Effect of Viruses on Platelet Aggregation and Platelet Survival in Rabbits


Thrombocytopenia may occur in association with some viral infections. The objective of the present study was to examine the mechanisms by which myxoviruses may induce thrombocytopenia via a direct effect on platelets. Newcastle disease virus (NDV) caused platelet aggregation and loss of granule and cytoplasmic constituents from washed rabbit platelet suspensions. This effect of NDV was abolished by heating the virus at 56°C. In contrast, influenza virus did not cause these platelet reactions. Pretreatment of platelets with NDV abolished their ability to respond to NDV and reduced their response to ADP and thrombin, but treatment of platelets with heated NDV or with influenza virus did not have a major effect on their function in vitro. NDV and influenza virus, which contain neuraminidase activity, removed platelet sialic acid. When platelets pretreated with these viruses were injected into normal rabbits, they were rapidly removed from the circulation. Since platelets pretreated with purified neuraminidase are also rapidly cleared after injection, it seems likely that the loss of platelet sialic acid caused by these viruses is responsible for the removal of the platelets from the circulation. In contrast, platelets treated with inactivated NDV, inactivated influenza virus, or chikungunya virus (which does not have neuraminidase activity) survived normally. Thus there may be at least two mechanisms directly involved in virus-induced thrombocytopenia: (1) paramyxoviruses such as NDV may cause intravascular platelet aggregation, and (2) platelets exposed to viruses with neuraminidase activity may lose surface sialic acid and be rapidly cleared from the circulation.

Several pathogenetic mechanisms have been postulated for the occurrence of virus-associated thrombocytopenia, including (1) disseminated intravascular coagulation with the consumption of platelets as well as plasma coagulation factors, (2) impaired platelet production due to the alteration of megakaryocytes by viruses, (3) a direct interaction between viruses and circulating platelets resulting in phagocytosis or in platelet aggregation, release, and lysis, (4) increased platelet utilization as a result of virus-endothelial cell interaction, vasculitis, and the formation of mural thrombi, (5) immunologically mediated platelet destruction by viral antigen-antibody complexes or by antibodies directed against specific platelet antigens (e.g., anti-i) that may act as cold agglutinins, and (6) hemolysis of red cells by...
viruses with the release of procoagulant materials (e.g., ADP, thromboplastin-like material).1

We previously showed that Newcastle disease virus (NDV), a paramyxovirus, can induce platelet aggregation, release, and lysis in vitro in stirred suspensions of human or rabbit washed platelets.2 In contrast, influenza virus (A/PR8), an orthomyxovirus, does not cause platelet aggregation, release, or lysis when used at an equivalent hemagglutinating titer in washed platelet suspensions.11

The removal of more than 15% of the total sialic acid from rabbit platelets by purified neuraminidase results in the rapid clearance of these platelets from the circulation when they are injected into rabbits, and the removal of up to 65% of the surface sialic acid has only a slight (enhancing) effect on platelet aggregation and release in vitro.12 Because both NDV and influenza virus contain neuraminidase activity, we examined the effects of these viruses on the survival of rabbit platelets in vivo and on their aggregation response in vitro. Chikungunya virus (CGV), a togavirus that does not contain neuraminidase activity or cause platelet aggregation or release, was used as a control virus.

MATERIALS AND METHODS

Virus propagation and titration. NDV (Hickman strain) and influenza virus (A/PR8) were grown in embryonated hens' eggs and assayed for both hemagglutinating activity (HA) and infectivity as previously described.11 Each was suspended in phosphate-buffered saline (PBS) pH 7.1. CGV was propagated in baby hamster kidney (BHK 21) tissue culture13 and suspended in borate saline (BS) pH 9.0. NDV (HA 10,240 U/ml) and influenza virus (HA 360,000 U/ml) were obtained at a strength of 10^8 and 10^11 egg-infective doses (EID_{50})/ml, respectively, and CGV at a strength of 10^9 tissue-culture-infective doses (TCID_{50})/ml.14 For experiments with inactivated virus, the virus suspensions were heated in a water bath at 56°C for 1 hr.

