

# Cloning of Rat Interleukin-3 Receptor $\beta$ -Subunit from Cultured Microglia and Its mRNA Expression *in vivo*

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The high-affinity receptors for interleukin-3 (IL-3), GM-CSF, and IL-5 are composed of a ligand binding ( $\alpha$ -) and a transducing ( $\beta$ -) subunit. Two distinct transducing subunits (clones AIC2A and AIC2B) have been cloned from mouse, whereas in humans, only one (common)  $\beta$ -subunit ( $\beta_c$ ) has been found. A PCR-based cloning strategy was used to obtain a full-length cDNA sequence from rat microglia including 5'-untranslated regions. Sequence analysis revealed a number of features indicative of the presence of only one  $\beta$ -subunit in the rat. Most likely, the new rIL-3R $\beta$  cDNA is the rat equivalent of human respective murine (AIC2B)  $\beta_c$  subunits. Regulation of rIL-3R $\beta$  mRNA expression was investigated in cultured microglia and *in vivo*. Purified microglia expressed significant amounts of rIL-3R $\beta$  mRNA. Addition of lipopolysaccharide (LPS) resulted in a marked upregulation of rIL-3R $\beta$  mRNA within approximately 4 hr. No downregulation was observed within 1 week's treatment. No rIL-3R $\beta$  mRNA was detectable in normal rat brain. However, 3 hr after a single injection of LPS into the tail vein of a rat, a marked induction of receptor mRNA occurred in a variety of brain regions. Transcriptional rates subsided significantly after 24 hr. rIL-3R $\beta$  mRNA was visualized by *in situ* hybridizations with cRNA antisense probes in ramified cells formerly characterized as microglial cells. rIL-3R $\beta$  mRNA was also induced in rat brain after occlusion of middle cerebral artery (MCAO). Time course of induction was slower than in lipopolysaccharide (LPS)-treated animals and lasted for more than 24 hr until a significant downregulation became apparent. In centers of infarcted areas, receptor-positive cells likely were blood-borne macrophages and microglia, whereas in areas distant from lesions, cells with morphologies typical of microglia stained positive with digoxigenin (dig)-labeled cRNA probes. It is concluded, that induction of rIL-3R $\beta$  mRNA in brain microglial cells is a very early marker of microglial activation *in vivo*.

[Key words: microglia, interleukin-3, receptor, fever induction, middle cerebral artery (MCAO), RT-polymerase chain reaction, RACE]

Microglia are the immunocompetent cells of the brain. Most likely, they derive from mononuclear cells of the blood but adopt special features in brain tissue. Extensive investigations on this cell type in recent years have revealed a variety of characteristics related to or distinct from blood monocytes (for reviews, see Giulian, 1987; Jordan and Thomas, 1988; Perry and Gordon, 1991). A special feature of microglia apparently is their expression of genes typical for cells of the lymphoid lineage, such as a lipopolysaccharide (LPS)-inducible K<sup>+</sup> channel (Nörenberg et al., 1993), or interleukin-3 (Gebicke-Haerter et al., 1994). Furthermore, it has been shown that these cells respond to a number of cytokines including granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3 with enhanced proliferation (Giulian and Ingeman, 1988; Ganter et al., 1992). The responsiveness to IL-3 suggests the concomitant expression of IL-3 receptors in microglia. These receptors belong to a larger family of cytokine receptors (Bazan, 1990; 1991; Stahl and Yancopoulos, 1993; Sato and Miyajima, 1994), generally composed of two subunits ( $\alpha$  and  $\beta$ ). The murine IL-3 receptor  $\beta$ - (Gorman et al., 1990; Itoh et al., 1990) and  $\alpha$ -subunits (Hara and Miyajima, 1992) have been cloned recently. The results revealed the presence of two  $\beta$ -subunits in this species. One subunit (clone AIC2A) appears to transduce only the IL-3 signal (Wang et al., 1992), whereas the other subunit (clone AIC2B) is required to transduce signals from IL-3-, IL-5, and GM-CSF (Takaki et al., 1991; Miyajima et al., 1992a). This common subunit likely corresponds to the  $\beta_c$ -subunit that has been identified in humans (Hayashida et al., 1990; Kitamura et al., 1991; Kitamura and Miyajima, 1992). AIC2B-derived gene product as well as the human  $\beta_c$ -subunit do not bind the ligand (Wang et al., 1992). Typically,  $\alpha$ -subunits are responsible for (low-affinity) binding of the cytokines. Ligand-activated  $\alpha$ -subunits associate with  $\beta$ -subunit. This high-affinity form of the receptor transports the signal across the plasma membrane.

Hence, in mice, IL-3 is endowed with a dual signal transduction option through two homologous but distinct  $\beta$ -subunits. Until now, no information about two comparable subunits in the rat or any sequence data about the rat IL-3 receptor ( $\alpha$ - and  $\beta$ -subunits) have been available. The present study was aimed at gaining more insight into the molecular biology of the rat IL-3 receptor. Based on the cDNA sequence of the murine IL-3-specific transducing unit (AIC2A), we sought to identify a homologous subunit in rat microglia by RT-PCR and to obtain its full-length cDNA sequence. The findings strongly suggest the lack of such a subunit and reveal the presence of only one common  $\beta_c$ -subunit. Using part of the new cDNA as a rat-specific probe, we studied the question of whether or not this subunit is

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**Table 1. Oligonucleotide primers used in this study**

	5'-Primer	3'-Primer	Size of PCR product	PCR conditions
I	5'-AAGCGGCTTCAGGACTCC	AGGTAGGGCCCCATTGAAG-3'	1253 bp	35 cycles 54°C
II		CGGGCCACATAGATACTGTT-3'		
III		GACCCAGAGGTTGCAAGTGT-3'	766 bp	35 cycles 62°C
IV	5'-CTGGCAGACCCAGGAAGCAA	CTGTGGTGATGCTCAGGCTC-3'	1140 bp	30 cycles 58°C
V	5'-ATGGAGCCCAGAGGTACTACT	TACTGGAGAAGGTCACCGTG-3'	829 bp	30 cycles 60°C
VI	5'-ACGTCAACACTGCTCTACA	CTTGGCCATAGTCTTAAC-3'	311 bp	35 cycles 54°C

subject to LPS-mediated regulation in cultured microglia. Finally, we have chosen two pathological paradigms to investigate its gene induction and mRNA localization *in vivo*. These investigations clearly show a very rapid induction (within a few hours) of rIL-3R $\beta$  mRNA in brain microglial cells. Strategies aimed at inhibiting induction of this receptor (e.g., anti-inflammatory therapies) might be suitable to better contain brain damage.

