**Annett Duemmler,[1]**
**Aurea Rosalía Montes-Vizuet,[2]**
**Julio Santiago Cruz,[2]**
**Luis Manuel Terán[2]**

# CXCL5 into the upper airways of children with influenza A virus infection

## RESUMEN

Introducción: la infiltración por neutrófilos es una característica importante en la patogenia de la infección por influenza. Los factores que regulan la afluencia de neutrófilos no están completamente establecidos. Nuestro objetivo fue estudiar la liberación de CXCL5 , potente quimioatrayente de neutrófilos, en niños con influenza naturalmente adquirida.

Métodos: la concentración de CXCL5 fue investigada mediante ensayo inmunoenzimático en aspirados nasales de niños (*n* = 18) con síntomas respiratorios desencadenados predominantemente por el virus de la influenza *A*.

Resultados: se encontró incremento en los niveles de CXCL5 en aspirados nasales obtenidos cuando los niños presentaban síntomas respiratorios, comparados con muestras de los mismos niños obtenidas después de cuatro semanas sin síntomas (medianas 1850 pg/mL *adversus* 30 pg/mL, $p < 0.005$). CXCL5 fue purificada de las muestras y se demostró actividad biológica quimiotáctica para neutrófilos.

Conclusiones: se sugiere un papel importante para CXCL5 en la atracción de neutrófilos a las vías aéreas superiores en infección respiratoria desencadenada por virus de la influenza, y podría ser un blanco para la intervención terapéutica.

## SUMMARY

Background: neutrophil infiltration is a major feature in the pathogenesis of influenza infection. The factors regulating the neutrophil influx are not fully understood. The chemokine CXCL5/ENA-78 is a potent neutrophil chemoattractant, that has been implicated in several inflammatory diseases. Our objectives was to study the release of CXCL5 in children with natural acquired influenza.

Methods: CXCL5 concentration was investigated by immunoenzyme assay in nasal aspirates of children (*n* = 18) in whom respiratory symptoms were precipitated predominantly by influenza *A* virus.

Results: there were increased CXCL5 levels in nasal aspirates when children were symptomatic as compared with samples from the same children when they had been asymptomatic for four weeks (medians 1850 pg/mL *vs.* 30 pg/mL, $p < 0.005$). We purified CXCL5 from these samples, and demonstrated biological neutrophil chemotactic activity.

Conclusions: it is the first *in vivo* data that suggest an important role for CXCL5 in neutrophil influx in proven upper respiratory influenza infection. We suggest that CXCL5 might provide a target for therapeutic intervention in influenza induced respiratory diseases.

[1]University of Bremen, Germany
[2]Instituto Nacional de Enfermedades Respiratorias, Secretaría de Salud, Distrito Federal, México

Comunicación con:
Luis Manuel Terán.
Tel/fax: (55) 5487 1740.
Correo electrónico:
lmteran@iner.gob.mx

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## Introduction

Influenza viruses (IV) are major cause of respiratory infection in humans and result in substantial illness, death and economic burden throughout the world.[1] There are two major types of human influenza viruses, *A* and *B*; the influenza A strains are responsible for seasonal or pandemic influenza. Influenza illness is characterized by fever, lower and upper respiratory symptoms, myalgia, malaise and occurs in temperate climates between late fall and spring. The average "flu season" in the United States is marked by 30,000-40,000 deaths per year primarily in elderly patients with significant comorbidity and in the very young. It is now well established that neutrophils play an important role in influenza respiratory infections.[2] To date, the 2009 Influenza *A* virus (IAV) (H1N1) has killed over 7820 people worldwide.[3] Increased number of neutrophils count in blood has been found, but also in nasal secretions, bronchoalveolar lavage and

sputum of patients suffering IAV infection.[3-5] Animal studies have shown an induced neutrophil influx into the lung of IAV infected mice.[6,7] H5N1 infection causes excessive infiltration of neutrophils into the respiratory tract of mice.[8] The year 1918 pandemic IAV strain increased the influx compared to infection with other human strains of IAV.[9] In this model, depletion of neutrophils before infection reduced survival.

Although the role of neutrophils is not well defined, it is known that they act as effectors cells in defense and eliminate viruses from sites of infection.[10,11] Electron microscopy studies have shown that, neutrophils incorporate IAV particles into phagosoms and lyse them.[7] Similarly, a specific binding of neutrophils to IAV infected cells was found.[12] Hence neutrophils may play a direct role in viral clearance.

