Brief Definitive Report

Immunoglobulin E Plus Antigen Challenge Induces a Novel Intercrine/Chemokine in Mouse Mast Cells

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Summary

In an attempt to characterize genes participating in the allergic late phase reaction, we have isolated a novel intercrine/chemokine (called MARC) from a cDNA library of the stimulated mouse mast cell line, CPII. As measured by Northern blotting, it is strongly upregulated at the mRNA level after the physiological challenge of the cells with immunoglobulin (Ig)E plus antigen. Unstimulated cells completely lack significant, stable expression, as do a number of other, different cell lines (uninduced and induced) and mouse tissues. In contrast to the Northern blot analysis, a polymerase chain reaction (PCR) analysis, performed on CPII cells and on Percoll gradient purified mouse peritoneal mast cells, revealed a basal level of transcription in the uninduced stage. After 2 h of IgE plus antigen challenge, a quantitative reverse transcriptase-PCR, using a spiked in MIMIC, showed a level of transcripts more than 100-fold higher in the CPII cells and 5–20-fold higher in purified mouse peritoneal cavity mast cells. This rapid induction after the $Fc_\epsilon RI$ challenge, the identification of the gene as a member of the chemokine family, and its upregulated expression in peritoneal mast cells, all suggest an involvement in certain acute and chronic pathological mast cell-driven diseases.

Intercrines/chemokines are small soluble proteins that regulate the physiological trafficking and the partial activation of leukocytes (1). In contrast to most other known cytokines and lymphokines, chemokines show a considerable homology (identity and similarity) at the amino acid level. Additionally, they are characterized by a common protein structure of two loops formed via disulfide bridges of four highly conserved cysteines. Based on the location of the two NH_2-terminal cysteines, this superfamily is subdivided into a CXC (IL-8 or PF4 family) and CC (RANTES/sis family) branch (2, 3). In the last, six members in the human system and five corresponding mouse genes are currently isolated (4, 5).

Specific sites of production in the body and the low amount produced in vivo make the direct isolation and characterization of these proteins from healthy individuals nearly impossible. A few members, like macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemotactic protein (MCP)1, have been purified at protein level from overexpressing tumor cell lines by functional monitoring, and the corresponding genes were subsequently identified (6, 7). However, the majority in the CC branch were isolated by induction-specific differential hybridization of cDNA libraries (see reference 4). This reflects the fact that most members are strongly upregulated at the transcriptional level after cell activation (4). Supernatants from transiently transfected cell lines were used for a detailed functional analysis afterwards (8).

Type I allergic reactions are characterized at the level of mast cells by a biphasic response. In an immediate reaction (up to several minutes) preformed low molecular weight substances, like histamine and serotonin, are released after IgE plus antigen binding to the cell (degranulation). Then, a less defined late phase reaction (starting after several hours) needs de novo transcription and protein synthesis and is probably mediated via certain lymphokines and chemokines. In an attempt to characterize genes participating in this late phase reaction, we have isolated a new member of the RANTES/sis superfamily using the approach of uninduced vs. induced differential hybridization of a cDNA library from a mouse mast cell line. The new member has a significant homology to the mouse JE gene and protein (9), the human MCP1 gene and protein (10, 11), and two, only recently at protein level described human isolates, designated MCP2 (identical to former partial isolate HC14) and MCP3 (12, 13).

Materials and Methods

With minor modifications, the RNA isolation, the Benton Davis screening, the Northern blot analysis, and the sequencing were done as recently described (14, 15).

The cDNA Library and the Differential Screening. A cDNA library was prepared starting with 5 μg poly(A)$^+$ endoplasmic reticulum mRNA of the 8-h PMA plus ionomycin-induced CPII mast

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cell line. As a vector, the λZapII phage was used (Uni-ZAP XR; Strategene Inc., La Jolla, CA). Packaging of the recombinant phages was done with the Gigapack Gold system (Stratagene Inc.). About 2×10^6 primary clones were generated and amplified. 10^4 amplified clones were then differentially screened using radioactively labeled cDNA probes generated from 5 μg total RNA of uninduced and 8 h PMA plus ionomycin-induced CPII cells. Probes were synthesized using a preamplification kit system (Superscript; Bethesda Research Laboratories, Gaithersburg, MD) with 10 mM dATP, dGTP, TTP, and 100 μCi dCTP (Amersham International, Amersham, Bucks, UK).