Preparation and labeling of platelet suspensions. Suspensions of washed platelets from rabbits were prepared as described previously.15,16 The final suspending medium was Tyrode solution containing albumin and apyrase. Apyrase was used at a concentration that converted 0.25 μM ATP to AMP in 120 sec at 37°C. Platelets were labeled in vitro in the first washing fluid as previously described17 using Na_251CrO_4 (specific activity approximately 160 mCi/μg chromium; Amersham/Searle, Arlington Heights, Ill.) or 4C-serotonin (3'-14C-5-hydroxytryptamine creatinine sulfate, 14C-SHT, specific activity approximately 50 mCi/mmol; Amersham/Searle).

Incubation with virus and recovery of platelets. For studies of platelet survival, rabbit platelet suspensions were incubated at 37°C with either NDV (1 part virus suspension to 5 parts platelet suspension), influenza virus (1:20), or CGV (1:10), or with an equivalent concentration of the respective inactivated virus or with virus-suspending medium only. The platelet suspensions were gently mixed with the virus until visible platelet aggregation occurred (for the platelet-NDV mixture only) or for an equivalent time period for influenza and CGV. Prostaglandin E_2 at a final concentration of 1 μM and apyrase were then added. After 30 min the platelets were washed once in calcium-free Tyrode-albumin solution and resuspended at 37°C in Tyrode-albumin solution containing apyrase.

For studies of platelet aggregation and release, the preincubation with the virus suspensions was similar except that the proportion of NDV suspension to platelet suspension was 1:20.

In separate experiments in which the extent of removal of N-acetylneuraminic acid (sialic acid) was evaluated, the platelets were suspended in glucose-free Tyrode-albumin solution to facilitate the subsequent estimation of sialic acid.

Platelet survival. The survival in vivo of virus-treated platelets was studied as previously described14 using 51Cr-labeled platelets. Radioactivity was determined in the whole blood samples. Platelet half-life (PHEL) was calculated assuming an exponential pattern of platelet disappearance.18

Platelet aggregation. Following treatment of the platelet suspension with virus, inactivated virus, or virus-suspending fluid, the platelets were resuspended to a concentration of 500 x 10^9/
Platelet aggregation was studied in a turbidimetric device as previously described\textsuperscript{12} and is expressed as a percentage of the maximum deflection on the chart paper. The aggregating agents used were NDV suspension, ADP, and bovine tendon collagen suspension (Sigma Chemical, St. Louis, Mo.),\textsuperscript{19} and topical bovine thrombin (Parke Davis, Detroit, Mich.). All concentrations given represent the final concentration in the platelet suspension.

\textbf{Measurement of the loss of platelet constituents.} The extent of loss of N-acetylneuraminic acid (sialic acid) during incubation of the virus with platelets was assayed by the thiobarbituric acid method.\textsuperscript{20} Both free and total (after hydrolysis with H\textsubscript{2}SO\textsubscript{4}) sialic acid were determined in the platelet supernate; the results are expressed as the percentage of total platelet sialic acid.

The release and/or loss of platelet constituents were determined after virus incubation and, for each aggregating agent, at the time at which maximum aggregation had occurred with the control platelet suspension as previously described.\textsuperscript{22} In samples that contained both \textsuperscript{14}C and \textsuperscript{51}Cr the radioactivity due to each isotope was estimated by the method of Sheppard and Marlow.\textsuperscript{22} The results were analyzed using a paired difference analysis and Student’s two-tailed \textit{t} test.

