

Mast cells express connexins on their cytoplasmic membrane

Harissios Vliagoftis, MD,^{a,b,*} Anne M. Hutson, BSc,^{a,*} Salahaddin Mahmudi-Azer, MSc,^b Hansoo Kim, MD,^a Vanitcha Rumsaeng, MD,^a Chad K. Oh, MD,^c Redwan Moqbel, PhD,^b and Dean D. Metcalfe, MD^a *Bethesda, Md, Alberta, Canada, and Torrance, Calif*

Background: Because of the close association between mast cells and fibroblasts in the microenvironment and the importance of connexins (Cx) in fibroblast communication with other cells, we hypothesized that mast cells also express Cxs, allowing them to similarly communicate with other cells through gap junctions.

Objectives: We sought to identify the expression of Cxs (particularly Cx43, Cx32, and Cx26) by murine mast cells.

Methods: The expression of Cxs was studied by RT-PCR, Northern blot analysis, Western blot analysis, flow cytometry, and confocal laser scanning microscopy.

Results: In this report we demonstrate that murine bone marrow cultured mast cells and the growth factor-independent murine mast cell line C57, express Cx43 and Cx32 as assessed by RT-PCR, Northern blot analysis, Western blot analysis, and flow cytometry, but do not express Cx26. We also show, by confocal laser scanning microscopy, that Cx43 localizes to the cytoplasmic membrane of mast cells in a pattern similar to that seen in fibroblasts.

Conclusions: Mast cells express Cx43 and Cx32, and Cx43 is associated with the cytoplasmic membrane, suggesting that mast cells have the potential to communicate with other cells in their microenvironment in part through gap junctions. (*J Allergy Clin Immunol* 1999;103:656-62.)

Key words: Mast cells, connexins, gap junctions, fibroblasts, cell communication

Mature mast cells reside within tissues and may generate inflammation after activation. It is also known that microenvironmental interactions of mast cells with stromal cells, growth factors, and connective tissue matrix components are fundamental for mast cell differentiation, proliferation, and survival. In particular, there is a close approximation in vivo between mast cells and connective

Abbreviations used

BMCMC:	Bone marrow cultured mast cell
Cx:	Connexin
DMEM:	Dulbecco's modified Eagle's medium
2-ME:	2-Mercaptoethanol
PMA:	Phorbol 12-myristate 13-acetate
SCF:	Stem cell factor

tissue fibroblasts, and fibroblasts are believed to promote mast cell survival and differentiation through the production of membrane-bound and soluble stem cell factor (SCF), which interacts with c-kit on the surface of mast cells.^{1,2} The interaction between fibroblast membrane-bound SCF and mast cell c-kit also serves to promote adhesion between these 2 cell types.³

In turn, mast cells influence the production of connective tissue matrix components by fibroblasts.^{4,5} However, it is not clear that all mast cell-fibroblast interactions are accounted for by soluble factors or by the interaction between SCF and c-kit.⁶ For instance, the ultrastructure of mast cells within a fibroblast monolayer is distinctly reminiscent of mast cells within connective tissues and cannot be duplicated simply by the addition of SCF.^{7,8}

These observations led us to question whether there might be additional mechanisms by which fibroblasts and mast cells could interact. One such system believed to allow interactions between fibroblasts consists of gap junctions.⁹ Gap junctions are pores or channels that link the interior of the 2 junction-forming cells and are structured by the end-to-end abutment of 2 hemichannels or connexons, which are comprised of 6 protein subunits called connexins (Cxs).¹⁰ The pore size limits permeability to substances less than 1 kd, and therefore most ions and small molecules may traverse the channels, although macromolecules cannot. Over 12 Cxs have been described.

We thus hypothesized that mast cells might express Cxs and have the potential to form gap junctions. To explore this possibility, we examined mast cells for their ability to synthesize Cx43, Cx32, and Cx26 and express Cx proteins on their cytoplasmic membrane. As will be shown, murine mast cells express 2 different Cx proteins, Cx43 and Cx32, and at least Cx43 localizes to the cytoplasmic membrane.

From ^athe Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda; ^bPulmonary Research Group, Department of Medicine, University of Alberta, Alberta; and ^cthe Department of Pediatrics, Harbor-UCLA Medical Center, Torrance.

