Anticardiolipin Antibody Aggravates Cerebral Vasospasm After Subarachnoid Hemorrhage in Rabbits

Hiroaki Nomura, MD; Yutaka Hirashima, MD; Shunro Endo, MD; Akira Takaku, MD

Background and Purpose—We previously reported that patients with antiphospholipid antibodies (aPLs) frequently demonstrate cerebral infarction due to cerebral vasospasm after subarachnoid hemorrhage (SAH). To examine the participation of aPLs in the pathogenesis of vasospasm after SAH, we studied the relationships of aPLs and SAH in an animal model.

Methods—SAH was produced in 34 rabbits that received two subarachnoid injections of autologous arterial blood. The animals were divided into four experimental groups: SAH was induced in group A (n = 9), intracutaneous injection of cardiolipin (CL) was performed before the induction of SAH in group B (n = 5), intravenous injection of CL was performed before SAH in group C (n = 12), and cyclosporin A was infused intravenously after the intravenous injection of CL and induction of SAH in group D (n = 8). Enzyme-linked immunosorbent assay identifying the titer of IgG CL antibodies, neurological evaluation, cerebral angiography, and histological examination were performed in all four groups.

Results—A significant elevation of anti-CL antibodies, aggravation of neurological deficit, and reduction of caliber of the basilar artery were observed in rabbits that received the intravenous immunization of CL (group C). The administration of cyclosporin A reduced the titer of anti-CL antibody, aggravation of neurological deficit, constriction of basilar artery, and the incidence of cerebral infarction (group D).

Conclusions—Anti-CL antibodies may therefore be involved in the deterioration of cerebral vasospasm after SAH. (Stroke. 1998;29:1014-1019.)

Key Words: antibodies, anticardiolipin ■ cyclosporin ■ cerebral ischemia, transient ■ subarachnoid hemorrhage ■ rabbits

The pathogenesis of cerebral vasospasm after SAH is not fully understood, but attention has been focused on the roles of inflammation and immunologic reaction.1-3 Severe inflammation and vasoconstriction were observed after the subarachnoid injection of latex beads4 or talc5 in experimental models. The activation of complement components C3a and C4a in cerebrospinal fluid and plasma5 and deposition of IgG in the arterial wall6 have been reported in respect to the relationship between humoral immunity and cerebral vasospasm. Neopterin concentrations that reflect T-cell macrophage activation were observed to be elevated in cerebrospinal fluid, which supports a relationship between cellular immunity and cerebral vasospasm.7 Experimental and clinical studies have thus been performed to evaluate the efficacy of immunosuppressive agents such as steroids8-11 and cyclosporin A12,13 in preventing cerebral vasospasm after SAH.

aPLs, including the biologic false-positive serologic test for syphilis (BFP-STS), LA, aCLs,14,15 and others, often cause thrombotic events.16-19 The mechanism of thrombotic events caused by aPLs remains obscure. It is suspected that they interfere with the production or release of prostaglandin I₂ by vessel wall endothelial cells. Such an inhibition of prostaglandin I₂ production and release could explain the recurrent thrombotic events seen in patients who have received LA.20 LA may also reduce the activation of protein C on the endothelial surface by interfering with the thrombin-thrombomodulin complex.21,22 This could also result in the loss of regulation in the procoagulant-anticoagulant system. Recurrent thromboembolism could also be due to the inhibitory activity of LA on prekallikrein and antithrombin III.23,24 We recently reported that patients who suffered cerebral infarction due to vasospasm after SAH demonstrated the presence of aPLs in the period of vasospasm.25,26 To investigate the association between aPLs and cerebral vasospasm, we introduced experimental SAH using rabbits that were immunized by CL, and we then evaluated their neurological symptoms, cerebral angiography, and histology. We attempted to evaluate the effect of immunosuppressive agents that restrain the production of antibodies on the occurrence of vasospasm after SAH.

