

Raised immunoglobulin concentrations in bronchoalveolar lavage fluid of healthy granite workers

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ABSTRACT Immunoglobulin concentrations and lymphocyte counts were determined in bronchoalveolar fluid obtained from nine symptomless, healthy, non-smoking granite workers (mean age 45.6, range 22-56 years) and nine normal, non-smoking, non industrial controls (mean age 22.8, range 21-32 years). The proteins were measured in unconcentrated lavage fluid by means of a solid phase, enzyme linked immunosorbent assay. IgG and IgA concentrations were three times greater in lavage fluid from granite workers than the samples from non-industrial controls ($p < 0.02$). Eight of nine normal volunteers (89%) had no detectable IgM (< 30 ng/ml) in the lavage fluid whereas eight of nine (89%) granite workers had detectable IgM ($\chi^2 = 8$, $p < 0.01$). Lymphocyte counts in lavage fluid from the workers were significantly greater (15.5%) than control counts (5.6%; $p < 0.05$). The normal albumin concentration suggests that differences in permeability do not account for all of the increased immunoglobulin concentrations found in granite workers' lavage fluid and that some immunoglobulin is locally synthesised. It is concluded that occupational exposure to granite dust is associated with an increased proportion of lymphocytes and an increased concentration of immunoglobulin in lavage fluid that may reflect a subclinical immune inflammatory response.

Many humoral immune abnormalities have been described in patients with silicosis. Polyclonal increases in serum immunoglobulins, antinuclear antibodies, immune complexes,¹⁻³ and autoimmune diseases occur with increased frequency in patients with silicosis.⁴ Caplan⁵ reported the presence of serum rheumatoid factor in Welsh coalminers. These findings suggest non-specific B lymphocyte hyperactivity. Various soluble mediators secreted by stimulated macrophages have direct effects on immune cells and their products, and could be causally related to these abnormalities. These cytokines, chemotactic factors, and fibrogenic factors such as interleukin 1 have also been suggested as mediators of silicotic fibrogenesis.⁶⁻¹⁰ Lowrie¹¹ pointed out that macrophages exposed to silica may not die, as previously suggested,² but may survive with altered metabolism.

Differentiation of epiphenomena from relevant pathogenic mechanisms has, however, been difficult. Study of early disease, and of subjects with exposure to granite dust or crystalline silicon dioxide but without disease, may provide insight into the relevance of these immune processes to the production of silicosis. The Barre, Vermont, granite sheds provide an opportunity to study a stable industrial population chronically exposed to levels of a biologically active and potentially injurious agent that are below the current threshold limit value (TLV).

In a study of normal, healthy granite workers we found a considerable quantity of granite dust in alveolar macrophages recovered by bronchoalveolar lavage fluid. Macrophage phagocytic function was preserved.¹² Although the total numbers of cells recovered in lavage fluid were not different, there were increases in the proportions and numbers of lymphocytes in lavage fluid that were not mirrored in blood. Analyses of T and B fractions of T cell subsets were not performed. We speculated that the immune system played a part in the biological response to inhaled

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granite dust (silica), and that the reported B cell hyperactivity might be related to the pathogenesis of disease. The increase in lymphocytes in the lavage fluid suggested a local immune response. We formed the hypothesis that a humoral immune response accompanied the cellular influx. To test this hypothesis, we have analysed cell free supernatants of the same bronchoalveolar lavage fluid specimens from nine granite workers and nine non-industrial controls for immunoglobulins G, A, and M and for albumin and total protein concentrations.

Methods

STUDY POPULATION

Nine workers actively employed in the Barre, Vermont, granite industry and nine contemporary non-industrial control subjects volunteered to undergo bronchoalveolar lavage. All provided informed consent in a manner approved by the University of Vermont committee on human research. The study population has been described in detail.¹² All subjects were non-smokers. All 18 volunteers had normal findings on physical examination; normal spirometric performance; and normal chest radiographs, electrocardiograms, and complete blood counts. None of the workers therefore had clinical evidence of silicosis. Workers were older (mean age 45.6 years, range 22–56 years) than the control volunteers (mean age 22.8, range 21–32 years), and all were male.

BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage was carried out as previously described.^{12–14} Four 60 ml aliquots of sterile 0.9% saline (Travenol Laboratories) were instilled and immediately aspirated through a bronchofibrescope wedged in a subsegment of the right middle lobe or lingula. All of the fluid for each subject was pooled for analysis. A cell free supernatant was prepared by centrifugation at 400 g for 15 minutes at room temperature and then frozen at -70°C until analysed. Details of the cellular analysis are published elsewhere.¹² Briefly, air dried cytocentrifuge smears were stained with May-Grünwald-Giemsa and at least 300 cells were identified as alveolar macrophages, polymorphonuclear leucocytes, or lymphocytes.

IMMUNOGLOBULIN, ALBUMIN, AND TOTAL PROTEIN ASSAYS

IgG, IgA, IgM, and albumin were measured by a double antibody, enzyme linked immunosorbent assay (ELISA) modified from that described by Ershler.¹⁵ Flat bottomed polystyrene 96 well plates were coated overnight with commercial antibody raised in goats against human IgG, IgA, IgM, or albumin (Cappel Laboratories, West Chester, Pennsylvania) to form a

base coat. Serial dilutions of each serum or lavage fluid samples were made and wells were filled in triplicate with each dilution. Antibody raised in goats was obtained commercially either conjugated to alkaline phosphatase (IgG, IgA, IgM—Tago, Burlingame, California) or unconjugated (albumin—Cappel). The antialbumin was conjugated in our laboratory with alkaline phosphatase (Sigma Chemical, St Louis) by the use of glutaraldehyde.¹⁶ The enzyme conjugate was applied and allowed to incubate overnight. Finally, the plates were incubated at room temperature with *para*-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer) for 30 minutes; the reaction was stopped with 3 N NaOH. We measured absorbance at 405 nm with a semiautomated ELISA plate reader. A standard curve relating optical density to known protein concentration was constructed for each individual assay by a least squares linear regression. We derived protein concentration from the mean of triplicate optical density measurements at one or two dilutions within the linear range of a simultaneous standard curve for each sample. Standard curves for ELISAs were constructed from dilutions of a standard pooled serum, which had been analysed by routine clinical assays for the proteins in question. Our anti-IgA antibodies had specificity for the α chain, and hence detected both monomeric and dimeric forms of IgA. Total protein in lavage and serum samples was measured by the method of Lowry,¹⁷ and was measured against a bovine serum albumin standard.

STATISTICAL METHODS

Comparisons between control and worker lavage fluid and serum were tested by an unpaired *t* test. The distribution of lavage IgM concentrations was non-normal. Thus all data on lavage IgM were analysed by means of a Wilcoxon rank sum test or χ^2 analysis.¹⁸ Except as noted, results are expressed as means with standard errors in parentheses.

Results

LAVAGE FLUID CHARACTERISTICS

Analysis of the cellular phase of these lavage fluid samples has already been reported.¹² There was a significantly higher proportion of lymphocytes in lavage fluid from workers (15.5% (3.3%) of total) than in control samples (5.6% (0.9%); $p < 0.02$). The fraction of alveolar macrophages was 82.0% (3.9%) in the controls, and 92.9% (1.8%) in the workers ($p < 0.02$). A higher proportion of alveolar macrophages from workers than from controls contained dust as measured by polarised light microscopy (76% (4%) v 6% (1%); $p < 0.01$) and confirmed by scanning electron microscopy with x ray energy spec-

trosopy. Total cell recovery was not different ($11.1 (2.1) \times 10^6$ in workers, $12.6 (1.1) \times 10^6$ in controls), and polymorphonuclear cells averaged less than 2% of the total cells in both groups. The fractional volume recovery was slightly greater in the control group ($76.4\% v 72.1\%$; $p < 0.02$); this small difference does not account for the protein changes described below.

LAVAGE FLUID PROTEINS

The results of measurement of protein concentration in lavage fluid are depicted in figure 1. The mean IgG concentration was $10.3 (2.0) \mu\text{g/ml}$ in granite workers and $3.3 (0.5) \mu\text{g/ml}$ in non-industrial controls ($p < 0.01$). Fluid from seven of nine workers contained IgG in excess of the control mean concentration + 2 SD. IgA showed a similar increase; the mean IgA was $1.78 (0.66) \mu\text{g/ml}$ in controls and $6.63 (3.27) \mu\text{g/ml}$ in workers ($p < 0.02$). In four of the nine workers the concentration of IgA exceeded the control mean value + 2 SD. IgM was present at less than 1% of the concentration of other immunoglobulins. IgM was undetectable ($< 30 \text{ ng/ml}$) in eight of nine control specimens, but was present ($\geq 30 \text{ ng/ml}$) in eight of nine samples obtained from workers ($p < 0.01$, χ^2