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*Harissios Vliagoftis, MD, and Anne M. Hutson, BSc, shared equally in the essential nature of this paper.

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Reprint requests: Harissios Vliagoftis, MD, Pulmonary Research Group, Department of Medicine, 574 HMRC, University of Alberta, Edmonton, AB, T6G 2S2, Canada.

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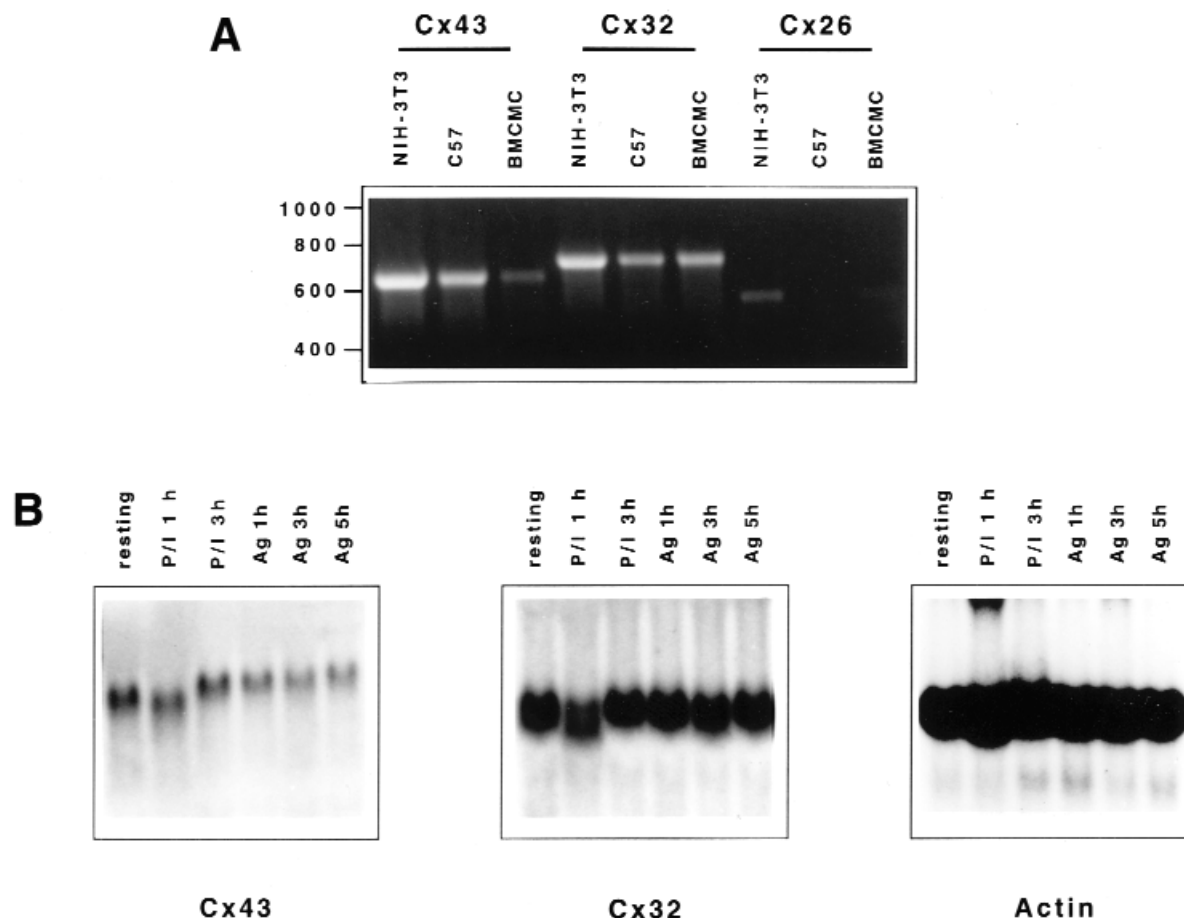


FIG 1. Mast cell Cx mRNA expression. **A**, RT-PCR for Cx43, Cx32, and Cx26. Cx43 (lanes 1 to 3), Cx32 (lanes 4 to 6), and Cx26 (lanes 7 to 9) were amplified from NIH-3T3 fibroblasts (lanes 1, 4, and 7), C57 mast cells (lanes 2, 5, and 8), and BMCMCs (lanes 3, 6, and 9). **B**, Northern blot analysis of Cx expression in C57 mast cells. Cx43 is shown in the left panel, Cx32 in the middle panel, and actin in the right panel. *Resting* indicates non-activated cells; *P/I* indicates PMA (50 ng/mL) and A23187 (0.5 μ g/mL) activation for 1 hour and 3 hours, respectively, and *Ag* indicates Fc ϵ R1 aggregation for 1 hour, 3 hours, and 5 hours, respectively.

