Assessment of Autoimmune Responses Associated with Asbestos Exposure in Libby, Montana, USA

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Asbestos-related lung disease (ARD), including fibrosis, pleural plaques, and cancer, continues to be a serious and significant problem despite increasing awareness of the health hazards of asbestos inhalation. Although the exact mechanisms leading to the progression of these conditions have not been fully explained, there is evidence that some of the lung pathologies seen with both asbestos and silica exposures are immunologically mediated (Hamilton et al. 1996; Holian et al. 1997; Perkins et al. 1993). Silica and asbestos exposures also both appear to exacerbate autoimmune responses. Epidemiologic studies have shown strong associations between silica exposure and several autoimmune diseases, including scleroderma, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (Koeger et al. 1995; Parks et al. 1999; Powell et al. 1999; Steenland and Goldsmith 1995). Increased serum immunoglobulins, positive antinuclear antibody (ANA) tests, and immune complexes have been reported in small cohorts of individuals exposed to asbestos (Lange 1980; Nigam et al. 1993; Zerva et al. 1989), but to our knowledge no comprehensive study has been undertaken to assess the prevalence, specificity, and significance of autoantibodies associated with asbestos exposures. The population in Libby provides a unique research opportunity because of significant exposures that occurred as a result of the mining of asbestos-contaminated vermiculite near the community. Exposures have been documented not only in the miners but also in their family members, as well as anyone who used the vermiculite or played near the mine tailings. Therefore, the Libby asbestos exposures were both occupational and environmental throughout the community (Peipins et al. 2003).

In addition to the ARD in Libby, there have been anecdotal reports of an increased prevalence of systemic autoimmune disease (SAID), but verification of these diagnoses is still in progress. When the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry (ATSDR) performed its screening in Libby during 2000–2001, 494 (6.7%) of 7,307 screening participants indicated that they had been diagnosed with either SLE, scleroderma, or RA (Larson T, personal communication). In contrast, a prevalence of < 1% for these three conditions combined would be expected based on pooled estimates from 43 prevalence studies (Jacobson et al. 1997). These data, along with extensive evidence of silica-associated autoimmunity, provided the impetus to initiate a multifaceted assessment of the impact of asbestos exposures on the population of Libby, Montana, including possible autoimmune responses.

In this article we report on a study designed to assess whether there were humoral alterations in serum samples from an asbestos-exposed population (Libby samples) that might indicate autoimmune responses, providing the rationale for future full-scale studies. Serum samples of subjects from Libby and from a Montana community with no reported asbestos exposure were assayed for a variety of immune parameters, including ANA, immunoglobulin A (IgA), rheumatoid factor (RF), and antibodies to extractable nuclear antigens (ENA).

Materials and Methods

Human samples. All samples were acquired according to approved University of Montana institutional review board protocols, protecting the well-being and confidentiality of all subjects. Appropriate informed consent was obtained from all subjects, and a questionnaire was administered regarding overall health, smoking status, asbestos exposure, age, sex, and recontact information.

Two sample pools were obtained through other studies at the Center for Environmental Health Sciences. Subjects were recruited through flyers and ads in Missoula, Montana, for a study of immune function (E. Putnam), and serum samples were collected at the same time for convenience. Missoula is similar to Libby in that it is located in a mountain valley subject to similar climatic conditions, including winter inversions, dry summers, and exposure to smoke from fall forest fires. Because prevailing winds in Missoula are from the west and Libby is well to the north, there would be
no transfer of asbestos from the Libby airshed to the Missoula airshed. Therefore, on a relative basis, although one cannot exclude the possibility that there could have been some minimal asbestos exposure by the Missoula population, it is an acceptable reference population for the Libby subjects who were definitively exposed to asbestos. For this study, we selected 50 samples from subjects with no reported asbestos exposure from the Missoula pool, excluding any who had lived or worked in Libby. Concurrently, subjects were being recruited from Libby, Montana, for a genetic study of ARD susceptibility (E. Putnam), and the samples were drawn at the Center for Asbestos Related Diseases Clinic in Libby when subjects came for screening or responded to subject recruitment advertisements. From this pool, 50 Libby subjects were selected that were matched to the 50 Missoula subjects to give similar mean age and sex ratios for the two subject sets. Permission was obtained to acquire information about prescription drug use and ARD status from medical records, and these data were inserted into a coded database.

