Actively Induced Platelet-bound IgG Associated With Thrombocytopenia in the Marmoset

By Nazareth Gengozian and Carla L. McLaughlin

Interspecies platelet immunizations among marmosets lead to antibody formation to the donor platelets and a profound thrombocytopenia, which when associated with anemia may result in death of the animal. This actively induced immunologic thrombocytopenia closely resembles two clinical disease entities manifesting autoimmune thrombocytopenia, posttransfusion purpura and idiopathic thrombocytopenic purpura. Although antibody to donor-type platelets could be demonstrated readily, antihost activity was most often nondetectable or, when present, was in very low titer. A consistent finding was the appearance of IgG on the host's platelets shortly after immunization and concomitant with the appearance of antidonor platelet antibody. In 3 of 13 immunized animals thrombocytopenia did not occur even though antibody was formed and the host's platelets became IgG positive. In those animals that recovered from the induced thrombocytopenia IgG-positive platelets were found for periods ranging from 30 to greater than 100 days. Splenectomy before or after immunization did not alter the sequential development of antibody formation, appearance of IgG-positive platelets, and thrombocytopenia. Eluates prepared from IgG-positive platelets contained IgG and platelet antigens; the eluted IgG could attach nonspecifically to platelets of host or donor (immunizing) type, in contrast to the species specificity demonstrated for IgG eluted from platelets that had been reacted in vitro with specific antibody. Platelets in a few normal, nonimmunized marmosets were found to have significant amounts of IgG on their surface, comparable to that observed in the immunized animal; interestingly, such IgG-positive platelets were found among imported but not laboratory-bred marmosets.

We previously reported that immunization of Saguinus oedipus marmosets with platelets from another species of marmoset, S. fuscicollis, resulted in a profound thrombocytopenia of the recipient that, when associated with marked anemia, led to death of the animal. Examination of the sera of the immunized animals revealed agglutinating antibody directed toward donor platelets but not to those of host type. This phenomenon closely resembled the clinical condition of posttransfusion purpura described initially by Shulman et al. wherein patients receiving one or more transfusions of whole blood for postoperative procedures developed an acute and fulminating thrombocytopenic purpura lasting anywhere from 3 to 21 days. As in our animal studies, the patients lacked antibody to their own platelets but had developed high-titered serum antibody to antigens of the donor platelets. This observation led Shulman and co-workers to speculate that the thrombocytopenia resulted...
from attachment of antigen-antibody complexes to the host platelets, which then led to their rapid sequestration and destruction by the reticuloendothelial system.

The present study was aimed at defining more closely the immunologic events associated with the disease process in the marmoset. The demonstration by Dixon et al. of significant amounts of platelet-associated IgG in patients with idiopathic thrombocytopenic purpura (ITP) prompted us to examine our experimental model for the active induction of IgG attachment to the platelet surface following immunization. Although several normal, nonimmunized marmosets were found to have significant amounts of IgG on their platelets, it was found that an interspecies immunization of platelets among marmosets initially lacking platelet-bound IgG invariably led to deposition of IgG on the host's platelets. The development of IgG-positive platelets coincided with the appearance of serum antibody and preceded the onset of thrombocytopenia. Surprisingly, however, this attachment of IgG on the host’s platelets did not always lead to thrombocytopenia, although the IgG-positive platelets were found for periods of 30 to greater than 100 days. Finally, in a few experiments, splenectomy before or after platelet isoimmunization did not alter the sequential development of antibody formation, platelet-bound IgG, and thrombocytopenia.

MATERIALS AND METHODS

Animals. Adult male and female marmosets of three species were used: S. o. oedipus, S. fuscicollis species, and Callithrix jacchus. Imported and laboratory-bred animals served as recipients and platelet donors with no significant effects of immunization being attributable to the source. Maintenance and handling procedures were described in detail elsewhere.5

Preparation of platelets and method of immunization. Procurement of platelets from the whole blood was described previously.1 Briefly, 2-3 ml anticoagulated blood was layered on a methylcellulose-sodium diatrizoate gradient, specific gravity 1.115-1.118. Following centrifugation at 400 g for 30 min the mononuclear cells remaining at the interface were collected and washed twice with Ca2+- and Mg2+-free Hanks' balanced salt solution (incomplete HBSS). This procedure eliminated almost all red blood cells but retained platelets and white blood cells (WBC). Our previous studies showed that the presence of WBC in the immunizing inoculum played no part in the induction of the disease; however, in certain experimental procedures (see below) the WBC were removed from the platelet preparation by differential centrifugation. In general, we used heparin as the anticoagulant when preparing platelets for immunization and EDTA (1.5%, disodium-ethylenediaminetetraacetate) or ACD (0.8%, citric acid, 2.2%, sodium citrate, 2.45%, hydrous dextrose) when the platelets were to be used for various assays in vitro.