## Materials and Methods

### Microglial cultures

Floating microglial cells were harvested from confluent mixed astrocyte cultures and plated in new culture dishes. After adherence, media was removed and more culture supernatants from mixed astrocyte cultures were added to reach higher cell densities. Isolated cells were pure as tested previously by a variety of cell type specific markers (Gebicke-Haerter et al., 1989). Lipopolysaccharide (*S. typhimurium*, SEBAK, Aidenbach, Germany)-treated and control cells were lysed in guanidinium isothiocyanate/mercaptoethanol solution, and total RNA was extracted according to Chomczynski and Sacchi (1987).

### PCR-based cloning and sequencing of the rat IL-3 receptor $\beta$ -subunit

Primer pair I (Table 1) was selected from the published murine sequence (Gorman et al., 1990) and used in reverse transcriptase-polymerase chain reaction (RT-PCR) with total microglial RNA. Multiple bands were obtained after electrophoretic separation of PCR mixture. A DNA band of approximately 1.2 kb size was excised from the gel and PCR amplified again. The resulting cDNA band was cloned into pCR<sup>II</sup> plasmid (Invitrogen, San Diego, CA) and sequenced on an automatic laser fluorescence sequencer (ALF). Sequence information of this cDNA fragment was used to select two inverse primers (II, III; Table 1) for the 5' rapid amplification of cDNA ends (RACE) method (purchased from GIBCO-BRL, Eggenstein, Germany). The cDNA product was cloned and sequenced as above. To obtain the 3' end, a new primer selected from the first rat sequence and an inverse complementary primer selected from a region downstream of the AIC2A stop codon and highly conserved between mouse and human (IV; Table 1) were used in RT-PCR (Fig. 1). All subsequent studies on gene regulation in the rat were carried out with an additional primer pair selected from the rat sequence (V; Table 1). Specific PCR conditions are listed in Table 1.

For semiquantitative estimation of PCR products, cDNA<sup>512</sup> (a ribosomal protein) was amplified from the same RT mixtures using the primers listed in Table 1 (VI) (Ayane et al., 1989). It is assumed that this mRNA does not change significantly upon the treatments employed in this study, and that it allows for an estimation of the integrity of the RNA.

### Animal treatments

**Lipopolysaccharide (LPS) administration.** Male Sprague-Dawley rats (180–200 gm) were injected intravenously with 5 mg/kg LPS (*E. coli*, 055 B5 Westphal, Difco) dissolved in sterile saline (Buttini and Bodecke, 1994). Control animals received injections of 0.9% saline alone. At different times after LPS injections (1 hr, 3 hr, 6 hr, 1 d, 2 d), animals were deeply anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital and blood was removed through intracardial perfusion with sterile, heparinized PBS. Brains were removed, freed from meninges, and dissected on ice. Tissue from cortex, hippocampus, striatum, diencephalon, cerebellum, brainstem, and the pituitary gland was prepared, immediately homogenized in solution D (Chomczynski and Sacchi, 1987), snap frozen, and stored at  $-70^{\circ}\text{C}$  until processed for RNA extraction.

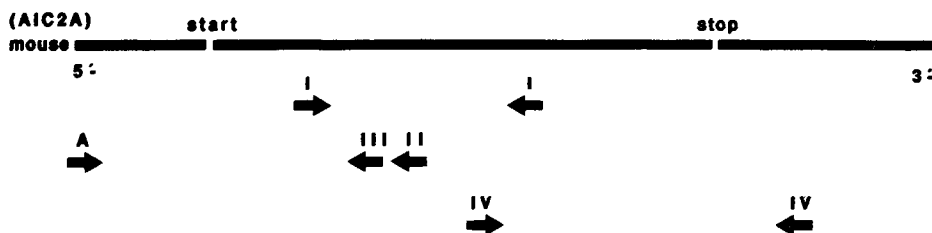
**Middle cerebral artery occlusion (MCAO).** Spontaneously hypertensive, male rats (SHR) weighing 250–300 gm were used. Under isoflurane anesthesia, the left middle cerebral artery (MCA) was permanently occluded with a bipolarator, as described in detail elsewhere (Tamura et al., 1981; Sauter and Rudin, 1986). Sham-operated animals were treated in the same way, except for closing the MCA. SH rats were used, since MCAO induces a highly reproducible total infarct size in these rats that exceeds that of normal Wistar-Kyoto rats by 60%. This allows a better assessment of histopathological changes (Sauter and Rudin, 1995).

At different times after MCAO (30 min, 1 hr, 3 hr, 6 hr, 8 hr, 1 d, 2 d, 4 d), rats were killed by decapitation, brains were quickly removed, and ischemic and nonischemic hemispheres were separated. Brainstem and cerebellum were discarded. Since blood of brain regions supplied by the MCA cannot be removed by intracardial perfusion after occlusion, MCA-occluded rats were not perfused. Brain tissue was homogenized in solution D (Chomczynski and Sacchi, 1987), snap frozen, and stored at  $-70^{\circ}\text{C}$  until further processing.

