

Aus der Neurologischen Klinik des Universitätsklinikums Düsseldorf

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Orts- und phasenabhängige Gliaaktivierung nach fokaler Ischämie des Rattenhirns

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Zusammenfassung

Die fokale Hirnischämie induziert eine komplexe inflammatorische Reaktion. In drei pathogenetisch unterschiedlichen Modellen fokaler Ischämie wurden die Expression von Aktivierungsmarkern und Zytokinen auf Mikroglia und Astrozyten analysiert und den Veränderungen nach KCl-induzierter Spreading Depression gegenübergestellt. Während die Gliaaktivierung in der ischämischen Hirnläsion direkte Folge des ischämischen Ereignisses ist, hat sich die NMDA-Rezeptor vermittelte Spreading Depression als ideales Modell läsionsferner Gliaaktivierung im intakten ipsilateralen Kortex erwiesen. Daneben kommt es mit deutlicher zeitlicher Verzögerung zur Mikrogliaaktivierung in sekundär degenerierenden subkortikalen Fasertrakten und Kerngebieten.

Gliareaktionen einer Infarktfrühphase, die die ersten 48-72 Stunden nach Infarktinduktion umfasst, sind von Veränderungen einer Infarktspätphase 72 Stunden bis 7 Tage nach Ischämiebeginn sowie einem sich anschließenden chronischen Infarktstadium abzugrenzen.

Mikroglia ist die primär auf den ischämischen Reiz reagierende nichtneuronale Zellpopulation. Die Mikrogliaaktivierung ist abhängig vom Grad der eintretenden Gewebeschädigung räumlich und zeitlich differenziert und heterogen: eine rasche und vollständige Aktivierung zu phagozytosefähigen Zellen im pannekrotischen Infarktkern steht einer inkompletten und möglicherweise frustranen Mikrogliaaktivierung in Bereichen mit selektivem neuronalen Untergang sowie einer transienten Mikrogliaaktivierung durch Spreading Depression gegenüber.

Mit einer Verzögerung von Tagen tritt eine astrogliale Aktivierung und die Infiltration hämatogener Zellen auf. Es findet sich eine anisomorphe Astroglie im Infarkttrand, dagegen eine isomorphe Gliose in Arealen mit inkompletter ischämischer Zellschädigung, sowie eine transiente Astrozytenaktivierung durch Spreading Depression im exofokalen ipsilateralen Kortex.

In der Infarktspätphase infiltriert eine gemischte Mikroglia/Makrophagenpopulation wallartig den Infarkttrand, die durch verschiedene immunozytochemische Aktivitätsmarker gekennzeichnet ist. Die in den vorgelegten Arbeiten erstmals beschriebene Expression von CD8 auf einer Makrophagensubpopulation ist charakteristisch für die ischämische (Pan-) Nekrose.

In Assoziation zu der heterogenen glialen Aktivierung führt die fokale Ischämie zu einer differenziellen Induktion proinflammatorischer Zytokine im Infarkt, aber auch im gesamten ipsilateralen Kortex. In der Infarktfrühphase induzieren Spreading Depression die Expression

von TNF- α und IL-1 β auch außerhalb der Infarktregion, während in der Infarktspätphase die Expression von IL-18 zusammen mit seinem bioaktivierenden Enzym, Caspase-1, auf den Infarktkern beschränkt bleibt.

In der chronischen Infarktphase kommt es nach flächiger Phagozyteninfiltration des Infarktes zur pseudozystischen Degeneration und Defektbildung sowie Ausbildung einer astrozytären Narbe.

Mittels multimodaler Kernspintomographie ist es möglich, die genannten Infarktstadien, nicht jedoch einzelne Aspekte der inflammatorischen Veränderungen nicht-invasiv darzustellen.

Die vorgelegten Arbeiten geben Einblick in ein komplexes Netzwerk inflammatorischer Reaktionen nach fokaler Ischämie. Diese Reaktionen laufen noch über Tage nach Eintreten des Infarktes ab und verändern dynamisch den Infarkt selbst, haben aber auch ausgedehnte Effekte auf nicht infarziertes Gewebe. Die beschriebenen glialen Reaktionen könnten zu einer sekundären Gewebsschädigung führen, aber auch Regeneration und Plastizität fördern sowie Ischämietoleranz für weitere Insulte vermitteln. Gelänge es, selektiv zytotoxische Effekte zu unterdrücken, aber Zytoprotektion und Regeneration zu fördern, eröffnete dies neue Perspektiven bei der Behandlung des menschlichen Schlaganfalls in einem Zeitfenster, in dem heute keine therapeutische Option zur Verfügung steht.

1. Einleitung

Der ischämische Hirninfarkt steht nach der Koronaren Herzerkrankung und bösartigen Neubildungen beim Menschen an dritter Stelle der Erkrankungen mit der höchsten Morbidität und Mortalität. Schon lange ist bekannt, dass es nach einem Schlaganfall sowohl zu einer raschen klinischen Besserung als auch zu einer sekundären Verschlechterung der klinischen Symptome kommen kann. Die moderne zerebrale Kernspintomografie (MRT) hat in der klinischen Situation des menschlichen Schlaganfalls zu neuen pathophysiologischen Einsichten ermöglicht. Es konnte nachgewiesen werden, dass das perfusionsgestörte Areal regelhaft erheblich größer ist als der definitive Infarkt. Außerdem weicht das initiale Ischämieareal in seiner Ausdehnung durchaus vom dem nach 7 Tagen demarktierten Infarkt ab (Neumann-Haefelin et al. 2000), so dass die Infarktentwicklung auch jenseits weniger Stunden beeinflussbar sein sollte.

Mit der Wiedereröffnung akut verschlossener Gefäße durch eine Lyse mit rekombinanten Gewebs-Plasminogenaktivator (rt-PA) steht für den akuten Schlaganfall in einem sehr begrenzten Zeitfenster von drei Stunden eine kausale, jedoch nur mäßig effektive Therapie zur Verfügung. Jenseits der initialen drei Stunden, in denen nur eine Minderzahl der Patienten die Klinik erreicht, besteht bislang keine effektive Therapiemöglichkeit.

Aus tierexperimentellen Untersuchungen ist bereits länger bekannt, dass es nach fokalen Hirninfarkten zu einer leukozytären Infiltration des Infarktgebietes kommt (Hallenbeck et al. 1986). Die Forschung der letzten Jahre hat in zunehmenden Maße belegt, dass es sich hierbei nicht um einen unspezifischen Einstrom von Leukozyten in infarziertes Gewebe (Garcia et al. 1994), sondern um ein differenziertes System spezifischer humoraler und zellulärer inflammatorischer Reaktionen handelt (Arvin et al. 1996; Stoll et al. 1998). Es ist inzwischen weitgehend akzeptiert, dass inflammatorische Veränderungen einen wesentlichen Einfluss auf die definitive Infarktgröße haben. Die therapeutische Modifikation inflammatorischer Kaskaden kann die Infarktgröße noch in einem Zeitfenster von Tagen nach Infarkteintritt verändern (Dirnagl et al. 1999; Becker 2001). Für eine Vielzahl von Faktoren gilt allerdings, dass sie abgänglich von den gewählten Bedingungen protektive oder zytotoxische Effekte haben. Ausweg aus diesem Dilemma erscheint die differenziertere Analyse von immunologischen Prozessen nach Schlaganfall in Bezug zur zugrundeliegenden Gewebsschädigung, die eine bessere Definition therapeutischer Angriffspunkte nach eingetretenem Schlaganfall verspricht. Diese These motiviert die vorgelegten Arbeiten.

Die Vorstellung, dass das Zentralnervensystem nicht der Überwachung des Immunsystems unterliege („immunologisches Privileg des ZNS“), ist überholt. Dagegen belegen zahlreiche Untersuchungen, dass hirneigene Zellen im gesunden Gehirn aktiv Immunprozesse unterdrücken, andererseits bei pathologischen Prozessen eine autochthone Immunreaktion initiieren (Neumann 2001).

Mikroglia ist die residente immunkompetente Zellpopulation des ZNS. Nachdem ihre Herkunft lange Zeit umstritten war, sprechen derzeit zahlreiche Befunde für eine Abstammung von hämatogenen Monozyten/Makrophagen. Aktivierte Mikroglia ist eine außerordentlich pluripotente Zellpopulation mit sowohl protektiven als auch zytotoxischen Eigenschaften, die unter *in vitro*-Bedingungen eine Vielzahl von Entzündungsmediatoren sezernieren kann (Abb.1).

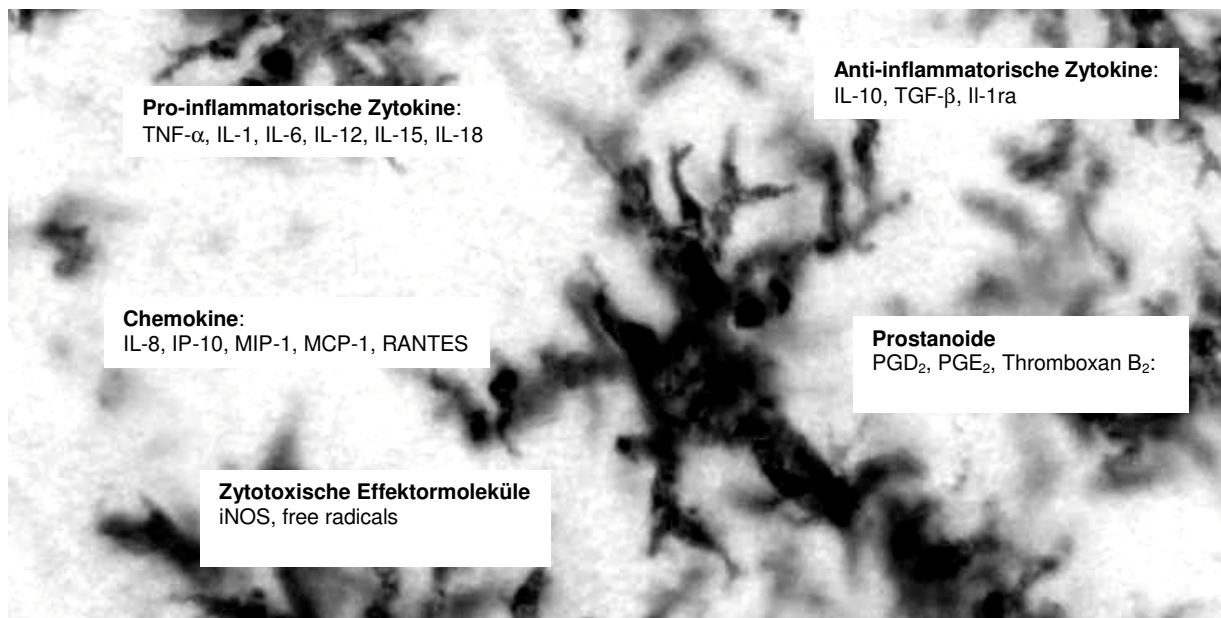


Abb.1: Mikroglia sezerniert eine Vielzahl immunologisch aktiver Faktoren. Modifiziert nach (Aloisi 2001)

Unter Aktivierung durchlaufen Mikrogliazellen eine typische Sequenz morphologischer Veränderungen (Streit et al. 1999). Dabei runden sich verzweigte (ramifizierte) Mikrogliazellen über einen sternförmigen Zustand („stellate cells“) zunehmend zu amöboider Mikroglia ab (Abb.2). Letztere sind neben den morphologischen Kriterien *in situ* immunzytochemisch nicht mehr von hämatogenen Makrophagen unterscheidbar. Parallel zur morphologischen Transition exprimiert aktivierte Mikroglia vermehrt bzw. *de novo* immunzytochemisch detektierbare Moleküle (Salimi and Humpel 2002). Hierzu gehören die Haupthistokompatibilitätsantigene (MHC) Klasse I und II, als Marker für die Funktion von

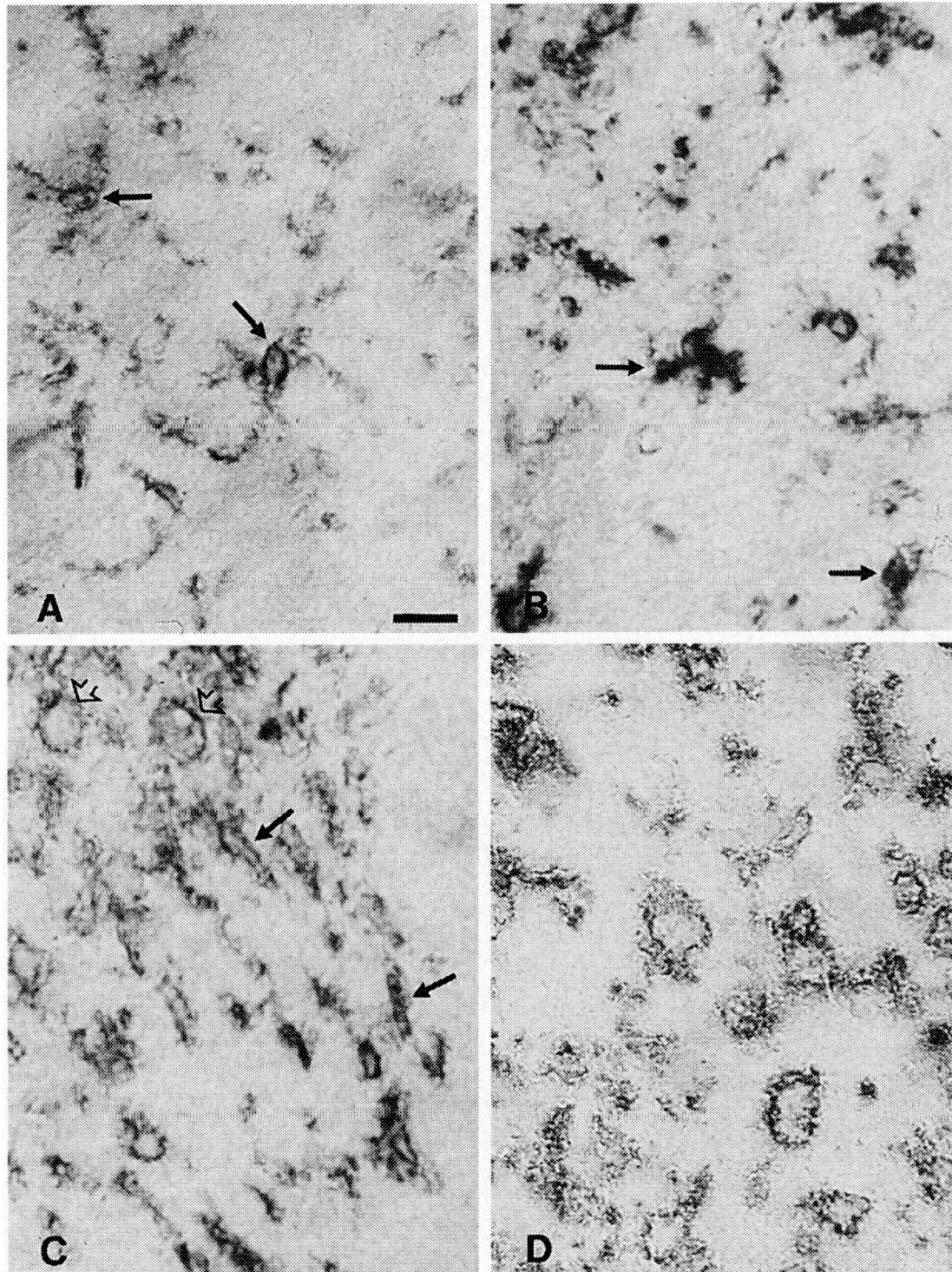


Abb.2: Morphologische Veränderungen mikroglialer Zellen unter Aktivierung. Die verzweigte (ramifizierte) Mikrogliazelle (A) erfährt unter Aktivierung eine Verplumpung der Zellfortsätze (sternförmige Zelle, B), wobei in der weißen Substanz die Zellfortsätze sich entlang degenerierender Fasern ausrichten und als sog. Stäbchenzellen (rod cells, schmale Pfeile in C) imponieren. Weitere Aktivierung führt zur vollständigen Abrundung als amöboide Mikroglia, die nicht mehr von hämatogenen Makrophagen zu unterscheiden ist (D).

aktivierter Mikroglia als Antigen-präsentierende Zellen (Ref.1,3,5) und die Immunmoleküle wie das CD4-Antigen (Ref.1,3,5,7). Der Antikörper ED1 detektiert bei der Ratte Phagolysosomen in Mikroglia/Makrophagen und gibt damit ein qualitatives Maß phagozytischer Aktivität dieser Zellen (Ref.1,3,5).

Ebenso ubiquitär wie Mikroglia reagiert Astroglia mit Hypertrophie und Proliferation auf pathologische Zustände im ZNS. Das gliale fibrilläre saure Glykoprotein (GFAP), ein für Astrozyten spezifischer Zytoskelettbestandteil, wird in reaktiven Astrozyten hochreguliert und eignet sich damit als Aktivierungsmarker (Bignami und Dahl 1974). Wie Mikroglia sind aktivierte Astrozyten in der Lage, eine Vielzahl von Oberflächenmolekülen zu exprimieren und lösliche Faktoren zu sezernieren (Ridet et al. 1997). Die Ansammlung aktivierter Astrozyten wird allgemein als Gliose bezeichnet. Nach mechanischen Läsionen werden modellhaft zwei grundsätzlich verschiedene Ausprägungen der Gliose unterschieden (Ridet et al. 1997): Die läsionsnahe Astrozytenaktivierung führt zu einer Narbenbildung, die die Gewebestruktur bleibend verändert (anisomorphe Gliose). Dagegen ist fern solcher Läsionen eine „zarte“ astrogliale Aktivierung zu sehen, die die vorbestehenden Strukturen respektiert, also nicht durch eine Narbenbildung ersetzt (isomorphe Gliose; Fernaud-Espinosa et al. 1993). Im erwachsenen Gehirn ist das Auftreten von Vimentin+ Astrozyten relativ spezifisch für eine anisomorphe Gliose (Ridet et al. 1997). Funktionell kann die reaktive Gliose einerseits ein Wiederaussprossen von Axonen hemmen (non-permissive Gliose), andererseits aber auch ein Substrat für die axonale Regeneration bilden (permissive Gliose; Mansour et al. 2002).

Astrozyten wie Mikroglia unterliegen dem Einfluß von Zytokinen und sind ihrerseits wichtige Quellen von Zytokinen im Nervensystem. Zytokine wurden initial als humorale Faktoren in immunkompetenten Zellen wie Lymphozyten und Makrophagen entdeckt, wo sie eine wegweisende Rolle in der Regulation von Immunprozessen spielen. Zwischenzeitlich ist klar geworden, dass Zytokine darüber hinaus auch von Neuronen produziert werden können und wesentliche Regulatoren von neurotoxischen und neuroprotektiven Prozessen im Nervensystem sind. Damit sind Zytokine potente Moleküle, die prinzipiell eine enge Wechselwirkung zwischen Neuronen und Gliazellen herstellen und eine differenziell regulierte inflammatorische Reaktion des ZNS vermitteln können.

Interleukin-1 (IL-1) und Tumor Nekrose Faktor- α (TNF- α) sind zwei pro-inflammatorische Zytokine, deren pathogenetische Relevanz nach fokalen Hirnischämien eindrucksvoll belegt ist (Liu et al. 1994; Rothwell and Luheshi 1996; Wang et al. 1997; Boutin et al. 2001). Im Infarktgebiet induzieren IL-1 und TNF- α die Expression von induzierbarer NO-Synthase (iNOS), einem Effektormolekül mit erheblicher zytotoxischer Potenz. Die Ausschaltung der induzierbaren NO-Synthase (iNOS) reduziert nicht nach 24 Stunden, sondern erst verzögert nach 4 Tagen die Infarktgröße (Iadecola et al. 1997).

Dies unterstreicht, dass durch Modifikation inflammatorischer Kaskaden die Infarktgröße auch in einem subakuten Zeitfenster noch beeinflusst werden kann, in dem beim Menschen zur Zeit keine therapeutischen Möglichkeiten zur Verfügung stehen (Becker 2001).

Der menschliche Hirninfarkt ist ätiopathogenetisch heterogen. Emboligene Verschlüsse der Arteria cerebri media bzw. ihrer Äste wie auch Verschlüsse kleinerer hirneigener Gefäße gehören zu den häufigsten Ursachen. Die in der Ischämieforschung eingesetzten Tiermodelle können demzufolge immer nur Teilaspekte der Pathophysiologie menschlicher Schlaganfälle abbilden. Deswegen werden in den vorgelegten Arbeiten mehrere Schlaganfallmodelle parallel verwendet.

Bei der mikrochirurgischen permanenten Okklusion der distalen Arteria cerebri media (MCAO; Ref.1; Bederson et al. 1986) wird durch Verschluss der Arteria cerebri media mittels elektrischer Koagulation ein Infarkt erzeugt. Da die Koagulation distal des Abganges der Stammgangliengefäße vorgenommen wird, entsteht ein überwiegend kortikaler Infarkt. Die Ausdehnung des Infarktes wird durch die individuelle Anatomie der Kollateralisierung bestimmt (Duverger und MacKenzie 1988). Die ischämische Koagulationsnekrose ist von einem unterschiedlich ausgeprägtem Saum inkompletter Ischämie mit selektivem neuronalen Tod umgeben. Auch wenn das Infarktareal bereits nach vier Stunden makroskopisch erkennbar ist, zeigt der Infarkt ein typisches Fortschreiten der Pannekrose in den ersten 72 Stunden (Clark et al. 1993; Garcia et al. 1995). Damit sind hämodynamisch und von der Infarktausdehnung die Ähnlichkeiten zu einem Mediateilinfarkt beim Menschen offensichtlich.

Die Arteria cerebri media kann ebenso durch das intraluminale Vorschieben eines Fadens über die Arteria carotis interna in die Aufteilungsstelle von Arteria cerebri media und anterior erreicht werden (Ref.5, Zea Longa et al. 1989). Charakteristikum dieser Infarkte ist eine große Zone kritischer Ischämie, die durch Zurückziehen des Fadens mit konsekutiver Reperfusion

des Medialstromgebietes „gerettet“ werden kann (Memezawa et al. 1992). Anderenfalls kommt es mit zunehmender Ischämiezeit zur fortschreitenden Infarzierung zunächst subkortikaler (Corpus striatum), dann kortikaler Areale bis zum kompletten Mediainfarkt. Damit bestehen Parallelen zur klinischen Situation des (transienten) Mediahauptstammverschlusses. Eine besondere Situation entsteht, wenn nach besonders kurzer Ischämiezeit der Faden wieder entfernt wird: dann kommt es neben einem subkortikalen Infarkt nur zu kleinen, segmentalen Koagulationssnekrosen im Kortex, aber ausgedehnten Bezirken inkompletter Ischämie mit wochenlang fortschreitendem selektiven neuronalen Untergang („mild focal ischemia“; Ref.5, Endres et al. 1998).

Deutlich von den Mediaokklusionsmodellen zu unterscheiden ist der kortikale Infarkt durch fotothrombotische Okklusion kleinerer Gefäße (Fot thrombose; Ref.2,3,4; Watson et al. 1985; Domann et al. 1993). Die Aktivierung eines intravenös applizierten fotosensitiven Farbstoffs, Bengal Rosa, im Bereich eines durch die Kalotte applizierten Lichtkegels führt zu einer Endothelzerstörung und konsekutiver Thrombose kleinerer Gefäße (Dietrich et al. 1987). Es resultiert ein scharf begrenzter kegelförmiger Infarkt. Kleine, scharf vom Gesunden abgegrenzte Ischämien finden sich beim Menschen aufgrund einer zerebralen Mikroangiopathie. Wesentlicher Vorteil der Fot thrombose ist die Platzierung der Läsion an stereotaktisch definierter Position mit konstanter Geometrie und Größe, die eine sichere Differenzierung des ischämischen Fokus von Ferneffekten durch Faserbahndegeneration oder Spreading Depression zulässt. Damit eignet sich die Fot thrombose insbesondere auch für Untersuchungen von Ferneffekten zerebraler Ischämien (Ref. 2,3,7).

Im Tierexperiment ist nachweisbar, dass fokale zerebrale Ischämien zum Auftreten von bestimmten elektrophysiologischen Veränderungen führt, die als Perinfarktdepolarisationen bezeichnet werden (Iijima et al. 1992; Dietrich et al. 1994). Spreading Depression (Leao 1944) sind durch einen Anstieg der extrazellulären Kaliumkonzentration über einen bestimmten Schwellenwert (ca. 12 mmol/l) ausgelöste negative Schwankungen des epikortikal ableitbaren Gleichspannungspotenzials mit Unterdrückung der normalen hirnelektrischen Aktivität („Depression“), die sich wie eine Welle über den Kortex der ipsilateralen Hemisphäre ausbreiten („Spreading“), jedoch die Mittellinie nicht queren. Während die Spreading Depression Welle durch den Kaliumexzess ausgelöst wird, ist ihr Fortschreiten („Propagation“) an die Funktion des glutamatergen N-Methyl-D-Aspartat (NMDA)-Rezeptors gebunden und durch NMDA-Antagonisten, für die MK-801 der Prototyp darstellt, pharmakologisch blockierbar (Lauritzen and Hansen 1992). Damit kann der Effekt

von Spreading Depression funktionell durch pharmakologische NMDA-Rezeptorblockade untersucht werden (Ref. 2,3,6). Im intakten Kortex bleiben Spreading Depression ohne neuronalen Schaden, können i.S. eines Präkonditionierungsreizes jedoch Zytoprotektion bei darauffolgenden ischämischen Insulten vermitteln (Kobayashi et al. 1995; Yanamoto et al. 1998). Treten dagegen Spreading Depression im Rahmen fokaler Ischämien auf („ischämische Depolarisationen“) können sie den resultierenden Infarkt vergrößern (Mies et al. 1993).

Kaliumchlorid (KCl)-induzierte Spreading Depression können morphologisch sowohl eine mikrogliale als auch astrogliale Aktivierung hervorrufen (Kraig et al. 1991; Caggiano und Kraig 1996; eigene unveröffentlichte Daten, s. Abb. 3).

In den vorgelegten Arbeiten wird die gliale Aktivierung und Induktion pro-inflammatorischer Zytokine durch Spreading Depression beschrieben (Ref. 2,3,6). Aus funktioneller Sicht ist die Untersuchung dieses Paradigmas deshalb bedeutsam, als es belegen kann, dass gliale Aktivierung und inflammatorische Reaktionen nicht zwangsläufig Zytotoxizität vermitteln, sondern durchaus positiv in Prozesse funktioneller Plastizität und Gewebereorganisation nach Schlaganfall eingreifen können.

Zur Evaluierung möglicher neuer therapeutischer Möglichkeiten sollte idealerweise eine nicht-invasive Untersuchungsmethode zur Visualisierung postischämischer inflammatorischer Reaktionen zur Verfügung stehen. Die MRT hat in der Situation des akuten menschlichen Schlaganfalls einen erheblichen Wissenszuwachs über die Pathophysiologie fokaler Ischämien erbracht. Hierzu gehört die Diffusionsgewichtete Bildgebung (DWI), die ohne Zeitverzögerung ein ischämisches Areal sichtbar macht, konventionelle T2- und T1-Wichtungen, die mit hoher Auflösung Informationen über die Morphologie des Gehirns detailgenau abbilden und die Perfusions-gewichteten Bilder, die Aufschluß über die lokale (Mangel-) Durchblutung des Gehirns geben. Bislang ist unklar, inwieweit diese Sequenzen in der Lage sind, die stadienspezifischen Gewebsveränderungen nach fokaler Hirnischämie, insbesondere im Hinblick auf inflammatorische Prozesse, abzubilden.

Basierend auf diesen Grundlagen wurden folgende Fragestellungen untersucht:

1. In welchen räumlichen und zeitlichen Mustern kommt es nach fokaler Ischämie zu einer Aktivierung von Mikroglia und Astroglia?
2. Können Subpopulationen der aktivierten Glia anhand des morphologischen und immunzytochemischen Phänotyps differenziert werden?
3. Wie unterscheiden sich Mikroglia- und Astroglia-subpopulationen im Hinblick auf den zugrundeliegenden Schädigungsmechanismus („Targeting“ glialer Aktivierung)?
4. Welche Wechselbeziehungen sind zwischen elektrophysiologischen Veränderungen, Mikroglia und Astroglia zu erkennen?
5. Welche Aspekte postischämischer Inflammation können nicht-invasiv in der multimodalen Kernspintomografie sichtbar gemacht werden?
6. Welche funktionellen Schlüsse hinsichtlich einer möglichen zytotoxischen oder zytoprotektiven Wirkung glialer Aktivierung sind möglich?

2. Zusammenfassende Darstellung der Ergebnisse und Diskussion

Die Zusammenschau der Ergebnisse der vorgelegten Arbeiten lässt eine Frühphase des Infarktes, die etwa die ersten 48-72 Stunden nach Ischämiebeginn umfasst, von einer Infarktspätphase, etwa 72 Stunden bis 7 Tage nach Infarktinduktion, abgrenzen. Schließlich sind chronische Veränderungen im Infarktgebiet zu analysieren, die ab der zweiten Postinfarktwoche auftreten. Im weiteren wird auf die Begriffe Infarktfrühphase und Spätphase Bezug genommen. Tabelle 1 stellt Charakteristika beider Phasen gegenüber, die im Folgendem erläutert werden.

	Frühphase	Spätphase	Referenz
Histologisches Bild	Infarktentstehung	Infarktmarkierung vom Gesunden	(Clark et al. 1993), Ref. 4, 5
Herkunft der Phagozyten	Mikroglia	Überwiegend hämatogene Makrophagen	(Schroeter et al. 1997), Ref. 5
Mikrogliale Aktivierungsmarker	Morphologische Transition; ED1 (beginnend)	ED1, MHC I+II, CD4, CD8	Ref.1,3,5
Typische proinflammatorische Zytokine	IL-1 β , TNF- α ;	IL-18	(Jander et al. 2000); Ref. 6,7
Expression von ICAM-1 (Adhäsion, Migration)	Endothel	Infiltratzellen	Ref. 1
Astrozyten	Verlust GFAP+ Zellen im Infarkt	GFAP+, +/-Vimentin Astrogliose	Ref. 2,5
Multimodales MRT	ADC-Absenkung, T2-Werterhöhung	ADC-Wert normal, T2-Werterhöhung rückläufig	Ref. 4
Neuronale Stimuli exofokaler glialer Aktivierung	Spreading Depression	Degeneration von Faserbahnen und subkortikalen Kerngebieten	Ref. 2,3,6,7

2.1. Infarktfrühphase

Mikrogliaaktivierung in der Infarktfrühphase

In der Infarktfrühphase waren drei unterschiedlichen Muster mikroglialer Aktivierung abgrenzbar: (1.) eine rasch erfolgende und komplette Mikrogliaaktivierung in der Zone der ischämischen Pannekrose mit Transformation zu phagozytierenden Zellen (Ref.1,3); (2.) eine parallel zum verzögerten neuronalen Untergang auftretende inkomplett bleibende Mikrogliaaktivierung in Arealen selektiven neuronalen Zelltodes (Ref.5) und (3.) eine durch

Spreading Depression induzierte, inkomplette und transiente, aber räumlich ausgedehnte Aktivierung, sichtbar als hyperramifizierte Zellen (Ref.3).

Nach Fotothrombose kommt es prompt und einzeitig zu einer Infarzierung, die eine ebenso rasche Mikrogliaaktivierung nach sich zieht. Der Infarktkern bleibt relativ lange zellarm. Bereits 4 Stunden nach Fotothrombose waren erste Zeichen der Mikrogliaaktivierung im Infarktrand der fotothrombotischen Läsion fassbar (Ref.3). Nach 24 Stunden exprimierte amöboide Mikroglia im Infarktrand ED1 als Marker phagozytischer Aktivität (Ref.3).

Nach distaler MCAO kommt es ebenfalls zu einer raschen Mikrogliaaktivierung. Allerdings ist schon früh der gesamte Infarkt diffus von Phagozyten durchsetzt. Eine Ursache hierfür mag die Infiltration des Infarktes aus oberflächlichen (pialen) Gefäßen sein, die im Gegensatz zur Fotothrombose nicht in die Läsion mit einbezogen sind (Ref.1).

Nach transientser MCAO entwickelt sich der Infarkt über die ersten 72 Stunden mit früher Infarzierung subkortikaler Areale und verzögerter Entwicklung einer kortikalen Läsion. Dabei spiegelt die Dynamik der Mikrogliaaktivierung die fortschreitende Infarktentstehung entlang dieser subkortikal-kortikalen Achse wieder. Letztere Ergebnisse wurden in ähnlicher Weise durch eine andere Arbeitsgruppe berichtet (Ref. 5, Lehrmann et al. 1997).

Damit zeichnet die Mikrogliaaktivierung im ischämischen Fokus ohne wesentliche Zeitverzögerung den entstehenden histologischen Schaden nach. Im Infarktrand kommt es in allen drei Modellen zunehmend zu einer kontinuierlich fortschreitenden Mikrogliaaktivierung. Morphologisch war eine gradientenförmige Zunahme der Mikrogliaaktivierung zum Infarktrand hin darstellbar, die wir auch in charakteristischer Weise in Autopsiepräparaten menschlicher Schlaganfälle wiedergefunden haben (Stoll et al. 2001). Am Modell der Photothrombose konnten wir zeigen, dass die Phagozyten der frühen Infarktphase sich aus Mikroglia rekrutiert, während hämatogene Makrophagen erst mit einer Verzögerung von Tagen einwandern (Schroeter et al. 1997). Damit konnten wir zeigen, dass die Akkumulation von Phagozyten im Infarktareal nicht auf einem passiven Influx leukozytärer Zellen beruht (Hallenbeck et al. 1986; Garcia et al. 1994), sondern durch einen aktiven Prozess hirneigener Zellen initiiert wird.

Untersuchungen am Fotothrombosedmodell haben darüber hinaus gezeigt, dass die Mikrogliaaktivierung nicht auf die Infarktregion beschränkt war. Vielmehr kam es zur einer diffusen Mikrogliaaktivierung im Kortex der gesamten ipsilateral zur Läsion gelegenen Hemisphäre (Ref.3). Im Gegensatz zur amöboiden Mikroglia des Infarktrandes zeigte diese

Mikroglia eine hyperramifizierte Morphologie, typisch für ein transientes Aktivierungsstadium (Streit et al. 1999). Durch Gabe des nicht kompetitiven NMDA Antagonisten MK-801 konnte das Auftreten der Mikrogliaaktivierung im ipsilateralen Kortex zumindest partiell geblockt werden, so dass funktionell der Zusammenhang mit Spreading Depression hergestellt werden konnte. In diesen Arealen bildete sich die Mikrogliaaktivierung später wieder zurück.

Nach Faden-MCAO war in Arealen selektiven neuronalen Untergangs (Ref.5) parallel zur Manifestierung des Nervenzellverlustes eine Mikrogliaaktivierung zu sehen. Hier fand sich überwiegend sternförmige Mikroglia, amöboide Zellen wie im Randbereich der ischämischen Pannekrose waren selten zu sehen.

Die unterschiedlichen Infarktmodelle führen zu typischen Infarktmustern mit kongruenter Mikrogliaaktivierung. Im Gegensatz zu den Schlußfolgerungen anderer Arbeitsgruppen ist in den vorgelegten Arbeiten ein Einfluss der Reperfusion *per se* auf die gliale Aktivierung und inflammatorische Reaktionen nicht zu erkennen. Die Mikrogliaaktivierung erscheint nicht dadurch determiniert, *wie* der ischämische Schaden eintritt, sondern durch Ausdehnung und Grad der ischämischen Zellschädigung bestimmt (Ref. 1,3,5, Clark et al. 1994).

Die funktionelle Bedeutung der Mikrogliaaktivierung wird durch Arbeiten belegt, bei denen eine funktionelle Blockade der Mikrogliaaktivierung mittels Minocyclin durchgeführt wurde. Hierdurch konnte eine Reduktion der Infarktgröße erreicht werden, die Expression proinflammatorischer Zytokine wurde reduziert (Yrjanheikki et al. 1999; Tikka et al. 2001). Die vorgelegten Arbeiten zeigen jedoch, dass Mikrogliaaktivierung nach fokaler Ischämie nicht ein uniformer Prozess ist, sondern räumlich und zeitlich differenziert und heterogen ist, so dass funktionell ein Nebeneinander protektiver als auch sekundär toxischer Effekte mikroglialer Aktivierung wahrscheinlich ist.

Astrogliaaktivierung

4 Stunden nach Fotothrombose war im Infarktareal eine Auslöschung der GFAP+ Strukturen zu sehen. Die Aktivierung von Astrozyten im den Infarkt umgebenden Gewebe wurde mit einer beachtlichen Verzögerung von 48-72 Stunden in der GFAP-Immunzytochemie nachweisbar (Ref.2). Die mikrogliale Aktivierung ging der astroglialen Aktivierung somit eindeutig voraus (Ref.2, 5).

Zytokine der frühen Infarktphase

Zytokine haben eine grundlegende Funktion als Botenstoffe zwischen Zellen des Immunsystems, indem sie Immunantworten auslösen (Arai et al. 1990; Arvin et al. 1996).

Bekannt war, dass bestimmte Zytokine nach fokaler Ischämie exprimiert werden. So konnte nachgewiesen werden, dass IL-1 β mRNA früh in den ersten Stunden nach fokaler Ischämie exprimiert wird (Liu et al. 1993; Buttini et al. 1994; Arvin et al. 1996; Boutin et al. 2001). Die funktionelle Bedeutung dieser Befunde wurde dadurch unterstrichen, dass die pharmakologische Blockade pro-inflammatorischer Zytokine zu einer Verkleinerung der Infarktgröße führen konnte, allerdings ohne dass Klarheit über die zelluläre Quelle oder den detaillierten pathophysiologischen Mechanismus bestand (Relton und Rothwell 1992; Nawashiro et al. 1997; Stroemer und Rothwell 1998; Pearson et al. 1999; Boutin et al. 2001). Wir hatten nachweisen können, dass IL-1 β und TNF- α nicht nur im ischämischen Fokus, sondern im gesamten ipsilateralen Kortex nach Fotothrombose auftritt (Jander et al. 2000).

Da diese exofokale Zytokinexpression durch MK-801 blockierbar war, konnte eine Abhängigkeit von Spreading Depression vermutet werden (Jander et al. 2000). In den vorgelegten Arbeiten konnten wir beweisen, dass KCl-induzierte Spreading Depression tatsächlich eine Expression von IL-1 β und TNF- α mRNA auslöste, die in Analogie zum Fotothrombosemodell den gesamten ipsilateralen Kortex erfasste (Ref.6). Auch diese Zytokininduktion war durch MK-801 blockierbar, so dass ein enger funktioneller Zusammenhang zwischen Ischämie induzierten Spreading Depression und dem Auftreten der raschen Induktion der pro-inflammatorischen Zytokine IL-1 β und TNF- α im ipsilateralen exofokalen Kortex hergestellt werden konnte (Ref.6; Jander et al. 2000).

Wir haben versucht, die zelluläre Lokalisation dieser Zytokinexpression nachzuweisen. Es gelang, das IL-1 β Protein auf Mikrogliazellen zu lokalisieren (Ref.7). Diese mikrogliale IL-1 β Protein Expression fand sich sowohl nach Fotothrombose als auch nach KCl-induzierter Spreading Depression und war in beiden Paradigmen mit ähnlicher Dynamik nach 8 Stunden maximal nachweisbar (Ref. 6, 7).

Zumindest *in vitro* besteht Anhalt dafür, dass die Ischämie selbst direkt die IL-1 β Produktion induzieren kann, die sich durch einen autokrinen Mechanismus selbstverstärken könnte (Shreenivas et al. 1992). IL-1 ist ein potenter Induktor einer Astrozytose (Giulian et al. 1988; Ban et al. 1993), so dass ein induzierender oder verstärkender Effekt auf die verzögert nachweisbare astrogliale Aktivierung wahrscheinlich ist.