\section*{RESULTS}

\textbf{Effect of Incubation In Vitro of Viruses With Platelets}

Only NDV caused visible platelet aggregation. Preincubation of platelets with a low concentration of NDV (1:20) in the presence of glucose was accompanied by the loss of 4.9\% \pm 2.1\% of \textsuperscript{14}C-5HT and 7.1\% \pm 2.1\% of \textsuperscript{51}Cr. A higher concentration of NDV (1:5) caused the loss of 21.6\% \pm 0.2\% of \textsuperscript{14}C-5HT and 8.7\% \pm 3.2\% of \textsuperscript{51}Cr. In contrast, inactivated NDV and the buffer in which the virus was suspended had no apparent effect. No aggregation, release, or loss of cytoplasmic constituents occurred during incubation of platelets with influenza virus or inactive influenza virus.

The extent of removal of platelet sialic acid by concentrations of virus equivalent to those used for the above experiments is shown in Table 1. In separate experiments, platelets were incubated with virus at the same concentration but in a suspending medium containing no glucose (see Materials and Methods). NDV (1:5) caused the loss of 18.1\% of total platelet sialic acid as free sialic acid and 36.7\% when both free and bound sialic acid were assayed in the supernate after incubation. Under these glucose-free conditions, however, almost 50% of the total \textsuperscript{14}C from prelabeled platelets appeared in the supernate together with 44% of the total \textsuperscript{51}Cr, indicative of extensive platelet lysis. During incubation with influenza virus, less free and total sialic acid was removed (5.3\%)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Virus Treatment} & \textbf{Sialic Acid Removed*} & \textbf{Loss of Platelet Constituents*} \\
\cline{2-6}
\textbf{Concentration} & \textbf{(Total Platelet Sialic Acid, %)} & \textbf{\textsuperscript{14}C-5HT} & \textbf{\textsuperscript{51}Cr} & \textbf{\textsuperscript{14}C-5HT} & \textbf{\textsuperscript{51}Cr} \\
\hline
NDV (1:5) & 18.1 \pm 3.8 (14) & 36.7 \pm 10.4 (13) & 48.6 \pm 5.0 (15) & 43.6 \pm 2.7 (13) & 1.4 \pm 0.5 (7) & 1.1 \pm 0.5 (7) \\
NDV (H)\textsuperscript{t} (1:5) & 2.1 \pm 1.4 (5) & 4.9 \pm 3.9 (7) & 0.7 \pm 0.7 (7) & 0 (7) & 0 (7) & 0 (7) \\
Influenza (1:20) & 5.3 \pm 2.3 (9) & 9.0 \pm 4.4 (11) & 1.1 \pm 0.5 (11) & 0.6 \pm 0.4 (9) & 0.7 \pm 0.7 (7) & 0 (7) \\
Influenza (H)\textsuperscript{t} (1:20) & 2.4 \pm 2.4 (5) & 0.7 \pm 0.7 (7) & 0 (7) & 0 (7) & 0 (7) & 0 (7) \\
\hline
\end{tabular}
\caption{Effect of Incubation of Washed Rabbit Platelets With Viruses on the Loss of Platelet Sialic Acid}
\end{table}

For these experiments the platelet suspensions contained no glucose and were used solely for the estimation of sialic acid removed by the viruses and the loss of granule and cytoplasmic constituents. Total sialic acid removed represents the amount measured after hydrolysis and includes both free and bound sialic acid.

*Mean \pm SEM (number of determinations).
\textsuperscript{t}NDV (H) and influenza (H) designate the inactivated (heated) viruses.
and 9.0\%\,\textit{a}, respectively) but no release or loss of cytoplasmic constituents occurred. Incubation of platelets with inactivated NDV or inactivated influenza virus did not remove sialic acid or cause release or lysis.

**Response In Vitro of Virus-treated Platelets to Aggregating Agents**

Platelets pretreated with NDV did not aggregate, release, or lose cytoplasmic constituents in response to subsequent exposure to this virus at a final concentration of 1:10 (Table 2). In contrast, the response of platelets pretreated with inactivated NDV did not differ from that of platelets previously exposed to control buffer. In both instances, however, the loss of granule contents (36.7\textit{a} and 36.5\textit{a} of 14C-SHT) and of cytoplasmic constituents (21.7\textit{a} and 18\textit{a}) was greater than that observed during the original preincubation studies with NDV. It should be pointed out that the conditions differed in these experiments; in the aggregation studies the platelets were stirred with the virus for approximately 3 min, whereas during preincubation the platelets were not stirred. Platelets pretreated with influenza virus responded to NDV to the same extent as platelets pretreated with inactivated influenza virus or control buffer (Table 2).

