Failure of FK-506, a new immunosuppressant, to prevent cerebral vasospasm in a canine two-hemorrhage model


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In order to clarify the possible role of immunological reaction in the pathogenesis of cerebral vasospasm, the authors examined the prophylactic effect of the immunosuppressant agents FK-506 and cyclosporin A on chronic vasospasm in a canine two-hemorrhage model. While a mean constriction of the basilar artery to 81.0% ± 4.0% (= standard error of the mean) occurred on Day 2 and to 63.8% ± 3.5% on Day 7 in the untreated group, constriction to 77.9% ± 3.4% on Day 2 and 62.8% ± 3.0% on Day 7 was demonstrated in the FK-506-treated group (difference not significant). This tendency was also noted in the cyclosporin A-treated group, with basilar artery constriction to 81.8% ± 3.7% and 56.3% ± 2.7%, respectively (difference not significant). The histological changes of the basilar artery, including corrugation of the elastic lamina, detachment of endothelial cells, and vacuolar formation in the smooth-muscle layer were not different in the two treated groups and the one control group. Since these immunosuppressant agents are known to inhibit the release of interleukin-2 (IL-2), the level of IL-2 was examined in the cerebrospinal fluid of patients with cerebral vasospasm. While interleukin-1 gradually increased in level as time passed, the level of IL-2 was consistently low during the course of the study, indicating less participation of IL-2 in the pathogenesis of cerebral vasospasm. This clinical observation matched the experimental results. The authors conclude that cell-mediated immunoreaction, initiated mainly by IL-2, plays little role in the pathogenesis of cerebral vasospasm.

Key Words • cerebral vasospasm • immunosuppressant • inflammation • complement system • immunohistochemistry • dog

The role of immunological reaction in the pathogenesis of cerebral vasospasm has gained attention ever since 1981, when Pelletier, et al. reported that a higher frequency of circulating immune complexes were found in the blood. Following several similar clinical reports, Peterson, et al. demonstrated that the immunosuppressant agent cyclosporin A reduces the degree of cerebral vasospasm in a canine subarachnoid hemorrhage (SAH) model. On the other hand, extravascular red blood cells are known to be simply scavenged by macrophages and polymorphonuclear leukocytes without the effect of immunoglobulin. Thus, controversy still surrounds the issue of whether the scavenging of autologous blood actually requires an immunological reaction.

The main purpose of this study was to examine the role of immunological reaction in the occurrence of cerebral vasospasm, using a canine SAH model treated with a new, strong immunosuppressant agent, FK-506. Furthermore, both cyclosporin A and FK-506 are known to inhibit the release of lymphokines, especially interleukin (IL)-2, resulting in the prevention of cytotoxic T-cell generation. In order to examine the role of IL-2 in the pathogenesis of cerebral vasospasm, the levels of IL-2 in cerebrospinal fluid (CSF) were also measured clinically in several patients with SAH.

Materials and Methods

Effect of Immunosuppressants on Cerebral Vasospasm

Animal Preparation. Thirty-seven adult beagle dogs, each weighing 9.0 to 13.5 kg, were used for these studies. All experiments were performed according to the Na-
Osaka, of tional exam "Guide for the Care and Use of Laboratory Animals, Revised 1985." The animals were anesthetized with intravenous sodium pentobarbital (30 mg/kg), orally intubated, and fixed in a stereotactic frame. As a preliminary experiment, the effects of arterial pCO2 and the injection speed of contrast material on the basilar arterial diameter were examined. It was found that the basilar arterial diameter was not significantly changed when pCO2 was within 30 to 45 mm Hg. The diameter was also not influenced by the speed of contrast material injection when the rate was 1.5 to 5 ml/sec.