Both communities from which subjects were drawn are fairly homogeneous in terms of ethnicity; most residents are of northern European descent, with 94.7% white in Lincoln County (Libby) and 93.6% white in Missoula County (Missoula) according to Montana census data (U.S. Census Bureau 2004). The mean age for both sample sets was 55 years (Missoula, 54.8 ± 2.5 years; Libby, 55.0 ± 2.1 years), and the male:female ratio was 25:25 for both sets. An exclusion criterion was the use of medications strongly associated with drug-induced autoimmunity (Fritzler 1994). The presence of diagnosed autoimmune disease did not exclude individuals from either sample set but was noted on intake. None of the Missoula subjects recruited had diagnosed SAID. Of the Libby subjects recruited initially for the genetics study, the percentage with diagnosed SAID was 5.9%, and in the final Libby sample set of 50, there were two with SLE, 1 with multiple sclerosis, and 1 with RA. Because the individual with RA also had SLE, this means that three people in 50 had SAID (6%). These values are consistent with the ATS/DR screening data for this community (Larson T, personal communication).

The lung diseases in this community have been previously described (Peipins et al. 2003) and include primarily pleural abnormalities (17.8%) and interstitial abnormalities (<1%). In our Libby sample set, 12 (24%) had no reported abnormalities, 27 (54%) had pleural abnormalities, 8 (16%) had interstitial abnormalities, and 3 (6%) had a combination of pleural and interstitial abnormalities.

Sample and data collection. The blood samples were collected, and serum samples were obtained and frozen by standardized clinical methods to prevent differences due to handling. The samples were blinded with only sex and age noted, and stored at −80°C until assayed. After testing, coded information regarding disease status and exposure was obtained from the questionnaire and ATS/DR screening data (ATS/DR 2002).

ARD and asbestos exposure rankings. ARD status, based on data recorded in the database primarily as a result of the ATS/DR screenings, was ranked on a scale of 0–3, as described in Table 1. The rankings were intentionally simplified, based on radiographic evidence of single versus extensive plaques or interstitial abnormalities, as well as spirometry evidence of functional deficits. For example, a subject with a single pleural plaque and no functional deficit would be scored at 1, whereas a subject with bilateral plaques and effects on spirometry was scored at 3. To further break down the sample sets by disease types would have made the subsets too small for statistical analysis. Exposure status was ranked on a scale of 0–4, as described in Table 2. These scores were also simplified in order to focus on duration of exposure and the existence of occupational and/or environmental exposure to asbestos. The rankings of the Libby subjects were performed independently by two of the researchers (J.C.P. and E.A.P.).

Autoantibody testing. A clinical test for nuclear antigens (ANA assay), used to screen for antibodies commonly seen in SAID, was performed at a screening dilution of the sera. All serum samples were diluted 1:40 in phosphate-buffered saline (PBS) and tested by indirect immunofluorescence (IIF) on a single lot of commercially prepared and fixed HEp-2 cells (ImmunoConcepts Inc., Sacramento, CA), according to manufacturer’s instructions. The staining pattern and relative fluorescence intensity were compared with known positive and negative controls using a Zeiss fluorescence microscope with 40× objective and recorded as positive (1+ to 4+) or negative (0). The staining pattern was also noted and recorded. The same microscope and settings were used for all samples, and the slides were read by two independent readers. Samples showing homogeneous staining patterns were reevaluated using the Crithidia luciliae substrate (ImmunoConcepts), which specifically detects antibodies to double-stranded DNA (dsDNA), and by enzyme-linked immunosorbent assay (ELISA) to detect antibodies to chromatin (INOVA Diagnostics, San Diego, CA), both according to the manufacturers’ instructions. Samples with positive ANAs were also evaluated using a modified ANA test to determine whether any of the anti-ANA antibodies were of the IgA isotype. The samples were tested on HEP-2 slides as above, but instead of the anti-human IgG fluorescein isothiocyanate (FITC) conjugate included with the slides, we used goat anti-human IgA FITC conjugate (Southern Biotech, Birmingham, AL). The slides were read as described above.

