The Interaction of Human Plasma Glycosaminoglycans with Plasma Lipoproteins

II. Hemagglutination Studies

PATRICIA V. DONNELLY, NICOLA DI FERRANTE, AND RICHARD L. JACKSON

SUMMARY Formalinized, tannic acid-treated sheep erythrocytes coated with low density lipoproteins (LDL) or apoprotein B (apo-B) are agglutinated by anti-apo-B immunserum. Those coated with high density lipoproteins (HDL) or apoprotein A-I (apo-A-I) are agglutinated by anti-apo-A-I immunserum. These coated formocells have been used to study the interactions of lipoproteins and apoproteins with plasma glycosaminoglycans (GAG). The sulfate-rich species of plasma GAG agglutinates cells coated with LDL, HDL, apo-B, and apo-A-I at ionic concentrations above 0.15 M. The less-sulfated species of plasma GAG does not agglutinate the coated cells but inhibits the agglutination caused by the sulfate-rich species. Treatment of the sulfate-rich GAG with papain causes a reduction in molecular weight by one-half and also causes a loss of its agglutinating activity. These results suggest that the sulfate-rich plasma GAG, consisting of two glycan chains linked to a peptide backbone, cause agglutination by binding to two or more formocells. In contrast, the less-sulfated plasma GAG, consisting of single, short glycan chains, are incapable of causing agglutination but may prevent it by covering specific binding sites present on the coated cells.

IN a previous report we have described the use of pyrene-excimer fluorescence techniques to study the interaction of human plasma lipoproteins with the glycosaminoglycans (GAG) extracted from human plasma. These consist essentially of chondroitin-4-sulfate, which may be separated into two different species on the basis of ester sulfate content. The sulfate-rich species, representing 15% of the total, has an average molecular weight of 37,100 and a hexuronate-hexosamine-sulfate molar ratio of 0.87:1:00:0.88. The less-sulfated species represents 85% of the total, has an average molecular weight of 2,800, and a hexuronate-hexosamine-sulfate molar ratio of 0.99:1.00:0.48. Our results demonstrated that the less abundant, sulfate-rich species of plasma GAG binds low density lipoproteins (LDL) and high density lipoproteins (HDL) and causes changes of their conformation even at ionic concentrations well above the physiological one (0.15 M NaCl). In contrast, the more abundant, less-sulfated species of plasma GAG does not bind LDL or HDL. However, when the less-sulfated species is added to LDL or HDL prior to the addition of the sulfate-rich species, it prevents the conformational changes caused by the latter. Moreover, treatment of the sulfate-rich plasma GAG with activated papain eliminates their interaction with LDL and HDL. Thus, the results suggested that a proper ratio of the two main species of plasma GAG might be relevant to the rheological properties and metabolic fate of plasma lipoproteins.

Although the pyrene-excimer fluorescence method is sufficiently sensitive to allow the use of physiological amounts of the various reactants, it has the disadvantage of requiring frequent preparations of pyrene-labeled lipoproteins and optical equipment which may not be available in most laboratories. Moreover, the method is not suitable for the study of the interaction of GAG with the various apoproteins, since the pyrene label is bound within the hydrocarbon layers of the lipoproteins.

In the present report, we describe a method for coating sheep erythrocytes with LDL or HDL (or with apolipoprotein B (apo-B) or apolipoprotein A-I (apo-A-I)). Using these coated cells, we demonstrate that the interaction of lipoproteins with GAG, when studied with methods of passive hemagglutination, yields results similar to those obtained previously using pyrene-labeled lipoproteins. Moreover, the technique indicates that the interaction occurs with the apolipoprotein components of the lipoproteins. Scott and co-workers have used a similar method with collagen-coated, tanned sheep erythrocytes to study the interaction between collagen and various GAG.

Methods

Preparation of Antigen-Coated Erythrocytes

Bacto-formocells from sheep (no. 3136-65-3; Difco Laboratories) were washed four times in the centrifuge with four volumes of 0.85% NaCl and then suspended in this saline solution to a final 2.5% suspension. Equal volumes

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Dr. Jackson is an Established Investigator of the American Heart Association.

