Autoantibodies and cancer among asbestos-exposed cohorts in Western Australia

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Running title: Autoantibodies among asbestos-exposed cohorts

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Abstract

Asbestos exposure is associated with many adverse health conditions including malignant mesothelioma and lung cancer as well as production of autoantibodies. Autoantibodies may serve as biomarkers for asbestos exposure in patients with cancer, and autoimmune dysfunction has been linked to increased rates of various cancers. The aim of this study was to examine the hypothesis that autoantibodies are more frequent in asbestos-exposed individuals with either lung cancer or mesothelioma than those without these conditions. Asbestos-exposed individuals from Western Australia who had lung cancer (n=24), malignant mesothelioma (n=24), or no malignancy (n=51) were tested for antinuclear autoantibodies (ANA) using indirect immunofluorescence and specific extractable nuclear autoantibodies (ENA) employing a multiplexed addressable laser bead immunoassay. Contrary to the hypothesis, data demonstrated that individuals without malignancy were more likely to be positive for ANA compared to those with cancer. However, autoantibodies to histone and Ro-60 were found to be associated with lung cancer. These results support a possible predictive value for specific autoantibodies in the early detection of lung cancer and/or in our understanding of the role of autoimmune processes in cancer. However, further studies are needed to identify specific target antigens for the antibodies.

Keywords: Asbestos, autoimmune antibodies, cancer, exposure, lung cancer, malignant mesothelioma

Introduction

Many studies have shown a link between autoimmunity and cancer (reviewed in Abu-Shakra et al. 2001). The innate and acquired immune systems are tasked with identifying and destroying malignant cells; dysfunction of the immune system affects its ability to perform this role and may even facilitate the growth of malignant cells (Azrielant et al. 2017). Accordingly, immune dysfunction in the context of autoimmunity has been linked to increased rates of various types of cancer (Giat, Ehrenfeld, and Shoenfeld 2017), and antinuclear autoantibodies (ANA) have been detected in sera of patients with cancer (Abu-Shakra et al. 2001). The association between autoimmune dysfunction and cancer varies by cancer type, autoimmune disorder, and population (Giat, Ehrenfeld, and Shoenfeld 2017). Environmental factors, including exposure to agents such as asbestos and silica, may also impact susceptibility to cancer and autoimmune disease, as well as the relationship between the two (Ferro et al. 2014; Lee and Lawrence 2018).

Associations between asbestos exposure and autoantibody responses were previously reported (Pernis, Vigliani, and Selikoff 1965; Pfau et al. 2018), with an elevated frequency of ANA (Pfau et al. 2005; Pfau, Serve, and Noonan 2014; Reid et al. 2018) and an increased risk for systematic autoimmune diseases (SAID; Bunderson-Schelvan et al. 2011; Noonan et al. 2006; Pfau, Serve, and Noonan 2014) observed in asbestos exposed cohorts. Studies in Libby, Montana USA found a higher relative frequency of positive ANAs among an asbestos-exposed compared with a reference population (Pfau et al. 2018; Pfau et al. 2005). Similarly, elevated odds of ANA positivity were detected among those exposed to asbestos in Wittenoom, Western Australia, compared with an unexposed reference population (Reid et al. 2018). These investigators also demonstrated higher ANA titers in the asbestos-exposed compared with reference populations (Pfau et al. 2018; Pfau et al. 2005; Reid et al. 2018). Both of these cohort studies examined populations that were exposed to an amphibole asbestos, Libby Amphibole in Libby and crocidolite or blue asbestos in Wittenoom. Amphibole asbestos types possess fibers that are long and straight, more readily inhaled, and thus the most carcinogenic form (Pfau,

Serve, and Noonan 2014). To date, a link between elevated ANA and cancer rates in the Wittenoom population has not been investigated. It is possible that autoantibodies may serve as early markers to detect or predict the severity of adverse health outcomes in asbestos-exposed populations (Pfau et al. 2019), making this study a critical step in understanding their potential as markers in cancer identification.

In order to determine whether asbestos-associated cancers were associated with ANA, this study aimed to investigate autoantibody profiles among asbestos-exposed cohorts in Western Australia. Due to the links between autoimmunity and cancer, and between asbestos and both autoimmunity and cancer, it was postulated that those with an asbestos-related cancer (malignant mesothelioma or lung cancer) might exhibit a higher frequency of ANA than those, also exposed, without a cancer diagnosis.

