The Role of Neutrophils and Platelets in a Rabbit Model of Thromboembolic Stroke

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Cerebral ischemia is accompanied by many of the cardinal features of acute inflammation such as neutrophil and platelet activation and accumulation. We sought to determine whether circulating neutrophils or platelets contribute to brain injury in a rabbit model of thromboembolic stroke that includes a fixed duration of superimposed systemic hypotension. We randomized 18 rabbits to receive either antineutrophil antisera (n=6), antiplatelet antisera (n=5), or nonimmune serum (n=7). We assessed brain ischemia by measuring cerebral blood flow, intracranial pressure, and infarct size. Following the intracarotid administration of an autologous clot, cerebral blood flow in all groups fell to <5 ml/100 g/min during induced hypotension. After restoration of baseline blood pressure, mean cerebral blood flow in neutropenic animals recovered to 20—30 ml/100 g/min while that in control and thrombocytopenic rabbits remained at <10 ml/100 g/min. Intracranial pressure in control animals rose steadily to a final value of 241% of baseline, while a much smaller increase (148% of baseline) was noted in the thrombocytopenic group; no change from baseline was evident in the neutropenic group. Infarct size was significantly (p < 0.05) reduced in the neutropenic group but not in the thrombocytopenic group. These results suggest that neutrophils may be important contributors to ischemia-induced brain injury whereas the role of platelets is more subtle. (Stroke 1991;22:44-50)

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lthough deprivation of oxygen and substrate is the initial event in ischemic stroke, secondary processes are increasingly recognized as contributing to the ultimate injury. With the recognition that cerebral ischemia is accompanied by many of the cardinal features of acute inflammation,1-7 attention has recently been focused on the role of circulating blood elements and their proinflammatory products in acute stroke. It has been demonstrated that a selective reduction in the number of either circulating neutrophils8,9 or platelets10 results in improvement in various indirect parameters of brain injury, although direct demonstration of ischemic brain salvage has been lacking. Specifically, a reduction in brain edema has been noted with platelet depletion10 while improvements in cortical sensory evoked responses and cerebral blood flow (CBF)8,9 have been noted in animals rendered neutropenic prior to the induction of cerebral ischemia. The mechanism by which the presence of platelets or neutrophils exacerbates ischemic brain injury is not well understood, but it may relate to rheologic3,4 or biochemical2,11,12 factors or both. Other data have implicated neutrophil–endothelial cell interactions2,13,15 as a source of rheologic deterioration in the microvasculature following ischemia, thus aggravating the local oligemia.

The aim of this study was to investigate the effects of specific antisera-induced thrombocytopenia and neutropenia in a rabbit model of thromboembolic stroke. The experiments were designed to measure the effects of selective depletion of neutrophils or platelets on infarct size, intracranial pressure (ICP), and regional cerebral blood flow (rCBF) following the unilateral injection of autologous clot into the carotid circulation followed by 45 minutes of hypotension.

Materials and Methods

Nineteen New Zealand White rabbits of either sex weighing 2.7–3.2 kg were anesthetized with a solution of 20 mg acepromazine and 50 mg/kg ketamine. In each animal, the right femoral artery was cannulated with PE90 tubing for sampling blood for arterial blood gas determinations and for monitoring mean arterial blood pressure (MABP). The right femoral vein was cannulated for drug infusion. A platinum-iridium electrode (Medwire Co., Mt. Vernon, N.Y.) was inserted into the left femoral artery and advanced to the aortic arch for monitoring aortic
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hydrogen concentration, used in determining rCBF by the hydrogen clearance technique.16

The skull was exposed through a midline scalp incision, and a diamond burr was used to create an epidural trough 8 mm long and 2 mm wide positioned 10 mm lateral to the midline originating at the coronal suture ipsilateral to the hemisphere to be embolized. A single 2-mm-diameter skull perforation was similarly created over the contralateral hemisphere. The dura was left intact, and 30-gauge platinum-iridium electrodes were inserted 2 mm into the cortical mantle through the skull perforations for monitoring rCBF. Three electrodes were spaced evenly in the epidural trough. These electrode positions were established by preliminary experiments as sampling the middle and posterior cerebral artery perfusion beds and the watershed region between them. A single electrode monitored rCBF in the contralateral hemisphere. A 4-mm-diameter burr hole was drilled over the frontal lobe ipsilateral to the side to be embolized for the placement of a fiberoptic (generous gift of Ladd Research Industries, Inc., Burlington, Vt.) epidural ICP monitor. All skull perforations were then hermetically sealed with fast-setting epoxy.

