

Local and Circulating Microchimerism Is Associated with Hypersensitivity Pneumonitis

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Rationale: Hypersensitivity pneumonitis (HP) is a lymphocytic alveolitis provoked by exposure to a variety of antigens. However, the disease occurs in only a subset of exposed individuals, suggesting that additional factors may be involved. Microchimerism has been implicated in the pathogenesis of autoimmune diseases, especially in those showing increased incidence after childbearing age.

Objectives: To evaluate the presence of circulating and local microchimeric cells in female patients with HP.

Methods: Male microchimerism was examined in 103 patients with HP, 30 with idiopathic pulmonary fibrosis (IPF), and 43 healthy women. All of them had given birth to at least one son, with no twin siblings, blood transfusions, or transplants. Microchimerism was examined by dot blot hybridization (peripheral blood), and by fluorescence *in situ* hybridization in bronchoalveolar lavage cells and lungs.

Measurements and Main Results: Blood microchimerism was found in 33% of the patients with HP in comparison with 10% in those with IPF ($p = 0.019$) and 16% in healthy women ($p = 0.045$). Patients with HP with microchimerism showed a significant reduction of diffusing capacity of carbon monoxide (DL_{CO} ; $53.5 \pm 11.9\%$ vs. $65.2 \pm 19.7\%$; $p = 0.02$) compared with patients with HP without microchimerism. In bronchoalveolar lavage cells, microchimerism was detected in 9 of 14 patients with HP compared with 2 of 10 patients with IPF ($p = 0.047$). Cell sorting revealed that microchimeric cells were either macrophages or $CD4^+$ or $CD8^+$ T cells. Male microchimeric cells were also found in the five HP lungs examined by fluorescence *in situ* hybridization.

Conclusions: Our findings (1) demonstrate that patients with HP exhibit increased frequency of fetal microchimerism, (2) confirm the multilineage capacity of microchimeric cells, and (3) suggest that microchimeric cells may increase the severity of the disease.

Keywords: allergic extrinsic alveolitis; hypersensitivity pneumonitis; microchimerism

Hypersensitivity pneumonitis (HP) is the result of immunologically induced inflammation of the lung parenchyma in response to inhalation exposure to a large variety of antigens (1). Importantly, HP occurs only in few exposed individuals, suggesting that other factors are involved in the development of the disease. However, the promoting factors that may play a role in triggering HP have not been elucidated, although genetic susceptibility associated with the major histocompatibility complex and viral infections have been implicated (2–4).

(Received in original form August 10, 2006; accepted in final form April 6, 2007)

Supported in part by Universidad Nacional Autónoma de México grant SDI.PTID.05.6. M.L.B. was funded by Consejo Nacional de Ciencia y Tecnología.

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This article has online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 176, pp 90–95, 2007

Originally Published in Press as DOI: 10.1164/rccm.200608-1129OC on April 12, 2007
Internet address: www.atsjournals.org

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Hypersensitivity pneumonitis occurs in few exposed individuals, suggesting that some promoting factors may be involved.

What This Study Adds to the Field

Patients with hypersensitivity pneumonitis exhibit an increased frequency of circulating and local microchimerism. Microchimeric cells may increase the severity of hypersensitivity pneumonitis.

Microchimerism is defined as the persistence of foreign cells in an individual (5). The most common source of microchimerism is pregnancy, and it is now known that fetal cells may persist in maternal circulation and tissues for many years (6). This event represents a widespread phenomenon, and the transference of fetal cells may start as early as 5 weeks gestation, as assessed by male DNA in the maternal circulation (7, 8). Other potential sources of microchimeric cells are blood transfusions, organ transplants, and the engraftment of cells from a twin that could occur early in pregnancy (9–12).

Fetal microchimeric cells have been implicated in the pathogenesis of some autoimmune diseases, especially in those that show increased incidence in women after childbearing age (5, 6, 13–20). For example, microchimeric cells have been identified in the peripheral blood and skin of patients with systemic sclerosis, a disease with a high incidence in females that frequently manifests after the childbearing years (21). However, the health consequences of persistent fetal cells in maternal tissues are still under debate.