analysis). The mean lavage fluid concentration of IgM in the workers was 144 ng/ml ($0.14 \mu\text{g/ml}$). The mean albumin concentrations did not differ significantly between workers and controls ($51.3 (5.0) v 43.6 (3.73) \mu\text{g/ml}$), but the total protein concentration was increased, averaging $81.9 (9.3) \mu\text{g/ml}$ in the workers and $58.9 (3.9) \mu\text{g/ml}$ in the controls ($p < 0.05$). Increased immunoglobulins accounted for half (52%) of the augmentation of the total protein in the workers, and measured proteins (IgG, IgA, IgM, albumin) comprised 85% of the increase in total protein.

Normalised concentrations

Expression of our data as proportions of albumin or as fractions of total protein present did not alter the relationship between the groups. Figure 2 depicts lavage fluid immunoglobulin as a fraction of albumin present. Mean IgG, IgA, and IgM concentrations for the workers normalised for total protein¹⁹ and for albumin²⁰ were significantly higher than the corresponding values obtained from non-industrial controls. Lavage albumin concentrations normalised for total protein did not differ significantly between the groups.

Correlations between selected protein and cellular indices are shown in table 1. Close correlation was seen between IgA and IgG concentrations in lavage fluid. There were also significant correlations between IgG, IgA, and albumin concentrations. In contrast, no significant correlation was found between IgM concentration and other lavage fluid values, or between the proportion of lymphocytes and any of the protein concentrations.

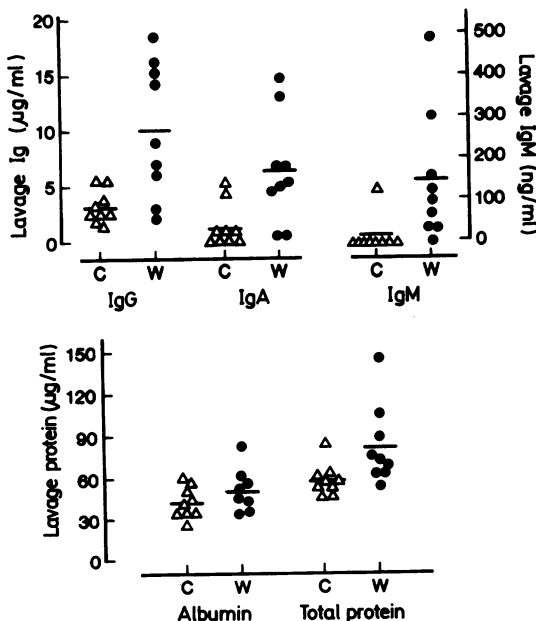


Fig 1 Bronchoalveolar lavage fluid proteins. Upper panel—lavage fluid immunoglobulin concentrations. Worker and control groups differ ($p < 0.02$) in concentrations of all three immunoglobulins. Lower panel—lavage fluid albumin and total protein concentrations. Worker and control groups differ ($p < 0.05$) in total protein only. c—control ($n = 9$); w—granite worker ($n = 9$).

SERUM PROTEINS

Serum protein values are given in table 2. Figure 3 shows serum immunoglobulin:albumin ratios graphically. Mean serum albumin and total protein concentrations were 33.7 and 74.6 g/l for control subjects and 33.4 and 76.0 g/l for granite workers, and did not differ significantly. Mean concentrations of IgG, IgA, and IgM in serum did not differ between control and worker populations. There were no significant differences between groups when raw concentrations, Ig:albumin ratios, or Ig:total protein ratios in serum were compared.

