The Journal of Immunology

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J Immunol 2000; 165:2943-2949; ;
doi: 10.4049/jimmunol.165.6.2943
http://www.jimmunol.org/content/165/6/2943

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cutting Edge: A Novel Chemokine Ligand for CCR10 And CCR3 Expressed by Epithelial Cells in Mucosal Tissues[1,2]

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Mucosae-associated epithelial chemokine (MEC) is a novel chemokine whose mRNA is most abundant in salivary gland, with strong expression in other mucosal sites, including colon, trachea, and mammary gland. MEC is constitutively expressed by epithelial cells; MEC mRNA is detected in cultured bronchial and mammary gland epithelial cell lines and in epithelia isolated from salivary gland and colon using laser capture microdissection, but not in the endothelial, hemolymphoid, or fibroblastic cell lines tested. Although MEC is poorly expressed in skin, its closest homologue is the keratinocyte-expressed cutaneous T cell-attracting chemokine (CTACK; CCL27), and MEC supports chemotaxis of transfected lymphoid cells expressing CCR10, a known CTACK receptor. In contrast to CTACK, however, MEC also supports migration through CCR3. Consistent with this, MEC attracts eosinophils in addition to memory lymphocyte subsets. These results suggest an important role for MEC in the physiology of extracutaneous epithelial tissues, including diverse mucosal organs. ***The Journal of Immunology*****, 2000, 165: 2943–2949.**

Chemokines are a family of leukocyte chemoattractants that regulate both inflammatory T cell recruitment and homeostatic trafficking of lymphocytes and dendritic cells. For example, recent studies have defined a critical role for CCR7 ligands, secondary lymphoid-tissue chemokine (SLC;[5] CCL21) and EBI1-ligand chemokine (ELC; CCL19), in lymphocyte and dendritic cell trafficking to secondary lymphoid tissues, including lymph nodes (1, 2), and have implicated CCR4 and its ligand, thymus and activation-regulated chemokine (TARC; CCL17) in lymphocyte-endothelial cell interactions and lymphocyte recruitment into nonintestinal tissues, particularly the skin (3). Of special interest in the context of the regional specialization of immune responses is the recent description of constitutive and selective expression of the CCR9 ligand, thymus-expressed chemokine (TECK; CCL25) by epithelial cells of the small intestine (4, 5) and of the CCR10 ligand CTACK by skin keratinocytes (6).

Here we describe mucosae-associated epithelial chemokine (MEC), a novel chemokine ligand for CCR10. MEC is most homologous to CTACK, displaying about 40% identity, and is expressed not in the skin, but, instead, in diverse mucosal tissues. MEC attracts subsets of memory lymphocytes as well as eosinophils. Analysis of cultured cell lines and laser capture microdissected epithelia reveals constitutive MEC expression by epithelial, but not endothelial, fibroblastic or lymphoid cells. Our findings suggest that MEC may play an important role in the physiology and/or recruitment of specialized cells into mucosal tissues, including the respiratory and oral mucosa and colon.

Materials and Methods

Cloning and sequencing of MEC

TBLASTN searches of the GenBank dbEST database with the sequences of known CC chemokines identified the expressed sequence tag for human MEC. IMAGE consortium clone 136910 was obtained from American Type Culture Collection (Manassas, VA) as an *Eco*RI-*Not*I insert in the pT7T3D-Pac vector. The nucleotide sequence was confirmed by automated sequencing. Protein similarity and dendrogram analysis were performed using ClustalW. BLASTN searches of the high throughput genome database using the MEC cDNA sequence revealed two BAC clones from human chromosome 5, CTD-2282F8 and CTD-2202K16, that encode the human MEC gene.

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Received for publication May 26, 2000. Accepted for publication July 14, 2000.

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[1] J.P., E.J.K., and U.G. are recipients of Arthritis Foundation Postdoctoral Fellowships. This work was supported by National Institutes of Health Grants GM37734, AI37832, HL57492, and AI47822, a grant from Aventis, and a Merit Award from the Veterans Administration.

[2] The human MEC sequence was submitted to GenBank under accession number AF266504.

[3] J.P. and E.J.K. contributed equally to this work.

