Leukocyte Invasion of the Brain After Experimental Intracerebral Hemorrhage in Mice

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Background and Purpose—Neuroinflammatory processes contribute to secondary neuronal damage after intracerebral hemorrhage. We aimed to characterize the time course of brain immigration of different leukocyte subsets after striatal injection of either autologous blood or collagenase in mice.

Methods—Intracerebral hemorrhage was induced by injection of either autologous blood (20 μL) or collagenase (0.03 U) in C57BI/6J mice. Hematoma volumetry was performed on cryosections. Blood volume was measured by hemoglobin spectrophotometry. Leukocytes were isolated from hemorrhagic hemisphere 1, 3, 5, and 14 days after intracerebral hemorrhage, stained for leukocyte markers, and measured by flow cytometry. Heterologous blood injection from CD45.1 mice was used to investigate the origin of brain-invading leukocytes.

Results—Collagenase injection induced a larger hematoma volume but a similar blood content compared with blood injection. Cerebral leukocyte infiltration in the hemorrhagic hemisphere was similar in both models. The majority of leukocytes isolated from the brain originated from the circulation. CD4+ T lymphocytes were the predominant brain leukocyte population in both models. However, cerebral granulocyte counts were higher after collagenase compared with blood injection.

Conclusions—Brain infiltration of systemic immune cells is similar in both murine intracerebral hemorrhage models. The pathophysiological impact of invading leukocytes and, in particular, of T cells requires further investigation.

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Key Words: cerebral hemorrhage • leukocyte-invasion

Intracerebral hemorrhage (ICH) accounts for 10% to 15% of all strokes, and its mortality and morbidity exceed that of ischemic stroke considerably.1-2 The lack of a specific target of therapy in ICH raises the need for new therapeutic options. Increasing evidence supports the involvement of neuroinflammatory mechanisms in brain injury and repair after ICH.4-8 Neuroinflammation comprises the response of brain resident cells as well as the impact of brain immigration by different systemic immune cells. In patients with ICH, leukocytes in the cerebrospinal fluid correlate with hematoma size.9 Exploration of the role of brain-invading leukocytes after experimental ICH so far has focused on the innate immune response including neutrophil and monocyte infiltration.8,10-12 Although the powerful protective and deleterious roles of different lymphocyte subsets have been elucidated in cerebral ischemia,13-17 infiltrating lymphocytes have received only little attention in ICH so far.18 Indeed, the kinetics of brain infiltration of lymphocytes and other immune cells and their individual pathophysiological roles largely remain to be elucidated in ICH.

Initially after ICH, leukocytes enter the brain as part of the inflow of blood after vessel rupture. Later, they migrate across the immunologic blood-brain barrier using activated cell adhesion pathways.19 An important obstacle for a better understanding of the contribution of systemic immune cells to the pathophysiology of ICH is the limitation imposed by the most widely studied experimental models of ICH.20-22 Injection of autologous blood allows studying the toxic and immediate inflammatory effects of extravascular blood components, but it fails to reflect the aspect of continuing bleeding. On the contrary, bacterial collagenase dissolves the extracellular matrix around capillaries resulting in active intraparenchymal bleeding because of vessel erosion. However, the vascular source of bleeding in collagenase-induced ICH models differs from...
most human ICH in which bleeding is of penetrating arterial origin. Importantly, the exact sequence of brain immigration and the pathophysiological contribution of the different leukocyte subpopulations have not been clarified in these models although characterization of differences between animal models is crucial for the evaluation of anti-inflammatory therapeutic strategies for ICH in the future.

The aim of the present study was to characterize and compare the temporal infiltration pattern of different leukocyte subsets in the autologous blood and the collagenase injection model. Furthermore, we determined the source of brain-invading leukocytes in the early phase of ICH in the blood injection model.

Materials and Methods

Animals
The study was conducted in accordance with national guidelines for the use of experimental animals. All experimental procedures were approved by the governmental committees (Regierungspräsidium Karlsruhe, Germany). Age-matched, 8- to 10-week-old male mice (C57BL/6; Charles River Laboratories) were used for the experiments. For heterologous blood injection experiments, congenic CD45.1 mice on a C57Bl/6 background (B6.SJL-Ptprca Pepcb/BoyJ) were kindly provided by A. Cerwenka (German Cancer Research Center). All mice were kept on a standard 12-hour light/dark cycle and had free access to food and water.

