Immune Response in Human Cerebral Cavernous Malformations

Changbin Shi, MD, PhD; Robert Shenkar, PhD; Hongyan Du, MS; Edward Duckworth, MD; Harish Raja, BS; H. Hunt Batjer, MD; Issam A. Awad, MD

Background and Purpose—Preliminary observations suggesting the presence of B and plasma cells and oligoclonality of immunoglobulin (Ig) G in cerebral cavernous malformations (CCM) have motivated a systematic study correlating the infiltration of the immune cells with clinical activity and antigen-triggered immune response in surgically excised lesions.

Methods—Infiltration of plasma, B, T, and human leukocyte antigen-DR–expressing cells and macrophages within 23 excised CCM was related to clinical activity. Relative amounts of Ig isotypes were determined. IgG clonality of mRNA from CCM was assessed by spectratyping, cloning, and sequencing.

Results—Infiltration of the immune cells ranged widely within CCM lesions, and cells were generally coexpressed with each other. Immune cell infiltration did not associate with recent bleeding and lesion growth. Significantly more B lymphocytes in CCM lesions were associated with venous anomaly. More T cells were present in solitary lesions. More T cells and less macrophages were present in CCM from younger subjects. IgG isotype was present in all CCM lesions. Most lesions also expressed IgM and IgA, with IgM predominance over IgA correlating with recent CCM growth. Oligoclonality was shown in IgG mRNA from CCM, but not from peripheral blood lymphocytes, with only 8 complementary-determining region 3 sequences observed among 134 clones from 2 CCM lesions.

Conclusions—An antigen-directed oligoclonal IgG immune response is present within CCM lesions regardless of recent clinical activity. Apparent differences in immune response in younger patients and in lesions with recent growth will need confirmation in other series. The pathogenicity of oligoclonal immune response will require systematic hypothesis testing in recently available CCM murine models. (Stroke. 2009;40:1659-1665.)

Key Words: cerebral cavernous malformations ■ immunohistochemistry ■ immune response ■ lymphocytes ■ oligoclonality

Cerebral cavernous malformation (CCM) is a prevalent vascular phenotype affecting up to 0.5% of the population,1–5 predisposing patients to a lifetime risk for stroke and epilepsy.4 The CCM lesions consist of clusters of dilated brittle capillaries that proliferate in the setting of repetitive hemorrhages. Lesions occur sporadically, in association with venous anomalies,6 or may be inherited as an autosomal dominant trait, with 3 known gene loci,7 but the genesis and proliferation of individual CCM lesions are largely unpredictable even among well-defined genotypes.8–11 There is no available therapy to prevent lesion genesis or clinical sequelae.

Immunoglobulin and other related genes are markedly unregulated within human CCM lesions.12 Preliminary work suggests the infiltration of B and plasma cells within the lesions, and the presence of oligoclonal immunoglobulin (Ig) G in lesions but not in paired sera from the same patients.13 A potential role of the immune response in CCM has not been demonstrated previously but would be compelling, given the unique antigenic milieu of CCM lesions with sequestered thrombi and leaky blood–brain barrier, and the numerous examples of immune modulation of angiogenesis in other disease states.

In this study we systematically assess the spectrum of immune cell infiltration and predominant Ig isotype in CCM lesions, in correlation with clinical behavior and other lesion features. We aim to define the immune response in CCM lesions by assessing the distribution of lengths, cloning, and sequences of complementary-determining region 3 (CDR3) of the IgG variable heavy chain (IgGVH) genes in comparison to peripheral blood lymphocytes (PBL) from the same patients.

Received September 28, 2008; accepted October 27, 2008.
From Division of Neurosurgery (C.S., R.S., E.D., H.R., H.H.B., I.A.A.) and Center on Outcomes Research and Education (H.D.), NorthShore University HealthSystem, Evanston, Ill; Department of Neurological Surgery (C.S., R.S., E.D., H.H.B., I.A.A.), Feinberg School of Medicine, Northwestern University, Chicago, Ill. C. Shi and R. Shenkar contributed equally to this work.
Correspondence to Robert Shenkar, NorthShore University HealthSystem, Division of Neurosurgery, 2650 Ridge Avenue, Evanston, IL 60201. E-mail rshenkar@northshore.org
© 2009 American Heart Association, Inc.
Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.108.538769
Methods

Subjects
We used consecutive subjects who had undergone CCM excision unrelated to this research. The diagnosis of CCM was established by typical histopathologic criteria in every case. The research was approved by the Institutional Review Board of Evanston Northwestern Healthcare, and the subjects gave informed consent.