[4] Address correspondence and reprint requests to Dr. Eugene C. Butcher, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305-5324. E-mail address: ebutcher@stanford.edu

[5] Abbreviations used in this paper: SLC, secondary lymphoid-tissue chemokine; MEC, mucosae-associated epithelial chemokine; DEPC, diethylpyrocarbonate; CLA, cutaneous lymphocyte Ag; ELC, EBI1-ligand chemokine; TECK, thymus-expressed chemokine; TARC, thymus and activation-regulated chemokine; SDF, stromal cell-derived factor; CTACK, cutaneous T cell-attracting chemokine.

0022-1767/00/$02.00

Synthesis of MEC

The signal sequence of MEC was predicted by the SignalP server (http://www.cbs.dtu.dk/signal/cbssignalp.html). The predicted 105-aa mature protein (aa 23–127 of the coding sequence) was synthesized by F-moc chemistry (automated 433 peptide synthesizer, PE Biosystems, Foster City, CA). A biotin moiety (aminocaproic acid-biotin) was added to lysine 124. The purity (>95%) and molecular integrity of the purified folded chemokine were determined by analytical C_{18} reverse phase HPLC as well as by mass spectrometry. Synthesized MEC had an experimentally determined m.w. of 12408.7 (vs a theoretical weight of 12409.5). CTACK, TECK, and other chemokines were synthesized, and stromal cell-derived factor (SDF)-1α, TARC, and eotaxin were purchased from PeproTech (Rocky Hill, NJ).

Blood cells and cell lines

A panel of 15 known chemokine receptor and 12 orphan G protein-coupled receptor stable transfectants were generated in the murine pre-B lymphoma cell line L1/2 as previously described (7, 8). The CCR10 (GPR-2) full-length sequence was provided by Dr. Craig Gerard (9). L1/2 cells were stimulated overnight with 10 mM butyric acid before performing chemotaxis assays. Peripheral blood mononuclear leukocytes and eosinophils were isolated from whole blood as described previously (8, 10).

Chemotaxis of L1/2 transfectants and blood lymphocytes

Chemotaxis assays were performed using 24-well Transwell plates (Corning Costar, Cambridge, MA; 3-μm pores for L1/2 cells and eosinophils and 5-μm pores for lymphocytes) as previously described (8, 10). L1/2 cells were incubated from 5 h to overnight, and lymphocytes and eosinophils were incubated for 1.5 h. Migrated cells were quantitated by flow cytometry, where FACS cell number corresponds to events acquired in a fixed time of 30 s. Migrated cells were stained with CD4-APC or CD8-APC, CD45RA-Cy, cutaneous lymphocyte Ag (CLA)-FITC, and $\alpha_4\beta_7$-PE to analyze T cells, IgD-FITC, CD19-PE, and CD45RA-Cy for B cell analysis or CD49d-PE and CD16-FITC for eosinphil analysis (all conjugated Abs purchased from PharMingen, San Diego, CA). Four-color flow cytometry was performed on a FACSCalibur (Becton Dickinson, San Jose, CA) using CellQuest software, version 3.1 (Becton Dickinson). For gradient disruption experiments, chemotaxis was conducted identically, except that 3 times the optimal chemotactic dose of chemokine was added to the cells in the top well, and for Ab blockade experiments, 50 μg/ml of blocking mAb (clone 7B11, mouse IgG2a) or control mAb (isotype control or eosinophil-binding anti-L-selectin mAb) was added to the top well.

Laser capture microdissection

Eight-micron sections from normal human salivary gland and colon (obtained from patients undergoing elective surgery at Stanford University Medical Center, Stanford, CA) embedded in OCT (Miles, Elkhart, IN) were cut and immediately fixed in 70% ethanol/diethylpyrocarbonate (DEPC)-treated water for 30 s. Sections were counterstained with toludine blue (Sigma, St. Louis, MO)/DEPC-treated water for 30 s, dehydrated in increasing concentrations of ethanol/DEPC-treated water, and cleared in xylene. Laser capture microdissection was performed on a PixCell II (Arcturus Engineering, Mountain View, CA). Epithelial and nonepithelial cells were captured from each tissue, lysed in guanidinium buffer, and placed on ice for later RT-PCR and Southern analysis. Total RNA was isolated and subjected to a semiquantitative RT-PCR analysis as previously described (11). The PCR primers for human MEC were 5′-CCATCGTGGCCTTG GCTGTCTGTG-3′ and 5′-GCCGTATGTTTCGTGTTTCCCCTG-3′.

