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Corticotropin-Releasing Factor Receptor 1 in Mouse Spleen: Expression After Immune Stimulation and Identification of Receptor-Bearing Cells

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A specific polyclonal Ab against the N-terminal domain of corticotropin-releasing factor (CRF) receptor, type 1 (CRF-R1), was employed to an immunohistochemical analysis of the spleen from naive mice and mice exposed to an immune challenge. Cell types stained with anti-CRF-R1 Ab were identified by their nuclear shapes and colocalization with the cell type-specific markers ER-MP58, ER-MP20, Moma-1, Moma 2, anti-CD3ε mAbs, and anti-Ig Ab. Only a few clusters of CRF-R1+ cells were found in spleen sections of naive mice at sites typical for granuloopoietic islands. However, a 17-fold increase in the mean number of CRF-R1+ cells was noted within hours following a challenge of acute systemic inflammation induced by i.p. administration of LPS. The majority of these cells were identified as mature neutrophils. CRF-R1 was shown to mediate suppression of the IL-1β secretion by these cells. However, at later time points a large number of granulocyte-macrophage precursors was strongly labeled with anti-CRF-R1 Ab. Western blot analysis of splenic membranes from animals treated with LPS revealed a m.w. of approximately 70,000 for CRF-R1. Subcellular staining patterns were suggestive for the predominant localization of CRF-R1 on granule membranes. CRF-R1 mRNA was detected in spleen but not in bone marrow and peripheral blood leukocytes from naive mice. Thus, it was indicated that CRF-R1 was not produced constitutively by mature or immature neutrophils. Its production was rather triggered by inflammatory stimuli. The Journal of Immunology, 1999, 162: 3013–3021.
Therefore, we pursued the objective to identify and characterize immune cells bearing CRF-R1 with recently developed Abs directed against the N-terminal domain of rat CRF-R1 (rCRF-R1-N; Ref. 11). Production of the CRF-R1 protein was examined in spleens of naive mice and after i.p. application of potent inflammatory stimuli such as LPS or keyhole limpet hemocyanin (KLH)/CFA. A functional role of CRF-R1 found on neutrophils was also studied.

Materials and Methods

**Abs and peptides**

ER-MP20 mAb (specific for a subset of granulocyte-macrophage (GM) precursors) and ER-MP58 mAb (all GM precursors) were purchased from BMA Biomedicals (Augst, Switzerland). Anti-mouse 3Fe-CTIC mAb, clone 145-2C11 (T cells), biotinylated GR-1 mAb, clone RB6-8C5, and FITC-labeled anti-mouse Ig polyclonal Abs (B cells) were obtained from Pharmingen (San Diego, CA). Moma-1 (metallophilic macrophages) and Moma-2 (monocytes and macrophages) were obtained from Serotec (Oxford, U.K.). Polyclonal affinity-purified rabbit anti-rCRF-R1-NT Ab was generated as previously described (11). Ovine CRF (oCRF) and astressin were synthesized as previously described in detail (26).

**Treatment of experimental animals**

Groups of four to five 8- to 10-wk-old male C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were injected i.p. either with 100 μg LPS from *Escherichia coli* (serotype 0127:B8; Sigma, St. Louis, MO) in PBS or 100 μg KLH (Boehringer-Mannheim, Mannheim, Germany) in PBS emulsified with CFA (Sigma). Spleens were removed and postfixed for 12 h, 24 h, 3 days, 5 days, 7 days, and 13 days following LPS or KLH/CFA injections, and their spleens processed for immunostaining.

