West Nile Virus Adheres to Human Red Blood Cells in Whole Blood

Maria Rios,1 Sylvester Daniel,1 Caren Chancey,1 Indira K. Hewlett,1 and Susan L. Stramer2

1Laboratory of Molecular Virology, Division of Emerging and Transfusion Transmitted Diseases, Office of Blood Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, and 2American Red Cross, Gaithersburg, Maryland

Background. West Nile virus (WNV) is endemic in the United States. It is transmissible by blood transfusion, and the nation’s blood supply is currently screened for WNV. Documented transmission of WNV infection through red blood cell (RBC) units in which the plasma co-component had a low viral load could be explained, in at least 1 instance, by cell-association of WNV; in this case, the RBC unit was released as negative by minipool nucleic acid testing (NAT) performed on plasma but was intermittently NAT-positive when subsequently tested as an individual sample. We hypothesized that a proportion of WNV bound to blood cells and was not measured by NAT performed on plasma samples. We have investigated whether WNV binds to RBCs, leading to reduction of WNV RNA detection by NAT performed on plasma samples.

Methods. Equal volumes of leukoreduced RBCs and their corresponding plasma components from 20 blood donors with NAT results that were positive for WNV were tested in 5 replicates by reverse-transcriptase polymerase chain reaction TaqMan for WNV. In addition, aliquots from 8 of the RBC units were tested by infectivity assays using Vero cells.

Results. The reverse-transcriptase polymerase chain reaction TaqMan assay showed that the viral load in the RBC components exceeded that in the corresponding plasma units by 1 order of magnitude. In addition, viruses associated with the RBCs were infectious in Vero cell cultures.

Conclusions. These observations reinforce the notion that extraction of viral RNA from whole blood could improve assay sensitivity for blood donor screening and further reduce the residual risk of WNV transmission through transfusion.

West Nile virus (WNV) is a flavivirus that is transmitted to humans primarily through mosquito bites. Birds are the primary amplifying host and reservoir. Although direct transmission can occur among vertebrates, the amount of infectious virus required for transmission is unknown. WNV has spread throughout the United States since 1999, infecting >1 million people, and has become the most common cause of viral encephalitis in the country [1]. Since the first outbreak of WNV in the United States, 8 consecutive epidemics have occurred, causing at least 23,000 documented human cases of disease and at least 930 human deaths (reported to the Centers for Disease Control and Prevention) [2].

Human-to-human transmission of WNV through organ transplantation, mother-to-child transmission (through breastfeeding or transplacental transmission), and blood transfusion were identified in 2002 [3–7]. The documentation of WNV transmission through transfusion led to the rapid development of investigational nucleic acid tests (NATs) for blood screening. The implementation of nationwide clinical studies in July 2003 was allowed by the US Food and Drug Administration, as a potential measure to prevent viral spread through transfusion of blood and blood components. Screening of blood donations for WNV has been performed year-round in mini-pools (MP) of 6 or 16 plasma samples (by MP-NAT) [8]. Since blood screening for WNV was implemented, there have been 7 instances in which recipients of blood components were infected with low-level viremic donations, as indicated by nonreactive test results when tested by MP-NAT and reactive test results when tested neat by
individual donation NAT (ID-NAT) [7, 9]. In all cases, blood components were released after a WNV nonreactive MP-NAT result. Transmissions were identified during retrospective studies that were performed to evaluate the sensitivity of MP-NAT for blood donor screening, when retention samples from MP-NAT–nonreactive units were retested by ID-NAT and found to be positive for WNV RNA. Lookback studies were performed, and ID-NAT–reactive units that had been transfused were found to have transmitted WNV infection. To date, a total of 30 cases of WNV transmission through transfusion have been documented [7, 9–11], including the 7 cases that occurred after implementation of blood donor screening for WNV under an investigational protocol.

Breakthrough WNV transmission through transfusion demonstrates that the current MP-NAT platform for WNV testing, performed in pools of 6 and 16 plasma specimens, lacks adequate sensitivity to detect units containing low viral loads that are capable of infecting recipients. Currently, because of logistical and technical reasons, routine blood donor screening is performed using MP-NAT. The sensitivity of NAT that is performed in pools of 6 and 16 plasma specimens, lacks adequate sensitivity to detect units containing low viral loads that are capable of infecting recipients. Currently, because of logistical and technical reasons, routine blood donor screening is performed using MP-NAT. The sensitivity of NAT that is required to detect units with low levels of virus is, at this time, only attainable by ID-NAT [7, 9]. To reduce the risk of transmission from units with low levels of virus, the blood collection and testing facilities switch from MP-NAT to ID-NAT in areas where the activity of WNV in humans is high, as defined by detection of WNV RNA in blood donations during MP-NAT screening [12–14]. Although the strategy of switching from MP-NAT to ID-NAT has improved screening sensitivity, alternative approaches to increase assay sensitivity in an MP-NAT platform are still desirable. In this study, the potential of increasing sensitivity for viral detection by examining blood components other than plasma for the presence of infectious WNV and viral RNA was investigated.