IgA ELISA. For detection of serum IgA, the mucosal antibody isotype, 96-well polystyrene plates (Nunc, Rochester, NY) were coated with 1 µg/mL of anti-human kappa light chain (Southern Biotech) in carbonate coating buffer overnight at 4°C. Wells were then blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hr. Subject samples were diluted 1:4,000, 1:8,000, and 1:16,000 in diluent buffer (PBS, 1% BSA, 0.1% Tween-20). Samples, standards, and blanks were added to wells to give 100 µL/well. After 1 hr, plates were washed with three changes of PBS containing 0.1% Tween-20. The detection antibody [goat anti-human IgA alpha chain coupled to horseradish peroxidase (HRP); Caltag, Burlingame, CA] was added and the plates were incubated for 1 hr at room temperature. The plates were washed again and developed using HRP-tetramethylbenzidine (TMB) substrate (Zymed, San Francisco, CA). The reaction was stopped with 2N H2SO4, and the plate was read on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). All data were evaluated against a standard curve, using human IgA (Sigma, St. Louis, MO).

RF ELISA. RF in the subjects’ serum was measured with an ELISA kit according to the
manu...ctor’s protocol (INOVA Diagnostics). The plates were read on the SpectraMax plate reader. Optical density (OD) values were compared with known controls provided with the kit and rated as negative or positive (marginal, moderate, or high).

**ENA array.** Analyses of antibodies to five extractable nuclear proteins commonly seen in SAID (Sm, RNP, SS-A, SS-B, and Scl-70) were performed using an addressable bead array kit (QuantaPlex ENA-5; INOVA Diagnostics) according to the manufacturer’s instructions, on a Luminox multiplex system (MiraBio, Alameda, CA). The values were compared by using MasterPlex software (MiraBio) to negative and graduated positive control reagents provided with the kit, and determined to be low, moderate, or high positive, or negative.

**Statistics.** In this study we included analysis of several different types of data, including percentages/frequencies (e.g., ANA frequencies), ordinal (e.g., disease status assessed on a 4-point scale), and scale (e.g., mg/mL IgA) data. Consequently, we used the following statistical methods: a) differences in percentages were tested using raw frequencies with Fisher’s exact test; b) contingency tables with 4- and 5-point ordinal level frequency comparisons were made via the chi-square test; and c) independent sample t-tests were used for scale measures. In the Libby samples, comparisons (correlations) between ANA levels and disease and exposure rankings were made using the nonparametric Spearman rank correlation. In all analyses we used two-tailed, unpaired analyses, and reported 0.05 type I error levels. Data reported in the text are mean ± SEM.

**Results**

**Frequency and fluorescence intensity of positive ANAs.** The 50 serum samples in each set were tested by IIF and were determined to be positive or negative for ANA based on known controls. Figure 1A shows that the relative frequency of positive ANAs was 28.6% higher in the Libby sample set than in the Missoula set (p = 0.006). Because low-titer positive ANAs are fairly common in normal populations, the ANA slides were scored for fluorescence intensity. The scored mean fluorescence intensity of positive ANAs, rated on a scale of 1–4 against known controls, was higher in the Libby sample set (mean = 2.34 ± 0.153) compared with those from Missoula (1.76 ± 0.194; p = 0.02), and the distribution of subjects receiving the various scores was shown to be significantly different between the sample sets (p = 0.004; Figure 1B). The scored fluorescence intensity is not a direct quantification of autoantibodies but generally suggests a higher titer. Therefore, the positive samples were subsequently titered to 1:1,280 and further analyzed for ANA. In both groups, the ANA scores and titers were highly correlated, suggesting that the scoring provides a close estimation of titer (Missoula: correlation coefficient = 0.502, p < 0.001; Libby: correlation coefficient = 0.828, p < 0.001; Table 3). We found that the distribution of titers for samples in the two sample sets were significantly different (p = 0.036; data not shown). The percentage of subjects having a positive ANA at a titer ≥ 320 is shown in Figure 1C, again showing a significant difference between the two sample sets.