**Immunostaining studies**

Naive and LPS- or KLH/CFA-injected animals were anesthetized and transectally perfused with ice-cold PBS followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Spleens were removed and postfixed for 48 h in the same fixative and then cryoprotected for immersion for 24 h consecutively in 10, 20, and 30% sucrose in 0.1 M sodium phosphate buffer. After the tissue was frozen in vapor of liquid nitrogen, 5-μm thick sections were cut on the cryostat. Elimination of endogenous peroxidase activity was accomplished using 3% hydrogen peroxide in methanol for 30 min, followed by five rinses with PBS. Five percent goat serum in PBS or 100 μg/ml for 1 h with a rabbit Ab that was generated against amino acids 24–121 of rCRF-R1-NT (11), Specificity of immunostaining was confirmed on sections that were incubated with the same Ab preadsorbed overnight at 4°C with a 10-fold molar excess (twofold with CFA). Controls included the overnight incubation of CRF-R1-specific Ab with a 30× (w/w) excess of rCRF-R1-NT (11) or incubation with normal rabbit IgG in the same concentration as anti-rCRF-R1-NT Ab.

**RT-PCR**

Bone marrow cells and spleen were homogenized for 60 s with a Polytron homogenizer (Kinematica) in guanidinium thiocyanate solution (RLT buffer; Qiagen, Santa Clarita, CA) supplemented with 0.1 M 2-ME. Bone leukocytes were homogenized in RLT buffer by vortexing. Erythrocytes from peripheral blood were lysed before homogenization by incubation in EL buffer (Qiagen) for 5 min on ice. Total RNA was then isolated with the RNeasy blood mini kit (Qiagen). One microgram of total RNA was reverse-transcribed with ‘Ready to Go’ kit (PharMingen) by use of dT primers. A cDNA equivalent corresponding to 20 ng of total RNA was amplified in each reaction. The primers used for PCR were 5′-GGTCTTCTCCAGAGGTACACCTGTTGAGG-3′ (mouse CRF-R1, sense); 5′-GGTAGTTGATGATGGCAATGCATGGG-3′ (mouse CRF-R1, antisense) and 5′-AAGAGTGACCAGATCATGTGAGCAC-3′ (β-actin, sense); 5′-CTGCTGCCTGAGCACTTCGTTGAG-3′ (β-actin, antisense). Primers specific for mouse CRF-R1 were designed to amplify a fragment spanning from nucleotides 100–404 of the mRNA coding for rat CRF-R1. As a control, a cDNA input, β-actin mRNA levels were determined for each sample in separate RT-PCR reactions. The PCR reactions contained deoxynucleoside triphosphates and buffer as supplied by the manufacturer, 500 pM of each specific primer, and 2.5 U Taq polymerase (Takara, Seta, Japan). Transcripts were amplified for 35 cycles with CRF-R1 primers (30 s at 94°C, 30 s at 67°C, 30 s at 72°C) and 20 cycles for β-actin primers (30 s at 94°C, 30 s at 65°C, 45 s at 72°C), followed by 7 min at 72°C. The PCR products were analyzed in 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination. For β-actin amplification, PCR was performed with different cycle numbers to ensure that the amplification was occurring in the linear range.

**Examination of nuclear shapes**

Spleen sections were prepared and stained for CRF-R1 with the Elite ABC-peroxidase kit (Vector Laboratories) as described above. A fluorescent marker was introduced by incubation with a 50× dilution of rhodamine-tyramide (New England Nuclear–Life Sciences, Boston, MA) in amplification buffer (New England Nuclear–Life Sciences) for 10 min. After washing in PBS, the nuclei were stained with 4 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma) in PBS for 10 min. For photographing, the sections were dehydrated through increasing ethanol concentrations, cleared with xylene, and coverslipped with Eukitt (O. Kindler). For quantification, at least 300 cells were counted per section except for sections from spleens of naive animals. These sections contained a low number of CRF-R1+ cells, and thus the minimal number of cells counted was decreased to 100.