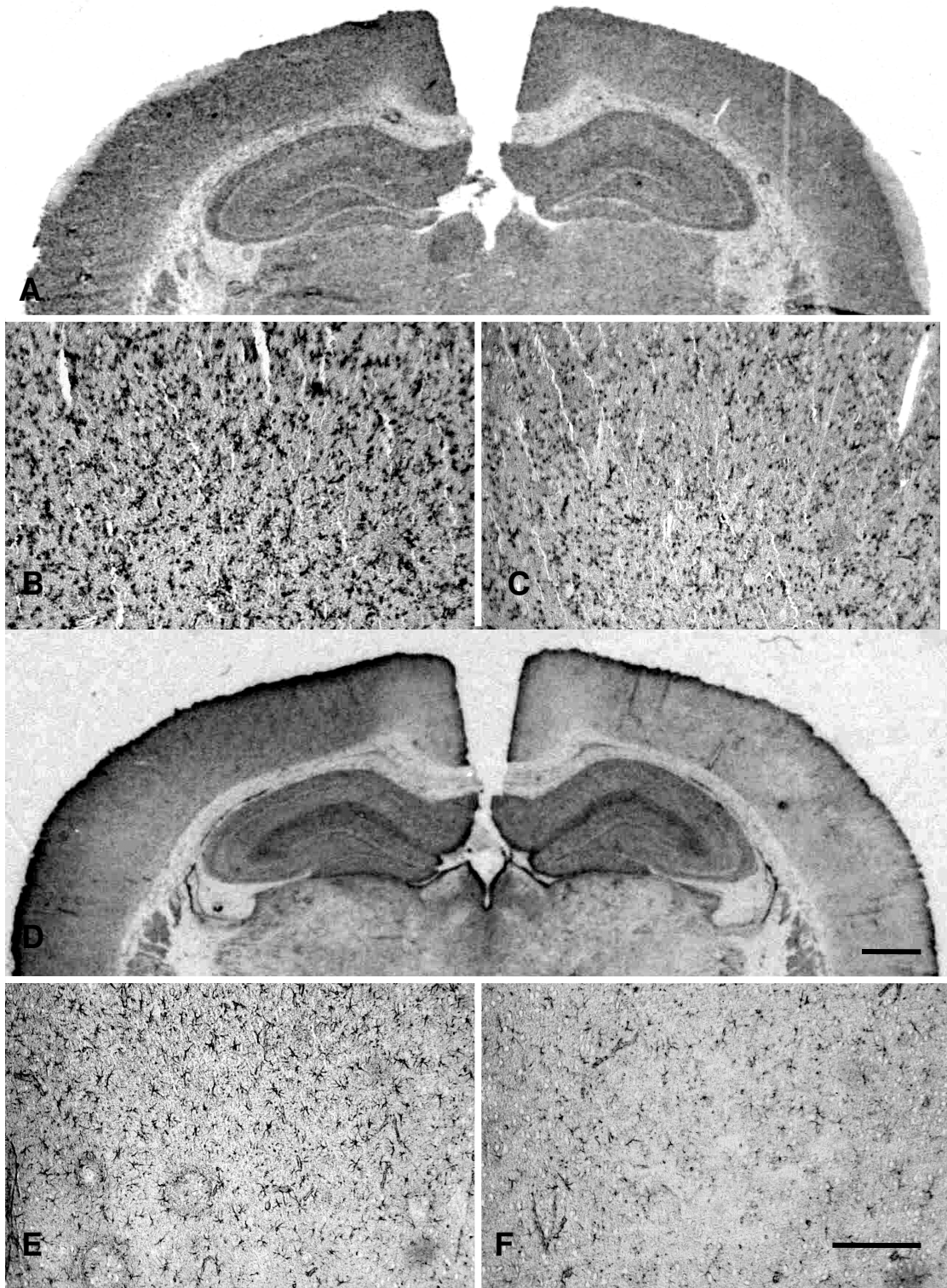


Abb.3: Gliale Aktivierung 3 Tage nach Spreading Depression. Applikation von 3M KCl führt zur mikroglialer (Ox42; A) und astroglialer (GFAP, D) Aktivierung des gesamten ipsilateralen Kortex (links im Bild). Detailvergrößerungen (B bzw.E) zeigen gegenüber Kortex nach ipsilateraler NaCl-Applikation (Sham-Operation; C bzw.F) Ox42+ hyperramifizierter Mikroglia mit erhöhter Dichte (B gegenüber C) und Astrozyten mit vermehrter GFAP Immunoreaktivität (E gegenüber F). Maßstab in D: 1mm in A, D; Maßstab in F: 150µm in B, C, E, F.

Parallel zur IL-1 Expression kommt es zu einer frühen transienten Expression von TNF- α mRNA in den ersten Stunden nach fokaler Ischämie (Liu et al. 1994; Arvin et al. 1996; Jander et al. 2000). Ähnlich wie für IL-1 β stimmten Kinetik und Ausmaß der TNF- α mRNA Expression weitgehend im exofokalen ipsilateralen Kortex nach Fotothrombose und nach KCl-induzierten Spreading Depression überein; beide konnten durch MK-801 pharmakologisch blockiert werden (Ref.5; Jander et al. 2000). Allerdings gelang uns im Gegensatz zu IL-1 β nicht, immunzytochemisch eine reproduzierbare zelluläre Lokalisation des TNF- α Proteins darzustellen. Dieses ist wahrscheinlich technisch bedingt. Hierzu sind auch die Literaturangaben bemerkenswert widersprüchlich (Buttini et al. 1996; Botchkina et al. 1997; Gong et al. 1998).

Nach Fotothrombose und MCAO war die IL-1 β und TNF- α Induktion im Läsionsbereich stärker und länger andauernd als durch Spreading Depression (Ref.6,7). Während es im Läsionsbereich zu einer Expression von iNOS kam, war in Arealen mit Spreading Depression zu keiner Zeit eine iNOS Expression zu detektieren. Dies erscheint aufgrund der Belege für eine iNOS vermittelte sekundäre Zytotoxizität besonders relevant (Ref.7; Iadecola 1997).

Insgesamt konnte mit den vorgelegten Arbeiten ein enger funktioneller Zusammenhang zwischen dem Auftreten von Spreading Depression, der Induktion der proinflammatorischen Zytokine IL-1 β und TNF- α mit direktem Nachweis von IL-1 β Protein in Mikrogliazellen sowie dem Auftreten einer typischen transienten Mikroglia- und Astrogliaaktivierung hergestellt werden (Ref.2,3,6,7; Abb.3). Die Induktion von Zytokinen durch ein elektrophysiologisches Phänomen zeigt einen neuen Mechanismus neural-immuner Wechselwirkung auf. Sie stellt zugleich einen Mechanismus dar, wie Spreading Depression Ischämietoleranz vermitteln könnten. Diese Ergebnisse legen nahe, dass die Aktivierung von Mikroglia und Astroglia sowie die Induktion von proinflammatorischen Zytokinen durchaus positive (protektive) Effekte nach fokaler Ischämie vermitteln könnte.

2.2. Spätphase des Infarktes

Differentielle Mikrogliaaktivierung und Rekrutierung hämatogener Makrophagen

Die frühe Mikrogliaaktivierung und deren Transformation in Phagozyten wird mit zunehmendem Infarkalter von einer Infiltration hämatogener Makrophagen überlagert. Unter maximaler Aktivierung sind residente Mikroglia und hämatogene Makrophagen morphologisch und immunzytochemisch nicht mehr unterscheidbar (Flaris et al. 1993). Wir hatten in einer Depletionsstudie nachgewiesen, dass in der frühen Infarktphase die ED1+

Ox42+ Zellen ganz überwiegend mikroglialer Herkunft waren. Dagegen infiltrierten in der späten Infarktphase, d.h. ab dem 3. Tag nach Fotothrombose, hämatogene Makrophagen den Infarkt (Schroeter et al. 1997; Ref.5). Am Ende der ersten Postinfarktwoche überwogen hämatogene Makrophagen zahlenmäßig Zellen mikroglialer Herkunft.

Der Antikörper ED1 markiert Phagolysosomen als Marker phagozytischer Aktivität (Damoiseaux et al. 1994). ED1+ Phagozyten infiltrierten in zunehmendem Maß den Infarkt vom Rand zum Zentrum hin. Dabei entstand zum Ende der ersten Postinfarktwoche ein charakteristisches Bild eines wallartig dicht infiltrierten Infarktrandbereichs, der einen zellärmeren Infarktkern umgab (Ref.1,3,4; Jander et al. 1995). 14 Tage nach Infarktinduktion war das Infarktareal flächig von Phagozyten infiltriert. Die weitere phänotypische Charakterisierung der Mikroglia-/Makrophagenantwort ergab überraschenderweise eine Differenzierung in CD4+ und CD8+ Mikroglia/Makrophagen.

Erstmals konnten wir diese Population nicht-lymphozytärer CD8+ Zellen nach fokaler Ischämie beschreiben, die 48-72 Stunden nach Infarktinduktion nachweisbar waren, an Tag 7 ihr Maximum erreichten, dann rasch abnahmen und nach 14 Tagen nur noch vereinzelt nachweisbar waren (Ref.1). Da Lymphozyten und natürliche Killerzellen (NK-Zellen) die einzigen bei der Ratte bekannten CD8+ Zellpopulationen waren, war zunächst davon auszugehen, dass es sich bei diesen CD3 und CD5 negativen Zellen um NK-Zellen handelt (Ref.1). Weitere Untersuchungen mit Doppelimmunfluoreszenz und konfokaler Lasermikroskopie konnten jedoch diese Zellen als eine Subpopulation ED1+ Phagozyten identifizieren (Jander et al. 1998). CD8+ Mikroglia/Makrophagen fanden sich bei allen drei verwendeten Modellen fokaler zerebraler Ischämie ausschließlich im Bereich der ischämischen Pannekrose (Ref.1,4,5). Dagegen waren Areale mit inkompletter ischämischer Schädigung und verzögertem selektiven neuronalen Zelltod ohne CD8+ Phagozyten, ebenso wie Bereiche mit sekundärer Faserbahn- oder Kerndegeneration (Ref.3,5). Für das Auftreten der CD8+ Makrophagen war also entscheidend, *ob* eine ischämische Pannekrose auftritt, nicht jedoch, *wie* diese in den unterschiedlichen Infarktmodellen induziert wird oder ob eine Reperfusion des Infarktgebietes auftritt (Ref. 1,3,5, (Barone et al. 1992; Clark, Lee, White, Jonal, Feuerstein, and Barone 1994).

In ähnlicher Weise konnten nach Quetschläsion des optischen Nerven CD8+ Zellen nur an der nekrotischen Quetschstelle nachgewiesen werden, während die Mikrogliaaktivierung durch Faserdegeneration im Verlauf des optischen Nerven ohne CD8+ Zellen blieb (Jander et al. 2001). Im Modell der durch Immunisierung mit Myelin Basischem Protein induzierten

Experimentell Allergischen Encephalomyelitis, die sich durch eine perivaskuläre Entzündung ohne relevante Gewebsdestruktion auszeichnet, fehlten CD8+ Makrophagen ebenfalls weitgehend (Jander et al. 1998). Hieraus ergab sich, dass die Rekrutierung CD8+ Makrophagen spezifisch an Orte einer nekrotischen Gewebeschädigung erfolgt.

Zur weiteren Charakterisierung der Herkunft CD8+ Phagozyten wendeten wir zwei Depletionsparadigmen an. Mit einem systemisch injizierten Biphosphonat ist es möglich, systemisch zirkulierende Makrophagen zu depletieren, während die Population (hirneigener) Mikroglia unbeeinflusst bleibt (vanRooijen 1989). Die Depletion hämatogener Makrophagen reduzierte deutlich das CD8+ Infiltrat in der späten Postinfarktphase im Vergleich zu einer Kontrollgruppe, verhinderte es aber nicht vollständig, so dass von einem überwiegenden, nicht jedoch ausschließlichen hämatogenen Ursprung der CD8+ Zellen auszugehen war (Ref.5). Einzelne CD8+ Zellen zeigten auch mikrogliale Morphologie (sternförmige Zellen), so dass auch residente Mikroglia zur Population der CD8+ Phagozyten beiträgt (Ref.5). Als zweites Depletionsparadigma wendeten wir die systemische Gabe eines depletierenden anti-CD8-Antikörpers an (Holmdahl et al. 1985). Die systemische Depletion CD8+ Zellen konnte im Gegensatz zur Depletion hämatogener Makrophagen das CD8+ Infiltrat nicht verändern (Ref.5). Dies legt nahe, dass die infiltrierenden Phagozyten erst sekundär nach Infiltration in das nekrotische Areal CD8 hochregulieren.

Unabhängig von den hier vorgestellten Ergebnissen isolierten Hirji und Kollegen unter bestimmten pathologischen Bedingungen CD8+ Peritoneal- und Alveolarmakrophagen. Sie wiesen nach, dass die CD8 Expression mit einer NO-Produktion gekoppelt ist (Hirji et al. 1997; Hirji et al. 1998). Dagegen stimmen nach fokaler Ischämie iNOS Expression und das Auftreten der CD8+ Mikroglia/Makrophagen in ihrer Dynamik nicht überein (Ref.1,3; Jander et al. 2000).

Auffällig war, dass sich unmittelbar angrenzend zum CD8+ Infiltrat des Infarktes in allen drei Ischämieparadigmen eine anisomorphe Astroglieose ausbildete, während sich in Bereichen ohne CD8+ Mikroglia/Makrophagen stets eine isomorphe Gliose fand. Diese enge räumlich-zeitliche Beziehung zwischen CD8+ Phagozyten und anisomorpher Gliose fand sich auch nach mechanischer Hirnläsion (intrakortikaler Implantation einer Stahlnadel) sowie nach Quetschläsion des Optischen Nerven, so dass unabhängig vom Schädigungsmechanismus ein enger funktioneller Zusammenhang zwischen dieser Makrophagensubpopulation und der Ausbildung einer anisomorphen Gliose zu vermuten ist (Schroeter et al., Manuskript in Vorbereitung).

Schon länger war bekannt, dass aktivierte Mikroglia und Makrophagen der Ratte CD4 exprimieren (Perry und Gordon 1987). Bis zum 14. Tag war ein zunehmender Anteil von Mikroglia/Makrophagen CD4+. Mit Doppelimmunfluoreszenzuntersuchungen hatten wir nachgewiesen, dass CD4+ und CD8+ nicht überlappende Mikroglia-/Makrophagensubpopulationen darstellen (Jander et al. 1998). *In vitro* konnte nachgewiesen werden, dass durch IL-1 α und TNF- α die Expression von CD4 auf Mikroglia induziert wird (Yu et al. 1998).

Die Expression von MHC Klasse I- und II-Antigenen zeigt einen immunologisch aktivierten Zustand der Mikroglia an. Das MHC Klasse I Molekül wurde diffus auf verschiedenen Zellpopulationen im Infarkt nachgewiesen. Dagegen war die Expression von MHC Klasse II weniger abundant. Während konstitutiv MHC Klasse II Expression nur auf perivaskulärer Mikroglia zu finden war (Ref.3), war es auf aktivierter Mikroglia in der späten Postinfarktphase maximal exprimiert und nahm dann im Infarkt, im Gegensatz zu sekundär degenerierenden Arealen, bereits an Tag 14 wieder ab (Ref.3).

Die Rekrutierung hämatogener Leukozyten in pathologisch veränderte Gewebe wird über einen prinzipiell gut erforschten dreischrittigen Mechanismus vermittelt. Leukozyten separieren aus der Strombahn und rollen an der Gefäßwand entlang („rolling“). Dieser erste Schritt wird überwiegend durch Selektine vermittelt. In einem zweiten Schritt kommt es zur festen Adhäsion von Leukozyten an die Gefäßwand. Dieses wird durch Adhäsionsmoleküle vermittelt. In einem dritten Schritt kommt es zur transendothelialen Migration der Leukozyten (Springer 1994). ICAM-1 ist Prototyp der Adhäsionsmoleküle und vermittelt überwiegend die Adhäsion von Makrophagen. Funktioneller Rezeptor für ICAM-1 ist das „leukocyte functioning antigen-1“, LFA-1. Wir haben die Expression von ICAM-1 und LFA-1 nach MCAO untersucht (Ref.1). Während in der Infarktfrühphase sich die Expression von ICAM-1 auf Endothelien fand, war in der Infarktspätphase ab dem dritten Tag nach Ischämieinduktion die Expression von ICAM-1 auf intraparenchymal lokalisierten Zellen zu beobachten (Ref.1). Parallel hierzu kam es zu einer Aufregulation von LFA-1 (Ref.1). Dieser Befund hat zusätzlich an Bedeutung gewonnen, nachdem wir nachweisen konnten, dass IL-18 das die Infarktspätphase dominierende Zytokin ist (Ref.7, s.u.). Ausgehend von Befunden, die eine IL-18 vermittelte ICAM-1 Expression beschreiben (Kitching et al. 2000) ist damit die Makrophagenakkumulation im Infarkt als ein sich selbst verstärkender Mechanismus aufzufassen: akkumulierende Mikroglia und Makrophagen exprimieren IL-18, das eine

vermehrte ICAM-1 Expression induziert. Dies vermittelt eine weitere Akkumulation von Makrophagen aus dem Blut.

Astroglie

Das gliale fibrilläre saure Glykoprotein (GFAP) ist ein für Astrozyten spezifischer Zytoskelettbaustein, der immunzytochemisch detektiert werden kann (Bignami und Dahl 1974). Es wird konstitutiv in ruhenden Astrozyten exprimiert. Unter Aktivierung nimmt die GFAP-Immunreaktivität zu. Vimentin ist ein weiterer immunzytochemischer Marker (Schiffer et al. 1986). Er wird von prämaternen und proliferierenden Astrozyten exprimiert, ist jedoch im normalen adulten Gehirn nicht auf Astrozyten zu finden. Bei der Darstellung aktivierter Astrozyten gilt Vimentin als relativ spezifischer Marker einer anisomorphen Glie (Ridet et al. 1997).

Nach 48-72 Stunden konnte im MCAO- und Fotothrombosemodell eine vermehrte Expression von GFAP in den Infarkt umgebenden (perifokalen) Astrozyten beobachtet werden, die mit der Koexpression von Vimentin Charakteristika einer anisomorphen Glie zeigte (Ref.2,5). Diese Astrozytenaktivierung nahm in den folgenden Tagen kontinuierlich zu. Im Infarktgebiet selbst war es dagegen früh zu einem Verlust GFAP+ Zellen gekommen.

Dagegen blieben in Arealen inkompletter ischämischer Gewebsschädigung mit selektivem neuronalen Verlust stets GFAP+ Zellen nachweisbar. In der Infarktspätphase kam es hier zu einer fortschreitenden Astrozytenaktivierung, jedoch blieb in histologischen Färbungen die neokortikale Struktur erkennbar, so dass es sich um eine isomorphe Glie handelte. Im Einklang hiermit wurde kein Vimentin exprimiert.

Im gesamten nichtischämischen ipsilateralen Kortex kam es darüber hinaus zu einer geringgradigen Zunahme der GFAP-Immunreaktivität (Ref.2), die transient an Tag 3 nachweisbar war, an Tag 7 nach Fotothrombose jedoch bereits rückläufig war. Durch MK-801 war diese exofokale Astrozytenaktivierung pharmakologisch blockierbar und stand damit im engen funktionellen Zusammenhang mit dem Auftreten von Spreading Depression (Ref.2). Damit konnten wir drei astrozytäre Reaktionsmuster differenzieren: (1.) eine Astrozytenaktivierung um die ischämische Pannekrose herum, die in eine anisomorphe Glie mündete; (2.) eine isomorphe Glie im Bereich des selektiven neuronalen Untergangs und (3.) eine reversible transiente Astrozytenaktivierung durch Spreading Depression. Die anisomorphe Glie grenzt den ischämischen Fokus vom Gesunden ab („encasement“), wie dieses auch nach inflammatorischen ZNS-Läsionen beschrieben ist (Matsumoto et al. 1992).

Der dichte Astrozytensaum ist eine mechanische Barriere für das Aussprossen von Axonen. Andererseits können aktivierte Astrozyten ein permissives Substrat für axonales Wachstum darstellen und damit zur Regeneration beitragen. Allerdings ist hierfür ein gewisses Ausmaß astrozytärer Aktivierung erforderlich, das möglicherweise im Bereich anisomorpher Gliose, wahrscheinlich nicht jedoch im Bereich isomorpher Gliose erreicht wird (Mansour et al. 2002). Interessant ist, dass GFAP, nicht jedoch Vimentin zur Non-Permissivität für axonales Wachstum in Neuronen-Astrozyten Kokulturen beiträgt (Menet et al. 2001).

Zytokinmediatoren

Wie oben dargestellt kommt es nach fokaler zerebraler Ischämie zu einer schnellen Expression, aber auch wieder Herunterregulation der proinflammatorischen Zytokine TNF- α und IL-1. Bei der weiteren Analyse der mit der Spätphase assoziierten Zytokine konnten wir eine inflammationsassoziierte Induktion des proinflammatorischen Zytokins IL-18 nachweisen.

Interleukin-18 (IL-18) ist ein noch relativ neues Mitglied der IL-1-Zytokinfamilie. Es ist in der Lage, die Expression von IFN- γ zu induzieren (alte Bezeichnung: Interferon- γ inducing factor) und kann eine T-Zell-vermittelte Immunantwort modifizieren (Kohno und Kurimoto 1998). Damit ist IL-18 als Bindeglied zwischen sog. unspezifischer („innate“) Immunität und spezifischen T-Zell vermittelten Immunantworten zu sehen. Schließlich kann es unabhängig von IFN- γ die Expression von Zelladhäsionsmolekülen, z.B. ICAM-1, fördern (vgl. Ref.1; (Kohka et al. 1998; Kitching et al. 2000)).

Iba1 ist ein für aktivierte Mikroglia und Phagozyten tyisches Calcium bindendes Protein, das nach fokaler Ischämie in der Zone aktivierter Mikroglia und von Phagozyten immunzytochemisch nachweisbar ist (Ito et al. 2001). Es konnte gezeigt werden, dass in IL-18 Gen-defizienten Mäusen der Mikroglia-Aktivierungsprozess unterbrochen und das Iba1 vermindert nachweisbar ist (Mori et al. 2001). IL-18 stimuliert damit die Aktivierung von Mikroglia und die Differenzierung in phagozytierende Zellen. Damit ist IL-18 ein guter Kandidat für die Regulation der Phagozytenakkumulation in der Infarktspätphase.

Ähnlich wie IL-1 wird IL-18 als inaktiver Vorläufer sezerniert, der durch Caspase-1 enzymatisch in die aktive Form überführt wird (Ghayur et al. 1997). Die Expression von IL-18 und Caspase-1 nach Fotothrombose und MCAO wurde mittels semiquantitativer und quantitativer PCR und Immunzytochemie gegen IL-18 untersucht (Ref.7). Während die IL-1 mRNA Expression bereits 4 Stunden nach Ischämie ihr Maximum erreichte, war IL-18

mRNA nach 6 Tagen maximal exprimiert. Parallel zur IL-18 mRNA Expression kam es zum einem Anstieg der Caspase-1 mRNA. Obwohl Caspase-1 auch das bioaktivierende Enzym für IL-1 ist, war kein Anstieg der Caspase-1 zu einem frühen Zeitpunkt zu detektieren. Damit korrelierte die Caspase-1 mRNA Expression zu IL-18, nicht jedoch zur IL-1 mRNA Expression (Ref.7). Im Gegensatz zur IL-1 und TNF- α mRNA, die infolge von Spreading Depression in der Frühphase im gesamten ipsilateralen Kortex vermehrt nachzuweisen waren (Ref.6), war die IL-18 mRNA Expression auf den ischämischen Fokus beschränkt. Immunzytochemisch konnte das IL-18 in der aktivierten Mikroglia der unmittelbaren Infarktumgebung, mehr jedoch in den Phagozyten des Infarktes nachgewiesen werden. Mittels Doppelimmunfluoreszenz konnte eine extensive Kollokalisierung von ED1 und IL-18 Protein bewiesen werden (Ref.7).

IL-18 ist damit wahrscheinlich Mediator der massiven Phagozyteninfiltration des Infarktes in der Spätphase nach Ischämie. Wir haben neben der mRNA Expression das Auftreten des Proteins in Mikroglia/Makrophagen der Infarktumgebung und insbesondere Phagozyten des Infarktes nachweisen können. Die parallele Expression der mRNA für das bioaktivierende Enzym, Caspase-1, unterstreicht die biologische Relevanz des Befundes. Neben dem Transforming Growth Factor- β (TGF- β) ist IL-18 das einzige bisher beschriebene Zytokin, dessen Auftreten zeitlich in die späte Infarktphase fällt. Wie bereits erwähnt, kann IL-18 über eine Induktion von ICAM-1 zur Attraktion hämatogener Makrophagen führen (Kohka et al. 1998; Kitching et al. 2000). Nach MCAO und Fotothrombose korrelierte das räumlich-zeitliche Muster der ICAM-1 Expression auf parenchymal lokalisierten Phagozyten mit der Kinetik der IL-18 Expression. Dies unterstreicht den engen funktionellen Zusammenhang zwischen IL-18 und ICAM-1 Expression und der fortschreitenden Phagozytenakkumulation im Infarkt (Jander et al. 1995; Ref.1,7).

2.3. Sekundäre Effekte des chronischen Infarktstadiums

Nach Schlaganfall kommt es schließlich zur pseudozystischen Defektbildung und Ausbildung einer astroglialen Narbe im infarzierten Areal. Die vorgelegten Arbeiten haben erstmals systematisch inflammatorische Veränderungen und gliale Aktivierung in diesem bislang wenig beachteten chronischen Infarktstadium untersucht.

Aus der Neurophysiologie war bekannt, dass fokale kortikale Infarkte zu elektrophysiologischen Veränderungen auch in weit vom Infarktareal entfernten Regionen mit Phänomenen elektrophysiologischer Entkoppelung (sog. transkortikaler Diaschisis) führen

(Buchkremer-Ratzmann et al. 1996; Witte et al. 2000). Die vorgelegten Arbeiten zeigen, dass auch im chronischen Infarktstadium die gliale Aktivierung weit über das eigentliche Infarktgebiet hinausgeht.

Anisomorphe Gliose und Pseudozystische Degeneration des Infarktes

Nachdem in der ersten Woche nach Ischämie Phagozyten präferentiell im Infarkttrand zu finden waren, wanderten die Phagozyten in der zweiten Postinfarktwoche zentripetal in den Infarkt ein, der nach 14 Tagen flächig von Phagozyten infiltriert war. Dies trifft für die Photothrombose und die permanente MCAO, aber auch für die Areale zu, die nach transients MCAO der ischämischen Pannekrose anheimfallen (Ref.1,3 bzw.5).

Die Phagozytose des Fett- (Myelin-)reichen und relativ eiweißarmen Gewebes führte wie beim Menschen zur Kolliquation der Nekrose und Ausbildung von Zysten, die durch gliomatöse Reaktion der nun in den Infarkt einwandernden Astrozyten und einen dichten Phagozytenwall begrenzt waren (Storchdorph 1986; Ref.1). Die mikrogliale Aktivierung und astrogliale Aktivierung in der Umgebung der Läsion war nach 14 Tagen weitgehend abgeklungen. Der Infarkttrand blieb durch einen persistierenden Saum ED1+ Phagozyten und GFAP+ Astrozyten markiert, die eine anisomorphe Gliose formten (Ref.1,2).

Faserbahn Degeneration

Seit Gudden (Gudden 1870) ist bekannt, dass fokale kortikale Läsionen zur Degeneration des Thalamus führen. Auch nach humanen Hirninfarkten ist eine Degeneration weit entfernter Abschnitte des ZNS bekannt (Russell 1958; Chung 1985; Nagasawa et al. 1994). Nach Fotothrombose kommt es zur einer Faserdegeneration im Corpus callosum, in kortikospinalen und thalamokortikalen Faserbahnen (Abb.4). Die Fotothrombose bietet den Vorteil, primär ischämische Läsion und sekundär degenerierende Areale direkt auf einem Koronarschnitt vergleichen zu können (Ref.3, s.a. Abb. 4). Mit deutlicher Verzögerung zum ischämischen Fokus fand sich beginnend 7 Tage, deutlich zunehmend zu Tag 14 eine Aktivierung von Mikroglia entlang degenerierender Faserbahnen. Mikroglia zeigte in der weißen Substanz die charakteristische Morphologie von Stäbchenzellen (rod cells), deren primäre Zellfortsätze sich parallel zum Faserverlauf ausrichten. Die aktivierte Mikroglia zeigte eine vermehrte Expression von Komplement Rezeptor 3, und eine *de novo* Expression von MHC II und CD4. Mikroglia im sekundär degenerierenden Thalamuskernen zeigte eine sternförmige Morphologie mit Expression von MHC Klasse I und II sowie CD4 Antigenen. In beiden

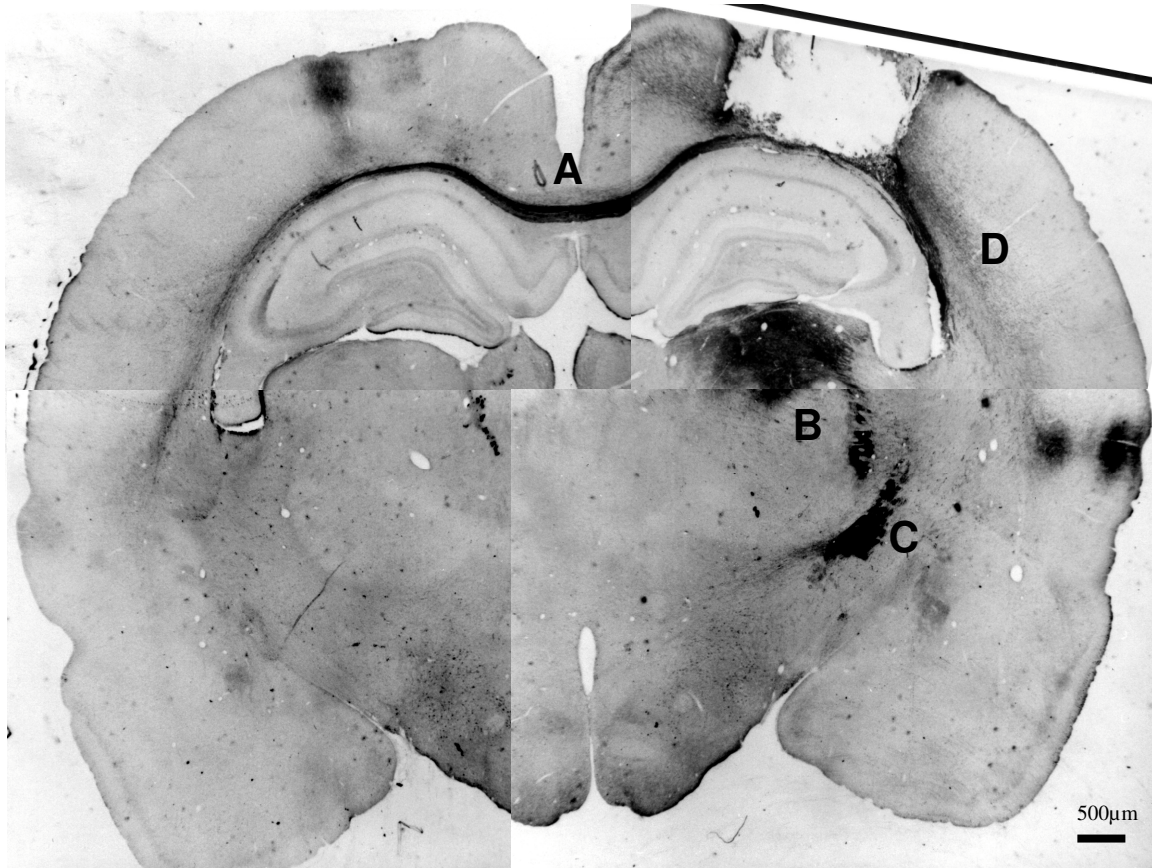


Abb.4: Kupfer-Versilberung (NeuroScience Associates, Knoxville, Tennessee, USA) zur Darstellung degenerativer Faseränderungen und Kerndegenerationen 2 Tage nach Fotothrombose. Die Läsion im sensomotorischen Kortex führt zu ausgedehnten Degenerationen im Bereich des Corpus callosum (A), thalamischer Kerne (B), der Pyramidenbahn (C) und kortiko-kortikaler Verbindungen (D). Interessant ist, dass diese Degenerationen früh nachweisbar sind, jedoch erst mit erheblicher Verzögerung zu einer glialen Aktivierung führen (s.Text, Ref.3).

Bereichen fand sich eine milde astrogliale Hypertrophie im Sinne der isomorphen Gliose. CD8 Expression fand sich im Gegensatz zum ischämischen Fokus nicht. Insgesamt muss im Bereich sekundär degenerierender Strukturen von einer stark verzögerten und inkompletten mikroglialen Aktivierung ausgegangen werden. Es bleibt unklar, ob dieses auf eine qualitativ oder quantitativ insuffiziente Stimulation durch Zytokine zurückzuführen ist. Im Gegensatz zum ZNS kommt es im peripheren Nervensystem zu einer guten Zellfortsatzregeneration nach peripherer Axondurchtrennung. Voraussetzung hierfür ist u.a. der rasche Einstrom von Makrophagen mit konsekutiver Phagozytose des Debris‘ (Stoll et al. 1989; George and Griffin 1994). Damit liegt nahe, dass die inkomplette Mikrogliaaktivierung zur Non-Permissivität des ZNS für Zellfortsatzregeneration beiträgt.

2.4. Untersuchungen zur nicht-invasiven Darstellung glialer Aktivierung und der Leukozyteninfiltration in der multimodalen Kernspintomographie (Ref.4)

Infarktverläufe können zwischenzeitlich auch beim Menschen sehr detailliert mit der Kernspintomographie dokumentiert werden, wobei bislang unklar ist, ob über allgemeine Gewebetexturstörungen hinaus Gliareaktionen oder Leukozyteninfiltration erfassbar sind. Deshalb wurden Histologie und Immunzytochemie (Darstellung der Astroglia, Mikrogliaaktivierung und infiltrierender Makrophagen) mit den zuvor erzeugten hochauflösenden MRT-Bildern verglichen (Ref.4). Die MRT-Parameter wurden entsprechend der multimodalen Kernspintomographie, wie sie in der klinischen Situation des akuten Schlaganfalls beim Menschen angewendet wird, gewählt.

In der Infarktfrühphase zeigte die Kernspintomographie die typischen Signalalterationen des akuten Infarktes (Norris et al. 1994; Hoehn-Berlage et al. 1995). Die Infarktausdehnung wurde durch das Areal der ADC-Absenkung exakt wiedergegeben. Dagegen ging infolge des lokalen Ödems das Gebiet der T2-Wertanhebung darüber hinaus. Die perfusionsgewichtete Bildgebung zeigte einen scharf begrenzten Perfusionsausfall des Infarktes. Die schwere Blut-Hirn-Schranken-Störung des fothrombotischen Areals war durch die lokale Gadolinium-Aufnahme nachzuweisen.

In der Infarktspätphase zeigte dagegen die ADC-Wichtung normale Werte im Infarkt. Die T2-Werterhöhung war rückläufig. Die Konstellation aus normalisierten ADC-Werten und reduzierter T2-Werterhöhung ist für die Spätphase des Infarktes charakteristisch. Der Infarkt im chronischen Stadium ließ sich durch heterogener ADC- und T2- Werterhöhungen abgrenzen. (Ref.4). Durch Überlagerung histologischer und immunzytochemischer

Serienschnitte auf korrespondierenden kernspintomographischen Bildern versuchten wir, Subareale des Infarktes mit oder ohne inflammatorische Reaktionen und den umgebenden Saum glialer Aktivierung typischen Signalalterationen im MRT zuzuordnen. Jedoch zeigte das MRT kein differenzielles Signalverhalten von Subarealen mit inflammatorischen Veränderungen oder glialer Aktivierung. Ebenso unterschied sich das den Infarkt umgebende vitale Gewebe mit ausgeprägter glialer Aktivierung nicht von anderen vitalen Kortexarealen, z. B. der kontralateralen Hemisphäre (Ref.4).

Insgesamt zeigte das MRT dynamische Veränderungen, die Informationen über das Infarktstadium gaben. Es gelang mit den aktuell beim menschlichen Schlaganfall angewendeten Wichtungen jedoch nicht, einzelne Aspekte der inflammatorischen Reaktionen sichtbar zu machen oder inflammatorisch veränderte Subareale des Infarktes abzugrenzen.

Hierfür bietet die Anwendung von „ultrasmall paramagnetic iron oxide“ (USPIO) eine interessante Perspektive. Dieses eisenhaltige Kontrastmittel, das sich derzeit auch beim Menschen bereits in Erprobung befindet, wird von Makrophagen phagozytiert und macht diese dann im MRT sichtbar. Durch Anwendung von USPIO gelingt es nach Fotothrombose, den Makrophagensaum im Infarkt kernspintomographisch sichtbar zu machen (Schroeter et al., Manuskript in Vorbereitung; s. Abb. 5)

3. Perspektiven

Die hier präsentierten Untersuchungen sind motiviert durch die These, dass durch Modifikation der postischämischen Inflammation die Folgen eines Schlaganfalls gemildert werden können. In diesem Sinne sind die Ergebnisse der Beginn einer genaueren Analyse der Mechanismen postischämischer Inflammation, die in die Entwicklung neuer therapeutischer Strategien münden sollen. Ziel ist es, inflammatorische Kaskaden zu modifizieren, um eine sekundäre Schädigung des ischämischen Areals durch proinflammatorische Moleküle, z.B. IL-1, TNF- α und iNOS zu vermindern, andererseits protektive Effekte und den Vorgang der Infarktdemarkation und Wundheilung zu fördern.

Der Vergleich verschiedener Läsionsparadigmen (Ref.1,3,5) half den Einfluss unterschiedlicher Schädigungsmechanismen auf die gliale Aktivierung zu beschreiben. Dieses sollte durch die Analyse glialer Aktivierung nach therapeutischer Beeinflussung der

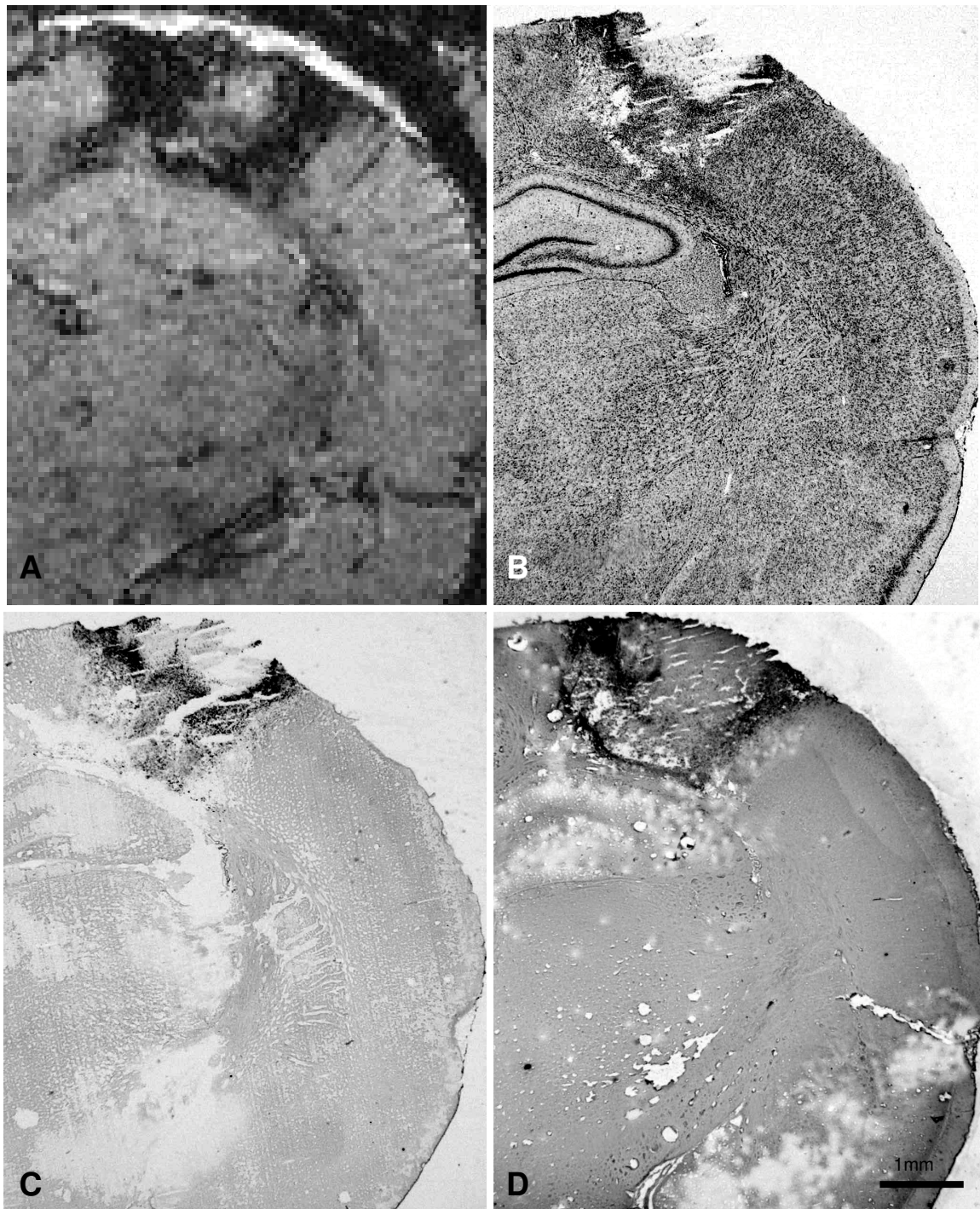


Abb.5: Visualisierung der Makrophagenakkumulation im hochauflösenden 7T MRT nach Gabe von USPIO. Die Kernspintomografie zeigt in T2* gewichteten Bildern eine Signalabsenkung in der äußeren Infarktzone des ischämischen Infarktes 6 Tage nach Fotothrombose (A). Kongruent hierzu findet sich im korrespondierenden histologischen Schnitt (Kresylviolet-Färbung; B) eine zellreiches Infiltrat, das immunzytochemisch als ED1+ Phagozyten identifiziert werden kann (C). In diesen Phagozyten kann histochemisch mit einer verfeinerten Berliner Blau Reaktion Eisen nachgewiesen werden (D). Maßstab in D entspricht 1mm in A-D.

Schlaganfallsgenese, v.a. nach Lyse oder dem Einsatz von Thrombozytenaggregationshemmern, ergänzt werden.

Die Untersuchungen zur astroglialen und mikroglialen Aktivierung nach Fotothrombose und Spreading Depression (Ref.2,6) machen eine enge Interaktion von Astrozyten und Mikroglia wahrscheinlich, die zum Großteil noch unverstanden ist.

