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Immunoglobulin Responses to Experimental Silicosis

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Silicosis is a crippling fibrotic lung disease induced by inhalation of crystalline silica. One feature of silicosis is systemic and pulmonary immune dysfunction characterized in part by elevations in serum and bronchoalveolar lavage (BAL) immunoglobulins. A major specific aim of the current report was to demonstrate that an experimental model of silicosis previously well characterized for the development of pulmonary inflammation and fibrosis would also exhibit increased levels of serum and BAL IgG and IgM similar to those of human silicosis. We also sought to document the anatomic compartments responsible for these immunoglobulin responses. To address these specific aims, we compared levels of IgG and IgM in serum and BAL from rats with experimental silicosis induced by inhalation of silica with levels of these immunoglobulins in titanium dioxide (TiO_2)- and sham (air)-exposed controls. The ability of mononuclear cell populations from lung, lung-associated lymph node, and spleen to produce IgG and IgM *ex vivo* were also compared. We found that experimental silicosis was associated with elevated IgG and IgM levels in blood and BAL relative to the control groups. Our findings also suggested that draining lung-associated lymph nodes (LALN) were the most important sites for increased IgG and IgM production in experimental silicosis, with lungs contributing to a lesser degree. Increased production in the LALN appeared related to marked expansion in total numbers, but not relative proportion, of B lymphocytes.

Key Words: **silicosis; quartz; titanium dioxide; IgG; IgM; bronchoalveolar lavage; lymphocyte; rat.**

Silicosis is a crippling lung disease induced by inhalation of crystalline silica. Although several silicates induce silicosis, alpha quartz (a type of crystalline silica) is the major cause of silicosis worldwide (Silicosis and Silicate Disease Committee, 1988). Miners working in the metal and coal mining industries (including surface coal miners), as well as workers in a variety of other occupations such as sandblasting, silica flour production, or any other occupation with potential for inhalation of dust high in silica content, are at increased risk for the subsequent development of silicosis (American Thoracic Society, 1997; Weissman and Banks, 1998). Chronic silicosis develops over a period of years, as retained intrapulmonary dust induces a sequence of events including inflammation, fibrogenesis, and, ultimately, end-stage pulmonary fibrosis (Adamson, 1992; Velan *et al*., 1993; Weissman and Banks, 1998). Stimulation of macrophages by silica and subsequent cytokine networking between macrophages, lymphocytes, neutrophils, fibroblasts, and potentially other cell types is an important mechanism for fibroblast stimulation and the development of pulmonary fibrosis (Davis, 1986; Li *et al*., 1992; Sjostrand *et al*., 1991; Vanhee *et al*., 1995).

Another result of silicosis not necessarily related to fibrosis is immune dysfunction, both systemically and in the lung (American Thoracic Society, 1997; Weissman and Banks, 1998; Davis, 1986). Clinical manifestations of immunologic dysfunction associated with chronic silicosis include autoimmune diseases such as progressive systemic sclerosis and increased susceptibility to pulmonary mycobacterial infections such as tuberculosis. A particularly striking immunological feature of individuals with histories of silica exposure or silicosis is increased immunoglobulin levels both in blood and at the lung level (Doll *et al*., 1981; Idel *et al*., 1990; Karnik *et al*., 1990; Nigam *et al*., 1990; Nagaoka *et al*., 1993). Increased serum IgG and IgM concentrations have been reported in sandblasters (Doll *et al*., 1981); increased serum IgG and IgA concentrations in silicotic stone masons (Idel *et al*., 1990); increased serum IgG, IgM, and IgA concentrations in slate-pencil workers (Karnik *et al*., 1990); increased IgG in quartz crushers (Nigam *et al*., 1990); and increased IgG, IgA, and IgE in silicotic miners (Nagaoka *et al*., 1993). In general, IgG is the antibody class most affected by silica exposure or silicosis (Doll *et al*., 1981; Idel *et al*., 1990; Karnik *et al*., 1990; Nigam *et al*., 1990; Nagaoka *et al*., 1993). Bronchoalveolar lavage (BAL) data demonstrate increases in immunoglobulin levels and probably immunoglobulin production in the lungs of silica-exposed nonsmokers and nonsmoking silicotics (Calhoun *et al*., 1986; Lusuardi *et al*., 1990). In BAL obtained from nonsmoking granite workers, IgG, IgM, and IgA concentrations, as well as ratios of these concentrations to BAL albumin, are reported to be increased relative to nonindustrial control sub-

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jects (Calhoun *et al*., 1986). In another report, nonsmoking silicotics' BAL IgG/albumin, but not IgA/albumin, ratios were found to be greater than those of controls. Smoking was noted as an important confounding factor in this study, as it obliterated a number of differences between silicotic and control BAL (Lusuardi *et al*., 1990).

Based on these considerations, we hypothesized that inhalation of crystalline silica initiates a sequence of events leading not only to fibrosis, but also to immune dysfunction. Furthermore, we hypothesized that better characterization of immunoglobulin responses to silicosis would help to elucidate mechanisms underlying other manifestations of silicosis-associated immune dysfunction. Our specific aims were to demonstrate that a rat model of silicosis previously well characterized in the literature for the development of pulmonary inflammation and fibrosis would also demonstrate immunoglobulin responses mirroring those of human silicosis, and to document the anatomic compartments responsible for these immunoglobulin responses. To address these specific aims, we compared immunoglobulin responses in rats with experimental silicosis induced by inhalation of silica with responses in titanium dioxide (TiO_2)- and sham (air)-exposed controls. We found that experimental silicosis was associated with elevated IgG and IgM levels in blood and BAL paralleling those of human silicosis. Our findings also suggested that draining lung-associated lymph nodes (LALN) were the most important sites for increased IgG and IgM production in experimental silicosis, with lungs contributing to a lesser degree.

MATERIALS AND METHODS

Animals. Male Fischer F344 rats were purchased from Charles River Laboratories, Wilmington, MA. Rats weighed 150 to 175 g upon arrival and were housed in filter-topped polycarbonate cages and kept in HEPA-filtered laminar-flow animal isolators. Animals were allowed food and water *ad libitum* and held for 2 weeks prior to use. All protocols involving the use of animals were approved by the West Virginia University Institutional Animal Care and Use Committee.