Neutrophils are derived from primitive precursor cells in the bone marrow. Once mature, they circle in the peripheral blood. The recruitment of neutrophils to the site of infection is regulated by chemokines which lead to adhesion of neutrophils to endothelial cells, transendothelial migration, chemotactic movement, degranulation and stimulation of respiratory burst.[13] CXCL5, also known as ENA-78 (Epithelial neutrophil activating protein 78), member of CXC chemokine family, is a neutrophil chemoattractant[14,15] whose role in virus influenza infection has not been investigated in detail. Our objective was to investigate the release of CXCL5 in nasal aspirate of children with natural acquired influenza infection.

**Table I**
**Differential and total cell counts (x 105/mL) in nasal aspirate (NA)***

| | Influenza (*n* = 18) | Without virus infection (*n* = 12) |
|---|---|---|
| Lym | 12.0* | 0.2 |
| | (3 -53) | (0-4) |
| Macro | 8.2* | 0.3 |
| | (0-19) | (0-2) |
| Epi | 10.5* | 2.0 |
| | (2.8 - 45) | (0-10) |
| Neut | 220* | 1.3 |
| | (190.3) | (0.5-5) |
| Total | 242* | 2.8 |
| | (26-126) | (0-6) |

**p < 0.001 compared to asymptomatic (Kruskal-Wallis test); asymptomatic,*
*Lym = lymphocytes, Macro = macrophages, Epi = epithelial cells, Neut = neutrophils*
*Number of cells in NA is expressed as medians with ranges shown in parentheses*

## Methods

Nasal aspirates of 18 children (aged 6-12 years) with upper respiratory symptoms caused by IV (*n* = 18) were analyzed. These 18 children were selected from a longitudinal study on virus respiratory infection conducted at the *Instituto Nacional de Enfermedades Respiratorias.* Nasal control samples were obtained from 12 children when they were symptom free. The hospital's ethic committee approved the study. We obtained written and verbal consent from parents and children.

Subjective respiratory symptoms' children were recorded in a diary and the peak flow rates measured daily. Assessments of respiratory symptoms were as follows-0: absent; 1: mild; 2: moderate; 3: severe. Upper respiratory tract symptoms included: runny/blocked nose, sneezing, sore throat, hoarse voice, headaches, pains elsewhere, shivers, chills or fever. Lower respiratory tract symptoms were: cough, wheeze and/or breathlessness during day or night. Scores were added to give daily upper and lower respiratory tract scores.

When upper respiratory tract score was 1, nasal aspirates were collected with a suction pump and homogenized in virus transport medium [L-15 (GibcoBRL) (9 volumes) supplemented with 1000 IU/mL penicillin (PISA), 1000 µg/mL streptomycin (PISA), 0.5 % bovine serum albumin (BSA) (Sigma)], and immediately transported in ice bath to the laboratory where it was centrifuged (2500xg, 10 min, 10 ºC). Supernatant was recovered and used for cytokine detection; cell pellet was used to study nasal recruited cells. Controls were obtained from the same children (*n* = 12) when they were free of symptoms.

### Viral detection

It was identified IAV infection by viral isolation technique and immunofluorescence assay as previously described.[15] Briefly, cell suspension (0.5 mL) and respective sample supernatant (0.5 mL) were added to Madin-Darby Canine Kidney cell mono layers, incubated (60 min, 37 °C, 5 % $CO_2$), washed with phosphate buffered saline (PBS) and incubated for ≥ 10 days with minimum essential medium (GibcoBRL) containing 1000 IU/mL penicillin (PISA), 1000 mg/mL streptomycin (PISA) and 250 mg/mL amphotericin *B* (Squibb). When cytopathic effect (CPE) was extensive, 5 mL of detached

cell suspension was centrifuged (200xg, 10 min), pellet resuspended in 5 mL PBS, spread onto immunofluorescence slides (15 µL) and airdried. Uninfected and infected cell controls were included. From CPE negative cultures a second successive culture was made. Cell smears were processed using immunofluorescence kit (Chemicon) according to provider's description.

### Differential cell counts

Cells obtained from nasal aspirates were resuspended in PBS ($10^6$ cells/mL). An aliquot of cells (50 µl) was subjected to cytocentrifugation (Wescor 7620), airdried and stained with Diff-Quick Stain kit (Dade Behring). Four hundred cells per cytospin were counted.

### CXCL5 in nasal aspirates

Concentration of CXCL5 in nasal aspirate was determined using a double antibody enzyme immuno-assay (EIA). Nasal aspirates were concentrated 2-fold and added in duplicate wells to EIA trays pre-coated with monoclonal anti-human CXCL5/ENA-78 antibody (R&D Systems). Secondary antibody was the biotinylated polyclonal anti-human CXCL5/ENA-78 (R&D Systems). Concentration of CXCL5 was calculated from a standard curve using recom-binant human CXCL5/ENA-78 (R&D Systems) (range 15.0-2000 pg/mL).