In sehr unterschiedlichen Läsionsparadigmen läßt sich eine enge räumlich-zeitliche Beziehung zwischen dem Auftreten der CD8+ Mikroglia/Makrophagenpopulation mit der folgenden anisomorphen Gliose herstellen. Unter geeigneten Bedingungen kann eine anisomorphe Gliose permissiv für ein axonales Wachstum als morphologisches Substrat funktioneller Plastizität sein. Durch die gezielte Beeinflussung CD8+ Makrophagen könnten somit reparative Funktionen in der Infarktumgebung moduliert werden.

IL-18 wurde als das die Spätphase des Infarktes dominierende Zytokin identifiziert. Die funktionelle Bedeutung des IL-18 könnte durch Verwendung von knock-out-Mäusen bzw. durch Blockade eines neutralisierender Agenzien wie dem „IL-18 binding protein“ näher analysiert werden (Yatsiv et al. 2002).

Die Spreading Depression (Ref.2,6) ist ein hervorragendes Paradigma, um die Dichotomie sowohl zytotoxischer als auch zytoprotektiver Potenz näher zu analysieren, die für viele der hier analysierten Faktoren eine große Rolle spielt. Die vergleichende Untersuchung von Ferneffekten der Fotothrombose und KCl-induzierter Spreading Depression (Ref.2,3,6,7) konnte eindeutig Spreading Depression als Ursache verschiedener Ferneffekte der ischämischen Läsion identifizieren. Zukünftige Untersuchungen sollten sich deswegen grundsätzlich nicht auf das Infarktareal beschränken, sondern immer auch Ferneffekte der Läsion in Betracht ziehen. Die Forschung der letzten Jahren hat in zunehmenden Maße gezeigt, dass die Ferneffekte entscheidend zur funktionellen Erholung nach Schlaganfällen im Sinne der Plastizität des Gehirns beitragen (Witte and Stoll 1997; Witte et al. 2000). Gliale Aktivierung und Zytokin-vermittelte Effekte sind nicht nur im Bereich des Schlaganfalls für die Gewebereparation und Abtransport nekrotischen Debris verantwortlich. Sie spielen offenbar auch eine wichtige Rolle bei den Ferneffekten fokaler Ischämie, bei plastischen Vorgängen und der Vermittlung von Ischämieprotektion.

Die hier vorgestellten Ergebnisse belegen, dass die Fotothrombose aufgrund des einzeitigen Eintretens des ischämischen Infarktes (Ref.3), der eindeutigen Differenzierung lokaler und Ferneffekte (Ref.2,6), und der vergleichenden Analyse von Effekten durch sekundäre Faserdegeneration im selben histologischen Schnitt (Ref.3) Vorteile gegenüber anderen

Infarktmodellen bietet, die in weiteren Studien genutzt werden sollten. Inzwischen konnte das Fotothrombose-Modell auch für die Maus etabliert werden, was in Zukunft auch den Einsatz dieses Modells an genetisch veränderten Tieren möglich machen wird (Schroeter et al. 2002). Schließlich ist für die Entwicklung neuer Therapien des menschlichen Schlaganfalls die nicht-invasive Visualisierung postischämischer Inflammation unbedingt erforderlich. Unsere Befunde zeigen, dass die derzeit angewandten kernspintomographischen Wichtungen inflammatorische Reaktionen und gliale Aktivierung nicht direkt darstellen können (Ref.4). Hierzu bedarf es der Entwicklung neuer Sequenzen oder des Einsatzes spezifischer Kontrastmittel. Eisenhaltige Kontrastmittel („*ultra small paramagnetic iron oxide*“, USPIO's; Dousset et al. 1999), die durch Makrophagen phagozytiert werden und diese in der Kernspintomographie sichtbar machen, bieten hierfür eine interessante Perspektive. Die Leistungsfähigkeit der USPIO's für die Visualisierung des Phagozyteninfiltrates nach fokaler Ischämie wird derzeit von verschiedenen Arbeitsgruppen – auch unserer – untersucht.

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Abkürzungsverzeichnis

ADC	apparent diffusion coefficient
CD	clusters of differentiation
DWI	diffusion weighted imaging
EAE	Experimentell Allergische Enzephalomyelitis
GFAP	Gliales Fibrilläres Saures Glykoprotein
ICAM-1	Interzelluläres Adhäsionsmolekül-1
IFN- γ	Interferon- γ
IL	Interleukin
iNOS	induzierbare Stickstoffmonoxid-Synthase
KCl	Kaliumchlorid
LFA-1	Leukocyte Functioning Antigen-1
MCAO	Okklusion der Arteria cerebri media
MHC	Hapthistokompatibilitätsantigene
MK-801	Dizocilpin
mRNA	Messenger-Ribonukleinsäure
MRT	Magnetresonanztomografie
NaCl	Natriumchlorid
NK-Zellen	Natürliche Killer-Zellen
NMDA	N-methyl-D-Aspartat
NO	Stickstoffmonoxid
TGF- β	Transforming growth factor- β
TNF- α	Tumor Nekrose Faktor- α
RT-PCR	Reverse-Transkriptase Polymerase Kettenreaktion
USPIO	ultrasmall particles of iron oxide
ZNS	Zentralnervensystem

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Zusammengefasste Arbeiten

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- Ref. 2: Schroeter M, Schiene K, Kraemer M, Hagemann G, Weigel H, Eysel UT, Witte OW, Stoll G (1995) Astroglial responses in photochemically induced focal ischemia of the rat cortex. *Exp Brain Res*; 106: 1-6.
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Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion

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Abstract

This study describes local immune responses in cerebral ischemia induced by permanent occlusion of the middle cerebral artery (MCAO) in the rat. The temporal and spatial pattern of leukocyte infiltration was characterized immunocytochemically using monoclonal antibodies against CD5, a pan T cell marker, against CD4 and CD8 for subtyping of T lymphocytes, and ED1, a marker for macrophages. CD5⁺ T cells were present in some animals on the pial surface at day 1 and with increasing numbers mainly at the edges of the infarcts at days 3 and 7. By day 14 their number had significantly decreased. Subtyping of T lymphocytes revealed that CD4⁺ helper/inducer T cells were rare, while CD8⁺ lymphocytes were abundant. Moreover, CD8⁺ lymphocytes outnumbered CD5⁺ T cells indicating the presence of CD5[−]/CD8⁺ natural killer (NK) cells. ED1⁺ macrophages primarily infiltrated the core of the infarct starting on day 1. Infiltrating leukocytes expressed leukocyte function associated antigen-1 and MHC class I and II antigens. Early after infarction, increased expression of the intercellular adhesion molecule-1 was found on vessels and leukocytes. In conclusion, this study shows that lymphocytes enter the nervous system not only in autoimmune diseases, but also in response to primarily 'non-immune' neuronal damage such as stroke.

Keywords: Cerebral ischemia; T cells; Macrophages; Natural killer cells; Intercellular adhesion molecule-1

1. Introduction

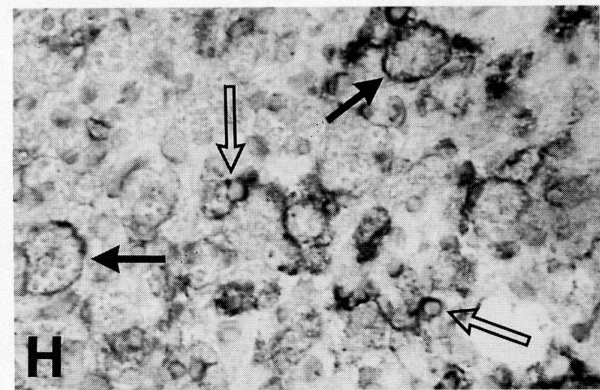
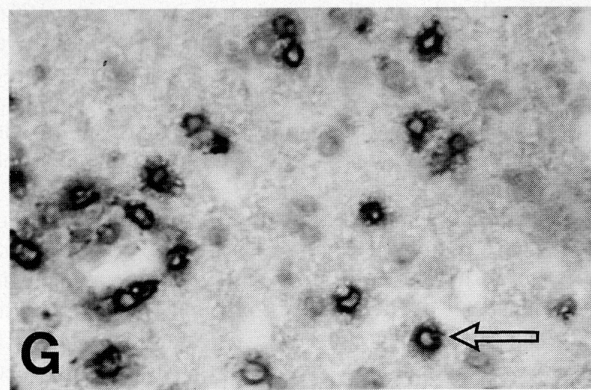
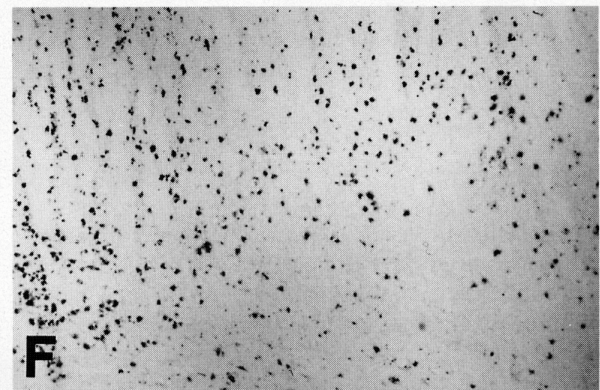
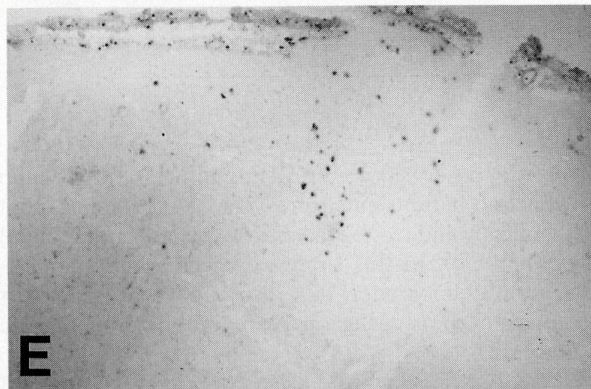
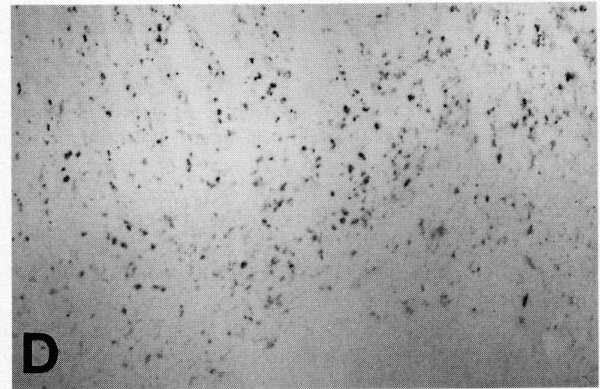
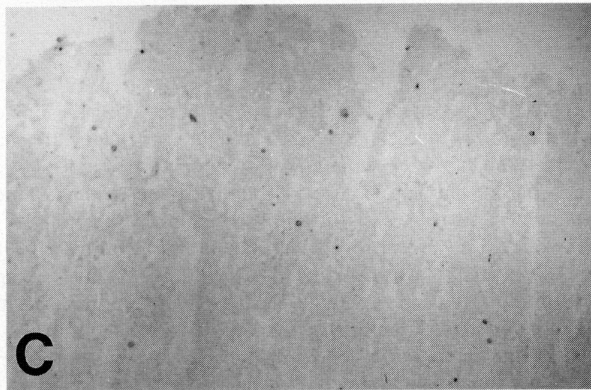
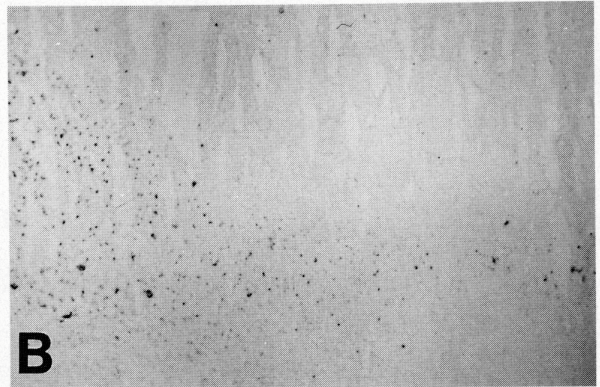
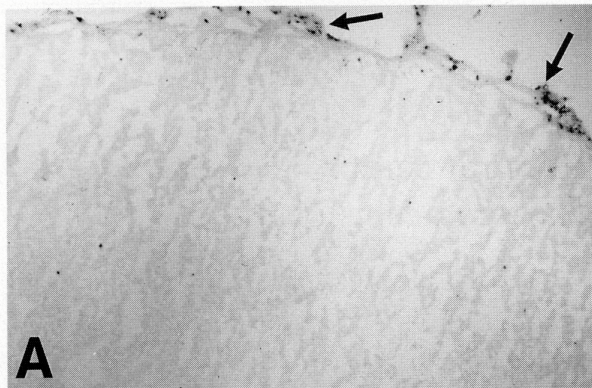
There is increasing evidence that the immune system could be involved in ischemic brain damage: (i) in spinal cord ischemia secondary deterioration can be prevented by blocking of macrophages (Giulian and Robertson, 1990); (ii) immune cell adhesion molecules such as P-selectin and the intercellular adhesion molecule-1 (ICAM-1) are upregulated early after focal ischemia in the baboon (Okada et al., 1994); (iii) ICAM-1 expression was detected in human infarcts of recent age (Sobel et al., 1990); and (iv) in some models treatment with antibodies against cell adhesion molecules reduced ischemic injury (Clark et al., 1991). ICAM-1 plays an important role in the trafficking of leukocytes through vessels and in T cell/macrophage interactions (reviewed by Springer, 1990). We have

recently described a long-lasting infiltration of lymphocytes and a transient expression of ICAM-1 in photochemically induced ischemia of the rat cortex (Jander et al., 1994). In this study we asked whether this is a general phenomenon that also applies to other stroke models such as occlusion of the middle cerebral artery (MCAO).

MCAO is a widely used experimental model for stroke. The histopathological changes and the infiltration of the infarcts by polymorphonuclear granulocytes (PMNL) and macrophages have been well described (Hallenbeck et al., 1986; Kochanek and Hallenbeck, 1992; Garcia et al., 1993, 1994). However, the question whether lymphocytes are involved has so far not been addressed systematically.

In the present study, focal ischemia in the rat cortex was induced by permanent occlusion of the middle cerebral artery. Infiltrating leukocytes were identified by immunocytochemistry using monoclonal antibodies (mAbs) against lymphocyte surface antigens CD5, CD4, and CD8 and against ED1, a marker for macrophages.

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Concomitantly, the expression of major histocompatibility complex (MHC) class I and II antigens and the cell adhesion receptor ICAM-1 and its ligand leukocyte function-associated antigen-1 (LFA-1) was studied at various stages after infarction. We provide evidence for a strong local immune response after local ischemia.

2. Materials and methods

2.1. Surgery

Thirty-two male rats (Wistar and Lewis strain) weighing 280–320 g underwent permanent middle cerebral artery occlusion (MCAO) as described by Tamura et al. (1981) and modified by Bederson et al. (1986).

Rats were anesthetized with halothane and rectal temperature was maintained at $36.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ with a heating blanket. The skin was cut coronally between eye and ear and the temporal muscle was retracted saving the facial nerve, the arterial supply and the contents of the orbita. The temporal skull was exposed just above the zygoma and periosteum and muscle insertions were removed. The skull was thinned microsurgically with a drill (Meisinger, Düsseldorf, Germany) and opened with a forceps just above the MCA. The dura was opened with the tip of a cannula (27G) and retracted with a thin glass stick. The artery was then gently lifted from the brain surface and coagulated with a microsurgical coagulation forceps (Leibinger, Freiburg, Germany) proximally of the inferior cerebral vein and distally of the olfactory tract. Finally, the artery was cut by coagulation at a third point in between to assure permanent interruption. Afterwards, temporal muscle and skin were closed in separate layers and animals were allowed to recover from anesthesia under a heating lamp. This procedure produced infarcts comparable with the data reported by Bederson et al. (1986) and Duverger and MacKenzie (1988).

2.2. Tissue processing and immunocytochemistry

1, 3, 7, 14 and 30 days after MCAO rats were decapitated in deep halothane anesthesia (at least five animals at each time point), whole brains were removed, immediately frozen in isopentane at -50°C

Table 1

Semiquantitative assessment of cellular infiltration by polymorphonuclear leukocytes, CD5^{+} and CD8^{+} lymphocytes, and macrophages

Time point after MCAO	Polymorphonuclear leukocytes	CD5^{+} lymphocytes	CD8^{+} lymphocytes	Macrophages
Day 1	++	(+)	(+)	+
Day 3	+++	+	++	+++
Day 7	(+)	++	+++	+++
Day 14	–	+	+	+++
Day 30	–	(+)	–	++

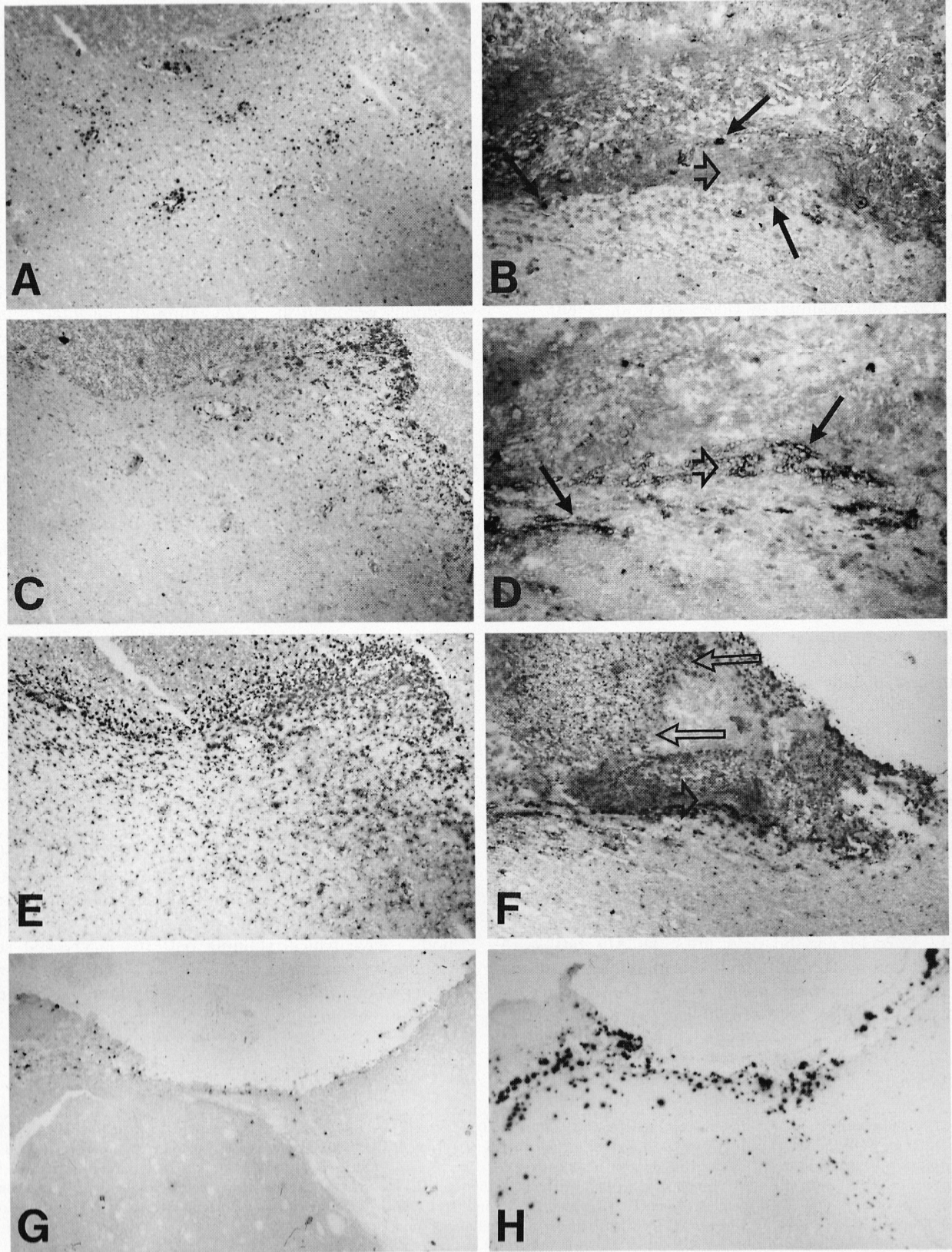
+, slight; ++, moderate; +++, massive infiltration.

and stored at -80°C . Serial $5\text{-}\mu\text{m}$ coronal cryostat sections were cut at -22°C through the infarcts and fixed in acetone for 10 min at -20°C . Some of these sections were stained with Hematoxylin and Eosin (H & E) to estimate the size and gross morphology of the infarcts.

Serial sections were stained by immunocytochemistry by the avidin–biotin–peroxidase complex technique. After preabsorption with 3% normal goat serum in 0.01M PBS, sections were incubated overnight at 4°C with the following mAbs (dilutions in parentheses): mAb ED-1 (Serotec, Oxford, UK; 1:1000), a marker for macrophages; mAb anti-rat-CD5 (clone R1-3B3, Seikagaku, Tokyo, Japan, 1:1000) recognizing all T cells; mAb against lymphocyte subsets CD4 (helper/inducer; clone 15-8A2, Holland Biotechnology (Hbt), Leiden, Netherlands; 1:1000) and CD8 (suppressor/cytotoxic T cells, NK cells; two different mAbs were used and gave identical results: clone 15-11C5, Hbt, 1:1000; clone R1-10B5, Seikagaku Kogyo, Tokyo, Japan, 1:1000), mAb against rat B cells (clone RLN-9D3, Seikagaku, 1:1000); mAb against rat killer cells and polymorphonuclear granulocytes (clone 3.2.3, Serotec; 1:200), mAb anti-rat-ICAM-1 (clone 1A29; 1:1000) and mAb anti-rat LFA-1 (clone WT-1; 1:500, both Medac, Hamburg, Germany), mAb OX6 (1:200) and mAb OX 18 (1:1000, both Serotec) directed against MHC class II and I antigens, respectively. These antibodies gave typical staining patterns in cryostat sections of rat spleen.

As secondary antibody, affinity-purified biotinylated rat absorbed anti-mouse IgG (Vektor; Burlingame, CA)

Fig. 1. Cellular infiltration of the infarcts at days 1 (A, C, E) and 3 (B, D, F) after MCAO. (A, B, G) are $5\text{-}\mu\text{m}$ cryostat sections stained for CD5, (C, D, H) for CD8 and (E, F) with mAb ED1, a marker for macrophages. At day 1, CD5^{+} T cells are present at the pial surface (arrows in A), very few CD8^{+} lymphocytes (C) and a considerable number of ED1⁺ macrophages (E) have infiltrated the core of the infarct. At day 3 the number of CD5^{+} T cells (B), CD8^{+} lymphocytes (D) and ED1⁺ macrophages (F) has increased. Note the different number and distribution of CD5^{+} T cells in (B) and CD8^{+} lymphocytes in (D). (G) shows CD5^{+} and (H) CD8^{+} lymphocytes at higher magnification. CD5^{+} cells represent small lymphocytes (open arrow), while CD8^{+} cells encompass two populations: small lymphocytes (open arrows), probably cytotoxic/suppressor T cells, and large cells, presumably NK cells (dark arrows). (A–F) $\times 33$; (G, H) $\times 300$.



was used. Non-specific labeling was checked by replacing primary antibodies with mouse mAbs against irrelevant antigens at working dilutions of primary antibodies. No unspecific labeling was observed.

3. Results

One day after MCAO, the infarct was clearly visible on cryostat sections stained with Hematoxylin. As described before (Bederson et al., 1986; Duverger and MacKenzie, 1988), there was a considerable variation in infarct volume which, however, did not affect the composition of the cellular infiltrate. The results on lymphocytic and macrophage infiltration are summarized in Table 1.

When coronal cryostat sections were stained with mAb against CD5, a pan T cell marker, T cells were seen at the pial surface of the ischemic hemisphere at day 1 after MCAO in about half of the animals, but only very few in the parenchyma (Fig. 1A). At the same time, a considerable number of ED1⁺ macrophages had infiltrated the core of the infarct (Fig. 1E). The number of CD8⁺ lymphocytes was low (Fig. 1C).

By day 3, CD5⁺ T cells were found in all animals examined and the number of CD8⁺ lymphocytes and ED1⁺ macrophages had increased (Fig. 1B, D, F). Some CD5⁺ T cells were scattered in the parenchyma of the infarct, but most of these cells appeared to be preferentially located at the edges of the infarct. In contrast, the entire infarct area was covered by CD8⁺ lymphocytes and ED1⁺ macrophages. CD8⁺ lymphocytes by far outnumbered CD5⁺ T cells at days 3, 7 and 14. As shown at higher magnification in Fig. 1G/H, mAb against CD5 labeled a homogeneous population of small T cells (open arrow in G), while mAb against CD8 stained similar small cells, probably suppressor/cytotoxic T cells (open arrows in H) and, in addition, larger lymphocytes (dark arrows in H), probably NK cells.

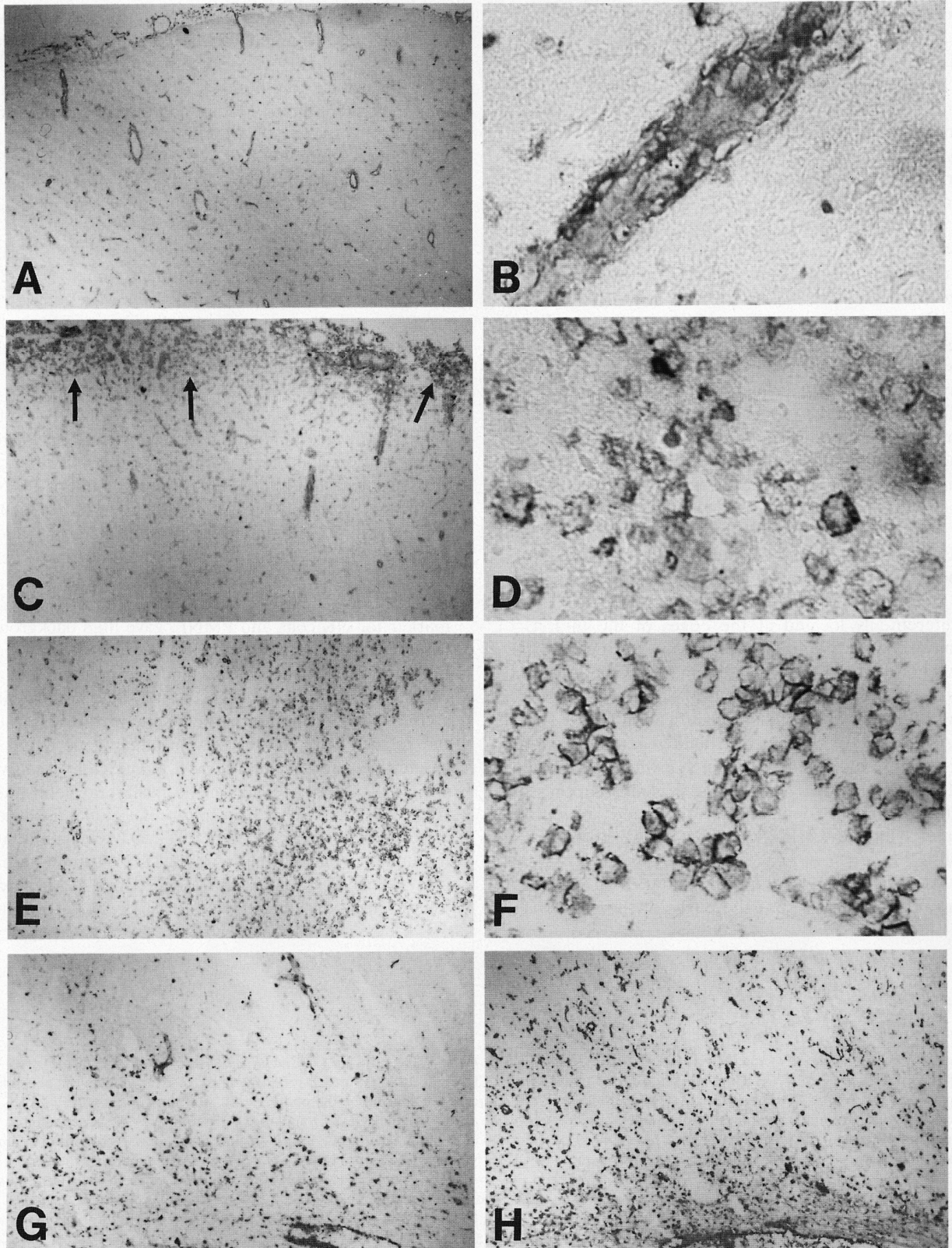
At day 7 after MCAO, the number of CD5⁺ T cells had further increased at the edges of the infarct (Fig.

2A). They were often found in close vicinity to vessels. CD8⁺ lymphocytes and ED1⁺ macrophages had massively infiltrated the core and, even more intensely, the edges of the infarct (Fig. 2C, E). By day 14, fewer CD5⁺ T cells remained (Fig. 2B, arrows) and concomitantly the number of CD8⁺ lymphocytes had decreased (Fig. 2D) which now were located preferentially at the margin of the infarcts. Macrophages were the predominant cell type and still distributed throughout the infarct and at the edges (Fig. 2F). Histologically, infarcts showed cytic degradation. By day 30 most of the infarcted tissue had been cleared off by macrophages and the cortex showed profound atrophy at the site of infarction. In this atrophic region, few CD5⁺ T cells (Fig. 2G) and a large number of macrophages (Fig. 2H) were still present. CD8⁺ lymphocytes were only rarely seen. Overall, the sequence and distribution of lymphocytic infiltration was identical in Wistar and Lewis rats. However, in the latter strain it appeared that more CD5⁺ T cells were present at days 3 and 7.

At all time points examined, CD4⁺ T cells of the helper/inducer phenotype and B cells were only rarely seen. As described in previous studies (Hallenbeck et al., 1986; Garcia et al., 1994), high numbers of PMNL, identified by their typical morphology in Hematoxylin-counterstained sections, were present at days 1 and 3 after MCAO, but their number rapidly declined thereafter. At day 7 they were only occasionally seen. As expected, these PMNL were CD5[−]. The mAb 3.2.3, a marker for NK cells and PMNL, gave high background labeling at high antibody concentrations. With a dilution of 1:1000, background had disappeared but no cellular labeling was seen in the ischemic brain region.

In normal brain and the hemisphere contralateral to the infarct, very faint ICAM-1 labeling occurred on cerebral vessels. Strong ICAM-1 staining of vessels was seen 1 day after MCAO on the ipsilateral hemisphere (Fig. 3A, B). At day 3, in addition, cellular infiltrates were ICAM-1-positive (Fig. 3C, D). Concomitantly, inflammatory infiltrates expressed LFA-1 immunoreactivity, the ligand for ICAM-1 (Fig. 3E, F). Moreover, many leukocytes were MHC class I- and II-positive

Fig. 2. Cellular infiltration of the infarcts at days 7 (A, C, E), 14 (B, D, F), and 30 (G, H) after MCAO. (A, C, E), (B, D, F) and (G, H) are 5- μ m serial sections, respectively. (A, B, G), are labeled for CD5, (C, D) for CD8, and (E, F, H) for ED1. Photographs were taken from the peri-infarct region with the infarcts located on top; in A, C, E the core of the infarct is not covered by the photographs. At 7 days, many CD5⁺ T cells are present in the peri-infarct region preferentially close to vessels, while CD8⁺ lymphocytes (C) and ED1⁺ macrophages (E) are located at the edges and the core of the infarct. At 14 days, few CD5⁺ T cells (arrows in B) are present at the transition zone from normal (bottom) to infarcted tissue (top of the section), while still a considerable number of CD8⁺ lymphocytes persists in this area (arrows in D). Note that the core of the infarct no longer contains CD8⁺ lymphocytes. The margin and the core of the infarct which shows cystic degradation are heavily infiltrated by ED1⁺ macrophages (open arrows in F). Open arrowheads in (B, D, F) denote identical regions that are shown at higher magnification in (B) and (D). At day 30, the atrophic cortex is still infiltrated by CD5⁺ T cells (G) and ED1⁺ macrophages (H). (A–F) are slightly counterstained with Hematoxylin to envisage morphology of the infarcts. (A, C, E–H) $\times 33$; (B, D) $\times 82$.



(Fig. 3G, H). Cortical vessels expressed MHC class I, but not class II antigens.

4. Discussion

This study shows that cerebral infarction after MCAO leads to a long-lasting infiltration of T cells, other CD8⁺ lymphocytes (probably NK cells), and ED1⁺ macrophages into the rat cortex. Concomitantly, immune activation markers such as MHC class I and II antigens, and the cell adhesion molecule ICAM-1 are expressed.

It is interesting to note that, compared to the contralateral hemisphere, ICAM-1 was expressed more strongly on ipsilateral vessels early after infarction. A similar upregulation of ICAM-1 and of another cell adhesion molecule, P-selectin, has recently been reported after MCAO in baboons (Okada et al., 1994). The recruitment of leukocytes from the blood to the nervous system depends on complex leukocyte–endothelial cell recognition processes via specialized adhesion receptors (Springer, 1990; Butcher, 1991). One of the best characterized pathways is the interaction of the CD18 complex that is constitutively expressed on leukocytes, with ICAM-1 as its ligand. ICAM-1 can be induced on a variety of immune and non-immune cells such as endothelial cells upon stimulation with cytokines like interferon- γ , interleukin-1 or tumour necrosis factor- α (Dustin et al., 1986; Pober et al., 1986). Since infiltrating leukocytes in our study expressed the ligand LFA-1, it is likely that ICAM-1 facilitates migration of leukocytes into the damaged brain after stroke. This pathway is mainly used by PMNL and macrophages. The signal that induces ICAM-1 on vessels after ischemia remains unknown.

It has been well established that cortical infarcts are transiently infiltrated by PMNL and for longer periods by macrophages (Hallenbeck et al., 1986; Kochanek and Hallenbeck, 1992; Garcia et al., 1994). The time course of PMNL and macrophage infiltration in the present study very closely resembles the findings reported recently by Garcia and colleagues (1994). However, the long-lasting infiltration by lymphocytes is surprising. All classical investigations on the histopathology of focal ischemia in the rat did not report on lymphocytic infiltration except one immunocytochemical study on microglial responses by Morioka and col-

leagues (1993), where they are briefly mentioned. This may mainly be due to the difficulties to unequivocally identify lymphocytes based on morphological criteria alone, even at the level of the electron microscope. In support of our present study we have obtained similar results in photochemically induced focal ischemia of the rat cortex (Jander et al., 1994). In the latter model, photoperoxidation of the endothelium leads to thrombotic aggregation with ensuing focal ischemia. It has been unclear so far whether the lymphocytic infiltration was unique to this model and solely due to endothelial damage rather than the cerebral ischemia itself. The fact that a long-lasting lymphocytic infiltration and expression of immune activation markers occurs in a similar fashion after photothrombosis and MCAO indicates that this is a general phenomenon in experimental stroke.

In autoimmune diseases of the nervous system like experimental autoimmune neuritis and encephalitis (EAE), antigen-specific T cells enter the brain and secondarily recruit other non-specific lymphocytes and macrophages (Raine, 1985; Lassmann et al., 1986). The very rapid recruitment of T cells within 3 days after MCAO makes a systemic immune reaction against cerebral antigens unlikely. It is important to note that in other 'non-immune' lesions with breakdown of the blood–brain barrier, such as Wallerian degeneration of the optic nerve (Stoll et al., 1989) or spinal cord injury (Dusart and Schwab, 1993), T cell infiltration has not been reported. The signal that attracts T cells and keeps them in the brain parenchyma for many days in ischemia is unknown at present. In EAE, T cell recruitment and clinical disease can be blocked with antibodies that interfere with the interaction between the $\alpha 4 \beta 1$ integrin of T cells and the vascular cell adhesion molecule (VCAM) (Yednock et al., 1992). This interaction plays an essential role in the adhesion of T cells to the endothelium, while ICAM-1 seems to be involved in the transendothelial migration (Oppenheimer-Marks et al., 1991). It is an attractive hypothesis that in addition to ICAM-1 and P-selectin other cell adhesion molecules such as VCAM are upregulated on vessels early after ischemia and facilitate T cell recruitment.

The composition of the lymphocytic infiltrate is of particular interest. Using two different well characterized mAb against CD8 (Matsuura et al., 1984) we found that CD8⁺ lymphocytes by far outnumbered CD5⁺ T cells similar to our findings in the pho-

Fig. 3. Localization of immune activation markers at days 1 (A, B) and 3 (C–H) after MCAO. Expression of ICAM-1 (A–D), LFA-1 (E, F), MHC class II (G) and I (H) antigens. At day 1 ICAM-1 is strongly expressed on cerebral vessels in the region of the infarct (A, B), whereas on day 3 in addition infiltrating leukocytes are ICAM-1-positive (C, arrows). (D) ICAM-1 staining on the cellular surface at higher magnification. (E, F) Infiltrating leukocytes also express LFA-1, the ligand for ICAM-1 (G, H). Serial sections showing strong MHC class II and I expression on infiltrating leukocytes. (A, C, E, G, H) $\times 33$; (B, D, F) $\times 330$.

tothrombosis model (Jander et al., 1994). CD5 is expressed by all T cells, but not by NK cells (Lawetzky et al., 1990), while both cell types express CD8 (Lawetzky et al., 1990; Hickey et al., 1992). Therefore CD5⁺/CD8⁺ lymphocytes are suppressor/cytotoxic T cells, while CD5⁻/CD8⁺ lymphocytes most likely represent NK cells. A similar cell population has been described by Sethna and Lampson (1991) after intracerebral injection of interferon-gamma. To further characterize CD5⁻/CD8⁺ lymphocytes, we tried to stain them with mAb 3.2.3, a marker for NK cells and PMNL (Chambers et al., 1989, 1992), but did not observe cellular staining in the infarcts despite typical results in spleen. The lack of staining after ischemia with mAb 3.2.3 can be interpreted in two ways: CD5⁻/CD8⁺ lymphocytes could represent a subpopulation of NK cells expressing the antigen of mAb 3.2.3 below detection level of immunocytochemistry, or, alternatively and less likely, represent a new, not yet defined lymphocyte subpopulation. NK cells can destroy target cells including neurons rapidly by a non-MHC-restricted and antigen-unspecific mechanism (Hickey et al., 1992). Thus, the presumable NK cells found after cerebral ischemia could contribute to tissue damage early after infarction.

While it is likely that macrophages degrade debris after infarction as usual after injury to the nervous system (Stoll and Hartung, 1992; Perry et al., 1993), the particular role of T cells and NK cells in cerebral ischemia is unclear at present. As reviewed by Nedergaard (1988) and Siesjö (1992) there is evidence that the perifocal tissue around an ischemic focus contains critically damaged but potentially viable cells whose survival depends on secondary lesion mechanisms such as edema formation, compromised vascular flow, and possibly cytotoxic factors produced by inflammatory cells (Piani et al., 1991; Lees, 1993). Recently, Garcia and colleagues (1993) have shown that brain lesions induced by MCAO in the Wistar rat grow and mature as a function of time. Pan-necrosis developed over about 72 h. Inflammatory cells characterized in the present study could be involved in this process. Identification and blocking of cytokines expressed by lymphocytes and interference with locally expressed cell adhesion molecules will help to elucidate the functional contribution of immune cells in focal ischemia. From an immunological standpoint it is of interest that a long-lasting lymphocytic infiltration and expression of immunological cell adhesion molecules can be triggered in the CNS without a preceding systemic sensitization process as it occurs in autoimmune demyelination by immunization with myelin antigens. Thus cerebral ischemia may serve as a primarily 'non-immune' lesion model that provides new insights into the initiation mechanisms of local immune responses in the CNS.

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RESEARCH ARTICLE

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Astroglial responses in photochemically induced focal ischemia of the rat cortex

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Abstract This study investigated astroglial responses after focal cerebral ischemia in the rat cortex induced by photothrombosis. Astrocyte activation was studied at various time points by immunocytochemistry for glial fibrillary acidic protein (GFAP) and vimentin (VIM). We found a dual astrocytic response to focal ischemia: In the border zone of the infarct, GFAP-positive astrocytes were present within 2 days and persisted for 10 weeks. These astrocytes additionally expressed VIM. Remote from the ischemic lesion, cortical astrocytes of the entire ipsilateral hemisphere transiently expressed GFAP, but not VIM, beginning on day 3 after photothrombosis. This response had disappeared on day 14. By recording DC potentials, five to seven spreading depressions (SD) could be detected on the cortical surface during the first 2 h after photothrombosis. Treatment with MK801, a non-competitive NMDA-receptor antagonist, completely abolished SD and remote ipsilateral astrocytic activation, while the reaction in the border zone of the infarct remained unchanged. Functionally, persistent astrocytosis around the infarct might be induced by leukocyte-derived cytokines, while NMDA-receptor-mediated SD might cause remote responses.