Northern analysis for MEC mRNA

Human epithelial cells derived from bronchus-associated epithelium or mammary gland epithelium were obtained from Clonetics (Walkersville, MD) and cultured in a complete medium according to the manufacturer's instructions. EA.hy926, a hybrid HUVEC line, was a gift from Dr. Edgell (University of North Carolina, Chapel Hill, NC). Poly(A)$^+$ RNA from these cell lines was isolated using a Fasttrack Kit (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA from various human tissues was purchased from Clontech (Palo Alto, CA) or Clemente Associates (Madison, WI). RNA was separated on a 1.5% formaldehyde-agarose gel and quantitated by Northern blot analysis according to a standard protocol.

Results and Discussion

MEC is a novel chemokine ligand for CCR10 and CCR3

A BLAST search of the GenBank human EST database with the coding region of CTACK revealed an EST encoding a novel chemokine, here termed MEC. The MEC-coding sequence displays 49 and 45% nucleotide identity with CTACK and TECK, respectively (Fig. 1*A*). Interestingly, unlike CTACK on chromosome 9 and TECK on chromosome 19, MEC is encoded on human chromosome 5 by at least four exons separated by large introns (Fig. 1*B*). At the protein level, MEC displays ~40% amino acid identity in the common homology region with CTACK and is also closely related to TECK, but MEC has a longer C terminus than CTACK and contains six cysteines (Fig. 1*C*).

Synthetic MEC was screened in Transwell chemotaxis assays for activity on L1/2 lymphoid cell lines transfected with known and orphan chemokine receptors. Transfectants expressing CCR10 (GPR-2) and CCR3 migrated efficiently to MEC with an optimal concentration of about 300 nM (Fig. 2*A*), whereas transfectants expressing other chemokine receptors (CCR1–2, CCR4–9, and CXCR3–5) failed to respond even though they chemotaxed to known chemokine ligands for their receptors. L1/2 cells transfected with 12 orphan G protein-coupled receptors (RDC-1, APJ, FEG-1/LyGPR, STRL33/BONZO, GPR-15/BOB, FLPR-2, TDAG8, CRTH2, ChemR23, GPR1, GusB, and AF015524/CRAM) also failed to respond to MEC.

CTACK has previously been shown to attract circulating skin-homing memory CD4 T cells identified by expression of the CLA (6). To determine whether MEC might also be able to recruit memory T cells, human blood T cells were migrated to MEC, CTACK, and SDF-1α (CXCL12), and the phenotype of the starting population and that of the chemokine-recruited cells were analyzed by flow cytometry. As shown in Fig. 2*B*, the patterns of T cell migration to MEC and CTACK were indistinguishable. Although MEC and CTACK exhibited similar optimal chemotactic doses (~300 nM), MEC was more efficacious at this dose (Fig. 2, *A* and *B*). MEC was not detectably chemotactic for naive (IgD$^+$) or memory (IgD$^-$) blood B cells (data not shown). Migration of T cells to MEC was inhibited by a competing gradient of CTACK (and vice versa), but not by a gradient of TARC (a ligand for CCR4), consistent with use of a common receptor for MEC and CTACK on circulating memory CD4 lymphocytes (data not shown).

