Interaction Between Vaccinia Virus and Human Blood Platelets

By Tova Bik, Israel Sarov, and Avinoam Livne

The objective of the present study was to characterize the interaction between human platelets and vaccinia virus and to examine possible impairment of platelet functions. The vaccinia virus was selected for our model system because it lacks detectable neuraminidase activity. Platelets were incubated with purified viral particles labeled with ^{3}H-thymidine and binding parameters were analyzed. Binding reached saturation with an average of 5 particles/platelet. It was not affected by the plasma but was sensitive to temperature and to metabolic inhibitors. ^{3}H-thymidine-labeled vaccinia virus and formaldehyde-fixed platelets were used to measure viral adsorption. The adsorption was temperature-independent but was affected by ionic strength, indicating electrostatic interactions. Treatment of the fixed platelets with neuraminidase or with alkaline phosphatase reduced viral adsorption, indicating that sialate and phosphate residues on the platelet surface may be involved in the adsorption. Platelet activities were markedly affected by vaccinia virus. The virus caused a dramatic ^{14}C-serotonin release with no added inducer. The release was inhibited by aspirin, a known inhibitor of serotonin release related to prostaglandin synthesis. Furthermore, the virus inhibited platelet aggregation, induced by either ADP, collagen, or thrombin. This study demonstrates that although vaccinia virus lacks neuraminidase activity, it does bind to platelets and affects their function.

THROMBOCYTOPENIA may occur during the acute phase of viral infections. Many possible mechanisms have been proposed for thrombocytopenia related to viral infection. The proposed mechanisms include disseminated intravascular coagulation with the consumption of platelets and plasma coagulation factors,[1] impaired platelet production due to alteration of megakaryocytes by viruses,[2] and a direct interaction between viruses and circulating platelets resulting in phagocytosis or in aggregation, release, and lysis of platelets.[3-6] Other suggestions include platelet destruction by viral antigen–antibody complexes or by antibodies directed against specific platelet antigens (e.g., anti-i).[7-11] Hemolysis of red blood cells by viruses with the release of procoagulant materials (e.g., ADP, thromboplastin-like material)[1] and removal of surface platelet's sialic acid residues by viruses that contain neuraminidase activity were also proposed.[12] The interaction of platelets with Newcastle disease virus and with influenza virus was studied in considerable detail.[3-5,12] These myxoviruses shorten platelet survival. The enhanced clearance of circulating platelets is associated with removal of platelet sialic acid by neuraminidase activity, which is common to myxoviruses. Therefore, it was of special interest to examine a virus–platelet interaction independent of neuraminidase activity. Vaccinia virus was selected for our study because it lacks neuraminidase activity, it causes thrombocytopenia after intravenous injection into mice,[6,13] and it is well characterized.[14]

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MATERIALS AND METHODS

Preparation of Platelet Suspensions

Venous blood was obtained from healthy donors who had not taken any drugs in the preceding 10 days. The blood was collected into plastic tubes. One part of acid citrate dextrose (ACD) anticoagulant solution was added to 6 parts of blood ($6.0 < pH < 6.5$) or 1 part of sodium citrate anticoagulant solution was added to 10 parts of blood ($7.2 < pH < 7.4$). Washed platelet suspensions were prepared by the method described by Mustard et al.[15] Heparin was used in the first washing fluid to prevent thrombin generation, and apyrase was used to prevent adenine nucleotides accumulation. Platelet suspensions were stored at 37°C till used. To prepare platelet-rich plasma (PRP), whole blood with ACD anticoagulant was centrifuged at 120 *g* for 10 min at 37°C. PRP was removed and stored at room temperature. Platelets were counted in a coulter counter Fn Model. Freshly prepared platelets were used within 4 hr from time of preparation.