Aerosol exposures. Exposures were conducted as reported for a well-documented experimental model of silicosis (Absher *et al*., 1989; Mohr *et al*., 1992). The model was specifically chosen for its ability to reproduce the pulmonary manifestations of human silicosis. Inhalation exposure of F344 rats to crystalline silica using the specified doses and schedule have been reported to result in the development of chronic inflammatory and fibrotic changes in the lungs paralleling those of human chronic silicosis after a latent period of approximately 4 months and persisting at 12 months. The timing of pulmonary changes also parallels human chronic silicosis, which usually manifest itself years after exposure (Silicosis and Silicate Disease Committee, 1988; American Thoracic Society, 1997; Weissman and Banks, 1998). Rats were exposed 5 h per day for 8 days (Tuesday to Friday and then Monday to Thursday) to aerosols of either α-quartz (Min-U-Sil 5; U.S. Silica Products, Berkeley Springs, WV), TiO_2 ($< 5\ \mu m$, Aldrich, Milwaukee, WI), or diluent air. Exposures were performed in horizontal flow chambers and dusts were aerosolized using a TSI 9310 fluidized bed aerosol generator (TSI, St. Paul, MN) (Lantz *et al*., 1989). The mean concentration of aerosolized silica in the exposure chamber was 39.3 ± 3.3 mg /m^3. Evaluation of the aerosol by cascade impactor documented that the mass median aerodynamic diameter (MMAD) of silica was 0.95 μm with a geometric standard deviation of 1.7 μm. The mean concentration of titanium dioxide in the exposure chamber was 40 ± 3 mg /m^3. Subsequent to exposures, the particle size distribution of titanium dioxide aerosolized from our fluidized bed was characterized using an aerodynamic particle sizer (Model 3320, TSI, St. Paul, MN) with an associated diluter (Model 3302A, TSI, St. Paul, MN) and documented to be 2.2 μm with a geometric standard deviation of 1.4 μm. Because there were only two chambers in our exposure apparatus, silica and air groups were exposed simultaneously, and rats used in the TiO2 group were purchased and exposed 20 days later. Subsequent studies of the TiO_2 group were performed approximately 20 days later than silica and air studies.

Experimental protocol. Immunoglobulin responses to experimental silicosis were characterized by comparison of findings in silica-exposed rats with those of titanium- or air-exposed rats. To document the presence and time course of changes in serum immunoglobulin levels, serum IgG and IgM were measured at 2, 3, 4, and 5 months after exposure. Rats were first sacrificed to evaluate immunoglobulin responses in specific anatomic compartments at 5.5 months after exposure. This time interval was chosen because pulmonary fibrotic and inflammatory changes paralleling those of human chronic silicosis were expected to be well established after this interval (Absher *et al*., 1989; Mohr *et al*., 1992), and changes in serum IgG and IgM levels had been documented to be well established at this time. Numbers of animals used for various studies are detailed in tables and figures. Animals were sacrificed over intervals ranging from 5.5 to 11.6 months after exposure, a period when pulmonary changes paralleling human chronic silicosis have been documented to be present in this model (Absher *et al*., 1989; Mohr *et al*., 1992). Care was taken to sacrifice animals in groups representing each exposure condition so overall duration since exposure in each of the three groups would be similar. *In vitro* immunoglobulin production by mononuclear cells derived from lung-associated lymph nodes (LALN), lung, and spleen was measured to assess potential for *in vivo* production of IgG and IgM in these anatomic compartments. For each of the three groups, *in vitro* immunoglobulin production was studied at a mean of 174 days after exposure. Changes in local pulmonary levels of IgG and IgM paralleling human silicosis, as well as local pulmonary inflammation, were evaluated by BAL in the silica-, titanium-, and air-exposed groups at mean time intervals of 245, 239, and 250 days after exposure, respectively. Enumeration of cell populations relevant to IgG and IgM production and silica-induced inflammation was evaluated in the tissues of silica-, titanium-, and air-exposed groups at mean intervals of 265, 261, and 265 days after exposure, respectively.

Serum immunoglobulins. Blood was collected by tail vein incision at 2, 3, 4, and 5 months after exposure. IgG and IgM levels were measured in serum using a sandwich enzyme-linked immunosorbent assay (ELISA). 1μg/ml of goat anti-rat IgG or IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD) were used as capture antibodies. Standards were prepared from rat IgG (Sigma Chemical Co., St. Louis, MO) or IgM (Bioproducts for Science, Indianapolis, IN). Goat anti-rat IgG or IgM peroxidase conjugates diluted 1:250 in PBS/BSA (both from Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD) were used as detecting antibodies. The chromogenic substrate used was 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate] (ABTS; Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD). Color development was detected as optical density at 405 nm using an automated ELISA plate reader (Bio-tek Instruments, Inc., Winooski, VT) and immunoglobulin concentrations were determined by comparison of sample color development to standard curves (Kineticalc, Bio-tek Instruments, Inc., Winooski, VT).

Histopathology. Five months after exposure, three rats from each exposure group were sacrificed by intraperitoneal (ip) injection of Ketamine/Xylazine followed by cardiac exsanguination. LALN known to receive lymphatic drainage from the lungs of Fischer-344 rats, cervical lymph nodes, lungs, and spleens were removed and weighed. LALN recovered included two nodes found close to the thymus (referred to here as parathymic) and a lymph node found in the right paratracheal area (referred to here as paratracheal) (illustrated in Bice *et al*., 1979). Tissues were fixed in 10% neutral buffered formalin. Right lungs were fixed by infusion of 10% neutral buffered formalin through the trachea at a pressure of 20 cm H_2O. Left lungs were removed prior to fixation and saved for other studies. Specimens were paraffin embedded and sections stained with hematoxylin and eosin (H & E) for examination by light microscopy. Photomicrographs were obtained using an Olympus AX70 photomicroscope (Olympus America Inc., Lake Success, NY) equipped with a Quantix 2000 $\times$ 2000 pixel digital camera (Photometrics Ltd., Tucson, AZ). Magnifications of photomicrographs are indicated using size bars.

Hydroxyproline determination. Five months after exposure, rats were sacrificed by ip injection of Ketamine/Xylazine followed by cardiac exsanguination. Lungs were removed, major airways dissected away, and the lungs weighed. Right lungs were used for silica determination (described below) and left lungs were used for hydroxyproline determination (Kivirikko *et al*., 1967).