Consistent with its ability to attract CCR3 transfectants, MEC also attracts blood eosinophils (Fig. 2*C*). The optimal chemotactic concentration of MEC for both CCR3 transfectants (Fig. 2*A*) and eosinophils (Fig. 2*C*) was in the range of 300–400 nM. Eotaxin-3 (PTEC, CCL26), another CCR3 ligand, is also maximally active at this concentration, as are several chemokines active on lymphocytes, including SLC and ELC for CCR7 (10) and TECK for CCR9 (12). Moreover, MEC completely inhibits [^{125}I]eotaxin (CCL11) binding to eosinophils with an IC_{50} of about 2–3 nM, whereas CTACK does not compete for eotaxin binding even at 500 nM (data not shown). Migration of eosinophils to MEC could be completely inhibited by a blocking mAb to CCR3 (Fig. 2*C*), but was unaffected by control mAbs, indicating that MEC activates eosinophils through CCR3 and not CCR10. This activity distinguishes MEC from CTACK, as CTACK fails to attract either eosinophils or CCR3 transfectants (Ref. 6 and D. Soler, unpublished observations).

Mucosal epithelial cell expression of MEC

Dot blot and Northern blot analyses revealed that MEC message is most abundant in salivary gland, but is also expressed in other

A

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  1 tgatcgaacagcctcacttgtgttgctgtcagtgccagtagggcaggcaggaatgcagca  60
                                                        M  Q  Q
 61 gagaggactcgccatcgtggccttggctgtctgtgcggccctacatgcctcagaagccat 120
    R  G  L  A  I  V  A  L  A  V  C  A  A  L  H  A  S  E  A  I
121 acttcccattgcctccagctgttgcacggaggtttcacatcatatttccagaaggctcct 180
    L  P  I  A  S  S  C  C  T  E  V  S  H  H  I  S  R  R  L  L
181 ggaaagagtgaatatgtgtcgcatccagagagctgatggggattgtgacttggctgctgt 240
    E  R  V  N  M  C  R  I  Q  R  A  D  G  D  C  D  L  A  A  V
241 catccttcatgtcaagcgcagaagaatctgtgtcagcccgcacaaccatactgttaagca 300
    I  L  H  V  K  R  R  R  I  C  V  S  P  H  N  H  T  V  K  Q
301 gtggatgaaagtgcaagctgccaagaaaaatggtaaaggaaatgtttgccacaggaagaa 360
    W  M  K  V  Q  A  A  K  K  N  G  K  G  N  V  C  H  R  K  K
361 acaccatggcaagaggaacagtaacagggcacatcagggggaaacacgaaacatacggcca 420
    H  H  G  K  R  N  S  N  R  A  H  Q  G  K  H  E  T  Y  G  H
421 taaaactccttattagagagtctacagataaatctacagagacaattcctcaagtggact 480
    K  T  P  Y  *
481 tggccatgattggttgtcctgcatactgatgaaactactgatgtcagctggtctgaagga 540
541 ccctaccagaagctaaatcatcaaagaatgcaatttccatatcctaatgattcaatctcc 600
601 cttaccctgaccaatcagtggcccaaattttccagccccttgcctcccagaaccccagcc 660
661 cagaactcttcagagatttaagaatctcctcctacctcctgactcagcaccatgtaatca 720
721 ttaaactctctgctgcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa             768
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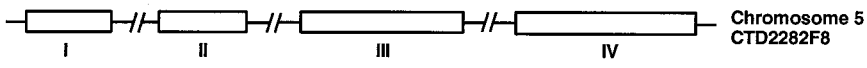
B



Exon			Sequence at intron-exon junction		Intron
	Location in cDNA	Length in bp	3' splice site	5' splice site	Length in bp
I	1-116	116	TGATCGAA--CTCAGAAGgtgagtgg..		> 22,000
II	117-243	127	..tcttttagCCATACTT--GCTGTCATgtgagtgc..		6296
III	244-495	252	..tctaacagCCTTCATG--GATTGGTTgtaagttt..		4857
IV	496-739	244	..tctttcagGTCCTGCA--GCTGCAAA		