LAVAGE:SERUM RATIOS

We compared lavage and serum values for specific protein measurements to investigate whether increased alveolar-capillary membrane permeability contributed significantly to the increased lavage fluid immunoglobulin. Lavage fluid immunoglobulin G, A and M normalised in relation to albumin is shown as a percentage of the corresponding normalised serum

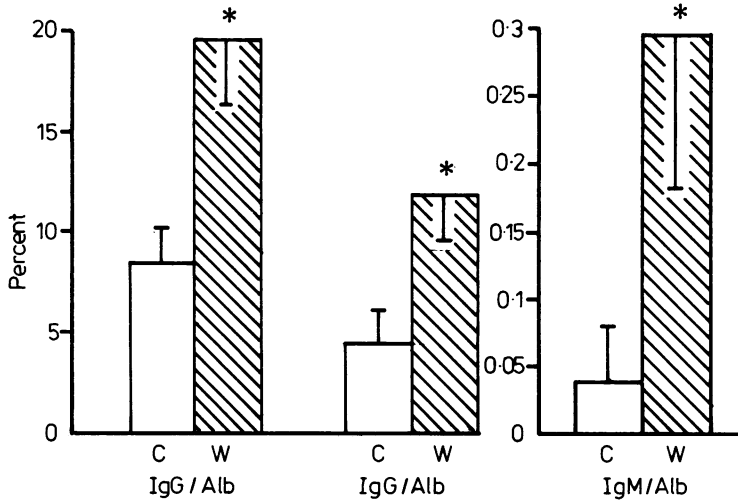


Fig 2 Immunoglobulin:albumin concentration ratios in lavage fluid. c—control; w—worker. Bars represent 1 standard error. *Worker and control groups differ at $p < 0.05$. Note the different scale of ordinate for IgM:albumin ratio.

Table 1 Correlations between concentrations of immunoglobulins and albumin and percentages of lymphocytes in lavage fluid (data from nine granite workers and nine controls)

	IgG	IgA	IgM	Alb	% LYS
IgG	—	0.85†	0.27	0.68*	0.002
IgA		—	0.44	0.71*	0.05
IgM			—	0.14	0.10
Alb				—	-0.19
% LYS					—

Data expressed as correlation coefficients, r , from least squares linear regression analysis.

* $p < 0.01$; † $p < 0.001$.

Alb: albumin; %LYS: percentage of lymphocytes in lavage fluid.

protein in figure 4. This ratio is numerically identical to the secretion:serum ratio of a constituent divided by the secretion:serum ratio of albumin. In figure 4 albumin is by definition 100%.

Figure 5 depicts lavage IgG, IgA, and IgM and albumin normalised to total protein (rather than albumin) as a proportion of the corresponding serum ratio, and is analogous to figure 4. A simple perme-

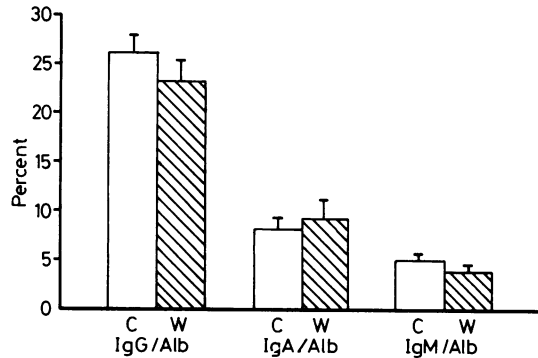


Fig 3 Immunoglobulin:albumin concentration ratios in serum. c—control; w—worker. Bars represent 1 standard error. Worker and control groups do not differ significantly.

ability model in which molecular weight is the sole determinant of distribution predicts that small molecules, such as albumin and transferrin, will show relative lavage enrichment but that proteins with higher

Table 2 Serum protein concentrations in nine granite workers and nine controls

	Mean (SE) protein concentration (g/l)				
	IgG	IgA	IgM	Albumin	Total protein
Controls	8.6 (0.9)	2.7 (0.5)	1.7 (0.3)	33.7 (2.5)	74.6 (1.1)
Worker	7.1 (0.3)	2.7 (0.4)	1.2 (0.2)	33.4 (2.7)	76.0 (0.8)

None of the differences between control and worker populations are statistically significant.