In our 20-year experience, HP induced by avian antigen (pigeon breeder's disease) occurs mostly in women, with a female-to-male ratio of 9:1, and most of them develop the disease after childbearing age (22, 23). Although microchimerism has been mostly associated with autoimmune disorders, several studies have indicated a possible association with nonautoimmune diseases, including polymorphic eruptions of pregnancy, infectious hepatitis, and nonautoimmune thyroid disorders (5). Recently, the presence of chimeric, maternally derived cells was reported in pityriasis lichenoides, a cytotoxic, T-cell-mediated skin disorder of unknown etiology, although infectious agents have long been suspected as etiologic factors (24, 25).

On the other hand, recent evidence suggests that alloreactive $CD8^+$ T cells are generated frequently after normal pregnancy and retain functional capability for years after pregnancy (26). Also, in the affected skin of patients with scleroderma, most of the chimeric cells are related to the immune response, including antigen-presenting cells, $CD8^+$ T cells, and B lymphocytes (28).

All of these cell subsets play an important role in the exaggerated immune response that characterizes HP (1, 27).

In this context, the current study was designed to investigate the frequency of male fetal microchimerism in peripheral blood, bronchoalveolar lavage (BAL), and lung tissues of female patients with HP, and compare them with those from female patients with idiopathic pulmonary fibrosis (IPF) and healthy women. Preliminary results of this study were previously reported in an abstract (29).

METHODS

Additional details are provided in the online supplement.

We studied 176 women who had previously given birth to at least one son, with no twin siblings, blood transfusions, or transplants: 43 healthy women (45.7 ± 9.1 yr [mean \pm SD]), 30 patients with IPF (60.7 ± 12 yr), and 103 patients with HP (48.8 ± 12.8 yr). Diagnosis of HP and IPF was based on established criteria (27, 30–32). Lung biopsy for morphologic evaluation was performed in 46% of the patients with HP and in 36% of the patients with IPF.

Patients with IPF had delivered more sons (3.1 ± 1.9 vs. 2.3 ± 1.1 and 1.4 ± 0.7 for the patients with HP and healthy female control groups, respectively; $p < 0.01$). Also women with HP had had an increased number of sons when compared with healthy women ($p < 0.01$). To explore possible clinical differences between patients with HP with and without microchimerism, clinical, functional, and BAL data were extracted from case records. The study was approved by the ethics committee of the National Institute of Respiratory Diseases, and informed, written consent was obtained from each subject.

BAL

BAL was performed as previously described (30). Cell aliquots were resuspended in fetal bovine serum/dimethyl sulfoxide and frozen in liquid nitrogen until use.

Microchimerism Assay

Genomic DNA was obtained from peripheral blood and BAL cells using the BDtract Genomic DNA isolation kit (Maxim Biotech, San Francisco, CA). The probe was made by amplifying the 198-bp testis-specific protein Y-encoded (TSPY) sequence from male DNA, and the product was analyzed by agarose gel electrophoresis, cut from the gel under ultraviolet light, and recovered using a DNA gel extraction kit (Millipore Corporation, Bedford, MA). The polymerase chain reaction (PCR) product was revealed using a digoxigenin DNA labeling and detection kit (Roche Molecular Biochemical, Mannheim, Germany). The product was sequenced as previously described (33), and the sequence results were analyzed by GeneBank Search (Bethesda, MD).

Male microchimerism was tested using nested PCR to amplify a sequence of the TSPY gene (34–36). Results were confirmed by direct random sequencing of some of the amplification products, showing that no false-positive results due to mispriming or nonspecific amplification products were present.

Dot Blot Hybridization

The nested PCR products were heat denatured, spotted on positively charged nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), hybridized with 25 ng/ml of the TSPY probe at 54°C overnight, and detected using digoxigenin DNA labeling and detection kit (Roche Molecular Biochemical). Each sample was run in four independent experiments, and we considered “positive” only those cases in which the test was positive at least twice. Patients showing one positive and three negative results were classified as “negative.”