Materials and Methods

Animal Preparation

Female rabbits weighing 2.5 to 3.0 kg each were used in this study. All surgical and angiographic procedures were performed with...
anesthesia induced intramuscularly with ketamine hydrochloride (50 mg/kg). All protocols were approved by the Animal Ethics Committee of the Toyama Medical and Pharmaceutical University. Five weeks before the planned induction of SAH, each rabbit was placed in the supine position with the head fixed so that the orbitomeatal line was horizontal. With a sterile technique, a midline cervical incision was made, the bilateral carotid arteries were isolated, and the common carotid arteries were ligated at the carotid artery bifurcation. Only rabbits that were asymptomatic 2 weeks after this carotid artery ligation were subjected to experimental SAH. Aortovertebral angiography was performed by manual injection of 7 mL ioxaglate (Hexabrix) into a catheter inserted retrogradely through the carotid stump into the aortic arch. An anteroposterior film obtained during the arterial phase 5 weeks after the carotid artery ligation and before the first SAH was used as a baseline. Repeated angiographic procedures were performed on day 4, ie, 2 days after the second SAH, and on days 7 and 14.

Preparation of Immunizing Antigen
CL antigen suspension, consisting of CL (Sigma Chemical Co), lecithin (Sigma), and cholesterol (Wako Pure Chemical Industries, Ltd) in the ratio of 1:10:30 (wt/wt/wt), was prepared according to the Venereal Disease Research Laboratory microflocculation technique. Twenty milliliters of this suspension was mixed with 30 mL of 0.1% methylated BSA (Sigma) and allowed to stand at 4°C overnight. The mixture was centrifuged at 13,300 g (0°C) for 1 hour and washed twice with cold saline, suspended in 2 mL of saline, and injected into animals. The CL antigen suspension described above was centrifuged before it was mixed with the methylated BSA solution. The CL antigen precipitate thus obtained was resuspended in methylated BSA solution, stored overnight at 4°C, and then used for injection.

Experimental Groups
The animals were divided into four experimental groups. In group A (n=9), SAH was induced 5 weeks after the carotid artery ligation. In group B (n=8), a freshly prepared suspension of antigens with Freund’s adjuvant was injected into rabbits intracutaneously once a week for 3 weeks before the induction of SAH, with each rabbit receiving a total of 0.9 to 1.8 mg CL. In group C (n=12), a freshly prepared suspension of antigens was injected into rabbits intravenously every other day for 3 weeks until the day before the induction of SAH, with each rabbit receiving a total of 3 to 6 mg CL. In group D (n=8), after the intravenous injection of antigens and the induction of SAH, 6.0 mg/kg cyclosporin A (Sandimmune IV, Sandoz Inc) with 0.3 mg/kg dexamethasone sodium phosphate as an adjunct low-dose steroid was infused intravenously from 14 hours before the second experimental SAH to day 7, according to the therapeutic regimen described by Peterson et al.

Method of Subarachnoid Injection
The animals were operated on in random order. They were placed in the prone position with the head down. With a sterile technique, a 1.5-cm vertical suboccipital incision was made to expose the craniopinal junction. Each rabbit received two subarachnoid injections 48 hours apart, ie, the first injection on day 0 and the second on day 2. A No. 26 needle inserted into the cisterna magna was used for the injection of autologous arterial blood. Manual injection was performed over a 2-minute period, with 0.5 mL/kg of blood used for the first and second SAH. During this procedure spontaneous respiration was maintained, and both PacO₂ and blood pressure were monitored. The PacO₂ was 35 to 45 mm Hg, and the mean arterial blood pressure before the induced SAH was 85 to 105 mm Hg in all animals. After the subarachnoid injection, a dramatic increase in systemic blood pressure occurred. Within 5 minutes, however, the blood pressure gradually returned to the preinjection level.

aCL-IgG
aCLs were identified by means of a sandwich-type enzyme-linked immunosorbent assay that identifies IgG CL antibodies. Polystyrene microtiter plates (96-well plates, Immulon-2; Dynatech Laboratories) were coated with 50 μL (10 μg/mL) CL. Serum samples diluted 1:320 in 0.1% egg albumin in PBS were added to the wells for 2 hours at room temperature. The plates were washed with 0.1% egg albumin in PBS three times and incubated for 2 hours with a 1:1000 anti-rabbit IgG antibody that conjugated with alkaline phosphatase (Vector Laboratories Inc) at room temperature. After six washes in 0.1% egg albumin in PBS, 100 μL of p-nitrophenyl phosphate (1 mg/mL in 10% diethanolamine) was added to each well, followed by incubation for 1.5 hours at room temperature. After termination of the reaction by addition of 3N NaOH, optimal density at 405 nm (OD₄₀₅) was measured with an enzyme-linked immunosorbent assay microtiter reader. The OD₄₀₅ of the sera of 20 untreated rabbits was measured, and mean±3 SD was determined as the normal range.