Cells and Viruses

HeLa cells for vaccinia virus production were cultivated in minimal essential medium (Bio-Lab, Israel), containing 10% fetal calf serum (Gibco, Grand Island, New York). Cells of 1 or 2 days were infected with vaccinia virus (WR strain) as previously described.[16] Radioactive virus was obtained by the inclusion of 10 μCi/ml of ^{3}H-thymidine (Nuclear Research Center, Beer Sheva, Israel) during virus propagation.[16] Viral particles were purified 48–72 hr postinfection by zone centrifugation in sucrose gradients as previously described.[17] Virus bands were collected and frozen at −70°C till use. The quantitation of viral particles was based on absorbance at 260 nm as follows:[18]

$$1\ A_{260nm} = 64\mu g\ \text{protein/ml} = 1.2 \times 10^{10}\ \text{virions/ml}$$

Binding Procedure

Binding of viral particles to platelets was done according to the scheme described in Fig. 1. Platelets (1.9 ml), either in plasma or washed, were incubated with ^{3}H-thymidine-labeled viral particles (0.1 ml). At time intervals thereafter, samples (0.25 ml) were centrifuged for 1 min in a microfuge (Beckman). The supernatant fluid and pellet were separated, and the pellet containing platelets with bound viral particles was suspended in 0.1 ml 0.5% sodium dodecyl sulphate and counted in a scintillation counter. The quantitation of virus binding to platelet was based on the radioactivity associated with platelets and equated with the equivalent number of particles.

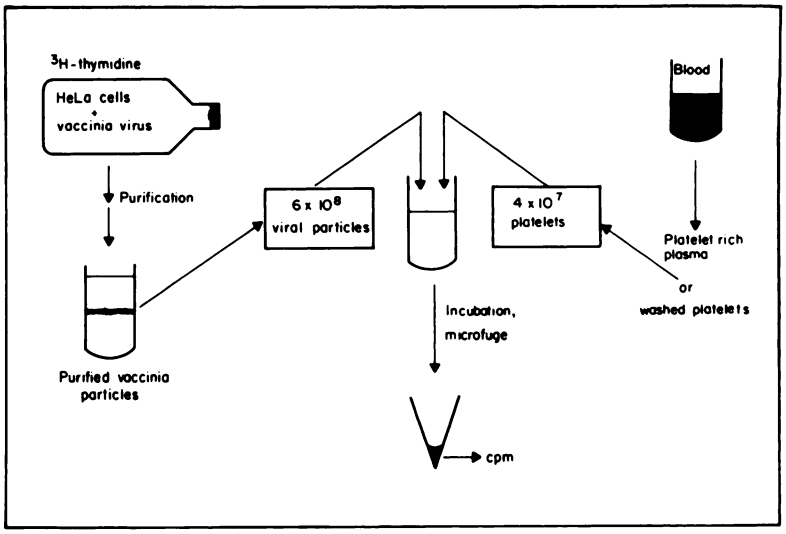

Fig. 1. An outline of the standard procedure for the binding of ^{3}H-thymidine-labeled vaccinia virus to human platelets. Temp: 22°C.

Platelet Fixation

Platelet fixation was done according to Kirby and Mills.[18] PRP was diluted with equal volume of 2% formaldehyde (Frutarum, Haifa, Israel) in saline. After storing the mixture at 4°C for 48 hr, the platelets were sedimented at 2500 *g* for 10 min at 4°C, and gently resuspended in a large volume of cold saline. Sedimentation and resuspension in fresh saline were repeated three times at 4°C, and the platelets were finally suspended at a platelet count of about 300,000/μl in buffered saline (pH 7.2) containing 0.05% sodium azide. The platelets were stored at 4°C till use.

Diminution of SH-Groups From Platelet Surface

Formaldehyde-fixed platelets were incubated with 10^{-3}*M* 5,5′-dithio-2,2′-dinitrodibenzoic acid (DTNB) (Sigma Chemical Center, St. Louis, Mo.), dissolved in sodium phosphate buffer (pH 7.0), and kept at room temperature for 1 hr. The platelets were then rinsed twice in a large volume of saline and finally resuspended in saline buffered with 50 m*M* phosphate (pH 7.2).