Fluorescence *In Situ* Hybridization

Male microchimerism in BAL cells was also tested by fluorescence *in situ* hybridization (FISH) using fluorescein-labeled X chromosome probe (DXZ1) and rhodamine-labeled Y chromosome probe (DYZ1) (Cytocell, Cambridge, UK), as previously described (37). In addition, BAL CD3⁺CD4⁺ and CD3⁺CD8⁺ T lymphocytes, and CD14⁺CD3⁻ alveolar macrophages were separated by sterile cell sorting in a flow

cytometer (30) (FACSAria; Becton Dickinson, San Jose, CA) and also assayed for FISH.

Male Microchimerism in Lung Biopsy Specimens by FISH

Lung tissue sections from five patients with HP and three patients with IPF were cut to a thickness of 5 μ m, and FISH was performed as described by Johnson and colleagues (38) using the XY dual-labeled probe (Cytocell). Hybridized slides were examined on an Olympus BX40 fluorescence microscope (Olympus, Tokyo, Japan) using triple band filters (4',6'-diamidino-2-phenylindole, fluorescein, Texas red), double band filters (fluorescein, Texas red), and single filters for fluorescein and Texas red. Male cells showed two different colored fluorescent signals: a green dot for the X chromosome, and a reddish-orange dot for the Y chromosome. Slides were counterstained with hematoxylin and eosin to evaluate the localization of the microchimeric cells.

Statistical Analysis

Interval variables were analyzed using Student's *t* test, whereas Fisher's exact test was used for categorical variables. The Bonferroni correction was applied for multiple comparisons when appropriate. We considered a final two-tailed *p* value of 0.05 or lower as statistically significant. Multivariate logistic regression analysis (backward approach), with microchimerism as the dependent variable, and diagnosis and age as independent variables, was also performed using SPSS, version 10.0, software (SPSS, Inc., Chicago, IL).

RESULTS

Baseline Characteristics of Patients with IPF and Those with HP

All patients exhibited clinical, radiologic, and functional evidence of interstitial lung disease, with decreased lung volumes, and hypoxemia at rest that worsened during exercise. In the HP group, differential cell count in BAL fluids was characterized by a marked lymphocytosis, whereas, in IPF, most BAL inflammatory cells were macrophages, with a moderate increase of neutrophils and eosinophils (data not shown).

Female Patients with HP Show Increased Frequency of Male Microchimerism in Peripheral Blood

The TSPY sequence was amplified from peripheral blood cell samples, and the PCR products were hybridized to the TSPY specific probe. The frequency of Y chromosome microchimerism was significantly higher in the HP group (34/103 patients [33%]) as compared with either the IPF group (3/30 [10%]; $p = 0.019$, Fisher exact test) or healthy control subjects (7/43 [16%]; $p = 0.045$, Fisher exact test). The multivariate logistic regression analysis ruled out the influence of age, and corroborated that patients with HP had a higher probability of presenting microchimerism than subjects from the other two groups (odds ratio, 3.1; 95% confidence interval, 1.4–6.8). False-positive results were discarded by sequencing the TSPY-specific probe, which had 98% sequence identity with the TSPY human sequence unique to the Y chromosome. Some PCR products were also randomly sequenced to verify that the amplification product was TSPY. The sensitivity of the test was determined by hybridization of the PCR amplification products, from serial dilutions of male DNA to aliquots of female DNA with the TSPY-specific probe. The equivalent of 1 male cell in 10,000 female cells could be detected.

Patients with HP with Microchimerism Showed Decreased Diffusing Capacity of Carbon Monoxide Compared with Patients with HP without Microchimerism

Demographic data, pulmonary function test results, and BAL differential cell counts from patients with HP with and without

TABLE 1. BASELINE DEMOGRAPHIC, CLINICAL, PHYSIOLOGIC, AND BRONCHOALVEOLAR LAVAGE CHARACTERISTICS OF PATIENTS WITH HYPERSENSITIVITY PNEUMONITIS WITH AND WITHOUT MICROCHIMERISM