Materials and methods

Participants

Serum samples were analyzed from three groups of asbestos exposed individuals: 24 diagnosed with lung cancer, 24 diagnosed with malignant mesothelioma, and 51 without malignancy. Samples were randomly selected within those three groups among participants in the Asbestos Review Program (ARP). The ARP was established in 1990 and monitors over 4,000 individuals who have been exposed to asbestos through either working or living at the Wittenoom asbestos mine in Western Australia or who have been otherwise occupationally exposed to asbestos (minimum of three months occupational exposure) (Armstrong et al. 1988). Participants are able to attend the program on an annual basis. Participants from Wittenoom were exposed solely to amphibole asbestos (crocidolite), while others were exposed to a mixture of amphibole and chrysotile asbestos. Asbestos fiber type was controlled for in all analyses.

Sera were collected in serum separation tubes, processed using standardized protocols, then stored at -80°C without repeat freeze/thaw cycles (Reid et al. 2018). For lung cancer and mesothelioma cases, the serum sample taken closest to (and prior to) the date of diagnosis was selected. For non-cancer subjects, the most recent serum sample was analyzed. There were no significant differences between groups in terms of time sera spent in the freezer. Demographic details including age, gender, smoking status, and asbestos exposure metrics (i.e. cumulative exposure, time since first exposure, exposure duration) were all recorded. Comprehensive information on how estimates of asbestos exposure were derived have been published elsewhere (Armstrong et al. 1988). All participants provided informed consent. This study was approved by the Curtin University Human Research Ethics Committee.

Antinuclear autoantibody (ANA) assays

ANA reactivity was determined by indirect immunofluorescence (IIF) using HEp-2000 slides and FITC reagent from ImmunoConcepts (Sacramento, CA USA) following the manufacturer's standard

protocols. Serum samples were diluted 1:80 in phosphate buffered saline. Positivity and negativity were determined using controls provided by the manufacturer, and only nuclear staining patterns (not cytoplasmic or mitotic) were considered positive. Positive samples were further diluted to determine ANA titer. Titers were classified into low (1:160 and 1:320) and medium-high (1:640 and above) for analysis. The pattern of ANA staining was determined based upon ICAP codes AC1-14 and AC29 (https://anapatterns.org/index.php); samples with cytoplasmic or cytoskeletal staining were classified as ANA-negative.

Immunoassays were performed by the Mitogen Diagnostics Laboratory (Calgary, Alberta, Canada). The levels of antibodies against 13 nuclear antigens were evaluated: dsDNA, Sm, histone (H2A, H2B, H3, H4), Jo-1 (histidyl tRNA synthetase), ribonucleoprotein (RNP), ribosomal P protein, proliferating cell nuclear antigen (PCNA), SSA/Ro60, SSB/La, Ro52/TRIM21, PM-Scl, Scl-70 (topoisomerase 1), centromere B (CENP-B). This multiplexed extractable nuclear antibody (ENA) profile utilised an addressable laser bead immunoassay (ALBIA) provided by TheraDiag (FIDIS: Paris, France). Cutoffs were established using internal calibrators provided by the manufacturers and control sera included with each assay run. Results were expressed as chemiluminescence intensity units (CIU) for ALBIA.

Statistical analysis

Cumulative asbestos exposure (f/ml-year) was non-normally distributed and transformed using $\log_{10}$ transformations. Individual antibody levels were transformed using $\log_e(x+1)$ transformations. Univariate statistical tests were used to investigate differences in demographic and exposure variables and ANA status by cancer diagnosis (no diagnosis, lung cancer, mesothelioma). Logistic regression models determined whether cancer diagnosis predicted ANA status. A series of linear regression models examined differences in levels of 13 individual antibodies by cancer diagnosis. Gender, age, smoking status, asbestos fiber type, and $\log_{10}$ transformed cumulative asbestos exposure were entered as covariates in all regression models. The criterion for significance was set at $p<0.05$.

Results

Demographics

The 99 participants included 70 males and 29 females (Table 1). There were no significant differences in terms of gender, age, or asbestos fiber type between groups. Lung cancer patients were significantly more likely to be current and ever smokers than either those with mesothelioma or with no cancer diagnosis. This may be attributed to the synergistic relationship between smoking, asbestos, and lung cancer (Klebe et al. 2019). Mesothelioma patients exhibited significantly higher total cumulative exposure; when separated by gender, this difference was only significant among males. There were no significant differences between groups in terms of exposure duration or time since first asbestos exposure.