To perform hydroxyproline determinations, left lungs were finely minced. Two milliliters of 6 N HCl was added to each sample and the tissues were hydrolyzed for 48 h at 110°C. Samples were neutralized with 10 N KOH and volume adjusted to 10 ml with distilled water and centrifuged (400 $\times$ *g*, 10 min);0.1 ml of each centrifuged hydrolysate was pipetted into a capped tube, and the volume was adjusted to 2.5 ml with borate-alanine buffer. Standards (trans-4-hydroxyl-L-proline; Sigma Chemical Co., St. Louis, MO) and blanks were also prepared in 2.5 ml of borate-alanine buffer; 0.5 ml saturated potassium chloride in borate-alanine buffer was added to each sample and vortexed. Resulting imino acid mixtures were oxidized at room temperature by adding 0.6 ml of 0.2 M chloramine T in methyl cellusolv. Thirty minutes later the oxidation was stopped with 2 ml of 3.6 M sodium thiosulfate. Samples were next mixed with 3 ml toluene and heated in a boiling water bath for 30 min to form pyrrole. After cooling, the tube was shaken vigorously, and centrifuged briefly at low speed. Finally, 0.4 ml of Ehrlich's reagent was added with stirring to the toluene layer and resulting color development was measured after 30 min at 560 nm. Hydroxyproline concentrations were determined by comparison of sample color development to a standard curve and results expressed as micrograms hydroxyproline per left lung.

Silica determination. Five days after exposure, three rats each from silica- and air-exposed groups were sacrificed and right lungs were removed for silica determination. Silica retention was also measured 5 months after exposure, using the right lungs of the rats described above under "Hydroxyproline determination." Silica determinations were done by infrared spectroscopy (Freedman *et al*., 1974). Briefly, right lung tissue was pooled, minced, and defatted with heptane in a glass vial at room temperature overnight and digested with 11.3 N HCl at 60°C while stirring for 2 h. Residues were washed twice in HCl followed by centrifugation at 1000 $\times$ *g* for 30 min and suspended in 1 ml of deionized water. The entire contents of the vial were layered onto 1 ml of 1.7 mg/ml Cs_2SO_4 and spun at 2000 $\times$ *g* for 20 min. After centrifugation the upper layer was discarded and the lower layer was vortexed and filtered through a DM-450 membrane filter. The filter was then washed with 10 ml distilled water. α-quartz standards (Min-U-Sil 5, U.S. Silica Products, Berkeley Springs, WV) were prepared by filtering known quantities through DM-450 membrane filters directly. Sample and standard filters were subjected to Fourier transform infrared (FTIR) spectroscopy using an ATI Mattson Genesis Series FTIR spectroscope between the frequencies of 400 and 1000 cm^{-1}. Silica content was measured as absorbance at the absorption peak of 799 cm^{-1} and experimental values were determined by comparison to the standard curve. A through-point weighted analysis and autoblank correction was made in the analysis of samples.

Samples and cell populations. Rats were sacrificed by ip injection of Ketamine/Xylazine followed by cardiac exsanguination. For each rat, LALN (parathymic and paratracheal lymph nodes) were identified, removed, and placed in cold RPMI 1640 supplemented with 25 mM HEPES, 50 μg/ml gentamycin, and 2 mM L-glutamine (RPMI; GIBCO, Grand Island, NY). Cervical lymph nodes (CLN) were harvested and processed separately as a control. Lymph node cell populations were obtained by passing the lymph nodes through a dounce tissue homogenizer. Resulting cell suspensions were pelleted by centrifugation at 300 $\times$ *g* for 10 min at 4°C, washed twice in RPMI, and suspended in RPMI containing 10% heat-inactivated fetal calf serum (CRPMI; HyClone Laboratories, Inc., Logan, UT).

Lungs were perfused with normal saline via the right ventricular outflow tract until oligemic. Animals were further exsanguinated by transection of the aorta. Bronchoalveolar lavage was performed by tracheal cannulation followed by infusion of 5 ml normal saline and recovery by gentle suction. Five such aliquots were instilled and recovered. BAL fluid recovered from each rat was centrifuged (300 $\times$ *g* for 10 min), and the resulting cell pellet was resuspended in RPMI for determination of cell count and differential. BAL supernatants were saved frozen at –20°C for measurement of IgG and IgM using the previously described ELISA. In addition, albumin determinations were performed by the bromocresol green technique (Sigma Chemical Co., St. Louis, MO).

After BAL, lungs were removed, dissected free of major airway, rinsed with normal saline to remove residual blood, and minced into cold RPMI. Minced lungs were digested in 10 ml RPMI containing type IV collagenase (150 U/ml) and type IIA elastase (10 U/ml) (both from Sigma Chemical Co., St. Louis, MO) over a shaking water bath at 37°C for 90 min. Enzyme-digested lung mince was dissociated into a cell suspension by passage through a stainless steel screen (Cellector, 100 mesh; Thomas Scientific, Swedesboro, NJ). Cells were pelleted by centrifugation and washed twice in RPMI. Pulmonary interstitial cells were enriched for mononuclear cells by density gradient centrifugation (Histopaque-1083; Sigma Diagnostics, St. Louis, MO). Cells at the gradient interface were harvested, washed twice in RPMI, then suspended in CRPMI.

Spleen cell suspensions were made by pressing spleens between two microscope slides in a petri dish filled with cold RPMI, then suspending the cells released by repeated aspiration through a sterile pasteur pipette. The resulting cell suspension was pelleted by centrifugation and then washed twice in RPMI. Splenic mononuclear cells were separated from contaminants such as red blood cells by density gradient centrifugation (Histopaque-1083; Sigma Diagnostics, St. Louis, MO). Cells at the gradient interface were harvested, washed twice in RPMI, then suspended in CRPMI.

Cell populations obtained from CLN, LALN, lung, BAL, and spleen were enumerated using a Coulter Counter. Cell differentials were performed by counting cells on cytocentrifuge preparations stained with Diff-Quik® (American Scientific Products, McGaw Park, IL). Cell viabilities were determined by trypan blue exclusion.