Cells and Stimulation. The CPII cell line is a recently established mouse mast cell line grown out of a spleen cell culture. It is able to respond with histamine release upon IgE plus antigen stimulation and upregulates TNFα, MIP1α, JE, and IL-3 at the transcriptional level. It is, however, negative for IL-4 transcriptional upregulation, which is characteristic for other mouse mast cell lines. Cells are cultured with 10% FCS and 10% WEHI cell-conditioned medium. Stimulation is performed with 20 nM PMA (Sigma Chemical Co., Deisenhofen, Germany) and 200 nM ionomycin (Sigma Chemical Co.) or with 2 μg/ml monoclonal mouse IgE (Biomakor, Rehovot, Israel) and 100 ng/ml antigen (DNP-BSA; Calbiochem Corp., La Jolla, CA). Mast cells from the peritoneal cavity were isolated from the peritoneal washes of 9-mo-old BALB/c female mice and further purified by a Percoll gradient as described (16). Purity was nearly 100%, as checked by Memacolor fast staining (Merck, Darmstadt, Germany).

Qualitative and Quantitative PCR Analysis. A 251-bp PstI fragment containing the 5′ end of the IL-4 receptor gene (generous gift of K. Koettnitz, Sandoz Forschunginstitut) was inserted into the NsiI restriction site (position 106) of our newly isolated chemokine clone to generate a MIMIC for our PCR analysis. As primers for this analysis, we used a 23 mer (5′ CCAAGTGCTA GCCCAACCAGATG 3′) and a 21 mer (5′ GTGACATGAGGT-CTCCAGAGC 3′) at positions 77 and 552 of our clone, respectively. The underlined sequence in the 23 mer represents a 3-bp mutation in the MARC sequence (original TGG) which was introduced to generate an NheI restriction site for further cloning. As the control, β-actin primers were used that were purchased from Clonetech (Palo Alto, CA). They amplify fragments of 476 bp (chemokine), 540 bp (β-actin), and 727 bp (chemokine MIMIC), respectively. The cycle length for amplification was 1 min each, temperatures were 94, 60, and 72°C, respectively. The cDNA for this type of analysis was generated using the Superscript kit system (Bethesda Research Laboratories). One third of each reaction was analyzed on an agarose gel and quantified with the help of an image analyzer (Appligene, Illkirch, France).

Results and Discussion

Cloning and Structural Analysis of MARC. It is our aim to isolate novel transcriptionally upregulated genes that could potentially play a role in acute and chronic diseases with mast cell involvement (allergic reactions type I, asthma). We therefore differentially screened an induced cDNA library from the mouse mast cell line CPII. The library was made from the endoplasmic reticulum mRNA of cells stimulated with PMA/ionomycin, for 8 h to achieve maximal induction. Radioactively labeled cDNA probes generated from the total RNA of unstimulated and 8-h stimulated cells were used for the screening. Clones resulting in differential signals specific for the induced stage over two rounds of plaque purification were immediately subjected to a sequence analysis. So far, computer-aided sequence comparisons of 50–100 nucleotides of this first sequence analysis identified several MIP1α- (17), ferritin- (18), and VL30 retrotransposon (19) sequences. One currently unknown cDNA clone was also purified and its complete nucleotide sequence and a translation of its open reading frame is given in Fig. 1. The clone is 746 bp long and codes for a small 97 AA very basic protein. At the NH_2-terminus of the protein, hydropathy analysis according to both the Kyte Doolittle and Hoop and Woods algorithms, indicated a leader peptide with a cleavage site prediction in a von Heijne analysis (20) at position 23/24. The lack of additional hydrophobic stretches that could serve as transmembrane domains immediately suggested that the gene might encode a small novel secretory protein. At the nucleotide level, two AUUUA sequences, postulated to be involved in the mRNA stability of protooncogenes and lymphokines, are found (21, 22). A slightly modified polyadenylation signal (5′TATAAA3′) is present 20-bp upstream of the poly(A) stretch (23). Computer comparisons of the whole nucleotide and deduced amino acid sequences to the EMBL database (Version 30.0, June 1992) and the Swissprot. database (Version 23.0, June 1992) pointed out identities at the nucleotide (around 65%) and amino acid (around 50%) level to the mouse (m) JE gene and protein, and the human (hu) MCP1 gene and protein. The homology at nucleotide level to both genes is clustered in the coding region and is virtually absent in the 3′ part of the sequences. A significant, but decreased identity at protein level is seen with other members of the CC branch (Table 1). An amino acid comparison to the mJE protein, the huMCP1 protein, and two very recently only partially at protein level sequenced human isolates, designated MCP2 (identical to former isolate HC14) and MCP3, is given in Fig. 2. The degree of homology and the conservation of the four important cysteines in the characteristic conformation clearly group our gene into the RANTES/sis chemokine family. It confirmed the initial