We hypothesized that a large proportion of WNV virions bind to cellular components of blood, such as RBCs, and current NAT performed on plasma samples fails to detect WNV RNA. Current NAT assays for infectious agents are performed using plasma as the test sample, because plasma and serum samples have been historically used for viral marker testing in the blood and plasma donor context. Although plasma-pool and individual donation testing have vastly reduced the residual risk of WNV transmission through transfusion, it is likely that virions bound to the cellular components of blood could potentially not be detected by current screening assays. Virions may attach directly to cell membranes or as immune complexes bound to Fc and complement receptors present in the

| Table 1. Viral loads in RBC and plasma co-components as determined by RT-PCR TaqMan for the West Nile virus (WNV) 3 NC region and antibody test results obtained from the corresponding plasma samples. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Unit            | Viral load in RBCs, copies/mL ± SD | Viral load in plasma, copies/mL ± SD | Plasma WNV IgM | Plasma WNV IgG | Viral load ratio, RBCs:plasma |
| ARC01           | 1694 ± 295      | 177 ± 60        | Neg            | Neg            | 9.6             |
| ARC02           | 1642 ± 250      | 175 ± 67        | Neg            | Neg            | 9.4             |
| ARC03           | 6087 ± 1965     | 3757 ± 506      | Neg            | Neg            | 1.6             |
| ARC04           | 1114 ± 27       | 107 ± 6         | Neg            | Neg            | 10.4            |
| ARC05           | 5261 ± 417      | 457 ± 173       | Neg            | Neg            | 11.5            |
| ARC06           | 7304 ± 868      | 475 ± 71        | Pos            | Pos            | 15.4            |
| ARC07           | 7337 ± 743      | 709 ± 147       | Neg            | Neg            | 10.3            |
| ARC08           | 6282 ± 261      | 198 ± 140       | Neg            | Pos            | 31.7            |
| ARC09           | 5562 ± 672      | 174 ± 90        | Pos            | Neg            | 31.9            |
| ARC10           | 149 ± 36        | 229 ± 58        | Neg            | Neg            | 0.5             |
| ARC11           | 4200 ± 2983     | 458 ± 20        | Neg            | Neg            | 9.2             |
| ARC12           | 908 ± 54        | 590 ± 169       | Neg            | Neg            | 1.5             |
| ARC13           | 548 ± 277       | 371 ± 34        | Neg            | Neg            | 1.5             |
| ARC14           | 26,700 ± 13,650 | 5050 ± 354      | Neg            | Neg            | 5.3             |
| ARC15           | 3170 ± 1361     | 1390 ± 1153     | Neg            | Neg            | 2.3             |
| ARC16           | 55,300 ± 28,378 | 5800 ± 3111     | Neg            | Neg            | 9.5             |
| ARC17           | 19,700 ± 7506   | 7600 ± 141      | Neg            | Neg            | 2.6             |
| ARC18           | 37,700 ± 25,423 | 1390 ± 1632     | Pos            | Neg            | 27.0            |
| ARC19           | 27,000 ± 8000   | 7050 ± 2192     | Neg            | Neg            | 3.8             |
| ARC20           | 1230 ± 267      | 427 ± 22        | Neg            | Neg            | 2.9             |

**NOTE.** Values in bold denote units that were also positive for antibodies to WNV. Neg, negative; Pos, positive.
membrane of various blood cells. We believe that the association of WNV with cellular blood components may be a mechanism that could account for undetectable viral loads in plasma at certain periods during the course of infection. This hypothesis is based on observations involving HIV, indicating that a substantial proportion of HIV virions in the circulation are attached to or have been engulfed by platelets [15] and RBCs [16]. The HIV load detectable in platelets frequently exceeded that detectable in plasma by ≥10-fold [17]. In a study involving 82 patients with AIDS [18], 23 were found to have an undetectable HIV load (<20 copies/mL) in plasma samples for up to 32 months, and the corresponding whole blood samples contained ~5 log copies/mL of HIV load. A similar phenomenon could occur with WNV and may explain transfusion transmission that was observed in at least 1 instance, in which the plasma sample from an RBC unit that was released as nonreactive when tested by MP-NAT was intermittently NAT reactive during retrospective testing, indicating a very low viral load. Our experiments were designed to test the hypothesis that a proportion of viable WNV virions are bound to RBCs, resulting in potential lack of detection of WNV by current NAT methods.