**Colocalization studies**

To identify CRF-R1+ cells, binding of anti rCRF-R1-NT Ab in spleen sections was visualized with rhodamine-tyramide as described above, while standard mAbs were incubated simultaneously and detected by appropriate secondary Abs labeled with FITC. When necessary, staining with FITC was further enhanced by the addition of peroxidase-labeling anti-FITC Abs (New England Nuclear–Life Sciences) after removal of residual peroxidase activity by incubation with 3% H2O2 in methanol for 30 min. Peroxidase was visualized by incubation with FITC-tyramide (New England Nuclear–Life Sciences). Nuclei were subsequently stained with DAPI. Multicolor immunofluorescence was observed with a triple band-pass filter (Appligene, Oncor, Illkirch, France). For quantification, at least 300 cells were counted per section from spleens of challenged animals. For the analysis of naive mice, at least 100 cells were counted.

**Isolation of splenic neutrophils**

Splenocytes were gently pressed out of the spleen with a forceps on ice and resuspended by pipetting in cold PBS supplemented with 10 mM glucose.
(GPBS) and 0.1% BSA. The osmolality of PBS was always adjusted with 2 M NaCl to 310 mOsm to match the osmolality of mouse plasma. After centrifugation at 200 × g for 8 min at 4°C, splenocytes were incubated in 1.5 ml of 0.8 μg/ml biotinylated GR-1 mAb in GPBS-0.5% BSA for 10 min at 8–10°C. After the addition of 20× excess (v/v) of GPBS-0.1% BSA, the cells were centrifuged again and resuspended in 11× diluted magnetic cell separation streptavidin microbeads (Miltenyi-Biotec, Bergish Gladbach, Germany) in GPBS-0.5% BSA and incubated for 10 min at 8–10°C. After an additional washing step, the cells were resuspended in 10 ml of GPBS-0.5% BSA and separated on a positive selection magnetic column, type LS⁺ (Miltenyi-Biotec) according to the manufacturer’s instructions. The cells were allowed to pass through the column, which then was washed five times with 3 ml of GPBS-0.5% BSA. The column was then removed from the magnetic field. Magnetically labeled cells were eluted with 5 ml of GPBS-0.5% BSA. The purity of neutrophils in the GR-1⁺ fraction was consistently 90% or higher as assessed by Giemsa-stained cell smears. The viability of GR-1⁺ cells was above 85% as judged by trypan blue exclusion. The GR-1⁻ fraction was observed to be quantitatively depleted of neutrophils.

Measurement of IL-1β secretion

Neutrophils (10⁷/ml) isolated from spleens of animals that were injected i.p. 12 h earlier with 100 μg LPS were incubated in GPBS-0.1% BSA/1 mM CaCl₂/1 mM MgSO₄ for 5 h at 37°C with different concentrations of oCRF. The cells were then pelleted by centrifugation at 200 × g for 5 min. Where noted, the cells were preincubated with different concentrations of astressin for 10 min at 37°C before the addition of 10 nM oCRF. Polysorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 6 μg/ml of anti-IL-1β mAb (R&D Systems, Minneapolis, MN) in PBS/0.04% merthiolate overnight at room temperature. Saturation was achieved with 1% BSA for 1 h at room temperature. Plates were then washed five times with PBS. Subsequently, the cell supernatants were added in triplicate and incubated for 2 h at room temperature. Biotinylated anti-IL-1β Ab (R&D Systems) was added at 100 ng/ml in PBS/1% BSA. The mixture was incubated for 2 h at room temperature. Streptavidin-peroxidase (New England Nuclear–Life Sciences) was diluted 750 times in PBS/0.1% Tween 20 and incubated for 30 min. Tetramethylbenzidine hydrochloride solution (Sigma) was added after five washes with PBS. The reaction was stopped with 2 M sulfuric acid. The OD was read at 450 nm.