After washing, 600-800 x 10⁶ platelets were resuspended in 0.1 ml Eisen's medium and emulsified in an equal volume of complete Freund's adjuvant. This preparation was injected intramuscularly along the outer aspects of the thigh, each recipient receiving either one or three weekly injections from the same donor.

Hematology. At varying intervals after the initial or single injection the animals were bled for serum or routine hematology. Platelet counts were made by phase-contrast microscopy.

Antiserum reagents. Marmoset IgG was obtained from ammonium sulfate (33%) fractionated serum chromatographed on a DEAE-Sephadex A-50 column for isolation of the IgG component. The various fractions were concentrated and tested with a broad spectrum goat anti marmoset whole serum reagent, and those showing the presence of only IgG by immunoelectrophoresis were used to immunize a goat and rabbit. The goat anti-IgG antiserum was fractionated with ammonium sulfate (33%) and then conjugated with fluorescein isothiocyanate (FITC) by the method of The and Feltkamp.6 The same batch of FITC-conjugated goat anti marmoset IgG (FITC-GAlgG), having an F/P ratio of 5.1, was used throughout the study. Although this reagent and the rabbit anti-marmoset IgG (RAIgG) antiserum reacted only with IgG when tested by immunoelectrophoresis against whole marmoset serum, they were not made heavy chain specific.
A rabbit antiplatelet antiserum (RAPS) was obtained by immunization of a rabbit with thoroughly washed platelets (WBC-depleted) of each marmoset species. The immunization protocol consisted of several intramuscular and footpad inoculations of platelets suspended in saline or complete Freund's adjuvant. When tested with the supernatant of sonicated platelets of either species, four precipitin bands were observed in the immunodiffusion reaction of this antiserum.

**Immunodiffusion.** Agarose, 1.5%, and pH 8.6, was used in the immunodiffusion (ID) analyses. The reactions were performed with the supporting gel on microscope slides.

**Assays for Serum Antibody**

Two procedures were used routinely in vitro to test for serum antibody to either donor- or host-type platelets.

*Platelet cytotoxicity in the presence of antibody and complement.* Approximately 1 x 10⁹ platelets free of WBC and suspended in 0.1 ml incomplete HBSS were mixed with 100 µCi ⁵¹Cr (sodium chromate, Amersham/Searle, Arlington Heights, Ill.) and incubated for 30 min at room temperature. The platelets were then washed twice with incomplete HBSS solution and resuspended to a concentration of 20 x 10⁶/ml in guinea pig complement diluted 1:9 in Eisen's medium containing optimal amounts of Ca²⁺ and Mg²⁺. Then 0.1 ml ⁵¹Cr-labeled platelets were added to tubes containing 0.05 ml twofold serially diluted serum to be tested. After incubation for 1 hr at 37°C, 2 ml cold Eisen's medium was added and the tubes centrifuged to separate supernatant and the platelet button. These were then counted in a Tracerlab Spectro/matic gamma counter and the percentage radioactivity released into the supernatant determined. Control background release, i.e., from platelets suspended in normal serum, was consistently less than 5% of the total radioactivity incorporated into the platelets. Therefore the endpoint in titration was taken as that dilution of serum causing release of at least 10% of the total radioactivity. Maximum release from labeled platelets was determined by three rapid freeze-thaw cycles; this yielded 75% of the total radioactivity into the supernatant fluid. That our test system was operating near optimal efficiency was shown by release of greater than 95% of the total releasable radioactivity when platelets were incubated in high concentrations of antiserum in the presence of complement.