Quantification of B cells. B-lymphocyte subpopulations of cells obtained from LALN, lung, and spleen were quantified by flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Immunofluorescence staining was performed using a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (Clone OX-33; Pharmingen, San Diego, CA). Lymphocyte populations were identified by forward and side scatter and percent lymphocytes binding the monoclonal antibody by fluorescence intensity. Gate settings for analysis of fluorescence intensity were determined using cells stained with an irrelevant FITC-conjugated monoclonal antibody (Pharmingen, San Diego, CA).

In vitro immunoglobulin production. *In vitro* immunoglobulin production by LALN, lung, and spleen cell populations was measured using a modification of a previously described method (Weissman *et al*., 1992). Cell suspensions were adjusted to a concentration of 2 $\times$ 10^6 cells/ml in CRPMI and cultured in sterile, 12 $\times$ 75 mm, polypropylene test tubes (37°C, 5% CO_2) for 8 days. Conditioned culture supernatants were centrifuged (300 $\times$ *g*, 10 min) to remove cells and debris and were stored frozen at –70°C. IgG and IgM levels in culture supernatants were measured using the ELISA described above. To control for passive release of preformed immunoglobulin from cultured cells, parallel cultures were performed with and without 50 μg/ml cycloheximide (Sigma Chemical Co., St. Louis, MO) and *in vitro* immunoglobulin production expressed as antibody concentration in cultures without cycloheximide, less the antibody concentration in the cultures with cycloheximide (Weissman *et al*., 1992). To allow comparison of *in vitro* immunoglobulin production by lymphocytes from different organs and different treatment

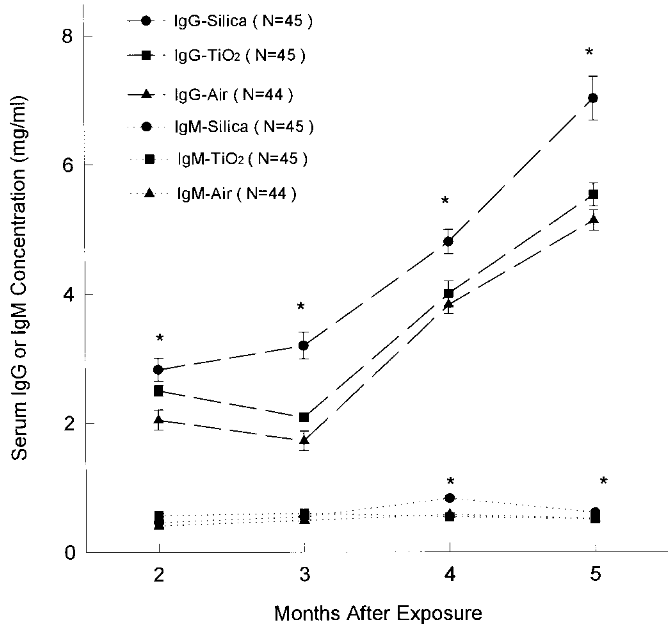

FIG. 1. Organ weights 5 months after exposure to silica, TiO_2, or air. Lungs and lymph nodes receiving drainage from the lungs are significantly heavier after silica exposure. Two remote lymphoid organs, cervical lymph node and spleen, are not affected. Data are expressed as mean ± SEM; * silica > TiO_2 and air controls; $p < 0.05$.

groups, results are expressed as nanograms immunoglobulin per 10^6 lymphocytes.

Statistics. Results are expressed as mean ± standard error except where indicated. Relative coefficient of excretion (RCE) for BAL IgG was calculated as follows:

RCE = [BAL IgG (μg/ml) / BAL albumin (mg/dl)] / [serum IgG (μg/ml) / serum albumin (mg/dl)].

The RCE IgG has been proposed as an indicator of the degree to which BAL IgG is derived from passive diffusion from blood versus local production (Mascart-Lemone *et al.*, 1987). RCE IgM was calculated in a similar fashion as shown for IgG.

Multiple comparisons between silica-, titanium-, and air-exposed rats were done by one-way analysis of variance followed by a multiple comparison procedure (either Student-Newman-Keuls or Dunn's; Sigmastat, SPSS, Chicago, IL). Differences were considered significant at the $p \leq 0.05$ level. Correlations between various parameters were analyzed by Pearson Product Moment Correlation (Sigmastat, SPSS, Chicago, IL). For correlations, serum IgG and IgM values were normalized by log transformation. RCE IgG and IgM were normalized by adding 1 prior to log transformation.

RESULTS

Induction of Disease by Silica Inhalation

Rats in all groups gained weight normally after silica, TiO_2, or sham-air exposures, with no significant differences in body weight between the three groups before or at 1 to 5 months after exposure (data not shown). Despite similar body weights, lungs and LALN were strikingly heavier in the silica-exposed group than in the air or TiO_2 groups. By contrast, CLN (which do not receive lymphatic drainage from the lung [Bice *et al.*, 1979]) and spleens were not heavier in the silica group than in the two control groups (Fig. 1).

Histopathologic examination of lungs and LALN from silica-exposed animals demonstrated marked changes (Figs. 2 and 3). Lungs demonstrated multifocal, mild to moderate, lymphogranulomatous interstitial pneumonia, and multifocal, mild to moderate, epithelial cell hypertrophy and hyperplasia. Multifocal, mild, alveolar lipoproteinosis, lymphadenitis of BALT, and lymphocytic perivascular cuffing were also noted. In contrast to the silica-exposed group, lungs of TiO_2-exposed rats were remarkable only for the presence of many macrophages containing dark brown to black particles compatible with their inhalation exposures (Fig. 2). In the silica-exposed group, LALN were markedly enlarged and remarkable for multifocal, moderate, granulomatous lymphadenitis. In contrast, LALN of TiO_2-exposed rats were remarkable only for small numbers of dark brown to black particles present in macrophages and histiocytes (Fig. 3). Lungs and LALN obtained from sham (air)-exposed controls did not exhibit any significant histopathologic changes.