### In situ hybridization

**Brain tissue.** LPS-treated and MCA-occluded rats were killed by decapitation, brains were quickly removed, and frozen on dry ice. Cryostat

### Cloning strategy of the rat IL-3 receptor- $\beta$ subunit



**Figure 1.** Schematic representation of PCR-based cloning strategy of interleukin-3 receptor- $\beta$  from rat microglial RNA. I–IV, Oligonucleotide primers used for RT-PCR as listed in Table 1. Primers I were selected from the murine AIC2A sequence. Primers II and III were derived from the new rat sequence and used for RACE. A is the anchor primer supplied with the RACE kit. A stretch of 20 bp of the nontranslated, 3'-flanking region, highly conserved between murine and human sequences was selected to construct reverse primer IV.

rIL-3Rβ  
mAIC2A

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- 161  CCAATGACA GAGCACCAGA GATGTCAGAC T      G G      GATCATGAC C C T G      T GATGTCGAG CCAACCCCA CCAAGCTTT GACACAGAC CCGAGACAA TGGAGACCC
      C C      CA A      T      G G      GATCATGAC C C T G      T GATGTCGAG CCAACCCCA CCAAGCTTT GACACAGAC CCGAGACAA TGGAGACCC
- 41  ACCCTGCCA TACCTCCCTG TGCAGCCAC TAAATGACA AATGAGACA GCACATGGCA TTTATCTGAG GCGTCTGCTA CATGAGCCCT GCGTCTCTCT GCTGGAGACA CCGATGACA
      C      T      T      A      A      A      A      C C CA      A      A      C C CA      A      A      C C CA      A      A      C C CA      A      A      C C CA
79  AAGCCAGAG AATCTCTCC TCTGAGACT CTGAGTCTC ACAAGACATA TGTCCAGGAC TGTCCAGGAC GCTGGAGACA CAGCAGGAC CCGCAGGAG TCTGTAGCT GAGCTC...
      G A      G      G      T      T      G      G      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
196  TATCTAGTC TACAGAGAA ACAGCCAGAT TCTCTGAGC TGTATGAGA CCTCATATG TCGAGTGGC CCGTATCCCA CCGCTGTGTC CTTAGAGAT GTTCTCTCC CTTATCAGAC
199  CA      T T AT G      A A      A A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
316  TTTTCTCTC CAAGAGAGA CTACTACTCA CTCCAGCCG ATCTGTATCT GGTATCCAT CTCTGTGTC CCGTGGCCA GCATGTGAC CAGACCTTC CCAAGGATC CAGCATGAC
319  TATG G G TA C      C T      A      C      G C      G      A      C      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
436  CCTCTGGAG ACATCTCTC GCTGAGTGC G A      A      A      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
439  CCTCTGGAG ACATCTCTC GCTGAGTGC G A      A      A      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
556  GAGATGCTC CAGCCCTCA CACTCTCAAC CTCTGGTCA CTCTGAGCC AAAGCTCTC CTACCAACA GTATCTATC GCTCTCTCA CCGCTGTGTC TACCCAGAG ATGAGCTCT
559  T      A      A      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
676  TCCGAGAC CAGCCGATG GAGCCAGAG GTACACTGG ACTCTCCAG AGAGGAGAG GCTGGCCAC AAGACTTCA GTCTCTCTC GATGATCC ATCCCTCAA CTCTCTGTC
679  T G      A      A      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
796  GAGATGCTC CAGCCCTCA CACTCTCAAC CTCTGGTCA CTCTGAGCC AAAGCTCTC CTACCAACA GTATCTATC GCTCTCTCA CCGCTGTGTC TACCCAGAG ATGAGCTCT
799  C AC      G      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
916  CCGTCACTC CCGCTCAAA TGTCTCCAG CCGCTCAAC ACAAGCATA CAGCTCTCT GTTATGGCC TGGAGAGAG GATGTATCC GAGGCTTTA ACCATATCA GATGATCT
919  C G      T CC      C T G      AG      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1036 CCAACTTCA ACCTGACAA GACAGAGAC AGCTCAGCC TCAATGGAG GACTCAGAA ATCTCTCTC CATTACCCA GCATCATTC CAGTCTCTC ACAGAGAGA ACTGAGACC
1039 T      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1156 TGGAGTACA GAGACAGAA GAACTTAAAT CATCCGACA GCATGAGCT CCGCAGACT GAGCTCTCA CCTCATCTC CCGCAGACT AGGTCATCA CCACTCTCA ATGCAAGAG
      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1276 CTCTGAGAG AGTGGAGCAA TGGATCAGC TGGAGTACT ACTGGTATG GCAAGATTC TGGATATCC TATCTCTGT CTCTCTATC CTACCTTCC TCGTGGCTC CAGTCTCTC
      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1396 TCACTCTAG GGTGATGTT GTACAGAGA TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT
      C TG T      A G AC A      G AG      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1516 TCACTCTAG GGTGATGTT GTACAGAGA TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT
      G G C      G T      G G G      G G C      T T      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1636 AATATCTAG ATCATATC TGGCTGATG ACAGCCAGC CTCTCTATC TGAACCCAG GAGCAGCTC CCAATGTTA AGTAGACCA CCAATGTTA CTGGAGACC CAGAGAGAA
      A      C      CA G      T      CA      T      CA      T      CA      T      CA      T      CA      T      CA      T      CA      T      CA      T      CA      T      CA      T
1756 TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT
      G G C      G T      G G G      G G C      T T      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A      A
1976 GCTCTCTAG AGTATGATG TGTGCTCTC GAGGAGAG TCAACTGAT CCGTCTCTC CAGTCTCTC GAGGAGAG GATGTATCC GTCTCTCTC GTCTCTCTC GAGGAGACA
      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G
1996 GAGGAGACC CAGTCTCTC AAGGAGAGC CCGTCTCTC AGCTGAGAA GAGGAGAG GAGGAGAG AAGGAGAG GAGTCTCTC ATGACTCTC GAGGAGACA AAGGAGAG
      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G
2116 GAGGAGACC AATGATGAC AGCTCTCTC CTCTCTCTC CTCTCTCTC AGAGCCCTG TCTACTCTC TGGTCTCTC TGGAGTACT TGGAGTACT TGGAGTACT TGGAGTACT
      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G      G
2236 AAGTCTCTC GGTCTCTCT TGAATGAGC GCTCTCTCT CAGCAGCTC TGAAGTATC GTGAGTCTC CTCTCTCTC GAGGAGACC GCAATGTTA TGGAGTACT TGGAGTACT
      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C      C
2386 CCGTCTCTC CAGCAGCTC AATGAGACA GAGGAGACC GAGGAGAG AGGAGAGCC CCGCAGACC CTGAGGACC CCGTCTCTC CAGCAGCTC GAGGAGACC CTCTCTCT
      T G      G      A      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
2476 GCGTCTCTC CAGCAGCTC CTCTCTCTC AATGAGACC CCGTCTCTC GAGGAGACC CCGCAGACC CTGAGGACC CCGTCTCTC CAGCAGCTC GAGGAGACC CTCTCTCT
      TG T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
2586 CAGTCTCTC CAGTCTCTC TGGAGTACT CAGTCTCTC CAGTCTCTC CAGTCTCTC CAGTCTCTC CAGTCTCTC CAGTCTCTC CAGTCTCTC CAGTCTCTC CAGTCTCTC
      CC      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T      T
2714 GAGGAGACC GGTCTCTCT GTGAGTACT T CA GCTCTCTCT CAGCAGACA GCTCTCTCT GTCTCTCTC CCGTCTCTC CCGTCTCTC CCGTCTCTC CAGCAGACA
      G      A      G      T      CA      GCTCTCTCT CAGCAGACA GCTCTCTCT GTCTCTCTC CCGTCTCTC CCGTCTCTC CCGTCTCTC CAGCAGACA