METHODS

Materials

WEHI-3 conditioned medium (containing IL-3) (Collaborative Biomedical Products, Bedford, Mass); Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, HEPES, penicillin/streptomycin, L-glutamine, nonessential amino acids, and FCS (Biofluids Inc, Rockville, Md); Trizol, Superscript Reverse Transcription kit, and Taq polymerase (Life Technologies, Gaithersburg, Md); phorbol 12-myristate 13-acetate (PMA), DNP, 2-mercaptoethanol (2-ME), IgE, monoclonal anti-DNP antibody, clone SPE-7, propyl gallate, and human IgG (Sigma Chemical Company, St Louis, Mo); Tris-glycine 10% and 14% gels (Novex Technologies, San Diego, Calif); rabbit anti-Cx43, anti-Cx32, and anti-Cx26 antibodies, Cx43 and Cx32 immunizing peptides, and mouse monoclonal anti-Cx43 antibody (Zymed Laboratories Inc, South San Francisco, Calif); BODIPY-conjugated goat anti-mouse antibody (Pharmingen); and oligolabeling kits (Pharmacia Biotech, Piscataway, NJ) were purchased from the distributors and manufacturers as shown.

Cell cultures

C1.MC/C57.1 (C57) is an IL-3 independent murine mast cell line derived from the bone marrow of BALB/c mice,¹¹ which

was kindly provided by Dr Stephen Galli (Beth Israel Hospital, Boston, Mass). C57 mast cells and NIH-3T3 cells were cultured in DMEM supplemented with 4 mmol/L L-glutamine, 100 μ g/mL penicillin/streptomycin, 50 mmol/L 2-ME, and 10% heat-inactivated FCS (complete medium). Bone marrow cultured mast cells (BMCMCs) were obtained from the bone marrow of female BALB/c mice and cultured in RPMI-1640 medium supplemented with 10% WEHI-3-conditioned medium, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 4 mmol/L L-glutamine, 25 mmol/L HEPES, 100 μ g/mL penicillin/streptomycin, 50 mmol/L 2-ME, and 10% FCS as described.¹²

Fc ϵ R1 aggregation

C57 mast cells suspended at 10^6 cells/mL in DMEM were incubated for 2 hours at 37°C with 1 μ g/mL mouse IgE anti-DNP. Subsequently, the cells were washed twice with fresh medium, suspended at the desired concentration (2×10^5 to 4×10^5 cells/mL), and Fc ϵ R1 aggregated by the addition of 100 ng/mL DNP-human serum albumin. Granule exocytosis from the Fc ϵ R1-activated cells was measured by quantitation of β -hexosaminidase released to verify activation.¹³

