する
背景:ブルセラ症の発生率は依然として高いが、病態生理学、診断および治療、特に宿主内でのブルセラ属種の生存能については現在も不明な点が存在している。
方法:定量的リアルタイム・ポリメラーゼ連鎖反応法を用いて、ブルセラ症患者の複数の疾患ステージにおける細菌DNA量をモニターした。39名の急性ブルセラ症患者それぞれから3つ以上の末梢血検体を入手した(診断時1検体、治療終了後1検体、追跡調査時1検体以上)。
結果:大多数の患者(治療終了後では87%、治療終了6ヵ月後77%、治療終了2年以上後70%)は、無症候性であるにもかかわらず、持続的に細菌が検出可能であった。再発を経験した患者3名は、追跡調査中のどの疾患ステージにおいても細菌量に統計的有意差を示さなかった。
結論:適切な治療を行い回復したように見えても、ブルセラ菌DNAは残存する。この知見は、当該疾患の病態生理学に新たな洞察をもたらす。すなわち、ブルセラ菌は除去不可能な持続性の病原体である。

報告企業の意見	今後の対応
ブルセラ症に対して適切な治療を行い回復したように見えても、ブルセラ菌DNAは長期間体内に残存するとの報告である。	日本赤十字社では、全ての輸血用血液製剤について、平成19年1月より保存前白血球除去を実施している。今後も細菌やウイルスの検出や不活化する方策について情報の収集に努める。

使用上の注意記載状況・その他参考事項等

合成血-LR「日赤」
照射合成血-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

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MAJOR ARTICLE

An Eternal Microbe: *Brucella* DNA Load Persists for Years after Clinical Cure

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Background. Despite the continuing high incidence of brucellosis, vague aspects of pathophysiology, diagnosis, and treatment continue to exist, particularly with regard to the ability of *Brucella* species to survive inside the host.

Methods. A quantitative real-time polymerase chain reaction assay was used for monitoring bacterial DNA load in brucellosis-affected patients throughout different disease stages. Three or more specimens per patient were obtained (1 at diagnosis, 1 at the end of treatment, and at least 1 during the follow-up period) from 39 patients with acute brucellosis.

Results. The majority of patients (87% at the end of treatment, 77% at 6 months after treatment completion, and 70% at >2 years after treatment) exhibited persistent detectable microbiological load despite being asymptomatic. The 3 patients who experienced relapse did not exhibit any statistically significant difference in their bacterial load at any stage of disease or during follow-up.

Conclusion. *Brucella melitensis* DNA persists despite appropriate treatment and apparent recovery. This finding offers a new insight into the pathophysiology of the disease: *B. melitensis* is a noneradicable, persisting pathogen.

Brucellosis is a zoonosis that is prevalent worldwide [1]. *Brucella* species have recently garnered renewed attention because of their potential for use in biowarfare [2] and their reemergence as a significant cause of travel-related infection [3]. The complex pathophysiology of *Brucella* species [4] is dominated by their ability to manipulate immune response, targeting professional and nonprofessional phagocytes. Therein, *Brucella* species replicate without affecting cellular viability; in fact, the pathogen, by switching off cellular apoptosis, practically renders the cell immortal, thus allowing for its own further survival [5]. This intracellular localization of *Brucella* species in specialized compartments affects both the natural history and the diagnostic and therapeutic principles of brucellosis. The natural history of brucellosis is characterized by a frequently silent, protracted disease evolution. Therapeutically, the disease evolution imposes the need for a prolonged combined treatment that, even when administered in accordance with optimal recommendations, may lead to relapses. Diagnostically, the disease evolution hampers the usefulness of blood cultures and the use of microbiological eradication indexes [6].

Quantification of the microbiological burden may theoretically offer insight into the actual natural history of the disease, and it may allow for the evaluation of when and how the pathogen is eradicated from the human body (the term "microbiological eradication" being questionable for such a disease) [6]. Serological tests are useful for diagnosis [7], but the time required for results after treatment is disappointingly long. In addition, serological test results are usually inadequate in predicting the outcome. The latter may also apply to newer, sophisticated techniques such as ELISA [8]. The development of such novel molecular diagnostic techniques as PCR offered promise—technology preceded clinical application in the context of brucellosis, and even before traditional PCR assays were adequately evaluated clinically [9], novel assays emerged.

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