Silica determinations performed 5 days after exposure detected 937 ± 149 μg silica/right lung in silica-exposed rats. Silica could not be detected in air-exposed lungs. At 5 months

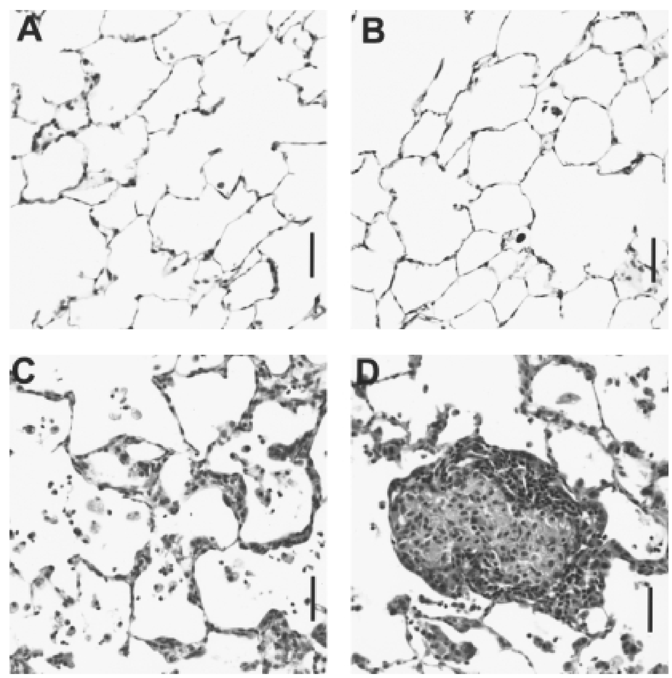

FIG. 2. Pulmonary histopathology 5 months after exposure to silica, TiO_2, or air (see text). Lung sections demonstrate interstitial pneumonia, alveolar epithelial cell hypertrophy and hyperplasia, and alveolar histiocytosis after silica exposure. A relative paucity of histopathologic changes is noted after control exposures. 2A: Air-exposed lung. 2B: TiO_2-exposed lung. 2C: Silica-exposed lung. 2D: Silica-exposed lung section showing silicotic granuloma. In each panel, vertical bar at the right border = 50 μM.

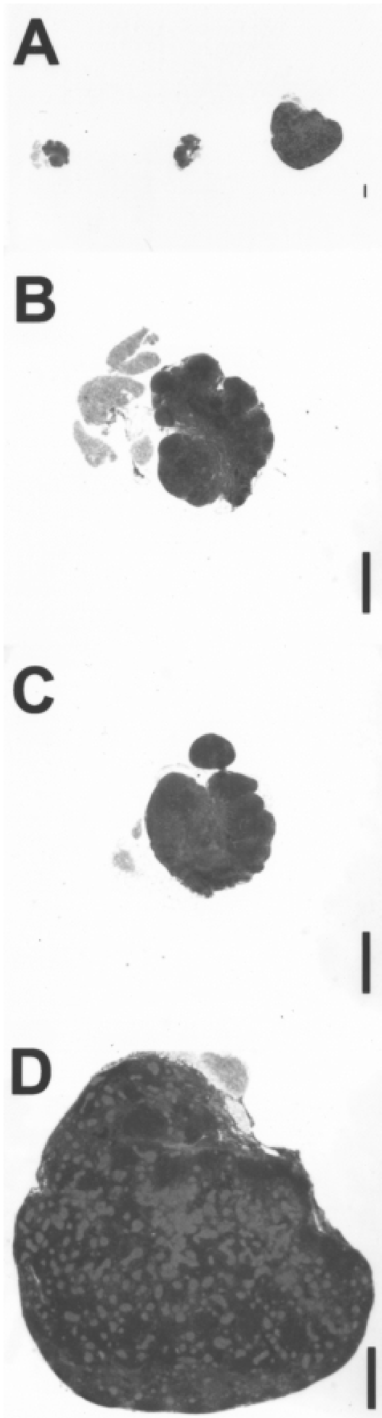

FIG. 3. Lung-associated lymph node histopathology 5 months after exposure to silica. Sections demonstrate marked hyperplasia and granulomatous lymphadenitis after silica exposure and relative paucity of histopathologic changes after control exposures. 3A: Scanned image of (from left to right) air-, TiO_2-, and silica-exposed LALN tissue sections mounted on a single microscope slide. Note massive enlargement of the silica-exposed LALN. 3B: Air-exposed LALN. 3C: TiO_2-exposed LALN. 3D: Silica-exposed LALN. Note massive enlargement and granulomatous inflammation. In all cases, vertical bar at the right border of each panel = 1 mm.

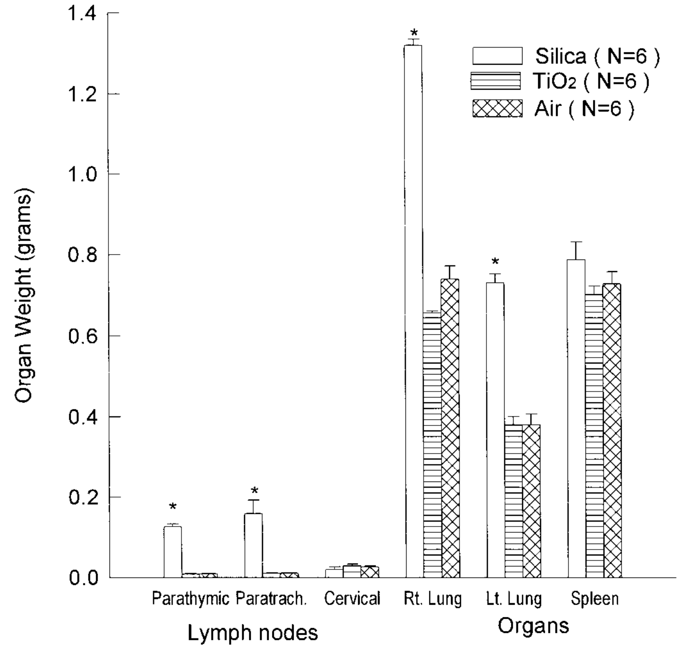

FIG. 4. Serum IgG and IgM concentrations before and up to 5 months after exposure to silica, TiO_2, or air. Silica exposure is associated with greater increases in serum IgG than serum IgM. Data are expressed as mean ± SEM; * silica > TiO_2 and air controls; $p < 0.05$.

after exposure, silica content of lungs of silica-exposed rats had markedly decreased, and the right lungs of three animals had to be pooled for silica detection. At this point, 10.5 μg silica/right lung was detected in pooled lung tissue of three silica-exposed rats. Silica was not detectable in the lungs of either air- or TiO_2- exposed rats at the 5-month time interval.