**IgA levels and RF.** Because other studies of asbestos-exposed populations have shown differences in serum IgA compared with unexposed subjects, serum IgA levels were analyzed by ELISA in our sample sets. The Libby samples showed significantly higher levels of serum IgA than the Missoula samples (Figure 2). Although both sample sets had mean IgA concentrations within normal ranges (~ 0.9–4.5 mg/mL), the Libby mean was at the high end of the range (4.2 mg/mL). The ANA tests were subsequently modified to detect IgA rather than IgG, and all of the ANA tests were negative in both sample sets, indicating that the autoantibodies were most likely primarily IgG and not IgA (data not shown).

RF consists of IgM or IgG antibodies directed against the constant domain of immunoglobin. These autoantibodies can lead to immune complex deposition in tissues and are associated with a variety of infectious and inflammatory disorders such as RA. The samples were evaluated for IgM RF by ELISA, and there were no differences in either the mean OD calculated for each sample set or the frequency of positive tests for RF (Figure 3).

**Staining patterns on ANAs.** To determine the specific targets for the IgG autoantibodies, we analyzed the patterns visible on the ANA tests. In the Missoula samples, a nuclear speckled staining pattern was most common, as expected in a normal population (spackled, 14%; homogeneous, 12%; nucleolar, 10%). Other staining patterns were relatively rare, as expected. However, in the Libby samples, homogeneous (indicative of antibodies to chromatin) and nucleolar staining patterns were more prevalent, although the differences were not statistically significant (spackled, 18%; homogeneous, 22%; nucleolar, 18%). We tested for specific antibodies related to these IIF patterns using an ELISA for chromatin and nuclear staining.

### Table 3. Correlation coefficients (CC) with immune parameters in the Libby cohort.

| Parameter analyzed | Age | ANA titer |
|--------------------|-----|-----------|
|                    |     |           |
| ANA score          |     | 0.399**   |
|                    |     | 0.828**   |
|                   | 50  | 50        |
| ANA titer          |     | 0.381**   |
|                   | 50  | 0.006     |
|                   | 50  | 0.399**   |
|                   | 50  | 0.828**   |
|                   | 50  | 0.381**   |
|                   | 50  | 0.006     |
|                   | 50  | 0.399**   |
|                   | 50  | 0.828**   |

*Correlation is significant at the 0.05 level (two-tailed Spearman’s rho test). **Correlation is significant at the 0.01 level (two-tailed Spearman’s rho test).

![Figure 1. Comparison of ANA in Libby samples with the Missoula samples.](image)

**Figure 2.** Serum IgA levels were significantly higher in the Libby group. Serum IgA was measured by ELISA using anti-human IgA capture and detection (HRP-conjugated) antibodies from Southern Biotech and Caltag, respectively. The samples were tested in duplicate, developed using TMB, and read on a SpectraMax plate reader. OD was compared with a standard curve (human IgA, Sigma) to calculate values (mg/mL, n = 50).

*Correlation is significant at the 0.05 level (two-tailed Spearman’s rho test). **Correlation is significant at the 0.01 level (two-tailed Spearman’s rho test).
with ARD, we tested that correlation using our scoring system. The correlation between disease and exposure was 0.239, consistent with the hypothesis (Table 4).

The analysis for asbestos exposure was performed using the graded system described in Table 2, but in looking at the data, it was apparent that the largest effect on ANA titer was seen in terms of length of exposure rather than the source (occupational or environmental). Figure 6A shows the mean ANA titers for the Libby samples when subgrouped by duration of exposure and demonstrates that the titers were significantly higher for those subjects exposed to asbestos for > 5 years. When samples were separated according to whether the subject was exposed environmentally or occupationally, no significant difference in mean ANA titer was observed in those two groups (Figure 6B). This suggests that the duration of exposure had a greater impact on autoimmune responses than the source of exposure. It should be noted that none of the subjects reported solely occupational exposures; all also had environmental exposures as well.

Discussion

By demonstrating an association between asbestos exposure and measures of autoimmune responses, this study supports and augments other existing evidence that, like silica, asbestos is an agent of systemic autoimmunity. Asbestos-contaminated vermiculite from Libby has been shipped and processed in many sites in the United States, and this material is still used in many applications. It therefore remains a significant health risk to humans both occupationally and environmentally, and an awareness of an association with autoimmunity could impact necessary monitoring, testing, and treatment regimens for exposed individuals or populations.