Immunohistochemistry
Paraffin-embedded sections of excised CCM lesions from 23 subjects were immunostained for the immune cells by a method previously described.14 Antihuman primary antibodies included anti-CD138 (BC/B4), anti-CD20 (L26), anti-CD3 (PS1), and anti-human leukocyte antigen (HLA)-DR (LN3) obtained from Biocare Medical; anti-CD79a (JCB117) from Dako; anti-IgA, -IgG, and -IgM from CellMarque Corporation; and anti-CD45RO (UCHL-1) and anti-HLA-DR (KP-1) from Ventana Medical Systems, Inc. Tonsil was used as a positive control.

The numbers of clumps of cells per area and total cells per area for plasma cells (CD138), B lymphocytes (CD20, CD79a), T lymphocytes (CD3, CD45RO), and HLA-DR antigen-presenting cells (HLA-DR) were determined in coded unidentified specimens.

For anti-CD68 (monocytes/macrophages), serial sections of stained and negative controls from up to 10 representative fields for each specimen were captured in grayscale using the Magnafire inverted images was acquired with NIH Image J program.15 The intensity for each specimen were captured in grayscale using the Magnafire

RNA Isolation From CCM Lesions and PBL
CCM lesions were surgically excised from 5 subjects, rinsed with saline, and snap-frozen in liquid nitrogen. The parts of the CCM lesions were homogenized separately with zirconia/silica beads. PBL were isolated by a method published previously.16 RNA was isolated from CCM specimens and PBL using TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. Table 1 lists the primers used for cDNA synthesis, specific amplification of VH regions of IgG, and sequencing.17 Spectratyping and sequencing was conducted on an ABI 3730 Sequencer (Applied Biosystems). The distribution of CDR3 sizes was assessed via a method previously reported17 with modifications.

Spectratyping and Sequencing of CDR3 Regions
Spectratyping and sequencing was conducted on an ABI 3730 Sequencer (Applied Biosystems). The distribution of CDR3 sizes was assessed via a method previously reported17 with modifications. GeneScan 500 LIZ Size Standard (Applied Biosystems) was performed in the same well with the sample labeled with 6-FAM conjugated primers.

RNA from the parts of 2 CCM lesions shown to be oligoclonal (≥5 different sizes) for both VH3 and VH4 by spectratyping was sequenced. Nested polymerase chain reaction products for CDR3 were purified using the Wizard SV Gel and polymerase chain reaction clean-up system (Promega) and ligated into pGEM-T Easy Vector using T4 DNA ligase (Promega). Between 30 and 38 clones from the VH3 or VH4 family from CCM mRNA were selected. Recombinant plasmid DNA was purified with Wizard Plus SV Miniprep DNA Purification System (Promega) and sequenced with T7 promoter primer. Alignment analysis of CDR3 sequences to every single clone obtained from VH3 or VH4 families and translation of amino acid were performed using Vector NTI 10.3.0 (Invitrogen).

Statistical Analysis
Spearman correlations were computed and Wilcoxon 2-sample test was applied for skew-distributed variables. Independent 2-sample t test and linear regression were used in univariate analyses on normal distributed variables and log-transformed values for skew-distributed variables. Multivariate analyses were conducted using mixed-effects models with McKeon F approximation via restricted maximum likelihood estimate. Two-sided P<0.05 was considered significant; false discovery rate-adjusted probability values from multivariate models were reported.

Results
Immunostained B, T, plasma, and HLA-DR antigen–presenting cells were identified in nearly all specimens, predominantly in perivascular clumps around caverns and lesional vessels (Figure 1). There was a wide range of clumps per area and cells per area among lesions, with skew distributions (Figure 2). Density of B cells per area and clumps per area were significantly correlated (Spearman correlation coefficients 0.441–0.788; P<0.05) with T and plasma cells in the same lesions. Clumps per area of plasma cells correlated with HLA-DR antigen–presenting cells (P=0.0164). The mean difference and mean ratio for intensity of CD68 staining were inversely correlated with clumps per area of CD79a-stained