Superoxide generation

The extracellular production of superoxide was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c over a 5-min period. Neutrophils (1 × 10⁶/ml in GPBS-0.1% BSA/1 mM CaCl₂/1 mM MgSO₄) were preincubated for various periods of time (2 min to 5 h) with different concentrations (0.01–1000 nm) of oCRF. Before the addition of the stimulus, such as formyl hexapeptide (1–1000 nm) or PMA (1–100 ng/ml), cytochrome c was added to a final concentration of 100 μM. The cells were incubated with the stimulus for 5 min, centrifuged for 10 s at 12,000 × g, and the OD of the supernatant was then determined by the difference of the absorbance measured at 550 and 540 nm. The reduced cytochrome c was determined as described previously (29). Each assay was performed in the

![FIGURE 1](http://www.jimmunol.org/). Immunostaining of CRF-R1 in spleens of naive mice and mice after LPS administration. A, Naive mouse. B–F, Mice 3 h (B), 7 h (C), 12 h (D), 24 h (E), and 5 days (F) after i.p. administration of 100 μg LPS. Typical sections from groups of four to five mice are shown. One of four comparable experiments is shown. Magnification, ×100. Bars, 100 μm.
absence and presence of 20 \( \mu \text{g/ml} \) superoxide dismutase to correct for nonspecific cytochrome c reduction.

**Assays for marker enzymes**

In degranulation assays, \( 10^7 \) neutrophils per milliliter in GPBS-0.1% BSA/1 mM CaCl\(_2\)/1 mM MgSO\(_4\) were preincubated at 37°C in 1.5-ml plastic tubes for various periods of time (2 min to 5 h) with different oCRF concentrations (0.1–1000 nm). Then, cytochalasin B was added to a final concentration of 5 \( \mu \text{g/ml} \). After 5 min of incubation with cytochalasin B, exocytosis was initiated by the addition of formyl hexapeptide in final concentrations ranging from 3–100 nM. Five minutes later, the cells were centrifuged at 12,000 \( \times \) g for 10 s, and 20 \( \mu \text{l} \) of supernatant was transferred to 130 \( \mu \text{l} \) of tetramethylbenzidine hydrochloride solution (Sigma) for measurement of myeloperoxidase activity. After 10–20 min, the color development was stopped by the addition of an equal volume of 2 M sulfuric acid. The OD was read at 450 nm. For the measurement of lysozyme release, the incubation with cytochalasin B was omitted, and the amount of lysozyme was determined with a modification of a previously described method (29), as a decrease in turbidity of a suspension of lysodeikticus (0.3 mg/ml in 50 mM phosphate acetate buffer, pH 6.0; Sigma) at 450 nm. Chicken egg-white lysozyme (Sigma) was used as a standard.

**Results**

**Time-dependent appearance of CRF-R1\(^+\) cells**

The number of CRF-R1\(^+\) cells on mouse spleen sections was estimated by immunostaining with anti rCRF-R1-NT Ab. A high degree of cross-reactivity between rat and murine CRF-R1 (AtT-20 cells) was found for these Abs (11), in agreement with a high degree of identity on the amino acid level (96%) between the N-terminal domains of mouse and rat CRF-R1. With anti-CRF-R1 NT Ab, a small number of clusters of CRF-R1\(^+\) cells were identified in normal mouse spleen (Figs. 1A, 2A, and 3), mainly under the splenic capsule. After 3 h, the number of CRF-R1\(^+\) cells was significantly higher than the corresponding cell number in spleens from naive animals and reached a maximal, seventeenfold increase.
12 h after LPS administration (Fig. 3). After a decrease in the number of CRF-R1\(^+\) cells 24 h after LPS application (Fig. 1E), clusters of CRF-R1\(^+\) cells appeared at day 3 (Fig. 3). Maximal cluster formation was observed at day 5 (Figs. 1F and 3). These clusters were located under the splenic capsule, like in spleens of naive animals, but were much larger than the clusters in spleens of naive mice and also spread throughout the red pulp, mainly along the trabeculae. An even stronger effect at the same time points was observed in KLH/CFA-injected mice (Figs. 2, C–E and 3). On the other hand, the acute effect (3–12 h after injection) was by far less pronounced after KLH/CFA than LPS (Fig. 3). In KLH/CFA-injected mice, the number of CRF-R1\(^+\) cells on spleen sections increased significantly at day 3 compared with naive animals, was maximal at day 7, and persisted until day 13 (Figs. 2 and 3). KLH, a soluble macromolecular Ag, was used together with CFA to achieve even stronger activation of immune cells. Because similar production of CRF-R1 occurred after administration of BSA/CFA instead of KLH/CFA (data not shown), it was concluded that this effect was not specifically dependent on KLH. Evidence that the immunostained protein was CRF-R1 was provided by the demonstration of the specificity of the used Ab, which did not stain after preincubation with a 10-fold molar excess of purified Ag (Fig. 2F). This effect was best observed by comparison between neighboring sections (Fig. 2, D and F). Similar results were obtained with sections representing other time points (not shown). In addition, when normal rabbit IgG was employed instead of anti-CRF-R1-NT Ab in matched concentration, only weak and uniform background staining was observed at all time points (not shown). No detectable staining of CRF-R1 could be demonstrated on peritoneal exudate cells 12, 24, and 48 h after injection of 100 \(\mu\)g LPS with the staining protocols employed (not shown).