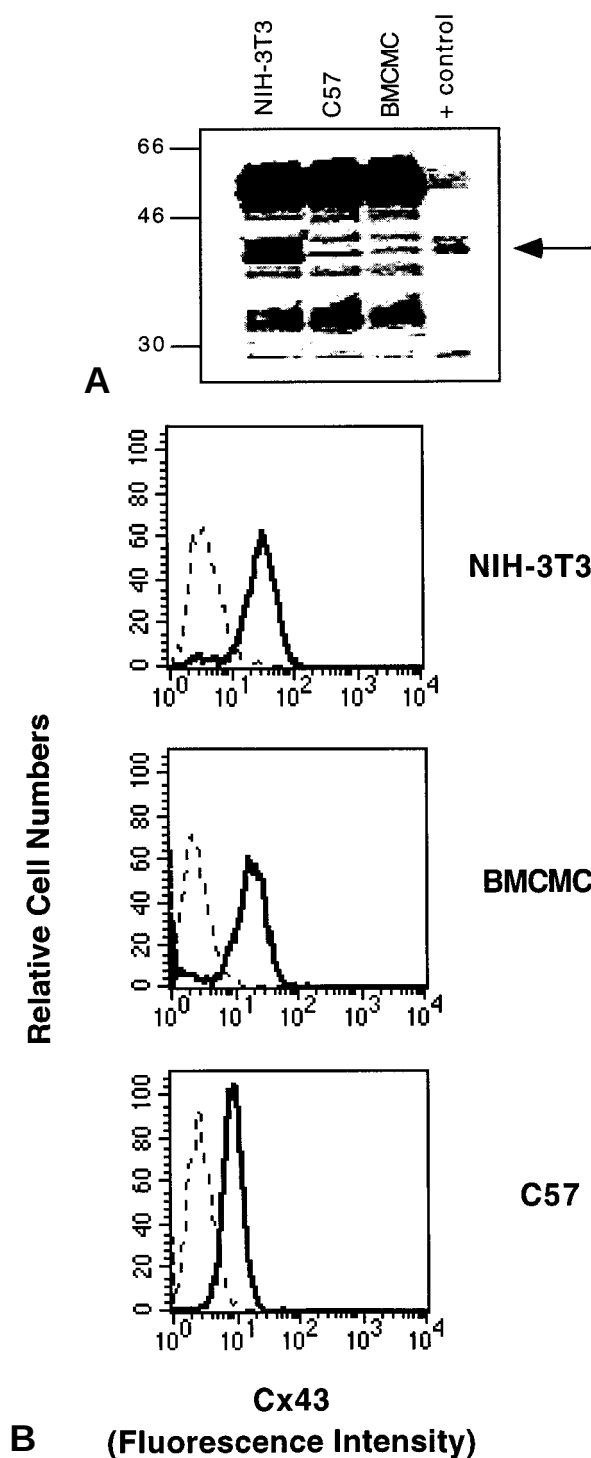
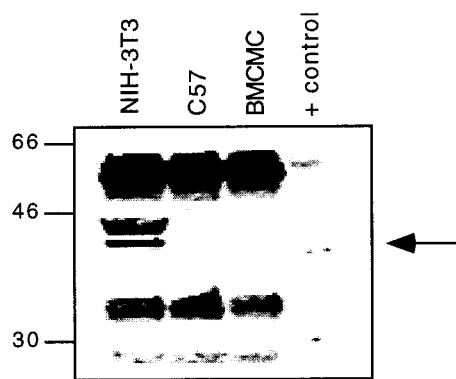


FIG 2. Mast cell expression of Cx43 protein. **A**, Western blot analysis of Cx43 expression in NIH-3T3 cells, C57 mast cells, and BMCMCs. *Far right lane* shows Cx43 expression in mouse brain lysate as a positive control. *Left panel* shows Western blotting with rabbit anti-Cx43 antibody, and *right panel* shows Western blotting with the same antibody preincubated with the immunizing peptide. **B**, Flow-cytometric analysis of Cx43 in NIH-3T3 cells (*top panel*) BMCMCs (*middle panel*), and C57 mast cells (*lower panel*). Isotype control staining is indicated by dotted lines, and anti-Cx43 antibody staining is indicated by dark lines.



RT-PCR

Total RNA was isolated from NIH-3T3 fibroblasts, C57 mast cells, and BMCMCs with Trizol according to the manufacturer's instructions. Five micrograms of total RNA was used for reverse transcription of first-strand cDNA with the Superscript-RT kit. One tenth of the total cDNA was used to amplify Cx26, Cx32, and Cx43 mRNA with 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute with a Perkin-Elmer 9600 automatic cyler (Perkin-Elmer, Norwalk, Conn). The following primers were used for Cx amplification: 5'-GGGAGTTGGAGATGGTGCTTCGGC-3' and 5'-AGGAGCTCAAAGTGGCGCAGACCGACGGGG-3' (Cx43, product of 662 bp); 5'-GGGGAGGCAGCATGACTATGCC-3' and 5'-CACCACCTCCGCCACGTTGAGGAT-3' (Cx32, product of 732 bp); and 5'-TGGCTTCTTCATGCAACGTVTG-TTG-3', 5'-CCTTCCTCAGGACCGGGTTCCTCTC-3' (Cx26, product of 576 bp). The PCR product was electrophoresed on a 1% agarose-TAE gel, stained with ethidium bromide, and photographed with Polaroid film under fluorescent light.

