The Kinetics of Lymphocyte Subsets and Macrophages in Subarachnoid Space After Subarachnoid Hemorrhage in Rats

Tetsuya Kubota, MD; Yuji Handa, MD; Akira Tsuchida, MD; Masanori Kaneko, MD; Hidenori Kobayashi, MD; Toshihiko Kubota, MD

Background and Purpose: Although it has been suggested that humoral immunity plays a role in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage, there has been no quantitative assay for cellular immunity. We studied the kinetics of immune cells in the subarachnoid space after subarachnoid hemorrhage in the rat.

Methods: One hundred fourteen Sprague-Dawley rats were used in this study. The animals were divided into two groups and injected with either autologous blood (0.3 mL) or saline into the major cisterns. They were killed at the specified time: 10 minutes or 1, 2, 3, 5, or 7 days after subarachnoid hemorrhage. For immunohistochemical analysis, the rats' whole brains were frozen, and cryostat sections were prepared. For flow cytometric analysis of immune cell presence, their whole brains underwent enzymatic digestion.

Results: Histopathologic study revealed pathological change of the arterial wall, and immunohistochemical study revealed the existence of macrophages and T cells in the subarachnoid space in animals with a survival time of 2 to 5 days after subarachnoid hemorrhage. A flow cytometric study revealed the peak of appearance of T cells and macrophages 2 days after subarachnoid hemorrhage. The helper-suppressor T cell ratio also reached a peak 2 days after subarachnoid hemorrhage.

Conclusions: A serial response of immunoreactive cells, which resembles that of the chronic allergic reaction observed in autoimmune diseases or delayed-type hypersensitivity, exists in the subarachnoid space after subarachnoid hemorrhage. The present results suggest that the initial response in cellular immunity, which is followed by humoral immunity and eicosanoid reactions, plays a role in eliciting the development of cerebral vasospasm. (Stroke. 1993;24:1993-2001.)

Key Words • cerebral vasospasm • subarachnoid hemorrhage • rats

Although a number of studies have been performed to clarify the pathophysiology of chronic cerebral vasospasm following subarachnoid hemorrhage (SAH), it still remains obscure. It has been emphasized that the immunologic response plays an important role in the pathogenesis of vasospasm.1-7 With respect to humoral immunity, an increase in immune complex1 and complement activation in cerebrospinal fluid2 and deposition of immunoglobulin in the spastic arterial wall3 were observed in clinical cases with vasospasm. In experimental studies, the deposition of immunoglobulin in the arterial wall was closely correlated with the time course and the degree of cerebral vasospasm in primates.4

Concerning the cellular component of immunologic responses, the appearance of inflammatory cells in the subarachnoid space has been reported both in autopsied cases5 and in experimental animals,6 and cyclosporine A, which is known to suppress cell-mediated immunity, has been reported to reduce the severity of vasospasm in experimental animals.6,10 These results strongly suggest that SAH elicits an immunologic process that includes cellular immunity and subsequent humoral immunity, and this process may have a role in the pathogenesis of vasospasm. When the response of cellular immunity exists, it must be characterized by serial changes in the subpopulation of lymphocytes, as observed in other immune/inflammatory reactions. However, no study has quantitatively investigated the kinetics of lymphocyte subsets, including T cells. In the present study, we investigated the serial changes in T-lymphocyte subsets and macrophages in the subarachnoid space after SAH in the rat by means of immunohistochemistry and laser flow cytometry. The possible roles of these immunoreactive cells in developing chronic cerebral vasospasm are discussed.

Materials and Methods

A total of 114 male Sprague-Dawley rats weighing 200 to 400 g were used in this study. Of them, 108 animals underwent insertion of a Silastic cannula (outer diameter, 1.0 mm; inner diameter, 0.5 mm) (Dow Corning Inc, Kanagawa, Japan) into the cisterna magna before induction of SAH. The rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and allowed to
breathe spontaneously. Under an operating microscope, the scalp was incised, and a small hole was made in the midline just rostral to the interparietal-occipital suture. The occipital bone and the atlanto-occipital membrane were exposed. The cannula was passed through the hole and advanced into the cisterna magna, sliding along the inner table of the occipital bone. The cannula was then sealed with a 3-0 nylon string, and the scalp was sutured. The animals were allowed to survive for more than 7 days.

Six animals underwent no surgical procedure as normal control. Two were used for hematoxylin-eosin staining, 2 for an immunohistochemical study, and the remaining 2 for a flow cytometric study.