### Heparin-sepharose chromatography

Pooled nasal aspirate (20 mL) from 16 children with IV acute respiratory infection was concentrated using Amicon filters and run over a heparinsepharose affinity column (HiTrap: Pharmacia). Bound material was eluted using 2 M NaCl in equilibration buffer.

### Purification of neutrophil chemotactic activity from nasal aspirate

Eluted fractions were concentrated using Amicon filters, resuspended in 0.1% trifluoracetic acid (pH 3.0), and applied onto a reversed phase-(RP-8) column (9.4 × 250 mm,$C_8$ Nucleosil) in an HP 1100 High Performance Liquid Chromatography System (HPLC). Bound material was eluted at a flow rate of 3 mL/min using a linear gradient of acetonitrile containing 0.1 % trifluoroacetic acid. Absorbance was measured at 280 nm. Fractions were freezedried, redissolved in 0.1 % bovine serum albumin, and subsequently assayed for neutrophils chemotaxis.

### Neutrophils chemotaxis assay

Chemotaxis assays were performed in modified Boyden chambers (Neuroprobe). Neutrophils were purified from blood of healthy volunteers, by centrifugation onto Polymorphprep (Axis-Shield POC) and adjusted to $10^5$ cells/mL in RPMI-1640 medium. Samples, positive and negative controls (90 µl each) were added separately into lower compartments of chemotaxis chamber. A polycarbonate filter (Nucleopore 3 µm) separated upper and lower compartment. The chamber was firmly clamped. Upper compartments were filled with neutrophils suspension (100 µl) and chamber was incubated in humidified

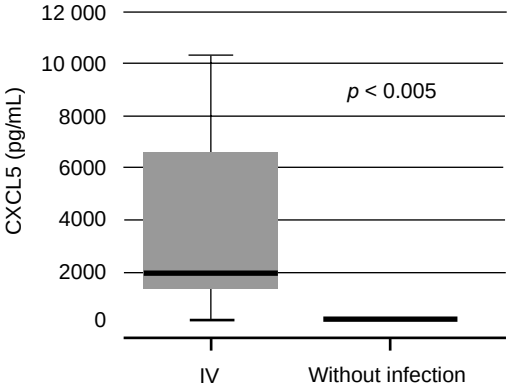


**Figure 1. Measurements of CXCL5 by EIA in nasal aspirate of children infected with influenza virus (IV) and non infected (without infection) (*n* = 18 )**

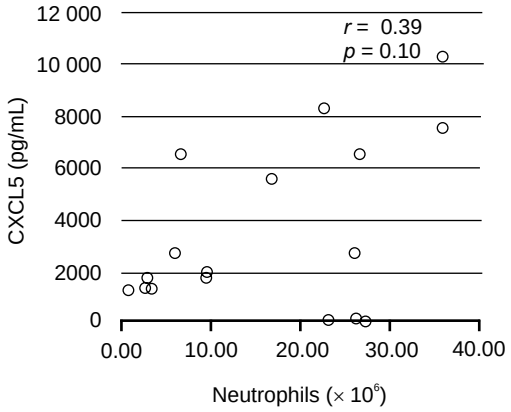


**Figure 2. Correlation between neutrophil numbers and levels of CXCL5 in nasal aspirate of influenza-infected children**

atmosphere (60 min, 37 ºC, 5 % $CO_2$). Attracted cells were lysed by adding 1 % Triton-X 100 (v/v) (10 µl) with subsequent incubation (10 min, 37º C). Samples were incubated with 0.01 M p-nitrophenyl-β-D-glucuronide (100 µl) in 0.1 M sodium acetate buffer (pH 4.0) for 18 h. Enzymatic reaction was stopped with 0.4 M glycine solution (pH 10.0) (200 µl) and p-nitrophenolate determined at 405 nm. Chemotactic activity is expressed as chemotactic index: stimulated migration/random migration.