Based on the results of this preliminary experiment, all of the dogs were allowed to breathe spontaneously, and their arterial pCO2 was maintained at 33.3 to 44.9 mm Hg. With an aseptic technique, the right cervical vein was exposed in the lower neck and cannulated with a 1.9-mm vinyl chloride tube for drug administration. The right vertebral artery was also exposed and cannulated with a 0.86-mm polyethylene catheter through which vertebral angiography was performed using 8 ml meglumine diatrizoate introduced at approximately 3 ml/sec by manual injection. Subsequently, 0.6 ml/kg of autologous arterial blood was injected percutaneously into the cisterna magna over 1 minute, followed by removal of one-half of that volume of CSF. The cisternal injection of autologous arterial blood was repeated 2 days later, also under general anesthesia. A further angiogram was taken on Days 2 and 7, and the basilar artery diameter was calculated as follows. The luminal diameter of the basilar artery was measured at five prefixed locations on the angiograms, and the mean value was defined as the calculated basilar arterial diameter. All films were randomly measured three times by a single investigator, and the relationship between the arterial pCO2 and the basilar artery diameter was examined. The error range of measuring the caliber on angiograms was evaluated, and the coefficient of variation was 3.7%, which suggested that the measurement was reproducible.

Drug Protocol. The animals were divided into three groups: an untreated group which included 11 dogs; a group treated with FK-506* which included 15 dogs; and a cyclosporin A-treated group which included 11 dogs. The FK-506 was freshly prepared for each infusion and suspended in normal saline to obtain a final concentration of 2 mg/ml. It was given intramuscularly at a dose of 0.16 mg/kg/day, the same formula as previously reported in the study of renal transplantation.13 The treatment was initiated just after the cisternal blood injection (Day 0), and continued once a day for 7 days. Among the 15 FK-506-treated dogs, eight dogs received a supplementary intravenous injection of prednisolone (0.5 mg/kg) twice a day for 7 days. Since the prednisolone had no significant effect, all data for FK-506-treated dogs were averaged together.

In the group treated with cyclosporin A,† the drug was freshly prepared for each infusion and dissolved in polyethylene glycol to obtain a final concentration of 6.0 mg/ml. It was given intravenously at a dose of 6.0 mg/kg/day, according to the protocol of Peterson, et al.17 The time schedule for drug administration was the same as for FK-506. In all of the cyclosporin A-treated dogs, a supplemental low-dose steroid was administered as described above.

Serum Complement Level. In order to examine the change in serum complement level (CH50), venous blood samples were obtained on Days 0, 2, and 7, in which the CH50 was measured via the method reported previously by Mayer.12 The animals were sacrificed by exsanguination on Day 7. Following infusion of 300 ml heparinized physiological saline into the vertebral artery, the brain and cervical cord were dissected free. The exposed basilar artery including the brain stem was fixed in 10% neutral buffered formalin solution. The tissue block was then processed routinely and embedded in paraffin. Each section (3 µm) was stained with hematoxylin and eosin for histological study. Other tissue blocks were also immediately frozen in dry ice-acetone, and cryosections (5 µm) were obtained for the immunohistochemical procedure. Each section was then fixed in periodate-lysine-paraformaldehyde solution at 4°C for 10 minutes, after which it was processed using the avidin-biotin-peroxidase complex method‡ with diaminobenzidine as a chromogen. Endogenous peroxidase was eliminated in 3% H2O2 in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 20 minutes. Three kinds of primary antibodies, rabbit anti-dog immunoglobulin (IgG, IgM, and complement component 3 (C3))§ (each at a dilution of 1:500 and maintained at room temperature for 2 hours) were used. Finally, the sections were counterstained with Hanzen's hematoxylin. Control slices were treated via the same procedure, in which 0.01 M PBS (pH 7.4) or normal rabbit serum was substituted for the primary antibody for a negative control and the mesenteric lymph node in the same animal (instead of the basilar artery) was used as a positive control.

Cerebrospinal Fluid IL-2 Levels in SAH Patients

Four patients with severe aneurysmal SAH (Hunt and Kosnic2 Grades III and IV) were selected for a study of IL-2 levels in the CSF. All had undergone aneurysm clipping within 24 hours post-SAH. Several days after surgery, all four patients exhibited symptomatic vasospasm. Cisternal drains were placed at the prechiasmatic cistern, and 3-ml samples of bloody CSF were collected on Days 1, 3, and 5. The samples were stored at -20°C until analysis. The levels of IL-1α, IL-1β, and

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* FK-506 provided by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.
† Cyclosporin A synthesized by Exploratory Research Laboratory, Fujisawa Pharmaceutical Co., Ltd., Tsukuba, Japan.
‡ ABC kit supplied by Vector Laboratories, Burlingame, California.
§ Antibodies supplied by Bethyl Laboratories, Montgomery, Texas.
IL-2 were measured with enzyme-linked immunosorbent assay.