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Figure 2. Nucleotide sequence of rat interleukin-3 receptor-β cDNA and changes in murine AIC2A receptor sequence. Putative signal sequence, transmembrane region, and ligand binding domain are *overlined*, or *double overlined*, respectively. WS X WS motif of cytokine receptor family is indicated in *italics*, and respective stop codons are marked \*\*\*.

sections (20 μm) were thaw mounted onto Vectabond-coated slides and stored at -70°C until use. Sections were thawed, air dried, and fixed for 20 min by immersion in 4% paraformaldehyde in PBS (2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 136 mM NaCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) at room temperature. Then they were washed once in 3× PBS, twice in 1× PBS, for 5 min each, and then incubated for 10 min in 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride. After two washes in 1× PBS, 5 min each, sections were processed for nonradioactive *in situ* hybridization.

**RNA probes and in vitro transcription.** The pCR<sup>®</sup> II plasmid (INVITROGEN, San Diego, Ca.) with cloned fragment of rIL-3Rβ (see above) was used to generate cRNA transcripts. Linearization of plasmid with *Not I*, followed by transcription with SP6 RNA polymerase (Boehringer, Mannheim, Germany), generates rIL-3Rβ antisense riboprobe; linearization with *BamH I* and transcription using T7 RNA polymerase generates the corresponding sense probe. Probes were digoxigenin (dig)-labeled using dig-labeled rUTP (400 μM, Boehringer), 2 μg linearized DNA template, 100 μM dithiothreitol (DTT), 4 U RNase inhibitor, 40 mM Tris-HCl (pH 7.5), 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 5 mM NaCl, 1000–2000 U DNA-dependent RNA polymerase. The transcription reaction was carried out for 45 min at 37°C, then 1000 U of the respective polymerase were added and the reaction mixture was incubated for additional 45 min. The DNA template was degraded by addition of 2 U DNase I and subsequent incubation at 37°C for 10 min. DNase reaction was stopped by addition of 80 μl 25 mM EDTA. Dig-labeled probes were purified on a Bio-Spin 30 column (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. Before hy-

bridization, labeled transcripts were ethanol precipitated, degraded to an average length of 150 base pair (bp) by partial alkaline hydrolysis (Cox et al., 1984), ethanol precipitated again, and resuspended in 80 μl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4) containing 0.1 M DTT.

**Hybridization and posthybridization treatment.** Hybridization solutions were composed of dig-labeled transcripts (400–500 ng/ml 4× SSC hybridization buffer), 50% formamide, 10% dextran sulfate, 1× Denhardt's (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 250 μg/ml yeast tRNA, 400 μg/ml salmon sperm DNA, 500 μg/ml heparin Na salt). Prehybridization was performed for 3 hr at room temperature. For hybridization, each slide was overlaid with 70 μl hybridization solution containing the appropriate amount of labeled transcript and incubated over night at 55°C in a chamber humidified with 4× SSC. After hybridization, sections were washed for 10 min in two changes of 2× SSC at room temperature and 30 min in 0.1× SSC at 70°C. They were then treated with RNase A (20 μg/ml in RNase buffer: 0.5 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) for 30 min at 37°C. After a wash in RNase buffer for 30 min at room temperature, sections were subjected to two additional high-stringency washing steps in 0.1× SSC at 70°C, 40 min each. Then sections were rinsed in 2× SSC and subjected to immunological detection as described below.

**Immunological detection of dig-labeled hybrids.** Slides were washed in 1× PBS for 10 min at room temperature. Nonspecific binding sites were blocked by incubation in PBS containing 3% sheep serum and 0.3% Triton X-100 for 45 min at room temperature. Slides were then incubated in the same buffer with 1:300 diluted alkaline phosphatase-

conjugated anti-dig antibody for 2 hr at 37°C. After three washes in PBS, 10 min each, and incubation in TBS, pH 9.2, containing 50 mM MgCl<sub>2</sub>, for 5 min, color was developed with 0.3 mg/ml nitroblue tetrazolium (NBT) and 0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the same buffer containing 0.24 mg/ml levamisole for 14–16 h at room temperature. Color reaction was stopped by transfer of slides into PBS. Sections were then covered with Crystal-Mount medium (Biomedica), dried for 20 min at 50°C, and coverslipped with Depex (Merck, Darmstadt, Germany).