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of cells suspension and of a 1:2000 solution of tannic acid in pH 7.2 buffered saline (100 ml saline + 23.9 ml 0.15 M KH₂PO₄ + 76 ml 0.15 M Na₂HPO₄) were incubated at 37°C for 10 minutes, with mixing. The cells were pelleted by centrifugation, washed once with saline solution, and then suspended in pH 6.4 buffered saline (100 ml saline + 32.2 ml 0.15 M Na₂HPO₄ + 67.7 ml 0.15 M KH₂PO₄) to a final 5% suspension.

Coating of tanned cells with lipoproteins was performed by adding to 10 ml of their suspension 5 mg of either HDL or LDL in saline solution [1-10 mg (as protein)/ml saline solution containing 0.01% sodium azide and 0.01% EDTA] and mixing the suspension for 45 minutes at room temperature. The coated cells were collected by centrifugation, washed once with two volumes of 1:200 normal rabbit serum (NRS) in saline solution and eventually suspended in 10 ml of 1:200 NRS.

Coating of tanned cells with apoproteins was performed as follows: Ten milligrams of apo-A-I apoprotein (prepared as described previously) were dissolved in 5 ml of pH 6.4 buffered saline, added to 10 ml of tanned cells suspension, and incubated for 90 minutes at 37°C with mixing.

Apo-B apoprotein (prepared as described previously) was dissolved in a saline solution containing 100 mM sodium decyl sulfate (SDS). The solution was dialyzed exhaustively against pH 6.4 buffered saline containing 1 mM SDS, and 5 ml of the retentate (containing 10 mg of apo-B) were added to 10 ml of tanned cells suspension. Incubation, with mixing, was performed as above.

The coated cells were collected by centrifugation, washed once with two volumes of 1:200 NRS, and suspended in 10 ml of 1:200 NRS. Control samples of tanned cells were similarly coated either with bovine serum albumin (BV 0162, crystallized, lot 16; Pentex) or with human Cohn Fraction IV (1+4) (provided by the American Red Cross National Fractionation Center*).

**Passive Hemagglutination of Coated Cells with Specific Antisera**

Anti-apo-B or anti-apo-A-I immunsera were raised in goats, as described previously. Anti-bovine albumin and anti-human globulins immunsera were purchased from Difco Laboratories. Fifty microliters of antigen-coated cells were added to 0.5-ml dilutions of corresponding antisera in 1:200 NRS. After the addition of 0.5 ml of 1:200 NRS to each tube, the tubes were mixed well and allowed to settle at room temperature for 1.5-2 hours. The sedimentation pattern of the cells was read from negative (uniform button) to 4+ (compact granular mat covering the bottom of the tube). Control tubes were prepared with coated cells in the absence of antisera and with noncoated cells in the presence of antisera.

**Passive Hemagglutination of Coated Cells with Purified Preparations of Plasma GAG or with Standard GAG**

Fifty microliters of antigen-coated cells were added to 0.5-ml dilutions of purified preparations of plasma GAG (either sulfate-rich or less-sulfated species, prepared as described previously) or of standard GAG. Dilutions were made with 1:200 NRS containing NaCl, from 0.15 to 0.4 M, to give final GAG concentrations varying between 60 and 0.02 μg of hexuronate/ml. Control tubes were prepared using coated cells in absence of GAG and noncoated cells in the presence of GAG. After the volume in each tube was brought to 1 ml with the appropriate concentration of NaCl in 1:200 NRS, the tubes were mixed, allowed to settle for 1.5-2 hours, and read as described.

**Hemagglutination Inhibition Studies**

**Interference of Plasma GAG in the Agglutination of Coated Cells by Specific Antisera**

Fifty microliters of antigen-coated cells were added to 0.5-ml dilutions of sulfate-rich or less-sulfated species of plasma GAG. Dilutions of the sulfate-rich species were made with 0.3 M NaCl in 1:200 NRS; those of the less-sulfated species, with 0.15 M NaCl in 1:200 NRS. The tubes were mixed and incubated at 37°C for 2 hours. Thereafter, the antiserum specific for the coating antigen was properly diluted in the corresponding concentration of NaCl in 1:200 NRS, and 0.5 ml was added to each tube. The tubes were mixed, allowed to settle at room temperature for 1.5-2 hours, and the settling pattern read, as described.