Hydroxyproline levels 5 months after exposure were significantly higher in the lungs of silica-exposed rats relative to the other two groups ($p < 0.05$). Levels in the silica, TiO_2, and air groups were 3.81 ± 0.23, 1.00 ± 0.05, and 1.17 ± 0.11 mg/left lung, respectively.

Serum Immunoglobulins

Maturational increases in serum IgG concentration were noted in each of the three exposure groups over the 5 months of observation (Fig. 4). At each time point studied, serum IgG concentrations were greater in the silica-exposed group than in the two control groups (Fig. 4). Serum IgM concentrations did not differ between groups nearly as much as did IgG concentrations. Although significantly greater serum IgM concentrations were noted in the silica-exposed group relative to the control groups at 4 and 5 months after exposure, these differences were of relatively small magnitude (Fig. 4).

Bronchoalveolar Lavage

Table 1 compares BAL characteristics in the three exposure groups. IgG and IgM levels in BAL fluids of silica-exposed

TABLE 1
Soluble and Cellular Constituents of Bronchoalveolar Lavage Fluids

	IgG (μg/ml)	IgM (μg/ml)	Alb (mg/dl)	RCE IgG	RCE IgM	Total Cells (x 10^6)	PAM ($\times 10^6$)	Lym ($\times 10^6$)	PMN ($\times 10^6$)
Silica ($n = 10$)	21.0 ± 12.8*	0.72 ± 0.34*	28.4 ± 14.5*	0.457 ± 0.245**	0.186 ± 0.088*	17.0 ± 4.6*	7.1 ± 3.0*	2.6 ± 1.8*	7.7 ± 2.9*
TiO_2 ($n = 11$)	3.06 ± 3.22	0.21 ± 0.51	9.57 ± 7.44	0.331 ± 0.257	0.108 ± 0.125	3.2 ± 2.4	3.1 ± 2.3	0.055 ± 0.067	0.021 ± 0.013
Air ($n = 11$)	2.24 ± 1.39	0.064 ± 0.040	9.81 ± 5.73	0.190 ± 0.093	0.052 ± 0.032	2.31 ± 0.85	2.28 ± 0.85	0.016 ± 0.009	0.018 ± 0.021

Note. Data expressed as mean ± standard deviation. Alb, albumin; RCE, relative coefficient of excretion; PAM, pulmonary alveolar macrophage; LYM, lymphocyte; PMN, neutrophil.

* Significantly different from TiO_2 and air groups, $p < 0.05$; **significantly different from air group, $p < 0.05$.

rats were markedly greater than those of sham (air)-exposed and TiO_2-exposed rats. Albumin concentrations were approximately 3-fold greater in the BAL fluids of silicotic rats than in the two control groups. Relative coefficient of excretion for BAL IgG (RCE IgG) was significantly greater in the silica group than the air group, and about 50% greater in the silica group than the TiO_2 group, although that difference did not attain statistical significance. RCE IgM was significantly greater in the silica group than in either of the comparison groups.

BAL cell counts also differed between the silica group and the two control groups (Table 1). Total cell recovery and recovered alveolar macrophages, lymphocytes, and neutrophils were all significantly and markedly increased in the silica group relative to the TiO_2 and air groups.

After pooling data from the three study groups, weak to moderate but statistically significant correlations were noted between several cellular measures of BAL inflammation and immunoglobulin levels in serum and blood (Table 2). After normalization by log transformation, serum IgG correlated significantly with BAL total cells, lymphocytes, and neutrophils, but not macrophages. Log-transformed serum IgM concentrations correlated weakly but significantly with BAL lymphocytes. BAL RCE IgG did not correlate significantly with any BAL cellular parameters, although trends for weak correlations with total cells and neutrophils were present. RCE IgM correlated significantly with BAL total cells and neutrophils. In contrast to the weak to moderate correlations with serum and BAL immunoglobulins, BAL total cells correlated strongly with cell numbers recoverable from LALN ($n = 19$; $r = 0.907$, $p < 0.001$).

Interstitial Cell Populations and in Vitro Immunoglobulin Production

Characteristics of cell populations derived from LALN, lung, and spleen are shown in Table 3. More cells were recovered from LALN and lungs of silica-exposed rats than LALN and lungs from either TiO_2- or air-exposed rats. Expansion of LALN cell populations was largely due to an almost 30-fold increase in lymphocyte numbers. Significantly increased recovery of interstitial pulmonary total cells, macrophages, and neutrophils was noted in the silica-exposed group relative to the two comparison groups. Neutrophil recovery was particularly increased. No significant differences in spleen cell recoveries were noted between the three groups.

No differences were noted in the relative proportion of B cells in lymphocyte populations derived from LALN, lung, or spleen of the three exposure groups (Table 4).

Interstitial cell populations derived from LALN, lung, and spleen were examined for their ability to produce IgG and IgM *in vitro* (Figs. 5 and 6). For each organ, IgM production per 10^6 lymphocytes did not differ between the three exposure groups (Fig. 5). IgG production per 10^6 lymphocytes also did not

TABLE 2
Correlations between Immunoglobulins and Inflammatory Cellular Constituents of BAL

	vs.	r	p
Log (serum IgG)			
	BAL total cells	0.424	0.018*
	BAL macrophages	0.254	0.168
	BAL lymphocytes	0.485	<0.01*
	BAL neutrophils	0.459	<0.01*
Log (serum IgM)			
	BAL total cells	0.306	0.095
	BAL macrophages	0.273	0.137
	BAL lymphocytes	0.362	0.046*
	BAL neutrophils	0.256	0.164
Log (RCE IgG + 1)			
	BAL total cells	0.329	0.071
	BAL macrophages	0.260	0.158
	BAL lymphocytes	0.224	0.225
	BAL neutrophils	0.340	0.062
Log (RCE IgM + 1)			
	BAL total cells	0.379	0.036*
	BAL macrophages	0.185	0.318
	BAL lymphocytes	0.318	0.081
	BAL neutrophils	0.463	<0.01*

Note. RCE, relative coefficient of excretion; $n = 31$.

* Significant at a level of $p < 0.05$.