*Indirect immunofluorescent tests for attachment of antibody (IgG) to platelets.* Platelets to be used in this test were known to be negative for surface-bound IgG by direct immunofluorescence with the FITC-GA IgG reagent (see below). To 0.05 ml of twofold serially diluted serum was added 0.05 ml platelets at a concentration of 500 x 10⁶/ml. The mixture was incubated for 30 min at 37°C and then washed three times with incomplete HBSS. The supernatant was removed and 0.05 ml of a 1:9 dilution of the FITC-GA IgG reagent added to the platelet button. After incubation for 45 min at 37°C the platelets were washed twice with phosphate-buffered (pH 7.4) saline containing 0.1% sodium azide. The platelet suspension was then examined as a wet preparation on a microscope slide with a Zeiss Universal microscope. Illumination was obtained with a xenon arc lamp using a FITC exciter filter (Zeiss No. 910031) and barrier filters 65 and 60. A rabbit antiplatelet antiserum (RAPS) was obtained by immunization of a rabbit with thoroughly washed platelets (WBC-depleted) of each marmoset species. The immunization protocol consisted of several intramuscular and footpad inoculations of platelets suspended in saline or complete Freund's adjuvant. When tested with the supernatant of sonicated platelets of either species, four precipitin bands were observed in the immunodiffusion reaction of this antiserum.

*Sucrose density gradients.* Selected serum samples were subjected to fractionation on sucrose density gradients to obtain some idea of the molecular size (heavy, ~19S, or light, ~7S) of the antibody formed. Sucrose gradients from 10%, to 37%, 4.6 ml volume, were made with an automatic mixing device, and 0.4 ml diluted serum (1:1) was layered on the gradient surface. The gradients were centrifuged for 16 hr at 35,000 rpm at 4°C in a Spinco L-2 preparative ultracentrifuge with a swinging bucket rotor (SW 39). Gradient fractions were obtained by piercing the bottom of the celluloid tubes; each fraction consisted of 25 drops. Fractions were assayed for antiplatelet antibody by both the ⁵¹Cr release test and indirect immunofluorescence for IgG binding to target platelets.

*Direct immunofluorescent tests for IgG on marmoset platelets.* Platelets from a number of marmosets were examined for surface-bound IgG before and after platelet immunization. This was done in a manner similar to that described above for testing for attachment of serum antibody to platelets. Thus excluding incubation with any serum the washed platelets were treated directly with the FITC-GA IgG reagent and examined for fluorescence under the conditions described. Although no attempts were made to quantitate the amount of IgG found on the platelets, examination of several preparations independently by two investigators permitted a 0, +, ++, +++ scoring system based on intensity of fluorescence and extent to which the platelet surface was covered with IgG. Although several animals were thus classified as bearing no platelet-bound IgG, it is recognized that a more quantitative and sensitive technique such as that described by Dixon...
et al. would show platelets from all animals to bear some surface IgG. The method of scoring in the present study was chosen because the number of platelets that could be obtained safely from animals experiencing severe thrombocytopenia precluded attempts to quantitate the amount of IgG on their surface such as those reported by others in various clinical studies. Thus for routine testing of experimental animals only 0.05-0.2 ml of whole blood was drawn for platelet separation and examination by direct immunofluorescence for surface-bound IgG.

RESULTS

Tests for surface-bound IgG on marmoset platelets. Platelets from imported and laboratory-bred marmosets of two species, S. o. oedipus and S. fuscicolis, were examined for surface-bound IgG by direct immunofluorescent tests with the FITC-GA IgG reagent. As shown in Table 1, a striking difference was noted in each species, dependent upon the source of the animal. Only 2 of a total of 42 laboratory-bred marmosets were found to bear any detectable IgG on their platelets. In contrast, significant numbers of imported members of both species were found to have IgG-positive platelets. When examined over a period of several months, there was little variation in the extent (i.e., +, ++, or ++++) to which this surface-bound IgG was found on the platelets of individual animals. Platelets testing negative for IgG were found to maintain this state.

The presence of highly positive (+++++) IgG-bearing platelets in several of the imported marmosets prompted us to examine their sera for the presence of humoral factors that would show attachment of IgG to IgG-negative platelets from animals of each species. Indirect immunofluorescent tests failed to reveal any such factors. Sera from these animals were also tested for cytotoxic antibodies directed toward platelets of IgG-negative animals of each species; using the 51Cr release assay, there was no indication of any natural circulating anti-platelet antibody. Identical results were obtained when similar tests were performed with sera from animals having IgG-negative platelets.