**Interference of Less-Sulfated Plasma GAG in the Agglutination of Coated Cells by Sulfate-Rich Plasma GAG**

Serial 0.5-ml dilutions of less-sulfated plasma GAG (from 66 to 1 μg of hexuronate/ml) were prepared with 0.2 M NaCl in 1:200 NRS. To each set of dilutions, 50 μl of antigen-coated cells were added, and the tubes were mixed and incubated at 37°C for 2 hours. Thereafter, the volume in each tube was brought to 1 ml with 0.2 M NaCl in 1:200 NRS containing sulfate-rich plasma GAG at concentrations of 0.25 μg of hexuronate/ml. The tubes were mixed, allowed to settle, and read, as described.

**Effect of Papain Digestion on the Hemagglutination of Coated Cells with Sulfate-Rich Plasma GAG**

Papain digestion of the sulfate-rich species was performed as described previously. Equal dilutions of papain-treated and non-papain-treated GAG (from 30 to 2 μg of hexuronate/ml) were prepared in 0.15 M NaCl in 1:200 NRS. Fifty microliters of LDL-coated cells and sufficient NRS to bring the final volume to 1 ml were added to each tube. The tubes were mixed, allowed to settle, and read.

**Results**

The coating of tanned formocells with LDL, apo-B, HDL, apo-A-I, bovine serum albumin, or Cohn Fraction

† Standard preparations of highly purified glycosaminoglycans (hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, and heparin) were obtained from Drs. J. Anthony Cifonelli and Lennart Roden, University of Chicago.
IV was demonstrated by their agglutination by the corresponding antisera.

The data in Table 1 indicate that anti-apo-B immunserum agglutinates LDL-coated cells at a titer one order of magnitude greater than that required for the agglutination of apo-A-I-coated cells. This difference may be due to the low solubility of apo-B and to a consequent lower concentration of this antigen on the cell surface. Cells coated with HDL or with apo-A-I apoprotein, vice versa, are agglutinated by similar titers of anti-apo-A-I immunserum. The higher antisera concentrations required for the agglutination of HDL or apo-A-I-coated cells suggest that this antisera is weaker than anti-apo-B immunserum.

The data in Table 2 show that cells coated with LDL, apo-B, HDL, or apo-A-I are agglutinated by very low concentrations of sulfate-rich plasma GAG, at ionic concentrations greater than the physiological one (0.15 M NaCl). The bond between sulfate-rich plasma GAG and HDL or apo-A-I-coated cells seems to be stronger than that occurring with LDL or apo-B-coated cells, since it is still present at much higher ionic concentrations. Cells coated with bovine serum albumin or with Cohn Fraction IV were not agglutinated by sulfate-rich plasma GAG (0.8 μg of hexuronate/ml) in 0.15 M NaCl (not shown in Table 2).

Similar experiments were carried out with larger concentrations (from 60 to 10 μg of hexuronate/ml of the less-sulfated species of plasma GAG and with the standard preparations of GAG. None of them caused agglutination of LDL, apo-B, HDL or apo-A-I-coated cells.

The data in Table 3 demonstrate that sulfate-rich plasma GAG at concentrations of 0.25 μg/ml inhibit the agglutination of LDL-coated cells by anti-apo-B immunserum at 1:320 dilution, but not that of apo-B-coated cells.

The less-sulfated plasma GAG, even at much higher concentrations (60 μg of hexuronate/ml), do not interfere with this hemagglutination system. However, the less-sulfated plasma GAG, in appropriate concentrations, inhibit the agglutination of LDL, apo-B, HDL or apo-A-I-coated cells (Table 4) by a concentration of sulfate-rich plasma GAG (0.25 μg of hexuronate/ml) which caused their agglutination in the experiments described in Table 2.

The treatment of sulfate-rich plasma GAG with activated papain, which causes a decrease of their molecular weight from 37,100 ± 2,000 to 18,500 ± 1,500 daltons, also causes complete loss of agglutinating activity of LDL-, HDL-, or apo-A-I-coated cells (Table 5).