Release of Phosphate Groups From Platelet Surface

Fixed platelets (10^8) were incubated with 2 mg alkaline phosphatase (orthophosphoric monoester phosphohydrolase, Sigma) in 1 ml 0.1 *M* glycine buffer (pH 10) for 1 hr at 37°C. Afterwards the platelets were washed twice with excess volumes of saline and the supernatant fluid was analyzed for released phosphate according to the assay developed by Fisk and Subarrow.[20] The platelets were finally suspended in buffered saline pH 7.2.

Release of Sialate Groups From Platelet Surface

Fixed platelets (10^8) were incubated with 0.02U *Vibrio cholera* neuraminidase (Behring, Marburg) in 1 ml 0.1 *M* acetate buffer (pH 5.5) for 90 min at 37°C. The reaction was stopped by adding 5% phosphotungstic acid (v:v). The platelets were removed by centrifugation, and the supernatant fluid was assayed for free sialic acid (as N-acetyl neuraminic acid) using the thiobarbituric acid assay.[21]

Neuraminidase Activity of Vaccinia Virus

Dialyzed viral particles (4.2–21 x 10^8) or neuraminidase (1–20 x 10^{-3}U), for comparison, were incubated with washed platelets (10^8) suspended in glucose-free Tyrode-albumin solution or with mucin (1.1 mg/ml) for 90 min at 37°C. The extent of loss of N-acetylneuraminic acid was determined using the thiobarbituric acid assay.[21] No neuraminidase activity was observed in the purified vaccinia viral preparation.

Platelet Aggregation

Aggregation was followed turbidumetrically in an aggregometer at 37°C. PRP was used for ADP and for collagen-induced aggregation, while washed platelets were employed for thrombin-induced aggregation. Viral particles (5 μl) in sucrose (0.01 *M* final concentration in the aggregation assay) or in 0.155 *M* NaCl were incubated with the platelets for 5 min at a final volume of 0.5 ml and then the appropriate inducer of aggregation was added. The medium in which the virus was added did not change the effect of the virus.

^{14}C-Serotonin Release

PRP was incubated with 5-hydroxytryptamine-3′-^{14}C-creatinine sulphate (the Radiochemical Centre, Amersham, England) at a final concentration of 0.7 μ*M* for 1 hr at 37°C. Then, viral particles or collagen (for comparison) were added to initiate serotonin efflux. To stop the reaction, samples of 0.2 ml were centrifuged for 1 min in the microfuge. Then, 0.1 ml of the supernatant fluid was added to the scintillation vials and radioactivity was counted.

RESULTS

Binding of Vaccinia Virus to Platelets

The pattern of binding of vaccinia virus to platelets, either in plasma (PRP) or in synthetic medium (washed platelets), was similar (Fig. 2): in both cases saturation was reached within 15–20 min of incubation with an average level of 5 viral particles per platelet. Thus, plasma did not modify the binding. This very same level of saturation of virus binding to platelets was observed either at a range of virus density (3–20 x 10^8 particles incubated with 5 x 10^7 platelets) or at a range of platelet density (1.34–13.4 x 10^7 platelets incubated with 6 x 10^8 virus particles). The binding of ^{3}H-viral particles to platelets was essentially irreversible and was practically halted by the subsequent