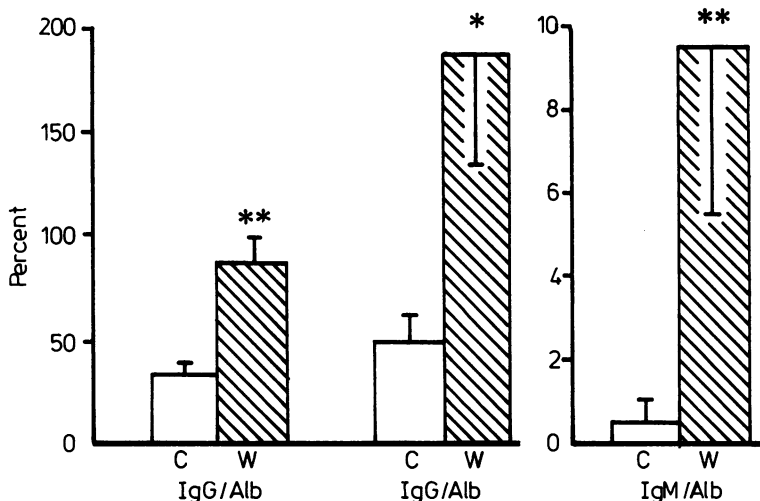


Fig 4 Lavage fluid:serum concentration ratios for immunoglobulins, normalised in relation to albumin (Alb). This ratio is calculated as $100 \times A/B \div C/D$, where A = lavage fluid component (IgG, IgA, etc), B = lavage fluid albumin, C = same serum component, and D = serum albumin. It is numerically equal to the secretion:serum ratio of a constituent divided by the secretion:serum ratio for albumin. c—control; w—worker. Bars represent 1 standard error. * $p < 0.05$; ** $p < 0.01$.

molecular weights, such as IgM and α_2 macroglobulin, will be relatively excluded. A ratio of 100% implies that a given protein comprises equal proportions of the total protein in lavage and serum. Ratios over 100% reflect relative enrichment in lavage fluid and those less than 100% relative exclusion. Figure 5 shows that for control subjects the lavage:serum (L:S) ratio for albumin was 171%. Lavage fluid was rela-

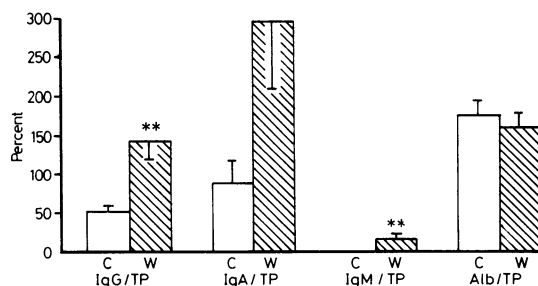


Fig 5 Lavage fluid:serum concentration ratios for immunoglobulins, normalised in relation to total protein (TP). This ratio is calculated as in figure 4, where A and C are defined, B is lavage fluid total protein, and D is serum total protein. c—control; w—worker. Bars represent 1 standard error. * $p < 0.05$; ** $p < 0.01$.

tively enriched with albumin, as predicted by a permeability model. The control lavage:serum ratio for IgG was 51%. The larger molecule showed relative exclusion from lavage fluid. The very large IgM molecule was almost totally excluded.

Protein ratios in workers showed a different pattern. Although the L:S ratio for albumin was not significantly different than the control value at 157%, the L:S ratio for IgG showed enrichment, in contrast to the exclusion seen in controls ($p < 0.001$). Similar enrichment was seen for IgA ($p < 0.05$); IgM showed significantly less exclusion ($p < 0.01$).

As a means of validating our assay, we measured serum immunoglobulins and albumin from normal, non-industrial control patients. The values shown in table 2 fall within the range of normal.²¹ The coefficient of variation was in all cases less than 15%. In addition, we analysed another set of unconcentrated lavage specimens from normal control subjects for albumin concentration, and showed good agreement between values obtained by radial immunodiffusion and those determined by ELISA analysis. There was no systematic error difference between these two methods, and the slope of the line of best fit was 0.98. The accuracy of the albumin assay is similar to that of the immunoglobulins (data not shown).

Discussion

Exposure to crystalline silicon dioxide remains a problem of occupational and public health. Since the institution of dust control measures in 1938–40, the prevalence of clinical silicosis and silicotuberculosis in Vermont granite shed workers has declined sharply. Although it has been suggested that deterioration in pulmonary function in these workers is accelerated by low levels of granite dust exposure,²² these findings have been questioned.²³ In addition, there is no evidence of an association between employment in the industry and a higher proportional mortality rate than in the United States population in general.²⁴ Craighead,²⁵ however, showed fibrotic lesions containing silicon in all the postmortem lung specimens from 15 granite workers who died of diseases other than pneumoconiosis. None of those workers had premortem clinical evidence of silicosis. Thus granite dust may provoke a pathological response in the lung, despite the lack of change demonstrated by relatively insensitive indicators such as pulmonary function tests or chest radiography.