In addition, this study establishes the Libby population as an excellent cohort for further study of the immunologic aspects of asbestos toxicology. It is a unique population with both occupational and environmental exposures, excellent ongoing monitoring and demographic data, enthusiasm for participation in these studies, and sufficient numbers of exposed individuals to develop sample sets with adequate power for statistical analyses of many parameters. The objective of this study was to compare the frequency of serum autoantibodies in two matched populations, one of them having significant asbestos exposure. It was designed as an initial study in order to explore the feasibility and justification for a more extensive study of autoimmunity in the Libby population.

Previous studies have measured several immune parameters in populations exposed, primarily occupationally, to asbestos. Nigam et al. (1993) demonstrated increased IgG and IgA and positive ANAs in asbestos-exposed individuals compared with controls, even in the absence of apparent ARD. This finding suggested that immune alterations may precede the onset of ARD. A high frequency of positive ANAs was also found in a Japanese group of asbestos plant workers (Tamura et al. 1996); this suggests that autoimmunity may play a role in ARD. Because that important observation needs to be confirmed, the present study also forms the basis for a similar progressive study of the Libby population to explore the temporal relationship between autoimmunity and ARD. If autoimmune responses play a role in the progression of ARD, this would become an important target for therapeutic strategies.

A higher frequency of positive ANA was seen in the Libby sample set compared with the samples from Missoula, even though the...
Missoula ANA positive frequency was fairly high (40%). This high “normal” frequency may be due to the age of the population, the low dilution used (1:40) for the screening, or other unidentified population considerations. Nevertheless, the Libby sample set showed a 28.6% increase above that seen for the Missoula samples. In addition, the mean fluorescence intensity of the ANAs, as well as the titers, were higher in the Libby samples compared with the Missoula samples. Although higher titers of autoantibodies are not necessarily correlated with an autoimmune disease process, in some cases increased titers can indicate an exacerbation, relapse, or stage of an autoimmune process.

Other immune parameters showed differences in the Libby group as well, including increases in serum IgA. Serum IgA is generally found at relatively low levels, but increased levels are associated with some chronic inflammatory disorders, such as occupational lung disease, psoriatic arthritis, Crohn’s disease, and ankylosing spondylitis (Hoffman et al. 2003; Lindqvist et al. 2002; Zhestkov 2000). The significance of increased serum IgA levels in the Libby samples is not clear, but it is consistent with other studies of asbestos-exposed subjects (Nigam et al. 1993; Zerva et al. 1989). We demonstrated that the autoantibodies detected by ANA were not of the IgA isotype, so it is possible that the IgA antibodies are simply elicited by nonspecific chronic inflammatory processes in these individuals. This possibility is supported by the lack of correlation between IgA titers and either ANA or asbestos exposure; however, a correlation between IgA titers and disease status was also lacking in our analysis.

We found no difference between the two sample sets in terms of frequency of positive tests for RF, and in general the positive samples in both groups were rated marginal to moderate when compared with known controls (data not shown). There was also no correlation between RF and asbestos exposure. These results suggest that asbestos exposure is not associated with increases in RF, especially because previous studies of asbestos-exposed populations have not consistently reported elevated RF. Interestingly, we found a positive association of RF with lung disease status. This may suggest that the presence of RF is more dependent on the chronic inflammation resulting from the ARD than on asbestos exposure itself. Alternatively, because there were positive correlations between RF and both ANA titer and ARD, the threshold of asbestos exposure impacting development of RF may simply be too low to be detected using the simplified exposure scale (Table 2). Therefore, the pathophysiologic significance of these results remains to be determined with further study. Although assaying RF is a good screening tool, it is not specific for RA. An alternative test would be an assay for cyclic citrullinated peptide (CCP), a more specific epitope characteristic of autoantibodies in RA (Bombardieri et al. 2004; Saraux et al. 2003) because CCP can antedate clinical RA.