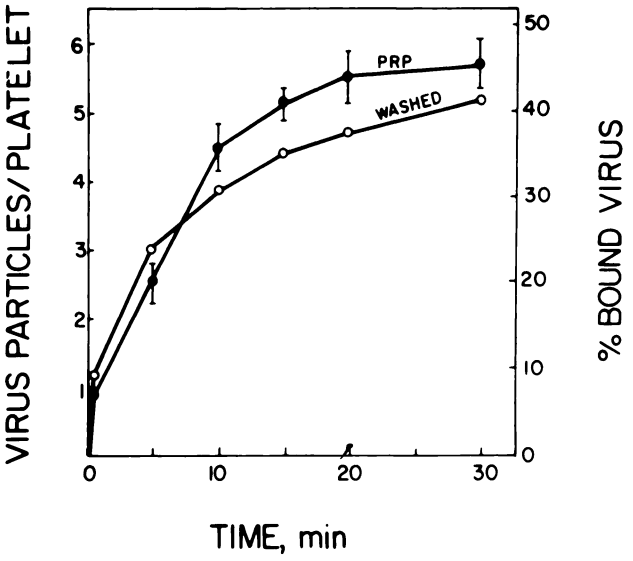

Fig. 2. Time course of binding of ^{3}H-thymidine-labeled vaccinia virus to either washed platelets or platelets in plasma (PRP). Details as in Fig. 1. The vertical lines denote standard deviation based on 6 individual measurements. The SD of the binding to washed platelets was similar.

addition of four-fold excess of nonlabeled viruses, indicating competition for limiting platelet sites. The binding of vaccinia virus to platelets was temperature sensitive, notably at the range of 15°C–22°C (Fig. 3).

Adsorption of Vaccinia Virus to Platelets

The overall binding measurement includes virus adsorption and further processing (such as internalization). The latter is apparently prevented when the platelet metabolic activity is impaired. Indeed, a typical pattern of limited association of ^{3}H-virus with

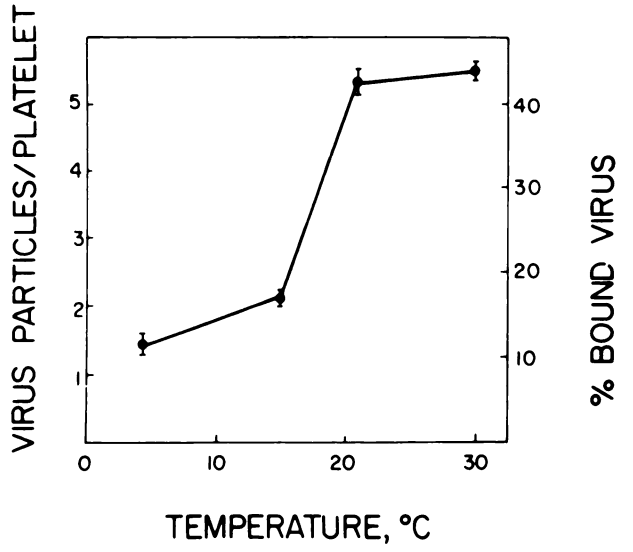

Fig. 3. The effect of temperature on the binding of ^{3}H-thymidine-labeled vaccinia virus to human blood platelets. Details as in Figs. 1 and 2. Incubation time: 30 min.

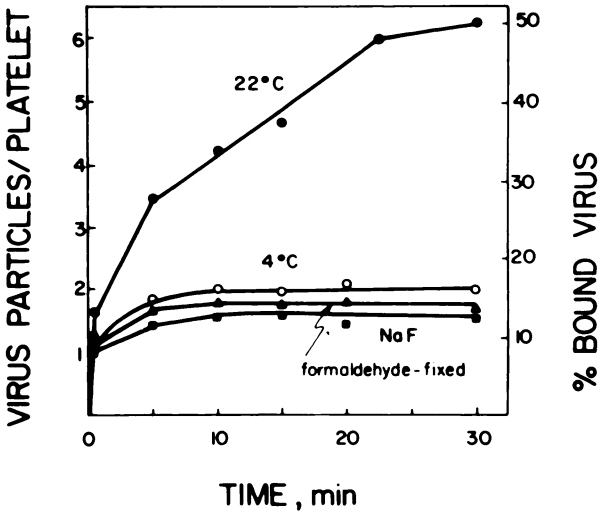

Fig. 4. Virus–platelets interaction under metabolic restrictions. Details as in Fig. 1. (●) Control; (○) platelets exposed to 4°C for 10 min and the interaction took place at 4°C; (▲) platelets fixed with formaldehyde as described in Materials and Methods; (■) platelets treated with 10 m*M* NaF for 30 min and washed by centrifugation. Each value is an average of at least 6 measurements. The differences between the 3 lower curves are not significant.