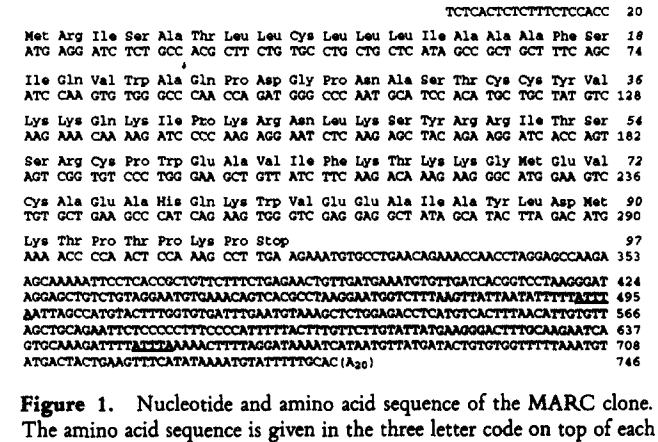

Figure 1. Nucleotide and amino acid sequence of the MARC clone. The amino acid sequence is given in the three letter code on top of each codon. (*Arrow*) Position of the predicted cleavage site of the signal peptide. (*Underlined*) Two ATTTA sequences speculated to be involved in mRNA stability in the 3′ end of induced genes. Nucleotide and amino acid positions are always given to the right; amino acids are continuously numbered, including the leader peptide. These sequence data are available from EMBL under accession number Z12297 as clone P3-6.

Table 1. *Homology of MARC to the RANTES/sis family*

Murine	Percent	AA	Human	Percent	AA
JE	48.5	99	MCP1 (total)	58.2	98
			MCP1 (mature)	56.0	75
			HC14 (MCP2)	49.4	77
			MCP3	56.5	69
RANTES	39.6	91	RANTES	36.3	91
MIP-1α	36.3	91	MIP-1α	33.0	94
MIP-1β	32.3	93	MIP-1β	34.4	93
TCA3	34.1	82	I-309	39.5	81

The comparison shows percent amino acid identity of the complete 97 AA MARC protein to all the other complete members of the CC branch. Only in the case of the MCP1 (mature) and the MCP2 and MCP3 chemokine were mature forms of the protein compared with the mature MARC protein (data for the leader peptide of MCP2 and MCP3 are not currently available). The numbers given under AA show the length of the proteins that were compared. AA sequences were taken from the following references: mJE (9); mRANTES (5); mMIP-1α (17); mMIP-1β (24); TCA3 (25); huMCP1 (10); huMCP2 (13); huMCP3 (13); huRANTES (26); huMIP-1α (27); huMIP-1β (28); I-309 (29); and huHC14 (12).

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            -23                  -1
MARC          MRISATLLCLLLIAAAFSIQVWA
mJE           MQVPVMLLGLLFTVAGWSIHVLA
huMCP1        MKVSAALLCLLLIAATFIPQGLA
huHC14 (MCP2)                      A
huMCP3
              *...  ** **.  *    .  *

            +1
MARC          QPDGPNAS-TCCY-VKKQKIPKRNLKSYRRITSSRCPW  36
mJE           QPDAVNAPLTCCYSFTSKMIPMSRLESYKRITSSRCPK  38
huMCP1        QPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPK  38
huHC14 (MCP2) QPDSVSIPITCCFNVINRKIPIQRLESYTRITNIQCPK  38
huMCP3            KSTTCCYRFINKKIPKQRLESYRRTTSSHCPR  32
              ***. . . ***.    . *.   * ** * *.  **

MARC          EAVIFKTKKGMEVCAEAHQKWVEEAIAYLDMKTPTPKP  74
mJE           EAVVFVTKLKREVCADPKKEWVQTYIKNLDRNQMRSEP  76
huMCP1        EAVIFKTIVAKEICADPKQKWVQDSMDHLDKQTQTPKT  76
huHC14 (MCP2) EAVIFKTKRGKEVCADPKERWVRDSMKHLDQIFQNLKP  76
huMCP3        EAVIFK   DKEICADPTQKWVQDFMKHLDKKTQTPKL  67
              ***.*     *.**..   **   .  **

MARC          ----------------------------------------
mJE           TTLFKTASALRSSAPLNVKLTRKSEANASTTFSTTTSS  114
huMCP1        ----------------------------------------
huHC14 (MCP2) ----------------------------------------
huMCP3        ----------------------------------------