### Discussion

Formol-treated sheep erythrocytes are easily coated with LDL, HDL, or their major apoproteins, as demonstrated by the fact that they may be readily agglutinated by the corresponding antisera. Higher titers of agglutination of apo-B-coated cells by anti-apo-B immunserum may be achieved by extending the coating period or by increasing the concentration of antigen in the SDS solution (see Methods). The coated cells, suspended in 1:200 NRS, are routinely stored at 4°C for periods of 6 months or longer, without loss of agglutinability.

Cells coated with LDL, apo-B, HDL, or apo-A-I are agglutinated by extremely low concentrations of sulfate-rich plasma GAG at ionic concentrations higher than the physiological one. The specificity of this binding is demonstrated by the fact that, under identical conditions, formol-treated cells, tanned cells, or cells coated with bovine serum albumin or Cohn Fraction IV are not agglutinated. The finding that the interaction of sulfate-rich plasma GAG is stronger with apo-B-coated cells than with LDL-coated cells (see Table 2) suggests that the interaction involves the apoprotein rather than the lipid component of LDL. This hypothesis is consistent with

### Table 1: Agglutination Titers of Antigen-Coated Formocells with Anti-apo-B or Anti-apo-A-I Immunsera

<table>
<thead>
<tr>
<th>Coating antigen and titer</th>
<th>LDL</th>
<th>Apo-B</th>
<th>HDL</th>
<th>Apo-A-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-apo-B</td>
<td>1:500,000</td>
<td>1:41,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-apo-A-I</td>
<td>0</td>
<td>0</td>
<td>1:1600</td>
<td>1:1280</td>
</tr>
</tbody>
</table>

### Table 2: Agglutination of Antigen-Coated Formocells with Sulfate-Rich Plasma GAG

<table>
<thead>
<tr>
<th>Coating antigen and ionic concentration</th>
<th>Minimal concentration of sulfate-rich plasma GAG, as μg hexuronate/ml giving agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL 0.15 M NaCl</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL 0.25 M NaCl</td>
<td>0.3</td>
</tr>
<tr>
<td>Apo-B 0.15 M NaCl</td>
<td>0.02</td>
</tr>
<tr>
<td>Apo-B 0.25 M NaCl</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL 0.15 M NaCl</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL 0.25 M NaCl</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL 0.30 M NaCl</td>
<td>0.04</td>
</tr>
<tr>
<td>HDL 0.40 M NaCl</td>
<td>0.04</td>
</tr>
<tr>
<td>Apo-A-I 0.15 M NaCl</td>
<td>0.3</td>
</tr>
<tr>
<td>Apo-A-I 0.40 M NaCl</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Performed with a different batch of sulfate-rich plasma GAG.

### Table 3: Agglutination of LDL- or Apo-B-Coated Formocells with Anti-apo-B Immunserum

<table>
<thead>
<tr>
<th>Coating antigen and immunserum dilution</th>
<th>μg hexuronate/ml</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-anti-apo-B  (1:320)</td>
<td>0.25</td>
<td>—</td>
</tr>
<tr>
<td>Apo-B-anti-apo-B (1:320)</td>
<td>4.6</td>
<td>3+</td>
</tr>
</tbody>
</table>

*Inhibition by sulfate-rich plasma GAG

<table>
<thead>
<tr>
<th>LDL-anti-apo-B (1:320)</th>
<th>66</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-B-anti-apo-B (1:320)</td>
<td>66</td>
<td>3+</td>
</tr>
</tbody>
</table>

*Inhibition by less-sulfated plasma GAG

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the stronger affinity of sulfate-rich plasma GAG for HDL, since their apoprotein components are relatively more abundant and more exposed.\textsuperscript{10} It is also consistent with the previously demonstrated lack of interaction with very low density lipoproteins,\textsuperscript{1} whose relatively low content of apoprotein B\textsuperscript{10} (even though localized near the outer surface of the particle) might not be adequate for the binding with the GAG. Since the agglutination of apo-A-I-coated cells was performed with a different batch of sulfate-rich plasma GAG, the results cannot be compared with those obtained with HDL-coated cells.