Table 1. Sequences of Oligonucleotide Primers Used for IgGVf Region Amplifications and Sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cγ5</td>
<td>CTCCTCACCCACTTTCTGTTCC</td>
<td>cDNA priming</td>
</tr>
<tr>
<td>Cμ1</td>
<td>GTTGGCACCGGGTGGTCTG</td>
<td>Primary PCR</td>
</tr>
<tr>
<td>Vg3</td>
<td>CACAGGAGGTGGTGGCGGACC</td>
<td>Vg3 leader primer</td>
</tr>
<tr>
<td>Vg4</td>
<td>ATGAAACCACCTGTTCTTTC</td>
<td>Vg4 leader primer</td>
</tr>
<tr>
<td>Vκ3FR3</td>
<td>MGATTCCACCTCCTMARGG</td>
<td>Vκ3 framework 3</td>
</tr>
<tr>
<td>Vκ4FR3</td>
<td>CGACTCCATRGCGTAGAC</td>
<td>Vκ4 framework 3</td>
</tr>
<tr>
<td>Jκ1,2,4,5</td>
<td>TACCTGAGAACGCTGAGCC</td>
<td>Jκ6 primer</td>
</tr>
<tr>
<td>Jκ6</td>
<td>ACTCTACGTACGCTATAGGG</td>
<td>Sequencing primer</td>
</tr>
<tr>
<td>T7 primer</td>
<td>TAATACGACTCACTATAGGG</td>
<td></td>
</tr>
</tbody>
</table>

(1 minute), 58°C (1 minute), and 72°C (2 minutes), ending with an incubation at 72°C (7 minutes).

(mean ratio for intensity of CD68 staining were inversely correlated with clumps per area of CD79a-stained

(mean ratio for intensity of CD68 staining were inversely correlated with clumps per area of CD79a-stained
B cells (Spearman correlation coefficients were \(-0.431, P=0.0401\) and \(-0.443, P=0.0343\), respectively).

Recent bleeding and lesion growth did not correlate with density of any cell type (Figure 3). In univariate analysis, CCM with associated venous anomaly had more clumps per cm\(^2\) (\(P=0.0335\)) and cells per cm\(^2\) (\(P=0.0408\)) of B lymphocytes stained with anti-CD20 antibody than in CCM without this anomaly. Log transformation gave a similar result that CCM with venous anomaly had more CD20-stained and CD79\(^\alpha\)-stained B cells per area (\(P=0.018\) and \(P=0.024\), respectively), and more clumps per area of CD20-stained cells (\(P=0.026\)) in univariate analysis. A multivariate analysis with all 6 types of cells per area as dependent variables further supported that CCM with venous anomaly had more CD20-stained and CD79\(^\alpha\)-stained B cells per area (false discovery rate-adjusted \(P=0.0458\)). There were more clumps of CD3-stained T lymphocytes per area (in log value) in cases with single lesions than from subjects with multiple CCM lesions (\(P=0.0071\)) in univariate analysis. Regression analysis revealed an inverse correlation between numbers of clumps of CD3 positive T cells per area (in log value) and the age at diagnosis (\(P=0.0318\); regression coefficient \(-0.0562, P=0.0401\)). Regression analysis gave a positive correlation between the age at surgery and the mean difference and mean ratio for intensity of CD68-stained cells (regression coefficients were 0.6350, \(P=0.0463\) and 0.0069, \(P=0.0329\), respectively), indicating greater CCM infiltration by those cells in older patients.

IgG isotype was expressed in lymphocytes in all lesions, and was predominant (IgG>IgA or IgM) in 15 of 23 lesions. IgM-stained cells were present in 18 of 23 lesions and IgA-stained cells were present in 19 of 23 lesions. IgM-stained cells predominated over IgA-stained cells in 15 of 23 lesions, and were positively associated with recent growth of the CCM, whereas all 10 CCM with recent growth had more IgM-stained than IgA-stained cells, and 5 of 8 CCM without recent growth had more IgM-stained than IgA-stained cells (\(P=0.0065\)).

We subsequently analyzed B-cell clonality by focusing on the CDR3 region in VH3 and VH4 families of IgGV\(^H\) gene using the spectratyping, cloning, and sequencing technology. In addition, to avoid IgG contamination from PBL, we used paired PBL from the same patient as an internal control. Our immunostaining results revealed B and plasma cells were predominantly identified in perivascular clumps. Therefore, to detect the clonality in the different lesional locations from

Figure 1. Positive immune cells in CCM lesions: (A) B cells (CD20), (B) plasma cells (CD138), (C) T cells (CD3), (D) monocytes/macrophages (CD68), (E) antigen-presenting cells (HLA-DR), and (F) negative control from the same area of the same specimen. IgG-positive cells (G) and IgM-positive cells (H) from 2 other specimens. Original magnification is 50× (A to F) and 132× (G, H). Scale bars are 100 μm.

Figure 2. Spectrum of immunity in CCM lesions. Distribution of the number of (A) clumps per area and (B) cells per area for the indicated immune cells in CCM lesions.
the same patient, the CDR3 spectratyping from 2 different regions of the same patients was determined, except for 1 patient with a small tissue.