Key words Spreading depression · GFAP · Astrocytes · Focal ischemia · Rat

Introduction

Astrocytes play a pivotal role in the CNS by maintaining ion and pH homeostasis and by regulating volume and glucose levels (Walz 1989). They are essential constituents of the blood-brain barrier (Risau and Wolburg

1990). Astrocytes respond to a wide range of pathological conditions in the CNS by cell hypertrophy and proliferation (Bignami and Dahl 1976; Latov et al. 1979; Eng 1985; Schiffer et al. 1986). Glial fibrillary acidic protein (GFAP), an established marker for astrocytes, is constitutively expressed in fibrous astrocytes of the white matter, but at much lower levels in protoplasmic astrocytes, which account for most astrocytes in the gray matter (Bignami and Dahl 1976; Miller and Raff 1984). The passage from the quiescent to the reactive state is accompanied by an increase in intermediate filaments, predominantly in GFAP and, under certain conditions, also vimentin (VIM) (Schiffer et al. 1986).

Astrocytes are activated after global cerebral ischemia (Petito et al. 1990; Schmidt-Kastner et al. 1993). In this study, we examined whether this also applies to focal ischemia. Focal ischemia in the rat parietal cortex was induced by local illumination after systemic application of rose bengal according to the method of Watson et al. (1985). This leads to photochemically stimulated platelet aggregation with ensuing occlusion of small intracerebral vessels. With this technique, focal cortical infarcts can be induced which are highly reproducible in location and size (Watson et al. 1985; Domann et al. 1993). Paraffin sections were stained immunocytochemically for GFAP and VIM at various stages after photothrombosis. We show that two types of astroglial responses occur which are differentially influenced by spreading depression (SD).

SD is characterized by a transient depression of EEG activity and a negative shift in the DC potential that spreads across the cortical surface (Leao 1944). MK801, a noncompetitive *N*-methyl-D-aspartate receptor antagonist, abolishes SD (Marrannes et al. 1988; Gill et al. 1992). SD also occurs after photothrombosis (Dietrich et al. 1994). After application of KCl to the cortical surface, SD can trigger astroglial activation (Kraig et al. 1991). To examine the contribution of SD to the activation of astrocytes after photothrombosis, SD was registered and astroglial responses were compared between sham- and MK801-treated animals.

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Materials and methods

Induction of photothrombosis and tissue processing

Focal ischemia was induced in the rat parietal cortex at the same location as in two previous studies (Domann et al. 1993; Jander et al. 1995) according to the method described by Watson et al. (1985). Briefly, male Wistar rats (250–300 g) were anesthetized with 1.3% halothane in O₂/N₂ (1:2) and placed in a stereotactic frame. A fiberoptic bundle was placed stereotactically onto the skull at 3.5–4 mm posterior to bregma and 3.5–4 mm lateral from the midline for a 20-min period of illumination. During the first minute of illumination, rose bengal (1.3 mg/100 g body weight at a concentration of 10 mg/ml in 0.9% NaCl) was injected into the femoral vein.

For immunocytochemistry, rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer transcardially in deep anesthesia (number of animals examined in parenthesis) at 4 h (2), days 1 (2), 2 (2), 3 (4), 6 (3), 14 (2), and 60 (2) after photothrombosis. Whole brains were embedded in paraffin. Coronal and sagittal serial sections 5 µm thick were cut through the center of the lesion and stained with monoclonal antibodies (mab) against GFAP (1:50) and VIM (1:20) (both mab Boehringer-Mannheim, Germany) using the avidin-biotin-peroxidase method as described elsewhere (Jander et al. 1995).

Registration of spreading depression

In another set of experiments, the astroglial responses in MK801- and sham-treated rats were compared. One group received 2 mg/kg MK801 intravenously 30 min prior to illumination; the control group received saline. For the detection of SD, a trepanation was made ipsilaterally 4 mm anterior to the illumination site. The dura was opened, and an electrode for DC recording was placed onto the brain surface, which was filled with artificial cerebrospinal fluid (NaCl 125; NaHCO₃ 25; Na₂HPO₄ 0.5; CaCl₂ 1.1; MgCl₂ 0.8; KCl 3 [mmol/l]; pH 7.3). SD were recorded during illumination and within 2 h thereafter. Rats from each group were perfused at days 3 or 6, and paraffin sections stained for GFAP and VIM, as described above.

Results

Astroglial responses in photochemically induced ischemia

When paraffin sections of rat cortex were stained with mab against GFAP, only astrocytes located in the subcortical white matter and the glia limitans on the surface of the brain were labelled in untreated control animals. A similar staining pattern was observed with mab against VIM, except that in addition to astrocytes, ependymal, meningeal and some endothelial cells were VIM-positive. One day after photothrombosis, the GFAP and VIM staining pattern had not changed (Fig. 1A,B). By day 2, GFAP/VIM-positive astrocytes started to form a ring around the ischemic lesion that was fully established at day 3 (Fig. 1C,D; Fig. 2C,D). At day 3, additional GFAP staining occurred on astrocytes in the entire ipsilateral cortex remote from the lesion (Fig. 2A). The contralateral hemisphere did not show any changes in GFAP immunoreactivity. GFAP-positive cortical astrocytes distant from the lesion were VIM-negative (Fig. 2B). Similar observations were made 6 days after photothrombosis,

except that the ipsilateral astrocytic GFAP response was slightly diminished. By day 14 the infarct was surrounded by a broad band of GFAP/VIM-positive astrocytes that had increased in comparison to day 6 (Fig. 1E,F). However, no more GFAP staining in the remote ipsilateral cortex was observed. In the upper zone of the infarct, no GFAP immunoreactivity was seen, despite intense VIM staining (arrows in Fig. 1F). VIM-positive cells in this region most likely represent fibroblasts derived from the meninges that organize the infarct to form a scar. By day 60, the region of the infarct had shrunk, cortical atrophy had occurred, and astrocytes in this atrophic region still expressed GFAP/VIM immunoreactivity (Fig. 1G,H).

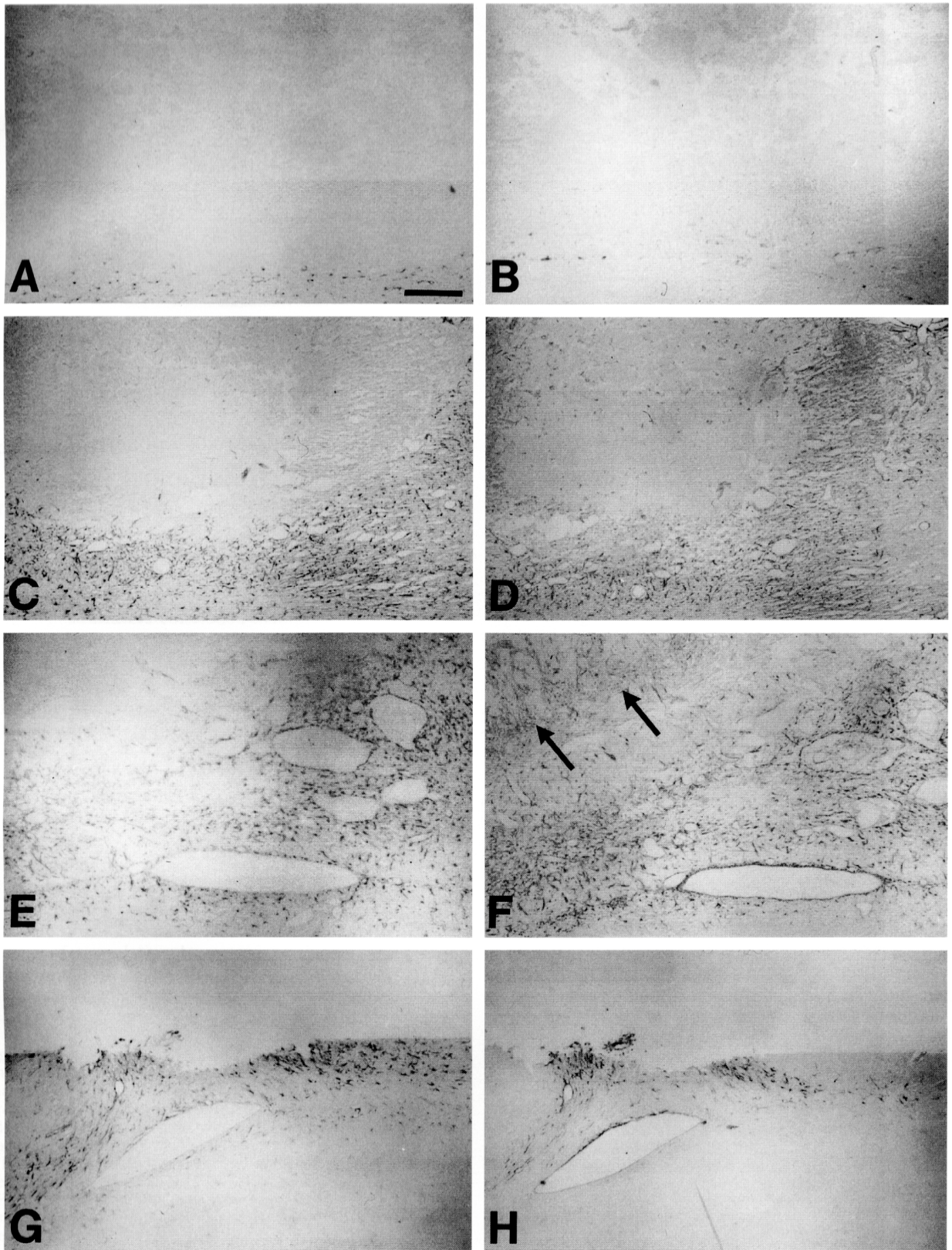
Influence of spreading depression on astroglial responses

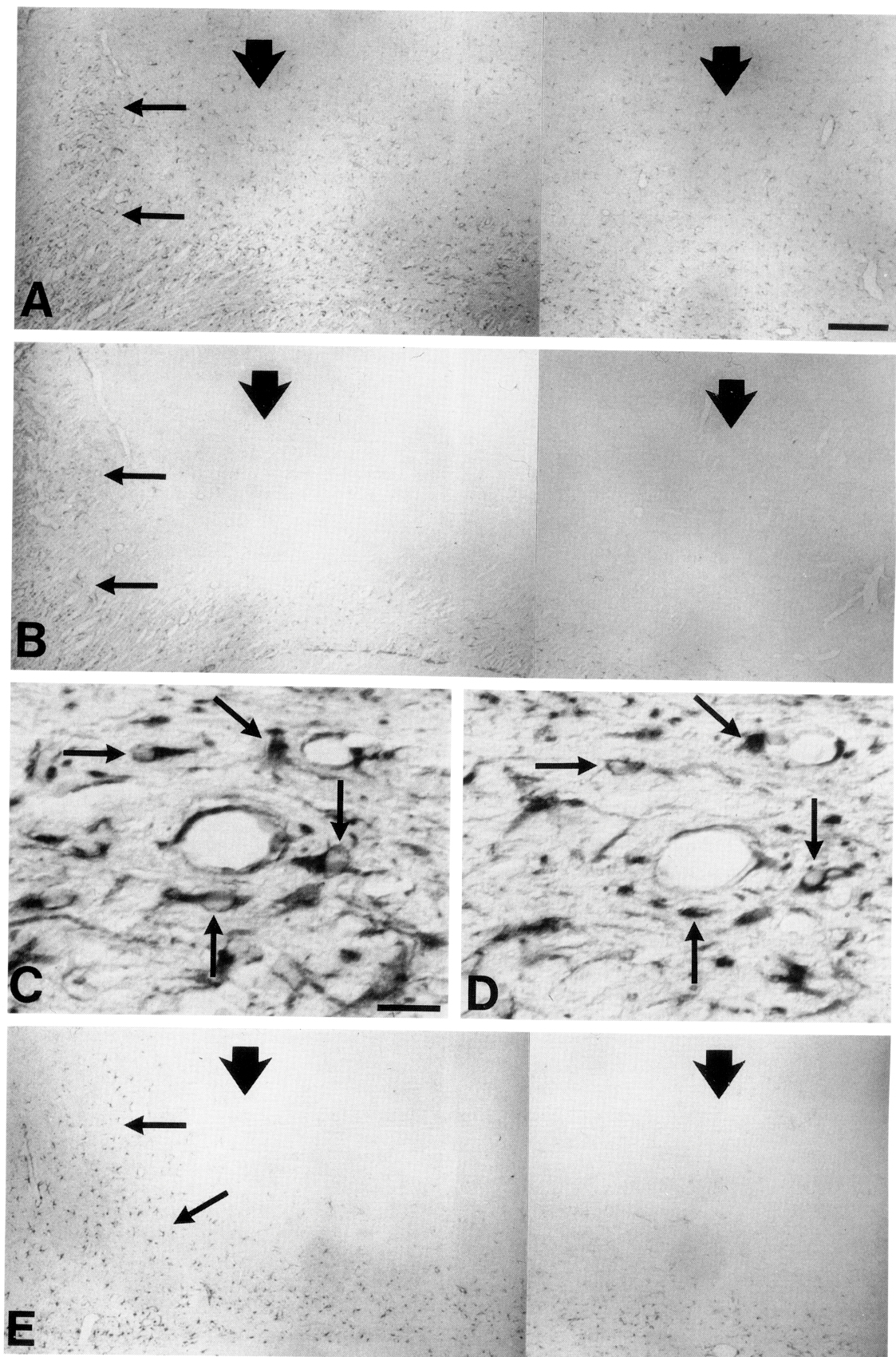
To elucidate the mechanisms that lead to the remote ipsilateral and transient activation of astrocytes after photothrombosis, spreading depressions were registered electrophysiologically. Within the first 120 min, five to seven SDs lasting about 2 min could be detected (Fig. 3A). Systemic application of MK801 abolished SD (Fig. 3B). When paraffin sections of rats with photothrombosis that were treated with MK801 were stained for GFAP, the remote ipsilateral GFAP staining of astrocytes had disappeared at day 3 (short arrows in Fig. 2E). In contrast, astrocytes surrounding the infarct still expressed GFAP-immunoreactivity (arrows in Fig. 2E). Similar observations were made 6 days after photothrombosis (not shown).

Discussion

This study shows that two different types of astroglial responses occur in photochemically induced focal ischemia of the rat cerebral cortex: (1) a long-lasting astrocytosis surrounding the infarct zone; these astrocytes, in addition to GFAP, expressed VIM typical for reactive astrocytes (Schiffer et al. 1986); (2) a transient astrocytic reaction remote from the lesion affecting the entire ipsilateral cortex; these astrocytes were VIM-negative. This dual astrocytic reaction in the cortex has also been observed in global cerebral ischemia (Petito et al. 1990), after laser irradiation (Schiffer et al. 1986) and after stab wounds (Takamiya et al. 1988), which means that this response is fundamental to CNS injury independent of the

Fig. 1 Localization of GFAP (A, C, E, G) and VIM (B, D, F, H) in the lesion area at days 1 (A, B), 3 (C, D), 14 (E, F) and 60 (G, H) after photothrombosis: 5-µm serial coronal paraffin sections. The surface of the cortex is always on top. At day 1 GFAP/VIM staining is limited to the subcortical white matter, while by day 3 GFAP/VIM-positive reactive astrocytes form a ring around the lesion. By day 14 more parts of the ischemic lesion are covered by GFAP/VIM-positive astrocytes. In addition, fibroblasts on the surface of the infarct express VIM (arrows in F). By day 60, there is persistent expression of GFAP and VIM in the atrophic cortex. Bar 300 µm





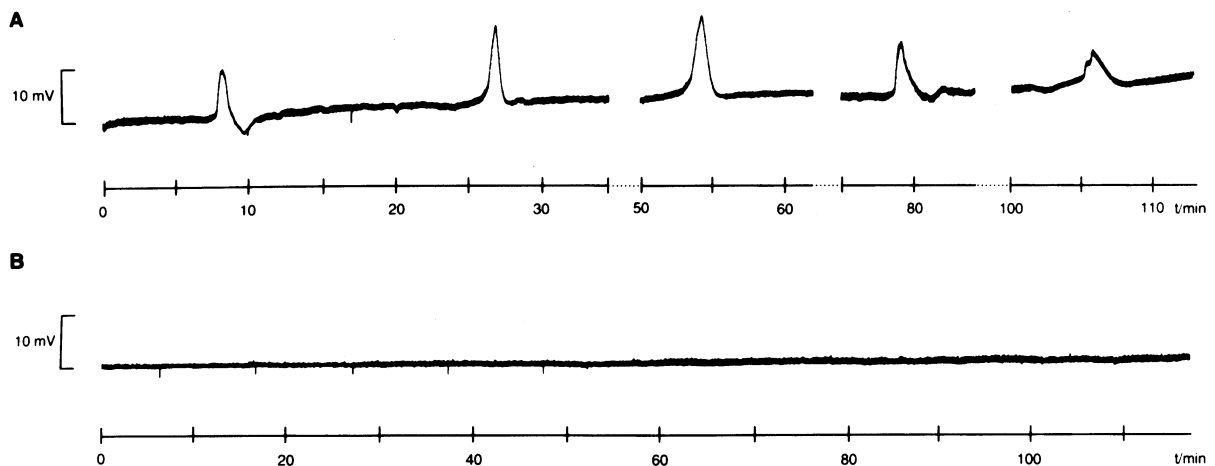


Fig. 3 Registration of spreading depressions (SDs) within 120 min after illumination onset. Note that the first DC deflection occurs as early as 7 min after beginning of illumination (A). After application of MK801, SDs are abolished (B)

mode of induction. It is interesting that only astrocytes close to the infarct expressed VIM in addition to GFAP. Similar results with limited colocalization of GFAP and VIM to areas close to the necrotic zone have been reported by Schiffer et al. (1986) after laser-induced CNS lesions. These authors concluded that VIM becomes stainable only in astrocytes that proliferate. Accordingly, it is conceivable that in photothrombosis astrocytes proliferate in the vicinity of the ischemic lesions, but show hypertrophy only in remote areas.

What are the mechanisms that trigger astrocyte activation after photothrombosis? The border zone of the infarct is heavily infiltrated by lymphocytes and macrophages, while the ipsilateral cortex remote from the lesion is spared (Jander et al. 1995). Macrophages can release a variety of cytokines, among them interleukin-1 (IL1) (reviewed in Nathan 1987). IL1 injected into mammalian brain stimulates astrogliosis and neovascularization (Guilian et al. 1988). Thus, astrogliosis in the border zone of the infarct may at least partly be the consequence of stimulation by cytokines derived from infiltrating leucocytes.

Fig. 2 Localization of GFAP and VIM in the ipsilateral cortex lateral to the ischemic lesion 3 days after photothrombosis. Same animal as shown in Fig. 1C,D. A, B At left the border zone of the infarct is shown (*thin arrows*). Note that astrocytes in the cortex outside the border zone of the lesion strongly express GFAP immunoreactivity (*thick arrows* in A), but are VIM-negative (*thick arrows* in B). C, D Representative astrocytes at higher magnification from the border zone stained for GFAP (C) and VIM (D). In this area GFAP and VIM are colocalized (*arrows* denote identical astrocytes in serial sections). After application of MK801, thereby suppressing SD (Fig. 3B), the ipsilateral astrocytic activation outside the lesion is abolished (*thick arrows* in E), while astrogliosis surrounding the lesion persists (*thin arrows*). Note that, in the area corresponding to A, GFAP staining is lost in this animal. Bars: A, B, E 300 μ m; C, D 30 μ m

After photothrombosis, cortical astrocytes not only express GFAP, but also the immediate early genes FOS and JUN are induced in the entire ipsilateral hemisphere (Gass et al. 1992). Moreover, electrophysiological investigations showed an impaired paired-pulse inhibition in this area (Domann et al. 1993). SDs occur in photochemically induced focal ischemia on the entire ipsilateral cortex, as described recently by Dietrich et al. (1994). These findings were confirmed in the present study. Treatment with MK801, a non-competitive NMDA receptor antagonist, abolished SD, but also remote astroglial responses. Similar results have been reported after topical application of KCl to the cerebral cortex, known to induce SD and activation of astrocytes in the entire ipsilateral cortex (Kraig et al. 1991; Herrera and Cuello 1992). Again, MK801 blocked SD and astrocytic activation. Since astrocytes do not possess NMDA receptors (Kettenmann and Schachner 1985), a direct effect of MK801 on astrocytes unrelated to blocking of spreading depression seems unlikely. It is conceivable that interstitial glutamate released by other cells after SD triggers activation of cortical astrocytes. In fact, astrocytes are able to rapidly metabolize glutamate (Hertz 1979). Alternatively, the activation of astrocytes is caused by the strong alterations of extra- and intracellular ion activities during SD which impose a strong metabolic stress upon astrocytes (Hansen and Zeuthen 1981).

In summary, there is concurring evidence from many studies that important differences exist in the reaction of astrocytes to a CNS lesion itself and to lesion-induced SD. Disclosure of the molecular mechanisms underlying these different responses will have implications for our understanding of astrogliosis in general.

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HETEROGENEITY OF THE MICROGLIAL RESPONSE IN PHOTOCHEMICALLY INDUCED FOCAL ISCHEMIA OF THE RAT CEREBRAL CORTEX

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Abstract—This study examined microglial responses after photochemically induced focal ischemia of the rat cortex. Microglial activation exceeded by far the area of the ischemic lesion. Based on morphological criteria and expression of immunomolecules three distinct patterns could be distinguished. (1) In the infarct core and the border zone microglia transformed into phagocytes and removed debris with the aid of hematogenous macrophages. Exclusively in this area a subpopulation of CD8+ microglia/macrophages was present. (2) In secondarily degenerating fibre tracts and nuclei with retrograde neuronal loss, microglia were activated with a delay of days and showed increased expression of complement receptor 3, major histocompatibility complex class II and CD4 molecules, but only low phagocytic activity. (3) In remote ipsilateral cortex devoid of neuronal damage, microglia transiently responded by increased complement receptor 3, but not by major histocompatibility complex class II and CD4 expression. Furthermore, the total number of microglia had increased. This remote response could partly be blocked by dizocilpine maleate, a non-competitive *N*-methyl-D-aspartate receptor antagonist, implicating a functional role of spreading depression.

Taken together, our findings point to a tight and differential regulation of microglial responses in the infarct core, degenerating fibre tracts and remote brain regions without neuronal loss. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: focal ischemia, microglia, macrophage, Wallerian degeneration, CD4, CD8.

Focal cerebral ischemia leads to complex cellular, molecular and electrophysiological changes in the brain.^{1,9,29,52} Severe ischemia induces necrotic cell death in the infarct core. Ischemic lesions are surrounded by a border zone in which neurons die with a delay of days by programmed cell death, apoptosis.^{4,35,36} After neuronal death, depending fibre tracts undergo anterograde Wallerian degeneration. Loss of neocortical target neurons after infarction is followed by retrograde degeneration of projecting neurons, for example in the thalamus.^{21,24,45} Within the first hours after ischemia negative depolarizations, so-called spreading depression,³² travel over the entire hemisphere and cause astrocytic activation.^{11,30,48} Spreading depression and the ensuing activation of astrocytes can be blocked by the non-competitive *N*-methyl-D-aspartate receptor antagonist dizocilpine maleate (MK-801). Focal cerebral ischemia, moreover, increases neuronal excitability remote from the lesion in the rat neocortex.^{12,46} These changes occur independently of spreading depression, but can be reversed by the investigational drug lubeluzole which interferes with the nitric oxide (NO)

cascade.^{6,7} NO plays multiple functional roles in the pathophysiology of cerebral ischemia.²³

Microglia are resident brain macrophages that strongly respond to cerebral ischemia.^{28,33,39,40,48} Parenchymal microglia are located in close vicinity to neurons in the gray matter and between fibre tracts in the white matter. Resting microglia exhibit highly arborized cell processes (ramified microglia) and can be distinguished from other glia of the CNS by immunocytochemistry with the monoclonal antibody (mab) Ox42 in the rat against the complement receptor 3 (CR3; CD11b) also present on monocytes/macrophages.¹³ Upon activation, microglia undergo morphological changes with development of stellate and ameboid microglia subpopulations.^{19,31} Stellate microglia are characterized by intensified Ox42 immunoreactivity, shortening of cell processes and loss of secondary arborizations.¹⁵ Further activation leads to complete loss of cellular processes and rounding of the cell body typical for phagocytes. These ameboid microglia become indistinguishable from hematogenous macrophages both on morphological grounds and based on the expression of surface and intracellular antigens. Activation leads to increased expression of major histocompatibility complex (MHC) type I and II molecules, CD4 antigen, an accessory molecule of T helper lymphocytes, and upon transition into phagocytes,

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Abbreviations: CR3, complement receptor 3 (CD11b); mab, monoclonal antibody; MHC, major histocompatibility complex; MK-801, dizocilpine maleate; NO, nitric oxide.

Table 1. Panel of monoclonal antibodies to characterize microglia activation

Antibody	Antigen	Dilution	Reference
Ox6 (MCA 46 G) (Serotec, Oxford, U.K.)	MHC class II	1:400	McMaster and Williams ³⁸
Ox8 (MCA 48 G) (Serotec)	CD8	1:2000	Brideau <i>et al.</i> ⁵
Ox18 (Serotec)	MHC class I	1:1000	Fukumoto <i>et al.</i> ¹⁴
Ox35 (22021 D) (Pharmingen, San Diego, CA)	CD4	1:1000	Pharmingen data sheet
Ox42 (MCA 275 G) (Serotec)	Complement receptor 3/CD11b	1:1600	Robinson <i>et al.</i> ⁴⁴
CD3 (G4.18) (Pharmingen)	CD3+ lymphocytes	1:1000	Pharmingen data sheet
ED1 (MCA 341) (Serotec)	Phagolysosomes	1:2000	Damoiseaux <i>et al.</i> ¹⁰
ED2 (MCA 342) (Serotec)	Perivascular microglia	1:1000	Graeber <i>et al.</i> , ²⁰ Harms <i>et al.</i> ^{20,22}

the phagolysosomal marker ED1.^{10,13,19,31,42} A specialized type of microglia, the perivascular microglia, is in close contact to blood vessels and can be distinguished from parenchymal microglia by immunocytochemistry with mab ED2.^{20,37}

Although there is evidence that microglia are activated after focal ischemia in the lesion area,^{41,48} secondary and remote microglial responses have not been addressed systematically. In the present study we provide evidence that microglia activation after photochemically induced focal ischemia is heterogeneous. In the photothrombosis model originally described by Watson and colleagues,⁵¹ infarctions are induced by a local photochemical reaction between the systemically injected dye Rose Bengal and a light beam transmitted through the intact skull, leading to endothelial alterations. These are followed by an early disruption of the blood-brain barrier, vasogenic edema, platelet activation, formation of clots and thrombotic occlusion of cerebral vessels. We describe the spatiotemporal activation pattern in the infarct core and the surrounding border zone, in the fibre tracts undergoing Wallerian degeneration and their target areas, and in the morphologically intact brain areas covered by spreading depression. We furthermore asked whether treatment of animals with MK-801 blocking spreading depression and lubeluzole would influence the microglial response.

EXPERIMENTAL PROCEDURES

Animal preparation

Animal experiments were performed according to German and European Communities Guidelines (86/609/EEC) with special efforts to minimize animal suffering, and to choose appropriate animal numbers. Focal cerebral ischemia was induced as described in detail previously.^{25,51} Briefly, enflurane-anaesthetized Wistar rats (University of Düsseldorf, Germany) received intravenous injections of the photosensitive dye Rose Bengal. The brain was illuminated through the exposed skull for 20 min at a stereotactically defined position 4 mm posterior to bregma and 4 mm laterally to the midline which resulted in local thrombosis of cortical microvessels and subsequent ischemic focal infarctions.

Groups of at least four animals were allowed to survive for 4 h, one, three, six and 14 days before they were decapitated under deep ether anaesthesia. Then brains were rapidly removed, frozen in isopentane and stored at -80°C .

Immunohistochemistry

Serial 5 μm coronal sections of the entire brain were cut at the level of the lesions on a Leica Cryotome (Leica, Bensheim, Germany) at -20°C and stained either immunocytochemically using Vector Elite ABC kit (Burlingame, CA, U.S.A.) and diaminobenzidine (DAB; Sigma) as the final reaction product or with Nissl, Hematoxylin-Eosin and Cresyl Violet staining. Parenchymal microglia were identified with mab Ox42 recognizing the CR3 (CD11b), perivascular microglia was stained with mab ED2. Microglia activation was assessed by staining for MHC class I and II antigen, CD4 and CD8 molecules²⁶ and with mab ED1, a marker for phagocytic activity. CD3 was used as a pan T-cell marker. Dilutions and sources of mab are given in Table 1.

Pharmacological treatment

MK-801 (Research Biochemicals International, Natick, MA, U.S.A.) was given intravenously in a single dose of 2 mg/kg body weight 30 min before induction of photothrombosis.

Lubeluzole (R087926, a kind gift from Janssen Research, Beerse, Belgium) was injected intravenously at a bolus of 0.31 mg/kg body weight 5 min after completion of illumination, and another 0.31 mg/kg body weight during the following hour.⁷ Survival time in both experimental groups ($n=4$) was three days. Normal Wistar rats and rats with three-day-old ischemic lesions without further treatment were compared to evaluate the effect of ischemia and further served as a baseline to elucidate the effects of pharmacological treatment.

From each animal, five sections at a distance of 30 μm were stained with mab Ox42 and quantified as follows. Ox42+ microglia in the ipsilateral cortex were counted in three non-overlapping 1 mm² squares at $\times 10$ magnification. Only complete cells with identifiable cell body and more than three cellular processes were included for analysis (Fig. 7B, circle). Counting squares were placed in an area reaching from 1 mm lateral to the ischemic lesion to the rhinal fissure as the ventral margin. Counting fields in the contralateral hemisphere were placed accordingly. Counts were performed blind to the treatment group by one investigator. Counts were compared with the help of paired two-tailed *t*-test testing H_0 at $P<0.05$.

RESULTS

Infarct core and boundary zone

Photothrombosis resulted in pure neocortical infarctions of about 2.5 mm diameter in cortical areas PAR1, OC2L, OC2M and HL according to the classification of Zilles.⁵³ A zone of pallor with loss of neuronal perikarya was already visible 4 h

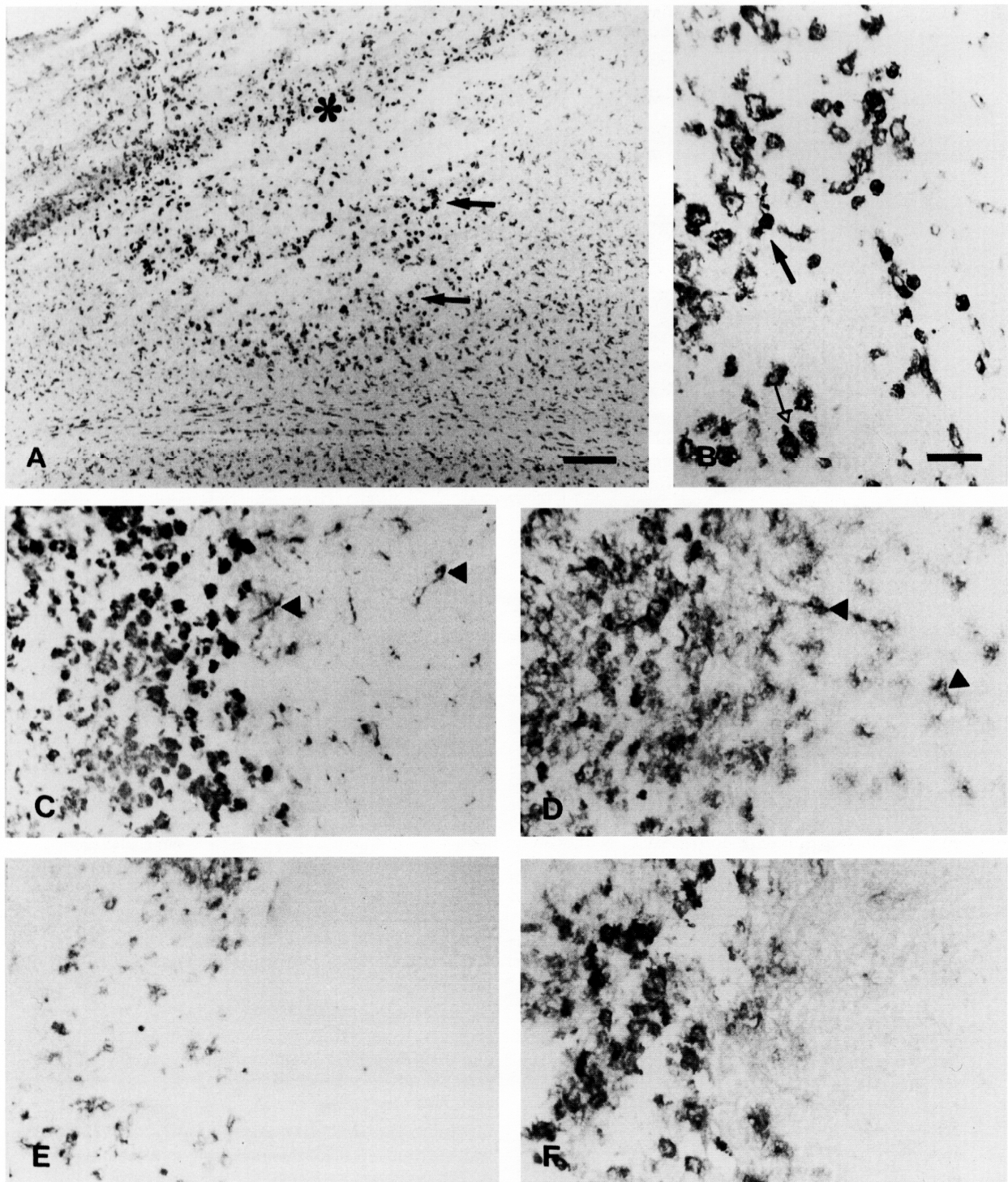


Fig. 1. Microglial responses at the infarct site three (A, B) and six days (C–F) after photothrombosis. Microglia were stained with mab Ox42 against the complement type 3 receptor (A, B) and for the activation markers ED1 indicating phagocytic activity (C), CD4 (D), MHC class II (E) and CD8 (F). (A) shows the entire infarct area, in (B–F) always the right border zone is shown (marked by arrows in A). Note that at day 3 both stellate microglia (closed arrow) and amoeboid microglia are present in the border zone (open arrow in B) suggesting transformation of microglia into phagocytes. At the core of the lesion (asterisk in A), avital cells exhibited a granular Ox42 immunosignal. At day 6 ED1+ phagocytes (C) which include transformed microglia and hematogenous macrophages (see text) are abundant and express activation markers MHC class II (E), CD4 (D) and CD8 (F). Note that CD4 and to a lesser extent ED1 are also up-regulated by ramified microglia in the non-ischemic cortex adjacent to the boundary zone (arrowheads in C and D), while CD8 expression is restricted to the demarcation wall (F). Scale bars: (A)=300 μ m, (B–F)=50 μ m.

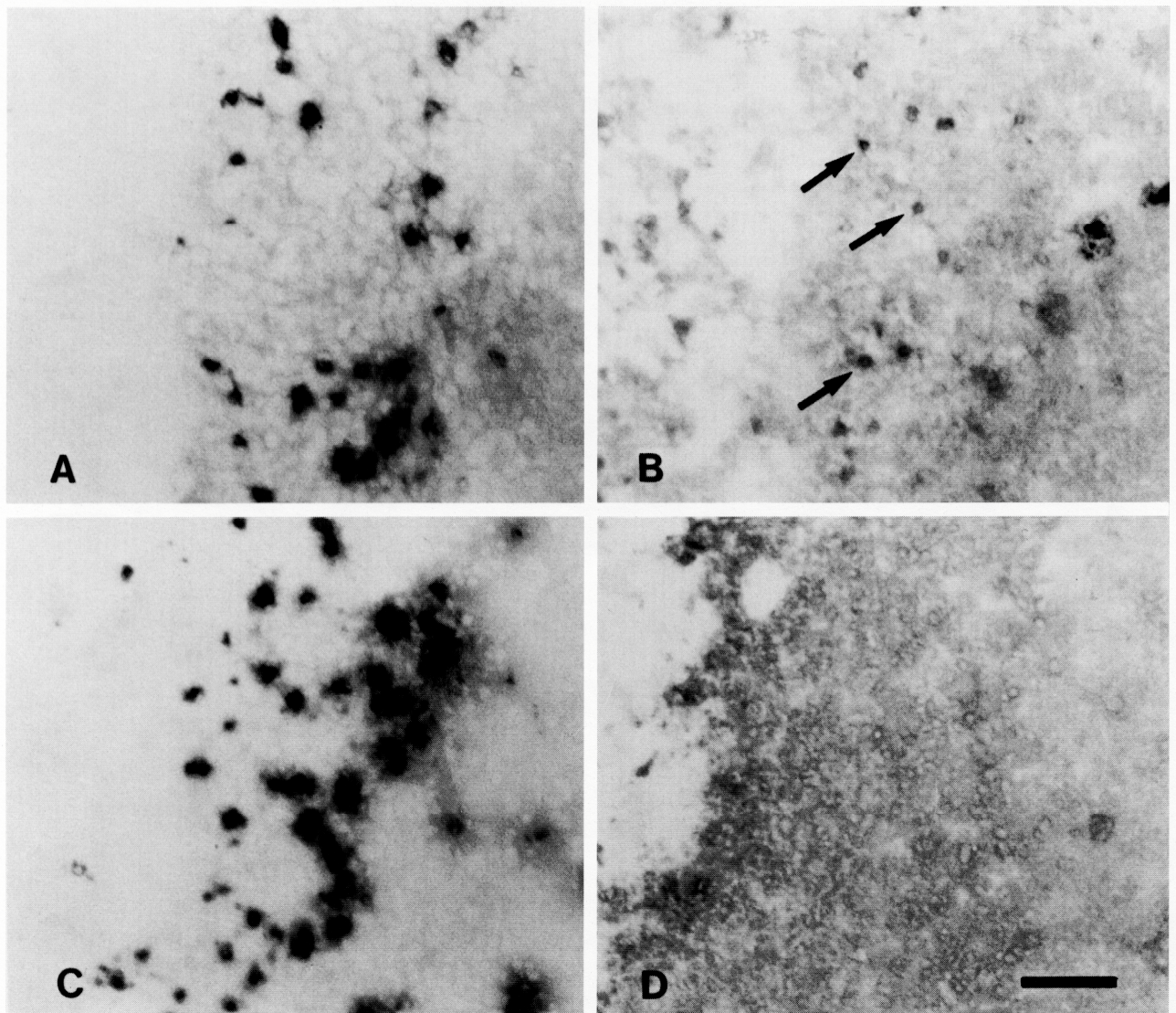


Fig. 2. Five-micrometer serial sections from the infarct border six days after photothrombosis. CD8+ microglia/macrophages (A) clearly outnumber CD3+ lymphocytes (B, arrows). A proportion of microglia/macrophages express MHC II (C) whereas MHC class I antigen (D) is diffusely expressed in the infarct border. Scale bar=50 μ m.

after induction of photothrombosis in Nissl, Hematoxylin-Eosin or Cresyl Violet-stained tissue sections. After 24 h, eosinophilic coagulation necrosis was fully developed. The infarct zone was sharply demarcated from the surrounding cortex that appeared morphologically intact. Beginning on day 2 and most prominent at day 6, a hypercellular infiltrate surrounded the necrotic infarct zone.