## Results

### *PCR-based cloning of the rat IL-3 receptor β-subunit (rIL-3Rβ) from isolated microglia*

Specific primers devised on the basis of the murine master sequence (AIC2A; Hara and Miyajima, 1992) were successfully used in RT-PCR amplifications of respective rat sequences (Table 1, primer pair I). Identity of PCR products was verified by cloning of the PCR product into pCR™II plasmid and subsequent sequencing of the insert. The new rat sequence was used to select and synthesize two appropriate reverse primers for the 5'-RACE method (Frohman et al., 1988). The PCR product was processed as described above. The 3'-end of rat IL-3 receptor-β was identified as described in Methods and Materials. Figure 1 summarizes the cloning strategy, and Figure 2 shows the complete cDNA sequence of rIL-3Rβ in comparison with murine AIC2A sequence.

### *Primary structure of cloned rat IL-3 receptor β-subunit (rIL-3Rβ)*

The new rat cDNA sequence encodes a polypeptide of 896 amino acid residues. It shows a single base pair exchange at the location of the murine (AIC2A) stop codon (TAG into CAG), which results in an elongation of the translated cDNA by 54 base pairs (Fig. 2). The stop codon in the rat sequence is located at a site corresponding to the stop site in the murine AIC2B. Despite sequencing a number of different clones and additional direct sequencing of the PCR product, the location of the stop codon was unchanged.

In Figure 3, amino acid sequences of murine AIC2A and AIC2B are compared to the rat sequence predicted from the new cDNA sequence. The rat sequence has 77.7% and 79.5% amino acid sequence identity with murine AIC2A and AIC2B, respectively. The N-terminal 22 amino acid residues constitute a hydrophobic amino acid sequence typical of signal peptides (Klein et al., 1985). The WSXWS motif typical for cytokine receptors (Miyajima et al., 1992b) preceding the membrane spanning region (double overlined) is put in italics. It is identical with the murine AIC2A, but differs from murine AIC2B. Another WSXWS-like motif is located beginning with amino acid 229 (PSXWS). Furthermore, the external domain contains eight conserved cysteine residues (39, 49, 77, 94, and 253, 263, 292, 309). Four potential N-glycosylation sites are found in positions 62, 262, 312, and 349, the first and last being conserved between murine and human sequences. One site critical for ligand binding (Wang et al., 1992) is printed in bold. In contrast to the murine sequences, it shows an insertion of one amino acid and resembles more AIC2B than AIC2A. According to the mutagenesis results by Wang et al. (1992) performed in mice, rIL-3Rβ likely is not suitable for specific ligand binding. Apart from 151 species-specific amino acid exchanges (exchanges in second and third lines), there are amino acids in the rat either identical with AIC2A (exchanges *only* in third line) or AIC2B (exchanges *only* in second line). Thirty-two amino acids or deletions are identical with AIC2A, and 31 are identical with AIC2B. Alto-

gether, these data suggest that in the rat there is only one IL-3 receptor β-subunit comparable to the β<sub>c</sub>-subunit in humans.

### *Occurrence of rIL-3Rβ mRNA in microglial cultures*

To investigate rIL-3Rβ mRNA expression in culture, isolated microglia were maintained up to 1 week in the absence or presence of LPS (100 ng/ml). Results in Figure 4 clearly show that the receptor was expressed in LPS-free conditions, and culture time had no significant influence on transcriptional rate. It is important to note that the number of PCR cycles had to be reduced to 28 when using RNA preparations from cultured cells. LPS substantially increased transcription of rIL-3Rβ mRNA beginning between 0 and 4 hr of LPS treatment. Elevated receptor mRNA was invariably found even after 1 week of LPS treatment. S12 mRNA, used as internal standard, was unchanged in all conditions (Fig. 4, bottom).

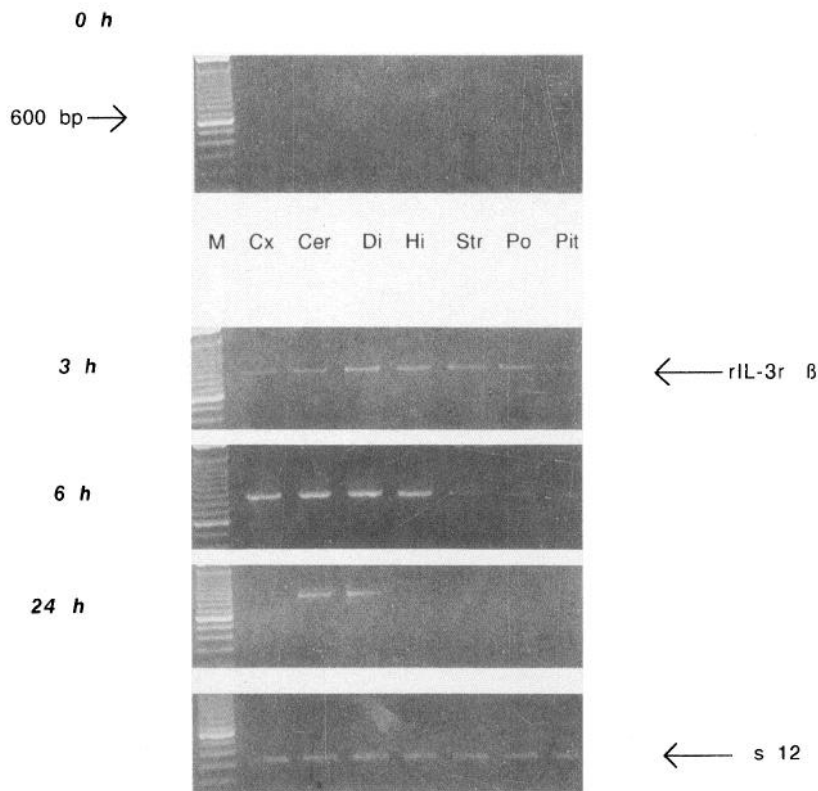
### *Occurrence of rIL-3Rβ mRNA in rat brain and its regulation by LPS*

A variety of brain areas were investigated before and after systemic administration of LPS. In untreated animals, rIL-3Rβ mRNA was undetectable by RT-PCR. Three hours after injection of LPS, however, mRNA was found in all brain regions investigated (Fig. 5). The highest amounts were observed in cerebellum, diencephalon, cortex, and hippocampus. Maximum expression was attained between 6–10 hr of LPS treatment. After 24 hr, mRNA levels had declined to baseline in most regions except in cerebellum and diencephalon. S12 cDNA amplified from each respective RT mixture served as control for an equal quality of RNA and comparable reverse transcriptions (bottom panel). *In situ* hybridizations with a receptor-specific antisense probe revealed occurrence of rIL-3Rβ mRNA throughout the brain as early as 3 hr after LPS injection. The scattered appearance (Fig. 6A) and morphologies of receptor-positive cells (Fig. 6B,C) were strongly reminiscent of microglia. No signal was obtained in parallel *in situ* hybridizations with sense probes (not shown).