C

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hMEC      -ILPIASSCCTEVSHH-ISRRLLERVNMCRIQRADGDCDLAAVILHVKRR--RICVSPHN
hCTACK    FLLPPSTACCTQLYRKPLSDKLLRKVIQVELQEADGDCHLQAFVLHLAQR--SICIHPQN
mCTACK    LPLPSSTSCCTQLYRQPLPSRLLRRIVHMELQEADGDCHLQAVVLHLARR--SVCVHPQN
hTECK     --QGVFEDCCLAYHYP-IGWAVLRRAWTYRIQEVSGSCNLPAAIFYLPKRHRKVCGNPKS
mTECK     --QGAFEDCCLGYQHR-IKWNVLRHARNYHQQEVSGSCNLRAVRFYFRQK--VVCGNPED

hMEC      HTVKQWMKVQAAKKNG---KGNVCHRKKHHGKRNSNRAHQGKHETYGHKTPY--------
hCTACK    PSLSQWFEHQERKLHGTLPKLNFGMLRKMG----------------------------
mCTACK    RSLARWLERQGKRLQGTVPSLNLVLQKKMYSNPQQQN----------------------
hTECK     REVQRAMKLLDARNKV-FAKLHHNMQTFQAGPHAVKKLSSGNSKLSSSKFSNPISSSKRN
mTECK     MNVKRAIRILTARKRLVHWKSASDSQTERKKSNHMKSKVENPNSTSVRSATLGHPRMVMM

hMEC      ----------
hCTACK    ----------
mCTACK    ----------
hTECK     VSLLISANSGL
mTECK     PRKTNN-----
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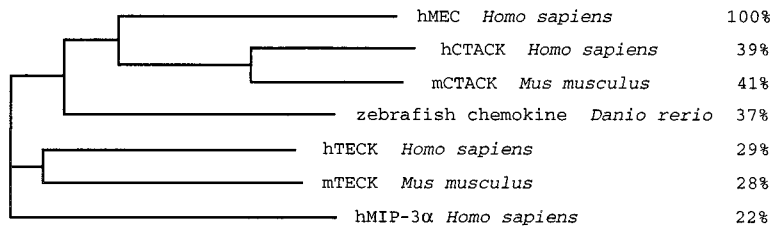

FIGURE 1. MEC, a novel chemokine homologous to CTACK and TECK. *A*, The nucleotide sequence of human MEC reveals a large 3′ untranslated region (the signal peptide is underlined). *B*, MEC is encoded on chromosome 5 by at least four exons separated by large introns. *C*, MEC protein is highly homologous to both CTACK and TECK (the percent identity to human MEC is shown).

tissues associated with mucosal epithelial surfaces, including trachea, mammary gland, colon and rectum (Fig. 3, *A* and *B*). Interestingly, MEC message was poorly expressed in skin (compare expression of CTACK and MEC in Fig. 3*B*), and appeared relatively less abundant in small intestine than in the colon or rectum (compare TECK and MEC expression in Fig.