Antinuclear autoantibodies (ANA)

Approximately one-third (n=32, 32.3%) of samples were positive for ANA. Males (n=27, 38.6%) were significantly positive for ANA compared to females (n=5, 17.2%). After controlling for gender, there was no marked association between ANA status and age, smoking status, asbestos fiber type, or cumulative exposure. A significantly larger number of samples from individuals without cancer (n=23, 45.1%) were positive for ANA than those with cancer (n=9, 18.7%). Those with no cancer diagnosis displayed 5-fold higher odds of being positive for ANA (OR=5.0, 95% CI 1.7-14.1) than those with cancer. When investigated by cancer type, both lung cancer (OR=0.2, 95% CI 0.1-0.7) and mesothelioma patients (OR=0.2, 95% CI 0.1-0.8) were less likely than those without cancer to be positive for ANA.

Over half of all samples positive for ANA (n=18, 56.2%) exhibited a speckled pattern (ICAP code AC-4,5) and a further one-third of positive samples (n=11, 34.4%) exhibited a homogeneous pattern (AC-1). The remaining samples exhibited centromere (AC-3; n=1, 3.1%), discrete nuclear dot (AC-6; n=1, 3.1%), and homogeneous nucleolar (AC-8; n=1, 3.1%) patterns.

Only 4 samples yielded cytoplasmic or mitotic staining (3 cytoplasmic, 1 mitotic). Including these as ANA positive in the analysis did not markedly change the results: subjects without cancer were still more significantly ANA positive. For those with cytoplasmic staining, 1 had mesothelioma while the other 2 did not. The individual with mitotic staining also displayed mesothelioma.

The majority of positive ANA samples had a low titer (1:160 to 1:320; n=28, 87.5%). Among positive samples, ANA titer differed significantly by cancer diagnosis, with a higher prevalence of low titer positive samples among those without a cancer diagnosis (n=22, 95.6%) and a higher prevalence of medium to high titer (1:640 to 1:5120) positive samples among those with lung cancer (n=2, 50%).

Differences in Extractable Nuclear Antigen (ENA) autoantibody levels

After controlling for gender, age, smoking status, asbestos fiber type, and cumulative exposure, cancer diagnosis was a significant predictor of levels of anti-histone and anti-Ro60>SSA, with mean levels of both being significantly higher in lung cancer patients compared to those without a cancer diagnosis (Figure 1). Gender did not markedly predict levels of either autoantibody, and autoantibody levels did not differ significantly by gender.

Discussion

Autoantibodies have been associated with both asbestos exposure (Pfau et al. 2018; Reid et al. 2018) and various cancers (Giat, Ehrenfeld, and Shoenfeld 2017). Therefore, autoantibodies can be explored as markers of disease (Pfau et al. 2019) or markers of asbestos exposure (Pfau et al. 2018). The current study is an extension of our previous work demonstrating an increase in positive ANA tests in the Wittenoom cohort of crocidolite exposures (Reid et al. 2018), with the hypothesis that some ANA autoantibodies might be involved in the development of the predominant diseases of this cohort (mesothelioma and lung cancer) and would therefore be markers for increased risk of cancer after exposure. If this were the case, more individuals with these cancers would have ANA autoantibodies. Contrary to the hypothesis, data demonstrated that individuals without malignancy were more likely to be positive for ANA compared to those with lung cancer or malignant mesothelioma. It was also found

that males were more likely to be positive for ANA than females, although gender did not emerge as a significant predictor of ANA status in the regression analysis.

Previous investigators reported that, in general, autoimmune diseases and autoantibodies are more prevalent in females than males (Dinse et al. 2016; Dinse et al. 2020; Ngo, Steyn, and McCombe 2014). Indeed, previously Reid et al (2018) investigating the prevalence of ANA positive results in Wittenoom workers and ex-residents found a 2-fold greater risk of being ANA positive among females than males. However, Pollard (2012) ntoed that autoimmune diseases associated with environmental exposures, including occupational exposures to agents such as silica and solvents, were more likely to show a higher proportion of males. This observed gender paradox may be attributed to exposure being greater among males, rather than autoimmunity itself (Pollard 2012). This is similar to findings reported for the cohort exposed to Libby Amphibole in Montana, USA, where positive ANA tests were not predominant in females, and males were more likely to have had occupational exposure (Pfau et al. 2005). This is consistent with the current study results, which showed that when controlling for asbestos exposure, gender did not predict ANA status.