Interspecies platelet immunizations. Animals used in this phase of the study were screened carefully for surface-bound IgG; only those found to be negative were used as recipients or donors. A total of 13 marmosets received either one or three weekly inoculations of platelets from another species of marmoset. Two were splenectomized after initiating the series of three inoculations, one on day 23 and the other on day 13; two were splenectomized before immunization. Information pertaining to antibody formation, thrombocytopenia, and the IgG status of the recipients' platelets are shown in Table 2. Figure 1 depicts

<table>
<thead>
<tr>
<th>Marmoset Species</th>
<th>Source</th>
<th>IgG on Platelets*</th>
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<tbody>
<tr>
<td>S. o. oedipus</td>
<td></td>
<td></td>
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<tr>
<td>Imported</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>Lab-bred</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>S. fuscicolis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imported</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Lab-bred</td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

*Numbers indicate number of animals tested.
†Based on the scoring procedure used (Materials and Methods), the majority of the animals bearing IgG-positive platelets were scored as +; however, eight S. o. oedipus and one S. fuscicolis were scored as ++ or ++++ in repeat examinations.
Table 2. Immunologic and Clinical Observations on Marmosets Receiving an Interspecies Platelet Immunization

| Platelet Isoimmunization* | Serum Antibody |  |  |  |  |  |
|---------------------------|----------------|--------------------------|---------------|--------------------------|---------------|
| No. | Recipient | Donor | Day of Detection | Maximum Titer† | IgG* Platelets (Days Detected) | Thrombocytopenia‡ (Day Detected) | Survival Status |
| 1 | S. fus. (3)§ | S. o. oed. | 20 | 12 | 23-25 | 23 | Dead (25)§ |
| 2 | S. fus. (3) | S. o. oed. | 14 (21)** | 10 (6)** | 18-38 | 21 | Dead (38) |
| 3 | S. fus.†† (3) | S. o. oed. | 21 | 11 | 24-60 | 24 | Alive |
| 4 | S. o. oed. (3) | S. fus. | 20 | 12 | 30-91 | — | || Alive |
| 5 | S. o. oed.†† (3) | S. fus. | 11 | 10 | 27-112 | 42 | Alive |
| 6 | S. fus. (1) | S. o. oed. | 26 | 10 | 33-102 | — | Alive |
| 7 | S. fus. (1) | C. jac. | 14 (30) | 13 (5) | 21-30 | 21 | Dead (30) |
| 8 | C. jac. (1) | S. fus. | 14 (24) | 13 (3) | 17-161 | 14 | Alive |
| 9 | S. o. oed. (1) | S. fus. | 14 | 14 | 13-26 | 14 | Dead (26) |
| 10 | S. o. oed. (1) | S. fus. | 21 | 11 | 21-115 | 34 | Alive |
| 11 | S. o. oed. (1) | S. fus. | 17 | 14 | 16-118 | — | Alive |
| 12 | S. fus. (3) | Freund | — | — | — | — | Alive |
| 13 | S. fus. (3) | Freund | — | — | — | — | Alive |
| 14 | S. o. oed. (3) | Freund | — | — | — | — | Alive |

*Fus., fusciolus; oed., oedipus; jac., jacchus; Freund, complete Freund's adjuvant.
†Log₂; indirect immunofluorescence test for IgG binding.
‡Thrombocytopenia defined as platelet count at least 50% below preimmunization level. Those experiencing thrombocytopenia ultimately showed platelet counts less than 50,000/cu mm at time of maximum platelet loss.
§Number in parentheses indicates number of weekly injections.
¶Day of death.
‖Laboratory-bred animal.
**Number in parentheses refers to tests made with host-type (Nos. 2 and 7) or autologous (No. 8) platelets.
††Animals splenectomized after initial injections, No. 3 on day 23 and No. 5 on day 13.
§§Negative test.

graphically the time course in development of these parameters of response for three of the animals.