Induction of ICH
See the online-only Data Supplement for details of blood and collagenase injection model.

Assessment of Hematoma Size
Hematoma size was determined on coronal cryosections. Mice were deeply anesthetized with intraperitoneal injection of ketamin/xylazin (100 and 10 mg/kg, respectively) and transcendally perfused with 15 mL of saline. Brains were removed and frozen in isopentane (−20°C). Forty-micrometer-thick coronal cryosections were taken at 400 μm intervals and scanned at 600 dpi. Hematoma size was analyzed using ImageJ software 1.46 (National Institutes of Health). The total hematoma volume (mm³) was obtained by multiplying the sum of the hemorrhagic area in each cryosection by the distance between sections.

Assessment of Blood Volume Within the Hemorrhagic Hemisphere
To assess not only the size of the hematoma but also the amount of extravasated blood in the different models, we performed hemoglobin spectrophotometry as previously described. Briefly, hemorrhagic and contralateral hemispheres were removed, mechanically homogenized in phosphate buffer solution, and sonicated on ice for 1 minute. After centrifugation, the supernatants were incubated with Drabkin reagent (Sigma) for 15 minutes. The optical density of the resulting fluid was measured at 540 nm using spectrophotometry. To calculate the absolute amount of blood within the hemorrhagic hemisphere, a calibration was performed by adding different volumes of whole blood (0.5/1/2/4/8/12 μL) to brain homogenates of transcendally perfused naive mice.

Flow Cytometric Analysis of Brain-Infiltrating Leukocytes
Hemorrhagic brain hemispheres and spleens were removed and mechanically homogenized. Tissue suspensions were incubated in dissociation buffer (10 mL RPMI-1640, 180 U collagenase IV, 250 U DNase) for 20 minutes and overlaid on Percoll gradients of 1.03 and 1.085 g/mL density. After gradient centrifugation, the collected mononuclear cells were used for flow cytometry. We stained the single-cell suspensions for antimmune CD45.1 (Clone A20), CD45.2 (Clone 104), CD3-ε (Clone 17A2), CD4 (Clone RM 4-5), CD8-α (Clone 53-6.72), CD69 (Clone H1.2F3), B220 (Clone RA3-6B2), NKp46 (Clone 29A1.4), Ly-6G (Clone RB6-8C5), CD11b (Clone M1/70), major histocompatibility complex class II (Clone M5/114.15.2), and the appropriate isotype control following the manufacturers’ protocols. We performed multicolor flow cytometry on a Becton Dickinson LSR II and analyzed the data by fluorescence-activated cell sorting Diva software. Gates were set according to unstained samples and isotype controls. Identically stained splenocyte populations were used to verify gating strategy. Compensation was adjusted using BD CaliBRITE Beads (BD Bioscience).

Immunohistology
See the online-only Data Supplement for details of immunohistochemistry of T lymphocytes.

Statistical Analysis
All values are expressed as mean±SD. Student t test was used for comparison between 2 groups and ANOVA for multiple comparisons with post hoc Tukey test after validating the normal distribution of these data sets by Kolmogorov–Smirnov test. The accepted significance level was P<0.05.

Results
Leukocyte Counts in the Hemorrhagic Hemisphere After Blood or Collagenase Injection
Leukocytes were isolated from the hemorrhagic hemispheres 1 day and 5 days after sham operation, collagenase injection, or whole-blood injection. In both ICH models, hematoma size decreased between day 1 and day 5 after surgery. No hematoma was evident in sham animals (Figure 1A–1C). Although the size of the hematoma was larger in the collagenase injection than in the blood injection model (Figure 1B), the blood content measured by hemoglobin spectrophotometry did not differ between ICH models (ie, bleeding was more dense in the blood injection model; Figure 1C).