The response of virus-treated platelets to ADP is shown in Table 3. The aggregation response of NDV-treated platelets was significantly less than that of platelets pretreated with inactivated NDV or control fluid. The response was not affected by the addition of fibrinogen (0.1\%) prior to ADP (results not shown). Treatment of platelets with influenza virus did not affect their subsequent responses to ADP when compared with the control platelets.

The response of virus-treated platelets to collagen is also shown in Table 3. Platelets pretreated with influenza virus showed an increased release of 14C-SHT (42.6\%) compared to those pretreated with inactive virus (33.9\%) or control buffer (32.3\%), but there was no significant difference in the extent of ag-

| Table 2. Response of Virus-treated Platelets to Newcastle Disease Virus (Final Concentration 1:10) |
|-------------------------------------------------|-------------------------------------------------|----------------------------------|
| Virus Pretreatment (Concentration) | Response to Addition of NDV |                          |
|                                   | Aggregation* (Percent of Maximum) | Loss of 14C-SHT* (Percent of Total) | Loss of Cytoplasmic Constituents* (Percent of Total LDH) |
| NDV (1:20)                        | 48.9 ± 6.7                          | 36.7 ± 2.4                     | 21.7 ± 3.7                     |
| NDV (H)t (1:20)                   | 51.4 ± 4.7                          | 36.5 ± 2.4                     | 18.0 ± 2.7                     |
| Control PBS                       |                                  |                                 |                                 |
| NDV versus control                | p < 0.001                          | p < 0.001                      | p < 0.001                      |
| NDV (H) versus control            | NS                                | NS                             | NS                             |
| Influenza (1:20)                  | 33.2 ± 5.3                          | 18.4 ± 1.9                     | 12.3 ± 2.0                     |
| Influenza (H)t (1:20)             | 60.5 ± 7.3                          | 27.4 ± 3.5                     | 16.0 ± 2.5                     |
| Control PBS                       | 63.2 ± 12.8                         | 22.4 ± 2.1                     | 12.7 ± 1.0                     |
| Influenza versus control          | NS                                | NS                             | NS                             |
| Influenza (H) versus control      | NS                                | NS                             | NS                             |

NS, not significant.

*Mean ± SEM of eight (NDV) and six (influenza virus) determinations.

1NDV (H) and influenza (H) indicate the inactivated (heated) viruses.
VIRUSES AND PLATELET SURVIVAL