The cannulated rats were divided into two groups: the SAH group and the saline group. At least 7 days after the insertion of cannula, the animals were reanesthetized with sodium pentobarbital (50 mg/kg IP). The femoral artery was cannulated with a 22-gauge elastic cannula. In the SAH group, the rat was held in a 20-degree head-down position, and 0.3 mL of nonheparinized autologous blood was withdrawn from the femoral artery and injected into the cisterna magna through the previously implanted cannula for approximately 3 minutes. In the saline group, 0.3 mL of physiological saline instead of autologous blood was injected through the cannula in the same manner.

The animals were killed at 10 minutes or 1, 2, 3, 5, or 7 days after SAH induction. In each group, 2 rats were used for hematoxylin-eosin staining, 2 for an immunohistochemical study, and the remaining 5 for a flow cytometric study at each survival time after SAH. All the rats were perfused transcardially with 300 mL of physiological saline, and the brain was removed from the skull. From the rats for flow cytometric analysis, 1 mL of peripheral blood was taken by cardiac puncture just before saline perfusion.

The experimental protocol was evaluated and approved by the Committee on Animal Experimentation of the Fukui Medical School. The care of the animals was performed in accordance with the Guidelines for Animal Experimentation of the Fukui Medical School, and all surgical procedures were carried out in a humane fashion. The animals were killed by intraperitoneal administration of 80 mg/kg of sodium pentobarbital.

For hematoxylin-eosin staining and the immunohistochemical study, the brain was cut axially to acquire two blocks: from the chiasmatic level to the hypothalamic level, and just caudal to the midbrain to the lower end of the medulla oblongata. These two parts contained major cerebral arteries covered with the arachnoid membrane. For hematoxylin-eosin staining these blocks were fixed in formalin and embedded in paraffin blocks. One of every 10 slices in the coronal plane with a thickness of 5 μm was prepared to study the histopathologic change of the subarachnoid space and major cerebral arteries.

For the immunohistochemical study, the blocks were embedded in Optimal Cooling Temperature Compound (Tissue-Tek Products, Miles Inc, Elkhart, Ind) and then placed in liquid nitrogen for 10 minutes and stored at −80°C in a deep freezer for 2 days. One of every 10 slices in the coronal plane with a thickness of 10 μm was prepared with a cryostat at −20°C. Thirty slices were obtained from each frozen block. The slices were placed on glass slides and air-dried for 1 hour. They were then incubated with normal rabbit serum for 10 minutes at room temperature. Twenty-four slices were followed by the application of primary monoclonal antibody for 24 hours at 4°C. The remaining six were used for negative control, and they therefore underwent no application of a monoclonal antibody. We used monoclonal mouse anti-rat pan-T cells (1:100) (R1-3B3, equivalent to human CD5) (Seikagaku Kogyo, Tokyo, Japan) and monoclonal mouse anti-rat granulocytes and macrophages (1:100) (R2-1A6a) (Seikagaku Kogyo) as the primary antibody (Table). Twenty-four hours after incubating with primary antibody, the specimens were incubated with biotinylated rabbit anti-mouse immunoglobulin (Nichirei Corp, Tokyo, Japan), followed by reacting with streptavidin anti–alkaline phosphatase (Nichirei Corp). Finally, the sections were counterstained with hematoxylin and observed under a light microscope.

For flow cytometric analyses, the cells of the whole brain were isolated and stained. We prepared an “enzyme cocktail” of deoxyribonuclease I (0.1 mg/mL), collagenase (1 mg/mL), hyaluronidase (2.5 U/mL), and 20 mL of Dulbecco’s modified Eagle’s medium. This modified method was reported by Suyu et al.\textsuperscript{11} These enzymes were manufactured by Sigma Chemical Co, St Louis, Mo. We used monoclonal mouse anti-rat helper T cells (1:20) (RTH-7, equivalent to human CD4) (Seikagaku Kogyo) antibodies and monoclonal mouse anti-rat killer suppressor cells (1:20) (R1-10BS, equivalent to human CD8) (Seikagaku Kogyo) antibodies in addition to the monoclonal antibodies (1:20) used in the immunohistochemical study (Table). They were