Northern blot analysis

Total RNA was isolated from resting FcεRI-activated and PMA-activated C57 mast cells with Trizol. Twenty micrograms of total RNA was separated on a 1.5% agarose-formaldehyde gel and then transferred to a nylon membrane overnight. The membrane was baked for 2 hours at 80°C and then used for hybridization.

A 1.0-kilobase (kb) clone of Cx43 isolated from a mast cell cDNA library¹⁴ and the PCR product of Cx32 were used to make a ³²P-labeled probe by the random hexamer method by using the oligolabeling kit according to the manufacturer's instructions. Blots were prehybridized at 42°C for 2 hours with Hybrizol and then hybridized at 42°C for 18 hours with the labeled probe in a hybridization oven. The blots were washed at 42°C in 0.1 × saline sodium citrate, 0.1% SDS, 0.1% Na pyrophosphate, 0.125 mmol/L Na₃ EDTA, and 10 mmol/L phosphate buffer. Blots were next exposed on Kodak X-OMAT-AR autoradiographic film with an enhancing screen.

Western blot analysis

Cells were lysed in borate-buffered saline with 1% Triton-X 100 plus 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 4 μg/mL leupeptin, and 10 μg/mL pepstatin. The protein concentration was measured with a colorimetric assay. Forty micrograms of total cellular protein was separated on a 10% Tris-glycine gel (for Cx43) or a 14% Tris-glycine gel (for Cx32 and Cx26) and then transferred to a nitrocellulose membrane. The membranes were blocked overnight at 4°C with 5% dried milk in Tris-buffered saline, and a Western blot was performed with rabbit anti-Cx antibodies (1 μg/mL) in Tris-buffered saline with 0.05% Tween-20. The membranes were subsequently incubated with goat anti-rabbit HRP sec-

ondary antibody (1/10,000) for 1 hour at room temperature. The proteins were visualized by enhanced chemiluminescence on Kodak film.

For blocking experiments, the rabbit anti-Cx43 antibody was preincubated with 50 µg/mL of the Cx43-immunizing peptide for 30 minutes at 4°C before being used for blotting, which was performed as above.

Flow cytometry with intracellular staining for Cxs

For flow-cytometric analysis of Cx expression, cells were fixed with 4% paraformaldehyde and made permeable with saponin in PBS (PBS-S) for 1 hour at 4°C, and 5% milk was added for blocking.¹⁵ Cells were then stained with rabbit anti-Cx43 and rabbit anti-Cx32 antibodies at 10 µg/mL in 5% milk in PBS-S for 30 minutes on ice. Finally, the cells were washed twice in PBS-S, incubated with goat anti-rabbit IgG phycoerythrin-conjugated antibody at 1:600 in 5% milk in PBS-S for 30 minutes on ice, and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif). For blocking experiments, antibodies were preincubated with 50 µg/mL of the immunizing peptides for 30 minutes at 4°C before being used to stain the cells.

Confocal microscopy

Cytospin preparations of BMCMCs or NIH-3T3 cells were fixed in 2% paraformaldehyde in PBS for 10 minutes. Slides were washed 5 times in Tris-buffered saline, followed by incubation in blocking solution (2% human IgG) in a humidified container for 1 hour. After a second wash step, mouse anti-Cx43 mAb (20 µg/mL) or isotype control was added, and slides were incubated for 1 hour. Slides were subjected to a wash step again before adding BODIPY-conjugated goat anti-mouse antibody (50 µg/mL) and incubating for 1 hour. After a final wash step, 10 µL of antibleaching agent (0.4% *n*-propyl gallate in 3:1 glycerol:TBS) was dropped onto each slide before coverslip attachment. Slides were examined under a Leica confocal laser scanning microscope (Heidelberg, Germany). Images were stored on computer and transferred to Adobe Photoshop for preparation of a composite image.

RESULTS

Mast cell Cx mRNA expression

To determine whether mast cells express Cx, we first examined Cx expression by RT-PCR. In addition to Cx43, which is expressed on monocytes and thymocytes, we also determined the expression of Cx32 and Cx26, which are transcribed in multiple tissues. As can be seen in Fig 1, A, BMCMCs and C57 mast cells express mRNA for Cx43 and Cx32. Mast cells did not exhibit Cx26 expression by RT-PCR. NIH-3T3 fibroblasts, which express mRNA for Cx43, Cx32, and Cx26, are shown as a positive control.