MARC          -----------
mJE           TSVGVTSVTVN  125
huMCP1        -----------
huHC14 (MCP2) -----------
huMCP3        -----------
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Figure 2. Amino acid comparison of MARC with mJE, huMCP1, huHC14 (now *MCP2*), and MCP3 (hu, human; m, murine). All five proteins were compared over the full length using the Clustal V program. (*) Identities of all five proteins; (·) similarities. (*Shaded*) Conserved four cysteines characteristic for the RANTES/sis family. The numbering of the mature proteins is given to the right, starting at Q (+1). The leader peptides are given in the first line numbered from −23 to A (−1). Information on the leader peptide of MCP2 and MCP3 is not available.

assumption that the clone codes for a secretory cytokine. Whether it is the mouse homologue of either the human MCP2 or MCP3 isolate cannot be decided on the basis of this amino acid comparison, because the identity/similarity values of MARC to both proteins are too similar. Clearly arising from the data of Van Damme et al. (13) and from this article is that in the CC branch a MCP-like subfamily (currently three members) exists, which has an analogous counterpart of JE-like proteins (now two members) in mouse.

Expression Analysis of MARC. Three parameters were important for a potential linkage of this new gene to allergic conditions. These are transcriptional upregulation after the allergic stimulus IgE plus antigen, a relatively restricted expression pattern, and the finding of an in vivo correlate to the situation in our cell line.

First, we determined whether an allergic provocation of our mast cell line also would be able to elicit an upregulation of this novel chemokine. Therefore, a Northern blot analysis was performed comparing the induction conditions used in the cDNA library construction with a stimulation with IgE plus antigen. As a probe, a cDNA fragment of our gene was used, which was first tested to show it had no significant crosshybridization to the murine JE gene under the conditions used, in spite of the 65% homology at nucleotide level. This Northern blot is shown in Fig. 3. Densitometric scanning and normalization to the β-actin control gene revealed that IgE plus antigen elicits a response identical to that of PMA/ionomycin over an 8-h incubation. The weak upregulation seen with ionomycin alone parallels effects in other mast cell lines and primary non-T and non-B cells with respect to the transcriptional induction of such cytokines as IL-3, IL-4, and GM-CSF (30–32).

To check the tissue distribution, an identical analysis was performed on six different cell lines either unstimulated or

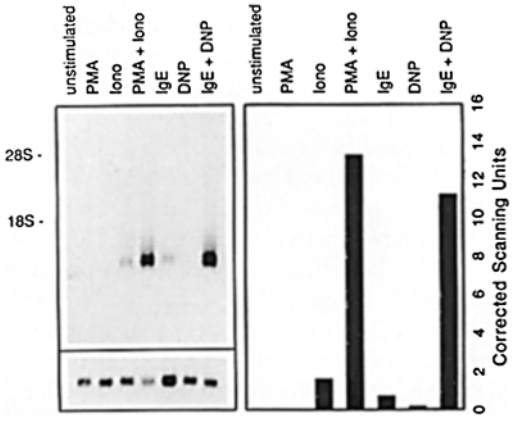

Figure 3. Northern blot analysis with MARC cDNA as a probe. (*Left*) 7.5 μg total RNA of either unstimulated or stimulated CPII cells were analyzed. The stimulus is indicated on top, 28S and 18S size markers are shown to the left. At the bottom, a control hybridization with the β-actin gene is shown. Stimuli were used at the following concentrations: PMA (20 nM); ionomycin (200 nm); IgE (2 μg/ml); DNP [DNP-BSA] (100 ng/ml). (*Right*) Laser densitometry giving corrected scanning units of MARC expression normalized to the β-actin control gene.

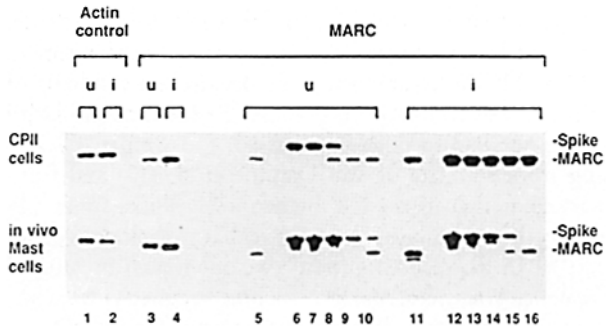

Figure 4. PCR analysis with MARC. Total RNA was transcribed into cDNA and equal aliquots were amplified either with β-actin primers (lanes *1* and *2*) or MARC-specific primers (lanes *3–16*). (*u*) RNA of unstimulated cells; (*i*) RNA of 2 h IgE plus antigen–stimulated cells. Lanes *1–4* and *5–16* are a side-by-side experiment. Lanes *6–10* and *12–16* contain a MIMIC plasmid of MARC (see Materials and Methods) spiked in 10-fold serial dilutions from left to right. Lanes *5* and *11* show the side-by-side reaction without the spike. The concentration of the spike in lanes *6* and *12* is 160 pg.