Antiplatelet antibody. All marmosets developed serum titers of at least 10 (log₂) against donor-type platelets, this antibody most frequently appearing within the second or third week after the initial or single inoculation. As shown in Fig. 1, greater antibody titers were recorded by the indirect immunofluorescent test than by the cytotoxic assay for complement-dependent antibody, a consistent finding throughout this study. Sucrose density gradient analyses of selected serum samples showed the antibodies to be primarily 7S immunoglobulins, although activity in the heavy or 19S zone of the gradient was detected in a few samples obtained early (days 14, 20) in the immunization scheme.

Selected serum samples from all animals were also tested for antibody toward autologous or host-type platelets by both assays. With the indirect immunofluorescence method, tests with autologous platelets could be made only after the animal had recovered from the thrombocytopenic state and when its platelets were known to be IgG negative (see below). Only three animals (Table 2, Nos. 2, 7, and 8) showed the presence of serum antibody directed toward host-type platelets; this activity could be detected only by the indirect immuno-
Fig. 1. Status of blood platelets in marmosets receiving an interspecies immunization of platelets suspended in complete Freund's adjuvant. Initial injection made at time zero on graph. *Number in parentheses, number of weekly platelet inoculations. ** IgG status of recipient's platelets indicated by - and + signs along ordinate correlating with time after immunization. Solid and open bars represent antibody titers as assayed by indirect immunofluorescence and 51Cr release assay, respectively. Data also tabulated in Table 2, Nos. 10 (Fig. 1A), 4 (B), and 3 (C). Animal No. 3 was splenectomized on day 23 after the initial injection (see arrow).
fluorescence assay. As noted in Table 2, the titers were low and the antibody was present in the serum at a time when the animals were severely thrombocytopenic. Death of marmosets 2 and 7 precluded test of their serum with autologous platelets. Animal No. 8 however, which experienced a marked thrombocytopenia for approximately 4 wk, eventually showed a recovery of platelets to normal levels, at which time the serum obtained during the period of thrombocytopenia could be tested for antibody activity against the autologous platelets. The indirect immunofluorescent test was positive with the autologous platelets, although the titer, log$_{10}$ 3, was much lower than that observed with the donor platelet antigen (Table 2). Serum samples from the remaining surviving marmosets showed no activity toward autologous platelets by either the $^{31}$Cr release assay or indirect immunofluorescence.

IgG-positive platelets and thrombocytopenia. Blood was obtained from each animal at varying times after receiving the initial or single inoculation of the interspecies platelets and the separated platelets examined by direct immunofluorescence for surface-bound IgG. As noted in Table 2 and Fig. 1, the platelets of all marmosets became IgG-positive shortly after the appearance of circulating anti-donor platelet antibody. The sequential development of the IgG-positive state varied for each individual animal, but platelets from all ultimately showed an intense immunofluorescent staining for this protein. Figure 2 offers a representative illustration of the appearance of IgG-positive and IgG-negative platelets when viewed through the fluorescent microscope. Figure 2A shows the fluorescent-staining platelets when the ultraviolet light (u.v.) is passed through exciter and barrier filters, and Fig. 2B shows the same field of platelets viewed through the dark-field condenser with no u.v. filtration. Figures 2C and 2D, under the same respective conditions, show the absence of fluorescence of IgG-negative platelets. The lymphocytes shown in these photographs are also stained with the FITC-GA IgG reagent and are assumed to be B lymphocytes; not shown here, T or thymus-derived lymphocytes are nonreactive with this reagent.

Four marmosets in this series died after experiencing severe thrombocytopenia, but in those surviving the platelets remained IgG positive for periods ranging from 30 to greater than 100 days after their initial appearance. Ultimately, the platelets of all animals reverted to an IgG-negative state, as they were before immunization. A primary observation made in this study is that three of the marmosets, although showing the development of IgG-positive platelets concomitant with the formation of high-titered antibody to the donor-type platelets, failed to experience any thrombocytopenia (Table 2, Nos. 4, 6, and 11). Thrombocytopenia among the other animals was usually evident within the third or fourth week and persisted for a period of at least 2 wk and in a few cases as long as 4 wk.