By Northern blot analysis, mast cells were verified to express a 3-kb message for Cx43 and a 1.6-kb message for Cx32 (Fig 1, B). Neither Cx43 mRNA levels (Fig 1, B, left panel) nor Cx32 levels (Fig 1, B, middle panel) were altered significantly after activation of C57 mast cells with PMA plus ionophore A23187 or with specific IgE plus antigen for up to 5 hours.

Expression of Cx43 and Cx32 protein

Mast cell expression of proteins corresponding to Cx43, Cx32, and Cx26 was next examined by Western

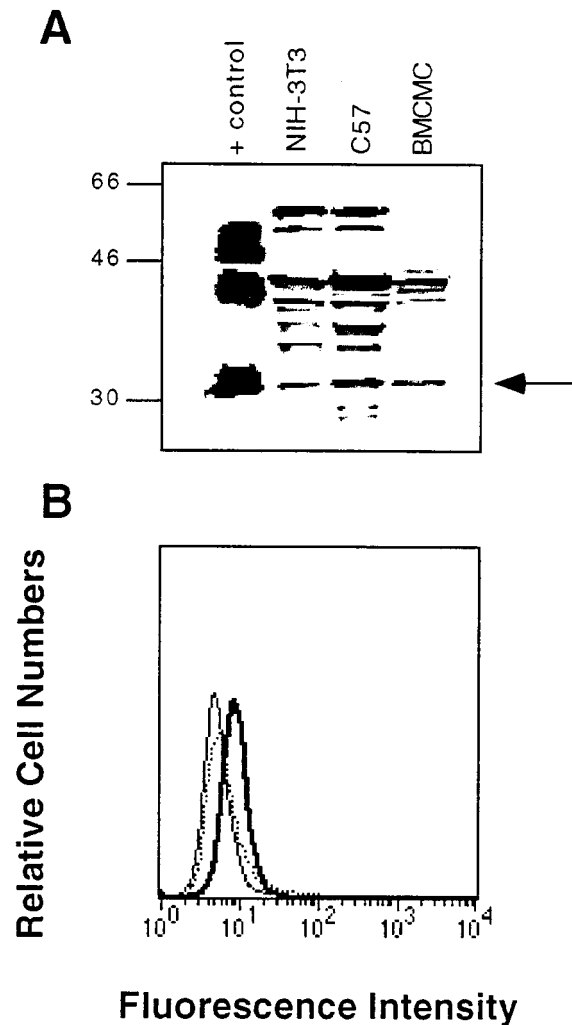


FIG 3. Mast cell expression of Cx32 protein. **A**, Western blot analysis of Cx32 expression in NIH-3T3 cells, C57 mast cells, and BMCMCs. *Left lane* shows Cx32 expression in mouse liver lysate as a positive control. **B**, Flow-cytometric analysis of Cx32 expression by BMCMCs. Isotype control staining is indicated by *light line*, anti-Cx32 antibody staining is indicated by *dark line*, and inhibition of staining by preincubation of the antibody with the immunizing peptide is indicated by *dotted line*.

blot analysis. C57 mast cells and BMCMCs expressed protein for Cx43 (molecular weight, 42 to 44 kD)¹⁶ (Fig 2, A) and Cx32 (molecular weight, 32 kD)¹⁷ (Fig 3, A). Cx43 presents as multiple bands between 42 and 44 kD, indicating different degrees of phosphorylation.¹⁶ The specificity of the bands for Cx43 was verified in that all the bands of the appropriate size disappeared when the anti-Cx43 antibody was preincubated with the Cx43-immunizing peptide before use in Western blotting (Fig 2, A; *right panel* vs *left panel*). Results of Western blotting of mast cell lysates with rabbit anti-Cx26 were negative (data not shown).

These results were consistent with flow cytometry with the same antibodies as above, which are directed against the C-terminal intracytoplasmic portion of Cxs.