In the infarct core morphological signs of microglial activation were visible as early as 4 h after induction of focal ischemia. Microglia showed shortening of cellular processes and more intense Ox42 staining. At 24 h some amoeboid microglia with rounded cell bodies were present. At three days the number of amoeboid microglia had increased preferentially in the border zone of the infarct, but also extending into apparently vital peri-infarct tissue

(Fig. 1A, B). A significant number of amoeboid microglia expressed ED1 immunoreactivity as an indication for phagocytosis. In the necrotic infarct core non-vital cellular debris exhibited a granular Ox42 immunosignal (Fig. 1A). At day 6, a dense cellular wall surrounded the ischemic lesion, and the number of ED1+ phagocytes had increased, now including both transformed microglia and hematogenous macrophages, as shown in a previous study⁴⁷ (Fig. 1C). Therefore phagocytes beyond day 3 are referred to as a combined microglia/macrophage response in the ischemic lesion. Proportions of microglia/macrophages expressed MHC class II molecules, whereas MHC class I molecule was expressed more widespread (Figs 1E, 2C, D). In confirmation of a recent study²⁶ two microglia/macrophage populations could be distinguished based on the expression

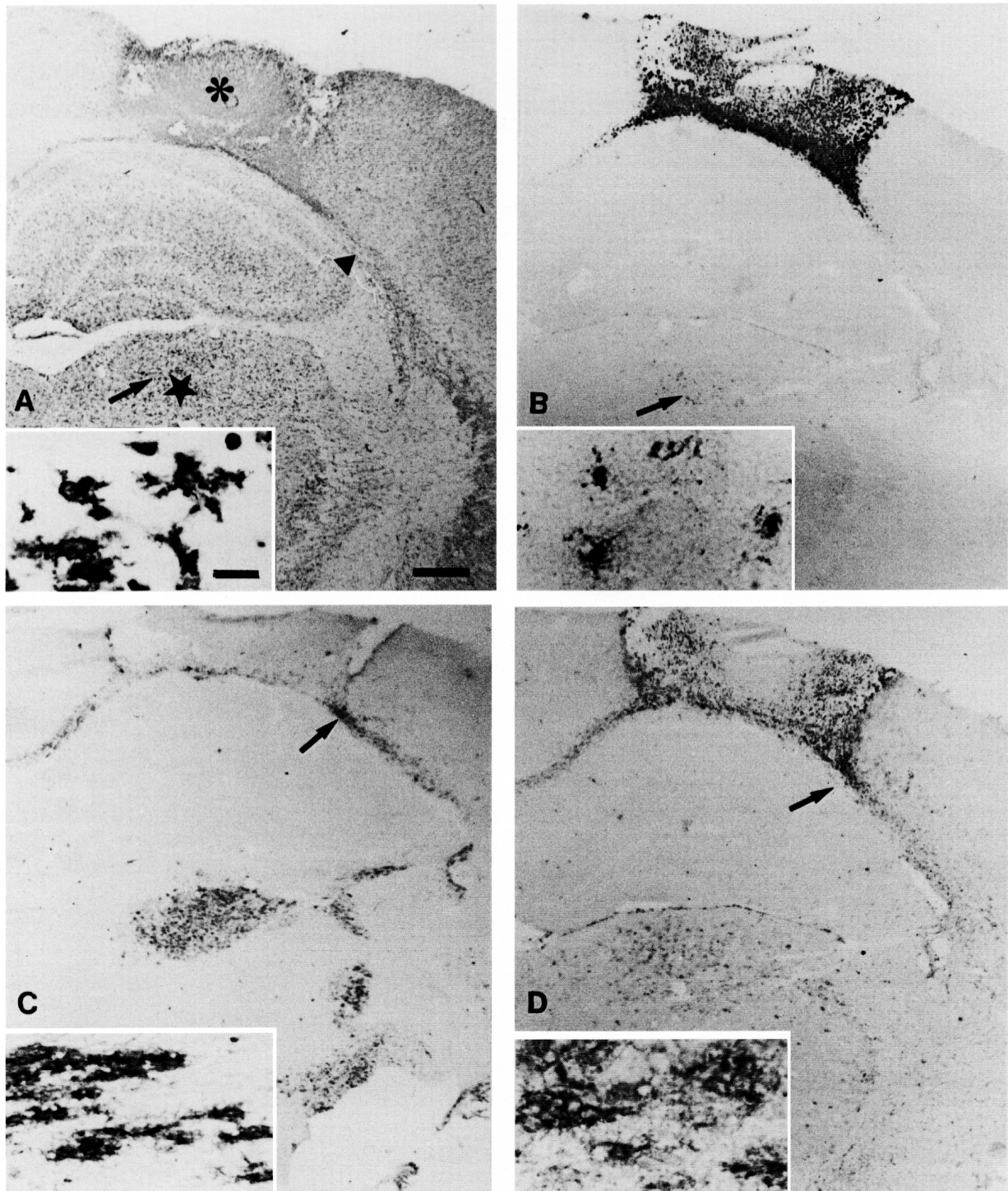


Fig. 3. Microglial responses in the ischemic core (asterisk in A) compared to the fibre tracts undergoing Wallerian degeneration (arrowhead in A) and the thalamus (star in A) at day 14 after photothrombosis. Serial sections stained for Ox42 (A), ED1 (B), MHC class II (C) and CD4 (D). Arrows in (A–D) mark areas where the inset is taken from to show labelled microglia at higher magnification. Note widespread and intense microglia staining in the ischemic lesion, the fibre tracts and the thalamus (inset) with mab Ox42 in (A). (B) is a serial section stained with mab ED1 showing that phagocytic activity is present in the ischemic lesion and to some extent in the thalamus (inset), but almost absent in degenerating fibre tracts (B). Contrastingly, MHC class II expression is low in the ischemic core at that late stage, but strong in degenerating fibre tracts (inset in C) and the thalamus (C). CD4 is predominantly up-regulated in the periphery of the ischemic core, degenerating fibre tracts (inset) and to a lesser extent in the thalamus (D). Scale bars: (A–D)=800 µm, (insets)=40 µm.

of CD4 (Fig. 1D) or CD8 molecules (Fig. 1F). Accordingly, CD8+ cells outnumbered by far CD3+ lymphocytes (Fig. 2A, B). Additionally, few ED2+ macrophages of meningeal origin had infiltrated the infarct from the surface of the brain (Fig. 4G). In the second post-infarct week, ED1/Ox42+ phagocytes spread towards the centre of the lesion so that the infarct was homogeneously infiltrated by phagocytic microglia/macrophages after 14 days (Fig. 3A, B).

Microglial activation in degenerating fibre tracts and nuclei undergoing retrograde degeneration

Microglia activation caused by Wallerian degeneration and retrograde degeneration of subcortical nuclei was seen in commissural, thalamocortical and corticospinal fibre tracts as well as in the posterior thalamic nuclei and lateral geniculate body (Fig. 3). Ramified microglia transformed into stellate microglia and expressed increased Ox42 immunoreactivity after day 3, an activation that was fully developed at day 14. The topography of these changes is depicted schematically in Fig. 5. In addition, a minor but significant proportion of microglia retracted processes, rounded up (inset in Fig. 3A) and expressed ED1 immunoreactivity, an indication of phagocytic activity (inset in Fig. 3B), in retrogradely degenerating nuclei, mainly the thalamus. Overall, this phagocytic response was much less in the thalamus compared to the ischemic focus in the neocortex (Fig. 3B). The vast majority of Ox42+ microglia in degenerating fibre tracts and thalamus still bore characteristic cellular processes and furthermore had up-regulated MHC class II (Figs 3C, 4F) and CD4 expression (Fig. 3D). Although CD8+ microglia/macrophages comprised a substantial proportion of the perilesional phagocytes, they were completely absent from secondary degenerating areas at any time-point studied. ED2+ perivascular microglia (Fig. 4A–C) showed no apparent change in the expression of CD4 or MHC class II molecules (Fig. 4D, E).

Microglial reaction in remote ipsilateral and contralateral neocortical areas

Remote from the photothrombotic lesion the cortex appeared morphologically intact and was devoid of neuronal loss as assessed by Hematoxylin–Eosin, Cresyl Violet and Nissl staining. At day 3 we observed a transient and highly significant increase of Ox42 immunoreactivity and microglial cell density (light grey area in Fig. 5; Figs 6B, E, 7) which was less prominent at day 7 and had disappeared at day 14. We did not observe concomitant expression of MHC class II molecules, CD4 and CD8 in this region. Counts of Ox42+ microglia were significantly increased both compared to the cerebral cortex of normal Wistar rats and compared to the contralateral hemisphere in ischemic animals (Fig. 7).



Fig. 5. Schematic drawing of the topography of microglial activation after focal ischemia. Black area depicts the infarct core, grey areas the degenerating fibre tracts and thalamus, and light grey ipsilateral cortical activation without obvious neuronal or fibre damage.

Perivascular microglia showed no change in the constitutive expression of ED2 and CD4 at any time-point studied.

Treatment with MK-801 significantly reduced numbers of microglia in the ipsilateral cortex remote from the lesion (Figs 6C, 7). However, there was still a statistically significant difference between cellular counts in normal and ischemic rats after MK-801 treatment (Fig. 7). Thus, microglia activation in remote ipsilateral cortical areas could be reduced, but not abolished by MK-801-mediated blockade of spreading depression. After treatment with lubeluzole no difference in microglial activation to naive ischemic rats was observed (Fig. 7). Both MK-801 and lubeluzole did not affect focal microglia activation at the lesion site.

DISCUSSION

This study confirms and extends previous investigations showing that microglia are activated after focal cerebral ischemia.^{33,41,48} Based on the present study three principal patterns of microglial activation can be distinguished: (i) microglial transformation into phagocytes in the border zone of infarction involving a unique CD8+ phagocyte population; (ii) a slowly evolving response in fibre tracts undergoing Wallerian degeneration and in nuclei with retrograde neuronal degeneration; and (iii) a transient ipsilateral activation remote from the infarction that can partly be blocked by pharmacological treatment with MK-801. ED2+ perivascular microglia did not significantly respond in our study.

In the boundary zone of infarction we observed a transition of ramified microglia into amoeboid phagocytic microglia with ensuing infarct demarcation and consecutive infiltration of the necrotic lesion core by phagocytes, a process that involved heterogeneous macrophages.⁴⁷ This combined microglia/macrophage response was accompanied by up-regulation of MHC class I and II molecules and

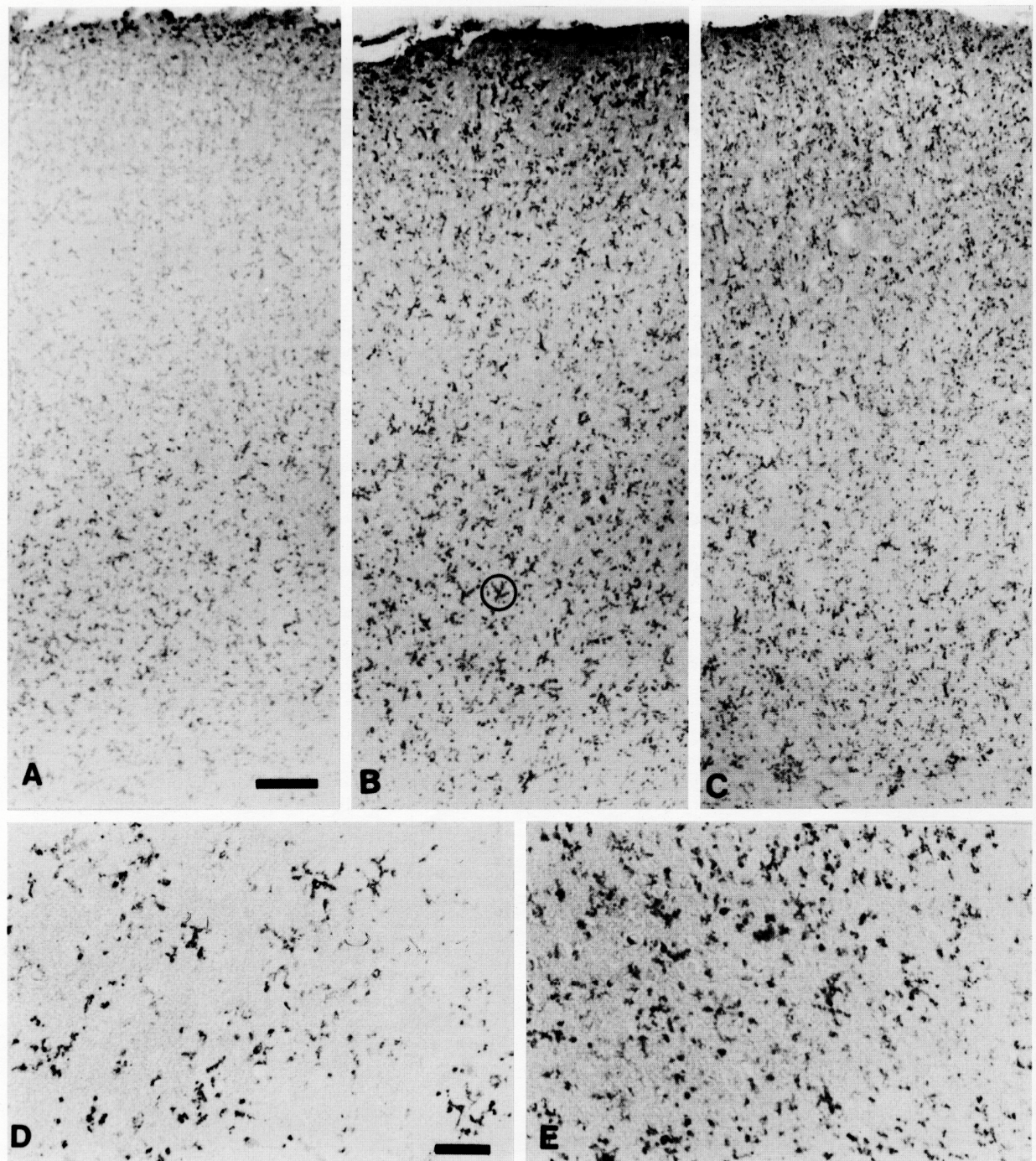


Fig. 6. Microglial activation remote from the lesion (light grey area in Fig. 5) as assessed by immunostaining with mab Ox42. (A and D) show Ox42 labelling of microglia in the normal neocortex. Note that three days after focal ischemia microglia increased in numbers and Ox42 immunostaining was more intense (B, E), a finding that could partly be blocked by MK-801 treatment (C). To further corroborate these findings quantitative data are given in Fig. 7. Circle denotes an example of a single microglial cell as used for counting. Scale bars: (A–C)=100 μ m; (D, E)=50 μ m.

the accessory molecules CD4 and CD8. In our previous studies^{25,26} we had shown that CD4+ and CD8+ microglia/macrophages form two non-overlapping subpopulations in the border zone of the infarct during the first week. CD4 and CD8 are

usually expressed on subsets of lymphocytes and serve as co-receptors in MHC-restricted T-cell responses.²⁷ The functional roles of CD4 and CD8 molecules on microglia/macrophages are unknown at present.

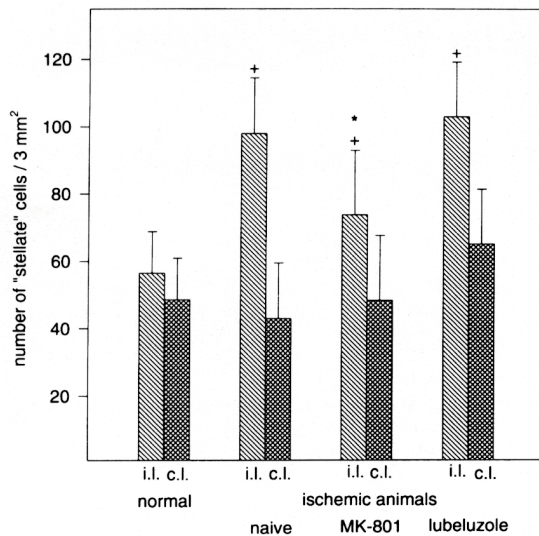


Fig. 7. Quantitative assessment of microglia in the ipsilateral cortex remote from the ischemic lesion based on immunostained sections as shown in Fig. 6. Ox42+ cell bodies were counted in corresponding ipsilateral (i.l.) and contralateral (c.l.) neocortical areas in normal rats and after focal ischemia, and compared to ischemic animals that were treated with either MK-801 or lubeluzole. Note that focal ischemia induces a statistically significant increase in the number of microglia ipsilaterally (+; $P < 0.001$) with no change in the contralateral hemisphere. Treatment with MK-801 partly suppressed this ipsilateral microglial activation (*; $P < 0.01$), while lubeluzole had no effect.

In extension of previous studies we now show that secondary neuronal and fibre degeneration elicits a microglial response different from the ischemic lesion. Similar to the findings at the lesion site, microglial CR3, MHC class II and CD4 expression was increased, although with a delay of days. In contrast, microglial activation was accompanied by a limited phagocytic response. Notably, the population of CD8+ microglia/macrophages was lacking. Microglial activation in fibre tracts after ischemia very closely resembled findings after transection of the optic nerve or fibre tracts of the spinal cord which showed a limited and delayed microglial response.^{17,26,43,50} This points to different signals involved in the activation of microglia. Focal ischemic brain lesions are characterized by a rapid breakdown of the blood-brain barrier and prompt up-regulation of proinflammatory cytokines such as interleukin-1 β and tumor necrosis factor- α in the immediate surrounding.^{1,3} Similarly, transection of a peripheral nerve leads to a strong phagocytic response with rapid clearance of myelin debris^{43,49} and increased steady-state levels of proinflammatory cytokine mRNAs.¹⁸ At present it is unclear whether the contrastingly delayed and ineffective microglial activation observed in secondarily degenerating fibre tracts in focal ischemia and after transection in the CNS is due to the lack of appropriate signalling by cytokines. However, it appears that tissue necrosis with breakdown of the blood-brain

barrier as seen in the infarct core can elicit a rapid microglia/macrophage response even in the CNS and involves a unique subpopulation of CD8+ microglia/macrophages.

Microglia responded not only in neocortical areas with primary or secondary structural damage, but also in the remote ipsilateral cortex that showed no histologically detectable neuronal damage. It is well established that focal ischemia elicits negative deflections, named spreading depression, within the first hours.^{11,48} Spreading depression induced either by focal ischemia or by focal application of KCl to the brain surface consistently led to transient astrocytic activation with increased expression of glial fibrillary acid protein and mRNA^{30,48} that could be blocked by MK-801 treatment. We extend these observations by showing that spreading depression also partly accounts for remote microglial activation in focal ischemia. Microglial responses included increased cell numbers, suggesting proliferation and increased expression of CR-3 immunoreactivity, but was not accompanied by up-regulation of the immunomolecules MHC class II, CD4 and CD8. In contrast to the present study, a previous study¹⁶ reported increased microglial MHC class II expression after KCl-induced spreading depression at 24 h that could be blocked by MK-801. Surprisingly, a concomitant astrocytic activation as consistently observed by others and in the present model,⁴⁸ was lacking in this investigation.¹⁶ In accordance with our findings, Caggiano and Kraig⁸ described increased Ox42/CR3 staining of microglia following spreading depression induced by focal application of KCl. In support of additional mechanisms to spreading depression involved, microglial activation could be augmented by the NO synthase inhibitor L-NAME and blocked by a NO donor. This points to a functional role of the NO cascade. Lubeluzole is an experimental drug that interferes with the NO cascade by inhibiting the NO-induced increase in cyclic GMP.³⁴ Lubeluzole treatment reversed hyperexcitability in the ipsilateral cortex after focal ischemia.⁷ However, lubeluzole did not affect remote microglial responses in our study.

CONCLUSIONS

Microglia can exert multiple functions. In culture systems activated microglia synthesize a variety of potentially harmful soluble factors. These include reactive oxygen and nitrogen intermediates, proteolytic enzymes, arachidonic acid metabolites and proinflammatory cytokines.² At present, it is unclear to what extent the various morphological and immunological activation states of microglia after focal ischemia reflect distinct functions. The spatiotemporal evolution of microglial activation described here may help to elucidate the functional roles of microglial subpopulations in the complex array of events following focal cerebral ischemia.

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REGULAR PAPER

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Dynamic changes of magnetic resonance imaging abnormalities in relation to inflammation and glial responses after photothrombotic cerebral infarction in the rat brain

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Abstract This investigation analyzed the potential of high-resolution magnetic resonance imaging (MRI) at a field strength of 7T to depict leukocyte infiltration and glial responses after focal cerebral ischemia induced by photothrombotic occlusion of cerebral microvessels. For this purpose we superimposed multiparametric MRI (apparent diffusion coefficient, T2, perfusion-weighted, and gadolinium-DTPA-enhanced T1-weighted imaging) on tissue sections stained for phagocytes and astrocytes and, moreover, assessed the regional distribution of tissue pH and ATP content by invasive biochemical methods. Comparing the histological data with the various MRI parameters, high-resolution MRI did not allow a spatial discrimination between distinct areas of phagocyte accumulation or astroglial scar formation, based on image contrast or even quantitative parameter value differences. However, MRI parameters underwent characteristic changes and differentiated distinct stages of tissue remodeling between days 3 and 14 after photothrombosis. Low apparent diffusion coefficient (ADC) and high T2 values indicated an early stage (3 days) with necrosis and beginning glial activation. Normal ADC and reduced T2 elevation characterized an infarct with advanced glial activation and infiltration of hematogenous cells at 7 days after photothrombosis. Heterogeneous ADC together with T2 elevation reflected a late infarct stage (14 days) when pseudocystic degeneration and scar formation had occurred.

Keywords Brain ischemia · Magnetic resonance imaging · Histology · Astrocytes · Microglia

Introduction

Focal cerebral ischemia leads to primary necrosis in the infarct core, but there is increasing evidence that delayed neuronal death by apoptosis contributes to further enlargement of infarcts during the first week [16, 27]. Magnetic resonance imaging (MRI) offers potent tools for following infarct development. A reduced apparent diffusion coefficient (ADC) signal is the most sensitive and earliest indication of focal ischemic brain damage and precedes T2 elevation at early time points after infarction. Between 48 and 72 h ADC normalizes again and previous T2 elevation declines [21, 23, 26]. Later, the area of increased T2 values usually shrinks and ADC increases above normal values [4, 15, 17, 31]. MRI dynamics have so far only been matched to standard tissue sections from focal cerebral infarcts. These sections provide histopathological information about gross infarct development, but lack detailed information about glial responses and inflammation. Glial activation and inflammation by granulocytes, macrophages and T cells are prominent features in the 1st and 2nd post-infarction week (for review see [1, 36]). On one hand these cells are essential components of the tissue remodeling process, on the other hand they may contribute to secondary infarct expansion. Accordingly, immune mediators that are produced by glia and inflammatory cells such as inducible nitric oxide synthase and interferon regulatory factor-1 have been associated with infarct expansion between days 1 and 3 ([18, 19]; for a review see [2]). With respect to their potential contribution to infarct development, we investigated whether glial activation and inflammatory responses can be delineated noninvasively by high-field high resolution MRI which allows structures down to about 100 μm or even below to be differentiated.

Small cerebral infarctions were induced by photothrombotic occlusion of cerebral microvessels [34]. This model was chosen because lesions are highly reproducible in size and location and show a predictable and sequential pattern of glial activation and leukocyte infiltration as im-

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portant prerequisites for the correlation of histological events with MRI changes. In photothrombosis the activation of the intravenously (i.v.) injected photosensitive dye, rose bengal, by a light beam through the intact skull results in free-radical formation, endothelium alterations and thrombus formation in small vessels of the illuminated cerebral cortex. Photothrombosis produces a circumscribed infarction without an appreciable zone of incomplete ischemia, a penumbra, which may interfere with clear visualization of the infarct boundaries in MRI (cf. [13]). Patterns of postischemic inflammation in photothrombosis are similar compared to focal ischemia induced by middle cerebral artery occlusion (MCAO; [20, 25, 32, 33, 36]). While previous MRI studies mainly followed the evolution of cerebral infarction during the first hours after onset, the focus of this correlative analysis was at later time points, i.e., 3, 7, and 14 days after induction of focal ischemia, when glial and inflammatory responses dominated the histological appearance of the infarcts [20]. Two major questions were addressed: (1) does multiparametric MRI allow monitoring of principle stages of infarct demarcation and tissue remodeling, and (2) can infarct subareas covered by activated glia or infiltrating phagocytes be delineated noninvasively?

Materials and methods

Animal preparation

All animal experiments were performed in accordance with NIH animal protection guidelines and had been approved by the local authorities.

Photothrombosis was induced in male Wistar rats (280–320 g) under anesthesia with enflurane in a 2:1 nitrogen/oxygen atmosphere, as previously described [33]. During anesthesia rectal temperature was kept at 36.5–37.5°C with the help of a servo-controlled heating blanket. In brief, the photosensitive dye rose bengal was injected i.v. via a femoral catheter and the brain was illuminated through the exposed skull with a cold light source centered at –4 mm from bregma and 4 mm laterally to the midline for 20 min. This resulted in a focal cerebral ischemia of reproducible shape and size in the sensorimotor cortex. Afterwards, wounds were sutured and rats allowed to recover.

For the MRI measurements, animals ($n=3$, each time point) were re-anesthetized once at days 3, 7 or 14, respectively, by halothane (1.5%) in 70/30 v/v N_2O/O_2 . Rectal temperature was monitored throughout the experiment and was kept constant at 37°C using a feedback-controlled heating pad with circulating warm water. The animals were tracheotomized, mechanically ventilated, and paralyzed with pancuronium bromide (0.3 mg/kg per h). After the beginning of mechanical ventilation the halothane concentration was reduced to 0.8–1.0%. A femoral artery and vein were cannulated for injection of drugs, monitoring of systemic blood pressure, and blood sampling. Blood gases were frequently analyzed and kept within physiological limits by appropriate settings of the respirator and/or by i.v. application of bicarbonate. Thus, mean arterial blood pressure was kept in a range of 80–130 mmHg.

MRI measurements

High-resolution multiparametric MR measurements were performed on a 7T BIOSPEC DBX experimental scanner (Bruker Medical, Ettlingen, Germany) with a 30-cm bore magnet and actively shielded gradient coils (200 mT/m; rise time <250 μ s). A 12-cm

Helmholtz coil was used for rf transmission; signal detection followed via a 23-mm inductively coupled surface coil placed over the skull of the animal. The rf coils were decoupled from each other, the transmitter coil actively, and the receiver coil passively.

Using gradient echo imaging, sagittal pilot scans were performed to assure accurate positioning of the animal in the magnet. For this purpose the animal was placed with the central image plane of multislice image experiments 5.9 mm posterior to the rhinal fissure. This position was identical with the isocenter of the magnet.

For a detailed comparison with histological data different NMR imaging techniques were used. Where not described otherwise, a field of view of 2.5 cm, a matrix of 256×256 and a slice thickness of 1.25 mm were used for all sequences.

Diffusion weighted imaging (DWI) was performed on seven contiguous coronal slices using a Stejskal-Tanner spin-echo sequence (echo time (TE)=25.8 ms, repetition time (TR)=2325 ms). To enable quantification of the ADC of brain water, two b values were used (30 and 1,500 s/mm^2). To minimize instrumental errors in the ADC determination, extensive data postprocessing, including correction for image-specific background noise and gradient crosstalk was performed as described in detail elsewhere [11]. ADC was calculated pixel-wise by solving the monoexponential intravoxel incoherent motion (IVIM) model of [24]. For this purpose, the MEMRIS software package [37], written in Interactive Data Language (IDL, Research Systems, Boulder, USA) was used.

Multislice T2-weighted imaging (T2WI) was performed with a multislice-multiecho CPMG sequence (TE=12.5 ms, TR=3000 ms) to record seven slices in positions identical to those of the DWI experiment. Sixteen echoes were collected for calculation of brain water T2.

Perfusion-weighted imaging was performed with an ultrafast version of the arterial spin tagging technique [9, 22]. Three coronal slices (slice thickness 1 mm) with an interslice gap of 1.25 mm were recorded, thus covering the larger part of the ischemic lesion. The perfusion-weighted imaging sequence consisted of two similar acquisition intervals separated by a recovery time of 10 s, each of which comprised a magnetization preparation step followed by snapshot FLASH (TE=4.4 ms, TR=7.6 ms, matrix=128 $\times$ 64). During the first image acquisition (the perfusion-sensitive image), spins of blood flowing through the neck vessels were inverted adiabatically through the combination of a magnetic field gradient applied in the z-direction and a B1 field set off-resonance to excite a slice through the neck approximately 2 cm upstream from the imaging plane. During the second acquisition phase (control image) the sign of the frequency offset of the B1 field was inverted so that inflowing spins were left undisturbed. Perfusion-weighted images (PWI) were obtained after subtraction of both images.

The status of the blood-brain barrier was qualitatively assessed at the end of the MR investigation by injection of gadolinium-DTPA (Gd-DTPA, i.v. injection, 0.1 ml, Schering, Germany). Gd-DTPA-enhanced images were obtained using a T1-weighted imaging sequence (TE=8.3 ms, TR=400 ms) before and after injection of the contrast medium. Contrast enhancement was identified by subtraction of the images obtained after the injection of Gd-DTPA from precontrast images.

Histology

After completion of MRI measurements the animals were killed under deep anesthesia by halothane/ N_2O/O_2 , entirely frozen in liquid nitrogen and stored at –80°C. Brains were carefully removed and serial coronal sections were cut through the lesion at –20°C. Thin sections (20 μ m) were processed for histology, ATP bioluminescence, and pH fluorescence, and 10- μ m sections for immunocytochemistry at 400- μ m intervals. Histology comprised staining with cresyl violet, hematoxylin-eosin, and a method for silver impregnation of cell bodies (slightly modified from Merker [28]) to depict necrotic areas, areas of hypercellular infiltrate and selective neuronal necrosis. Immunocytochemistry made use of biotinylated anti-mouse IgG secondary antibody (1:100; Vector, Burlingame,

Calif.), Vector Elite ABC kit, diaminobenzidine (Sigma), and the following primary antibodies: ED1 (1:2,000, Serotec, Oxford, UK) recognizing phagocytic microglia/macrophages and anti-gial fibrillary acidic protein (GFAP; 1:50, Boehringer, Mannheim, Germany), a marker for astrocytes.

Biochemical data

Pictorial analysis of ATP content and tissue pH were performed on serial 20- μ m cryostat sections. Briefly, the method of pH measurement followed the umbelliferone fluorescence technique by Csiba et al. [7]. The resulting pH images were calibrated with graded pH standards. The regional determination of ATP content used the substrate-specific bioluminescence method [8]. The local metabolite concentration was quantified by correlation of the optical densities of the bioluminescence images with metabolite values measured in small tissue samples by conventional enzymatic analysis.

Image processing and statistics

Histological and immunocytochemical sections were scaled, digitized with the help of a 256 $\times$ 256 bit CCD camera (Sony) and a DVS video processing unit (Hamamatsu, Hersching, Germany). The extent of ischemic necrosis, cellular infiltration within the necrosis, microglia and astroglia activation in the adjacent vital tissue were outlined and images were superimposed on MR images using NIH image software (NIH, Bethesda, USA) on a Macintosh Power PC (Apple, Cupertino, Calif.). For data correlation, all images were normalized to standard image size and resolution using linear interpolation.

Based on the analysis of the superimposed histological and immunocytochemical sections, regions of interest were outlined interactively on the MR parameter maps. Calculated mean values were normalized to the MR signal intensities of corresponding homotopic, contralateral area and expressed as percent values.

Data were expressed as mean values $\pm$ standard deviation (SD). Statistical differences between all three groups were calculated using Student's *t*-test (unpaired). Statistical significance was accepted for $P < 0.05$.

Results

Three days after infarction

Photothrombotic infarcts were analyzed at three different stages of maturation, i.e., at 3, 7, and 14 days after induction of the ischemic event. Three days after photothrombosis histology showed focal pannecrosis in a conical area of the sensorimotor cortex (Fig. 1A). Accordingly, biochemical data showed complete ATP loss (Fig. 1B) and sustained alkalosis (Fig. 1C). Cresyl violet and silver impregnation methods still revealed a few "ghost neurons", indicative of neuronal death at the border of the infarcted area (Fig. 1D, F, arrows).

By means of histology and immunocytochemistry three distinct subareas (α , β , γ) within the infarction could be differentiated as indicated in Fig. 1A. The core of the infarct (designated as " γ ") was virtually devoid of vital cells (Figs. 1A, 2D). Immunocytochemistry revealed accumulation of ED1⁺ phagocytes in a border zone (designated as " β "; Figs. 1A, 2F), which at day 3 represents transformed microglia as shown in a previous study [34]. In the adjacent vital tissue, GFAP staining depicted a rim of astrocytic hypertrophy designated as " α " (Fig. 2 H),

whereas GFAP⁺ immunoreactivity was completely absent within the infarction zones (β and γ). MRI depicted photothrombotic infarctions as circumscribed areas of reduced ADC and as hyperintensities after Gd-DTPA injection (Fig. 2A, B). ADC (Fig. 2C showing boxed detail of Fig. 2A) was lowered within the infarct core to $72 \pm 9\%$ of ADC values in the homotopic contralateral area, but preserved normal values in adjacent tissue. In contrast, the T2 relaxation time was increased ($129 \pm 12\%$ of contralateral side), and the corresponding area encompassed surrounding vital tissue (Fig. 2E). In PWI (Fig. 2G) the β and γ areas showed severe hypoperfusion. The zone α as well as the vital tissue immediately adjacent to it were slightly hypoperfused, but PWI further normalized within a short distance from the infarct (Fig. 2G). Within this zone of hypoperfusion, hyperperfused spots were congruent with dilated vessels in corresponding histological sections (arrows, Fig. 2D, G).

Seven days after infarction

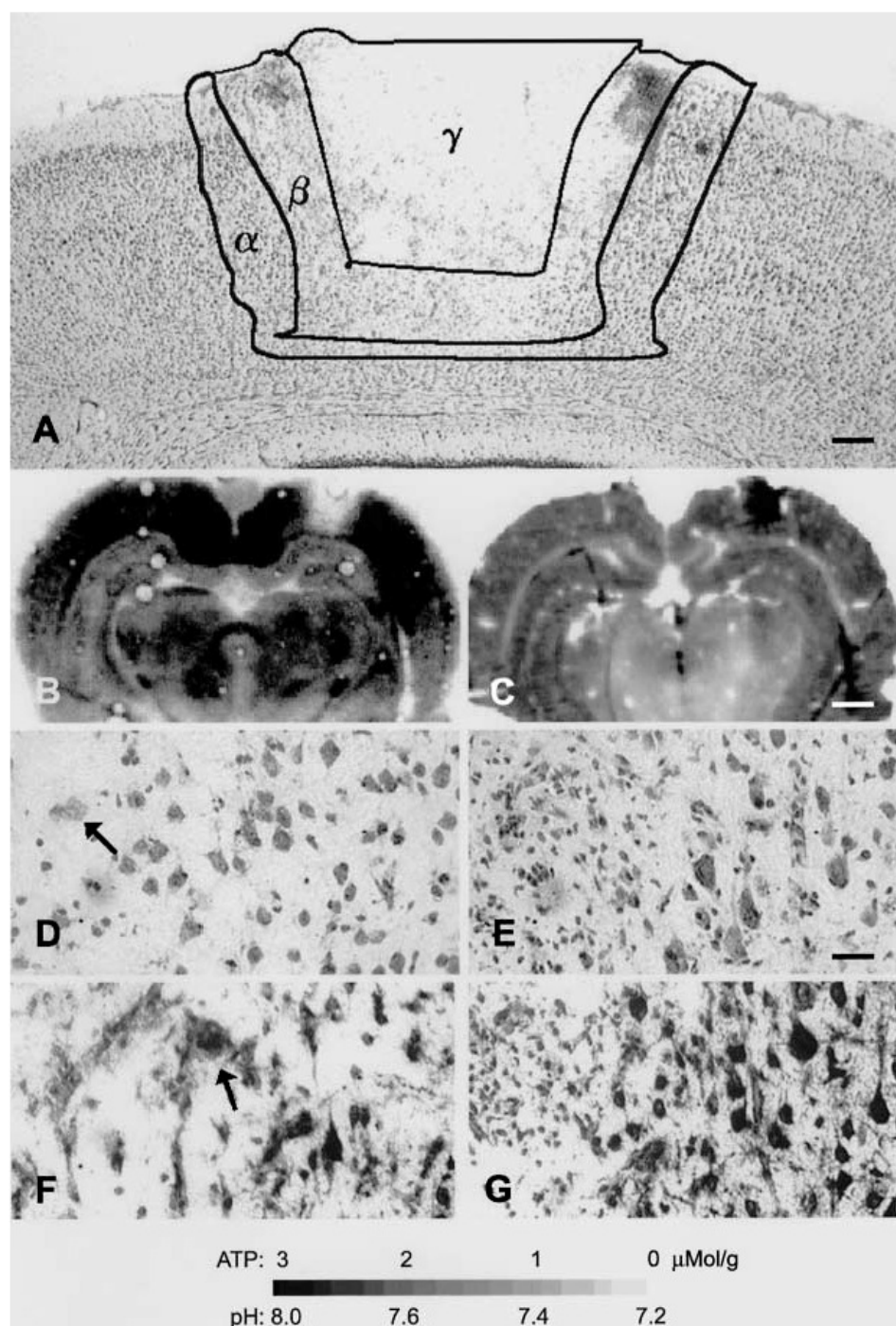
At 7 days after photothrombosis the infarct was well demarcated from the surrounding vital tissue in cresyl violet-stained sections (Fig. 3D). Histologically, the signs of ongoing neuronal loss at the immediate vicinity of the infarct that were visible at 3 days were no longer seen (Fig. 1F, G). A dense ring of phagocytes defined the β zone (Fig. 3D, F), now comprising both transformed microglia and hematogenous macrophages as shown previously [34]. Some phagocytes began to infiltrate the core of the lesion (γ). In surrounding vital tissue, activation of GFAP⁺ astrocytes (Fig. 3H) had further proceeded in comparison to day 3, defining an area of glial activation within vital tissue outside the phagocytic rim (α).

In corresponding sections the entire infarct lighted up after administration of Gd-DTPA (Fig. 3B). In contrast to day 3, ADC (Fig. 3A, C) had returned to normal values at 7 days ($102 \pm 30\%$ of contralateral values). The T2 relaxation time was still increased ($113 \pm 4\%$; Fig. 3E), but had significantly declined compared to the strongly increased T2 values at 3 days (Fig. 2E; $129 \pm 12\%$ vs $113 \pm 4\%$, $P < 0.01$). Detailed analysis showed ADC and T2 values only lightly elevated in area γ compared to areas β and α . PWI showed persisting hypoperfusion of the infarct with a gradual increase to normal values in α (Fig. 3G). Hyperperfused spots again corresponded to dilated vessels (Fig. 3D, G, arrows).

Fourteen days after infarction

At 14 days after photothrombosis the infarct area was entirely covered by ED1⁺ phagocytes (Fig. 4F). The infarcted cortex underwent atrophic changes and cystic degeneration, and scar formation occurred (Fig. 4D, F). GFAP staining revealed that the area of glial activation (α) had narrowed compared to earlier time points and astrocytes had begun to invade the infarct resulting in scar formation (Fig. 4H).

Fig. 1A–G Histopathological and biochemical characterization of infarcts at day 3 after photothrombosis. **A** Three sub-areas of cortical infarction are outlined on a cresyl violet-stained tissue section: a necrotic core region almost devoid of cells (γ), a pannecrotic border zone infiltrated by phagocytes (β), and a surrounding area of glial activation within vital tissue (α). **B** Note homogeneous loss of ATP within the entire lesion and the sharp demarcation towards normal tissue without an intermediate penumbra zone (see text). **C** pH measurements show severe alkalosis. **D–G** Sections stained by cresyl violet (**D,E**) or by silver impregnation (**F,G**) show neurons undergoing delayed degeneration at the infarct border at 3 day (arrows in **D,F**). Note that at day 7 there is no more histological evidence for ongoing neuronal death and the infarct area is sharply demarcated from normal cortex (**E,G**). *Grayscale depicts quantitative values of pH and ATP.* Bars **A** 175 μm ; **B,C** 350 μm ; **D–G** 20 μm



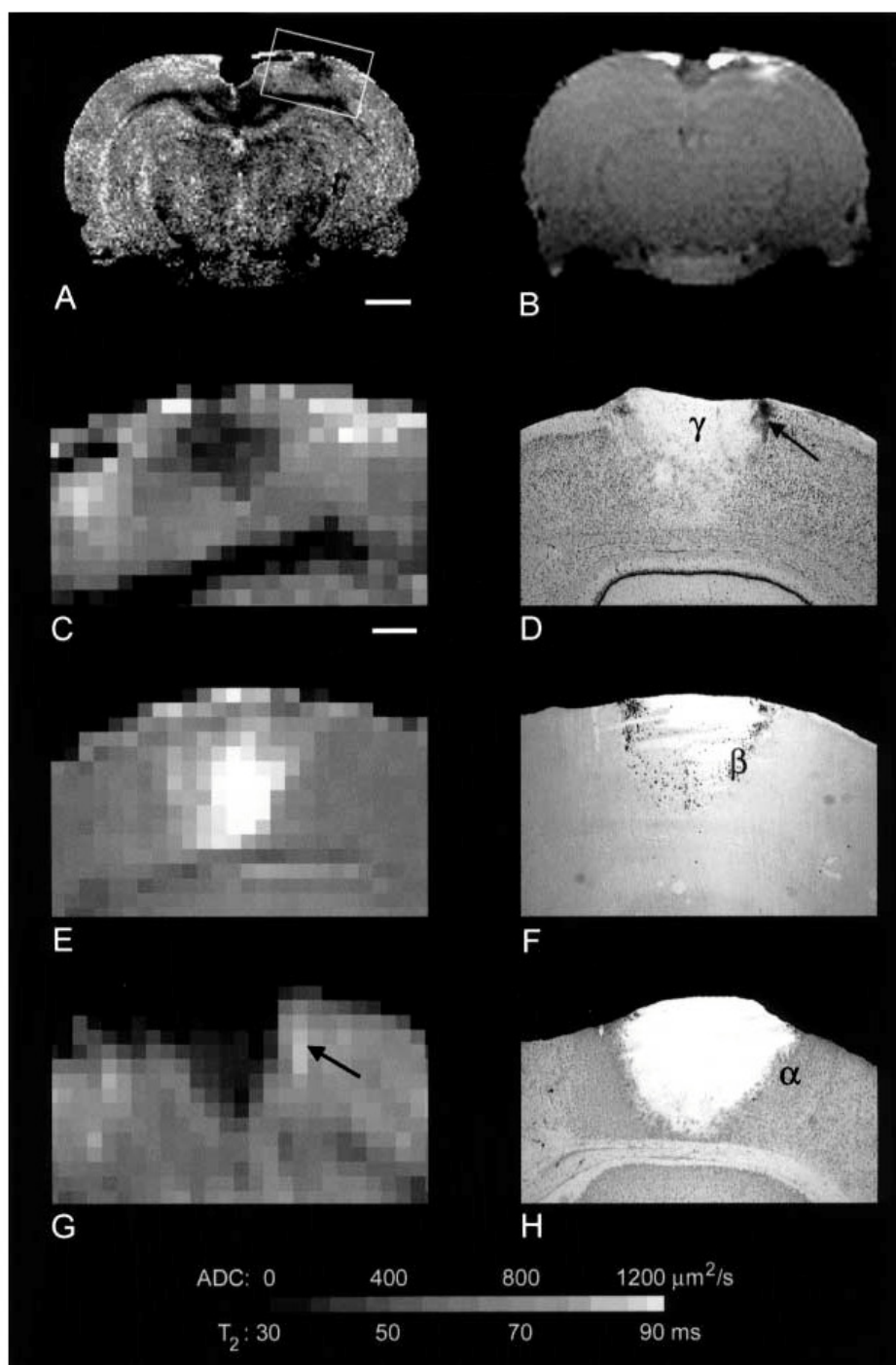
MRI showed that ADC values of the infarct had further increased compared to day 3 and 7 ($123 \pm 10\%$ of contralateral side) and showed a heterogeneous pattern (Fig. 4A, C). ADC elevation was distributed in a rim-like fashion in the outer zone of the infarct, where pseudocystic degeneration preferentially occurred. T2 images showed spot-like hyperintensities (Fig. 4E) that corresponded to pseudocysts in histological sections (Fig. 4D, F). Severe hypoperfusion of

the infarct persisted, but in adjacent tissue perfusion had recovered to normal values (Fig. 4G).