platelets was observed under 3 distinct conditions: following treatment with NaF, at 4°C, or with formaldehyde-fixed platelets (Fig. 4). Thus, we conclude that adsorption is measured under such conditions of impaired metabolism. Formaldehyde-fixed platelets were further studied to characterize the process of virus adsorption. Similar to the overall binding, the adsorption was essentially irreversible and the labeled particles were not dislodged by fourfold excess of unlabeled virus particles added 10 min later. Unlike the temperature-sensitive binding, adsorption was not affected by temperature at a wide temperature range, indicating that hydrophobic interactions were not dominant in the adsorption process. The involvement of electrostatic interactions in this process is seen in Fig. 5; with increasing ionic strength of the medium, the adsorption of virus to the fixed platelets was clearly elevated, even at 1 *M* NaCl. Control experiments established that pretreatment of either the virus or the fixed platelets with NaCl concentrations of up to 1 *M*, followed by dilution to 0.155 *M* NaCl during platelet–virus incubation, yielded adsorption level typical to 0.155 *M* medium.

What groups on the surface of the fixed platelets are involved in viral adsorption?

(A) *Sulphydryl groups.* Treatment of fixed platelets with 1 m*M* DTNB to eliminate free SH groups did not modify viral adsorption. Thus, SH groups on the platelet surface do not participate in the adsorption.

(B) *Phosphate groups.* Treatment of formaldehyde-fixed platelets with alkaline phosphatase resulted

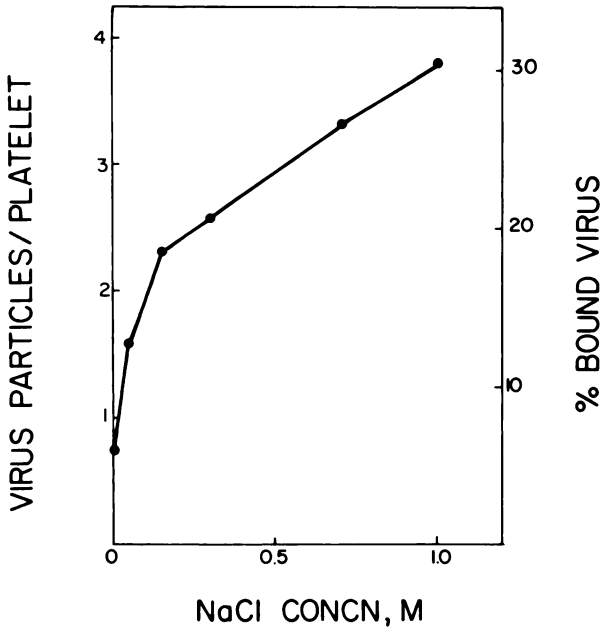

Fig. 5. Adsorption of vaccinia virus to platelets as affected by addition of NaCl. ^{3}H-thymidine-labeled viral particles were incubated with formaldehyde-fixed platelets for 30 min at 22°C. Each value is an average of 8 measurements.

in a gradual release of phosphate and a concomitant decrease in viral adsorption. One-hour treatment with phosphatase released 7 nmol Pi/10^9 platelets and decreased viral adsorption by 25%.

(C) *Sialate groups.* Neuraminidase treatment of fixed platelets hydrolyzed sialate groups and in parallel reduced viral adsorption. Up to 60% of total sialic acid were removed by neuraminidase. The corresponding maximal reduction in adsorption was 40%. Figure 6 compares the maximal effects of alkaline phosphatase and neuraminidase on viral adsorption. The figure shows that the two enzymes, applied consecutively, exerted additive effects.

Vaccinia Virus and Platelet Function

Serotonin release and aggregation of freshly prepared platelets were analyzed as parameters of platelet function. Vaccinia virus induced a rapid release of serotonin. The extent of the release, exceeding 45% of the serotonin content, was proportional to the viral dose (Fig. 7A) and was greatly inhibited by aspirin (Fig. 7B).