### *rIL-3Rβ mRNA expression after occlusion of the middle cerebral artery (MCAO)*

Receptor mRNA was traceable by RT-PCR 1 hr after artery occlusion in the affected brain hemisphere and reached peak values between 8 and 24 hr. A significant decline was observed at day 2 after occlusion, and there was barely any rIL-3Rβ mRNA detectable by RT-PCR at day 4 (Fig. 7). *In situ* hybridizations revealed scattered rIL-3Rβ mRNA-positive cells at 3–6 hr after onset of ischemia in brain regions originally supplied by the middle cerebral artery (frontal, fronto-parietal, and temporal cortex, caudate putamen, globus pallidus, part of hippocampus, lateral thalamus, and amygdala; not shown). One day after MCAO, increasing numbers of rIL-3Rβ mRNA-positive cells were observed in the infarcted brain areas as well as in parenchyma adjacent to infarct region. Additionally, morphologies of cells changed with time. Two days after MCAO, many cells in infarct core showed condensed morphologies with no or short stout extensions (Fig. 8B,E,F). Supposedly, it is a mixture of blood macrophages and brain microglia. In more remote areas of the affected hemisphere, positive cells displayed more ramified morphologies (Fig. 8A,C,D) suggestive of microglial cells in different states of activation. No positive cells were observed in hybridizations with sense probes (not shown).





**Figure 5.** IL-3 receptor- $\beta$  induction in rat brain upon LPS-induced fever. Substantial amounts of receptor mRNA were detectable by RT-PCR 3 hr after peripheral injection of LPS in cortex (Cx), cerebellum (Cer), diencephalon (Di), hippocampus (Hi), striatum (Str), pons (Po), and pituitary (Pit). Maximal receptor induction occurred at approximately 6 hr and declined to undetectable levels in most brain regions 24 hr after LPS injection. S12 cDNA was used as internal standard to make sure equal quantities of cDNAs were amplified. M, 100 bp DNA ladder.

## Discussion

### Receptor cloning

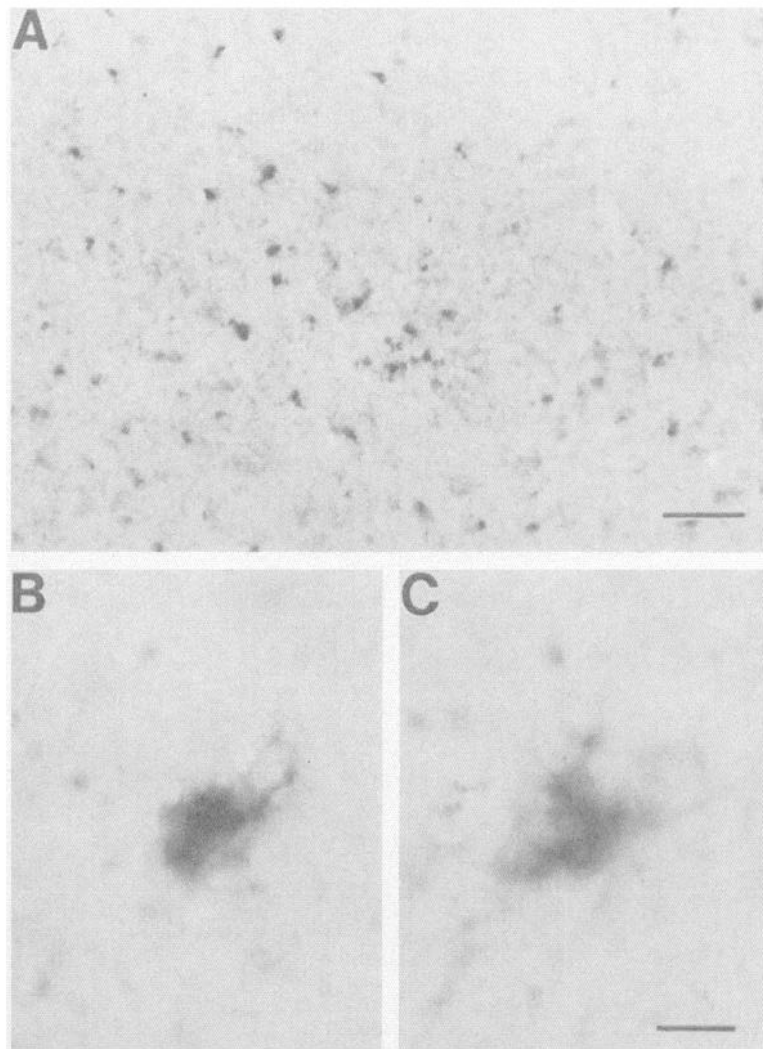
The cloned rIL-3R $\beta$  shares all the structural features known from related cytokine receptors including four conserved cysteine residues in its external domain and the unique WSXWS motif in close proximity to the transmembrane region. No kinase domain is found in the cytoplasmic region of the sequence. Moreover, the new sequence reveals a number of features interesting in both evolutionary and biochemical aspects. Apart from species-specific differences, there are almost 50% amino acid exchanges in AIC2A, where AIC2B and the rat sequences are identical, and 50% amino acid exchanges in AIC2B, where AIC2A and the rat sequences are identical. This finding strongly supports the likelihood of a developmental segregation of this gene between mouse and rat resulting in the lack of mRNA for a second  $\beta$ -receptor subunit in the rat. It was hypothesized previously, that closely related AIC2A and AIC2B genes were generated by gene duplication of a common ancestral gene (Hara and Miyajima, 1992), which raised the question why in humans (and now in rat) there is only one common  $\beta$ -subunit. The authors speculate, that  $\beta_c$  may be the prototype gene, and gene duplication has occurred after divergence between the mouse and humans. On the other hand, neither we nor others (Hayashida et al., 1990) can exclude the possibility of the presence of a second  $\beta$ -subunit both in rats or humans, since the expression of AIC2A mRNA in mice can be very low (Gorman et al., 1990). If a homologous mRNA in the rat or man is expressed at comparable or even lower level, it may escape analytical techniques.