The less-sulfated plasma GAG and the various standard GAG tested under similar experimental conditions failed to agglutinate cells coated with either LDL, apo-B, HDL, or apo-A-I.

The interaction between sulfate-rich plasma GAG and LDL is confirmed by the results (Table 3) of experiments in which LDL-coated cells were exposed to decreasing concentration of sulfate-rich plasma GAG at an ionic concentration (0.3 M NaCl) which did not allow agglutination to take place. Nevertheless, the interaction between GAG and LDL was demonstrated by the fact that the subsequent addition to the system of anti-apo-B immunserum at a dilution of 1:320 failed to cause agglutination.

These results suggest that the interaction of sulfate-rich plasma GAG with LDL-coated cells is sufficient to cover the antigenic sites normally recognized by anti-apo-B immunserum. This may be due to the paucity of GAG reactive sites, compared with the abundance of immunserum reactive sites, present on apo-B.

Despite the stronger affinity of sulfate-rich plasma GAG for the apo-B-coated cells (Table 2), their interaction in 0.3 M NaCl prior to addition of anti-apo-B immunserum did not prevent their agglutination.

### TABLE 4

**The Inhibition of Less-Sulfated Plasma GAG of the Agglutination of Coated Formocells by Sulfate-Rich Plasma GAG (0.25 \( \mu \)g Hexuronate/ml)**

<table>
<thead>
<tr>
<th>Coating antigen</th>
<th>Minimal concentration of less-sulfated plasma GAG, as ( \mu )g hexuronate/ml, inhibiting agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>16</td>
</tr>
<tr>
<td>Apo-B</td>
<td>16</td>
</tr>
<tr>
<td>HDL</td>
<td>4</td>
</tr>
<tr>
<td>Apo-A-I</td>
<td>8</td>
</tr>
</tbody>
</table>

The interaction between sulfate-rich plasma GAG with HDL or apo-A-I-coated cells could not be evaluated with this type of experiments because, even at very high concentrations of NaCl (0.4 M or higher), agglutination of the coated cells would occur immediately after exposure to the GAG. The results presented in the lower part of Table 3 also indicate that exposure of LDL- or apo-B-coated cells to the various concentrations of less-sulfated plasma GAG does not interfere with their agglutination by anti-apo-B immunserum.

Even though the less-sulfated plasma GAG fail to cause agglutination of cells coated with LDL, apo-B, HDL, or apo-A-I or fail to interfere with their agglutination by specific antisera, nevertheless, they interact with the coated cells, as demonstrated by the results presented in Table 4. It is evident that the incubation in 0.2 M NaCl of cells coated with LDL, apo-B, HDL, or apo-A-I with less-sulfated plasma GAG prevents the agglutination normally caused by the addition of sulfate-rich plasma GAG (0.25 \( \mu \)g of hexuronate/ml).

Finally, the data summarized in Table 5 clearly demonstrate that the products of papain digestion of sulfate-rich plasma GAG have lost the agglutinating activity toward LDL-, HDL-, or apo-A-I-coated cells which was present in the native preparation.

These results are in complete agreement with those obtained previously using pyrene-labeled lipoproteins and measuring variations of the fluorescence emitted by the monomer and excimer species of the bound pyrene upon interactions with the two species of plasma GAG.\textsuperscript{1}

Such an agreement, obtained with two completely different methods, lends more reliability to the conclusions that the less-sulfated, more abundant plasma GAG interfere with the reaction of LDL and HDL with the sulfate-rich, less abundant plasma GAG.

While these experiments confirm that the interaction between lipoproteins and plasma GAG is not simply electrostatic, they indicate that the interaction occurs with the apoprotein(s) moiety of the lipoproteins and it is, in fact, stronger when the lipid components are either scarce or have been removed with appropriate methods of extraction.

Finally, the confirmed sensitivity to papain treatment of the sulfate-rich plasma GAG allows the conclusions that these, because of their structural features, are capable of recognizing sites on two or more cells coated with LDL, apo-B, HDL or apo-A-I, thus causing their agglutination. The less-sulfated plasma GAG, consisting of short, single glycan chains with a lower anionic charge, cannot span between two coated cells and, therefore, cannot agglutinate them. However, by reacting with specific sites on single coated cells, they prevent their agglutination by subsequent exposure to sulfate-rich plasma GAG.