The rabbit was tracheotomized and mechanically ventilated (Bird Corp., Palm Springs, Calif.). The common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA) were isolated, and the ECA was ligated. Following a 30-minute equilibration period, baseline measurements of serum glucose concentration, hematocrit, ICP, arterial pH, PaCO2, PaO2, MABP, core temperature, and rCBF were recorded. Each reading was repeated to ensure reproducibility; ICP, MABP, and core temperature were recorded continuously. Twenty minutes prior to embolization, 1.5 ml of either antineutrophil antisemum (ANAS), antiplatelet antiserum (APAS), or nonimmune serum (control) was administered intravenously to each rabbit.

Prior to each experiment, autologous blood was obtained and mixed with tin powder (20 μm granular tin, 50 mg/ml blood). The blood/tin mixture was gently vortexed, and the suspension was injected into 12-inch lengths of PE90 tubing. Both ends of the tubing were sealed, and the tubing was constantly rolled while the clot formed, keeping the tin granules evenly distributed. The tubing containing the clot was then suspended in a 37°C water bath for 24 hours. Just prior to embolization, the tubing was cut into 5-cm lengths and the clot was irrigated from the tubing into a normal saline bath. The 5-cm-long cylindrical clot was then gently aspirated back into a saline-filled PE90 catheter, and a roentgenogram was made to document even dispersion of the tin prior to embolization.

Temporary vascular clips were then applied to the proximal ICA and CCA. A microarteriotomy was made in the CCA, and it was cannulated with the clot-containing PE90 tubing. The catheter was advanced to the origin of the ICA. The ICA clip was momentarily opened, the clot embolus was injected into the ICA under direct microscopic visualization, and the temporary clip was then reapplied. The cannula was withdrawn, and the CCA was immediately repaired with interrupted 10-0 nylon sutures. Both vascular clips were then removed, restoring blood flow to the CCA.

A submental vertex roentgenogram was taken to document the presence and location of the tin-impregnated clot at the base of the hemisphere. Any clot not visualized in the right ICA distribution necessitated termination of the experiment (one of 19 rabbits).

Each rabbit was then subjected to controlled exsanguination to achieve an MABP of 30 mm Hg for 45 minutes. This transient hypotension is necessary to produce consistently a cerebral infarct in both the middle and posterior cerebral artery distributions. During hypotension the CBF was reduced to <5 ml/100 g/min in all rabbits. At the end of this 45-minute hypotensive period, baseline MABP (55±5 mm Hg) and hematocrit were restored using autologous and homologous packed red blood cells. Baseline MABP was supported when necessary with homologous citrated blood (at most 20 ml/rabbit and typically <10 ml/rabbit was required throughout the experiment), which served to replace blood withdrawn for physiologic and cell count measurements. Core temperature was maintained within 1°C of the animal’s initial temperature with a heating blanket. Arterial pH, PaCO2, and PaO2 were maintained within the physiologic ranges by controlling the rate and depth of ventilation and/or the use of intravenous sodium bicarbonate. Blood gas determinations were made using a Corning Model 168 blood gas analyzer (Corning, N.Y.).

Each rabbit was supported for an additional 3.25 hours, that is, for a total of 4 hours after the embolic event. This duration was chosen to provide sufficient time for infarct delineation. Serum glucose concentration, hematocrit, ICP, arterial pH, PaCO2, PaO2, MABP, core temperature, and rCBF were recorded at five times at baseline, during the hypotensive period immediately prior to the restoration of normotension, and hourly during the subsequent normotensive period.

Four hours after embolization, the rabbit was killed with 150 mg/kg i.v. sodium pentobarbital. A calvariotomy was then performed, the brain was harvested, and the location of the clot was verified by visual inspection. The brain was then cut in a bread-loaf fashion into 2-mm slices and incubated in 1.5% buffered 2,3,5-triphenyltetrazolium chloride to delineate the infarct. The use of this dye to distinguish viable from infarcted tissue in various organs is well documented.17-21 Infarct size, as a percentage of the total hemispheric area, was determined planimetrically using an IBM image analyzer (Essex Junction, Vt.).