Provided there is only one (common)  $\beta$ -subunit expressed in the rat, one would anticipate cross-competition between GM-CSF, IL-5, and IL-3, which does not occur in murine tissues between IL-3 and the other cytokines (Kitamura et al., 1991),

and which may impact on cellular responsiveness. Furthermore, the 3'-elongated form of the transducing unit in the rat may influence subsequent intracellular signalling pathways in ways distinct from the murine system, although in humans, major sites for signalling appear to reside in closer proximity to the plasma membrane (Sakamaki et al., 1992).

### LPS-induced rIL-3R $\beta$ mRNA expression in cultured microglia and in vivo

The IL-3/IL-3 receptor system appears to become increasingly more interesting in brain research. The ligand reportedly has a beneficial effect for the survival of cholinergic neurons both *in vitro* and *in vivo* (Kamegai et al., 1990). Moreover, rIL-3R $\beta$  appears to be involved in certain pathological events, as shown here. Undoubtedly, the primary cell type expressing these genes is the microglial cell. We have shown recently (Appel et al., 1995), that IL-3 gene transcription can be upregulated by a variety of agents in cultured microglia, including LPS. The present data document that LPS also upregulates rIL-3R $\beta$  mRNA in the cultured cells. We also have preliminary data (Appel et al., in preparation) that IL-3 mRNA, after systemic injection of LPS, is induced within approximately 3 hr in rat brain. The present results document that within the same time frame, IL-3 receptor- $\beta$  mRNA is induced in microglia *in situ*. The short time frame of induction and superfusion of brain precludes a confusion with blood-derived mononuclear cells. Hence, there is a good agreement of data obtained in culture with data obtained *in vivo*. The receptor subunit, however, was not downregulated in cultured microglia, suggesting the lack of feedback mechanisms in the purified cell population. *In vivo*, LPS may be degraded more efficiently, or may induce additional factors in other brain cell types that counterbalance the LPS effect. It is presently unclear to what extent LPS (5 mg/kg body weight) is able to



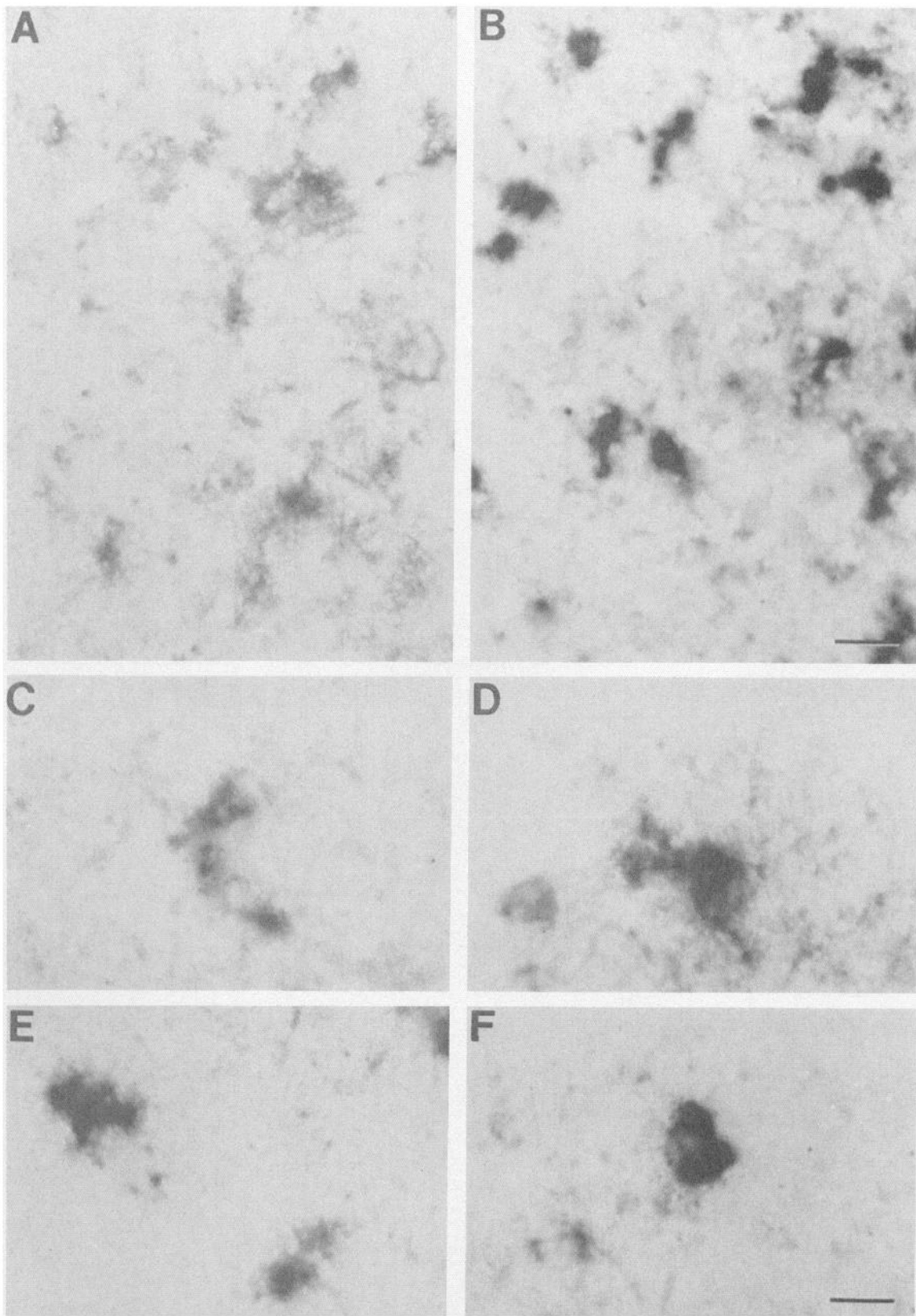
**Figure 6.** Visualization of IL-3 receptor- $\beta$  mRNA-positive cells in brain of LPS-treated rats (6 hr after peripheral administration of 5 mg/kg LPS) using a dig-labeled receptor-specific cRNA. *A*, Low magnification of receptor-positive cells in cortex. *B* and *C*, Higher magnification of receptor-positive cells. Note that ramified morphology of cells is reminiscent of microglia. No hybridization signal was obtained with sense probes (not shown). Scale bars: 14  $\mu$ m (*A*), 8  $\mu$ m (*B* and *C*).