Leukocytes were isolated from the hemorrhagic hemisphere and analyzed by flow cytometry. According to previous reports,27–32 CD45⁺/CD11b⁺ cells were defined as microglia and were excluded from the calculation of infiltrating leukocyte numbers. Although the hematoma volume was reduced by day 5, the number of brain-infiltrating leukocytes rose significantly between day 1 and day 5 in both ICH models (Figure 1D). The number of cerebral leukocytes did not differ significantly between the collagenase and the blood injection models at any time point after surgery. Sham groups had significantly lower numbers of brain-infiltrating leukocytes compared with either blood or collagenase injection at 1, 3, and 5 days after surgery. However, sham surgery also resulted in substantial leukocyte invasion compared with the healthy brain of naive animals (Figure 1D).

Leukocytes in the Hemorrhagic Brain Largely Do Not Originate From Injected Blood in the Blood Injection Model
To determine the source of cerebral leukocytes after ICH induction (hemorrhage-derived versus actively invaded), heterologous blood injection between congenic CD45.1 and CD45.2 mice as blood donors and recipients was applied (Figure 2A). Leukocytes isolated from the hemorrhagic
hemispheres were stained for CD45.1 and CD45.2 markers and analyzed by flow cytometry (Figure 2B). Additional CD11b stainings were performed to verify gate settings, and cells showing microglia-like uniformly high CD11b expression were excluded from the analysis (Figure I in the online-only Data Supplement). After cerebral injection of blood from CD45.1 mice into CD45.2 wild-type mice, the ratio of donor blood derived leukocytes was 39±11% at 24 hours after surgery. At 5 days, this proportion decreased to <5% (Figure 2B and 2C). These findings were corroborated by additional experiments injecting blood from CD45.2 wild-type mice into CD45.1 animals (Figure 2B and 2C).

**Temporal Changes in Distribution of Brain-Invading Leukocyte Subsets in the Blood Injection Model**

Leukocytes were isolated from the hemorrhagic hemisphere at different time points ≤14 days after blood injection. We
analyzed brain infiltration of the leukocyte subpopulations by flow cytometry after staining cell surface markers of leukocyte subsets (Figure 3). CD45<sup>low</sup>/CD11b<sup>high</sup> cells were considered as microglia and were excluded from further analysis. At each time point, CD4<sup>+</sup> T cells were the predominant brain-infiltrating leukocyte subpopulation. Except for CD8<sup>+</sup> T-cell counts that constantly increased during the observation period, all leukocyte subsets peaked 5 days after surgery (Figure 4A).

Lymphocytes (taken together T cells, natural killer cells, and B cells) increasingly contributed to brain invasion, whereas the proportion of granulocytes within the infiltrating cell populations was decreased between day 5 and day 14 (Figure 4B).

To characterize the spatial pattern of infiltration of the predominant brain-invading cell population in ICH, we performed CD3 immunohistochemistry 5 days after blood injection or sham operation (Figure 5A). After blood injection, T
lymphocytes were located in the area of the hemorrhage predominantly in the periventricular area. In sham mice, T cells surrounded the injection canal (Figure 5B).

**Distribution of Brain-Invading Leukocyte Subsets Is Similar in the Different ICH Models**

In the blood injection model, CD4+ T cells were the predominant leukocyte population during the whole observation period, whereas a similar infiltration rate of CD4+ T cells and granulocytes was found in the collagenase injection model (Figure 6; Figure II in the online-only Data Supplement). Both at day 1 and day 5, the absolute cell numbers of granulocytes, and at all time points their frequency of total brain-invading leukocytes, were significantly higher after collagenase injection than after blood injection. No other significant differences regarding the numbers of brain-invading leukocyte subsets were detected between the 2 ICH models. Blood as well as collagenase injection induced significant monocyte infiltration at 1, 3, and 5 days after surgery (Figure 6). No consistent ICH-induced changes of natural killer and B-cell numbers were detected. In sham mice, the distribution of brain-invading leukocyte subsets was similar to ICH models (Figure II in the online-only Data Supplement). In contrast to the predominant presence of CD4+ T cells, CD8+ T cells formed the smallest infiltrating leukocyte population. In additional experiments, CD3+/CD4−/CD8− were found to be NK1.1+ and, therefore, identified as natural killer T cells (Figure III in the online-only Data Supplement).