Variable	With Microchimerism (n = 34)	Without Microchimerism (n = 69)	p Value*
Age, yr	48.3 ± 12.5	49.0 ± 13	NS
Duration of symptoms before diagnosis, mo	28.1 ± 22.0	31.1 ± 23.6	NS
FVC, % predicted	53.8 ± 15.4	55.5 ± 15.8	NS
PaO ₂ , mm Hg [†]	50.8 ± 7.1	50.2 ± 11.0	NS
DL _{CO} , %	53.5 ± 11.9	65.2 ± 19.7	0.02
SaO ₂ rest	85.1 ± 6.6	84.6 ± 9.3	NS
SaO ₂ exercise	71.3 ± 9.8	74.2 ± 9.6	NS
BAL macrophages, %	34.0 ± 17.5	44.0 ± 16.2	NS
BAL lymphocytes, %	64.3 ± 16.7	54.8 ± 16.3	0.07
BAL neutrophils, %	1.1 ± 2.0	1.2 ± 3.1	NS
BAL eosinophils, %	0.8 ± 1.0	1.2 ± 2.4	NS

Definition of abbreviations: BAL = bronchoalveolar lavage; NS = nonsignificant.

* p Value corrected for multiple comparisons.

[†] Normal values at Mexico City altitude: 67 ± 3 mm Hg.

male microchimerism are summarized in Table 1. Patients with HP positive for microchimerism displayed a reduced diffusing capacity of carbon monoxide (DL_{CO}; 53.5 ± 11.9% vs. 65.2 ± 19.7%; p = 0.02), and a marginal but nonsignificant increase of BAL lymphocytes in the differential cell counts (64.3 ± 16.7% vs. 54.8 ± 16.3%; p = 0.07). No differences were found in the remainder variables.

Microchimerism in BAL Cells

To ascertain whether circulating male microchimeric cells enter into the lungs, we amplified a TSPY sequence from DNA extracted from BAL cells from 14 patients with HP and 10 patients with IPF, and the PCR products were hybridized to the TSPY-specific probe. We found that 9 of 14 (64%) of the patients with HP were positive compared with only 2 (20%) of the patients with IPF (p = 0.047, Fisher exact test). The repeatability of the results was verified by testing the BAL samples four times. Results were considered positive if at least two experiments displayed a positive signal. As detected in blood measurements, the sensitivity of the test was 1 male cell in 10,000 female cells.

To confirm the presence of male cells in BAL samples, we used FISH to examine 15,000 nuclei from BAL cells of 6 patients with HP. Male cells were observed in all of these samples (Figure 1), and the ratio of XY cells to XX cells ranged from 1/6,260 to 1/15,000.

To characterize the phenotype of BAL microchimeric cells, samples from seven patients with HP were separated by cell sorting in a flow cytometer and then examined by FISH. Microchimeric male cells were detected in macrophages and in CD4⁺ and CD8⁺ T lymphocyte subpopulations (Figure 2). In three cases, microchimerism was identified in more than one cell type (e.g., macrophages and CD8⁺ T cells or macrophages and CD4⁺ T cells).

Microchimerism in HP Lungs

FISH analysis of X- and Y-chromosome-specific DNA sequences was also performed in five paraffin-embedded tissue sections from patients with HP that showed circulating microchimeric cells. These patients were different from those in whom we evaluated BAL cells. In all these samples, the presence of microchimeric cells was revealed. Figure 3 illustrates three different cases demonstrating the presence of male cells, with reddish-

orange and green fluorescent signals in the nuclei, usually observed as single cells surrounded by female cells (two green signals) within the architecture of the affected tissue. Cells containing the X and Y chromosomes and, therefore, being of male origin were not found to occur in clusters. When these lung samples were counterstained to identify the localization of the microchimeric cells, these cells were observed in interstitial inflammatory areas, as exemplified in Figure 3, panels C1 and C2. In one case, the microchimeric cell seemed to correspond to a bronchiolar epithelial cell (panel C3). By contrast, microchimeric cells were not found in IPF lungs (see Figure E1 in the online supplement).

DISCUSSION

The incidence and prevalence of HP in the general population are essentially unknown. However, only a small percentage of individuals exposed to HP-related antigens seem to develop the disease. This observation strongly suggests that HP is probably the result of a double-strike process, but an unambiguous promoting factor has not been identified. Some evidence supports the notion that genetic susceptibility associated with HLA complex or respiratory viral infections may be implicated (2–4). Also, gene polymorphisms in the tumor necrosis factor- α promoter have been associated with increased susceptibility (2, 39). However, how antigen exposure, environment, and genetics interact to induce HP is unknown.