Figure 1. Viral loads in RBC and plasma co-components, as determined by RT-PCR TaqMan for the West Nile virus (WNV) 3' noncoding region

Figure 2. Viral loads in RBC and plasma co-components, as determined by RT-PCR TaqMan for the West Nile virus (WNV) 3' noncoding region
METHODS

Specimens. In this study, we used samples from 20 leukoreduced RBC units and from their corresponding plasma components that were ID-NAT reactive by an investigational screening assay. These units were confirmed to be positive according to the clinical trial protocol (i.e., the units were repeatedly reactive on the screening assay, as well as reactive on an alternate NAT assay and/or were reactive for the presence of WNV-specific antibodies).

Viral RNA isolation. Viral RNA was isolated from equal volumes of each RBC and plasma specimen, as described below.

**RNA from plasma.** Aliquots of 250 µL of the plasma specimens were extracted using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer’s instructions. RNA was eluted from columns in a volume of 50 µL of elution buffer (provided in the kit).

**RNA from RBC.** RBC aliquots from each unit were washed twice using double the volume of PBS before extraction. Aliquots of 250 µL of the washed RBCs from each unit were extracted using Trizol (Invitrogen), according to the manufacturer’s instruction, and RNA pellets were resuspended in 50 µL of RNase-free water.

We also attempted to recover the remaining leukocytes from 7 RBC units by standard gradient centrifugation in Ficoll Hypaque. For these 7 units, additional aliquots of 250 µL of the RBC fraction obtained after Ficoll gradient were also used for RNA extraction and RT-PCR TaqMan for WNV using the TaqMan One-Step RT-PCR (Applied Biosystems).

**RT-PCR TaqMan for WNV.** Purified viral RNA was tested for the presence of the WNV 3′ noncoding (3′NC) region by the TaqMan assay using the following sets of primers and probe under the same amplification conditions described previously [19]: WN3ncF, 5′-CAGGCCAGCTACGGC-3′; WN3ncR, 5′-CAGTGCCTGTCCGACTA-3′; and WN3ncP, 5′-TCTGGG-

GAGATCGAGTCTCGAT-3′. WNV RNA with known copy number concentration was used as standard for viral load determinations [20]. Each RNA sample was tested in 5 replicates. Assays were performed on 2 different days, using 10 µL of RNA per reaction; one round was run in duplicate, and the other was run in triplicate.

**Infectivity assays on Vero cells.** Aliquots from 8 RBC units were tested by infectivity assays on Vero cells. Briefly, Vero cells were plated in T75 flasks and grown to 85% confluence in Dulbecco's Modified Eagle Medium (Gibco BRL) containing 5% fetal bovine serum (HyClone) and 10 µg/mL of penicillin and streptomycin (Gibco). For the infectivity assay, the medium was removed, and 500 µL from each of the 3 plasma components and their corresponding RBC samples were added to 6 individual flasks; the volume was increased to 5 mL with fresh medium. Cultures were incubated at room temperature for 2 h under gentle rocking, after which 10 mL of fresh medium were added to each flask and cultures were incubated at 37°C in 5% CO₂ and observed for cytopathic effect on a daily basis.

RESULTS

**Viral load in plasma components.** The viral load determined by RT-PCR TaqMan showed a range of WNV RNA from 10² to 5 × 10³ copies/mL. The mean (±SD) for the viral loads from each plasma unit, tested in replicates of 5, are shown in table 1 and plotted in figure 1. These donations were identified as reactive by ID-NAT and were not tested by MP-NAT; therefore, it is unknown whether the viral loads would be detectable by MP-NAT.

**Viral load in RBC specimens.** The viral loads determined by the RT-PCR TaqMan assay showed a range of WNV RNA from 10² to 5 × 10⁴ copies/mL, and the range of variation in viral loads observed in replicates were within 0.5 log₁₀ copies/
mL. Moreover, the viral loads in the RBC components exceeded those of the corresponding plasma units—in the majority of cases (15 of the 20 pairs), by at least 1 order of magnitude (table 1 and figure 1).

Test results obtained from aliquots of the RBC portion after gradient centrifugation in Ficoll Hypaque were also positive for WNV RNA, and the viral loads in these aliquots were only slightly lower than those obtained from the same RBC units when cells were just washed with PBS (figure 2).