In this study polyclonal antibodies were produced by injecting CL. Two different types of aCLs have been established: one is induced by the aPL syndrome and another by infectious diseases. To determine which type of antibody was mainly produced in our study, the titers of aCLs were measured in the presence of β₂-glycoprotein 1. Wells to which sera of day 4 in group C were added were incubated with 50 μL of 0.1% egg albumin containing 12.5 μg/mL human β₂-glycoprotein 1 (Serbio Laboratory) for 2 hours at room temperature, and the change of the titer of antibody was estimated.

Neurological Examination
Neurological examination was performed before SAH and daily thereafter until the animals were killed. Rabbits were observed on a flat surface, and neurological status was graded according to a four-point system reported previously: grade 1, no neurological deficit (normal); grade 2, minimal or suspected neurological deficit; grade 3, mild neurological deficit without abnormal movements; and grade 4, severe neurological deficit with abnormal movements.

Evaluation of Caliber of Cerebral Arteries
The diameter of the basilar artery was measured blindly from the angiograms with a technique similar to that described previously. The average of three measurements was expressed as the percent reduction from the baseline diameter.

Histological Examination
The rabbits were killed on day 14 for histological examination of the brain. The brains, including five of the animals of group C that died between days 4 and 6, were fixed by perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brains were cut into coronal slices and stained with hematoxylin and eosin. Ischemic lesions were evaluated in a blinded manner with light microscopy.

Statistical Examination
The findings are reported as mean±SD. Data were analyzed with the use of Student’s t test or 1-way ANOVA followed by Fisher’s protected least-squares difference test for multiple comparisons to compare the titer of aCL-IgG and the diameter of basilar arteries. We also used Wilcoxon’s U test to compare the neurological grades and the χ² test to compare the occurrence of cerebral infarction among these four groups. A value of P<0.05 was accepted as significant.
Anticardiolipin Antibody Aggravates Vasospasm

Results

aCL-IgG
No difference of serum levels (OD$_{405}$) of aCL-IgG before the carotid artery ligation was observed among the four groups (Fisher’s test) (Table 1). When the normal range was determined as less than mean+3 SD, two animals (6%) demonstrated an elevation of serum aCL-IgG without any experimental stimulation. The aCL-IgG level of rabbits with carotid ligation was not different from that of rabbits without (data not shown). No significant elevation of the titer of the antibody was observed for 14 days after only SAH (group A) (Table 1). The immunization by intracutaneous injection also induced an elevation of serum aCL-IgG without any experimental stimulation. The aCL-IgG level of rabbits with carotid ligation was not different from that of rabbits without (data not shown). No significant elevation of the titer of the antibody until day 14 (Table 1). The administration of cyclosporin A reduced the intravenous immunized aCL-IgG levels (groups C and D) (P<0.01, Fisher’s test) (Table 1). However, the intravenous immunization performed in this study could not maintain the elevation of the antibody until day 14 (Table 1). The administration of cyclosporin A reduced the intravenous immunized aCL-IgG levels (groups C and D) (P<0.01, Fisher’s test) (Table 1). In group C animals that were immunized intravenously, the serum level (OD$_{405}$) of aCL-IgG before the addition of β$_2$-glycoprotein 1 was 0.22±0.33 (mean±SD), and that of aCL-IgG after the addition of β$_2$-glycoprotein 1 was 0.42±0.42. A significant elevation of OD$_{405}$ was observed after the addition of β$_2$-glycoprotein 1 (P<0.01, Student’s $t$ test).

Neurological Examination
A significant aggravation of neurological deficits was observed on day 4 in animals that were immunized intravenously (group C) compared with animals without immunization (group A) (P<0.05, Wilcoxon’s $U$ test), although no significant difference was observed between animals that were immunized intracutaneously (group B) and group A (Table 2). Moreover, five rabbits in group C died between days 4 and 6. The administration of cyclosporin A improved the neurological deficit grade of animals that were immunized intravenously (groups C and D) (P<0.05, Wilcoxon’s $U$ test) (Table 2). There was no difference of neurological deficit grade among the four groups on days 7 and 14 (data not shown). In the course of this study, three rabbits of group B were dead before the induction of SAH because of infection and pain.