Effect of splenectomy before or after platelet immunization. Since the spleen is considered to be one of the major organs involved in the sequestration and destruction of cellular elements bearing antigen-antibody complexes such as may occur in posttransfusion purpura and ITP, we were interested in determining if the disease as induced in the marmoset would be affected in animals splenectomized before or after platelet immunization. Although only four ani-
Fig. 2. Photomicrographs of platelets from (A, B) immunized and (C, D) normal marmosets. Platelets treated with fluorescein conjugated goat anti-marmoset IgG (FITC-Goat IgG) as described in Materials and Methods. (A) Fluorescence (IgG positive) of platelets when u.v. passed through exciter and barrier filters; (B) same field viewed through the dark-field condenser with no u.v. filtration. (C, D) Same respective conditions, showing absence of fluorescence (IgG negative) of platelets from normal marmoset. Note fluorescent-stained lymphocytes, presumed to be B lymphocytes, in A and C.

mals were involved in this phase of our study, the results indicate that the absence of the spleen does not alter the sequential development of antibody formation, appearance of IgG-positive platelets, and thrombocytopenia. Data for the two animals splenectomized after initiation of the immunization protocol are shown in Table 2 (Nos. 3 and 5), and Fig. 3 shows the kinetics of thrombocytopenia for all four along with two control nonsplenectomized animals (Nos. 1 and 2, Table 2). The primary observation is that thrombocytopenia occurred
in all the experimental groups in approximately the same time frame relative to
the immunization scheme, with the possible exception of a slight delay in the
animal splenectomized shortly before receiving the third injection (Table 2,
No. 5). The rebound effect in platelet recovery observed in the two animals
splenectomized after immunization, although quite pronounced, was observed
in nonsplenectomized marmosets (Fig. 1A versus 1C).

Host-donor relationship and immunization protocol. As is evident in Table 2,
immunologic thrombocytopenia could be induced readily in three species of
marmosets with no particular predilection for any one donor-host interspecies
combination. Thus S. o. oedipus and S. fusicollis could be interchanged as host
and donor, and, similarly, S. fusicollis and C. jacchus. As in our original
description of this phenomenon, a single inoculation of platelets suspended in
complete Freund's adjuvant appeared to be as effective as three weekly inoculations. That the induction of antiplatelet antibody, thrombocytopenia, and IgG-positive platelets was related to immunization with platelets is indicated by the failure of three control animals receiving complete Freund's adjuvant only to show any response with respect to these physiologic parameters when examined over a period of 2–3 mo (Table 2, Nos. 12–14).

**Eluates prepared from IgG-positive platelets.** Finding IgG on platelets from normal animals and the ability to actively induce deposition of this protein by a platelet immunization scheme prompted us to attempt to elute these proteins and examine their antigenic and immunologic properties. IgG-positive platelets (+++) were obtained from normal and immunized *S. o. oedipus* marmosets that had recovered from induced thrombocytopenia. Attempts at elution by heat or low pH were generally unsatisfactory in that these treatments frequently resulted in platelet disruption. It was subsequently found that incubation of the platelets suspended in tissue culture medium (1 x 10⁶ platelets in 0.2 ml) for a period of 8 days in the cold (4°C) yielded satisfactory material and yet did not alter platelet morphology. Eluates prepared in this manner were reacted with RA IgG and RAPS antisera in immunodiffusion plates (Fig. 4). The eluate obtained from IgG-positive platelets of an immunized marmoset contained IgG and two platelet proteins. The eluates from IgG-negative platelets, when reacted with the RAPS, revealed a faint precipitin band that appeared to be in identity with the leading band found in the IgG-positive eluate. IgG could not be detected in this and other eluates from IgG-negative platelets. Studies on several eluate preparations from platelets of immunized animals consistently showed IgG and platelet antigens, but the number of the latter precipitin bands varied from two to three. Similar findings were made with eluates of IgG-positive platelets of normal animals.

We next attempted to demonstrate the immunologic reactivity in vitro of the eluted IgG with platelets of *S. o. oedipus* and *S. fuscicollis*. This was accomplished by indirect immunofluorescence using the FITC-GA IgG reagent, which would reveal attachment of the IgG to IgG-negative platelets of either species. As shown in Table 3, eluates from the platelets of actively immunized *S. o. oedipus* marmosets (group I) showed attachment of IgG to platelets of each
Table 3. Species-specific Reactivity of Eluates From IgG-positive Platelets