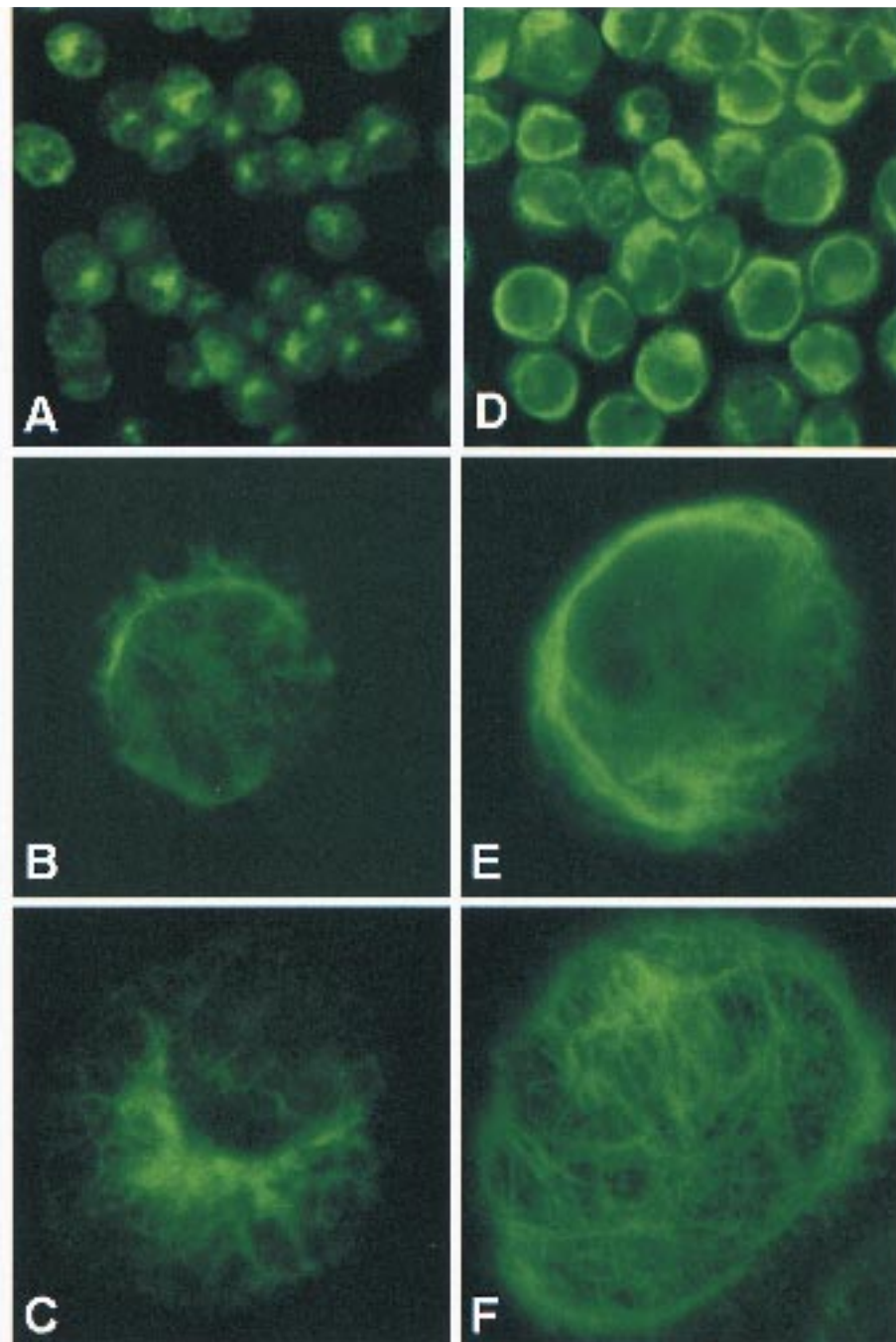


FIG 4. Confocal laser scanning microscopy images of BMCMCs and NIH-3T3 fibroblasts stained with mouse monoclonal anti-Cx43 antibody and BODIPY-conjugated goat anti-mouse antibody. Mast cells (**A**, **B**, and **C**) and NIH-3T3 cells (**D**, **E**, and **F**) are shown at low magnification ($\times 400$, **A** and **D**) and at high magnification ($\times 1000$) highlighting membrane (**B** and **E**) and cytoplasmic expression (**C** and **F**).