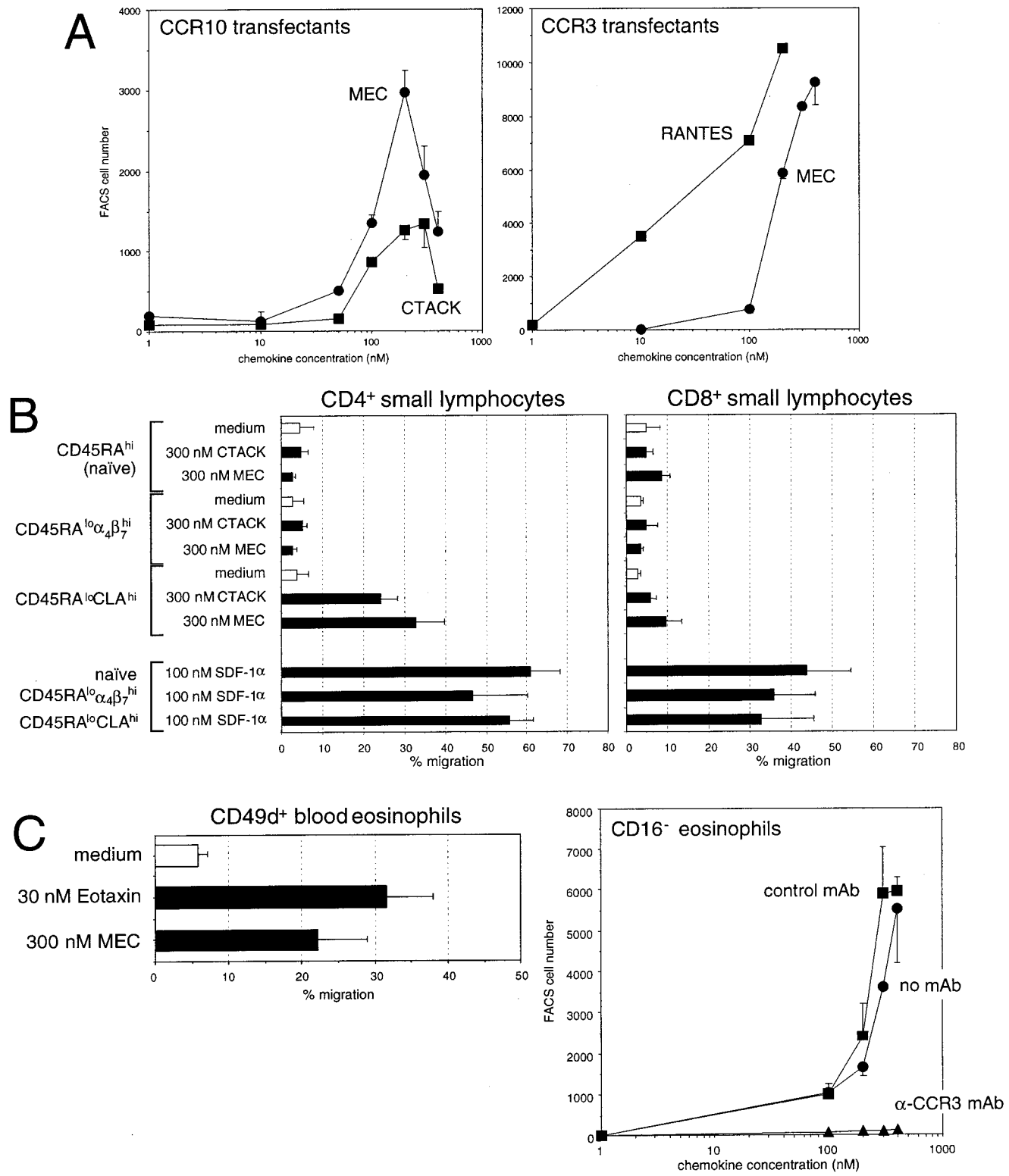

FIGURE 2. MEC is a ligand for CCR10 and CCR3 and is chemotactic for circulating CLA^+ memory CD4 T cells and eosinophils. *A*, MEC is chemotactic for L1/2 cells expressing CCR10 and CCR3. *B*, Similar to CTACK, MEC is chemotactic for memory CD4 T lymphocytes expressing CLA. *C*, Consistent with MEC binding to CCR3, MEC is chemotactic for human blood eosinophils, and its activity can be specifically blocked by an anti-CCR3 mAb, but not a binding control mAb.

3*B*). However, the dot blot results suggest that there may be variable low expression of MEC in different segments of the small intestine and in the stomach, pancreas, thyroid gland, and prostate as well (Fig. 3*A*). In the Northern blots, we found that our MEC cDNA probe detected five MEC transcripts of different sizes (0.8, 1, 3, 3.5, and 6 kb), with the 3-kb transcript being