**Discussion**

This is the first study directly comparing leukocyte invasion in 2 common models of ICH using flow cytometry. Our study has 4 new findings. (1) Experimental ICH evokes a similar temporal pattern of cerebral leukocyte infiltration independent of whether autologous blood or collagenase is injected as long as the blood volume is matched. (2) The majority of leukocytes found in the brain after experimental ICH originates from the systemic circulation. (3) CD4+ T lymphocytes are the predominant cerebral leukocyte population in the acute phase of ICH in mice. (4) Collagenase injection induces significantly higher numbers of infiltrating granulocytes than blood injection.

Blood and collagenase injection, respectively, led to a different size and morphology of the hematoma in the present and a previous study. We matched blood content determined by hemoglobin spectrophotometry. Collagenase injection has been reported to evoke a stronger inflammatory reaction. However, when we matched blood content in the 2 ICH models, overall leukocyte infiltration did not differ substantially.

After ICH induction, some leukocytes are part of the inflowing blood in the hematoma, whereas others actively migrate across the blood–brain barrier. We used the blood injection model to determine the source of leukocytes over time. Our
results show that already 24 hours after ICH induction, only 40% of leukocytes isolated from the brain originate from the injected blood, and that by day 5, when leukocyte numbers peak, almost all leukocytes are of peripheral origin. Therefore, in this respect these models are suitable for translational studies targeting leukocyte immigration in experimental ICH in analogy to cerebral ischemia.15

Understanding the time course of cerebral leukocyte infiltration after experimental ICH is another prerequisite for translation from preclinical studies targeting neuroinflammatory mechanisms to ICH in patients. Previous studies investigating leukocyte invasion after experimental ICH focused on early neutrophil and monocyte infiltration; they were mostly performed in the blood injection model; and temporal patterns of leukocyte infiltration were heterogeneous.11,33–38

We used flow cytometry for the analysis of brain-infiltrating leukocytes in 2 models of ICH 1, 3, and 5 days after surgery. Similar to several previous studies including 2 in experimental stroke using 2-photon imaging and immunohistochemistry, we distinguished microglial cells from infiltrating leukocytes based on their lower CD45 expression.27–32,39,40 A limitation of this approach is that CD45 can be upregulated on microglia as demonstrated by conversion toward the CD45high phenotype in experimental autoimmune encephalitis.41 We found a peak of all infiltrating leukocyte subsets 5 days after blood injection. In an extensive study using immunohistochemistry in 3 different ICH models, Xue and Del Bigio42 found more pronounced neutrophil invasion in the collagenase model than after blood injection, which is consistent with our findings. Characterizing the infiltration pattern of natural killer cells and B cells after ICH for the first time, we did not find a prominent brain infiltration of these populations.

Intriguingly, T cells formed the predominant brain-invading leukocyte population in ICH in our study. T lymphocytes are also present in the perihematomal tissue of ICH patients as early as 6 hours after ICH.43 In contrast, preclinical studies reported a more delayed infiltration of T cells 48 to 96 hours after blood injection.38,42 Remarkably, brain infiltration by CD8+ T cells in the present and a previous study by Loftspring et al37 was much less prominent in both ICH models compared with previous reports in cerebral ischemia.44,45 In view of the predominant proportion of T lymphocytes among all brain-infiltrating leukocytes, the pathophysiological role of T-cell subpopulations should be further elucidated in future studies.

In accordance with previous findings,37 we detected a similar distribution but lower absolute cell numbers of brain-infiltrating leukocytes in sham groups compared with ICH mice, indicating that the needle insertion alone causes tissue damage leading to a detectable but less intense inflammatory response than ICH.
A limitation of this study is that we did not examine the signaling pathways and pathophysiological consequences of individual leukocyte populations in the context of ICH. On the contrary, the use of the fluorescence-activated cell sorting technology allowed a multiparametric characterization and quantitative assessment of different leukocyte subpopulations in the brain after experimental ICH.

Conclusions

Our study provides detailed information about the temporal pattern of cellular neuroinflammation in the 2 most widely used murine ICH models. Brain infiltration of systemic immune cells is strikingly similar in both models. CD4+ T cells were identified as the predominant subpopulation invading the hemorrhagic brain suggesting a prominent role of T-cell–driven secondary neuroinflammation in ICH.