All extracts from paired PBL RNA showed a polyclonal CDR3 size distribution pattern with 10 peaks, whereas spectratyping profiles of CDR3 of IgGVH from all 5 extracts of CCM lesions demonstrated oligoclonal patterns when the VH3 family was tested. CCM lesions showed only 2 to 5 sizes in the VH3 family. There was a significant difference in the number of peaks for CDR3 of IgGVH in the VH3 family between CCM lesions and PBL (P<0.0001). Three of 4 CCM lesions showed oligoclonal profiles in 2 different parts from the same lesions, whereas 1 lesion had an oligoclonality in one part and polyclonality in the other part. Likewise, similar polyclonal profiles of CDR3 size distribution from PBL RNA with almost similar number of peaks were observed in VH4 family. However, spectratyping of CDR3 mRNA from 3 of 5 CCM lesions gave an oligoclonal pattern, with 2 to 6 sizes in the VH4 family. One lesion had an oligoclonal pattern in 2 separate parts. Another lesion had oligoclonality in one part and polyclonality in the other part. The remaining 2 lesions had polyclonality in 2 separate parts.

There were no peaks with identical sizes for the VH3 and VH4 families in lesions from different CCM patients. There was also a significant difference in the number of peaks of CDR3 in the VH4 family between CCM lesions and PBL (P<0.0001).

Discussion

Robust Infiltration of the Various Immune Cells in CCM Lesions

Macrophages have long been recognized in CCM lesions, in presumed response to lesional hemorrhage. The presence of other immune cells in CCM has only recently been described in preliminary observations but had not been previously examined systematically. Recent study of cerebral arteriovenous malformations revealed the presence of polymorphonuclear cells and macrophages, but no significant B-cell or plasma cell infiltration. Our results confirm a robust infiltration of antibody producing B-lymphocytes and plasma cells in CCM lesions, with predominant IgG response, with occasional IgM- or IgA-expressing lymphocytes in several
lesions. There was coexpression of T cells and of antibody-producing cells in the same specimens.

**Correlation With Clinical Activity and Lesional Features**

Surgically removed CCM may not reflect the biological spectrum of the majority of CCM. However, this large cohort included cases with a variety of lesion phenotypes and clinical activity, allowing preliminary correlations with infiltration of immune cells and Ig isotypes (supplemental Table I). Cell infiltration of the immune system studied, including antibody-producing cells, was not more prevalent in lesions with recent growth or hemorrhage. This finding is consistent with the hypothesis that cells of the immune system are a feature of CCM lesion phenotype regardless of recent clinical activity.

There was a greater T-cell infiltration and less prevalent macrophages in younger patients. Although it was previously shown that the fraction of collagen IV-expressing caverns in CCM lesions decreased with age, an explanation for a possible association between immunity and collagen IV in CCM is unknown. Other investigators found more macrophages/microglia were present inside and around a lesion, caused by intracerebral hemorrhage in older, rather than in younger, mice. Microglial activation also has been shown to increase with age in human brains. In mice, increased prostaglandin E2 production by macrophages has been shown to be responsible for decreased T-cell function with age. These are consistent with the possibility that the immune response may shift from acquired to innate with age and growth of CCM.

Associated venous anomaly, as determined by contrast-enhanced MRI, has been frequently documented with sporadic solitary lesions and is rare in familial CCM cases. B-cell infiltration was greater in CCM associated with venous anomaly. There was a greater T-cell infiltration in solitary lesions than in cases with multiple/familial CCM. CCM lesions with recent growth were significantly associated with greater IgM isotype response than other lesions, suggesting a more acute antigenic response in these lesions than the more chronic response associated with IgG isotype. However, selected surgical cases and their clinical and imaging features may not reflect accurate lesion age or maturity. These findings will require confirmation in other clinical series and in murine models of early and late stage CCM lesions.

**Antigen-Driven Immune Response in CCM Lesions but Not PBL**

An antigen-directed IgG immune response in multiple sclerosis and antigen-directed IgA immune response in the vascular wall in acute Kawasaki disease have been de-
Antigen-driven immune response in CCM could also be intriguing. Previous studies have suggested that oligoclonal bands from IgG proteins are present within CCM lesions, but not in serum from the same patients. However, it has not been previously determined if this reflects antigen-directed IgG immune response or an inflammation marker. To answer this question, we used the techniques of spectratyping, cloning, and sequencing to detect CDR3 region of the IgGVH gene. This region is unique to each B-cell clone and specific to antigen recognition, and CDR3 structure, independent from the VH framework, is sufficient to define the antigen binding specificity of an antibody. In addition, the sequence of CDR3 gene offers the useful clonal signature of an individual B cell. The high-frequency occurrence of a specific CDR3 sequence illustrates the expansion of its corresponding cell clone. The alternative method of CDR3 size distribution analysis is a quicker approach to characterize the presence of B-cell or plasma clonal expansion.