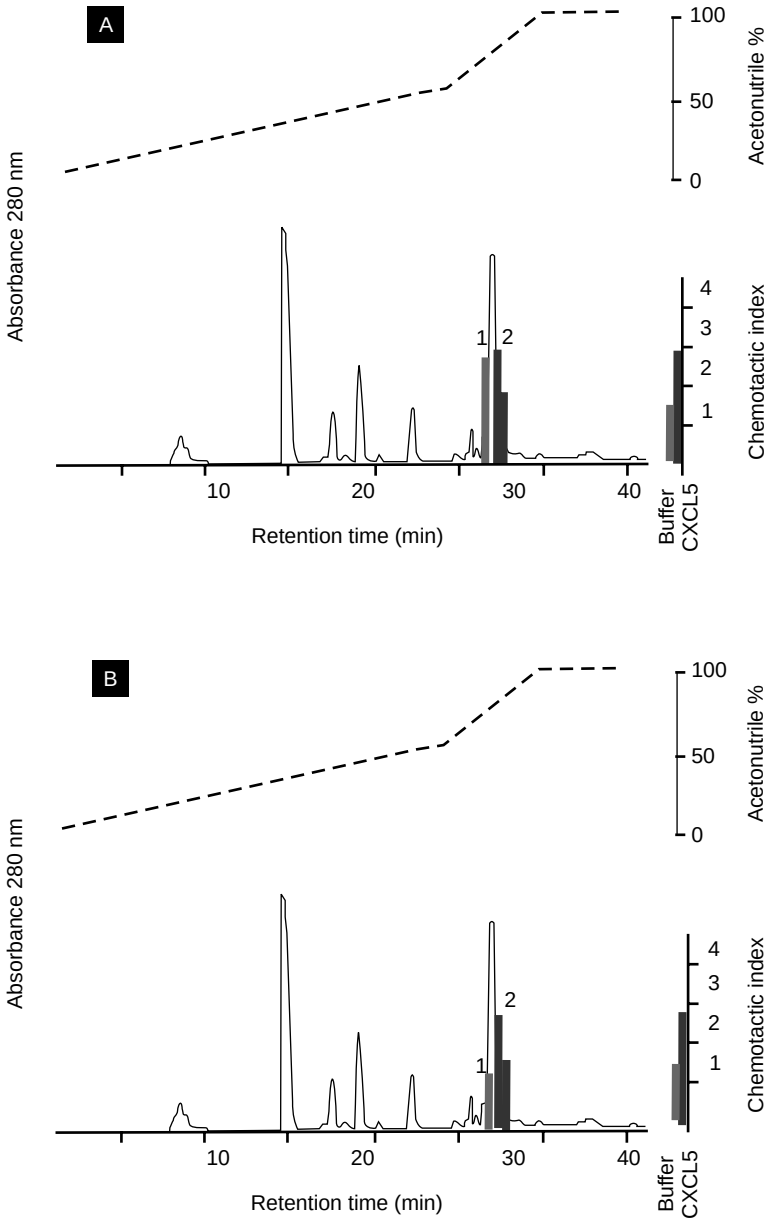


**Figure 3. Neutrophil chemotactic activity (NCA) in nasal aspirate of influenza-infected children. A) Separation of nasal proteins by reverse-phase HPLC chromatography shows two peaks (1 and 2) of NCA. B) Preincubation with anti-CXCL5 abolished peak 1 NCA. Neutrophil chemotaxis to individual fractions is given as a chemotactic index. Bars in the right side represent the neutrophilic chemotactic activity produced by buffer and recombinant CXCL5 and IL8**

### Statistical analysis

CXCL5 levels and neutrophils were analyzed using one-way ANOVA with Dunnett t-test as Post-Hoc analysis. Correlations between CXCL5 and cell counts were analyzed using Spearman's rank sum testing. A p-value < 0.05 was considered as statistically significant. Analysis of sample size using G-Power (version 3.0.10) showed that 18 influenza-infected subjects would have sufficient power (90 %) to test our hypothesis.

## Results

Eighteen children, mean age 10 ± 0.4 years, with respiratory symptoms were included. All children were infected with IV (16 IAV, 2 IBV). Median lower respiratory tract scores in the week before and after infection were 0.3 (0-1) and 2.3 (0.9-18) respectively. Inspection of the charts showed that only four children developed falls in peak expiratory flow followed by recovery to baseline over the following week.

### Differential cell counts in nasal aspirate

Total number of cells recovered from the influenza infected nasal aspirate was 86-fold higher when compared with control samples ($p < 0.05$) (table I). Neutrophils infiltrate upper airways most although monocytes/macrophages and lymphocytes were also increased in IV infected children compared with controls (table I). Neutrophil numbers derived from infected children were 150-fold when compared when they were asymptomatic. Cell comparison in nasal infected samples showed that neutrophils were 18 and 24-fold increased when compared with lymphocytes and macrophage numbers, respectively.

### CXCL5 concentration in nasal aspirate

EIA measurements of CXCL5 showed that this cytokine was increased significantly in nasal aspirate from influenza samples as compared with controls (1850 pg/mL *vs.* 30 pg/mL, $p < 0.005$) (figure 1). There was no significant correlation between neutrophil numbers and CXCL5 concentration of influenza -infected children ($r = 0.39$, $p = 0.10$) (figure 2).