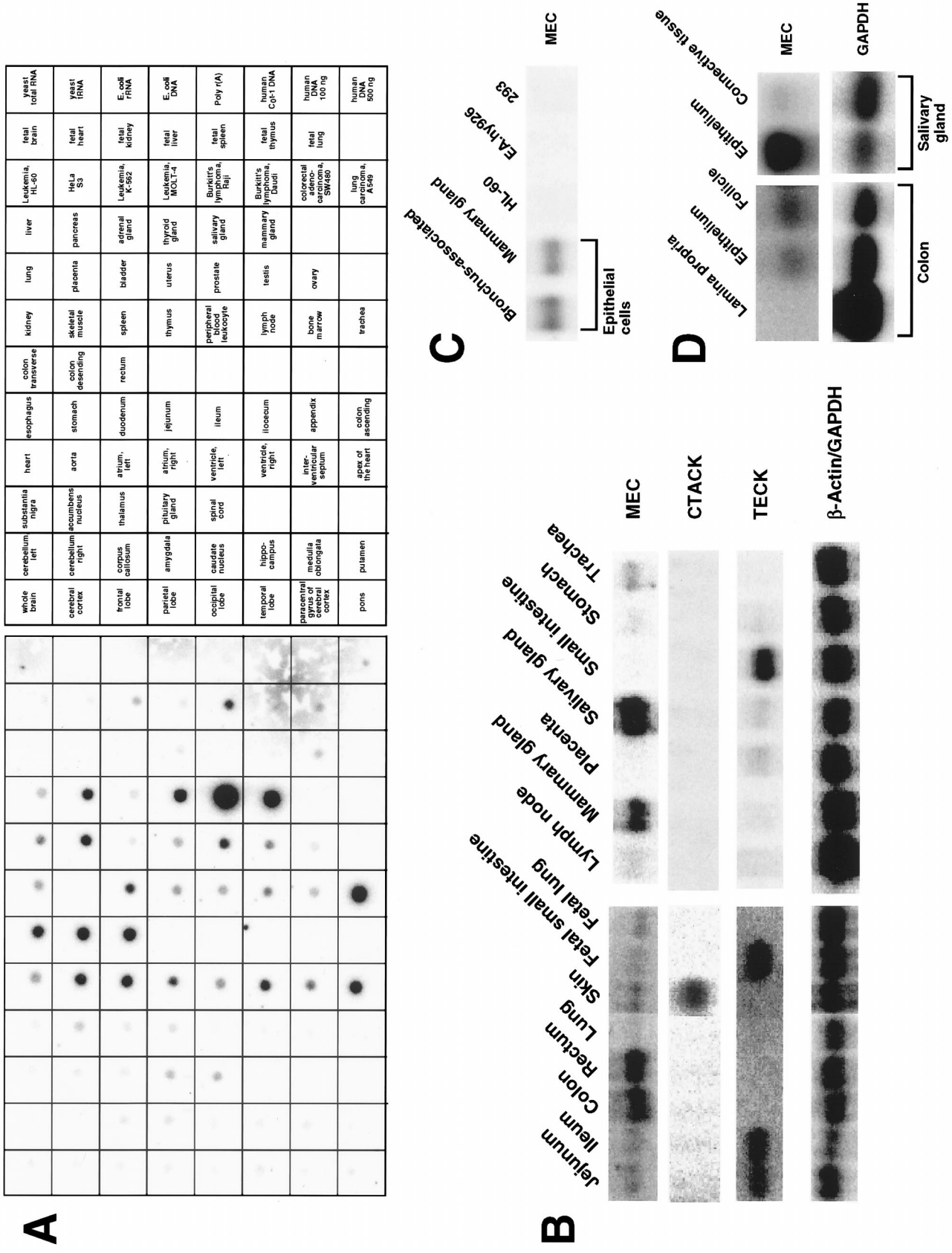

FIGURE 3. MEC is preferentially expressed by epithelial cells in diverse tissues. *A*, Dot blot analysis of a variety of human tissues shows high MEC message in diverse mucosal organs, such as salivary gland, mammary gland, trachea, colon, and rectum, with lower message levels in various segments of the small intestine. *B*, Northern analysis of mRNA from various human tissues confirms the high MEC message in salivary gland, colon, mammary gland, and trachea, in contrast to CTACK, which is only highly expressed in skin, and TECK, which is largely restricted to the small intestine. *C*, MEC is expressed in bronchial and mammary gland epithelial cells, but not myeloid (HL-60), fibroblastic (293), or endothelial (EA.hy926) cell lines. *D*, MEC mRNA is enriched in laser capture microdissected epithelial cells and colon follicles, compared with that in lamina propria or connective tissue.

the most abundant (data not shown). The abundance of the other transcripts is correlated with that of the 3-kb transcript, and there were no obvious tissue-specific transcripts in any tissues examined. These transcripts may represent splice variants of MEC and/or variations in polyadenylation sites.