Figure 8 shows that vaccinia virus particles impaired platelet aggregation induced by either ADP, collagen, or thrombin. Both the initial rate and extent of aggregation were clearly affected by the virus. Control platelets formed large aggregates in a clear medium, but the aggregates formed by the virus-affected platelets were much smaller, in a turbid medium, despite extended mixing. The extent of impairment was correlated with virus concentration

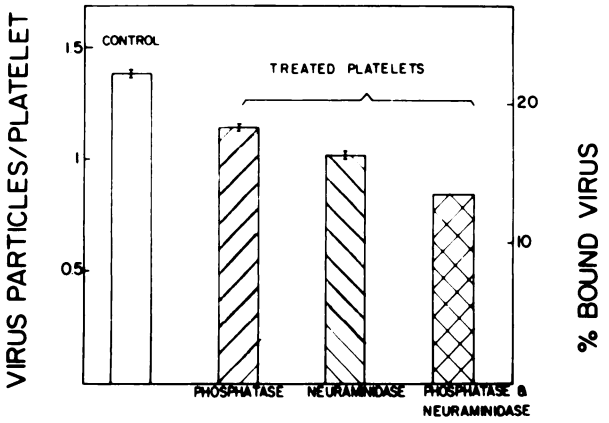

Fig. 6. Adsorption of ^{3}H-thymidine-labeled vaccinia viral particles to formaldehyde-fixed platelets following enzymatic treatment of the platelets. The treatments are described in Materials and Methods. The vertical lines denote standard deviation. The combined enzymatic treatments were tested 4 times; other treatments, 8 times.

and could be partly obviated by increasing the inducer concentration.

DISCUSSION

Vaccinia virus is one of the largest viruses (220 × 220 × 280 μm). We estimate that an "average" platelet is capable of accomodating on its surface up to 30 particles. Actually, just 5 ^{3}H-virus particles were found associated with a platelet at saturation (Fig. 2), including both adsorbed and internalized particles. Thus, the limitation in virus binding to platelets cannot be explained by steric interference only. The binding of ^{3}H-virus was inhibited by the addition of nonradioactive virus to the reaction mixture. Such type of interference has been interpreted as blocking of specific membrane binding sites in a number of virus systems.[22] Lycke et al. showed that herpes simplex type I (HSV-1) interfered with the adsorption of subsequently

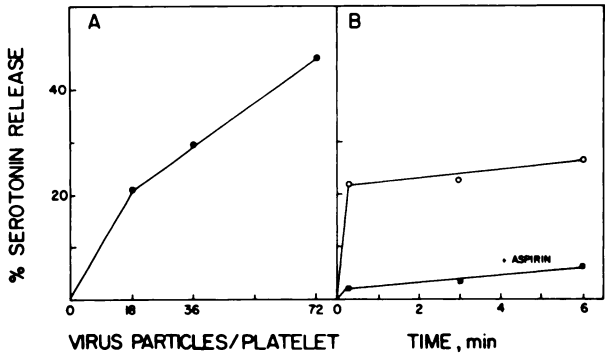

Fig. 7. Release of ^{14}C-serotonin from platelets (10^8) caused by vaccinia virus. The experiment was repeated 4 times in duplicate and average values are given. (A) Effect of viral concentration. Reactivation time: 6 min. (B) Time course of serotonin release as affected by aspirin. Number of viral particles: 1.8 × 10^9 (18 particles per platelet). The platelets were incubated with aspirin (0.7 m*M*) for 20 min.