cross the blood-brain barrier, if at all. Additional experiments performed in our laboratory with lower LPS concentrations (1 mg/kg body weight) also resulted in significant but much less pronounced microglial activations (Buttini et al., in preparation). Furthermore, investigations on endothelial cultures revealed enhanced permeability of plasma membranes in the presence of

LPS (Tunkel et al., 1991). These data point to a disturbance of the blood-brain barrier permeability caused by LPS. This might allow direct access of some LPS from the circulation to the brain parenchyma proper. The results could, however, also be interpreted as indicative of a transduction mechanism relaying the LPS signal across the blood-brain barrier. It is also a matter of



**Figure 7.** IL-3 receptor- $\beta$  mRNA induction in infarcted region of rat brain after occlusion of middle cerebral artery (MCAO). Receptor mRNA was already traceable by RT-PCR 3 hr after surgery and reached maximum between 8 and 24 hr. A marked decline was observed 2 d after occlusion. RT-PCR amplification using S12-specific primers is shown below.



**Figure 8.** Visualization of IL-3 receptor- $\beta$  mRNA-positive cells in brain of MCAO rats (2 d after onset of ischemia) using a dig-labeled receptor-specific cRNA. *A*, rIL-3R $\beta$  mRNA-positive cells in brain tissue adjacent to infarct region. *B*, rIL-3R $\beta$  mRNA-positive cells in infarcted area. Note that receptor-positive cells in *A* show morphology of partially activated microglia, whereas positive cells in *B* display morphology of fully activated microglia with retracted, stout processes or are round-shaped cells reminiscent of reactive microglia or macrophages. *C–F*, Higher magnification of different activation states of receptor-positive microglia/macrophages. *C* and *D* are receptor-positive cells in tissue adjacent to infarct area; *E* and *F* are cells in infarct zone. No hybridization signal was obtained with sense probes (not shown). Scale bars: 14  $\mu$ m, 8  $\mu$ m, respectively.



speculation why IL-3 and rIL-3R $\beta$  are induced under fever conditions and in what way these transient gene expressions affect microglial cell numbers and functions of other brain cells. Moreover, it seems reasonable to assume, that the rIL-3R $\alpha$ -subunit is also expressed upon LPS application. Experiments are under way to resolve this question. At any rate, the presence of IL-3 under pathological conditions suggests that rIL-3R $\beta$  is used as transducer for the IL-3 signal. GM-CSF or IL-5, if induced, may also use this pathway (Takaki et al., 1991; Vairo and Hamilton, 1991; Duronio et al., 1992). Although GM-CSF and IL-5 appear to be inducible by LPS in cultured murine astrocytes resp. microglia (Sawada et al., 1993; Mizuno et al., 1994), no data are available as concerns the occurrence of both cytokines under MCAO conditions.

#### *Induction of rIL-3R $\beta$ by occlusion of the middle cerebral artery (MCAO)*

Evidently, rIL-3R $\beta$  mRNA is not only induced in microglia upon LPS administration. MCAO-dependent lesioning also elicits (molecular or ionic) signals stimulating rIL-3R $\beta$  gene transcription. Although identification of these factors warrants further investigations, the involvement of LPS in this paradigm is highly unlikely. For instance, the time course of rIL-3R $\beta$  induction is significantly slower and longer lasting than in the fever model. In another paradigm of ischemia, microglial activation was investigated up to 7 d after occlusion by a number of immunocytochemical markers (Gehrmann et al., 1992). Those results show an onset of microglial reactions somewhere between 0 and 24 hr after surgery, and strongest stainings of some markers occurred only after 7 d. Whether or not this indicates a more delayed time course of microglial activation remains to be determined. In these cases, rIL-3R $\beta$  induction may, indeed, result in a marked increase of microglial cell numbers. It would be of great clinical interest to know what kind of anti-inflammatory measures are suitable to specifically inhibit rIL-3R $\beta$  expression and subsequent expansion of immunocompetent cells in the injured tissue. Since rIL-3R $\beta$  expression is a very early event of microglia activation, its inhibition would be a means of interference near the beginning of unwanted cell or tissue destruction.

Due to the type of surgery, perfusion prior to brain removal was omitted. Therefore, and because no microglia-specific markers are available, it was not possible to discern blood-borne monocytes from resident brain microglia in core or areas immediately adjacent to the infarct. Although data are available, that murine mast cell lines and macrophage cell lines express AIC2B mRNA (Gorman et al., 1990), it would be interesting to know whether or not blood monocytes from rat can also express rIL-3R $\beta$ .

In summary, appearance of rIL-3R $\beta$  (and ligand) mRNA within a few hours after a pathological stimulus demands a very fast transcriptional activation of these genes in brain microglia. The present results lead us to conclude that rIL-3R $\beta$  plays a crucial role in early events of microglial activation *in vivo*.

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