Because CTACK and TECK are constitutively expressed by keratinocytes and small intestinal epithelial cells, respectively, we asked whether MEC might also be expressed by epithelial cells. As illustrated in Fig. 3*C*, MEC mRNA was expressed in bronchus-associated epithelial cells and mammary gland epithelial cells, but not in HL-60 promyeloid cells, HUVEC, or human fibroblastic cells. To confirm epithelial cell expression of MEC in vivo, we used laser capture microdissection (13) to capture epithelial and nonepithelial cells from human colon and salivary gland and assessed these cells for the presence of MEC mRNA by low cycle RT-PCR followed by Southern blot analysis. As illustrated in Fig. 3*D*, captured epithelial cells from the colon and salivary gland expressed much higher MEC mRNA than adjacent soft tissue, although MEC mRNA was also expressed in gut Peyer's patches.

We conclude that MEC is a novel epithelial cell-expressed chemokine ligand for CCR10 and CCR3 with a unique pattern of tissue expression. Chemokines can modulate tissue physiology at multiple levels, including regulation of cell proliferation and differentiation as well as modulation of adhesion, locomotion, and chemotaxis. MEC may therefore participate in the physiology of mucosal tissues through any number of different mechanisms. The recent demonstration of CCR10 mRNA in Peyer's patches, colon, and stomach (9, 14) is consistent with the coordinate presence of MEC and $CCR10^+$ cells in these mucosal sites. Clearly, however, additional studies will be required to define the role(s) of MEC in the physiology of mucosal tissues and in the specialization of their immune responses.

The close relationship of MEC to CTACK suggests that these chemokines may be products of a relatively recent gene duplication event. Several other clusters or pairs of closely related chemokines that share a common receptor have been described: notably the CCR7 ligands ELC (15) and two nearly identical variants of SLC (16, 17), and the CCR4 ligands TARC and macrophage-derived chemokine (18). In each case, the homologous chemokines display highly distinctive patterns of expression in vivo, suggesting that the evolutionary pressure for gene duplication may have facilitated selective chemokine regulation and tissue or cell type-specific expression (19).

In addition to their distinctive patterns of tissue expression, MEC and CTACK differ in their ability to attract cells via CCR3. MEC, but not CTACK, stimulates migration of CCR3 transfectants as well as eosinophils. In this context, MEC may play a role, in conjunction with other regulatory elements, in the recruitment of eosinophils and, potentially, of rare $CCR3^+$ T cells into mucosal tissues, including trachea and bronchi.

In the context of chemokine regulation of lymphocyte trafficking, it is reasonable to postulate that MEC may play a role in the recruitment and/or microenvironmental localization of specialized CCR10-expressing memory and effector lymphocyte subsets. For example, the high levels of MEC expression in salivary glands may be relevant to the migratory patterns of a population of CLA^+ lymphocytes, as the buccal mucosa is known to be a site of localization of CLA^+ T cells (20, 21). (In contrast, it is unlikely that MEC recruits CLA^+ T cells to the colon, as CLA^+ T cells lack the necessary homing receptors to interact with and arrest on intestinal vascular endothelium.) It is likely that additional $CCR10^+$ lymphocyte populations exist, populations that may be relatively poorly represented in blood. Subsets of $CCR10^+$ gut-associated lymphocytes, for example, might express $\alpha_4\beta_7$ but lack CLA, and thus would have the opportunity to respond to gut-associated MEC (but not to skin keratinocyte-expressed CTACK). In the context of the multistep process required for lymphocyte-endothelial recognition, diapedesis, and recruitment from the blood (22), MEC and CTACK could thus play parallel roles in facilitating lymphocyte diapedesis into mucosal tissues and skin, but in each site would be acting on different lymphocyte subsets, whose availability would be determined by prior vascular interaction (i.e., capture, rolling, activation, and firm adhesion).

Another group has independently identified CCL28, reporting its expression by epithelial cells and activity on CCR10 (but not CCR3) (23).

In conclusion, its unique pattern of mucosal epithelial expression and of chemokine receptor usage suggests that MEC may play important roles in the physiology and pathophysiology of diverse mucosal tissues.

Acknowledgments

We thank E. Resurreccion for expert tissue sectioning for laser capture microdissection, and Ellen Triantafellow for excellent technical assistance.

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