It is now recognized that the placenta is not a strict barrier to cell traffic between the mother and fetus, and that fetal cells have the potential to persist long term in the maternal organism (40–43). The fetal cells transferred into maternal blood carry a complement of genes that are only haploidentical with the resident host, and, importantly, they are fetal stem cells that may engraft in marrow, where they may remain throughout life (44).

Because the clinical similarities between graft-versus-host disease after allogeneic hematopoietic stem cell transplantation (iatrogenic chimerism) and some autoimmune diseases, it has been hypothesized that microchimerism may be implicated in the pathogenesis of these disorders. In this context, a growing body of evidence suggests that microchimerism may play a role mainly in systemic sclerosis, although a mechanism by which these cells might contribute to the pathogenesis of the disease is still unknown (22). Similarly, women with other autoimmune diseases (i.e., Sjögren syndrome and Hashimoto thyroiditis) showed higher rates of male microchimeric cells than control subjects (15, 18, 45).

The incidence of HP in Mexico is in great excess in females compared with males (22, 23, 27), and the disease frequently manifests after the childbearing years. Therefore, in the present study, we aimed to examine the presence and possible influence of fetal cells on this disorder, initially seeking male cells in blood samples from female patients.

Our results show that one third of the female patients with HP had male cells in peripheral blood, which was significantly higher compared with patients with IPF and healthy women. All the studied women had previously given birth to sons, and, because we excluded other potential sources of microchimerism, the origin of the male cells was likely to be fetal. Because patients with IPF were roughly 10 years older than those in the HP and control groups, and had the lower percentage of microchimeric cells, we examined the putative effect of age. Our results show that the differences in microchimerism were not influenced by age. Likewise, our results suggest that the number of sons does not have an effect on microchimerism, as patients with IPF have the higher number and the lower percentage of microchimerism. This finding is in agreement with other reports, showing that

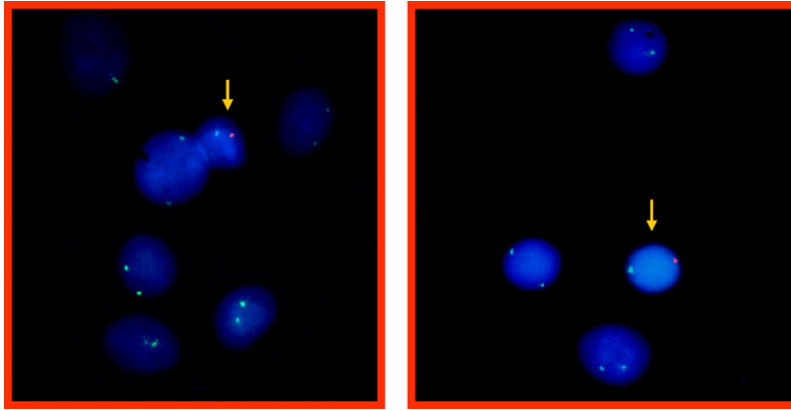
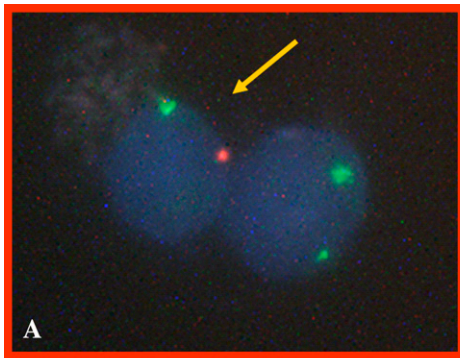
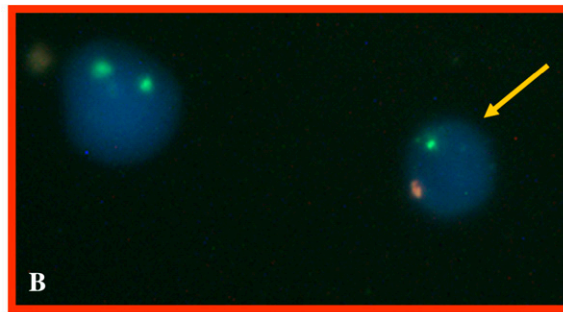


Figure 1. Detection of fetal male cells by fluorescence *in situ* hybridization in bronchoalveolar lavage samples from two patients with hypersensitivity pneumonitis. Male cells are recognized by the presence of the X (green) and Y (reddish-orange) chromosomes (arrows). Cell nuclei are identified with 4',6'-diamidino-2-phenylindole (DAPI; blue). Original magnification: $\times 100$.