**Vero cell infectivity assays.** To verify the viability of virus bound to the RBCs, we performed infectivity assays using Vero cells in culture. Eight of the 20 RBC units cocultivated with Vero cells resulted in Vero cell infection, as indicated by cytopathic effect formation within 5 days after infection (figure 3). This experiment indicates that WNV associated with RBCs remains capable of infecting Vero cells.

**Correlation of viral load in RBC specimens with viral load in plasma specimens and antibody to WNV.** Four of the 20 blood samples were positive for antibodies to WNV, ARC06 was positive for IgM and IgG, ARC08 was positive for IgG only, and both ARC09 and ARC18 were positive for IgM only. There was no observable correlation between the presence of antibody and viral load. However, the ratio of viral load in RBC to viral load in plasma from antibody-positive units (15.4:31.9) consistently exceeded the ratio of viral load in RBC to viral load in plasma from antibody-negative units (1.5:11.5) (table 1). This finding suggests that antibodies may facilitate viral attachment to RBCs.

**DISCUSSION**

We have shown that WNV virions bind to circulating RBCs and that bound virus is detectable by NAT. WNV virions that were bound to RBCs were capable of infecting Vero cells in culture, suggesting that the bound virus is infectious. This observation is not surprising, because RBC components of blood have been associated with transmission of WNV through transfusion, even in instances in which the viral load in plasma was very low and only erratically detected [7, 9]; this could be explained by the low infectious dose of WNV. However, there have been instances in which the transfusion of WNV RNA-positive blood did not result in recipient seroconversion (i.e., in the presence of WNV antibodies) [12, 13]. It is also possible that, in some cases, the WNV load bound to RBCs exceeds that in the corresponding plasma and that the RBC-bound WNV remains infectious, as our data demonstrate. This could explain the sporadic transmission of WNV through RBC units for which the corresponding plasma samples tested nonreactive by WNV MP-NAT and reactive by ID-NAT, indicating low viral loads in plasma.

Adherence of pathogens to RBCs is not a new concept and has been reported for a long time. Several studies and reviews have been published about this subject, reporting adherence and/or attachment of multiple pathogens to RBCs, including viruses, bacteria, and parasites. Additional information about this issue can be found in other articles [21–23].

There have been reports of the binding of HIV virions to several molecules present on the membrane of RBCs [24, 25], but the potential ligand(s) for WNV remains to be determined. Although the mechanisms of WNV binding to RBCs are currently unknown, the binding appears to be sufficiently strong to resist several washes with PBS. The attachment of WNV to RBCs is also strong enough to resist standard gradient centrifugation in Ficoll Hypaque, as revealed during the attempt to recover remaining WBCs from the packed RBCs. In these experiments, aliquots from 7 units were subjected to standard gradient centrifugation in Ficoll Hypaque, and the RBC portion was tested for WNV. These RBC aliquots had positive results and had only a slightly lower viral titer than that seen in the same RBC unit when cells were just washed (figure 2).

In addition, the ratio of viral load in RBCs, compared with that in the plasma co-component, was higher in specimens with antibodies than in those without measurable antibodies (table 1), suggesting that WNV antibodies may play a role in the attachment of the virus to RBCs. The association of WNV with RBCs could contribute to viral clearance, because antibody-virion immune complexes bind to molecules expressed in the membrane of RBCs and are cleared by the reticuloendothelial system. Unfortunately, the specificity of antibodies could not be verified, and some of the antibody present in the specimen may not be WNV specific; the positive test result could occur because of cross-reactivity. Nevertheless, the increased viral load associated with RBCs suggests that antibodies have a role in the binding of virus to RBCs. We plan to investigate this phenomenon further.

Although blood donor screening has become more sensitive because of the conversion of MP-NAT to ID-NAT during the peak season of the epidemic, failure to convert in a timely manner may allow transmissions to occur [9]. In addition, there was a recently documented case of WNV transmission through transfusion of RBCs that were negative for WNV by MP-NAT, for which retention plasma collected from the same donation produced erratic test results by ID-NAT for WNV [26]. Therefore, alternative approaches to increase assay sensitivity of WNV NAT platforms remain desirable.

We have shown that WNV bound to the RBC component of blood represents a large proportion of the circulating virus, which may be higher than the proportion of circulating virus in plasma. The viral load in the RBC components frequently exceeded that in the corresponding plasma unit by 1 order of magnitude, and WNV associated with RBCs remained infectious.

These findings reinforce the notion that future improvements
in assay sensitivity for blood donor screening by WNV NAT may be warranted. One such strategy would be the development of methods that use whole blood as the test sample and enable extraction of viruses bound to cellular components of blood, additionally reducing the residual risk of WNV transmission through transfusion.

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References