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Antibody} & \textbf{Subset} & \textbf{Antibody for Humans} \\
\hline
R1-3B3 & Pan-T & CD5 \\
RTH-7 & Helper T & CD4 \\
R1-10BS & Suppressor/killer T & CD8 \\
R2-1A6a & Macrophage/granulocyte & None \\
\hline
\end{tabular}
\caption{Antibodies Used for Immunohistochemical and Flow Cytometric Study}
\end{table}

\begin{figure}[h]
\centering
\includegraphics{figure1.png}
\caption{Dotgram of leukocytes in peripheral blood shown by laser flow cytometry. The cell population indicated by “1” demonstrates a cluster of lymphocytes and monocytes. G indicates a cluster of granulocytes; D, debris of hemolyzed blood cells; FS, forward scatter; and SS, side scatter.}
\end{figure}
followed by fluorescein isothiocyanate (FITC)–labeled goat anti-mouse immunoglobulin (Becton Dickinson, San Jose, Calif) (1:50) as the secondary antibody.

The preparations were immediately analyzed by laser flow cytometry (EPICS Profile Analyzer, Coulter Co, Hialeah, Fla). Appropriate settings of the forward and side light scattering gate were determined by using those for lymphocytes and monocytes in the peripheral blood for each animal. The cytogram of the peripheral blood showed the gated area occupied by lymphocytes and monocytes (Fig 1). The percentage of each type of antibody-positive cell was calculated from the histogram with a computer (Fig 2). Based on the assumption that all T cells were positive for R1-1A6a and for either RTH-7 or R1-10B5, or both, a higher value was cited between R1-1A6a(+) and the sum of RTH-7(+) and R1-10B5(+). The true percentages of T cells, T-cell subsets, and macrophages were obtained by subtracting the percentage of the cells stained with only FITC from that obtained after staining with FITC after each monoclonal mouse antibody. The ratio of RTH-7–positive cells to R1-10B5–positive cells, which is equivalent to the CD4–CD8 ratio in humans, was calculated from the values obtained by histogram analyses. Analysis of variance was used to compare the percentage of each type of labeled cells in each survival time between the two groups and within groups. Student's t test was applied to examine the difference of percentage values between the two groups and within groups. Values of \( P \leq .05 \) were considered statistically significant.

**Results**

In the specimens of the animals in the SAH group with a survival time of 10 minutes, the histopathologic study with hematoxylin-eosin staining demonstrated numerous erythrocytes around the basilar artery but no...
architectural change of the arterial wall. At 2 and 3 days after SAH, the most marked corrugation of the intima and thickening of the medial and adventitial layers of the basilar artery, showing a narrowing of the vascular lumen, were observed. Infiltration of mononuclear cells was observed in the adventitial layer and the subarachnoid space. The corrugation of the intima and thickening of the arterial wall were slightly alleviated 5 and 7 days after SAH. In animals in the saline group with any survival time, there was no or minimal pathological change in the subarachnoid space and cerebral arteries (Fig 3).

Immunohistochemical study demonstrated that in the SAH group there were positively stained mononuclear cells with either the anti-pan-T antibody or anti-macrophage antibody in the subarachnoid space, especially around the main cerebral arteries only in animals that survived 2, 3, or 5 days after SAH (Fig 4). No stained cells could be observed in the cerebral parenchymal tissue. In animals in the saline group with any survival time, no stained mononuclear cells could be observed in either the subarachnoid space or the brain parenchyma, the same as in normal control rats.

Flow cytometric study revealed that in the SAH group the percentages of macrophages were significantly ($P<.05$) higher in animals with a survival time of 2 to 7 days after SAH than in the saline group. The mean percentage value of macrophage reached a peak at 2 days after SAH (13.5±2.5% [mean±SEM]) and was significantly ($P<.05$) higher than in animals with other survival times. The distribution of pan-T cells in the SAH group was significantly ($P<.05$) higher in animals with a survival time of 1 to 7 days than in the saline group. The mean percentage value peaked at 2 days after SAH, but it was not significantly higher than in those with other survival times. The percentage of helper T cells in the SAH group was significantly ($P<.05$) higher in animals with a survival time of 1 to 3 days than in the saline group. The peak value was recorded 2 days after SAH, but it was not significantly higher than others. For suppressor/killer T cells, the percentage was significantly ($P<.05$) higher in SAH animals with a survival time of 2 days than in the saline group. The percentage was not significantly different in animals at each survival time (Fig 5).