<table>
<thead>
<tr>
<th>Group</th>
<th>Eluate Source</th>
<th>Eluate Reaction With Platelets from:†</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>IgG-positive platelets induced in vivo (S. o. oedipus platelets from an animal immunized with S. fuscicollis platelets)</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>Naturally occurring IgG-positive platelets (from S. o. oedipus)</td>
<td>+</td>
</tr>
<tr>
<td>III</td>
<td>Naturally occurring IgG-negative platelets (from S. o. oedipus or S. fuscicollis)</td>
<td>–</td>
</tr>
<tr>
<td>IV</td>
<td>Coated IgG-positive platelets in vitro (S. o. oedipus platelets treated with S. fuscicollis anti-S. o. oedipus antibody)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>S. fuscicollis platelets treated with S. o. oedipus anti-S. fuscicollis antibody</td>
<td>–</td>
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</table>

*Binding to platelets detected by immunofluorescent staining for IgG on platelet surface.

species, while the eluate obtained from IgG-positive platelets of a normal S. o. oedipus marmoset bound only to platelets of that species (group II). Eluates from IgG-negative platelets did not show any binding of IgG to platelets of either species (group III). As controls for the above studies, eluates were made from platelets that had been reacted in vitro with specific antibody, e.g., S. fuscicollis anti-S. o. oedipus platelet antibody reacted with S. o. oedipus platelets; then the species specific reactivity of this material was determined by indirect immunofluorescence (group IV). Each eluate showed attachment of IgG only to those platelets (species) that had been used in the reaction in vitro with the specific antibody.

DISCUSSION

The present study extended our initial observation on the induction of immunologic thrombocytopenia in the marmoset and more significantly, perhaps, documented further the parallelism of this disease to posttransfusion purpura (PTP) and ITP. As in PTP, thrombocytopenia in the marmoset is associated with active immunization with foreign platelets and the subsequent appearance of serum antibody directed toward the donor platelets with little (this study) or no (PTP patients) antibody toward host-type platelets. As in ITP, the disease in the marmoset is associated with platelet-bound IgG, a finding not yet reported for PTP.

A primary question in PTP has been how the autologous platelets are destroyed in the apparent absence of antibody directed toward their antigens. Shulman and co-workers promulgated the antigen-antibody complex thesis wherein a soluble form of the platelet antigen (PLA1) stimulates antibody formation and then combines with it to form a complex with a high affinity for the platelets of the host (PLA1 negative). These antigen-antibody coated autologous platelets are then eliminated through the reticuloendothelial system. Direct experimental evidence for this concept is lacking and its support is derived primarily from the following observations: (a) the failure to detect antibodies to the host’s platelets at any stage of the disease, and (b) induction of remission by
exchange transfusions in the face of considerable amounts of residual antibody and increasing antibody titers.\textsuperscript{2,10} An alternative explanation has been the formation of antibodies cross reactive with that of the host’s platelets; failure to detect them in the serum is due simply to a complete absorption by the host’s platelets.\textsuperscript{11} In this instance, it is argued that the amount of cross-reactive (i.e., toward self) antibodies needed to result in thrombocytopenia is quite small and consequently could be readily absorbed or not be present in sufficient quantities to be detected.

Data in this report do not point definitively to either possibility. In support of the cross-reaction thesis, antibody capable of binding to host-type platelets was found in 3 of the 13 immunized marmosets, and this of low titer and only at a time when the host platelets were virtually absent. In the single case in which the serum was found to contain antibody reactive to autologous platelets, this also occurred at the time of severe thrombocytopenia. Whether or not any of these factors reacting with host-type or autologous platelets, however, were indeed free “cross-reactive” antibody or residual antigen-antibody complexes of the nature speculated by Shulman and co-workers is not known. Relevant to the probability of inducing cross-reactive antibodies, some note must be made of studies in our laboratory bearing on the immunologic relationships of the marmoset species involved. In the course of producing specific reagents to red and white blood cell antigens by interspecies immunizations, the development of cross-reactive antibodies, i.e., antibodies reacting toward self or the same species undergoing immunization, has been virtually absent, even though the immunization protocols have been of greater duration and intensity than that used in this study.\textsuperscript{12,13} This comparison, although not exact because of the different antigens involved (platelet versus red and white blood cells), must be borne in mind in view of the ease in which the “autoimmune” thrombocytopenia is induced in the marmoset.