### TABLE 5

**Agglutination of Antigen-Coated Formocells with Sulfate-Rich Plasma GAG, before and after Papain Treatment**

<table>
<thead>
<tr>
<th>Concentration of sulfate-rich plasma GAG, as ( \mu )g hexuronate/ml</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LDL-coated cells</strong></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>2</td>
</tr>
<tr>
<td>After treatment</td>
<td>30</td>
</tr>
<tr>
<td><strong>HDL-coated cells</strong></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>2</td>
</tr>
<tr>
<td>After treatment</td>
<td>15</td>
</tr>
<tr>
<td><strong>Apo-A-I-coated cells</strong></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>2</td>
</tr>
<tr>
<td>After treatment</td>
<td>15</td>
</tr>
</tbody>
</table>

### References

Enhancement of Platelet Aggregation by Tranylcypromine in Mouse Cerebral Microvessels

WILLIAM I. ROSENBLUM AND FAROUK EL-SABBAN

SUMMARY Tranylcypromine, given intraperitoneally at doses ≥ 10 mg/kg, enhanced platelet aggregation in the arterioles on the cerebral surface in mice. Tranylcypromine inhibits prostacyclin synthesis in vitro. Iproniazid, which inhibits monoamine oxidase but not prostacyclin synthesis, failed to enhance platelet aggregation. The failure of iproniazid to enhance aggregation in this study rules out an effect on monoamine oxidase as the cause of tranylcypromine’s action. That iproniazid inhibited aggregation indicates it has an effect opposite to that of tranylcypromine. Imidazole, a drug known to inhibit synthesis of both prostacyclin and thromboxane, failed to affect platelet aggregation. All of our data are compatible with the hypothesis that prostacyclin is an inhibitor of platelet aggregation. This hypothesis has been based largely on in vitro data, to which we now add in vivo support.

RECENTLY it has been suggested that aggregation of circulating platelets is controlled by a balance of two factors derived from prostaglandin endoperoxides. These factors, thromboxane (TXA₂) and prostacyclin X (PGX, now known as PGI₂), have platelet-aggregating⁴ and platelet-aggregation-inhibiting⁵ effects, respectively. TXA₂ is synthesized by platelets, whereas PGI₂ is synthesized by vessel wall, perhaps from substrate supplied by platelets attempting to adhere to and/or aggregate adjacent to the vessel wall.⁵ This hypothesis concerning the physiological or pathological importance of a balance between TXA₂ and PGI₂ originally was based on in vitro data to which a brief report of in vivo work has been added recently.⁶ The following data come from a study employing a different in vivo model and a known inhibitor of PGI₂ synthesis. The data provide further support for the suggestion that PGI₂ inhibits platelet aggregation in vivo.

From the Division of Neuropathology, Medical College of Virginia, Richmond, Virginia.


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Methods

Production of Platelet Aggregation

Platelet aggregation was induced in microvessels on the surface of the brain using a model previously described in detail. Mice are anesthetized with urethane (2 mg/g, ip), a tracheotomy and craniotomy are performed, and the dura stripped. The exposed surface vessels (pial vessels) are cannulated. The model involves arboreal illumination of a selected area of the cerebral surface using a tungsten epi-illumination. After selection of an appropriate field, sodium fluorescein (0.2 ml of a 2% solution) is injected via a tail vein, and illumination switched to a filtered UV light source. The combination of light and dye induces platelet aggregation which is not observed with dye alone, or with the filtered UV illumination in the absence of dye. The aggregating platelets fluoresce. A variety of parameters can be used to assess the tendency for aggregation. In this study we measured the time between onset of UV illumination and time to the first visible aggregate ("time to first aggregate"). We also observed aggregates enlarging until the lumen was totally occluded and determined the time elapsing between the onset of the noxious stimulus and occlusion ("time to stop flow"). In our earlier study, we pointed out the availability of this latter parameter, but elected not to use it because it seemed to be less sensitive than time to first
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