![Image](http://stroke.ahajournals.org/)

**Fig 4.** Immunohistochemical staining by streptavidin-biotin anti-alkaline phosphatase method of the subarachnoid space including main cerebral arteries. A, Five days after subarachnoid hemorrhage. Arrow indicates R2-1A6a-positive cell (macrophage). B, Two days after subarachnoid hemorrhage. Arrows indicate R1-3B3-positive cells (T cells). W indicates cerebral arterial wall; M, arachnoid membrane. Bar=100 μm.
In peripheral blood, the percentage of pan-T cells in the SAH group was significantly (P<.05) higher in animals with survival time of 3 days than in the saline group. For percentage of helper T cells, there was no significant difference between the distribution of positive cells in the SAH group and the saline group. In the measurement of suppressor/killer T cells, mean values in the SAH group were significantly (P<.05) higher in animals with a 2- and 3-day survival time than in the saline group (Fig 6).

The ratio of helper T cells to suppressor/killer T cells in the whole brain was significantly (P<.05) higher in animals with 2 days' survival time after SAH than in animals with other survival times. The ratio calculated from the data obtained in the peripheral blood showed no significant difference between animals with various survival times (Fig 7).

**Discussion**

We used a flow cytometric technique to make a quantitative analysis of lymphocyte subpopulations. We prepared single cell suspensions of rats' whole brains by mincing the brain and initiating digestion with collagenase, hyaluronidase, and deoxyribonuclease, which
have a major effect on digestion of connective tissue and tissue debris. It has been reported that using the mincing and dilution technique, more than 80% cell viability could be recognized in rat brain tumor tissue.\textsuperscript{12} In the present study, however, the immunohistochemical study revealed that the immune cells existed in the subarachnoid space but not in the brain parenchyma. To evaluate a quantitative change of immune cells in the subarachnoid space, it was necessary to perform a flow cytometric study with single cell suspensions of whole brain. The damage to immune cells from the subarachnoid space after isolation into single cell suspensions is thought to be negligible, and thus the results by flow cytometric study are thought to reflect well the cell kinetics of immune cells in the subarachnoid space.

Although many studies revealed cell infiltration into the subarachnoid space in experimental SAH models,\textsuperscript{13,14} none has offered quantitative analysis of the subpopulation of infiltrated lymphocytes and the time course of their appearance. In the present study, the flow cytometric study revealed that the significant peaks in the distribution of both macrophages and helper and suppressor T cells were seen 2 days after SAH. We also demonstrated that histopathologic changes of wall thickening and narrowing of the vascular lumen in a basilar artery were most prominent at the same time points. In the same rat SAH model, Delgado et al\textsuperscript{15} also demonstrated the maximum phase of angiographic vasoconstriction 2 days after SAH. These results indicate that the peak appearance of immunoreactive cells is...
R1-10B5(+) ratio in brain

Days after SAH

0 1 2 3 5 7

0 2 4 6 8

A

RTH7(+)R1-10B5(+) ratio in peripheral blood

Days after SAH

0 1 2 3 5 7

0 2 4 6

B

Fig 7. Line graphs show time course of ratio of RTH7(+) to R1-10B5(+) in brain (A) and peripheral blood (B) after subarachnoid hemorrhage (SAH). Day 0 refers to 10 minutes after induction of SAH. Vertical bars indicate SEM. All five animals were used each day. *P<.05, intergroup comparison.

compatible with the period of maximum pathological change of the cerebral arteries, ie, the maximum phase of chronic vasospasm. Immediately after SAH only few leukocytes were observed in the subarachnoid space, as revealed by the flow cytometric study. This result suggests that the infiltration of immune cells into the subarachnoid space may start sometime after SAH.

The evaluation of the kinetics of pure T-cell subsets in the present study may suggest that the response in the subarachnoid space after SAH may be a nonspecific type of inflammation. Moreover, it has been reported that vasoactive cytokines released from platelets and leukocytes in autologous blood can provoke binding of leukocytes to endothelium. However, we also demonstrated the time course of infiltration of macrophages, which suggests that there are some antigens in the subarachnoid space after SAH. It was not determined whether there is a possible antigen after SAH. Peterson et al explained that an aged clot is suspected to become a nonautologous antigen within a few days after SAH. Immune responses caused hemolysis of erythrocytes, which resulted in the development of vasospasm. Another possible antigen was suggested by Handa et al to be derived from the damaged arterial wall after SAH, because they observed that immune complex was deposited in the medial layer of the artery when the arteries exhibited a severe degree of vasospasm. Although the present results suggest the existence of antigen and macrophages and the subsequent appearance of T-cell subsets, this study does not precisely reveal what antigen is involved in the subarachnoid space.

