International Journal of Hygiene and Environmental Health 217 (2014) 586–591

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

Lymphocyte activation in silica-exposed workers

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ARTICLE INFO

Article history:
Received 11 June 2013
Received in revised form
17 November 2013
Accepted 19 November 2013

Keywords:
Lymphocytes
Soluble IL-2 receptor alpha
Interleukin-2
Silica
Cytokines
Autoimmunity

ABSTRACT

Exposure to silica dust has been examined as a possible risk factor for autoimmune diseases, including systemic sclerosis, rheumatoid arthritis, systemic lupus erythematosus and ANCA-associated vasculitis. However, the underlying cellular and molecular mechanisms resulting in the increased prevalence of autoimmunity remain elusive. To clarify these mechanisms, we studied various markers of immune activation in individuals occupationally exposed to silica dust, i.e., serum levels of soluble IL-2 receptor (sIL-2R), levels of IL-2, other pro- and anti-inflammatory cytokines and lymphoproliferation. Our results demonstrate that silica-exposed individuals present important alterations in their immune response when compared to controls, as shown by increased serum sIL-2R levels, decreased production of IL-2 and increased levels of the pro-inflammatory (IFN-γ, IL-1α, TNF-α, IL-6) as well as anti-inflammatory (IL-10 and TGF-β) cytokines. Furthermore, silica-exposed individuals presented enhanced lymphoproliferative responses. Our findings provide evidence that the maintenance of immune homeostasis may be disturbed in silica-exposed individuals, possibly resulting in autoimmune disorders.

Introduction

Studies demonstrate that silica can act as an adjuvant to nonspecifically enhance the immune response. Many cases of autoimmune diseases in silica-exposed individuals have been identified in patients with silicosis and the risk of developing clinical connective tissue diseases has been reported to be greater among individuals with either silicosis or silica exposure (Rosenman and Moore-Fuller, 1999). In fact, silica dust exposure may be associated with a wide range of autoimmune diseases and abnormalities (as reviewed by Parks et al., 1999). However, the underlying mechanisms resulting in increased prevalence of autoimmunity remain elusive (Cohen Tervaert et al., 1998; Steenland and Goldsmith, 1995).

Peripheral mechanisms of self tolerance and immune homeostasis are essential to control self-reactive cells and the loss or reduction of these mechanisms may result in autoimmune disease. In particular, leukocytes may play a role in the generation of autoimmune diseases in silica-exposed individuals based on their involvement in the processing and presentation of silica particles (Pernis, 2005). Stimulation of lymphocytes is observed in human and experimental silicosis, as demonstrated by increased levels of autoantibodies (Conrad and Mehlhorn, 2000; Subra et al., 2001), immunoglobulins and immune complexes (Doll et al., 1981). Furthermore, silica was shown to stimulate the polyclonal activation of human T cells in vitro (Ueki et al., 1994). After activation, both T and B lymphocytes express in their membranes specific receptors for interleukin (IL)-2 (Loughnan et al., 1988). This membrane receptor is important for cell stimulation with interleukin-2 (IL-2) (O'Shea et al., 2002), a potent T cell growth factor. Besides the membrane IL-2 receptor expression, the release of these receptors in a soluble form takes place after leukocyte stimulation and the soluble alpha subunit of IL-2 receptor (sIL-2R) appears to possess

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http://dx.doi.org/10.1016/j.ijheh.2013.11.002

the best diagnostic value in a number of diseases associated with T-cell stimulation (Stegeman et al., 1993; Witkowska, 2005; Grutters et al., 2003; Kitaoka et al., 2003; Murakami, 2004).

Dysregulation of cytokine production or action is thought to have a central role in the development of autoimmunity and autoimmune disease (O'Shea et al., 2002). The pro-inflammatory cytokines are regarded to be pathogenic, whereas the anti-inflammatory cytokines are considered to be protective (Feldmann and Maini, 2001; O'Shea et al., 2002; Trinchieri, 2003; Kim and Moudgil, 2008) in autoimmune diseases such as multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), and rheumatoid arthritis (RA) (Kim and Moudgil, 2008).

To clarify the mechanisms by which silica exposure may disrupt immune tolerance and subsequently predisposing silica-exposed individuals to autoimmune processes, we evaluated in the present study various markers of immune activation, focused on the IL-2–IL-2R pathway, in individuals occupationally exposed to silica dust. Aiming to clarify the association between silica exposure and autoimmunity, the presence of silicosis in the population studied was not required.

Materials and methods

Study population

Brazilian silica-exposed workers ($n = 103$) and unrelated non-exposed, age-, sex- and ethnically-matched individuals ($n = 33$) were studied. All exposed individuals were recruited from the Ambulatory of Occupational Medicine of the Clinical Hospital of the State University of Campinas (UNICAMP, Brazil). The present study was approved by the ethics committee of the Faculty of Medical Sciences-UNICAMP, Campinas, Brazil in accordance with the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects and a written informed consent was taken from all the study subjects.