Analysis of lavage fluid macromolecules has been hampered by their very low concentrations. Our measured values, obtained by double antibody ELISA techniques for IgG, IgA, IgM, and albumin and a Lowry assay for total protein concentrations, were made in unconcentrated fluid. We detected higher concentration of albumin, and hence showed higher albumin:total protein and lower IgG:Alb ratios, than others have reported.^{14 20 26–28 32} Those workers, however, analysed fluids concentrated by various techniques, including membrane filtration. Possibly a fraction of albumin was lost in the concentration step. Afford *et al* have shown that such losses occur, and that the losses are different for each protein.²⁹ Our analysis, in contrast, was based on measurements of unaltered lavage fluid. Lavage technique and fractional volume recovery have also been shown to have important effects on lavage fluid analysis. Small lavage volumes tend to generate higher concentrations of protein, carbohydrate, and lipid, and larger lavage volumes generally produce lower values.^{30 31} The use of a uniform technique is essential.²⁰ Normalising the raw concentrations in relation to an internal standard such as albumin,^{14 20} potassium,³² or total protein¹⁹ also overcomes some of these difficulties. The use of protein ratios may, however, mask a multicomponent rise in protein constituents, and therefore ratios must be interpreted with caution. Thus some of the variability in reported immunoglobulin concentration in non-smoking controls is likely due to technical rather than biological factors.

We studied cell free supernatants of lavage fluid from non-industrial control subjects and normal,

healthy granite workers for immunoglobulins G, A, and M, and determined albumin and total protein concentrations. Concentrations of all classes of immunoglobulin studied were increased in the worker population. Albumin concentration was not significantly different between groups. Total protein concentration in lavage fluid from workers was increased, and half of the increase could be accounted for by increased immunoglobulins. It is likely that some of the increase in immunoglobulin concentration is likely to be due to local synthesis in the lung. Increased alveolar-capillary membrane permeability cannot account for the increase in concentrations that we observed. Albumin was the smallest molecule measured, and would be expected to rise the most if increased permeability alone were operative. The mean lavage fluid albumin concentrations did not, however, differ significantly between control and worker populations. The immunoglobulin:albumin ratios likewise do not support the hypothesis that permeability factors account for the alterations, but rather suggest local production or a local mechanism for concentrating the immunoglobulins. Various sites for immunoglobulin production exist in the lung, including plasma cells surrounding granulomas, bronchus associated lymphoid tissue and free air space cells. Hance³³ showed that lung cells can produce immunoglobulin in culture. We have shown that lavage cells produce IgM but not IgG in culture; moreover, alveolar macrophages stimulate both IgG and IgM production in a mitogen driven system, suggesting a role for macrophages in the regulation of immunoglobulin concentrations in air spaces.^{34 35} Our study design does not allow us to determine the site of immunoglobulin production. There was no correlation, however, between the proportion of lymphocytes and the immunoglobulin concentrations in lavage fluid (table 1). Rankin *et al*³⁶ showed a relationship between lavage IgG concentration and "IgG secreting cells" in sarcoidosis. Notably, only 0.03–3% of lavage lymphocytes were "IgG secreting." Our methods did not include enumeration of IgG secreting cells, which may account for the lack of correlation. Alternatively, the differences in the patient population may be responsible. Although some of the immunoglobulin is probably of local origin, the correlation between IgG and albumin and between IgA and albumin allows the possibility of some increased transudation as well; variable dilution may, however, have accounted for this relationship. These correlations exist both in the combined (control and worker) data (table 1) and in the data on the worker group alone (not shown). There were no inter-protein correlations among the non-industrial control data when these were analysed alone.

We suggest that increased concentrations of immu-

noglobulins in bronchoalveolar lavage fluid represent a part of an immune-inflammatory response initiated and perpetuated by inhalation of granite dust. The cellular mechanisms by which granite dust induces this response are not clear. Burger and colleagues³⁷ reported enhanced proliferation of mouse splenocytes in response to mitogen when exposed to silicon oxide. Soluble mediators such as interleukin 1 and prostaglandin E₂ secreted by macrophages containing silica may play a part. Furthermore we have suggested that there may be exogenous or altered endogenous antigens associated with the particulate silica. Delineation of these process will require further study. Occupational exposure to granite dust produces cellular and biochemical responses that can be quantified by analysis of lavage fluid. The changes we describe may be important in the pathogenesis of silicosis.

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