stimulated for 8 h with PMA/ionomycin (cell lines: YAC, WEHI, BAF, EL-4F, 3T3, and L292) and six different mouse tissues (brain, heart, liver, lung, spleen, and testis). We failed to detect any transcriptional activity of our gene in these cell lines and tissues. The constitutively expressed β-actin control gene, however, was detectable (data not shown). Although we cannot conclude from the limited number of samples (total 12) on a strict cell type specificity, the expression pattern of our gene seems to be narrow. This is in contrast to most other members of this chemokine superfamily which are broadly expressed after a variety of stimuli.

Even if it seems very unlikely that a cell line in the process of its establishment has gained a function in terms of a novel signal transduction pathway (induction of MARC via the $Fc_\epsilon RI$ in our case), it was important to investigate the in vivo situation of mast cells with respect to our new chemokine. Therefore, peritoneal mouse mast cells were isolated as described in Materials and Methods and, because of the low amount of cells available after purification, analyzed by the PCR technology. In parallel, an analogous experiment was also performed with the CPII cell line. The time of stimulation with IgE plus antigen was reduced, in comparison to the initial isolation and Northern blotting, to 2 h. This time point was determined to be optimal for expression in the CPII cells. A PCR analysis is shown in Fig. 4. Lanes *1–4* give a side-by-side reaction of β-actin control primers and specific MARC primers. It revealed minute levels of transcripts in the uninduced stage of the CPII cells (see lane *3*, *top*) which were not detected in the Northern blots. This suggests that a posttranscriptional stabilization might contribute to the upregulation of this gene, a fact that was also previously reported for the related mJE gene (33). It is likely that the same mechanism is partially the cause of the band seen in the unstimulated in vivo mast cells (lane *3*, *bottom*). However, a certain degree of preactivation, especially of the in vivo cells, might also be an alternative explanation. Differences in the amount of the β-actin control gene for the in vivo mast cells (lanes *1* and *2*; lower in the induced stage) are visible, which indicates less input of cDNA of the induced cells in our analysis. This fact is important for a later quantification. Because the exponential nature of the PCR method makes a direct quantification from these data impossible, a quantitative RT-PCR was performed on another cDNA aliquot. A plasmid, containing a 251-bp unrelated insert in position 106 of our clone, was constructed to serve as the MIMIC in such a reaction. This plasmid was then spiked into constant amounts of cDNA, generated from unstimulated and stimulated CPII cells and in vivo mast cells, in 10-fold serial dilutions. This is shown in Fig. 4, lanes *5–16*. For the CPII cells, this analysis revealed an at least 100-fold difference of transcript levels between the uninduced and the 2-h induced stage. The exact quantification in this analysis is hampered by the fact that there was not enough spike (lanes *12–16*) to efficiently compete for the amount of transcripts in induced CPII cells. The identical analysis for the in vivo mast cells revealed an ~10-fold induction with our IgE plus antigen stimulation ex vivo (compare lanes *9* and *10* with lanes *15* and *16*). As pointed out above, RNA/cDNA input levels in our analysis are lower for the induced stage and, therefore, we estimated a range of 5–20-fold for the upregulation of our gene in in vivo mast cells. This clearly shows that the new chemokine is transcribed from in vivo mast cells, which also respond with an upregulation of this gene to an $Fc_\epsilon RI$ challenge. We believe that a certain amount of preactivation, either already in vivo or in the process of the isolation of the cells (mainly via the Ca^{2+} concentration in the isolation buffer), is the reason for the smaller induction seen with these cells in comparison to the cell line.

Our findings add to the four RANTES/sis genes (TCA3, MIP1α, MIP1β, and JE), which are reported to be upregulated in different IL-3–dependent and –independent mouse mast cells after an $Fc_\epsilon RI$ challenge, another, novel member of this family (32). The production of most of the chemotactic factors of the CC branch by various mast cell lines and subpopulations suggests a central regulatory role, not only for this cell type but also for the RANTES/sis family, in initiating cell infiltrations to allergic sites after an IgE plus antigen provocation. This further emphasizes the emerging picture that the mast cell is not only a pure effector cell type, but that it also has central regulatory functions in allergic events. The finding that certain members of this family are more or less specific for the recruitment of hematopoietic subpopulations (8) not only explains why such a broad spectrum of factors is produced upon the same stimulus by one cell type, but also raises the question of for which subtype will MARC be the corresponding chemokine.

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