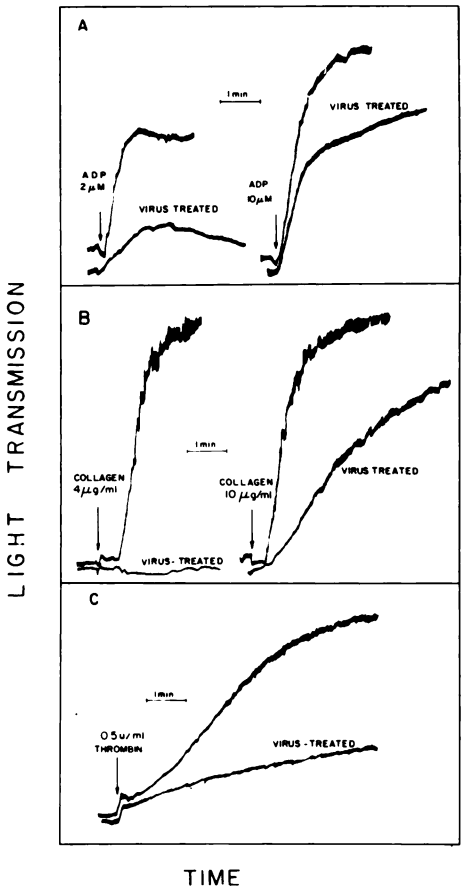

Fig. 8. Effect of vaccinia virus on platelet aggregation induced by (A) ADP, (B) collagen, (C) thrombin. The experiments were repeated at least 5 times, and representative tracings are given. PRP was used for ADP- and collagen-aggregation, while washed platelets were used for thrombin-induced aggregation. Number of viral particles 1.8×10^9 (18 particles per platelet). Additional details in Materials and Methods.

added homotypic but not heterotypic HSV.[23] Weissman et al. showed that the binding of MuLV to target cells was only blocked by homologous MuLV.[24]

Our results suggest an interaction between vaccinia virus particles and certain binding sites on the platelet membrane. To identify likely groups on the platelet membrane involved in the interaction with the vaccinia virus, we have examined conditions that limit virus penetration and thus allow estimation of viral adsorption. Three different treatments, both chemical or physical, which led to impaired platelet metabolism gave rise to a common pattern of viral association to platelets. This pattern was distinct in rate and extent from the overall binding. Formaldehyde-fixed platelets are particularly suitable for the characterization of the interaction because of several reasons: (A) The fixed platelets retain surface properties of the fresh platelets.[25,26] (B) The fixed platelets can be stored for several weeks for repeated and reproducible studies. (C) Following fixation the platelets are resistant to a very wide range of conditions, such as required for alkaline phosphatase treatment.

Viral adsorption was clearly affected by ionic strength (Fig. 5) but not by temperature, indicating the involvement of electrostatic rather than hydrophobic interactions. Our results are in agreement with the report of Allison and Vallentine, who demonstrated that vaccinia virus adsorbed to charged surfaces and that adsorption was dependent on the medium cation concentration. They proposed electrostatic interaction between phosphate groups of the host cells and amino groups of vaccinia virus.[27] Our data show that the virus adsorbs to either carboxyl or phosphate groups of the platelet surface (Fig. 6), while SH and amino groups apparently do not play an essential role.

The mechanism of impairment of platelet aggregation (Fig. 8) is not clear, and several possibilities exist. The virus did not induce aggregation by itself, but it did cause a marked serotonin release (Fig. 7A). Serotonin release was mediated by the mechanism related to prostaglandin metabolism, since it was sensitive to aspirin (Fig. 7B). The dual effects of the virus (namely, induction of serotonin release but inhibition of platelet aggregation) may be due to protease activity of the vaccinia virus, which cleaves surface proteins. Indeed, the involvement of proteolytic enzymes in the entry of viruses into animal cells was proposed.[28]

Alternatively, the virus may have interfered with accessibility of the platelets to the aggregating agents, particularly since it was possible to partially overcome the inhibition by raising the dose of the aggregation agents. However, this explanation is in some doubt as thrombin, ADP, and collagen affect platelet aggregation through different and distinct receptors. Additional possibilities related to calcium availability should be considered. Durham has shown that vaccinia virus makes "holes" in the membrane allowing leakage of calcium ions.[29] Thus, an effect of vaccinia virus on the platelet calcium economy, in possible analogy to the effect of EDTA,[25,26] may be detrimental to platelet aggregation.

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