Discussion

In the present investigation, we examined the evolution of photothrombotic cerebral infarcts in rats using multipara-

Fig. 2A–H Matching of MR signals by overlay with histologically and immunohistochemically stained tissue sections at day 3 after photothrombosis. **A, C** MRI: ADC signal, **B** Gd-enhanced T1 image, **E** T2 image, and **G** PWI. **D, F, H** Sections were stained with cresyl violet for histology (**D**), with mAb ED1 recognizing phagocytes (**F**) and mAb against GFAP (**H**), a marker for astrocytes. Phagocytes form a small ring around the necrotic infarct core (**D**) and are themselves surrounded by activated astrocytes (**H**). Note low ADC (**C**) and high T2 (**E**) values in the infarct area. The hypercellular rim itself does not lead to a distinctive MR signal. The hyperperfused spot at the right superficial margin of PWI (**G**) corresponds to a dilated vessel visible on the cresyl violet-stained section in (**D**). *Grayscale depicts quantitative values of ADC and T2 (MRI magnetic resonance imaging, ADC apparent diffusion coefficient, Gd gadolinium, PWI perfusion-weighted images, GFAP glial fibrillary acidic protein). Bars A (also for B) 2 mm; C (also for D–H) 500 μ m*



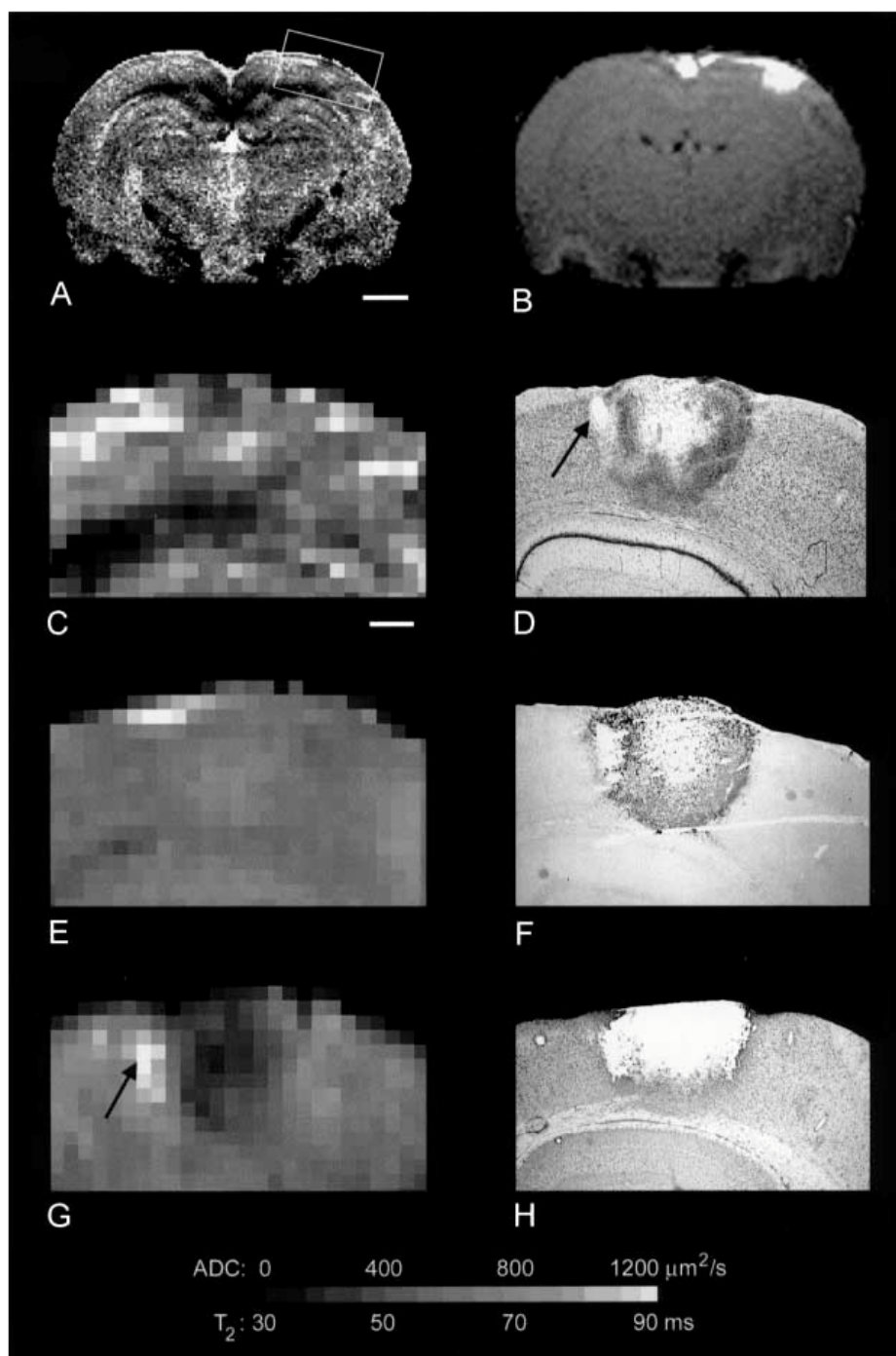
metric high resolution MRI, and compared these findings with evolving cellular responses. In contrast to previous investigations [12], we focused on delayed cellular responses at days 3, 7, and 14 which showed characteristic patterns of glial activation and inflammation by hematogenous macrophages [20, 33].

The MR images documented a reproducible sequence of changes after photothrombotic cerebral infarctions. The

following three stages of postacute infarct maturation could be distinguished.

1. At day 3, which was histologically characterized as necrosis with activated glia and few inflammation, infarcts showed homogeneously lowered ADC and increased T2 values. At this stage delayed neuronal death still occurred.

Fig. 3 Matching of MR signals by overlay with histologically (D) and immunohistochemically (F phagocytes, H astroglia) stained tissue sections at day 7 after photothrombosis (composed as in Fig. 2). While the ischemic lesion shows a uniform enhancement after Gd application (B), the infarction is hardly discernible in the ADC image (A, C), and the T2-weighted image is almost isodense (E). PWI still reveals hypoperfusion (G). The hyperperfused spot in G on the right infarct margin corresponds to a dilated vessel in (D). Cresyl violet (D) and ED1 (F) stainings now envisage a dense rim of phagocytes covering more than half of the lesion that again has no specific signal correlate on corresponding MR images. Grayscale depicts quantitative values of ADC and T2. Bars A (also for B) 2 mm; C (also for D–H) 500 μm

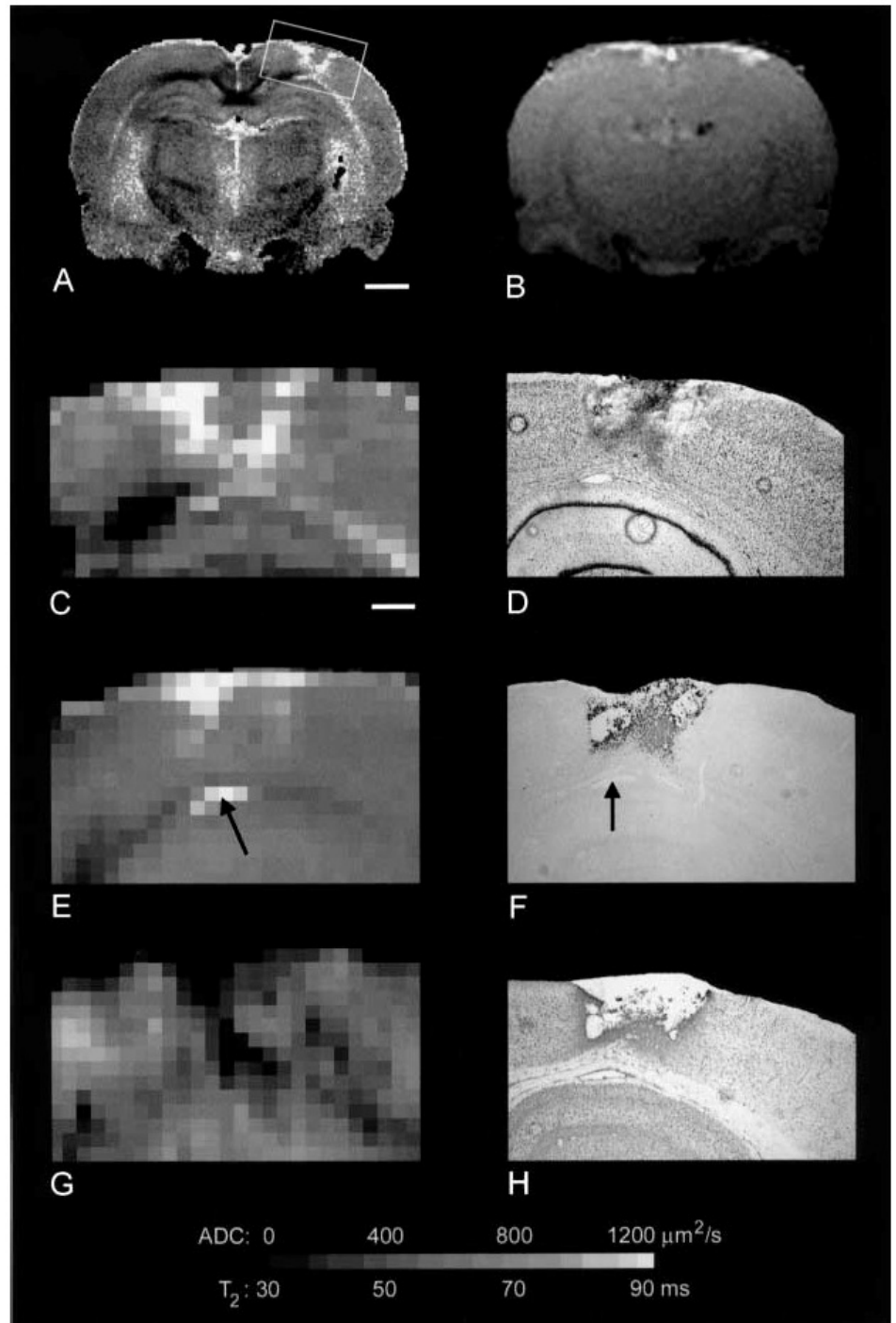


2. At the time of considerable macrophage infiltration in the infarct border zone at day 7, ADC had normalized in the entire infarct region and elevation of the T2 relaxation time had decreased.
3. At day 14, the latest stage of infarct development examined, infarcts were entirely covered by phagocytes and underwent colliquation, pseudocystic degeneration

and scar formation. At this time, ADC had increased preferentially in the outer zone of infarction and increased T2 values were restricted to areas of cystic degeneration.

Overall, our findings are in accordance with the dynamic evolution of MRI abnormalities reported after experimen-

Fig. 4 Matching of MR signals by overlay with histologically (D) and immunohistochemically (F phagocytes, H astroglia) stained tissue sections 14 days after photothrombosis (composed as in Fig. 2). The infarct now contains cystic structures (D) and is completely covered by phagocytes (F) surrounded by and partly overlapping with a well-developed astroglial scar (H). ADC has further changed towards increased levels preferentially at the outer circumference of the infarct zone (A, C), Gd enhancement has decreased (B) and T2 levels remained normal except in areas of pseudocystic degeneration (E) (corresponding regions are marked by arrows in D and E). Grayscale depicts quantitative values of ADC and T2. Bars A (also for B) 2 mm; C (also for D–H) 500 μm



tal MCAO in rats and in humans with two exceptions [4, 15, 17, 21, 23, 26, 31]. First, in contrast to our present study, MRI signal abnormalities are inhomogeneous after MCAO. Signal abnormalities of subcortical areas often precede changes of cortical areas which is explained by a different timing of neuronal death in these areas [14]. Second, changes of “tissue signatures” occur much faster af-

ter transient MCAO than after photothrombosis [21]. In studies of transient ischemia in rat brain ADC returned to normal values in subcortical necrotic structures already after 48–72 h [21, 23, 26]. These differences may be further accentuated by differences between models of transient and permanent ischemia. In fact, reperfusion accelerates and increases inflammatory responses [3, 6]. Inter-

estingly, in human stroke initial abnormalities of diffusion-weighted images persisted for weeks [31, 38]. Different severity of ischemia and/or species-dependent differences could account for these discrepancies. Thus, it is especially important to correlate MRI signals with histopathological changes to define MRI parameters that allow a model-independent prediction of the stage of infarct maturation.

We therefore investigated whether the changes in MRI parameters reflect specific cellular responses as an indication of different stages of infarct maturation. MR images were overlaid with corresponding histological sections stained for ED1, a marker for phagocytic microglia and macrophages, and GFAP to identify the distribution of scar-forming astrocytes. At the stage of phagocytosis microglia and macrophages are not distinguishable by immunocytochemical markers, but previous investigations have shown that the population of ED1⁺ phagocytes encompassed transformed microglia and infiltrating blood-derived macrophages [34]. As described in detail previously [20, 34] at day 3 phagocytes formed a characteristic small ring around the lesion which had significantly enlarged at day 7. At day 14, the entire lesion was covered by ED1⁺ phagocytes. This discrete distribution of the cellular components (astrocytes, phagocytes) was not reflected in readily identifiable MRI patterns that would permit the regional assignment to the various stages of inflammation within a given lesion. This lack of spatial correlation, however, is not due to the limited resolution of MRI technology used, because high field MRI was in fact able to identify substructures of dimensions with the range of a few micrometers such as dilated vessels (Fig. 2, arrows) or pseudocysts (Fig. 4, arrows). Rather, it emphasizes that the physical parameters, accessible and measured by MRI, do not primarily distinguish cell types but rather detect structural details of tissue types as well as, e.g., tissue water content and extracellular space structures.

In conclusion, with the current MRI parameters it is possible to determine noninvasively the overall stages of infarct development and tissue remodeling in well-defined settings such as photothrombosis. It is not yet feasible, however, to delineate responses of discrete cellular populations such as phagocytes and astrocytes. At present, this would require prelabeling of circulating cells by a magnetic contrast agent. Recently, a technique for magnetic labeling of human mononuclear cells has been established in vitro, but not yet applied in vivo, by exploiting their ability to incorporate monocrySTALLINE iron oxide nanoparticles [35]. In experimental animals suffering from experimental allergic encephalomyelitis, an iron oxide-containing contrast agent, when injected systemically, could be detected by MRI in the brain. Although MRI were not directly superimposed on histological sections T2 hypointense spots were distributed similarly to histologically verified macrophage infiltration. Additional electron microscopy studies revealed that macrophage-like cells had taken up the iron oxide particles [10]. As another approach it was possible to depict structural details such as granule cell layers and microscopic plaques with

MR microscopy in human hippocampal specimens taken from patients with Alzheimer's disease [5]. Thus, it appears possible to extend the spatial resolution of MRI under in vivo conditions to facilitate noninvasive monitoring of inflammation processes. This would be of clinical importance; increasing experimental evidence suggests that focal cerebral infarcts still grow beyond day 1. Although the molecular mechanisms of this prolonged infarct growth are not yet understood, immune mediators such as nitric oxide and interferon regulating factor, and possibly inflammatory cells have been implicated [18, 19, 36]. The noninvasive observation of the effects of therapeutic interventions at these posts ischemic immune-mediated reactions requires such development of new MRI sequences and further improvement of spatial resolution.

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REGULAR PAPER

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CD8⁺ phagocytes in focal ischemia of the rat brain: predominant origin from hematogenous macrophages and targeting to areas of pannecrosis

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Abstract We have recently described a novel population of CD8⁺ phagocytes that are strongly recruited to focal ischemic lesions of the rat brain but absent from axotomized central fiber tracts. To assess the relative contribution of infiltrating macrophages and resident microglia to the CD8⁺ phagocyte response, we selectively depleted peripheral macrophages by systemic administration of dichloromethylene diphosphonate-filled liposomes prior to the induction of permanent ischemia by photothrombosis of cortical microvessels. Macrophage depletion led to a dramatic reduction but not complete abolishment of CD8⁺ cells in the ensuing infarcts. Systemic administration of monoclonal antibody Ox-8 eliminated CD8⁺ cells from peripheral lymphoid organs but had no effect on CD8⁺ phagocytes in the ischemic brain lesions. To further characterize the lesion conditions inducing the recruitment of CD8⁺ phagocytes, we induced mild focal ischemia by transient occlusion of the middle cerebral artery that leads to a core infarction with ischemic pannecrosis surrounded by areas with selective neuronal cell death. Recruitment of CD8⁺ phagocytes was restricted to areas of ischemic pannecrosis. In areas undergoing selective neuronal loss microglia up-regulated complement receptor-3, exhibited ED1 immunoreactivity (indicating phagocytic activity), and to some extent expressed CD4, but not CD8 antigens. In conclusion our present study shows that CD8⁺ phagocytes in focal brain ischemia are predominantly derived from hematogenous macrophages and selectively target to areas of ischemic pannecrosis.

Keywords Focal ischemia · Microglia · Macrophage · CD8 · Selective neuronal loss

Introduction

Focal cerebral ischemia induces activation of resident microglia and astrocytes as well as recruitment of hematogenous granulocytes, T cells, and macrophages. The role of this inflammatory response in the exacerbation of neuronal death and in tissue remodeling after cerebral ischemia is incompletely understood (for a review see [42]). Microglia/macrophages secrete a number of both toxic and protective effector molecules and thereby play a key role in inflammatory CNS processes [1, 3, 4, 16, 28]. We and others have shown that microglia/macrophage responses following focal cerebral ischemia are heterogeneous with respect to the spatio-temporal pattern of cellular activation and the cell types recruited [25, 29, 39, 40]. In permanent focal ischemia microglia in the border zone of the infarction exhibit an early transformation into phagocytes, whereas hematogenous macrophages are recruited with a delay of several days and secondarily contribute to infarct demarcation and removal of debris [39]. This is accompanied by up-regulation of the complement receptor-3 (CR-3), expression of the lysosomal marker ED1 and increasing levels of CD4 expression. Using confocal microscopy and mRNA analysis by reverse transcriptase-polymerase chain reaction we recently defined a distinct population of Ox42/ED1⁺ microglia/macrophages that express the CD8 α/β molecules so far only described on cytotoxic/suppressor T cells. Interestingly, CD8⁺ phagocytes were not detectable in paradigms of either degenerative or immune-mediated phagocyte responses in the CNS [25], suggesting a specific involvement of these cells in the pathogenesis of ischemic brain damage. Independently from our *in vivo* findings, Hirji and colleagues [18, 19, 31] recently detected CD8 antigens on the surface of alveolar macrophages *in vitro*, thereby principally confirming the expression of CD8 antigen on cells of the mononuclear phagocyte lineage.

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Since activated resident microglia and infiltrating macrophages share all available immunological surface markers the relative contribution of each cell type to CD8⁺ phagocyte responses after focal brain ischemia is currently unknown. In our present study we therefore assessed the origin of CD8⁺ cells in ischemic brain lesions by the systemic administration of dichloromethylene diphosphonate (Cl2MDP)-filled liposomes that selectively deplete peripheral macrophages but leave resident brain microglia unaffected [5]. In additional experiments, CD8⁺ cells were depleted by systemic administration of the CD8-specific monoclonal antibody (mAb) Ox-8. In all depletion experiments, permanent focal ischemia was induced noninvasively by photothrombosis of cortical microvessels because the ensuing infarctions in this model show little variation in size and location and induce highly predictable microglia/macrophage responses [24, 39, 44] as a key requirement for the reliable assessment of changes occurring after depletion. To specifically define the lesion conditions inducing CD8⁺ phagocyte responses, we extended our previous studies in permanent brain ischemia to a model of transient ischemia that was induced by the intraluminal suture technique originally described by Zea Longa et al. [45]. This procedure leads to a core infarction with pannecrosis of neurons and glial cells surrounded by a clearly distinguishable zone of selective neuronal death with loss of large pyramidal neurons but preservation of endothelial and glial structures [9, 14, 30].

The main conclusion of this study is that divergent macrophage/microglia subpopulations appear as a general phenomenon in ischemic CNS lesions. Among these, CD8⁺ phagocytes are predominantly derived from hematogenous macrophages and selectively target to areas of ischemic pannecrosis.

Material and methods

Animal experiments were performed according to German and European Communities Guidelines (86/609/EEC) with special efforts to minimize animal suffering, and to choose appropriate animal numbers. Wistar rats were obtained from the breeding facility of the University of Duesseldorf. Two models of cerebral ischemia, transient occlusion of the middle cerebral artery (MCAO) and photothrombosis, were used. Focal ischemia was induced under enflurane/N₂O/O₂ anesthesia in spontaneously breathing rats. In each experimental group at least four animals were kept for 3, 6 (photothrombosis) or 7 (MCAO), and 14 days after ischemia before they were decapitated under deep ether anesthesia. Then, brains were rapidly removed, frozen in isopentane, and stored at -80°C before further histological and immunocytochemical processing.

Depletion experiments in permanent focal ischemia induced by photothrombosis

Photothrombosis of cortical microvessels was induced as described in detail previously [24, 39, 44]. Briefly, Wistar rats received intravenous injections of the photosensitive dye rose bengal (0.13 mg/kg body weight). The brain was illuminated through the exposed intact skull for 20 min at a stereotactically defined position 4 mm posterior to bregma and 4 mm laterally to the midline. This leads to local activation of the dye by the light beam and thrombus formation in small vessels with ensuing pure cortical infarcts of approximately 2.5-mm diameter (Fig. 1A).

Hematogenous macrophages were selectively depleted by the "macrophage suicide technique" using the systemic administration of Cl2MDP-filled liposomes according to van Rooijen [43]. In our present study to save animals we used stored brain tissue from our recently published experimental series [39] for the immunocytochemical analysis of CD4 and CD8 antigen expression following macrophage depletion. In those previous experiments, mannoseylated Cl2MDP-containing liposomes had been injected intravenously 2 days before, immediately after and 2 days after induction of photothrombosis. This resulted in an almost complete depletion of hematogenous macrophages at the time of ischemia onset and for the following 8 days. Control animals received saline-filled liposomes or saline solely.

Fig 1 Frontal cresyl violet-stained histological sections of rat brain after photothrombosis (A) and transient MCAO (B). α denotes areas of pannecrosis in A and B, β the area of selective neuronal loss in B, which is lacking in photothrombosis (A), and γ denotes apparently normal cortex within the ischemic MCA territory (MCAO middle cerebral artery occlusion). Bar 1 mm

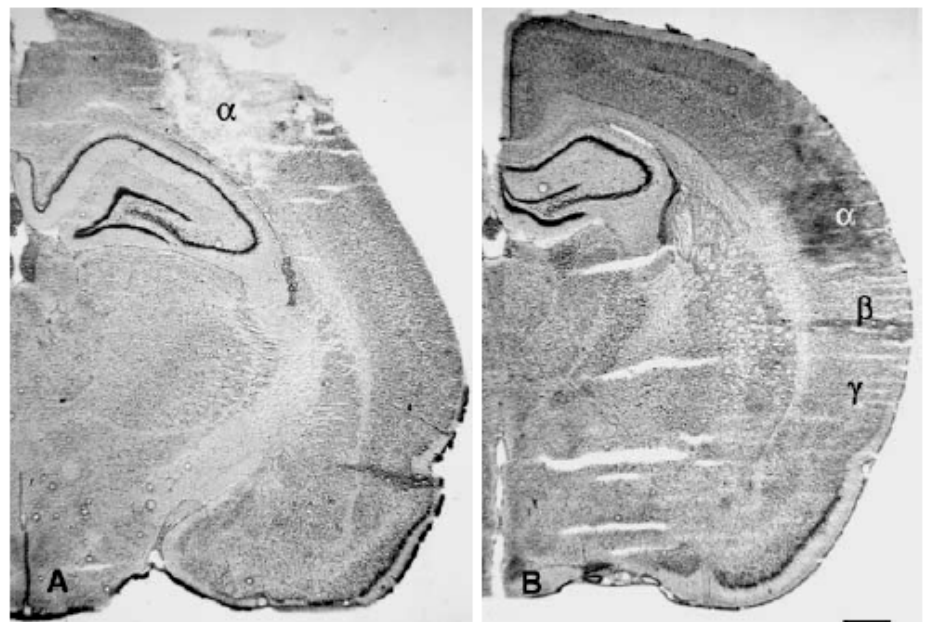


Table 1 Panel of monoclonal antibodies

Antibody	Antigen	Dilution	Reference
Ox8 (MCA 48 G) Serotec	CD8	1:2,000	Brideau et al. [7]
Ox35 (22021 D) PharMingen	CD4	1:1,000	PharMingen data sheet
Ox42 (MCA275 G) Serotec	Complement receptor 3/CD11b	1:1,600	Robinson et al. [36]
ED1 (MCA341) Serotec	Phagolysosomes	1:2,000	Damoiseaux et al. [11]
GFAP Boehringer Mannheim	Astrocytes	1:50	Bignami and Dahl [6]

Systemic depletion of CD8⁺ cells from blood and lymphoid organs was achieved by application of an azide-free, endotoxin-free, protein A-purified anti-rat CD8 IgG1 mAb (clone Ox-8, Serotec, Oxford, UK) as described previously [2, 21]; 0.5 mg was given intraperitoneally the day before induction of photothrombosis, and an additional 0.25 mg on days 3 and 5. Immunocytochemical staining of the spleens of treated rats verified complete depletion of peripheral CD8⁺ cells for at least 8 days. At day 14 only few repopulating CD8⁺ cells could be detected. Control animals received aliquots of an isotype control antibody (MCA 1209, Serotec).

Transient MCAO

Transient MCAO was induced using the intraluminal suture model originally described by Zea Longa et al. [45]. Wistar rats (260–300 g) were anesthetized and placed supine. A midline incision of the skin was performed and the right common carotid artery (CCA), the internal carotid artery (ICA) with its pterygopalatine branch, and the external carotid artery (ECA) were exposed under microsurgical conditions. After ligation of the proximal CCA, ECA, and temporal clipping of the ICA, a silicone-coated 4-0 monofilament (Ethilon, Ethicon) was inserted into the CCA just proximal of the carotid bifurcation. The thread was advanced into the ICA until a slight resistance was felt or the tip were approximately 17 mm distal of the bifurcation. The thread was then fixed, the skin closed, and animals allowed to awake. Animals showing typical circling behavior indicating ischemic brain damage were re-anesthetized and the thread was redrawn exactly 30 min after induction of ischemia. Animals without circling behavior as well as animals with evidence of subarachnoidal bleeding at autopsy were excluded from the study.

Immunohistochemistry

In all experiments serial 5- to 10- μ m coronal sections of the entire brain were cut at the level of the ischemic lesions on a Leica Cryotome (Leica, Bensheim, Germany) at -20°C . Sections were stained by routine procedures by immunocytochemistry for complement receptor-3 (CD11b) on all microglia/macrophages (mAb Ox42), phagocytic microglia/macrophages (mAb ED1), the astrocyte marker glial fibrillary acidic protein (GFAP) and for CD4 or CD8 antigens, respectively, using the ABC Elite kit (Vector, Burlingame, Calif.) with diaminobenzidine (Sigma, Deisenhofen, Germany) as the final reaction product. Dilutions, target antigens and sources of mAb are given in the Table 1. For histological evaluation additional sections were stained with cresyl violet.

Results

The origin of CD8⁺ phagocytes in permanent focal ischemia

Photothrombotic occlusion of cortical microvessels resulted in a circumscribed purely cortical infarction of approximately 2 mm in diameter. The zone of coagulation necrosis was sharply demarcated from surrounding vital

tissue (α in Fig. 1A), lacking an area of selective neuronal loss as typically observed in the transient ischemia model.

In previous studies we had identified the majority of CD8⁺ cells in cerebral ischemia induced by permanent MCAO and photothrombosis as Ox42⁺/ED1⁺ microglia/macrophages [24, 25]. A further distinction between microglia and macrophages was impossible due to lack of distinctive immunocytochemical markers [15]. In our present study we therefore selectively depleted hematogenous macrophages using C12MDP-filled liposomes and compared the composition of the cellular infiltrate in photothrombotic lesions between macrophage-depleted and sham-treated rats with particular reference to the expression pattern of the CD8 antigen at days 3 and 6 after ischemia. Immunocytochemical staining of spleen sections affirmed successful depletion of ED1⁺ macrophages (Fig. 2H, J) for up to 8 days after ischemia. In the lesions, compared with placebo-treated controls (Fig. 2A), systemic depletion of hematogenous macrophages resulted in gross reduction of ED1⁺ macrophages (Fig. 2B) [39] as well as a dramatic reduction of CD8⁺ cells (Fig. 2E) at 6 days after photothrombosis. Thus, the majority of CD8⁺ phagocytes in the lesions were derived from hematogenous macrophages. However, CD8 immunoreactivity was not completely abolished, indicating that local microglia had also up-regulated CD8 to some extent (Fig. 2E). Accordingly, in addition to round phagocytes (arrow in Fig. 2G) cells with the typical morphology of microglia exhibited CD8 immunoreactivity in the border zone of the infarcts (arrowhead in Fig. 2G).

In an additional set of experiments we specifically targeted CD8⁺ cells by repeated intraperitoneal application of Ox-8 antibodies. Previous studies had demonstrated that *in vivo* treatment with mAb Ox-8 efficiently depletes CD8⁺ lymphocytes in the blood and lymphoid organs [2, 21, 34] and, furthermore, inhibits the CD8⁺ infiltration of the CNS during the course of EAE in Lewis rats [41]. In line with these studies we found CD8⁺ cells to be greatly reduced in spleens of Ox-8-treated rats (Fig. 2K). The density of CD8⁺ cells in the ischemic brain lesions, however, was similar in Ox-8- and sham-treated animals (Fig. 2A, D versus C, F).

Spatio-temporal pattern of CD8⁺ phagocyte recruitment in transient MCAO

For transient MCAO, preliminary experiments showed that duration of ischemia of less than 30 min resulted in pure subcortical infarction with variable selective neuronal loss

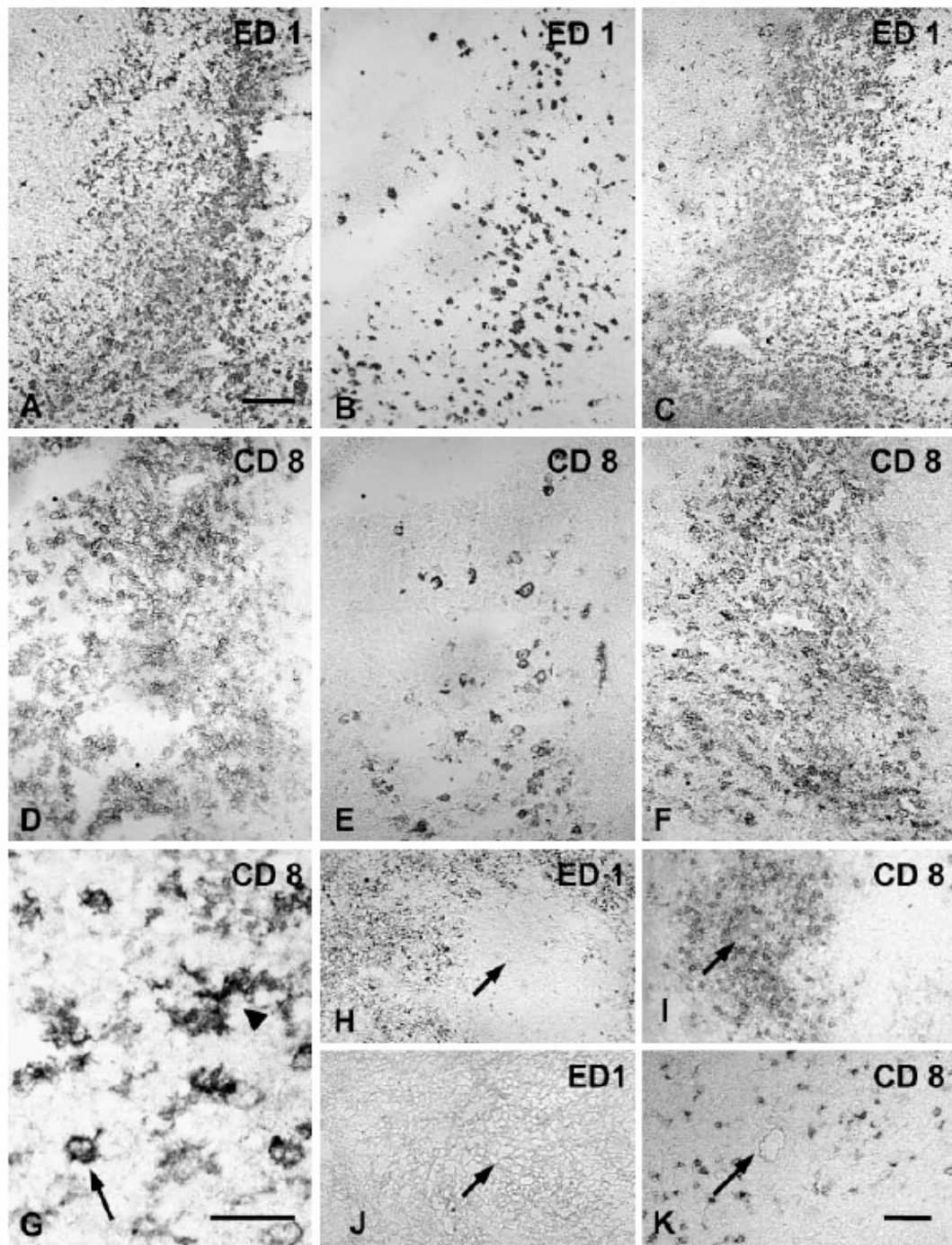


Fig. 2A-K Origin of CD8⁺ cells in ischemic photothrombotic lesions determined by depletion experiments. Rats were treated either with carrier solution/control mAb (A,D,G,H,I), mAb Ox-8 to eliminate peripheral CD8⁺ cells (C,F,K), or C12MDP-filled liposomes depleting all peripheral monocytes/macrophages (B,E,J). A-F are taken from frontal sections through the right border zone of photothrombotic lesions at day 6 (see also Fig. 1A) stained for either ED1⁺ phagocytes (A-C) or CD8 antigen (D-F). In sham-treated animals both ED1⁺ phagocytes and CD8⁺ cells are abundant in the border zone (A,D). There is no change in this pattern after Ox-8 treatment (C,F). In contrast, depletion of macrophages (B,E) leads to a dramatic reduction of ED1⁺ phagocytes (B) and concomitantly to reduction, but not abol-

ishment of the CD8⁺ cell population (E). According to their mixed microglial/macrophage origin some CD8⁺ cells exhibit a ramified morphology (G, *arrowhead*), while others are round phagocytes (G, *arrow*). H-K are taken from spleen sections that were stained to assure effective depletion of the respective cell type. H shows the distribution of ED1⁺ cells in the white pulp of the normal spleen. After macrophage depletion ED1⁺ macrophages are absent in spleen in liposome-treated animals (J) compared to placebo-treated animals (H). Compared to the abundance of CD8⁺ cells in white pulp of control animals (I), CD8⁺ cells are greatly reduced in spleen after Ox-8 treatment (K). *Arrows* denote central artery of the white pulp in H-K. *Bars* A (also for B-F) 80 μ m; K (also for H-J) 40 μ m; G 40 μ m

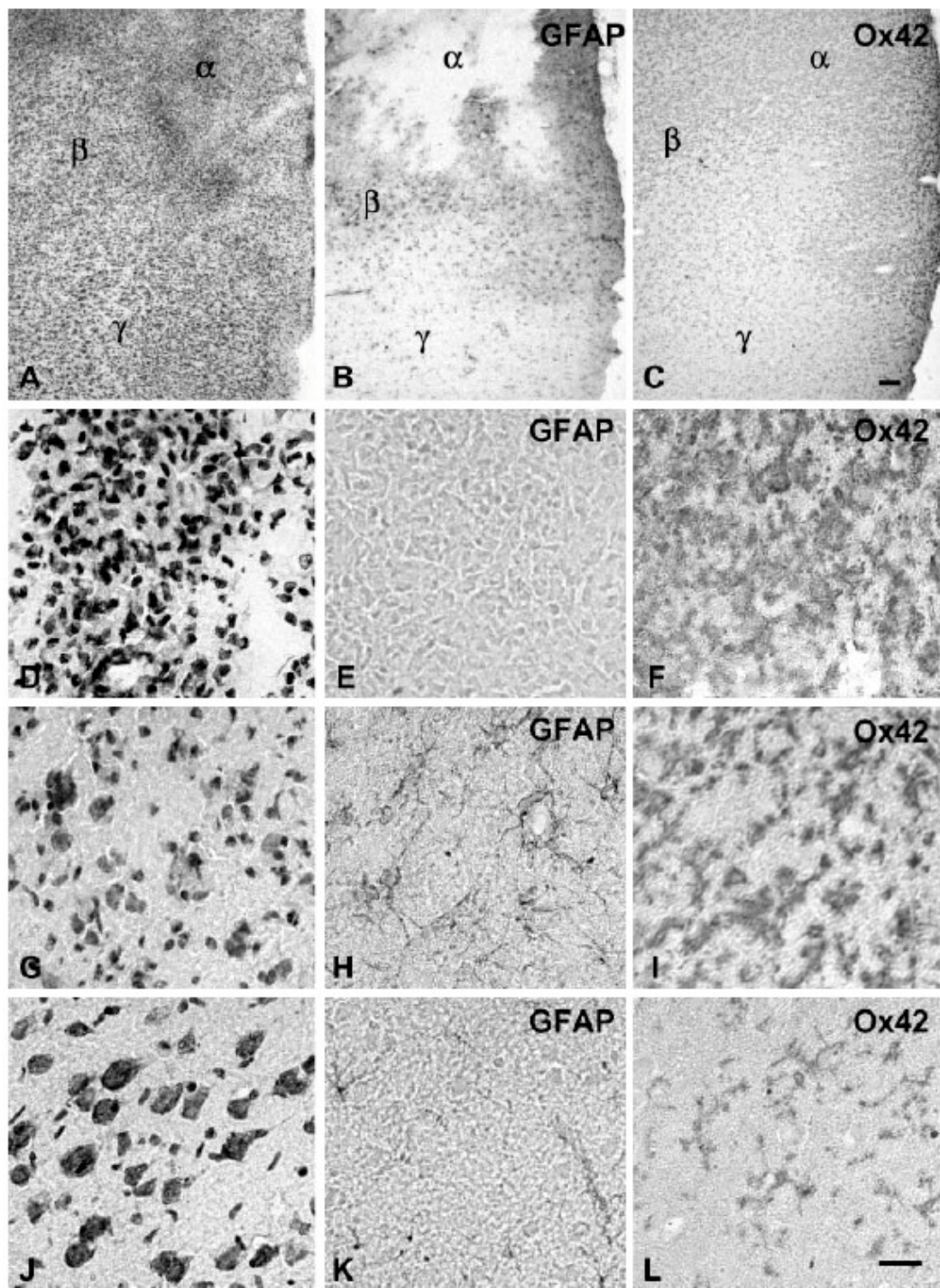


Fig.3A–L Histological and cellular changes in the area of pan-necrosis (α), in the area of selective neuronal loss (β), and in the ipsilateral cortex without neuronal loss (γ) at 7 days after transient MCAO. Cresyl violet staining (A,D,G,J), immunocytochemistry for GFAP to assess astrocytic responses (B,E,H,K), and OX42 immunocytochemistry for identification of microglia (C,F,I,L). The zone of coagulation necrosis corresponding to α appears hypercellular but lacks neurons (D). No GFAP⁺ astrocytes are present in

this area (E), which is covered by OX42⁺ microglia/macrophages (F). G Section corresponding to β shows loss of neurons with accompanying astrocytosis (H), and strong microglial activation (I) as revealed by up-regulation of CR-3 immunoreactivity, and decrease of ramification. Note normal appearance of neurons in the γ area (J) with barely detectable astrocytes (K) and hyperamplified microglia (L) (GFAP glial fibrillary acidic protein). Bars C (also for A–C) 100 μ m; L (also for D–L) 50 μ m

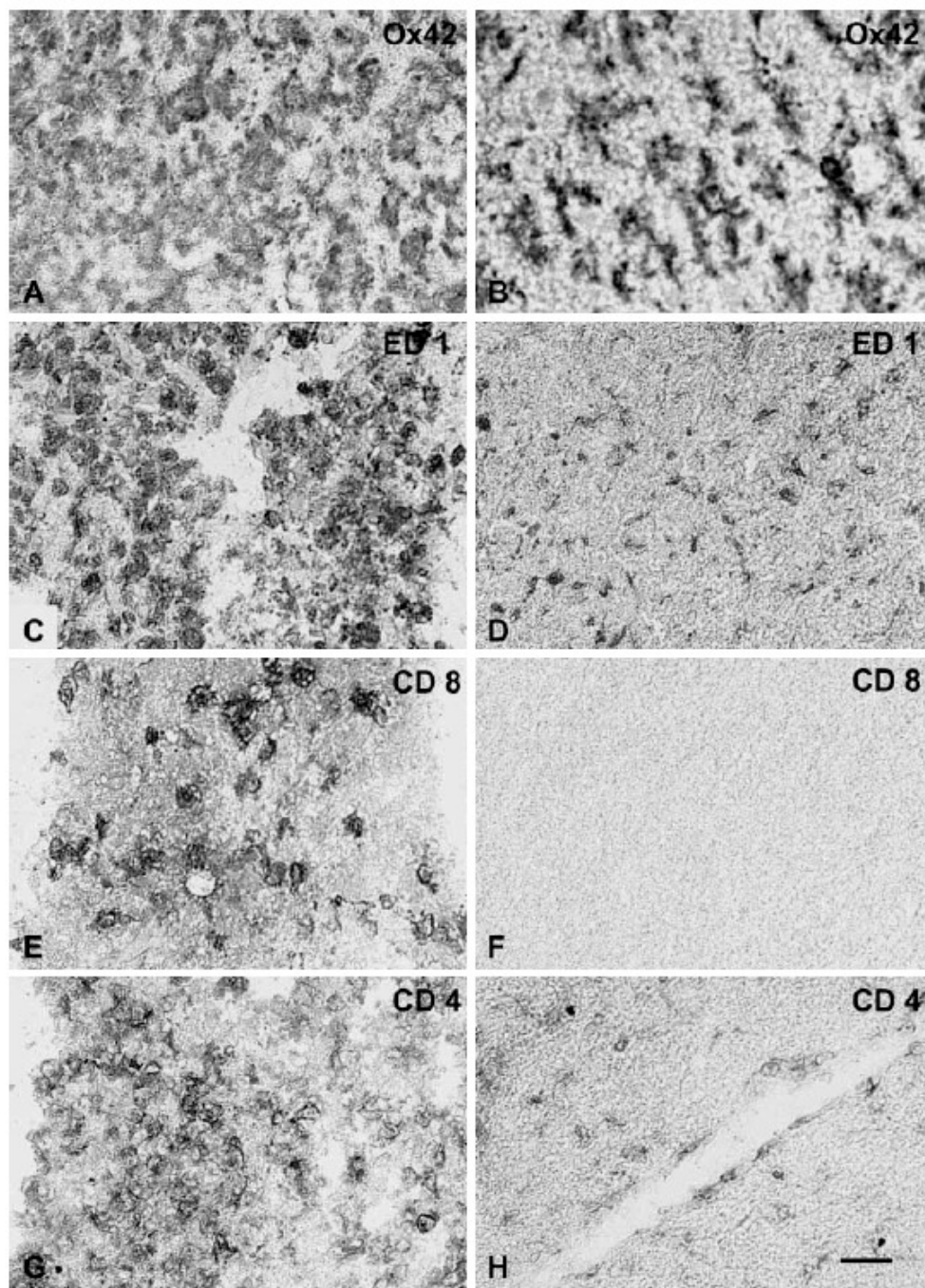


Fig. 4 Differential expression of CD4 and CD8 molecules in areas of pannecrosis (A,C,E,G) corresponding to α in Fig. 1 and selective neuronal loss (B,D,F,H) corresponding to β in Fig. 1. Sections were stained using mAb Ox42 recognizing CD11b⁺ microglia/macrophages (A,B), mAb ED1 as an indicator for phagocytic activity (C,D), CD8 (E,F) and CD4 (G,H) antigens at 7 days after transient MCAO. In the area of pannecrosis microglia/macrophages exhibit a round cell body typical for phagocytes (A, C) and

express CD8 (E) as well as CD4 (G). Note that the population of CD8⁺ cells is overall smaller than that of CD4⁺ cells. In areas of selective neuronal loss mAb Ox42 mainly stains activated microglia identified by their typical ramified morphology (B). These microglia exhibit relatively weak ED1 immunoreactivity as a sign for limited phagocytic transformation (D), and to some extent express CD4 (H), while CD8 immunoreactivity is virtually absent (F). Bar H (also for A–G) 50 μ m

in the cerebral cortex (Fig. 1B). Ischemia times longer than 30 min led to cortical infarcts with increasing and massive edema formation interfering with exact histological assessment of cellular damage. We therefore used an ischemia period of 30 min, which resulted in ischemic coagulation necrosis (pannecrosis) of most of the striatum and small parts of the cortex dorsal to the rhinal fissure (α in Figs. 1B and 3A–C). Selective neuronal loss was reliably seen in adjacent cortical areas dorsal and anterior of pannecrosis and in the entorhinal cortex (β in Figs. 1B and 3A–C). As outlined in Fig. 3, areas of coagulation necrosis were further defined by the absence of GFAP⁺ astrocytes (α in Fig. 3B, E). In contrast, the surrounding areas of selective neuronal loss (β in Fig. 3A) were characterized by selective loss of neurons (Fig. 2G), but increased staining of both microglial and astroglial surface markers and preservation of vascular structures (Fig. 3H, I). More remote from the pannecrotic core of the infarction, parts of the ischemic MCA territory displayed normal cresyl violet histology without any indication of neuronal loss (Fig. 3J). However, in line with previous studies [38, 40] astroglial and microglial staining intensities in these normal-appearing cortical areas were slightly increased above levels of the contralateral hemisphere (Fig. 2K, L) and microglia displayed the morphology of a hyperamified stage (Fig. 2L).