TABLE 3
Cell Populations Derived from LALN, Lung, and Spleen

	Group	Total Cells ($\times 10^6$)	Mono/Mac ($\times 10^6$)	Lymph ($\times 10^6$)	PMN ($\times 10^6$)
LALN	Silica (n = 13)	141.7 ± 99.0*	2.9 ± 3.6	137.7 ± 97.9*	0.378 ± 0.610
	TiO_2 (n = 12)	5.28 ± 2.51	0.17 ± 0.23	5.09 ± 2.36	0.0068 ± 0.011
	Air (n = 9)	5.39 ± 2.31	0.15 ± 0.11	5.23 ± 2.28	0.0052 ± 0.009
Lung	Silica (n = 8)	118.2 ± 61.8*	55.4 ± 29.4*	33.4 ± 27.9	26.0 ± 13.2*
	TiO_2 (n = 5)	31.3 ± 16.8	14.2 ± 7.1	15.0 ± 8.6	2.11 ± 1.69
	Air (n = 5)	31.2 ± 7.0	14.5 ± 5.0	14.6 ± 3.0	1.93 ± 1.16
Spleen	Silica (n = 13)	57.5 ± 43.7	0.71 ± 0.80	55.9 ± 42.7	0.50 ± 0.56
	TiO_2 (n = 15)	48.7 ± 38.2	0.49 ± 0.58	47.0 ± 36.4	0.13 ± 0.34
	Air (n = 13)	53.6 ± 27.7	0.55 ± 0.69	52.3 ± 27.9	0.31 ± 0.43

Note. Data expressed as mean ± standard deviation. Mono/Mac, monocyte/macrophage; Lymph, lymphocyte; PMN, neutrophil; LALN, lung-associated lymph node.

* Significantly different from TiO_2 and air groups, $p < 0.05$.

differ for lymphocytes obtained from LALN or spleens of the three exposure groups (Fig. 6). IgG production per 10^6 lung lymphocytes was significantly greater in the silica-exposed group than in either the TiO_2-exposed group or the air-exposed group. Although a trend was noted for increased IgG production in the TiO_2-exposed group relative to the air-exposed group, this difference did not attain statistical significance. IgG production per 10^6 silica-exposed lung lymphocytes was also significantly greater than IgG production per 10^6 silica-exposed LALN or spleen lymphocytes (Fig. 6).

DISCUSSION

In order to examine relationships between experimental silicosis and dysregulated immunoglobulin production, we used a rat model that has been reported as a good approximation of human silicosis (Absher *et al.*, 1989; Mohr *et al.*, 1992). In this model, rats develop silicotic changes in lung and lung-associated lymph nodes over a period of months after either quartz or cristobalite aerosol inhalation. Our data confirm the relevance of the model to human disease, both from the standpoint of histopathological changes and from the standpoint of pulmonary extracellular matrix protein accumulation.

A TiO_2 aerosol-exposed group was used to help differentiate between dust overload phenomenon and effects specific to silica inhalation (Driscoll *et al.*, 1991). TiO_2 was used as a control dust, because at relevant doses it is nonfibrogenic and fails to induce histopathological lesions such as those caused by quartz. In general, the TiO_2-exposed group exhibited features that were not significantly different from those of the air-exposed group. Where trends existed suggesting differences between the TiO_2 and air groups such as in RCE IgG and RCE IgM, changes in the TiO_2 group were not nearly as great as in the silica-exposed group. These findings suggest that changes in serum and BAL immunoglobulins in experimental silicosis were due primarily to silica exposure and not simply the result of pulmonary dust overload.

As in human silica exposure or silicotic disease, this experimental model is associated with elevated serum immunoglobulins after silica exposure with increases in IgG more pronounced than increases in IgM (Doll *et al.*, 1981; Idel *et al.*, 1990; Karnik *et al.*, 1990; Nigam *et al.*, 1990; Nagaoka *et al.*, 1993). Interestingly, serum IgG levels increased over time in all three groups, but levels in the silica-exposed group were consistently higher. Parallel increases in serum IgG noted in all three groups appear to be maturational, as the rats used in this study were young (approximately 70 days old) at the time of exposure. Serum IgG concentrations in humans do not reach adult levels until about 2 years of age (van Loghem, 1978). The

TABLE 4
B Cells as a Percentage of Lymphocytes Derived from Organs of Silica-, TiO_2-, or Air-Treated Rats

	LALN (%)	Lung (%)	Spleen (%)
Silica	35.9 ± 7.7 (n = 12)	22.7 ± 11.6 (n = 7)	41.8 ± 11.9 (n = 11)
TiO_2	34.2 ± 9.2 (n = 9)	18.6 ± 5.1 (n = 6)	37.7 ± 9.8 (n = 14)
Air	36.3 ± 5.9 (n = 7)	19.5 ± 4.7 (n = 6)	38.1 ± 8.8 (n = 10)

Note. Data expressed as mean ± standard deviation. LALN, lung-associated lymph node.

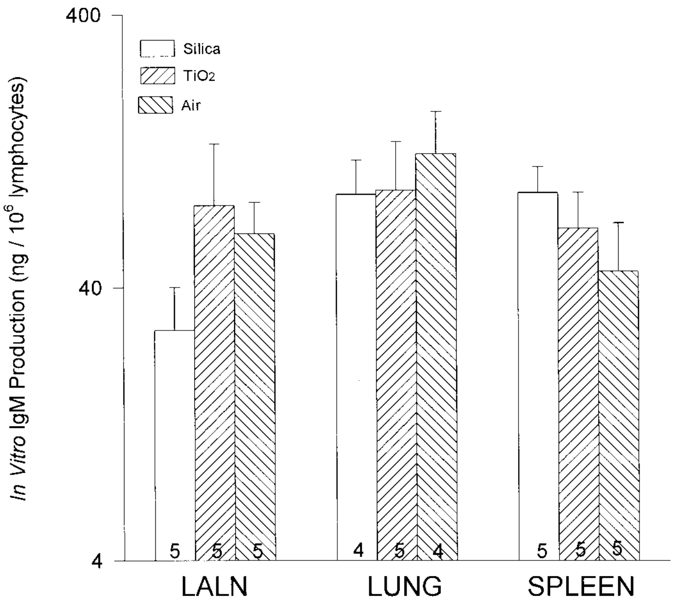

FIG. 5. *In vitro* IgM production by lymphocytes derived from LALN, lung, or spleen of silica-, TiO_2-, or air-exposed rats. Similar levels of IgM production per 10^6 lymphocytes occur in each exposure group. Data are expressed as mean ± SEM.

current data suggest that serum IgG concentrations in normal Fischer F344 rats continue to increase at least until the age of 7 months, as our animals were exposed at about 2 months of age and serum IgG levels monitored for another 5 months.