**Determination of the m.w. of the splenic CRF-R1**

The m.w. of splenic CRF-R1 was determined by Western blot analysis of splenocyte membrane extracts (Fig. 4). Extracts of spleens 12 h after LPS and 7 days after KLH/CFA administration, when the number of CRF-R1\(^+\) cells was maximal, contained CRF-R1 protein with a m.w. of 70,000. In agreement with immunohistochemical data, CRF-R1 was not abundant in extracts obtained from spleens of naive animals (Figs. 1A, 2A, and 3). Although the number of CRF-R1\(^+\) cells was shown to be similar on spleen sections 12 h after LPS and 7 days after KLH/CFA administration (Fig. 3), the intensity of the band indicating a m.w. of 70,000 was stronger in the extracts of the latter group. This finding was consistent with our observation that CRF-R1 density was higher in immature cells than in mature cells, as judged on the basis of the immunofluorescence determined under the conditions mentioned.

**RT-PCR**

RT-PCR was used to compare CRF-R1 expression in spleen to other major neutrophil pools such as bone marrow and peripheral blood leukocytes (Fig. 5). Positive amplification was only observed with cDNA from spleen but not from bone marrow and

![FIGURE 3. Kinetic study of the number of CRF-R1\(^+\) cells. Time zero represents CRF-R1\(^+\) cell count on spleen sections of naive mice. Cell numbers refer to the total area of 20 randomly selected nonoverlapping fields. Results are expressed as mean values \(\pm\) SEM of the representative experiment from the three performed. Statistical significant differences were determined by ANOVA: *, \(p < 0.05\); **, \(p < 0.0001\), in comparison to naive mice.](Image 110x618 to 239x733)

![FIGURE 4. Molecular weight determination of splenic CRF-R1. SDS PAGE and Western blot was followed by immunostaining with anti-cRF-R1-NT Ab. Lane 1, Membrane extracts (100 \(\mu\)g) from spleens of naive animals. Lane 2–4, Membrane extracts 12 h after LPS injection (lane 2), 7 days after KLH/CFA administration (lane 3), and 7 days after KLH/CFA administration stained with normal rabbit IgG (lane 4). Molecular weight markers are indicated on the left.](Image 364x641 to 502x733)

![FIGURE 5. Expression of CRF-R1 mRNA analyzed by RT-PCR. Lane 1, 20 ng of splenic RNA. Lane 2, Bone marrow. Lane 3, Peripheral blood leukocytes. Lane 4, Spleen. RT-PCR was performed using specific primer sets for CRF-R1 or \(\beta\)-actin. Total RNA was obtained from organs of the same naive animal. Samples were amplified for 55 cycles (CRF-R1 primer set) or 20 cycles (\(\beta\)-actin primer set). Data are representative of four naive animals.](Image 3017)
Table II. Frequency of different cell types within a population of CRF-R1<sup>+</sup> spleen cells of naive mice and following LPS or KLH/CFA administration<sup>a</sup>