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Disclosures

None.

References

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Supplementary Methods

Blood injection model. Mice were anesthetized with isoflurane (3% for induction; 1.2-1.4% for maintenance) in O₂/N₂O (1:2). The mouse was placed in a stereotactic frame (Stoelting, 51615) and a 1 cm long incision of the scalp was made over the midline. A cranial burr hole (1 mm) was drilled 2.2 mm lateral to the midline and 0.2 mm anterior to the bregma. The tail was disinfected with povidone-iodine and after puncturing the tail artery autologous whole blood was collected onto paraffin wax paper. Blood was drawn into a sterile 26 Gauge needle, which was connected to an infusion system containing a 50 μl microsyringe (Hamilton 705N). The 26 Gauge needle was slowly introduced 3.7 mm deep into the left striatum. At a rate of 1 μl/min, 5 μl of blood was infused over 5 min followed by a waiting period of 10 min to allow clotting of the injected blood within the injection site. Then, another 15 μl of blood was injected over 15 min. The needle was left in place for another 10 min and then gradually withdrawn.

In case of heterologous blood-injection experiments, blood was taken from CD45.1 expressing mice and injected into the brains of wild type (CD45.2 expressing) mice. For confirmatory experiments, heterologous blood-injection was performed vice-versa by obtaining blood from wild type (CD45.2) mice and injecting into CD45.1 mice.

Collagenase injection model. For the collagenase injection model, 0.03 U of bacterial collagenase VII-S (Sigma) dissolved in 0.5 μl of sterile saline was drawn into a 10 μl microsyringe (Hamilton, 1701RN) connected to a 26 Gauge needle. Using the same coordinates, 0.03 U of collagenase was infused at a rate of 1 μl/min. The needle was left in place for 10 min and then withdrawn.

Body temperature was maintained throughout the procedure at 37°C using a feedback-controlled heating device. Animals recovered for a period of 60 min under a heating lamp with free access to food and water. Sham operated mice were exposed to insertion of the needle at the same coordinates without injection.

Immunohistology
We performed immunohistochemistry of T lymphocytes on 12 μm coronal sections taken at the level of the injection canal (0.2 mm anterior from the bregma) after fixation with 4% paraformaldehyde for 20 min. Sections were
blocked with 10% goat serum for 1h at room temperature followed by incubation with monoclonal rat anti-CD3 (clone 17A2, eBioscience) at 4°C overnight. After blocking endogenous peroxidase, sections were incubated with anti-rat biotinylated secondary antibody (Vector) for 1h at room temperature. Immunoreactivity was visualized by the avidin-biotin complex (Vector) method, sections were developed in diaminobenzidine (Vector) and mounted on coverslips. We captured images on a Zeiss Axiovert 200M microscope, and cells from sections of 4 animals per group were marked on a topographic brain map.
Supplementary Figure I. Differentiation of blood-borne leukocytes and microglia in brains of CD45.1 and CD45.2 mice after blood injection from CD45.2 and CD45.1 donor animals, respectively. (A) In CD45.1 recipient mice, CD45.2+ population indicated leukocytes originating from the injected blood. Among the two distinct CD45.1+ cellpopulations, one population showed uniformly high CD11b expression suggesting to be microglia cells while CD45.1+ brain-invading leukocytes were mostly CD11b-. (B) In CD45.2+ recipients, CD11b\textsuperscript{high} cells could been likewise distinguished from blood-borne leukocytes.
Supplementary Figure II. Flow cytometric analysis of cerebral leukocyte populations in proportion of all brain-invading leukocytes 1d (A), 3d (B) and 5d (C) following sham operation, collagenase or blood injection. CD45$^{\text{low}}$/CD11b$^{\text{high}}$ cells were defined as microglia and were excluded from the rate calculation. (5 individual experiments with 4 pooled animals per group in each experiment, one-way analysis of variance for every population, * p<0.05). Data are expressed as mean ± SD.
Supplementary Figure III. Identification of CD3+/CD4- cell population in brain 5d after blood injection. SSC: side scatter channel.