We used the transient MCAO model to examine the appearance and distribution of CD4⁺ and CD8⁺ cell populations in areas of ischemic pannecrosis compared to areas with selective neuronal loss. In areas of pannecrosis a strong induction of the CD11b antigen on microglia/macrophages occurred that was accompanied by high expression levels of the lysosomal marker ED1, indicating phagocytic transformation (Fig. 4A, C). In addition, most of these activated phagocytes also expressed the CD4 molecule (Fig. 4G). Compared to the CD4 antigen, the CD8 antigen was expressed on a smaller subpopulation of cells in the pannecrotic core of lesions. CD8⁺ phagocytes appeared with a delay of 2–3 days and were most abundant at the end of the 1st week after ischemia (Fig. 4E). Until day 14, the number of CD8⁺ cells rapidly decreased, while CD4 expression increased. Thus, the time course of CD4 and CD8 antigen expression in the area of pannecrosis was similar to that observed previously in models of permanent focal ischemia [25, 40].

In areas with selective neuronal loss (β in Fig. 3A–C) considerable microglial activation with transition to a less ramified morphology and induction of CD11b as well as CD4 surface antigens occurred. In addition, relatively weak ED1 immunoreactivity as an indication of partial phagocytic transformation was detectable (Fig. 4D). However, in sharp contrast to the area of pannecrosis, CD8⁺ cells were virtually absent from the zone of selective neuronal death (Fig. 4F). Thus, in this model of transient ischemia recruitment of CD8⁺ phagocytes was restricted to areas of pannecrosis.

Discussion

In this study we provide further evidence that focal ischemia induces a spatio-temporally distinct recruitment of microglia/macrophage subpopulations that can be differentiated by their surface expression of CD4 and CD8 antigens. In extension of our previous studies, we show that accumulation of CD8⁺ phagocytes is a general phenomenon in ischemic CNS lesions since it occurs independently from the model used, e.g., photothrombosis [24, 25], permanent MCAO [25, 37] and transient MCAO (present study). In transient MCAO CD8⁺ phagocyte responses were restricted to areas of pannecrosis, while areas with selective neuronal loss were spared. Compared with the permanent MCAO model, reperfusion in the transient MCAO model did not augment the CD8⁺ infiltrate [10]. In accordance with findings reported by Lehrmann et al. [29], we found progressive activation of microglia characterized by retraction of cellular processes, up-regulation of CD11b antigen, the lysosomal marker ED1, and surface molecules MHC class I and II within “penumbral” regions. Interestingly, this local microglial response involved the up-regulation of CD4, but not of CD8 molecules. This indicates that the local molecular milieu leading to microglia activation and attraction of blood-borne macrophages must be different in areas of pannecrosis compared to areas with selective neuronal loss. Further evidence for site-specific macrophage responses following cortical ischemia is provided by the fact that in descending central fiber tracts undergoing anterograde Wallerian degeneration up-regulation of microglial CD4 antigens but no infiltration by CD8⁺ phagocytes occurs [40]. The signaling molecules involved await further elucidation.

Using depletion experiments in the photothrombosis model, this study clarifies the origin of CD8⁺ cells in focal cerebral ischemia. In a first experimental series we administered C12MDP-filled liposomes, which reliably deplete peripheral monocytes/macrophages in blood and lymphoid organs but do not affect resident microglia in the brain. Therefore, this technique allows the determination of the relative contribution of resident microglia and infiltrating macrophages to the phagocyte response in CNS lesions models. In our present study, the density of CD8⁺ phagocytes in the ischemic lesions was greatly reduced in macrophage-depleted rats, suggesting that the majority of CD8⁺ phagocytes in the lesions originates from hematogenous macrophages. However, a significant number of residual CD8⁺ cells persisted after liposome treatment, indicating that local up-regulation of CD8 antigen on resident microglia also occurs to some extent. In line with this assumption a considerable number of CD8⁺ cells in the lesions displayed the typical morphology of ramified microglia.

Interestingly, the expression of the CD8 antigen in peripheral lymphoid organs appears to be restricted to T cells, whereas macrophages at these sites are mostly CD8⁺. Therefore, even though the majority of CD8 anti-

gen was apparently expressed on infiltrating macrophages, its appearance is most likely due to secondary up-regulation in the CNS compartment. This may provide an explanation for our unexpected finding that by systemic administration of mAb Ox-8 we could effectively deplete peripheral CD8⁺ cells in the spleen, but failed to reduce the number of CD8⁺ macrophages in the ischemic brain lesions. Whether this failure is due to insufficient amounts of antibody diffusing into the lesions across the disturbed blood-brain barrier is an open question, which is currently addressed by experiments employing the direct intracerebroventricular administration of mAb Ox-8. Of note, Hirji et al. [20] showed that Ox-8 treatment of CD8⁺ alveolar macrophages *in vitro* led to significant induction of inflammatory gene expression, but not to a depletion of the cells, raising the possibility that the depleting properties of mAb Ox-8, as established for peripheral CD8⁺ lymphocytes *in vivo*, depend on so far unknown cell type-specific or microenvironmental factors.

What is the functional role of CD8⁺ microglia/macrophages in ischemic lesion development? To date both CD4 and CD8 molecules have been studied extensively as coreceptors in MHC class I-restricted T cell responses [27]. Although expression of the CD4 molecule on macrophages/microglia has been frequently described [35], Buttini et al. [8] have only recently shown that the transgenic overexpression of the human CD4 gene in normally CD4⁺ microglia/macrophages in mice leads to increased neurodegeneration. Whether similar neurotoxic effector functions of microglia/macrophages are induced by expression of the CD8 molecule remains to be studied. As already mentioned, Hirji and colleagues [19, 20, 31] have shown *in vitro* that signaling via the CD8 molecule leads to the activation of TNF- α , IL-1 β , and inducible nitric oxide synthase (iNOS) expression in alveolar macrophages with concomitantly enhanced cytotoxicity toward *Leishmania major*. Several studies showed that these inflammatory effector molecules are likewise expressed in focal ischemic brain lesions, where they may contribute to the delayed expansion of the lesion. However, at least based on mRNA data [17, 22, 33] and our own findings in the photothrombosis model [26], cytokine and iNOS induction after focal ischemia peaks during the first 12–24 h, i.e., well before the delayed onset of CD8⁺ phagocyte recruitment at 48–72 h after ischemia. At this stage immunocytochemical studies suggest already significant structural repair processes in which macrophages can participate by secretion of growth factors and matrix-degrading enzymes [12, 13, 23, 32]. Therefore, future studies into the functional role of CD8⁺ macrophages in brain ischemia should equally consider their possible contribution to exacerbation of ischemic brain damage as well as tissue remodeling and healing processes. Comparative analysis of the molecular environment of lesions under which CD8⁺ cells either are present or fail to appear, as demonstrated in the present study, may provide the crucial clue for elucidation of their role in the nervous system.

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Cortical Spreading Depression Induces Proinflammatory Cytokine Gene Expression in the Rat Brain

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Summary: Cortical spreading depression (CSD) is characterized by reversible neuronal dysfunction in the absence of cell death. Preconditioning by CSD induces tolerance against subsequent lethal ischemia. In this study, we used quantitative reverse transcriptase-polymerase chain reaction and immunocytochemistry to analyze proinflammatory cytokine expression after CSD induced by topical application of potassium chloride (KCl) to the cortical surface of rat brains. Relative to control cortex, we found an increase of tumor necrosis factor- α (mean 62-fold, $P < 0.001$) and interleukin (IL)-1 β (mean 24-fold, $P < 0.001$) mRNA levels within 4 hours ipsilateral to the site of KCl application. At 16 hours cytokine expression was decreasing toward baseline levels. Ipsilateral cytokine induction was abolished by pretreatment with the noncompetitive *N*-methyl-D-aspartate antagonist, MK-801. In contrast to focal cortical in-

farcion, cytokine induction in CSD was not accompanied by the expression of inducible nitric oxide synthase mRNA. In immunocytochemical studies, expression of IL-1 β protein was localized to ramified microglia in cortical layers I to III of the ipsilateral hemisphere. Our finding that NMDA receptor signaling without subsequent neuronal cell death is sufficient to induce inflammatory cytokine expression in the brain has basic implications for central nervous system immunoregulation. We postulate that cytokine expression in CSD forms part of a physiologic stress response that contributes to the development of ischemic tolerance in this and other preconditioning paradigms. **Key Words:** Central nervous system—Spreading depression—NMDA receptor—Cytokine—Nitric oxide synthase—Ischemic tolerance.

Proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are strongly induced after focal ischemic and traumatic brain injury (Arvin et al., 1996; Rothwell et al., 1997; Stoll et al., 1998). In several *in vitro* and *in vivo* models, cytokines exacerbate hypoxic as well as excitotoxic damage of neurons (Relton and Rothwell, 1992; Hewett et al., 1994; Chao and Hu, 1994; Barone et al., 1997). Neurotoxic cytokine effects appear to be mediated by the downstream activation of the cytokine-inducible nitric oxide synthase (iNOS) pathway (Hewett et al., 1994; Iadecola et al., 1997). However, depending on the time window and dosage of their administration, cytokines can also confer protection against neuronal injury both *in vitro* (Cheng et al., 1994; Strijbos and Rothwell, 1995) and *in vivo* (Bruce et al., 1996; Nawashiro et al., 1997).

Cortical spreading depression (CSD) is characterized by a transient suppression of all neuronal activity that repetitively extends from sites of increased extracellular potassium concentration to the entire ipsilateral hemisphere (Martins-Ferreira et al., 2000). The propagation of CSD is inhibited by pretreatment with the noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, and therefore is likely to be mediated by glutamatergic signaling through the NMDA receptor (Marrannes et al., 1988; Lauritzen and Hansen, 1992; Schroeter et al., 1995). In contrast with paradigms of lethal NMDA neurotoxicity (Choi et al., 1987), healthy cortical neurons can fully compensate for the massive ion shifts caused by CSD (Hossmann, 1996). Therefore, CSD proceeds without cell death (Nedergaard and Hansen, 1988), but induces neuronal tolerance against subsequent lethal ischemic challenge (Kobayashi et al., 1995; Yanamoto et al., 1998).

Although CSD has been shown to profoundly influence gene expression in the brain (Miettinen et al., 1997; Kariko et al., 1998), the molecular mechanisms underlying tolerance induction by CSD are not completely understood. To elucidate a potential contribution of

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proinflammatory cytokines, we used reverse transcriptase-polymerase chain reaction (RT-PCR) to study the expression of TNF- α , IL-1 β , and iNOS mRNA after CSD induced by the topical application of potassium chloride (KCl) to the cortical surface in rats. IL-1 β expression was also analyzed by immunocytochemistry. As a paradigm of lethal neuronal injury, we comparatively studied infarctions induced by photothrombosis of cortical microvessels (Watson et al., 1985).

MATERIALS AND METHODS

Animal experiments

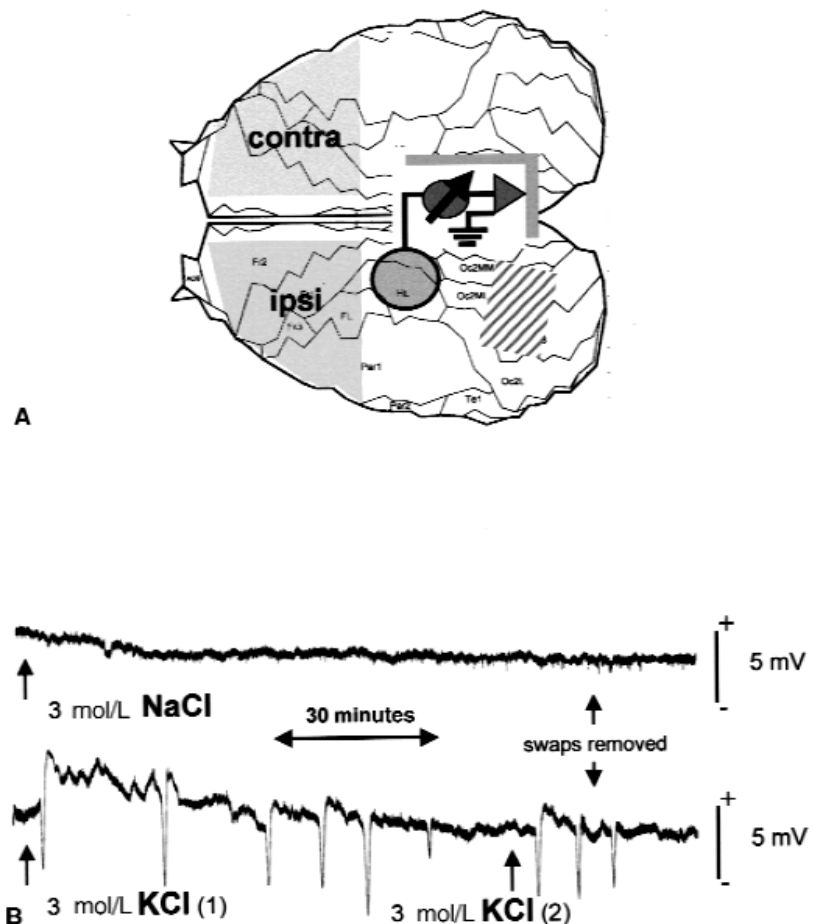
Male Wistar rats ($n = 16$; 270 to 320 g) were anesthetized with enflurane [2% in an O_2/N_2 (1:2) mixture] and fixed in a stereotactic frame. Body temperature was measured by a rectal probe and maintained at 36.5°C to 37.5°C with a feedback-controlled heating pad throughout all procedures. After exposure of the skull, two burr holes leaving the dura intact were made over the left hemisphere at the locations indicated in Fig. 1A. A recording electrode filled with artificial cerebrospinal fluid (NaCl 124 mmol/L, KCl 5 mmol/L, $CaCl_2$ 2 mmol/L, $MgSO_4$ 2 mmol/L, NaH_2PO_4 1.25 mmol/L, glucose 10 mmol/L, $NaHCO_3$ 26 mmol/L) was positioned upon the dura. Direct

current (DC) potentials were monitored using a home-built amplifier and an analog plotter. Swabs soaked with either 3 mol/L KCl or 3 mol/L NaCl solutions ($n = 8$ rats in each group) were placed upon the dura over the occipital cortex and in some experiments renewed once during the measurement. After recording of 7 DC deflections in KCl-treated animals, the swabs were removed (usually approximately 100 minutes after their application), and the burr holes flushed with 0.9% saline. In the next 10 minutes, a maximum of 1 or 2 more DC deflections occurred. Typical examples of DC potential recordings in KCl- and NaCl-treated animals are shown in Fig. 1B. For RNA isolation, animals were killed 4 and 16 hours, respectively, after the occurrence of the first DC deflection ($n = 4$ animals at each time point in both the KCl- and NaCl-treated group).

To clarify the role of NMDA receptor signaling, an additional experiment was performed in which $n = 4$ rats were injected intravenously through a femoral catheter with the non-competitive NMDA antagonist, MK-801 (dizolcipine; Research Biochemicals International, Natick, MA, U.S.A.) at a dosage of 2 mg/kg body weight 30 minutes before application of the KCl swab. As control, the remaining rats ($n = 4$) were pretreated with carrier solution. Cortical tissue was prepared 4 hours after the induction of CSD.

Photothrombosis of cortical microvessels in the right parietal cortex of male Wistar rats was induced as described previously (Jander et al., 1995). For isolation of RNA, animals were killed

FIG. 1. (A) Experimental setup for the induction of cortical spreading depression and localization of the tissue samples removed for reverse transcriptase-polymerase chain reaction analysis. Tissue samples (shaded areas) were removed from frontal cortex either ipsilaterally or contralaterally to the site of KCl or NaCl application (hatched box). Direct current (DC) potentials were recorded from ipsilateral cortex between the sites of KCl application and tissue sampling, respectively. (B) Direct current potentials registered after application of KCl or NaCl solutions, respectively. In KCl-treated rats, approximately one DC deflection was recorded every 10 to 20 minutes. No DC deflections occurred in NaCl-treated rats.



at 4 and 16 hours after the induction of ischemia, respectively ($n = 5$ at each time point).

Semiquantitative reverse transcription-polymerase chain reaction

Total RNA was prepared from cortical tissue samples using the TRIzol reagent (GIBCO BRL, Gaithersburg, MD, U.S.A.), according to the manufacturer's instructions. RNA was quantified spectrophotometrically. One microgram RNA isolated from each tissue sample was reverse transcribed using oligo(dT)₂₀ primers and SuperscriptII-Reverse Transcriptase (GIBCO BRL) essentially to the manufacturer's protocol. cDNA equivalent to 20 ng of total RNA was subjected to subsequent PCR analysis as detailed elsewhere (Jander et al., 1998). Rat TNF- α and iNOS primers were purchased from Clontech (Palo Alto, CA, U.S.A.) and Biosource (Camarillo, CA, U.S.A.), respectively. The sequences of the rat IL-1 β and glyceraldehyde-phosphate dehydrogenase (GAPDH) primers have been described previously (Gillen et al., 1998). For each primer combination, linear cycling conditions were determined in preliminary experiments as described previously (Jander and Stoll, 1998). Cycle numbers were as follows: TNF- α = 26, IL-1 β = 25, iNOS = 25, GAPDH = 18. Controls included RNA subjected to the RT-PCR procedure without addition of reverse transcriptase and PCR performed in the absence of cDNA that always yielded negative results. Representative samples were sequenced on an ABI PRISM 310 genetic analyzer (Perkin Elmer, Oak Brook, IL, U.S.A.) for each PCR product. Polymerase chain reaction products were separated on 1.5% agarose gels containing 10 μ g/mL ethidium bromide, photographed using a CSC-camera (Cybertech, Berlin, Germany), and densitometric analysis was performed with TINA 2.1 software (Raytest, Straubenhardt, Germany).

Competitive polymerase chain reaction

Competitive PCR analysis of TNF- α , IL-1 β , and GAPDH mRNA was performed using CytoXpress quantitative PCR kits from Biosource, essentially according to the manufacturer's protocol. In initial titration assays, serial dilutions of cDNA corresponding to 50, 5, 0.5 ng starting RNA were coamplified with 1000 copies of an internal calibration standard for 30 cycles and analyzed on 1.5% agarose gels. Dilutions with visibly equivalent intensities of the cDNA and internal calibration standard bands were used for subsequent quantitation in a microplate hybridization assay using colorimetric detection of biotinylated primer incorporated into both cytokine and internal calibration standard amplicons during the PCR reaction.

Statistical analysis

Analysis of variance (Tukey's Multiple Comparison Test) was performed using GraphPad Prism 3.0 software for statistical analysis of competitive PCR data.

Immunocytochemistry

At 4, 8, and 16 hours after induction of CSD, rats were deeply anesthetized and perfused with 4% paraformaldehyde in 0.15 mol/L phosphate buffer (pH 7.4) through the left ventricle. Whole brains were removed from the skull, postfixed in the same fixative overnight at 4°C, and cryoprotected by overnight infiltration with 20% sucrose in phosphate buffer at 4°C. Free-floating 50 μ m cryostat sections were washed three times in Tris-buffered saline containing 0.05% Triton X-100 (TBS-T). Endogenous peroxidase was blocked by 30 minutes incubation in 0.3% H₂O₂ in TBS-T. After three washes in TBS-T, sections were incubated with affinity-purified goat anti-rat IL-1 β polyclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.) at

0.5 μ g/mL in 2% normal horse serum in TBS-T for 72 hours at 4°C. After three washes in TBS-T, bound antibody was detected using biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, U.S.A.) and the ABC Elite kit (Vector) with diaminobenzidine as substrate. Control experiments in which the primary antibody was replaced by nonspecific goat IgG yielded negative results. Sections were mounted onto gelatine-coated slides, air-dried, dehydrated with ascending series of ethanol, cleared, and coverslipped with Entellan (Merck, Darmstadt, Germany).

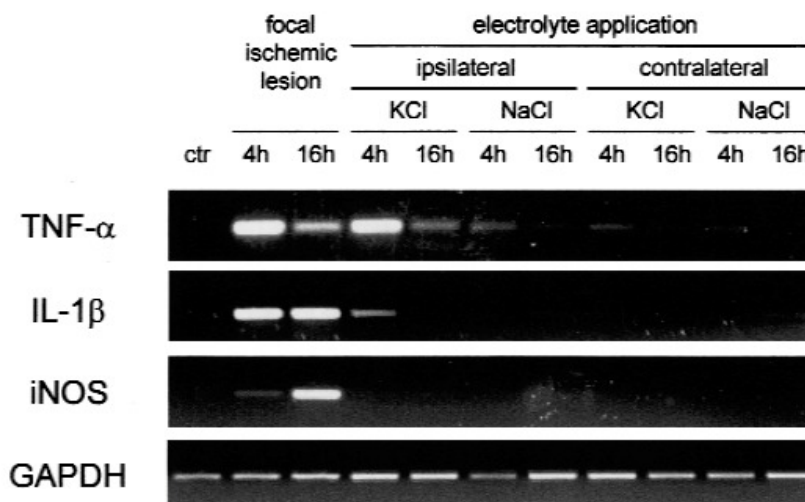
RESULTS

During the recording period of 100 to 120 minutes, topical application of 3 mol/L KCl solution onto the intact dura induced 7 to 9 DC deflections of ~ 5 mV typical for CSD (Fig. 1B). As a control, we applied 3 mol/L NaCl instead of KCl solution onto the cortex. No CSD-like DC deflections were detectable (Fig. 1B) in these animals. To characterize cytokine expression, we prepared cortical tissue samples from frontal cortex ipsi- and contralateral to the site of KCl or NaCl application and isolated total RNA for PCR analysis. At 4 hours after induction of CSD in KCl-treated rats, semiquantitative RT-PCR showed a strong increase of TNF- α and IL-1 β mRNA levels that was strictly confined to the ipsilateral cortex (Fig. 2). At 16 hours, cytokine mRNA levels were decreasing toward baseline levels. In NaCl-treated rats, no significant increase of cytokine mRNA levels greater than background levels was found (Fig. 2).

In contrast to TNF- α and IL-1 β mRNA, we found no induction of iNOS mRNA in remote cortex ipsi- or contralateral to the site of KCl or NaCl application (Fig. 2). As positive control for iNOS expression, we used total RNA from focal cortical infarctions induced by photothrombosis of cortical microvessels (Watson et al., 1985; Jander et al., 1995). In line with previous findings in lesions induced by middle cerebral artery occlusion (Iadecola et al., 1995; Galea et al., 1998), we found a clear increase in iNOS mRNA at 4 hours and even more pronounced at 16 hours after ischemia. Likewise, strong increases in TNF- α and IL-1 β expression occurred in the focal ischemia model (Fig. 2).

For the more precise quantification of cytokine mRNA levels, we performed competitive PCR analysis. In initial titration experiments (Fig. 3A), increasing dilutions of cDNA were coamplified with a fixed amount (1000 copies) of an internal calibration standard. At 4 hours after KCl application, approximately equal band intensities of TNF- α cDNA and the standard were observed at 5 ng of total starting cDNA, whereas cDNA from NaCl-treated animals only competed at 50 ng starting cDNA. Thus, KCl treatment induced considerable up-regulation of TNF- α mRNA. Similar findings were obtained in the analysis of IL-1 β mRNA.

FIG. 2. Semiquantitative reverse transcriptase-polymerase chain reaction analysis of tumor necrosis factor- α (TNF- α), IL-1 β , and inducible nitric oxide synthase (iNOS) transcripts after topical application of KCl or NaCl to the cortical surface of rats. Glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA served as a "housekeeping gene" control that is not modulated under the experimental conditions. Tissue samples were removed from frontal cortex ipsilateral or contralateral to the site of electrolyte application at 4 and 16 hours, respectively. For comparison, tissue samples from photochemically induced ischemic lesions 4 and 16 hours after ischemia were analyzed.



Quantification of the amplicons by microplate detection of biotinylated primer incorporated during the PCR reaction (Fig. 3B) showed a mean 62-fold increase in TNF- α copy numbers in the ipsilateral cortex of KCl-treated rats compared with untreated control cortex ($n = 4$ animals in each group, $P < 0.001$). After NaCl treatment, a mean 17-fold increase relative to control cortex was found although this difference did not achieve statistical significance. In the contralateral cortex of both KCl- and NaCl-treated animals, no major increase in TNF- α mRNA was found. IL-1 β mRNA levels likewise showed a significant increase in ipsilateral cortex of KCl-treated rats that was however somewhat less pronounced (mean 24-fold, $P < 0.001$) compared with TNF- α mRNA. Throughout, no differences in GAPDH mRNA levels were observed between any of the treatment groups (not shown).

To compare cytokine expression after CSD with a paradigm of ischemic neuronal cell death, we analyzed photochemically induced cortical infarctions by competitive PCR. In these lesions, mean mRNA induction at 4 hours after ischemia was 93-fold for TNF- α and 80-fold for IL-1 β , respectively. Thus, relative to the infarctions, induction in the CSD model reached 67% for TNF- α mRNA and 31% for IL-1 β mRNA, respectively.

The propagation of CSD can be inhibited by the non-competitive NMDA receptor antagonist, MK-801 (Marrannes et al., 1988; Lauritzen and Hansen, 1992). To delineate a role of NMDA receptor signaling in CSD-induced cytokine expression, we performed an additional experimental series in which a proportion of the animals received an intravenous injection of MK-801 (2 mg/kg body weight) 30 minutes before induction of CSD (Fig. 4). In MK-801-treated animals, cortical DC deflections after KCl administration were generally absent (not shown). Basic physiologic variables (blood gases, blood

glucose, and rectal temperature) were monitored throughout the experiment and revealed no differences between MK-801-pretreated and control rats. Animals were killed at 4 hours after CSD induction and total cortical RNA was analyzed for TNF- α and IL-1 β mRNA expression by competitive PCR. In vehicle-treated animals, a strong induction of both cytokines was found that was almost completely inhibited by MK-801 pretreatment (Fig. 4).

To extend the studies to the protein level, we performed immunocytochemical labeling studies with various commercially available antibodies against rat TNF- α and IL-1 β . While several different TNF- α antibodies yielded variable and overall inconclusive staining patterns, we obtained highly reproducible staining for IL-1 β using a rat IL-1 β -specific, antigen affinity-purified goat polyclonal antibody on free-floating sections of paraformaldehyde-fixed tissue. IL-1 β immunoreactivity was confined to cortical layers I to III of the ipsilateral hemisphere (Fig. 5A to 5C) where it appeared at 4 hours after SD induction, reached its maximum at 8 hours, and decreased toward baseline levels at 16 hours. At higher resolution, IL-1 β immunoreactivity appeared to be mainly, if not exclusively, expressed by ramified microglia (Fig. 5B).

DISCUSSION

The current study shows that CSD induced by the topical application of KCl to the cortical surface of the intact rat brain leads to strong induction of the proinflammatory cytokines TNF- α and IL-1 β . Although primarily obtained by quantitative RT-PCR analysis of total RNA extracted from defined cortical areas, we were able to corroborate our data at the protein level by the immunocytochemical localization of IL-1 β protein.

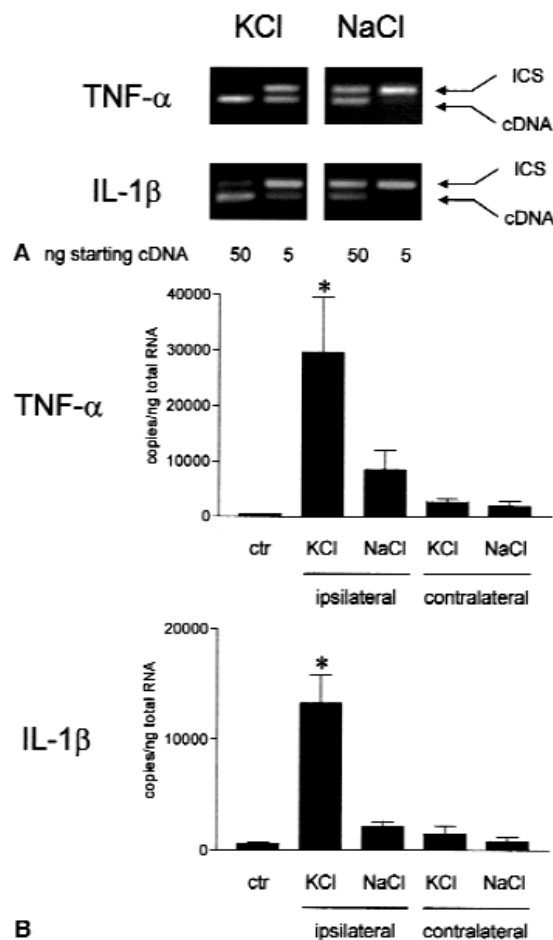


FIG. 3. Competitive polymerase chain reaction (PCR) analysis of cytokine mRNA levels at 4 hours after cortical spreading depression induction. **(A)** Representative original PCR findings in initial titration experiments. **(B)** Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) mRNA levels 4 hours after electrolyte application (mean $\pm$ SD; $n = 4$ animals in each group) determined by microplate analysis of cytokine and internal calibration standard (ICS) amplicons, respectively. * $P < 0.001$ compared with both untreated control cortex (ctr) and all other experimental samples from KCl- and NaCl-treated rats.

IL-1 β immunoreactivity was selectively induced in the ipsilateral hemisphere affected by CSD and was found to be mainly localized to ramified microglia in cortical layers I to III. Because of technical difficulties concerning antibody specificity and poor reproducibility of staining results, we were so far unable to obtain convincing immunocytochemical localization of TNF- α in CSD. Overall, however, the current data indicate that transient neuronal dysfunction without cell death (as it is characteristic for CSD) is sufficient to induce inflammatory cytokine gene expression in the brain. Our findings suggest a novel mechanism of neural-immune interaction that may have basic implications for the regulation and

pathophysiologic consequences of inflammatory processes in the central nervous system.

The initial rise of both TNF- α and IL-1 β mRNA at 4 hours after KCl administration was comparable to focal ischemia lesions induced by photothrombosis of cortical microvessels but was more rapidly decreasing at 16 hours. Likewise, immunocytochemical findings in photochemically-induced ischemia (Jander, Schroeter, and Stoll, unpublished observations) and the middle cerebral artery occlusion model (Davies et al., 1999) indicate that the expression of IL-1 β protein is more sustained in focal ischemic lesions than in CSD. As the main difference between the two paradigms, we found strong coinduction of iNOS mRNA in focal ischemia but not after CSD. Both the lack of iNOS expression after CSD and its strong induction in focal ischemia are in accord with previous immunocytochemical data (Iadecola et al., 1995; Caggiano and Kraig, 1998; Galea et al., 1998). Therefore, it appears that CSD as a model of transient neuronal dysfunction without cell death induces proinflammatory cytokines without concomitant iNOS expression, whereas the coinduction of cytokines and iNOS is characteristic of the pannecrosis evolving because of focal brain ischemia. Although protective effects of iNOS in tissue injury responses have been reported (Guo

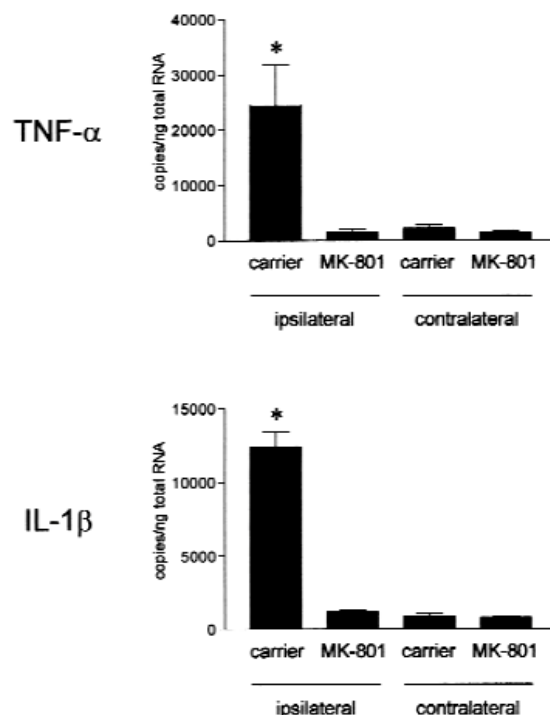
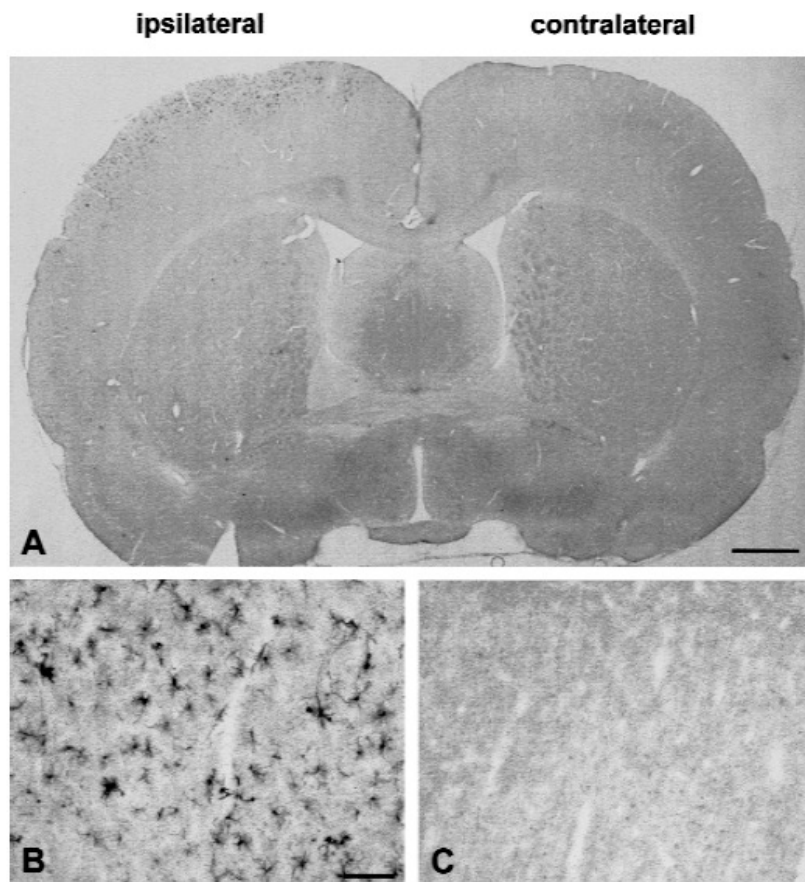


FIG. 4. Inhibition of ipsilateral cytokine mRNA induction by pretreatment with the noncompetitive *N*-methyl-D-aspartate antagonist, MK-801, assessed by competitive polymerase chain reaction ($n = 4$ animals in each group). * $P < 0.001$ compared with MK-801-treated animals.

FIG. 5. Immunocytochemical localization of IL-1 β protein at 8 hours after induction of cortical spreading depression. In line with the mRNA data, expression of IL-1 β immunoreactivity is restricted to the ipsilateral hemisphere (A). At higher magnification, IL-1 β expression appears to be mainly localized to ramified microglia in cortical layers I to III of the ipsilateral (B), but not contralateral (C), hemisphere. Scale bars = 1 mm (A), 100 μ m (B and C).



et al., 1999; Sinz et al., 1999), most findings in cerebral ischemia suggest an overall harmful role of iNOS in subacute stages after injury (Dimaghi et al., 1999). *In vitro*, the exacerbation of hypoxic and excitotoxic neuronal cell injury by inflammatory cytokines depends on the downstream action of iNOS (Hewett et al., 1994). Accordingly, studies in knockout mice showed a critical role of iNOS in delayed infarct growth after focal brain ischemia (Iadecola et al., 1997). The differential coinduction of iNOS may therefore critically determine the pathophysiologic consequences of inflammatory gene induction in CSD and focal ischemia, respectively.