Macrophages



CD4+ T-cells

Figure 2. Detection of male microchimerism in bronchoalveolar lavage cells sorted by high-speed flow cytometer in two patients with hypersensitivity pneumonitis. Two microchimeric cells found in the alveolar macrophages and in the CD4 T-cell subpopulations are indicated by arrows. Original magnification: $\times 100$.

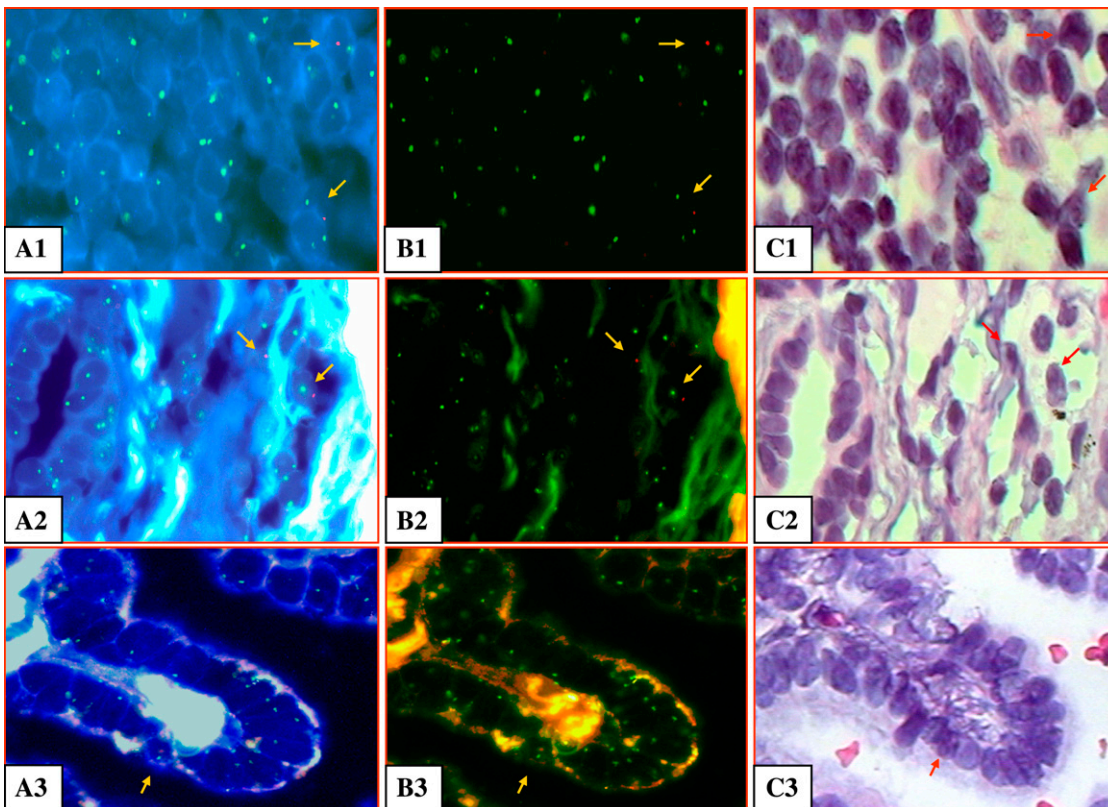


Figure 3. FISH for centromeric regions of chromosomes X (green) and Y (reddish-orange signal) in lung tissues from three different patients with hypersensitivity pneumonitis. Cell nuclei are identified with DAPI (blue). (A) Filter used: triple band (DAPI–fluorescein–Texas red). (B) Same field and technique as in (A), using double-band filter (fluorescein–Texas red). (C) Light microscopy of the same tissue sections as in (A) and (B) counterstained with hematoxylin and eosin. Original magnification: $\times 100$. Arrows indicate the fetal male cells with chromosomes X and Y surrounded by XX bearing maternal cells in the inflammatory infiltrate (A1 and A2) and in the bronchiolar epithelium (A3).

there is no correlation between male microchimerism and the number of sons each subject had delivered or the time elapsed since the birth of the most recent son (46, 47).