### Biochemical and biological characterization of neutrophil chemotactic activity from nasal aspirate

In order to investigate whether the CXCL5 immunoreactivity detected in nasal aspirate exhibited biological activity, 16 samples were pooled and affinity purified followed by RP-8 chromatography. Analysis of eluted fractions in chemotaxis assay allowed identifying two peaks of neutrophil chemotactic activity (NCA). Neutralizing experiments in the assay showed that the first peak of NCA was largely due to the presence of CXCL5 as preincubation of the NCA with a neutralizing antibody to CXCL5 for 15 min abolished the NCA associated with this fraction. EIA measurements determined that this peak of NCA contained CXCL5 (20.5 ng/mL) (figure 3). No chemotactic activity was detected after similar processing in nasal aspirate from control samples (data not shown).

## Discussion

The present study has shown that IV infection induces a profound neutrophil infiltration of the upper respiratory tract, which is associated with the release of the neutrophil attractant CXCL5. Partial purified CXCL5 from nasal aspirate by HPLC showed biological activity on neutrophils suggesting this cytokine recruits neutrophils during naturally acquired influenza. Neutrophil recruitment hallmarks influenza infection. Studies in mice have shown that neutrophils are recruited into the airways already 8 h post-infection and their presence is associated with suppressed virus replication early after infection.[7,9] Neutrophils increase has been found in blood and sputum of IAV infected patients.[16,17] Myeloperoxidase measurements have shown elevated neutrophil numbers in nasal aspirate from asthmatic children with cold.[18] The present study has extended these observations: neutrophils are the main cells infiltrating the upper airway of children suffering influenza. Increased numbers of monocytes and lymphocytes were also found in nasal aspirate. However, their numbers were lower as compared with neutrophils (18-, 24-times) suggesting a prominent role of neutrophils in influenza illness.

This is the first report showing that CXCL5 is associated with influenza infection. CXCL5 levels were 60-fold in influenza-infected nasal aspirates as compared with concentrations of this chemokine in the samples obtained when children were healthy. Since neutrophil numbers paralleled with CXCL5 increase it might be that CXCL5 is involved in recruiting neutrophils during IAV infection. This is supported by stimulation of neutrophil migration in the chemotaxis assays due to partially purified nasal CXCL5. It is established that CXCL5 activates neutrophils via the CXCR2 receptor.[19] CXCR2 deficient mice failed to recruit neutrophils during IAV exposure,[20] which supports the involvement of CXCR2/CXCR5 axis in influenza illness *in vivo*.

CXCL5 levels and neutrophil numbers do not correlate significantly. However, also other neutrophil attractants e.g. interleukin 8 (IL8) are linked to IAV infection. Increased IL8 levels have been found in nasal washings and sputum of influenza infected patients.[18,21,22] IL8 activates neutrophils via CXCR1 and CXCR2, which explains alternative mechanisms of neutrophil recruitment.[23] The chemotaxis experiments identified additional neutrophil activity, which showed that other neutrophil attractants are released in IAV infection. IV triggers, in human blood dendritic cell, a chemokine secretion with three successive waves[24] with, CXCL1, CXCL2, CXCL3 and CXCL8, being released during the first two waves which occurred after 2 to 48 h. Thus, CXCL5 together with other neutrophil activating chemokines may recruit neutrophils into the upper airways infected with seasonal influenza *A*. While analyzing the data of the present study, the novel 2009 influenza *A* (H1N1) virus emerged in Mexico. Future studies will define the role of CXCL5 in the respiratory infection caused by this novel virus.

Airway epithelial cells, monocytes/macrophages, fibroblasts and neutrophils produce CXCL5.[14,15,25-27] However, the precise CXCL5 source in nasal aspirate from infected children of this study remains unidentified. Since airway epithelial cells are the gates for viruses in upper respiratory infections, it is tempting to speculate that these cells release CXCL5 during initial phase of infection. Later monocytes and fibroblasts may contribute to the inflammation. This is relevant, since some of these cells infected with rhinovirus release CXCL5.[15,26] Moreover, neutrophil defense induces CXCL5 production from airway epithelial cells suggesting that these cells contribute to recruit more neutrophils in the virus mediated inflammation.[27]

In summary, our data have shown that IV infection induces neutrophil recruitment and CXCL5 release into the upper airways of 10-year-old children. Nasal CXCL5 showed biological activity on neutrophils suggesting that it has impact in vivo. Altogether these data suggest that CXCL5 may contribute to neutrophil recruitment in IV infection.

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