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Frequency (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Naive</th>
<th>12 h after LPS</th>
<th>7 days after KLH/CFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segmented and band cells</td>
<td></td>
<td>21.5 ± 14.1</td>
<td>89.7 ± 8.1</td>
<td>13.8 ± 6.5</td>
</tr>
<tr>
<td>GM precursors</td>
<td></td>
<td>76.2 ± 15.5</td>
<td>8.9 ± 7.4</td>
<td>85.9 ± 6.5</td>
</tr>
<tr>
<td>Mature mononuclear cells</td>
<td></td>
<td>2.3 ± 2.1</td>
<td>1.4 ± 0.6</td>
<td>0.3 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by examination of the nuclear morphology of CRF-R1<sup>+</sup> cells.

<sup>b</sup> Expressed as mean of percentage ± SEM of the total number of CRF-R1<sup>+</sup> cells.

Calculation was performed on the basis of data obtained from three animals per data point.

peripheral blood leukocytes. Control amplifications of β-actin demonstrated that all three cDNAs contained similar amounts of β-actin cDNA. Controls performed in the absence of cDNA or by adding 20 ng RNA that was not reverse transcribed (Fig. 5) did not yield detectable PCR products with any primer pairs.

Identification of CRF-R1-bearing splenocytes

CRF-R1-bearing immune cell types could be identified by their nuclear morphology. In addition, colocalization of anti-CRF-R1-NT Ab with Abs specific for markers of the main immune cell types was examined by two-color immunofluorescence. The majority of CRF-R1<sup>+</sup> cells in spleens of naive animals were identified as GM precursors on the basis of overlap observed between anti-CRF-R1-NT Ab and ER-MP58 mAb (Ref. 30 and Tables I and II). Isotype control (rat IgM), matched in concentration to mAb ER-MP58, did not show any staining (not shown). There was little overlap in staining (Table I) between CRF-R1<sup>+</sup> and Ab specific for metallophilic macrophages (Moma-1; Ref. 31), monocyte/macrophages (Moma-2; Ref. 31), T cells (anti-CD3ε), and B cells (anti-mouse Ig). This finding was in agreement with the nuclear morphology of the CRF-R1<sup>+</sup> cells, as not more than 2.3% of these cells appeared as mature mononuclear cells at all time points (Table II). Mature neutrophils (17.9%, Table I) also produced CRF-R1 in naive mice on a low level. However, 12 h after stimulation with LPS, mature granulocytes dominated the population of CRF-R1<sup>+</sup> cells (Tables I and II). They could be identified as neutrophils on the basis of their segmented nuclei (Fig. 6A). To examine whether any basophils and eosinophils were falsely identified as neutrophils due to similarities in nuclear shapes, sections were stained with Wright-Giemsa. By this procedure, no evidence for basophils and eosinophils was provided on the basis of differential staining of granules. This result confirmed that CRF-R1 was not produced by cells with segmented nuclei other than neutrophils. From day 3 (LPS) or day 5 (KLH/CFA), the majority of CRF-R1<sup>+</sup> cells were again identified as GM precursors on the basis of their nuclear shapes (Fig. 6B and Table II) and overlap with ER-MP58 mAb (Fig. 7 and Table I). It was evident that all CRF-R1<sup>+</sup> cells within granulopoietic clusters were also ER-MP58<sup>+</sup> (Fig. 7). A similar overlap was obtained with ER-MP20 mAb, which also binds to GM precursors (not shown).

Subcellular localization of CRF-R1

Uniformly distributed punctate staining of CRF-R1 throughout the cytoplasm of immature neutrophils (Fig. 6B) was observed. Mature neutrophils exhibited a similar pattern of punctate staining, but it was less visible through triple band-pass filter (Fig. 6A) due to their smaller size.