Immunoglobulin determinations in BAL fluid also paralleled those reported in human BAL specimens from silica-exposed granite workers (Calhoun *et al.*, 1986) and silicosis resulting from a variety of occupational exposures (Lusuardi *et al.*, 1990). The main difference between this model and reported findings in human BAL was the greater magnitude of increase in BAL IgG and IgM noted in the model system. Differences between the silica-exposed rats and the two control groups were far more marked than differences reported between silica-exposed humans and controls (Calhoun *et al.*, 1986; Lusuardi *et al.*, 1990). Several factors might have contributed to this difference from human findings, such as species differences, age at exposure, and severity of disease. Increased RCE IgG and RCE IgM in BAL of silica-exposed animals relative to the two control groups suggests that local production contributed to increased BAL IgG and IgM in the silica-exposed group. However, RCE values alone cannot prove the presence of local production. Other possibilities, such as impaired clearance from the alveolar space of immunoglobulin reaching the lung by passive diffusion, cannot be ruled out. Indeed, significant elevations in BAL albumin levels in the silica group document increased pulmonary vascular permeability and suggest that some proportion of BAL immunoglobulin reached the lung by passive transudation from blood (Burnett, 1986).

Moderate to weak but significant correlations noted between various BAL cellular components, serum IgG and IgM levels, and BAL RCE IgG and IgM suggest mechanistic relationships between pulmonary inflammation, production of immunoglobulin reaching the systemic circulation, and local pulmonary immunoglobulin levels. It has been suggested that local pulmonary antibody production in silicosis is the result of complex cytokine networking during pulmonary inflammation (Davis, 1986). A variety of cytokines with potential impact on immunoglobulin production, including IL-1β, TNF-α, IL-6, TGF-β, interferon-γ, and IL-10 have been reported to play important roles in responses to intrapulmonary deposition of silica (Davis, 1986; Garn *et al.*, 1997; Huaux *et al.*, 1998; Jagirdar *et al.*, 1996; Piguet *et al.*, 1990; Vanhee *et al.*, 1995; Davis *et al.*, 1998). A variety of cell types including alveolar macrophages, epithelial cells, fibroblasts, and infiltrating inflammatory cell types such as granulocytes and lymphocytes have the potential to engage in cytokine networking within the lung after silica exposure, with attendant collateral effects on B-cell function.

Evaluation of interstitial cell numbers and ability to produce immunoglobulins *ex vivo* suggest LALN as a particularly important site for increased IgG and IgM production in experimental silicosis. The massive enlargement in LALN and LALN cell numbers associated with silica exposure are consistent with other reports (Friedetzky *et al.*, 1998; Garn *et al.*,

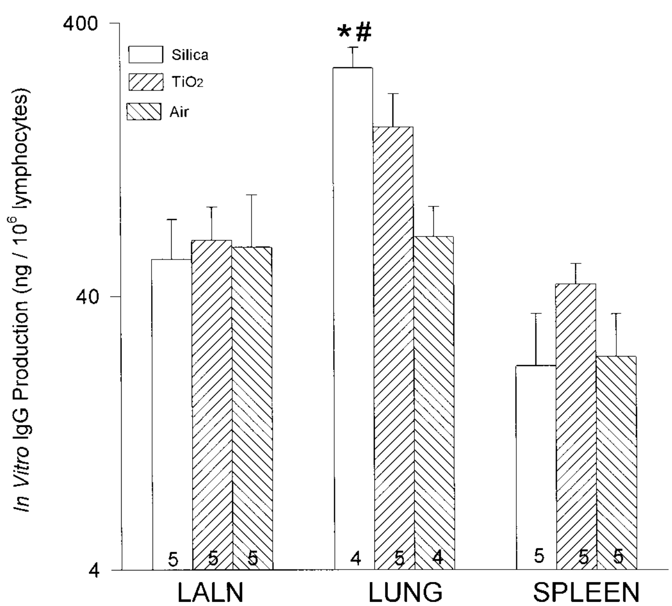

FIG. 6. *In vitro* IgG production by lymphocytes derived from LALN, lung, or spleen of silica-, TiO_2-, or air-exposed rats. IgG production by silica-exposed lung lymphocytes is significantly increased relative to TiO_2- and air-exposed lung lymphocytes, and relative to silica-exposed LALN and splenic lymphocytes. Number at the base of each bar denotes number of experiments. Data are expressed as mean ± SEM; * silica lung data > TiO_2 and air lung data; $p < 0.05$; # silica lung data > silica LALN and spleen data; $p < 0.05$.

1997). Furthermore, increases in total number, but not relative proportion, of LALN B lymphocytes are also consistent with previous reports (Friedetzky *et al.*, 1998). In the current study, silica-exposed LALN lymphocyte populations outnumbered those of the control groups by approximately 35 times. The ability of silica-exposed LALN to produce IgG *ex vivo* on a per-cell basis was similar to that of the control groups. This suggests an approximately 35-fold increase in potential for IgG production by LALN of the silica-exposed group *in vivo*.

Pulmonary interstitial lymphocyte numbers were also increased in silica-exposed animals relative to controls by about 2-fold, but this difference was not nearly as marked as in LALN and not enough to attain statistical significance. Unlike LALN, increased specific activity for IgG production by lung lymphocytes from silica-exposed rats was noted, despite similar proportions of B cells among lung lymphocytes derived from the three groups. A potential explanation might be increased terminal differentiation of silica-exposed lung lymphocytes into plasma cells, which are the most efficient producers of antibody. In this regard, plasma cells have a paracrine dependence on IL-6 (Henderson and Calame, 1998) for which increased gene expression occurs in the lungs in experimental silicosis (Piguet *et al.*, 1990).

In summary, we have used a well-characterized rat model of experimental silicosis to investigate altered immunoglobulin levels in this disease. Increases in serum and BAL IgG and IgM mirror those seen in human disease. Lung inflammation as measured by BAL appears related to the increased immunoglobulin levels present in serum and BAL. The most important site of increased antibody production appears to be lung-associated lymph node, although the data support increased antibody production in the lung as well.

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