**Results**

**Effect of Immunosuppressants on Cerebral Vasospasm**

As stated above, based on the results of a preliminary experiment, the dogs were allowed to breathe spontaneously during this experiment. Since their pCO₂ before each angiogram was always within 33.3 to 44.9 mm Hg, the effect of arterial blood gas on the basilar arterial diameter was thought to be negligible. In this study, every angiogram was taken with manual injection of contrast medium. Since the injection speed was estimated as approximately 3 ml/sec, the effect of injection speed was also considered negligible.

The change in basilar arterial diameter was expressed as a percentage of the original diameter before the blood injection. In the untreated group, the basilar artery was constricted to 81.0% ± 4.0% on Day 2 and to 63.8% ± 3.5% on Day 7 (mean ± standard error of the mean). The dogs that received FK-506 therapy showed a similar degree of vasospasm, with basilar arterial diameter constriction to 77.9% ± 3.4% on Day 2 and 62.8% ± 3.0% on Day 7. The difference between the untreated and FK-506-treated groups was not statistically significant. Basilar arterial constriction also occurred in the cyclosporin A-treated group. The diameter of this group's basilar arteries were reduced to 81.8% ± 3.7% on Day 2 and 56.3% ± 2.7% on Day 7 (Fig. 1). Thus, both FK-506 and cyclosporin A failed to reduce the delayed basilar arterial constriction in our model.

Serum complement level showed a wide individual variation (Fig. 2). The initial CH₅₀ in the untreated group was 38.0 ± 1.7 U/ml. The values on Day 2 and Day 7 were 39.2 ± 1.8 and 37.3 ± 2.5 U/ml, respectively. The slight decrease noted on Day 7 was not statistically significant. In the FK-506-treated group, this decrease in CH₅₀ on Day 7 was more prominent and was statistically significant (p < 0.001). The initial value was 39.3 ± 0.9 U/ml, and the values on Days 2 and 7 were 39.9 ± 0.7 and 31.9 ± 1.5 U/ml, respectively. In the cyclosporin A-treated group, a decrease in CH₅₀ on Day 7 was also observed but was not significant; the values were 34.4 ± 2.3 U/ml on the initial day, 37.5 ± 1.8 U/ml on Day 2, and 35.2 ± 0.8 U/ml on Day 7. Except for the low level initially, which was possibly due to individual variation, the tendency for a decrease in CH₅₀ on Day 7 was observed in each group.

Histological examination of the sections from the untreated SAH group revealed a severe constriction of the artery accompanied by folding and corrugation of the elastic lamina, several instances of endothelial cell detachment, and vacuolar formation in the smooth-muscle layer. These histological changes, which have been reported to be specific to SAH, were almost the same in the FK-506- and the cyclosporin A-treated groups (Fig. 3).

Immunohistochemical study showed a moderate degree of IgG, IgM, and C3 deposits on the endothelium and on the medial side of the smooth-muscle layer in all three groups (Fig. 4). Although these deposits might have been caused by the insufficient perfusion of the vessel with saline solution, it is noteworthy that no definite difference was observed among the three groups studied. Since the methods of irrigation before tissue fixation were the same in all of the dogs, this finding is not simply caused by the insufficient irrigation, but rather suggests the possibility that immunosuppressant agents scarcely affect the deposit of immunoglobulins and complements in the arterial wall after SAH.
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Levels of Interleukins in CSF After SAH in Human Patients

The level of IL-1α in the CSF on Day 1 was less than 7.8 pg/ml (under detection limit) and gradually increased to 27.2 pg/ml on Day 3 and 28.9 pg/ml on Day 5. The level of IL-1β in the CSF on Day 1 was also under the detection limit (< 15.6 pg/ml), then it increased more prominently, with levels at 110 pg/ml on Day 3 and 156 pg/ml on Day 5. On the other hand, the IL-2 level did not show a significant change, being 1.06 IU/ml on Day 1, 3.64 IU/ml on Day 3, and less than 1.0 IU/ml (under detection limit) on Day 5.