Evaluation of Caliber of Cerebral Arteries
The mean basilar artery constriction rate on day 4 was 20.4±10.0% (mean±SD) (n=9) in animals without immunization (group A), 23.3±10.4% (n=5) in animals with intracutaneous immunization (group B), 31.7±8.9% (n=12) in animals with intravenous immunization (group C), and 19.3±9.0% (n=8) in animals with the administration of cyclosporin A after intravenous immunization (group D). Significant differences between groups A and C were noted (P<0.01, Fisher’s test) as well as between groups C and D (P<0.001, Fisher’s test). Intravenous immunization induced significant vasoconstriction of the basilar artery on day 4, and this vasoconstriction was prevented by the administration of cyclosporin A. There was no significant difference among the four groups on days 7 and 14 (Figure).

Histological Examination
In light microscopic examination, cerebral infarctions were observed in 3 of the 9 rabbits in group A, 1 of the 5 in group B, and 8 of the 12 in group C. Animals with intravenous immunization (group C) showed a somewhat higher incidence of cerebral infarction than animals without immunization (group A); a significant difference was not observed (Table 3). However, the administration of cyclosporin A (group D) clearly reduced the incidence of cerebral lesions that were induced by intravenous immunization of CL (P<0.01, $\chi^2$ test) (Table 3).

Discussion
Clinical and experimental findings have suggested a role of immunologic reaction in the development of cerebral vasospasm.

<table>
<thead>
<tr>
<th>Group</th>
<th>Before Carotid Artery Ligation</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.018±0.015 (9)</td>
<td>0.018±0.025 (9)</td>
<td>0.037±0.041 (9)</td>
<td>0.068±0.067 (9)</td>
</tr>
<tr>
<td>B</td>
<td>0.066±0.076 (5)</td>
<td>0.064±0.049 (5)</td>
<td>0.077±0.066 (5)</td>
<td>0.057±0.049 (5)</td>
</tr>
<tr>
<td>C</td>
<td>0.013±0.019 (12)</td>
<td>0.220±0.21 (12)*</td>
<td>0.210±0.19 (7)*</td>
<td>0.100±0.095 (7)</td>
</tr>
<tr>
<td>D</td>
<td>0.027±0.037 (8)</td>
<td>0.060±0.053 (8)†</td>
<td>0.037±0.032 (8)†</td>
<td>0.072±0.068 (8)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of animals. Group A had only SAH; group B, intracutaneous immunization and SAH; Group C, intravenous immunization and SAH; and group D, administration of cyclosporin A after intravenous immunization and SAH.

*P<0.01 compared with group A.
†P<0.05 compared with group C (one-way ANOVA followed by Fisher’s protected least-squares difference test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Neurological Deficit Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>B</td>
<td>1 2 3</td>
</tr>
<tr>
<td>C</td>
<td>1 2 7</td>
</tr>
<tr>
<td>D</td>
<td>1 3 1</td>
</tr>
</tbody>
</table>

Groups are defined in Table 1.
*,†P<0.05 compared with groups A and C, respectively (Wilcoxon’s $U$ test).
vascular changes observed in the presence of this antibody, although the mechanism is not well understood, it may involve interference with the procoagulant-anticoagulant system. LA also reacts with platelet phospholipids. Platelets may be activated and release vasoactive substances. Endothelial damage and platelet activation may thus be induced by aPL. Therefore, the changes in the vessel wall endothelium and in platelets during vasospasm may be caused by aPL. The association of aPLs with thrombosis is well recognized. Although the mechanism is not well understood, it may involve interference with the procoagulant-anticoagulant system. LA also reacts with platelet phospholipids. Platelets may be activated and release vasoactive substances. Endothelial damage and platelet activation may thus be induced by aPL. Therefore, the changes in the vessel wall endothelium and in platelets during vasospasm may be caused by aPL. We reported previously that a reduction in platelet count, increased platelet aggregability, and an increased plasma PF4 concentration were observed in aPL-positive patients with symptomatic vasospasm.