Thus an intracytoplasmic staining protocol¹⁵ revealed that BMCMCs and C57 mast cells express Cx43 (Fig 2, *B*) and Cx32 (Fig 3, *B*). The specificity of the staining for Cx32 was verified in that complete inhibition of staining was obtained when the antibody was preincubated with the Cx32-immunizing peptide before use in flow cytometry (Fig 3, *B*).

Confocal microscopy

The above experiments demonstrate the expression of Cx43 and Cx32 by mast cells. However, antibodies against the extracellular portion of these proteins are not available to us. This precludes us from identifying protein expression on the surface of the cells by flow cytometry.

etry. To identify the expression of Cx43 on the surface of mast cells, we performed confocal microscopy with a mouse monoclonal anti-Cx43 antibody. As can be seen in Fig 4, A, BMCMCs expressed Cx43 as did NIH-3T3 cells (Fig 4, D). The staining pattern between the 2 cell types was similar. Staining was seen in both cell types associated with the cytoplasmic membrane (Fig 4, B and E, for mast cells and NIH-3T3 cells, respectively), as well as in the cytoplasm (Fig 4, C and F). The plasma membrane (rim) staining of mast cells is weaker than the staining of NIH-3T3 cells (compare Fig 4, B and E). However, mast cells showed intense cytoplasmic staining. This may indicate that mast cells have a large pool of cytoplasmic Cxs that could be transferred to the cell membrane given the appropriate stimulus.

DISCUSSION

Gap junctions are channels that are composed of oligomerized Cxs, which allow intercellular movement of ions and molecules of less than 1 kd. In this study we report that murine mast cells express mRNA (Fig 1) and protein (Figs 2 and 3) for Cx43 and Cx32 but not Cx26. Furthermore, at least Cx43 immunoreactivity appeared to be expressed on the cytoplasmic membrane of mast cells, and its localization was similar with that seen in fibroblasts (Fig 4).

Information concerning the expression of Cxs on hematopoietic and immune cells is limited. Mouse macrophage cell lines¹⁸ and LPS-activated hamster peritoneal leukocytes¹⁹ have been shown to express Cx43. Leukocytes that adhere to endothelium after ischemia reperfusion of the cheek pouch in hamsters show evidence of gap junction formation with endothelial cells by electron microscopy.¹⁹ Freshly isolated human peripheral blood monocytes do not express Cx43, whereas macrophage foam cells from atherosclerotic lesions do, although there is no evidence that they form functional gap junctions.²⁰ Thymic epithelial cells and thymocytes express Cx43.²¹ If the gap junction communication between thymic epithelial cells and thymocytes is inhibited, the production of the thymic hormone thymulin is also inhibited.

Fibroblasts have been shown to influence the differentiation and survival of mast cells in coculture. The phenotype of connective tissue mast cells is thus preserved by coculture with fibroblasts, but it is not preserved when connective tissue mast cells are cultured in contact with extracellular matrix proteins.²² The activation of c-kit on the mast cell surface through its interaction with fibroblast membrane-bound SCF is believed to be responsible for many of the effects of fibroblasts on mast cells. However, this interaction may not explain all the effects observed. The interaction of mast cells with fibroblasts through Cxs offers another mechanism that may allow a communication between fibroblasts and mast cells.

Cx43 knock-out mice show cardiac abnormalities,²³ and Cx32 knock-out mice show impairment of sympathetic nerve stimulation in the liver.²⁴ Whether any of

these abnormalities relates to heterologous gap junction formation between mast cells and other cell types is unknown. Mast cell-dependent events have not been studied in relation to Cx in these mice. However, because fibroblasts express multiple Cxs, it may be unlikely that the loss of any single Cx would significantly interrupt biologically relevant mast cell-fibroblast interactions that are Cx-dependent.

This article thus presents the first biochemical evidence that mast cells express Cxs on the cytoplasmic membrane and therefore have the potential to form functional gap junctions with neighboring cells. This observation is in accordance with electron microscopic observations of structures between mast cells and fibroblasts identified as gap junctions.²⁵ The physiologic relevance of gap junctions between mast cells and fibroblasts remains to be investigated. However, it would appear that these heterologous gap junctions may provide an unrecognized pathway for communication between mast cells and the cells in the local microenvironment.

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