The ANAS and APAS were generated using standard techniques22 in adult Wistar rats using rabbit
polymorphonuclear leukocytes (PMNs) stimulated with intraperitoneal glycogen or washed platelets isolated from rabbit platelet-rich plasma, respectively. Rabbit PMNs (2×10⁸) or rabbit platelets (2×10⁹) were administered intraperitoneally to adult male Wistar rats weekly for a total of four injections. Several days after the final injection, 5–8 ml whole blood was removed from each rat by direct cardiac puncture and collected in heparinized tubes. Serum was isolated by centrifugation and repeatedly incubated with rabbit hepatocytes, lymphoid tissue, and washed platelets (for ANAS) or PMNs (for APAS) to adsorb any antigens to these cells. The antisera were stored at −20°C until used.

A total of 18 rabbits were used in this study, randomly assigned to receive either ANAS (n=6), APAS (n=5), or nonimmune serum (n=7). The volume infused 20 minutes prior to embolization (1.5 ml) was determined by pilot studies demonstrating 80–90% reductions in neutrophil and platelet counts after injection of their respective antisera. All rabbits continued to receive ANAS, APAS, or nonimmune serum as 0.5 ml i.v. boluses every 30 minutes to maintain similar reductions in cell counts, which were confirmed throughout the remainder of the experiment.

Hemograms (erythrocyte and leukocyte counts, differential leukocyte count, and platelet counts) were obtained before (baseline) and 10 minutes after the initial antisera or nonimmune serum administration and approximately 2 hours after embolization. Cells were counted using a hemocytometer, with the differential count performed following Wright-Giemsa staining.

The hydrogen clearance method was used to allow for rapid and multiple determinations of rCBF by supplying 5% H₂ to the inspired gas mixture for 3–5 minutes until saturation was achieved. The hydrogen washout curves were then recorded on a Nicolet digital oscilloscope (model 4094, Madison, Wis.); this device immediately calculates rCBF, and the raw data are then stored on disk. Monitoring the aortic hydrogen concentration allowed for accurate initial slope washout measurements.

Hematocrit, arterial pH, PaCO₂, PaO₂, serum glucose values, ICF, rCBF, and infarct size were analyzed by repeated-measures analysis of variance. Further analysis of these values within each time was performed by Tukey's method for multiple comparisons and by analysis of covariance where the need for adjustment due to baseline variation was indicated. Paired t tests were used to compare the neutrophil and platelet counts between times within each group. Data were logarithmically transformed for analysis to stabilize variances and improve normality where such a need was indicated. Results are reported as mean±SD. All tests of hypotheses were performed at the α=0.05 level of significance.

**Results**

The ANAS significantly reduced the number of circulating neutrophils to 15.9±11% of the baseline value (p<0.01). This reduction was maintained throughout the experiment, with the neutrophil counts 22.7±12.2% of the baseline value 2 hours after embolization. The ANAS was relatively specific for neutrophils, with populations of the other leukocytes remaining at approximately 60% of baseline values throughout the experiment. Similarly, the platelet count in the ANAS-treated group remained at 73.2±15.1% of the baseline value, with platelet function assessed by adenosine diphosphate-induced aggregation remaining at 95.3±1.8% of the baseline response.

The APAS immediately reduced the platelet count to 23.0±15.0% of the baseline value (p<0.01), with a further reduction to 6.1±9.5% 2 hours after embolization. The circulating neutrophil count following the administration of APAS was 96.8±3.6% of the baseline value. Similar values were noted for mononuclear leukocytes.

No significant changes in the circulating leukocyte (including neutrophils) or platelet counts were noted in rabbits treated with nonimmune serum. Exsanguination-induced hypotension did result in small reductions in the circulating platelet and neutrophil counts of 29.2±10.1% and 11.1±5.8% of baseline values, respectively.

Controlled exsanguination lowered the hematocrit by 5–7% in all groups (Table 1); however, reinfusion of packed red blood cells at the end of the hypotensive period rapidly reversed this transient anemia in all groups to the same extent. Arterial blood gases were very similar in all groups at each time, with the exception of pH during the hypotensive period (Table 1). Both the control and the APAS-treated groups were more acidic than the ANAS-treated group. This acidosis was readily reversed, although both the control and the APAS-treated groups needed approximately 50% more bicarbonate than the ANAS-treated group to maintain physiologic pH for the remainder of the experiment.