We were not able to determine whether the mechanism for vasospasm after SAH might be related to aCLs. However, aCL-associated neurological worsening after SAH may be due not only to arterial constriction but also to impairment of microcirculation.

Microcirculation may be disturbed through the interaction of aPLs and blood cells, including platelets. Increased aPLs in patients with SAH may involve the same type of antibody as those of patients with aPL syndrome. The processes or mechanisms of increased antibodies are still unknown in patients with aPL syndrome, and elucidation will be helpful.

Experimental SAH in the present study did not introduce the production of aCLs, and therefore we could not elucidate the mechanism of the production of aCLs. However, aggravation of neurological deficit and a reduction of the caliber of basilar artery were observed in the presence of this antibody, and it is therefore possible that aCLs are involved in the deterioration of cerebral vasospasm.

### References

10. Chyatte D, Fode NC, Nichols DA, Sundt TM Jr. Preliminary report: effects of high dose methylprednisolone on delayed cerebral ischemia in...
The aPL syndrome is a relatively common cause of an acquired hypercoagulable state associated with cerebral ischemia and infarction.\(^1\) aPLs are antibodies directed at phospholipids and usually a protein cofactor. They include LA and aCLs. The latter were found and were suggested to be the cause of ischemic stroke in 10% of patients with first ischemic stroke.\(^1\) Hirashima et al\(^2\) previously reported that the presence of aPLs in patients with ruptured aneurysms is an adverse prognostic factor for good outcome. Eleven (34%) of 32 patients with SAH tested positive for these antibodies, a rate that is higher than would be expected in a random sample of the population or of ischemic stroke patients and suggests that SAH caused or was previously associated with aPLs. The antibodies disappeared 7 to 13 days after hemorrhage in some patients who first tested positively for antibodies. Patients with aPLs were more likely to develop cerebral ischemia and to have poor outcome.

In this experimental study, Nomura and colleagues raised aPLs in rabbits and then subjected the animals to SAH by two injections of blood into the cisterna magna. The vasospasm produced in this model does not usually cause neurological deficit, but both cervical common carotid arteries were ligated 5 weeks before the hemorrhage. This would be expected to make brain blood supply more dependent on the basilar artery that develops the most spasm in this model. It was found that SAH itself did not induce the formation of aPLs. When such antibodies were increased by immunization, however, the rabbits developed more severe vasospasm and worsened neurological condition 4 days after SAH. There were no differences by 7 days after hemorrhage. The findings are interesting and potentially clinically relevant but must be regarded as preliminary at this point. The blood injection models of SAH probably produce arterial narrowing by the same set of common mechanisms that occur after clot placement in other models or after SAH in humans, although it is the opinion (albeit speculative) of this reviewer that the relative importance of various mechanisms differs between models and that inflammation may be relatively more important in rabbits and dogs than in humans. The differences between groups were analyzed by pairwise comparisons between multiple groups where analysis of variance might have been more appropriate. The mechanism of the beneficial effect of cyclosporin A and dexamethasone is not worked out in these studies nor is the mechanism by which aPLs worsen vasospasm. Dexamethasone could decrease vasospasm and neurological deficits on the basis
of an anti-inflammatory action in this model. Cyclosporin A theoretically could worsen vasospasm on the basis of its action as an inhibitor of endothelium-dependent relaxation. Unfortunately, the studies of anti-inflammatory and immunosuppressive agents in humans with SAH have been marred by high complication rates and lack of demonstrated efficacy. One might have expected that aPLs would worsen neurological condition in the rabbits without affecting vasospasm, because they might be more likely to cause arterial thromboses in narrowed vasospastic arteries. But why would they increase the vasospasm? Furthermore, the 30% average basilar artery diameter reduction that was seen in the group with SAH and aPLs would not usually be associated with enough reduction in flow to cause cerebral ischemia.

Treatments for patients with aPLs include antiplatelet and anticoagulant drugs. The observation that patients who were taking aspirin before SAH were less likely to develop cerebral infarcts might support the hypothesis of these investigators. Certainly other explanations are possible. In any case, the test to detect the antibodies is simple, and the treatment probably carries little risk. More studies should be performed to confirm the hypothesis.

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References
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