The analysis of lymphocyte subsets is important in clarifying the entity of immune/inflammatory diseases and the quality of immunologic responses. An increase in the helper-suppressor T cell ratio has been observed in the active stage of autoimmune diseases, presenting a state of chronic inflammation.23 The phenomenon of graft-rejection response is also a severe inflammatory reaction induced by cellular immunity in which T cells may play the chief role. In experiments of graft rejection, the helper T subset is reported to be more important than the killer/suppressor T subsets in the initiation of graft-rejection reaction.20,21 In these studies the helper T cell is suggested to be the crucial cell inducing delayed-type hypersensitivity.21 The present results indicate that the kinetics of lymphocyte subsets in the subarachnoid space resemble those of the chronic allergic reaction observed in autoimmune diseases or delayed-type hypersensitivity.

On the basis of the present results and previous studies in our laboratory, we speculate that an immune process after SAH plays a role in the pathogenesis of vasospasm. First, a possible antigen following SAH may be recognized as an autoantigen by macrophages. They may stimulate T-cell migration into the subarachnoid space via chemical mediators, ie, eicosanoids. The eicosanoids also have been considered to play a role in the development of cerebral vasospasm because of their vasoconstrictive action, and they cause a breakdown of the arterial barrier.22 Helper T cells promote the production and release of leukotrienes, which induces arterial wall injury, from killer T cells. Helper T cells also promote the production and release of immunoglobulins from B cells. Once an immune complex forms, it may activate the cellular and mediator systems either directly or via the action of the complement, resulting in the development of an inflammatory reaction at the affected site. It is easily suspected that these interactions between cellular and humoral immunity via chemical mediators occurred in the subarachnoid space within a few days after SAH, and these processes may be followed by histopathologic change in the arterial wall as a result of chronic inflammation. In view of this, eliciting the cellular immunity response is of primary importance in the development of vasospasm.

The present results failed to reveal a significant change in the ratio of helper to suppressor T cells in the peripheral blood. It is supposed that the immune/inflammatory response was stronger in the subarach-
noid space than in the peripheral blood in the rat SAH model. Furthermore, the time course of vasospasm in rats was quite different from that in humans. In clinical cases, we need to analyze immune cell kinetics for a longer time because of the longer time course of vasospasm in humans. In human cases, a greater number of purer monoclonal antibodies to lymphocyte subsets for flow cytometric analysis can be acquired. We will then be able to analyze precisely the time course of the response of cellular immunity after SAH in humans. By evaluating whether the present results in rats are compatible with those in humans, we may demonstrate more clearly that the eliciting of cellular immunity is an initiating factor in the subsequent development of cerebral vasospasm. We might then suggest how various kinds of immunosuppressive therapies may be valuable in preventing the development of vasospasm during the time course of SAH.

References

Editorial Comment

Vasospasm of superficial cerebral arteries and cerebral injury are recognized consequences of subarachnoid hemorrhage. The appearance of chronic vasospasm has been taken as a poor prognostic sign in this setting. However, the pathogenesis of chronic cerebral artery vasospasm here remains unclear. Kubota and colleagues, drawing on previous observations in humans, have explored the possibility that leukocytes or mononuclear cells may participate in the genesis of chronic vasospasm. Their immunohistochemical and flow cytometric study indicated that T lymphocytes and macrophages may be found in cerebral tissue within 48 hours of experimental subarachnoid hemorrhage, at a time when both cell types are also seen in the subarachnoid space.

Several features of this work are interesting. With the demonstration that pial cerebral arterioles may respond to endothelium derived relaxing factors (EDRFs) or endothelium derived contracting factors,1 it is of interest to know whether the organizing thrombus in the subarachnoid space may elaborate substances that are themselves vasoconstrictors or inhibitors of the EDRF response. It would be of great interest to know whether the lymphocyte response per se, or individual T lymphocyte subsets, may modify or inhibit EDRF synthesis/release and therefore promote the chronic vasospasm...
The kinetics of lymphocyte subsets and macrophages in subarachnoid space after subarachnoid hemorrhage in rats.

T Kubota, Y Handa, A Tsuchida, M Kaneko, H Kobayashi and T Kubota

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