Effect of CRF on neutrophil function

oCRF significantly and dose-dependently reduced the secretion of IL-1β (by maximally 30%) from neutrophils purified from spleens of mice injected with LPS 12 h earlier (Fig. 8A). The number of CRF-R1<sup>+</sup> neutrophils in spleen was maximal at this time point after LPS injection (Figs. 1D and 3). The inhibitory effect of oCRF (10 nM) was reversed by increasing doses of astressin (32), an antagonist inhibiting CRF binding to CRF-R1 or CRF-R2 (Fig. 8B). Astressin alone showed no effect in the employed concentrations on the IL-1β secretion (not shown). On the other hand, oCRF did not exhibit a direct effect on superoxide production and exocytosis or modulate these neutrophil functions after the neutrophils were stimulated with formyl hexapeptide or PMA (not shown).
Discussion

CRF-R1 production was examined on spleen sections of naive mice and after in vivo immune challenge. The spleen was selected because of its abundance of mature and immature immune cell types. In addition, application of LPS or KLH/CFA, known to induce acute (LPS) or chronic (CFA) inflammation, has enabled us to examine the production of CRF-R1 under conditions when all types of spleen cells are activated. The results demonstrated that both LPS and KLH/CFA induced a massive up-regulation of the number of CRF-R1+ cells in the spleen, mainly on mature and immature neutrophils. The immunostained protein was further identified by its m.w., which corresponded to values previously determined for the splenic CRF-R (33). The interpretation of the immunostaining data was also supported with the identification of CRF-R1 mRNA in spleens of naive animals.

Although most of the CRF-R1+ spleen cells of naive mice were identified as GM precursors, CRF-R1 mRNA was not detected in bone marrow. This observation was an interesting finding in view of the fact that bone marrow is the main site of granulopoiesis, whereas the spleen of adult mice retains only a low level of granulopoietic activity in the absence of an inflammatory challenge (34). In view of the lack of CRF-R1 mRNA in bone marrow, it was concluded that CRF-R1 was not constitutively expressed by immature cells of the GM lineage.

In the acute phase of inflammation, LPS was more potent than KLH/CFA in triggering CRF-R1 production in the spleen. The finding that CRF-R1 was expressed on mature polymorphonuclear cells (neutrophils), accumulated around marginal zones and throughout the red pulp of the spleen correlated with the massive LPS-induced neutrophil tissue infiltration occurring in the acute phase of inflammation (35, 36). Therefore, it cannot be excluded that these cells have produced CRF-R1 before recruitment to the spleen.

A previously performed autoradiographic study with 125I oCRF (13) had also localized CRF-R near marginal zones and in red pulp of a mouse spleen. This anatomical distribution (13) was consistent with our identification of CRF-R1+ cells as neutrophils.

During endotoxemia, LPS (37) and inflammatory cytokines induce neutrophil priming that is accompanied by an increase of RNA and protein synthesis (38, 39). Therefore, it seems conceivable that the seventeenfold inrease in number of CRF-R1+ cells in the spleen observed after endotoxin administration resulted from neutrophil priming. This assumption was supported by the finding that CRF-R1 was not constitutively expressed in resting neutrophils, as judged by the absence of detectable levels of mRNA coding for CRF-R1 in the main pools of mature neutrophils, bone marrow, and peripheral blood leukocytes.

The increase of CRF-R1 observed 7 days after injection of KLH/CFA was in agreement with the time requirement for the stimulatory effect of CFA on splenic hematopoiesis (34). Accordingly, CRF-R1 was mainly present in GM precursor cells. The finding that KLH/CFA triggered stronger CRF-R1 production than LPS in the chronic phase of inflammation may be explained by the greater potency of CFA to stimulate splenic hematopoiesis (34). Abs available to date cannot accurately distinguish between granulocyte and macrophage precursors. However, the immature CRF-R1+ cells were mainly identified as granulocyte precursors by their nuclear shapes (40). Additionally, the anatomical distribution of CRF-R1+ cells, under the splenic capsule and along trabeculae, was typical of granulopoietic islands (41).