The Libby sample set had a significantly higher number of total positive ENA subjects (24% vs. 4% in the Missoula group). ENAs are defined target antigens in a variety of autoimmune diseases, including systemic lupus, mixed connective tissue disease, systemic sclerosis, and polymyositis (Pahor et al. 1998). In HEP-2 cells, most of these antibodies produce a speckled or atypical speckled pattern. Therefore, our combined data suggest that the major responses in asbestos-exposed individuals include antibody development to targets such as chromatin components (i.e., histone) or nucleolar components (fibrillarin, DNA topoisomerase I), which give the homogeneous and nucleolar patterns, and several ENAs that give the speckled pattern. There are autoantibodies—other than those detected by the assay used here—that could give the speckled patterns seen in both populations. Analysis of a more comprehensive array of autoantigens may provide more insight into the spectrum of autoantibodies related to asbestos exposure.

The presence of autoantibodies does not necessarily suggest a disease process. However, to begin to explore a possible role of these antibodies in the health of the population in Libby, data regarding the extent of ARD severity were gathered as described in “Materials and Methods.” Extent of exposure included both duration and site of exposure (work vs. recreational/home). The data showed that individuals with longer exposures, and especially if exposed both at work and at home, had higher ANA titer than those with shorter exposures (<5 years). Disease status considered the extent of fibrosis or plaquing, as well as functional deficits. Again, the data showed increasing ANA titer with increasing disease severity. The stronger correlation between ANA titer and disease than between ANA titer and exposure may be due to imprecise scoring systems. Exposure and ARD are assumed to be correlated (Peipins et al. 2003), even though this correlation was only at the 90th percentile using our scoring systems. Therefore, these data do not exclude the possibility that the correlation of ANA titer with exposure may be secondary to the chronic inflammation and tissue damage of the associated ARD. It is interesting to note, however, that of those 494 participants from the Libby Tremolite Asbestos Registry cohort with suspected SAID, 171 (35%) have had pleural and/or interstitial abnormalities indicated on chest radiographs and confirmed by two B-readers (Larson T, personal communication). These cross-sectional findings suggest that the proportion of radiographic abnormalities among participants with suspected SAID (35%) was almost double the proportion of

### Table 4. Correlation coefficients (CC) between immune parameters and scores both of asbestos exposure and of asbestos-related disease.

| Parameter analyzed | Exposure | ARD |
|--------------------|----------|-----|
| Age                |          |     |
| CC                 | -0.015   | 0.304* |
| No.                | 51       | 49  |
| ANA score          |          |     |
| CC                 | 0.252    | 0.295* |
| Significance (two-tailed) | 0.074 | 0.040 |
| No.                | 51       | 49  |
| ANA titer          |          |     |
| CC                 | 0.366**  | 0.392** |
| Significance (two-tailed) | 0.009 | 0.006 |
| No.                | 50       | 48  |
| RF (OD)            |          |     |
| CC                 | 0.129    | 0.389* |
| Significance (two-tailed) | 0.410 | 0.010 |
| No.                | 43       | 43  |
| Exposure           |          |     |
| CC                 | 1.00     | 0.239 |
| Significance (two-tailed) | 0.098 | 0.598 |
| No.                | 49       |     |
| IgA                |          |     |
| CC                 | -0.167   | -0.090 |
| Significance (two-tailed) | 0.283 | 0.567 |
| No.                | 43       | 43  |

*Correlation is significant at 0.05 (two-tailed Spearman’s rho test). **Correlation is significant at 0.01 (two-tailed Spearman’s rho test).
radiographic abnormalities among the entire Libby cohort (~ 18%) (Peipins et al. 2003). Further study is required to determine if, and how, autoimmune responses are related to ARD and whether autoimmunity influences ARD progression.

**Conclusion**

We have established that there are significant differences in frequency and titer of positive ANA tests, frequency of positive ENA tests, and higher levels of serum IgA when an asbestos-exposed Libby cohort was compared with one from Missoula with no reported asbestos exposures. We have also shown significant associations between asbestos exposure and ANA titer. These data support the hypothesis that asbestos exposure is associated with autoimmune responses. The correlations of ARD with ANA titer, ANA score, and RF suggest the possibility that autoimmunity could play a role in the progression of ARD. This study provides the foundation and justification for a larger and more extensive study, planned to explore the hypothesis that asbestos exposure and autoimmune responses are related to ARD progression.

**Autoimmunity**

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