The induction of cytokine mRNA after CSD was almost completely inhibited by pretreatment of the animals with the noncompetitive NMDA antagonist, MK-801. In line with this observation, numerous studies have implicated glutamatergic signaling through NMDA receptors with subsequent intracellular entry of Ca^{2+} ions in the initiation and propagation of CSD (Marrannes et al., 1988; Lauritzen and Hansen, 1992). Thus, CSD shares basic cellular mechanisms with excitotoxic cell death induced by high concentrations of glutamate (Siesjö and Bengtsson, 1989; Hossmann, 1996), although the

massive ion shifts occurring in CSD are compensated without leading to any irreversible neuronal damage (Nedergaard and Hansen, 1988). However, CSD activates a cellular stress response with induction of immediate early genes and heat shock proteins (Kobayashi et al., 1995; Kariko et al., 1998) and leads to the development of ischemic tolerance. The mechanisms of tolerance induction by CSD may involve neurotrophins such as brain-derived neurotrophic factor that show widespread up-regulation in the ipsilateral cortex after focal KCl application (Kokaia et al., 1993; Kawahara et al., 1997; Matsushima et al., 1998; Kariko et al., 1998). In CSD, it is an open question if the inhibition of cytokine synthesis by MK-801 reflects prevention of CSD propagation or a direct effect on cellular activation processes underlying inflammatory gene induction. Notably, similar to the *in vivo* situation in CSD, tolerance induction by sublethal doses of NMDA also has been shown *in vitro* (Marini et al., 1998; Grabb and Choi, 1999).

In certain *in vitro* models, both TNF- α and IL-1 β can protect neurons against subsequent hypoxic or excitotoxic injury (Cheng et al., 1994; Strijbos and Rothwell, 1995). In ischemic injury of both brain (Bruce et al.,

1996) and heart (Kurrelmeyer et al., 2000), increasing evidence suggests protective roles of endogenous TNF- α *in vivo*. Protective effects of TNF- α involve activation of NF- κ B with subsequent induction of manganese superoxide dismutase (Mattson et al., 1997; Van Antwerp et al., 1998). Furthermore, neurotrophins are highly inducible by inflammatory cytokines (Lindholm et al., 1987; Hattori et al., 1993; Stribos and Rothwell, 1995; Heese et al., 1998). In hippocampal slice preparations *in vitro*, long-term potentiation as a basic mechanism of neuronal plasticity was accompanied by substantial induction of IL-1 β that depended on NMDA receptor signaling and in turn enhanced the maintenance of long-term potentiation (Schneider et al., 1998). Taken together, it is therefore conceivable that under certain circumstances proinflammatory cytokines participate in a physiologic stress response that contributes to the development of tolerance and postlesional plasticity. Interestingly, tolerance induction by brief nonlethal brain ischemia (that is, ischemic preconditioning) recently has been shown to be likewise accompanied by significant up-regulation of TNF- α and IL-1 β mRNA (Wang et al., 2000a,b). Thus, preconditioning stimuli as different as CSD and mild transient ischemia apparently activate similar molecular effector pathways that may critically depend on the production of proinflammatory cytokines.

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Interleukin-18 Expression After Focal Ischemia of the Rat Brain: Association With the Late-Stage Inflammatory Response

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Summary: Interleukin-18, previously designated interferon γ -inducing factor, is a proinflammatory cytokine structurally related to interleukin-1 β and is therefore considered a member of the growing family of interleukin-1-like cytokines. Both interleukin-18 and -1 β are synthesized as inactive precursors that necessitate cleavage by caspase-1 for functional activity. In this study, the authors analyzed the expression pattern of interleukin-18, -1 β , and caspase-1 in focal brain ischemia induced in rats either by permanent middle cerebral artery occlusion or by photothrombosis of cortical microvessels. Using reverse transcriptase-polymerase chain reaction, they found a delayed increase of interleukin-18 mRNA starting at 48 hours and reaching its peak between 7 and 14 days after ischemia. In contrast, interleukin-1 β mRNA peaked within 16 hours and was downregulated thereafter. The time course of caspase-1

mRNA expression paralleled that of interleukin-18, but not of interleukin-1 β mRNA. Immunocytochemically, interleukin-18 expression was localized to ED1-positive phagocytic microglia/macrophages infiltrating the necrotic lesion between 3 and 6 days after ischemia. In contrast, interleukin-1 β immunoreactivity was expressed by ramified microglia in the infarct border zone and remote ipsilateral cortex during the first 16 hours postlesion. Induction of interleukin-18 was not accompanied by detectable expression of interferon- γ mRNA. Their data show spatial and temporal diversity in interleukin-1 and -18 cytokine family expression in brain ischemia, and suggest a role of the interleukin-18/caspase-1 pathway in late-stage inflammatory responses to focal brain ischemia. **Key Words:** Central nervous system—Cerebral ischemia—Cytokine—Inflammation—Macrophage.

Focal ischemic brain damage elicits a strong inflammatory response that involves both activation of resident microglia and recruitment of blood-derived macrophages and T cells (Arvin et al., 1996; Stoll et al., 1998). Inflammatory mechanisms can contribute to secondary neurotoxicity and the exacerbation of ischemic brain damage (Relton and Rothwell, 1992; Barone et al., 1997), but are also involved in neuroprotection, tissue remodeling, and wound-healing processes (Clark et al., 1993; Wang et al., 1998; Shohami et al., 1999). In rat models of focal ischemia, the early-stage inflammatory response is mainly governed by resident microglia, whereas hematogenous macrophages are recruited with a delay of 2 to 3 days (Schroeter et al., 1997). Active synthesis of proinflammatory cytokines, such as tumor

necrosis factor α and interleukin-1 β , is restricted to a narrow time window of 12 to 24 hours after lesion induction, and is thereby correlated with the activation of resident glia rather than hematogenous cell infiltration (Liu et al., 1993; Liu et al., 1994; Jander et al., 2000). Therefore, the cytokine profile and the functional implications of late-stage macrophage responses to focal ischemia are currently unknown.

Cytokines of the interleukin-1 family, along with their specific receptors and naturally occurring antagonists, have been critically implicated in mechanisms of acute and chronic neurodegeneration (Rothwell et al., 1997; Touzani et al., 1999). Recently, an additional member of the interleukin-1 cytokine family, interferon- γ -inducing factor, was identified in a mouse model of endotoxic shock and was subsequently designated *interleukin-18* (Okamura et al., 1995; Bazan et al., 1996). Similar to interleukin-1 β , interleukin-18 is synthesized as an inactive precursor molecule that necessitates cleavage by caspase-1 for functional activation (Ghayur et al., 1997; Gu et al., 1997; Akita et al., 1997). Interleukin-18 is a critical regulator of innate immunity that overall promotes the development of proinflammatory type-1 T cell responses hallmarked by the release of interferon- γ (Kohno and Kurimoto, 1998). Interleukin-18 also exerts

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proinflammatory effects, such as the upregulation of the intercellular adhesion molecule-1 adhesion receptor independent of the induction of interferon γ (Kohka et al., 1998; Kitching et al., 2000).

In the nervous system, interleukin-18 is induced in prototypic Th1-mediated central and peripheral nervous system diseases such as experimental autoimmune encephalomyelitis (Jander and Stoll, 1998) and neuritis (Jander and Stoll, 2001), respectively, in parallel with caspase-1 messenger RNA (mRNA). Functional studies suggest an important role of the interleukin-18/caspase-1 pathway in the activation of pathogenic T helper cells in the context of central nervous system autoimmunity (Wildbaum et al., 1998; Furlan et al., 1999). Although constitutive expression of interleukin-18 has been described in the brains of normal rats (Culhane et al., 1998) and in glial cell cultures (Conti et al., 1999), its potential role in degenerative and ischemic brain injury is unclear. In the present study, we investigated whether interleukin-18 is induced after focal ischemia in the rat brain. Using reverse-transcriptase polymerase chain reaction (RT-PCR) and immunocytochemistry, we analyzed the time course and cellular localization of interleukin-18 mRNA and protein in two models of permanent cortical ischemia: surgical occlusion of the middle cerebral artery (MCAO) and noninvasive photothrombosis of cortical microvessels. Our data provide evidence for a role of interleukin-18 in late-stage inflammatory responses to focal ischemic brain damage.

MATERIALS AND METHODS

Animal experiments

Focal cortical infarctions were induced in adult male Wistar rats either by permanent MCAO occlusion or by photothrombosis of cortical microvessels using inhalation anesthesia [halothane 1.3% in a 1:2 oxygen:nitrogen mixture] as described in detail elsewhere (Schroeter et al., 1994; Jander et al., 1995). All animal experiments were performed in accordance with institutional guidelines.

For MCAO, the skin was cut coronarily between the ear and eye and the temporal muscle was dissected. The temporal skull was opened under microsurgical conditions, the dura was opened with a sterile 27-gauge cannula, and the middle cerebral artery was coagulated twice (at the level of the inferior cerebral vein and distally) and was cut in between. The temporal muscle and the skin was closed in separate layers, which resulted in mainly cortical infarctions that could be macroscopically identified from 4 hours after induction onwards. For RNA isolation and subsequent RT-PCR analysis, infarcted and noninfarcted contralateral cortex was prepared 4, 16, and 24 hours and 3, 6, and 14 days after ischemia ($n = 3-4$ animals at each time point).

In the photothrombosis model, a fiberoptic bundle of a cold light source was centered stereotactically 4 mm posterior and 4 mm lateral from Bregma on the skull exposed by a dorsal midline incision of the skin. Then, 0.4 mL sterile-filtered Rose Bengal solution (10 g/L) was administered through a femoral vein catheter, and the brain was illuminated for 20 minutes. The

skin was sutured, which resulted in a cone-shaped pure cortical infarction that was readily visible 4 hours after induction of ischemia. For PCR analysis, ischemic lesions (A in Fig. 2a) and frontal noninfarcted cortex of the ipsilateral hemisphere (D in Fig. 2a) and contralateral cortex homotopic to the infarct (C in Fig. 2a) were prepared at 4 h, 16 h, 24 h, 2 d, 3 d, 6 d, 14 d, and 28 d ($n = 4$ per time point). Approximately 30 to 50 mg tissue (wet weight) were obtained from each site.

As a comparative paradigm of immune-mediated central nervous system inflammation, experimental autoimmune encephalomyelitis (EAE) was induced in 8-week-old female Lewis rats (weight, 180–200 g) by active immunization with a synthetic peptide (25 μ g per animal) corresponding to the amino acids 68 to 86 of guinea pig myelin basic protein (MBP68–86), as detailed elsewhere (Jander et al., 1998a). According to the previously defined time course of interferon- γ mRNA expression in this model (Jander et al., 1998a), animals were killed at early (day 11), peak (day 15), and recovery disease stages (day 21) by an overdose of ether ($n = 4$ rats at each time point). The lumbar and lower thoracic spinal cord (≈ 300 mg wet weight per animal) was rapidly prepared by air insufflation and immediately homogenized in 5 mL TRIzol Reagent (GIBCO BRL, Gaithersburg, MD), and stored at -80°C until RNA isolation.

Semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA was prepared using the TRIzol reagent according to the manufacturer's instructions and was quantified spectrophotometrically. One microgram RNA isolated from each tissue sample was reverse transcribed using oligo(dT)₂₀ primers and SuperscriptII-Reverse Transcriptase (GIBCO BRL) according to the manufacturer's protocol. The cDNA equivalent to 20 ng total RNA was subjected to subsequent PCR analysis, as detailed elsewhere (Jander et al., 1998b), using primer pairs specific for rat interleukin-1 β [sense, 5'-AGAAGAGCCC-GTCCCTGTGACTC-3'; antisense, 5'-TCGACAATTGCT-GCCTCGTGAC-3' (Gillen et al., 1998)], interleukin-18 [sense, 5'-ACTGTACAACCGCAGTAATACGG-3'; antisense, 5'-AGTGAACATTACAGATTTATCCC-3' (Conti et al., 1997)], caspase-1 (Biosource, Camarillo, CA), interferon- γ [sense, 5'-ATCTGGAGGAAGTGGCAAAGGACG-3'; antisense, 5'-CCTTAGGCTAGATTCTGGTGACAGC (Jander et al., 1998a)], and glyceraldehyde-phosphate dehydrogenase (GAPDH) [sense, 5'-CCTTCATTGACCTCAACTACATGGT-3'; antisense, 5'-TCATTGTCATACCAGGAAATGAGCT-3' (Gillen et al., 1998)], respectively. For each primer combination, linear cycling conditions were determined in preliminary experiments, as described elsewhere (Jander and Stoll, 1998). Cycle numbers were as follows: interleukin-1 β = 25, interleukin-18 = 25, caspase-1 = 25, interferon γ = 29, and GAPDH = 18. Controls included RNA subjected to RT-PCR without addition of reverse transcriptase and PCR performed in the absence of cDNA, which yielded negative results. For each PCR product, representative samples were sequenced on an ABI PRISM 310 genetic analyzer (Perkin Elmer, Oak Brook, IL).

Quantification and statistical analysis

The PCR products were separated on ethidium bromide-stained agarose gels and quantified densitometrically, as described previously (Jander and Stoll, 1998). Relative mRNA levels were obtained by normalization against the respective GAPDH mRNA level in each sample, and are presented as

mean $\pm$ SD throughout. To compare mRNA levels at different time points and between different cortical areas, analysis of variance (Tukey multiple comparison test) was performed using GraphPad Prism 3.0 software (San Diego, CA, U.S.A.). To analyze the relation between cytokine and caspase-1 mRNA levels in tissue samples, we performed linear regression analysis using Microcal (Northampton, MA, U.S.A.) Origin 5.0 software.

Immunocytochemistry

At 8 hours and 3, 6, and 14 days after induction of cortical photothrombosis ($n = 3$ at each time point), rats were deeply anesthetized with ether and perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) through the left ventricle. Whole brains were removed from the skull, postfixed in the same fixative overnight at 4 °C, and cryoprotected by overnight infiltration with 20% sucrose in phosphate buffer at 4 °C. Free-floating 50- μ m cryostat sections were washed three times in Tris-buffered saline containing 0.05% Triton X-100 (TBS-T). Endogenous peroxidase was blocked by 30-minute incubation in 0.3% hydrogen peroxide in TBS-T. After three washes in TBS-T, sections were incubated with affinity-purified goat polyclonal antibodies against rat interleukin-1 β (1:400 dilution, R&D Systems, Minneapolis, MN, U.S.A.) or rat interleukin-18 (1:2000, R&D Systems), respectively, in 2% normal horse serum in TBS-T for 16 hours at 4 °C. After three washes in TBS-T, bound antibody was detected using biotinylated horse anti-goat immunoglobulin G (Vector Laboratories, Burlingame, CA), respectively, and the ABC Elite kit (Vector) with diaminobenzidine as substrate. Control experiments in which primary antibodies were either omitted, replaced by irrelevant control immunoglobulin G, or preadsorbed with a 10-fold excess of recombinant cytokine proteins (R&D Systems) revealed complete loss of specific immunostaining. Sections were mounted onto gelatin-coated slides, air-dried, dehydrated with ascending series of ethanol, cleared, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Immunofluorescent double-labeling and confocal microscopy

Free-floating sections were pretreated as described previously and incubated for 16 hours at 4 °C with goat polyclonal antibody against rat interleukin-18 (1:500, R&D Systems) combined with mouse monoclonal antibody ED1 against phagocytic macrophages (1:2000, Serotec, Oxford, UK). Immunofluorescent detection of bound interleukin-18 antibody was performed with catalyzed reporter deposition (Shindler and Roth, 1996) using horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) followed by the TSA Plus Fluorescein system (NEN Life Science Products, Zaventem, Belgium) according to the manufacturer's instructions. Bound ED1 mouse monoclonal antibody was detected by conventional immunofluorescence using TexasRed (TXRD)-conjugated donkey antimouse immunoglobulin G (1:100; Jackson ImmunoResearch). The horseradish peroxidase- and TXRD-conjugated secondary antibodies were applied simultaneously for 2 hours at room temperature. After completion of staining, sections were mounted using Vectashield mounting medium (Vector Laboratories). Samples were analyzed using a Leica TCS-NT confocal laser scanning system with an argon-krypton laser on a Leica DM IRB inverted microscope. Images were acquired from two channels at 488 and 568 nm. To analyze the localization of different antigens in double-stained samples, images obtained from the appropriate excitation wavelength were collected and merged.

RESULTS

Delayed induction of interleukin-18 and caspase-1 mRNA after focal brain ischemia

We first used semiquantitative RT-PCR to compare the time course of interleukin-18, caspase-1, and interleukin-1 β expression in the MCAO model (Fig. 1). Both interleukin-18 and caspase-1 transcripts showed only slight, statistically insignificant increases at early stages (up to day 3) after ischemia. At day 6, however, both interleukin-18 and caspase-1 mRNA exhibited a significant increase compared with the control level. In contrast, interleukin-1 β mRNA increased rapidly with an early peak at 4 hours and a decrease thereafter.

To study the spatiotemporal distribution of interleukin-18 and caspase-1 mRNA induction in more detail, we used the model of photochemically induced cortical ischemia in the rat (Watson et al., 1985; Jander et al., 1995). With this method, relatively small, sharply demarcated cortical infarcts are generated that, in contrast to the MCAO model, reliably allow the differentiation between ischemic infarct and nonischemic intact surroundings of the ipsilateral hemisphere. The time course of infarct development and inflammation is similar in both models (Stoll et al., 1998; Jander et al., 2000). Using the photothrombosis model, we have shown previously that the induction of inflammatory gene expression after focal ischemia is not restricted to the primary lesion, but likewise involves the entire ipsilateral hemisphere with upregulation of proinflammatory cytokines (Jander et al., 2000) and activation of resident microglia (Schroeter et al., 1999). These remote responses are elicited by cortical spreading depression (Jander et al., 2000; Jander et al., 2001).

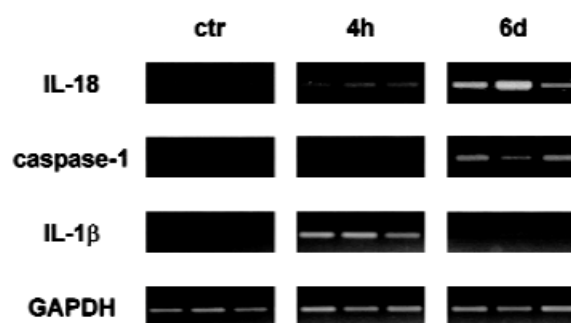


FIG. 1. Original reverse-transcriptase polymerase chain reaction findings showing differential induction of interleukin-18, caspase-1, and interleukin-1 β mRNA in cortical ischemic lesions induced by permanent occlusion of the middle cerebral artery. Representative results from three animals are shown at each time point. Whereas interleukin-1 β mRNA is induced strongly at 4 hours and downregulated at 6 days, both interleukin-18 and caspase-1 mRNA induction are considerably delayed with peak levels reached at 6 days after ischemia. Transcript levels of the house-keeping gene glyceraldehyde-phosphate dehydrogenase (GAPDH) remained constant at all time points.

In the photochemically induced infarctions, we found an early increase in IL-1 β mRNA and a delayed induction of both interleukin-18 and caspase-1 mRNA identical to the MCAO model (Fig. 2). Semiquantitative analysis revealed that the increase of interleukin-18 (Fig. 2B) and caspase-1 mRNA (Fig. 2D) was restricted to the evolving infarct, whereas interleukin-1 β (Fig. 2C) additionally showed a significant increase in the nonischemic cortex of the ipsilateral, but not contralateral, hemisphere. We subsequently related the transcript levels of caspase-1 in each tissue sample to those of interleukin-18 and -1 β , respectively (Fig. 3). This correlative analysis revealed a highly significant association of caspase-1 mRNA induction with interleukin-18 ($r = 0.91$, $P < 0.0001$, Fig. 3A), but not with interleukin-1 β mRNA levels ($r = -0.07$, $P < 0.54$, Fig. 3B). Thus, although caspase-1 has been implicated in the processing of both interleukin-1 β and -18 into their active forms, the spatiotemporal pattern of caspase-1 expression paralleled that of interleukin-18, but not of interleukin-1 β mRNA.

Interleukin-18 mRNA induction in focal ischemia is not accompanied by interferon γ expression

Because interleukin-18 was originally discovered as an interferon γ -inducing factor (Okamura et al., 1995), we asked if the increase in interleukin-18 mRNA after focal ischemia was accompanied by the induction of interferon γ mRNA. As positive control for interferon γ mRNA expression, we used the EAE model in which interleukin-18 plays a key role in the activation of pathogenic T helper cells producing high levels interferon γ (Jander and Stoll, 1998; Wildbaum et al., 1998). In pre-

vious studies, we have shown that the expression of both interleukin-18 and interferon γ in the spinal cord is maximal at the stage of active disease progression between 11 and 13 days after immunization (Jander and Stoll, 1998; Jander et al., 1998a). Accordingly, in our present study we could easily detect significant levels of interferon γ mRNA in the spinal cord of EAE rats 11 days after immunization with a moderate number (29) of amplification cycles (Fig. 4). Under the same conditions, no interferon γ mRNA was detectable in ischemic lesions 3, 6, and 14 days after photothrombosis (Fig. 4); even increasing the cycle number to 35 yielded no reproducible interferon γ signal from these samples (data not shown). Identical findings were obtained in the MCAO model.

Localization of interleukin-18 immunoreactivity in focal ischemia

To extend the mRNA data to the protein level, we performed immunocytochemical studies using an affinity-purified goat polyclonal antibody against rat interleukin-18 on free-floating sections of paraformaldehyde-fixed tissue (Fig. 5). In the brains of untreated control rats, we found weak constitutive expression of interleukin-18 immunoreactivity that was localized to ramified microglia-like cells. There was no significant increase of interleukin-18 immunoreactivity in the lesions up to 24 hours after ischemia. At day 3 and more strongly at day 6, increased interleukin-18 staining was found in the lesions and was most pronounced in round phagocyte-like cells in the inner border zone and necrotic core of the infarcts (Figs. 5a, 5b). Additionally, ramified microglia

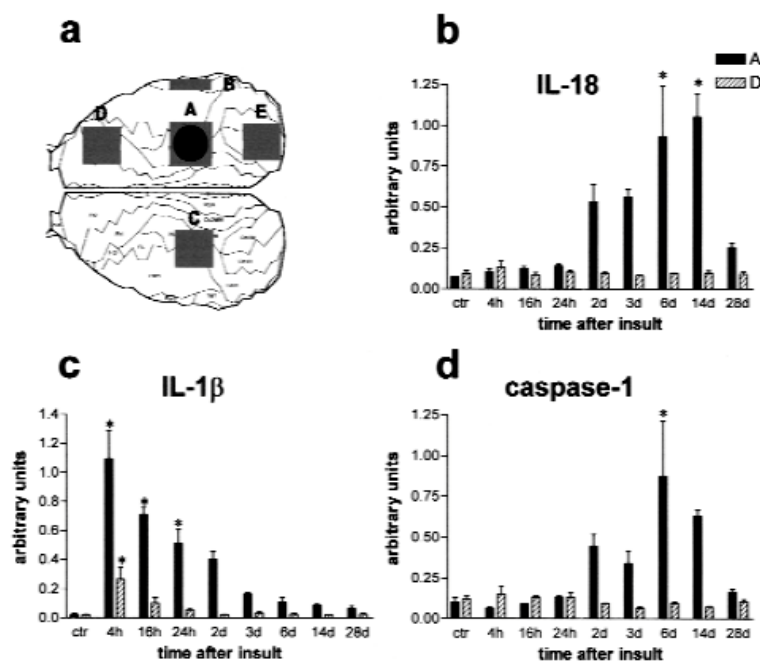


FIG. 2. Semiquantitative reverse-transcriptase polymerase chain reaction analysis of interleukin (IL)-18, -1 β , and caspase-1 mRNA levels in the photothrombosis model of focal brain ischemia. a: Localization of tissue samples removed from the ischemic lesion (A), nonischemic ipsilateral (B, D, E), and contralateral cortex (C). b–d: Relative levels of IL-18 (b), IL-1 β (c), and caspase-1 (d) mRNA exemplified for ischemic (A, filled bars) and nonischemic frontal (D, hatched bars) cortex after photothrombosis. Bars denote mean $\pm$ SD. * $P < 0.01$ compared with normal control cortex ($n = 4$ rats per time point). Note that significant induction in the nonischemic frontal cortex was found for IL-1 β , but not for IL-18 and caspase-1 mRNA. Findings in the other nonischemic ipsilateral areas (B and E; data not shown) were similar to those in the frontal cortex (D).

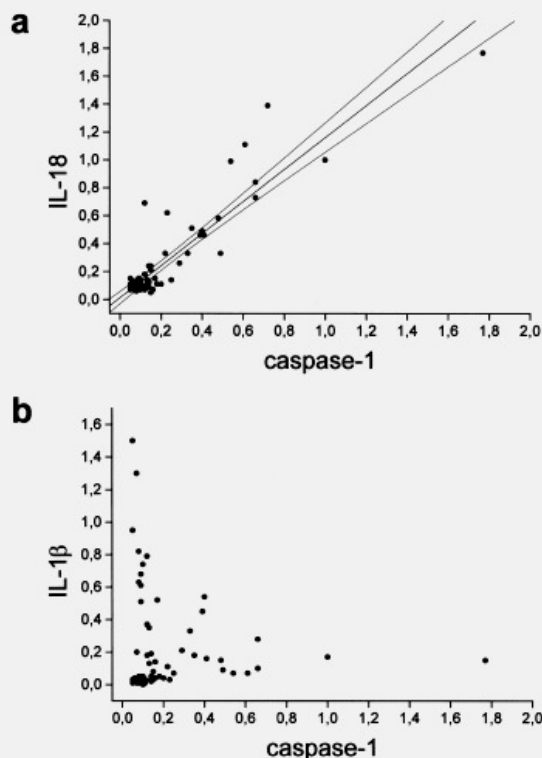


FIG. 3. Relation between caspase-1 mRNA and interleukin-18 (a) or interleukin-1 β (b) mRNA expression after photothrombosis. Correlative analysis at the level of each individual tissue sample across all time points and both ischemic and nonischemic cortex reveals a highly significant correlation of caspase-1 with interleukin-18 ($r = 0.91$, $P < 0.0001$), but not with interleukin-1 β mRNA expression ($r = -0.07$, $P < 0.54$)

in the outer border zone of the lesions expressed enhanced interleukin-18 immunoreactivity (Figs. 5a, 5c). At day 14, interleukin-18 immunoreactivity was decreasing. Thus, immunocytochemistry confirmed the delayed time course of interleukin-18 induction with a peak approximately 6 days after ischemia. Specificity of interleukin-18 immunostaining was proven by control experiments in which the primary interleukin-18 antibody was omitted or preadsorbed with excess recombinant rat interleukin-18 before staining. These procedures led to a complete disappearance of immunostaining in the ischemic lesions (Fig. 5d).

In contrast to interleukin-18, upregulation of interleukin-1 β immunoreactivity was evident 4 hours after ischemia, peaked between 8 and 16 hours (Figs. 5e, 5f), and decreased thereafter. After 6 days, no significant interleukin-1 β staining was visible. Morphologically, interleukin-1 β immunoreactive cells had the typical appearance of ramified microglia and were essentially restricted to the outer border zone of the lesion with considerable extension into the neighboring nonischemic cortex (Figs. 5E, 5F). In line with our previous findings (Jander et al.,

2000; Jander et al., 2001), significant numbers of interleukin-1 β expressing ramified microglia were found in the ipsilateral cortex far remote from the lesion (data not shown). In contrast, the remote nonischemic cortex was always devoid of significant interleukin-18 induction.

To clarify the cellular source of interleukin-18 in cerebral ischemia, we additionally performed immunofluorescent double-staining combining the polyclonal interleukin-18 antibody with the monoclonal antibody ED1 against phagocytic macrophages and microglia. Analysis by confocal microscopy revealed extensive colocalization of interleukin-18 with ED1-positive microglia/macrophages in the ischemic lesions (Figs. 6a–6c).

DISCUSSION

Interleukin-18 is a newly discovered cytokine that exhibits structural homology to the prototypic proinflammatory cytokine interleukin-1 β . Therefore, interleukin-18 is considered a member of the growing family of interleukin-1-like cytokines (Okamura et al., 1995; Bazan et al., 1996; Kohno and Kurimoto, 1998; Touzani et al., 1999). Our present study is the first to show that focal ischemia of the rat brain leads to local induction of interleukin-18 in infarcted cortex and its immediate surroundings. Interleukin-18 mRNA and protein expression exhibited a delayed time course of induction, commencing at 24 to 48 hours and peaking 6 days after ischemia. Thereby, interleukin-18 differs from interleukin-1 β , which is induced rapidly during the first few hours after ischemia and is dramatically downregulated after 2 days (Liu et al., 1993; Davies et al., 1999).

The initial inflammatory response to focal ischemia is mainly exerted by resident microglia, whereas hematogenous macrophages are recruited with a delay of at least 2 days (Schroeter et al., 1997). Accordingly, interleukin-1 β -immunoreactive cells around the ischemic lesion had the morphology of resident ramified microglia, whereas interleukin-18 was mainly localized to round phagocytes

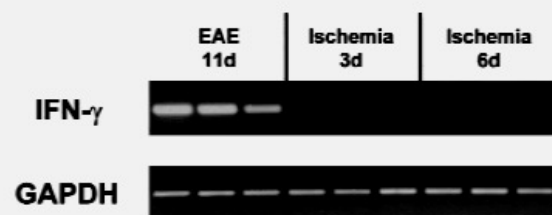


FIG. 4. Lack of interferon γ mRNA induction after focal brain ischemia. Total RNA from either spinal cord of animals in the progressive phase of experimental autoimmune encephalomyelitis (11 days after immunization, lane 1–3) or ischemic lesions 3 (lane 4–6) or 6 days (lane 7–9) after photothrombosis were analyzed by reverse-transcriptase polymerase chain reaction using rat interferon γ -specific primers. Interferon γ mRNA is easily detectable in the experimental autoimmune encephalomyelitis spinal cord, but not in the ischemic brain lesions. Representative original findings are from three animals per group.

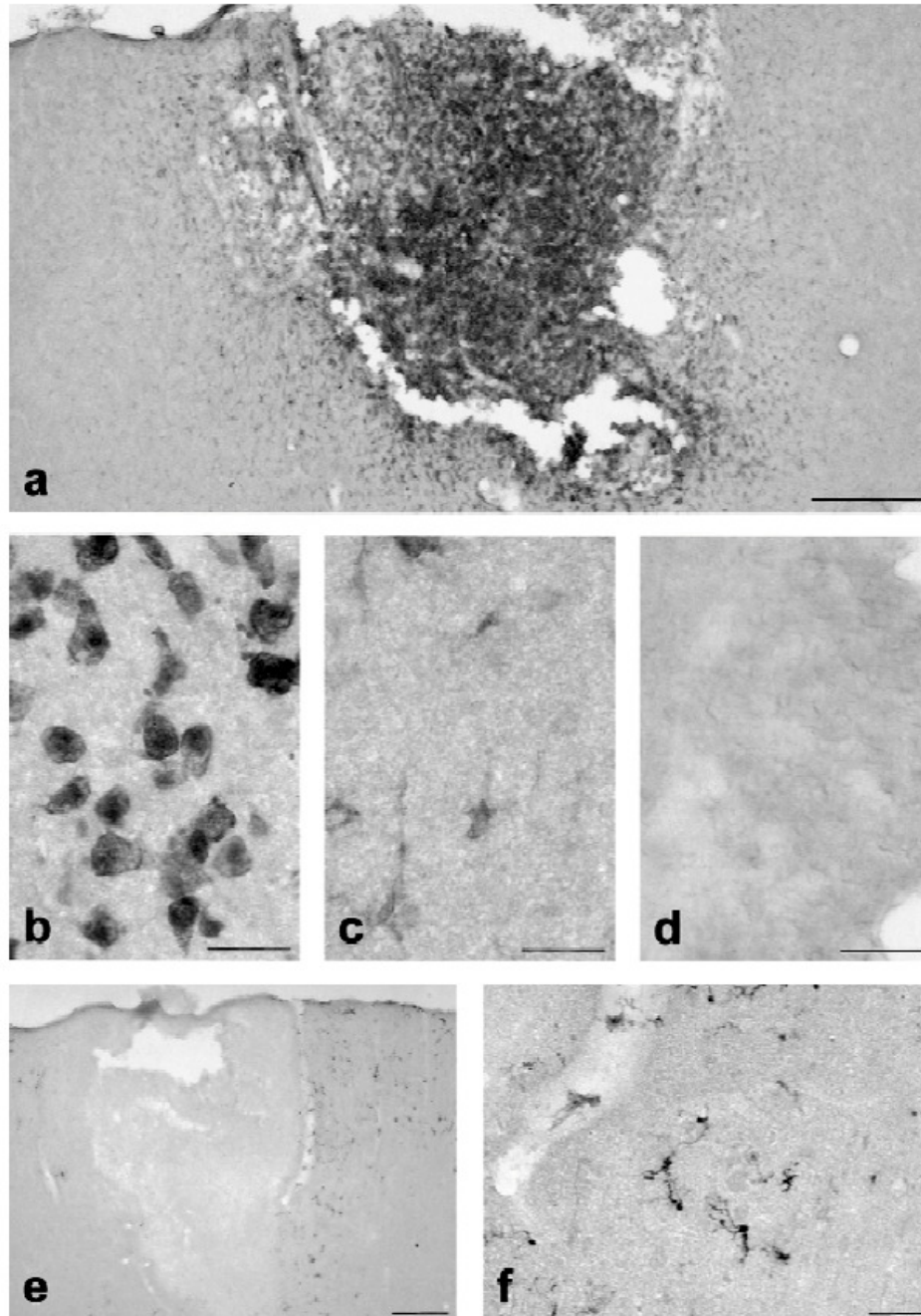


FIG. 5. (a–c) Immunocytochemical localization of interleukin-18 protein expression 6 days after cortical photothrombosis. Increased interleukin-18 immunoreactivity is detectable in round phagocytelike cells in the necrotic lesion (a and b) and in ramified microglia (a and c) in the outer border zone and immediately surrounding nonischemic cortex. (d) Lack of immunostaining after preadsorption of the primary interleukin-18 antibody with an excess of recombinant rat interleukin-18. (e and f) Localization of interleukin-1 β immunoreactivity 8 hours after ischemia. In contrast to interleukin-18, interleukin-1 β is expressed exclusively by ramified microglia in the outer border zone of the lesion with considerable extension into the surrounding nonischemic cortex, whereas the necrotic lesion core is spared. Bars = (a and e) 250 μ m, (b and c) 25 μ m, and (d and f) 50 μ m.

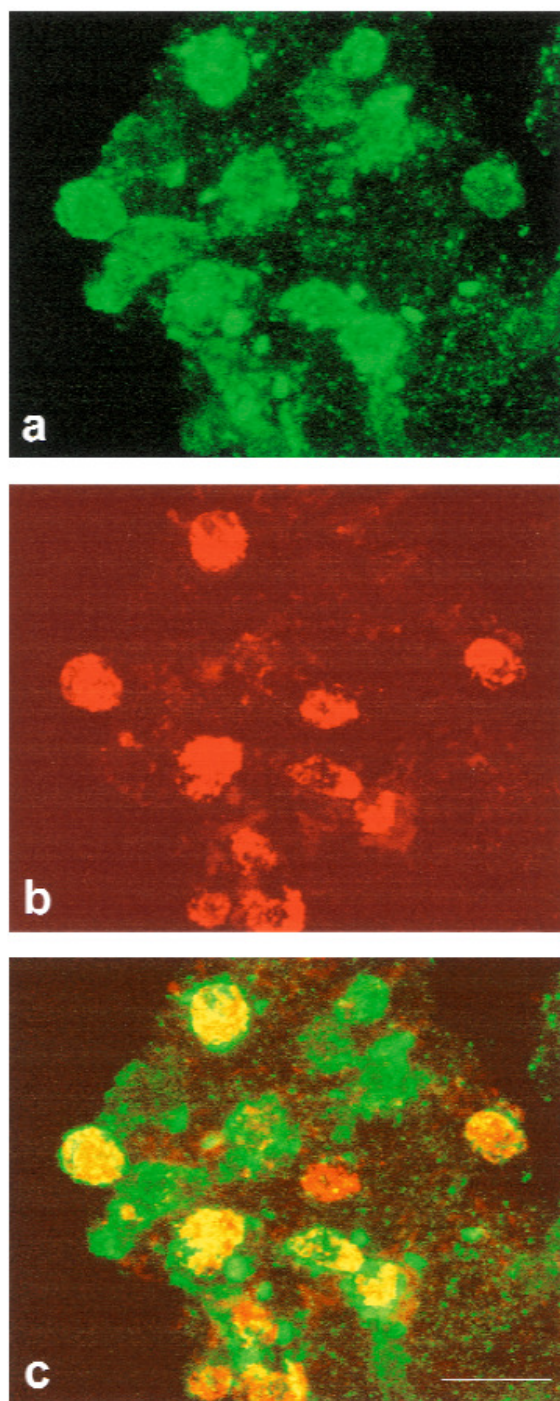


FIG. 6. Confocal analysis of double-labeling immunofluorescence for interleukin-18 (green in a) and the phagocyte marker ED1 (red in b) in ischemic lesions 3 days after cortical photothrombosis. (c) Sites of colocalization of both antigens appear yellow. Bars = μm .

infiltrating the infarct at the end of the first week after ischemia. Of note, by using mouse monoclonal antibody ED1 against a phagolysosomal antigen, activated resident microglia cannot be formally differentiated from hematogenous macrophages. Therefore, a contribution of both resident and blood-derived phagocytes to interleukin-18 synthesis during late stages after ischemia cannot be excluded. Similar to interleukin-1 β , other proinflammatory cytokines, such as tumor necrosis factor α and interleukin-6, are upregulated within a narrow time window of the first 12 to 24 hours after ischemia (Liu et al., 1994; Wang et al., 1995; Jander et al., 2000). Thus, interleukin-18 represents the first proinflammatory cytokine associated with the delayed inflammatory response to focal brain ischemia.

In previous studies, we have shown that induction of interleukin-1 β and tumor necrosis factor α after focal ischemia is not restricted to the infarcts, but is likewise observed to a significant extent in noninfarcted cortex of the entire ipsilateral hemisphere (Jander et al., 2000). Using pharmacologic blocking experiments with the *N*-methyl-D-aspartate receptor antagonist MK-801, we could show that this remote cytokine response in nonlesional cortex is elicited by cortical spreading depression (Jander et al., 2000; Jander et al., 2001). Spreading depression can be triggered by the local application of high-molar potassium chloride to the intact cortex, and is furthermore commonly observed in the surrounds of focal ischemic lesions (Hossmann, 1994). In our present study, interleukin-18 expression was restricted to macrophages and microglia in the ischemic lesion and the immediate surroundings, whereas the nonischemic cortex remote from the lesion was devoid of interleukin-18 immunoreactivity. Therefore, our data show highly differential spatiotemporal regulation of proinflammatory cytokines of the interleukin-1 family after focal brain ischemia.

Both interleukin-18 and -1 β are primarily synthesized as inactive precursor molecules that exhibit considerable structural homology (Bazan et al., 1996) and necessitate cleavage by caspase-1 (previously designated interleukin-1 β -converting enzyme) for conversion to the mature active cytokines (Ghayur et al., 1997; Gu et al., 1997; Akita et al., 1997). Thus, apart from a direct effect on early apoptotic cell death (Friedlander et al., 1997), blockade of caspase-1 may represent an intriguing way to downregulate inflammation as a potential mechanism of delayed lesion growth (Yang et al., 1999; Rabuffetti et al., 2000). In our present study, expression of caspase-1 mRNA was tightly correlated with the delayed upregulation of interleukin-18, but not with the early induction of interleukin-1 β mRNA. In fact, in both the MCAO and photothrombosis model, caspase-1 mRNA levels did not increase significantly until 2 days after ischemia. This finding is consistent with a previous Northern blot study

by Asahi et al. (1997) showing constitutive expression of caspase-1 mRNA that did not change within the first 24 hours after ischemia. Therefore, the early increase of caspase-1 bioactivity described by Rabuffetti et al. (2000) may be explained by proteolytic cleavage of caspase-1 precursor molecules rather than by *de novo* synthesis of caspase-1 mRNA. Activation of caspase-1 early after ischemia is also suggested by the beneficial therapeutic effect of interleukin-1 β antagonists in experimental stroke models (Relton and Rothwell, 1992).

Interleukin-18 was discovered based on its ability to stimulate secretion of interferon γ by T helper cells and was subsequently characterized as an important regulator of T helper cell differentiation, which overall promotes delayed type hypersensitivity reactions mediated by T helper type-1 cells (Kohno and Kurimoto, 1998). However, at least at the time points examined in our study, we did not find significant expression of interferon γ mRNA in the ischemic lesions under conditions that easily allow for its detection in the model of EAE as a classical Th1-mediated central nervous system autoimmune disease. This finding is consistent with previous findings in traumatic brain injury (Rostworowski et al., 1997) and cutaneous wound healing (Kampfner et al., 2000). However, independent from its interferon γ -inducing properties, interleukin-18 induces expression of the adhesion receptor ICAM-1 on endothelial and monocytic cells, which might provide a basis for interferon γ -independent proinflammatory actions of interleukin-18 (Kohka et al., 1998; Kitching et al., 2000). It remains to be studied whether interference with the expression of interleukin-18 or its activation by caspase-1 provides a means to alleviate late-stage inflammatory responses to focal ischemia.

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