The control group demonstrated marked increases in ICP following normalization of MABP (p<0.01), with ICP attaining a mean value of 241% of baseline 4 hours after embolization. The ANAS-treated group demonstrated no increase in ICP throughout the entire experiment (p>0.10), with values significantly lower than those of the control group 2 and 4 hours after embolization (Figure 1, p<0.05). The APAS-treated group experienced a steady although not significant increase in ICP throughout the experiment (p>0.16, to 148% of baseline); at each time the increase in ICP was less than that seen in the control group although the level of significance was not reached.

Baseline rCBF ranged from 62.2±19.5 ml/100 g/min in the control group to 88.2±26.6 ml/100 g/min in the APAS-treated group, with baseline rCBF in the ANAS-treated group 71.2±32.2 ml/100 g/min (Figure 2). These values did not differ significantly (p>0.24), and all were within the range reported in rabbits.28 All groups experienced very similar reduc-
tions in rCBF (to <5 ml/100 g/min) during the hypotensive period. At all times following the restoration of normotension, rCBF in five of the seven control rabbits and four of the five APAS-treated rabbits remained below 10 ml/100 g/min. In the ANAS-treated group, however, four of the six rabbits experienced a recovery of rCBF to levels above 18–20 ml/100 g/min. The ANAS-treated group demonstrated a significantly greater rCBF recovery than either the control or the APAS-treated group at 4 hours \( (p<0.02) \) after adjusting for variation in baseline rCBF and a significantly greater rCBF recovery than the APAS-treated group at 2 hours \( (p<0.03) \).

In preliminary experiments, only modest increases in serum glucose levels (maximum values <250 mg/dl) were noted with embolization or hypotension alone. When embolization and hypotension were combined, however, a marked hyperglycemic response was noted, with the mean serum glucose value in the control group being 516.4±169.9 mg/dl. Although this value was much higher than that seen in the other groups, significance was reached only when the APAS-treated group was compared with the control group at 2 and 3 hours after adjusting for the variation in baseline serum glucose concentration \( (p<0.05) \). In all groups, serum glucose levels subsequently declined, reaching baseline values by the conclusion of the experiment.

Infarct size for the control and APAS-treated groups did not differ significantly (Figure 3, 65.0±20.4% and 62.8±15.8%, respectively). Treatment with ANAS resulted in a significant \( (p<0.05) \) reduction in infarct size, to 34.9±24.5%. No infarcts were noted in the nonembolized hemisphere in any rabbit of the three groups.

**Discussion**

We describe a reproducible model of stroke in rabbits combining a carotid embolus with a limited...
period of superimposed hypotension. Critical to the development of this model was the incorporation of a period of systemic hypotension sufficient to reduce rCBF below the critical value needed to induce cerebral infarction. In support of this statement, preliminary studies demonstrated much smaller infarcts in rabbits receiving only the embolus and no infarcts in those undergoing a fixed period of systemic hypotension without an embolus. This model has been used in other paradigms in our laboratory, with cumulative control infarcts of 63±7% (n=22).

Using this rabbit model of brain ischemia, we demonstrated a reduction in infarct size and a restoration of rCBF in neutropenic but not thrombocytopenic animals. Several mechanisms could account for the reduction in infarct size seen with experimental neutropenia. First, cerebral ischemia results in impaired leukocyte filterability, which could result in capillary plugging and the no-reflow phenomenon as previously suggested in skeletal and cardiac muscle. In the ANAS-treated group, four of six rabbits experienced a restoration of rCBF to levels above 18–20 ml/100 g/min ≤30 minutes after the restoration of normotension. This is in the range associated with maintenance of neuronal electrical activity and is well above the published values of 10–12 ml/100 g/min found necessary to maintain membrane integrity. Only one APAS-treated rabbit and two control rabbits experienced an rCBF of >10 ml/100 g/min at any time following embolization. Second, activated neutrophils may release a variety of deleterious mediators during cerebral ischemia. The peptidoleukotrienes increase vascular permeability and are potent vasoconstrictors. Neutrophil-derived oxygen free radicals and platelet activating factor may also increase vascular permeability. The release of neutrophil-derived leukotriene C4 (LTC4) during ischemic stroke has been verified, and a direct relation between cerebral edema and the level of LTC4 has been noted. Although we did not directly measure edema and LTC4 concentration, it is notable that only the ANAS-treated group did not experience a rise in ICP, which, in the absence of hemorrhage, suggests a reduction in edema formation.