Clonal expansion may be caused by either specific antigen stimulation or inflammation (bystander effect). In this investigation, oligoclonality in CCM lesions, but not in PBL from the same subjects, has been confirmed by size distribution and sequencing of the gene for the CDR3 region of IgGVH gene. Spectratyping profiles for CDR3 regions of ≤6 different sizes, indicating oligoclonality for IgG in CCM lesions are comparable to those for IgA in the vascular wall from subjects with acute Kawasaki disease. Only 8 different IgG CDR3 nucleotide sequences (coding for only 7 amino acid sequences) were obtained from 134 clones from 2 CCM lesions in this study. These are fewer than the proportion of unique IgG CDR3 sequences found in multiple sclerosis plaques by other investigators using similar methods, suggesting that fewer epitope selections are involved in CCM than in other diseases. Our findings demonstrated predominant and reproducible CDR3 amplification in CCM lesions, but significantly different from PBL, suggesting CCM lesions are specific B-cell expansions, instead of a random B-cell response or a random bystander response. Further studies should determine if the oligoclonal antibody response is targeted against a foreign or self-antigen in the milieu of CCM lesions. Iron breakdown products, glial or neuronal cells, are hypothetical candidates, as are potential infectious agents. The relationship between the antigen-triggered immune response and leaky blood–brain barrier also needs to be determined. These hypotheses are amenable to investigation by determining the putative antigen trigger in immunoprecipitation experiments with lesional antibodies.

One CCM patient showed the mixed oligoclonality and polyclonality of CDR3 from the different lesional locations for V\textsubscript{H3} family. Likewise, another CCM patient also demonstrated the same mixed clonality of CDR3 from the different lesional locations for V\textsubscript{H4} family. This may be related to the different stages of the disease and, consequently, B or plasma cells in the different lesional areas reflecting distinct clonal expansion. Or it is possible that those heterogeneous clonal characteristics originated from PBL contamination. This discrepancy could be solved by capturing single or clumped plasma cells in CCM lesions via a technique of laser-captured microdissection. The other patients showed identical clonality from the different lesion locations, indicating homogeneous distribution of B-cell or plasma cell clonal expansion. From our immunostaining results of B and plasma cells, those cells were mostly distributed in clumps. Therefore, it is possible that each or several clumps may originate from clonal expansion of a few B or plasma cells in homogenous clonal lesions. Heterogeneous clonality of IgG may be linked to B or plasma cells in different clumps in CCM lesions. We have initiated studies examining clonality in microdissected plasma cell clumps from CCM lesions.

Among V\textsubscript{H} families (V\textsubscript{H1}1–7), V\textsubscript{H3} and V\textsubscript{H4} are the largest, accounting for ≈75% of repertoire in adult peripheral blood B cells and in the vascular wall in acute Kawasaki disease. Our results seem to demonstrate that there is more frequent usage of oligoclonality for V\textsubscript{H3} family than V\textsubscript{H4} family in CCM lesions. The V\textsubscript{H3} family had been reported as the most frequently used V\textsubscript{H} family in the synovial tissue of patients with rheumatoid arthritis, and V\textsubscript{H1} and V\textsubscript{H4} families are the most frequently used in multiple sclerosis brain.

Pathobiologic Implications and Future Directions

Taken together, these results suggest an antigen-directed oligoclonal IgG immune response in CCM lesions regardless of recent clinical activity. The role of this immune response in the genesis and progression of CCM lesions remains speculative.

Features of the immune response will be examined in recently characterized murine models, comparing primordial and more mature lesions and the reaction to experimental intracerebral blood. If CCM genesis is affected by immunosuppression or immunomodulation in existing animal models, this would suggest that an immune response may be part of the cause of CCM. Artificially created recombinant antibodies will be used to locate putative antigens in human specimens. This may offer novel strategies at therapeutic manipulation to modify CCM disease.

Sources of Funding

This study was partially supported by the National Institutes of Health (NS052285 to L.A.A.).

Disclosures

None.

References


Immune Response in Human Cerebral Cavernous Malformations
Changbin Shi, Robert Shenkar, Hongyan Du, Edward Duckworth, Harish Raja, H. Hunt Batjer and Issam A. Awad

Stroke. 2009;40:1659-1665; originally published online March 12, 2009;
doi: 10.1161/STROKEAHA.108.538769
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/40/5/1659

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office.
Once the online version of the published article for which permission is being requested is located, click
Request Permissions in the middle column of the Web page under Services. Further information about this
process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/