Discussion

The present study demonstrates that neither FK-506 nor cyclosporin A show a definite prophylactic effect on cerebral vasospasm in a canine two-hemorrhage model. The dose of FK-506 used in this study has already been reported to show a significant effect in renal allotransplantation in dogs. The protocol of drug administration was similar to that study, and the drug was prepared fresh before each injection; hence, the immunosuppressive effect of FK-506 in our experiment seems to be indisputable. The dose of cyclosporin A was the same as reported by Peterson, et al. While they started treatment with cyclosporin A at 14 ± 4 hours before the second experimental SAH, we started it just after the first blood injection. Thus, a stronger prophylactic effect might be expected in our experiment. The mechanism behind the discrepancy of the prophylactic effect of these immunosuppressant agents is unknown.

Neither of the two immunosuppressant drugs, cyclosporin A and FK-506, showed a prophylactic effect on cerebral vasospasm in our experiment. Two well-established mechanisms have been suggested to explain the effects of cyclosporin A: it inhibits the release of lymphokines, especially IL-2, and it prevents clonal expansion of helper and cytotoxic T cells. The effect of FK-506 is known to be almost the same, except that it suppresses the immune system at a concentration 100 times lower than cyclosporin A. The roles of interleukins on immunological reactions are as follows. While IL-1, mainly produced by macrophages, is an initiator of the following immunological reaction, IL-2 acts mainly as a T-cell growth factor. The T-cell replication is explained as the exclusive result of the interaction of IL-2 and IL-2 receptors. Thus, FK-506 and cyclosporin A prohibit the cell-mediated immune response by suppressing the production of IL-2 and the expression of the IL-2 receptor. Hence, the present study indicates that the role of cell-mediated immune response is scant in the pathogenesis of cerebral vasospasm. This view is also supported by our limited clinical observation: the high level of IL-1 and the low level of IL-2 in CSF after SAH with vasospasm.

Histological examination of the contractile artery could not discriminate the groups treated by immunosuppressant agents from the untreated group. Several specific histological changes, (corruption and folding of the elastCcalCminia, subintimal proliferation, and myonecrotic change in the smooth-muscle layer) were not inhibited by the immunosuppressant drugs. Thus, these histological changes also seem to be unaffected by a cell-mediated immune response.

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**FIG. 4.** Photomicrographs of basilar artery sections from an untreated animal (left), an FK-506-treated animal (center), and a cyclosporin A-treated animal (right). Upper: Indirect peroxidase-labeled antibody method using rabbit anti-dog immunoglobulin G (IgG), × 100. Lower: Indirect peroxidase-labeled antibody using rabbit anti-dog complement component 3 (C3), × 100.

Immunohistochemical study also failed to discriminate the treated groups from the untreated group. Deposits of IgG, IgM, and C3 in the arterial media were observed even in the groups with immunosuppressant treatment. Hoshi, et al., reported deposits of IgG and C3 in 74% of vasospasm patients; however, these deposits were also noticed in 42% of patients with hypertensive intracerebral hematoma, 44% with cerebral infarction, 42% with brain tumor, and 75% with meningoencephalitis. Thus, deposits of IgG and C3 do not appear to be specific to vasospasm, but rather to a nonspecific arterial reaction against several types of cerebral stress. The inflammatory process would, of course, not be excluded from the occurrence of this deposition.

The present study also showed the systemic activation of complement systems during vasospasm. This activation was not influenced by the administration of immunosuppressant agents. The extravascular homologous erythrocytes, implicating the injured cells by the change of circumstances, are known to be rapidly eliminated by macrophages. This recognition of the injured homologous erythrocytes by macrophages is explained by the loss of sialic acid from sialoglycoprotein in the membrane of erythrocytes. It is also reported that the erythrocyte-phagocyte interaction (adhesion) is initiated by this lectin-like recognition but is not necessarily followed by uptake (phagocytosis). Complete erythrophagocytosis is known to require a complement system. Thus, the elimination of extracellular homologous erythrocytes does not involve an immunological reaction, but requires the activation of systemic complements. This agrees with the theory that the change in the complement level during vasospasm, which was considered to be evidence for the involvement of immunological reaction against cerebral vasospasm, is a simple consequence of the homologous erythrophagocytosis.

Based on the present results, we conclude that cell-mediated immunoreaction, initiated mainly by IL-2, plays little part in the pathogenesis of cerebral vasospasm.
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References


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