It is also possible that neutropenia reduces platelet deposition in ischemic brain, as seen in other organ systems. This could account for a thrombocytopenia, which has been associated with a reduction in ischemic brain edema. However, since neutropenia was more effective than thrombocytopenia in ameliorating elevated ICP in our model, other causative mechanisms in addition to interference with platelet deposition are suggested for this phenomenon.

The significance of the reduced hyperglycemic response in the ANAS-treated group relative to the other two groups is unclear. However, it is generally (although not universally) accepted that hyperglycemia can exacerbate ischemic brain injury. The hyperglycemic response demonstrated in our study may have contributed to the increase in ischemic...
brain injury seen in the APAS-treated and control groups.

Salvage of ischemic brain in our study may also relate to the maintenance of systemic perfusion in neutropenic rabbits following exsanguination-induced hypotension. The ANAS-treated group was less acidic during the early ischemic period; only 50% as much bicarbonate was required to maintain pH in the physiological range in the ANAS-treated group and in the APAS-treated and control groups. It is of interest that very similar results were obtained by Dutka et al in a canine model of granulocytopenia and brain ischemia. Additionally, in a rabbit model of hemorrhagic shock and resuscitation, removal of significantly more blood was required to achieve similar reductions in MABP and cardiac output in animals pretreated with an antineutrophil adhesion antibody compared with control animals; also, compared with the experimental group in that study and similar to our study, the control animals demonstrated a significant acidosis. The significance of the apparent ability of ANAS to block the early and transient systemic acidosis seen in the other two groups is unclear. While this may contribute to the reduced infarct size and improved rCBF seen, we have demonstrated that similar levels of acidosis encountered in other protocols using this model have had no apparent impact on brain injury (unpublished observations). The acidosis was aggressively treated in this experiment, which would likely limit its impact.

The MABP in all groups consistently measured 55 ± 5 mm Hg. Although they are substantially lower than published values in both conscious and ketamine-anesthetized but nonventilated rabbits, the MABP values are virtually identical to those in a previous report using ketamine anesthesia in mechanically ventilated rabbits. This MABP is certainly at the lower limit of pressure autoregulation and may have contributed to the ultimate brain injury. We felt that the need for critical control of arterial blood gases and pH afforded in ventilated animals outweighed the impact of this response, which was, after all, equivalent in all groups.

Reducing the MABP transiently to 30 mm Hg has introduced the additional factor of a period of hemorrhagic shock to this model. The combination of hypotension and embolus resulted in sufficient ischemic brain injury to produce the acute increase in ICP seen without transient hypotension. This model enabled us to examine a possible role for neutrophils and platelets in the genesis of early ischemic brain injury. We felt that the need for critical control of arterial blood gases and pH afforded in ventilated animals outweighed the impact of this response, which was, after all, equivalent in all groups.

It should also be noted that the ANAS did react with mononuclear cells, with a 40% reduction in mononuclear cell counts. Thus, it is possible that part of the reduction in ischemic brain injury seen is secondary to a partial decrease in the number of circulating mononuclear cells. However, the late arrival of mononuclear cells to an ischemic region compared with neutrophils suggests that this is a much less likely possibility. The few eosinophils and basophils (0–8% in our model) preclude a definitive statement about their role in ischemic brain injury. The reduction in the number of eosinophils and basophils, although greater than the reduction in the mononuclear populations, was much less than that seen for neutrophils. It should also be noted that the ultimate level of neutropenia was not complete (3–28% of the baseline value), which might have served to underestimate the role of neutrophils in this model.

The fate of the blood elements following antisera treatment was not addressed in this study; however, it is well known that immunologic factors that lead to the disappearance of granulocytes from the circulation result in margination and aggregation of these elements mainly within the pulmonary circuit. Preliminary studies in our model support the fact that there is a subpopulation of neutrophils that can be demarginated (approximately 10% of baseline value) with epinephrine following the administration of ANAS.

In summary, evidence is presented demonstrating a role for neutrophils in increasing brain infarct size in a rabbit model of thromboembolic stroke with superimposed hypotension. Neutropenia also blocks the rise in ICP, partially restores rCBF, and blunts the hyperglycemic response seen in this model. A role for platelets in this model, although much more subtle, is also apparent since thrombocytopenic rabbits demonstrated much smaller increases in ICP than control rabbits. The relation and any interactions between neutrophils and platelets require further study.

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References


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