Table 3. Response of Virus-treated Platelets to ADP, Collagen, and Thrombin

<table>
<thead>
<tr>
<th>Virus Pre-treatment</th>
<th>ADP (5 × 10⁻⁶ M)</th>
<th>Collagen (1:10)</th>
<th>Thrombin*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aggregation</td>
<td>Release of ¹⁴C-5HT</td>
<td>Aggregation</td>
</tr>
<tr>
<td></td>
<td>(Percent of Maximum)</td>
<td>(Percent of Total)</td>
<td>(Percent of Maximum)</td>
</tr>
<tr>
<td>NDV (1:20)</td>
<td>26.8 ± 4.9 (7)</td>
<td>2.9 ± 1.3 (7)</td>
<td>68.2 ± 12.2 (7)</td>
</tr>
<tr>
<td>NDV (H) (1:20)</td>
<td>48.6 ± 6.4 (7)</td>
<td>1.9 ± 0.3 (7)</td>
<td>71.2 ± 11.7 (7)</td>
</tr>
<tr>
<td>Control PBS</td>
<td>52.0 ± 4.9 (7)</td>
<td>2.6 ± 1.1 (7)</td>
<td>76.5 ± 9.1 (7)</td>
</tr>
<tr>
<td>NDV versus control</td>
<td>p &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NDV (H) versus control</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Influenza (1:20)</td>
<td>61.9 ± 12.3 (4)</td>
<td>1.0 ± 0.2 (4)</td>
<td>93.8 ± 3.1 (5)</td>
</tr>
<tr>
<td>Influenza (H) (1:20)</td>
<td>50.5 ± 13.3 (4)</td>
<td>0.9 ± 0.2 (4)</td>
<td>72.3 ± 9.0 (5)</td>
</tr>
<tr>
<td>Control PBS</td>
<td>55.7 ± 12.5 (4)</td>
<td>1.7 ± 0.6 (4)</td>
<td>76.3 ± 10.8 (5)</td>
</tr>
<tr>
<td>Influenza versus control</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Influenza (H) versus control</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean ± SEM (number of determinations). NS, not significant. There was no loss of cytoplasmic constituents from any of the pretreated suspensions.

*The final concentration of thrombin was 0.05 U/ml for NDV and 0.02 U/ml for influenza virus pretreated platelet suspensions.

Mean ¹⁴C-5HT release was 6.6% for NDV-pretreated platelets, compared to 76.3% ± 9.6% for inactive NDV-pretreated platelets and 79.6% ± 6.1% for control suspensions (n = 3, p < 0.001). Treatment of platelets with influenza virus had no effect on their subsequent aggregation response to low concentrations of thrombin (0.02 U/ml), but less ¹⁴C-5HT was lost (6.6%) when compared to platelets pretreated with inactive influenza virus or control buffer (Table 3).

Recovery and Survival of Virus-treated Platelets

Approximately 50%–60% of the platelets exposed to NDV were recovered as single platelets after washing and resuspension. There was no loss of platelets during treatment with influenza or CGV or with the inactivated viruses. Platelets exposed to NDV and injected into rabbits were eliminated from the circulation almost immediately (Fig. 1). At 1 hr less than 5% of the injected radioactivity could be recovered in the circulation. In contrast, the recovery of platelets treated with inactive NDV (90%) was not decreased and the estimated platelet half-life (24.0 hr) was similar to that of untreated platelets (27.8 hr).

Similar results were obtained for influenza-treated platelets after their infusion into rabbits: 13% recovery for platelets preincubated with active virus compared with 81% recovery and a PHL of 21.1 hr for platelets exposed to inactivated virus.

When platelets treated with CGV were injected into rabbits, the percentage recovery was within the normal range and identical to that of the platelets treated with inactivated virus (Fig. 1). The estimated half-lives of both CGV and inactive CGV-treated platelets were slightly shorter than the half-life of untreated platelets but similar to the PHL for platelets treated with either inactive NDV or inactive influenza virus.
DISCUSSION

Thrombocytopenia may be a frequent manifestation of viral infections. We examined the mechanisms whereby myxoviruses may interact directly with platelets to affect platelet survival in vivo or platelet function in vitro. The results indicate that these viruses may cause thrombocytopenia by the formation of platelet aggregates and/or by the accelerated clearance of platelets from the circulation after the removal of platelet surface sialic acid.

Of the viruses studied, only NDV caused aggregation, release of granule contents, and the loss of cytoplasmic constituents in washed platelet suspensions. The hemolytic activity of NDV may be responsible for the loss of cytoplasmic constituents; the other viruses lack this activity. The loss of cytoplasmic constituents including ADP may then enhance the aggregation and release of granule contents caused directly by the virus.