Although the eluates obtained from platelets of actively immunized animals contained both IgG and platelet antigens, the variability observed among different preparations and the limited spectrum of platelet antigens detected by our antiplatelet antiserum does not permit firm conclusions regarding elution of any antigen-antibody complex. The strongest evidence pointing to such a complex was the observation that the IgG in eluates prepared from platelets of immunized marmosets could bind to either host- or donor-type platelets, while the IgG in eluates derived from platelets reacted with specific antibody in vitro could attach only to the specific platelet preparation. These latter findings are in keeping with the known specificity of such reactions in other systems. Thus the nonspecific attachment of unrelated antigen-antibody complexes to platelets with resultant injury to the latter elements is well documented.\textsuperscript{14,15} Israels et al.\textsuperscript{16} showed that the binding of such complexes may be mediated through an Fc receptor on the platelet membrane and suggested that platelet involvement with circulating complexes may not be a purely random event. Whether or not the nonspecific binding of the eluted IgG takes place through a similar platelet receptor site is yet to be determined for our experimental system.

The active induction of IgG deposition on the host’s platelets provides an excellent experimental counterpart to the clinical situation presented by ITP.
Thus Dixon et al. showed that the platelets of ITP patients have significant amounts of IgG on their surface and suggested this may be responsible for their destruction. In the present study, three of the animals never experienced thrombocytopenia even though their platelets showed high levels of surface IgG for periods of 30–100 days. Whether this reflects the presence of humoral protective factors or, more probably, the development of a compensated thrombolytic state is not known. The latter phenomenon was proposed by Karpatkin et al. to account for the increased number of megathrombocytes and normal platelet counts in patients with systemic lupus erythematosus (SLE) having shortened platelet survival times. That a compensatory mechanism can operate in our animals is suggested by the spontaneous recovery and even overshoot in the platelet counts among the few surviving animals in the face of the continuing presence of IgG-positive platelets.

Although the number of splenectomized marmosets used in this study was small, the results suggest that the spleen was not involved actively in the induction of the disease or its manifestations. The method and route of immunization would perhaps preclude significant participation of splenic cells in antibody formation, but the lack of any inhibition of platelet loss was rather surprising in view of the known phagocytic and sequestering potential of this tissue. Germane, however, may be the observations of Shulman et al. and Baldini suggesting that platelets heavily coated with antibody are removed primarily by the liver but that platelets lightly sensitized by antibody are filtered mainly by the spleen. Immunization of the marmosets involved three weekly injections of platelets in complete Freund’s adjuvant, a procedure that evoked significant antibody formation and an excellent coating of IgG on the host’s platelets. It is therefore conceivable that the liver was the primary site of platelet elimination in these animals.

A surprising observation was the IgG-positive platelets in some of the normal, nonimmunized marmosets. Although IgG levels were not quantitated, it should be noted that even with the scoring system used the amount of platelet-bound IgG in several of these animals appeared to be comparable to that observed in the experimental situation. Significantly, however, the IgG-positive platelets were found primarily in the imported group, occurring in a higher frequency in *S. o. oedipus* marmosets than in *S. fuscicollis*. These animals had normal platelet counts and lacked serum factors suggestive of antibody or complexes that would bind to platelets from the same or different species of marmoset. It is possible that parasitic or unsuspected viral infections provided a chronic stimulus for antibody formation toward antigens of the infective agent(s), this in turn leading to complexes binding to the host’s platelets. As yet, other than the fact that there is a clear distinction between the platelets of the imported and the laboratory-bred animals, the latter presumably “cleaner,” no real evidence for this speculation is at hand.

One may also consider the role that blood chimerism may play in the IgG-positive status of the platelets of the normal animals. Virtually all marmosets are hemopoietic chimeras, this condition resulting from the high frequency of fraternal twinning and the consistent development of placental vascular anastomoses between the twin fetuses. Although it may be argued that such
chimerism is present also in laboratory-bred marmosets, which show essentially IgG-negative platelets, this may simply be an age-related phenomenon, the imported animals having been received at an indeterminate age and conceivably older than the majority of the laboratory-bred marmosets tested. Thus a random breakdown of immunologic tolerance in combination with or perhaps facilitated by an age-related susceptibility to infectious agents may account for the differential IgG status of the platelets among the two sources of marmosets.

REFERENCES

Actively induced platelet-bound IgG associated with thrombocytopenia in the marmoset

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