However, the demonstration of microchimeric cells in the peripheral blood is not enough to attribute them with a role in the lung disease. Therefore, after our initial finding, we assessed the presence of male cells in the affected tissues using BAL cells and lung tissues from patients with HP and those with IPF. In these BAL samples, we found that two-thirds of patients with HP showed male cells, whereas most patients with IPF were negative. Furthermore, we also demonstrated the presence of male cells in lung tissues from another subgroup of patients with HP, indicating that exogenous male fetal cells migrate from the peripheral circulation into the lung of women affected by this disease, and that local microchimerism might be a relatively common phenomenon in HP. By contrast, no microchimeric cells were found in IPF lungs.

To our knowledge, this is the first demonstration of microchimerism in HP, although the finding of microchimerism in the affected lung tissue from some patients with systemic lupus erythematosus (48) and systemic sclerosis (49) has previously been noted.

The putative role of these microchimeric fetal cells in the HP lungs is presently unknown. In general, the functional relevance of microchimerism in health and disease remains poorly understood (5). The observation that these cells are also detected in the peripheral blood of healthy women raises the question of whether microchimeric fetal cells are really participating in the pathologic events, or are simply bystanders, or even whether they have beneficial effects for the host, as has been suggested (7, 22). Although studies on autoimmune diseases have been primarily focused on the effector arm of the immune system, increasing evidence indicates that microchimeric cells may differentiate into many lineages, raising additional possible roles for these cells. Thus, it has been indicated that microchimeric cells may develop from progenitor cells into parenchymal cells, and replace damaged host cells after tissue injury (5, 50). In addition, because the number of microchimeric cells is very low, other mechanisms may participate. For example, an exaggerated immune reaction may occur by indirect antigen presentation, a mechanism believed to be involved in chronic organ rejection. However, the precise role of microchimeric cells in tissue (e.g., reflecting a repair process or participating as a pathogenic mechanism) cannot be proven in human tissues that are usually examined at one point in time.

In our study, the finding that patients with HP with microchimerism showed a more severe reduction of DL_{CO} and a tendency to have more BAL lymphocytes suggests that a relationship between microchimerism and the inflammatory response, or a worse clinical course, may exist. However, these putative associations deserve further corroboration.

Microchimeric male cells, bearing epithelial, leukocyte, T-lymphocyte, and even mesenchymal stem cells markers, have been detected in blood and in a variety of maternal tissue specimens, suggesting that the fetal cells have multilineage capacity (14, 42, 44, 51–54). In a recent study performed in healthy women, microchimerism was found in lymphocytes, monocyte/macrophages, and even natural killer cells (55). In our study, using cell sorting, we determined that BAL microchimeric cells were macrophages and T lymphocytes (either $CD4^+$ or $CD8^+$ T cells), suggesting that they might be participating in the exaggerated immune response that characterizes this disease. In HP lungs, fetal male cells were found in inflammatory interstitial lesions, likely macrophages and lymphocytes, and in one case in the lung epithelium. These findings corroborate the multilineage

capacity of the microchimeric cells, and again raise the question of whether they are participating in tissue damage or repair.

In summary, we provide the first evidence for the increased frequency and the presence in the lung of fetal male cells in female patients with HP. The precise role of microchimeric cells in this disorder has yet to be elucidated, and warrants further research. However, our findings suggest that microchimeric cells may increase the severity of the disease. Further investigation will be necessary to elucidate whether there is a potential link between microchimerism and disease pathogenesis. The functional characterization of lung microchimeric cells could provide additional clues about their role in HP.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Silvia Sanchez and Bertha Molina for their technical assistance in the studies involving *in situ* hybridization.

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