The ability of platelets pretreated with NDV or influenza virus to respond to NDV or to ADP, collagen, and thrombin was determined. Pretreatment of platelets with NDV abolished their ability to respond a second time to NDV.
and reduced their aggregation response to ADP. Aggregation and release induced by thrombin were diminished, but the response to collagen was similar to that observed with control suspensions. It is likely that the initial interaction of platelets with NDV altered or abolished the virus receptors on the platelet surface so that a subsequent addition of NDV was unable to elicit a further response. It is possible that this effect was due to the viral neuraminidase activity, since it was not seen when platelets were pretreated with inactivated virus and NDV does not associate with platelets when the neuraminic acid receptors are removed using purified neuraminidase. However, it seems unlikely that the reduced responsiveness of NDV-pretreated platelets to ADP or thrombin is attributable solely to the neuraminidase activity of NDV, since treatment of platelets with purified neuraminidase slightly enhances their responsiveness to these aggregating agents.

Pretreatment of platelets with influenza virus did not affect their subsequent response to NDV or to ADP. Aggregation and release in response to collagen were slightly enhanced. This observation is compatible with an effect of neuraminidase on the platelet surface. In response to low concentrations of thrombin, the loss of $^{14}$C-5HT from prelabeled platelets was slightly decreased; this finding may be due to either decreased release of granule contents or to increased reuptake of 5HT by the platelet membrane altered by influenza virus. Support for the latter possibility comes from studies that showed an increased uptake of 5HT by human platelets after short-term incubation with neuraminidase.

The results from the platelet survival experiments indicate that treatment of platelets with NDV or influenza virus can cause the rapid removal of these platelets from the circulation following their injection into normal rabbits. This is probably due to the neuraminidase activity of these viruses, since pretreatment of platelets with purified neuraminidase results in their immediate clearance from the circulation. Heat inactivation of NDV and influenza virus destroys their ability to remove sialic acid and to shorten platelet survival. CGV, which does not have neuraminidase activity or cause platelet aggregation, also did not affect platelet survival.

In our earlier experiments with purified neuraminidase it was shown that incubation of rabbit platelets with this enzyme did not result in the release of granule contents or the loss of cytoplasmic constituents, and it was concluded that the sialic acid that appeared in the suspending fluid originated from the platelet membrane glycoproteins. In the present experiments it was observed that influenza virus did not cause the release of granule contents or the loss of cytoplasmic constituents from rabbit platelets. Hence the sialic acid removed from the platelets by this virus probably originated from the platelet membrane glycoproteins. In contrast, the loss of sialic acid from platelets treated with NDV cannot be attributed solely to viral neuraminidase activity because this virus also causes the loss of granule contents and cytoplasmic constituents from rabbit platelets. However, it seems likely that the removal of surface sialic acid by the neuraminidase activity of NDV is responsible for the rapid clearance of these platelets from the circulation. It is unlikely that their rapid elimination was a result of the aggregation and release caused by NDV because we pre-
viously showed that rabbit platelets that underwent these reactions in response to thrombin subsequently survived normally.25,26

The demonstration that viruses with neuraminidase activity shorten platelet survival is in agreement with previous studies with influenza virus5 and with the observations that anucleate red cells treated with either neuraminidase2730 or viruses with neuraminidase activity31,32 are rapidly cleared from the circulation. In addition, treatment of lymphocites with neuraminidase33 or with NDV or influenza virus34,35 results in an alteration of their normal pattern of distribution through lymphoid tissue. Although the present study was not designed to investigate the mechanism responsible for the removal of desialylated platelets from the circulation, it seems likely that they may be removed by the liver in the same manner as desialylated erythrocytes and lymphocytes.28,30,32

It was previously shown that NDV16 and influenza virus5 cause thrombocytopenia when injected into rabbits. The results of the present study indicate that there may be at least two intravascular mechanisms directly involved in virus-induced thrombocytopenia, (1) the ability of certain viruses such as NDV or other paramyxoviruses to cause platelet aggregation and (2) the enhanced clearance of circulating platelets associated with the removal of platelet sialic acid by viruses with neuraminidase activity such as influenza virus or NDV. In addition, the results indicate that platelets altered during viremia but that remain in the circulation may have an altered response to agents such as ADP, collagen, or thrombin.

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