In contrast to previous reports (6, 16, 17, 19), we could not detect CRF-R1 on a significant number of lymphocytes or monocytes under the conditions employed. However, the studies mentioned did not determine the type of the CRF receptor. Therefore, the effects of human/rat CRF could have been caused by its binding to CRF-R2 (42) or to an unknown CRF-R subtype. Cross-reactions of anti-CRF-R1-NT Ab used in this study to CRF-R2 (11) or CRF-binding protein (12) were previously excluded.

It may be assumed that neutrophils that accumulate in the peritoneal cavity 24–48 h after the i.p. injection of 100 μg LPS (Ref. 43 and our unpublished observation) were mobilized from the spleen, as the number of CRF-R1+ neutrophils in the spleen declined rapidly from 12–24 h. It is then unclear why neutrophils from the peritoneal exudate did not contain detectable levels of CRF-R1. Diffuse punctate staining of splenic neutrophils was suggestive that a significant proportion of CRF-R1 was localized intracellularly (Fig. 6). Such suggestion is in agreement with the localization of other neutrophil receptors, such as the receptors for N-formyl-peptides, platelet activating factor, C5a, thrombin, and IL-8 on granule membranes (44, 45). Therefore, it cannot be excluded that an extensive degranulation that occurs during and after transmigration of neutrophils to the peritoneum (46) resulted in a
considerable reduction of CRF-R1 of neutrophils from the peritoneal exudate to levels below the sensitivity of our detection system. However, an inhibitory effect of oCRF on the IL-1β secretion and its reversal by the CRF-R-specific antagonist astressin suggested that a sufficient amount of functional CRF-R1 was present on the cell surface. This inhibitory effect was mediated by the CRF-R1+ neutrophils, which represented a 46.1 ± 3.3% fraction of the total splenic neutrophil population (Table I). Therefore, it can be assumed that the suppression of the IL-1β production would be even more pronounced after separation of CRF-R1+ neutrophils from CRF-R1− neutrophils. A human/rat CRF-mediated modulation of IL-1β secretion was previously observed in monocytes (47). The inhibitory effects of glucocorticoids and CRF on the secretion of IL-1β from human mononuclear cells were shown to be additive (48).

In view of the absence of an oCRF effect on neutrophil superoxide production and exocytosis of primary or secondary granules, a selective role of CRF-R1 in the regulation of cytokine secretion was suggested. Accordingly, it was previously reported that IL-1β had no influence on degranulation and superoxide production of neutrophils (49). On the basis of IL-1 receptor blockade experiments, IL-1 has been shown to be one of the principal mediators of LPS-induced toxicity (50). Neutrophils have to be considered as a major source of IL-1β in view of the finding that, following intratracheal LPS injection, a predominant proportion of the IL-1β RNA from bronchoalveolar lavages is attributable to polymorphonuclear, as opposed to mononuclear cells (51). Similarly, after i.v. infusion of LPS into rats, a predominant proportion of the IL-1β RNA was detected in a fraction enriched by polymorphonuclear leukocytes harvested from the pulmonary vasculature (52). Therefore, by serving as a negative feedback to limit IL-1β secretion of CRF-R1+ neutrophils, CRF may contribute to the inhibition of the inflammation induced by endotoxin.

In addition to the finding that neutrophils synthesize CRF-R1, as demonstrated here, it is probable in view of the presence of mRNA coding for CRF (7) that neutrophils also produce CRF. This view is consistent with the assumption of CRF’s involvement in the autocrine regulation of inflammation (9).

In conclusion, CRF-R1 was shown to be produced by neutrophils upon inflammatory challenge. The results presented here provide new evidence for the cell population, receptor subtype, and mechanism that may mediate the previously reported anti-inflammatory effects of CRF (18, 23, 53).

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