The individuals studied are workers (21 women and 82 men; 57 ± 11 years old) occupationally exposed to respirable crystalline silica dust for 18 ± 9 years, mainly: workers of ceramic industries specially in the production of sanitary ware, but also some miners, stonecutters, rock drillers as well as metallurgic, foundry and quarry workers. The examined workers in this study did not protect themselves appropriately and did not give special attention to their workplace. The ventilation of these workplaces was rather poor and the mean respirable mass concentration was quite high. *Inclusion criteria*: retired workers exposed to silica (59 silicotic and 44 non-silicotic individuals); silica exposition was confirmed by a detailed and reliable occupational history based in: (1) employment papers: the registration of the professional on the books of the company; (2) the type of production and the period of exposure in the company: production of sanitary ware whose main raw material is silica (ceramic/porcelain) during several years in an workplace with poor conditions without effective actions regarding occupational hygiene; (3) epidemiological history: epidemiological data about numerous cases of silicosis among workers in the same company. The diagnosis of silicosis was based on the confirmation of exposure accompanied by the presence of pulmonary parenchyma alterations according to the International Labor Office (ILO) classification (ILO, 2000). *Exclusion criteria*: workers (a) exposed concomitantly to silica dust and other types of dust that might interfere in the immune response as asbestos, cobalt, beryllium, nickel, chromium, etc.; (b) under treatment for infectious diseases as tuberculosis, paracoccidioidomicosis or other pulmonary mycosis; (c) under treatment with immunosuppressive drugs. The control group consisted of non-related, non-exposed, age- (54 ± 10 years old), sex- and ethnically-matched healthy volunteers, which were not smoking, drinking alcohol, or using medicines, during the period of blood collection as well as not presenting renal and pulmonary diseases.

Sera and PBMCs isolation

Peripheral blood was obtained from both silica-exposed and control individuals. The serum was separated from coagulated blood for sIL-2R analysis; sera were frozen at $-80\,^{\circ}C$ until use. Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood by using a Ficoll–Hypaque gradient separation (density = 1.070 g/mL) and washed three times with Hanks' balanced salt solution medium before determination of viability by the trypan blue dye exclusion method. Next, cells resuspended in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated pooled human AB serum, 2 mM L-glutamine, 12.5 mM HEPES [pH 7.4], 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 0.2% $NaHCO_3$ and 1% penicillin–streptomycin [10,000 U/mL]) were adjusted to 1×10^6 cells/mL.

Soluble IL-2 receptor (sIL-2R)

The serum levels of sIL-2R were analyzed in commercially available Diaclone ELISA kits (Sanquin, Amsterdam, The Netherlands). Serum samples were diluted fivefold and analyzed in duplo. CV values of duplicate analyses were < 20%, otherwise the samples were reanalyzed. Concentrations were calculated from a 6 point standard curve ranging from 69 to 2200 pg/mL, taking into account the dilution factor. Concentrated samples (>2200 pg/mL) were diluted further as required.

Lymphoproliferation by the measurement of tritiated [^{3}H]-thymidine incorporation

PBMCs of each studied individual were cultured in triplicate for 3 days (72 h) at $37\,^{\circ}C$ in a 5% CO_2 incubator. Phytohemagglutinin (PHA, 1 μg/mL, DIFCO, Detroit, USA) mitogen was used as stimulant. Nonstimulated control wells contained just PBMCs in complete RPMI culture medium. At 18 h before harvest, the cultures in each well were pulsed with 1.0 μCi of ^{3}H-thymidine (Amersham GE Healthcare Life Sciences, UK); after the 18 h incubation, the cells were harvested on glass fiber filters (Cambridge Technology, USA) with a 96-well automatic cell harvester (Cell Harvester model 200 A, Cambridge Technology, USA). Radioactivity was measured by a scintillation counter (Beckman LS 6000 – Liquid Scintillation System). Data were expressed as the net counts (counts per minute for stimulated wells minus counts per minute for unstimulated control cultures).

Quantification of cytokines

Cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-10 and TGF-β) were measured in the PBMCs culture supernatant obtained after 48 h incubation in RPMI medium by using the Beadlyte® Human 22-Plex Multi-Cytokine Detection System (Upstate, Temecula, CA, USA) and performed according to the manufacturer's instructions with all kit reagents and assay wash buffers. Briefly, each sample was incubated with antibody-coated capture beads, then labeled with biotin-anti-human cytokine antibodies, followed by incubation with streptavidin-phycoerythrin. Beads were analyzed using Luminex 200™ (Luminex, Austin, TX, USA). Cytokine titers were expressed as pg/mL, calculated by reference to standard curves constructed with known amounts of recombinant cytokines.