There was also no marked association between ANA status and age. Commonly, the frequency of positive ANA tests increases with age (Solomon et al. 2002), as in a large US sample selected from the National Health and Nutrition Examination Survey (NHANES) (Satoh et al. 2012). Previously Reid et al (2018) in a Western Australian study compared an asbestos-exposed population with an unexposed reference population, and data demonstrated that increasing age was associated with the frequency of ANA positive results in unadjusted analyses; however, when controlling for other variables, no marked difference in prevalence by age was detected. Again, this suggests that there is an environmental factor triggering these positive ANA tests in younger individuals.

In this study, the relationship between ANA and asbestos associated cancers was examined, since amphibole asbestos exposures were shown to induce ANA (Pfau et al. 2005), and because of some evidence that autoimmune responsiveness is associated with, and may play roles in, development of

some cancers (Chapman et al. 2008; Macdonald, Parsy-Kowalska, and Chapman 2017; Noble et al. 2016). Anti-nuclear autoantibodies are strongly associated with, and sometimes diagnostic for, systemic autoimmune diseases including systemic lupus erythematosus (SLE), systemic sclerosis, Sjōgren Syndrome, and mixed connective tissue disease. Several investigators reported that ANA also occur in the serum of cancer patients (Abu-Shakra et al. 2001; Tan 2012; Vlagea et al. 2018), and the possibility that these autoantibodies may be related to DNA damage and cancer etiology was proposed (Noble et al. 2016; Vlagea et al. 2018). However, the plethora of different autoantibody specificities and challenges in their detection, plus inconsistencies with different cancer types, has made it difficult to test this hypothesis. A standardized method of ANA detection, the HEp-2000 indirect immunofluorescence test, was employed due to its reliability in detecting a wide range of autoantibodies, particularly in asbestos-exposed populations. The ALBIA method was also utilized to detect specific autoantibody targets associated in general with ANA in systemic autoimmune diseases (SAID), including those associated with amphibole exposure (Diegel et al. 2018).

The current study found that those without a cancer diagnosis were significantly (5-fold) more likely to be positive for ANA than cancer patients. This was true for both lung cancer and mesothelioma patients. While it is widely held that ANA might occur pre-clinically (Li et al. 2011), the specific cytokine environment plays a key role in their development. In some cancer patients, autoantibodies appeared only while individuals were on interferon therapy for their cancers (Abu-Shakra et al. 2001; Valencia, Egbukichi, and Erwin-Cohen 2019). Therefore, it is unclear whether there is an etiological relationship between ANA and cancer, or whether they co-exist in some patients due to the inflammatory phases of disease. While the *frequency* of positive ANA tests was lower in patients with cancer, the *titers* of positive ANA were higher in lung cancer patients compared to non-cancer subjects. It is possible that autoantibodies need to reach a certain threshold titer before they are significantly pathogenic. Noble et al (2016) suggested that ANA might enter cells and inhibit DNA repair mechanisms or directly induce DNA damage. While the existence of cell-penetrating ANA is

widely accepted, it is not thought that this phenomenon is common, nor is the frequency of cell penetration known. Therefore, it may be that ANA titers need to be high before there are adequate numbers of cell-penetrating antibodies.

Certain autoantibodies that are common in myositis and scleroderma appear with higher frequency in patients who have concurrent or go on to develop cancer, making them potential biomarkers for risk of cancer among autoimmune patients (Betteridge et al. 2018; Fiorentino et al. 2013; Igusa et al. 2018). However, some studies aver that autoantibodies do not occur frequently in cancer patients without a co-existing autoimmune disease (Betteridge et al. 2018; Igusa et al. 2018), such that they are poor predictors of cancer outcomes in non-autoimmune patients. Autoantibodies to RNA polymerase were also shown to occur in scleroderma patients with cancer, but no predictability for cancer in patients without autoimmune disease (Betteridge et al. 2018; Parker et al. 2008). Therefore, in asbestos-exposed patients without autoimmune disease, testing for specific SAID-associated autoantibodies may not be valuable on its own for prediction of lung cancer. In addition, ANA testing by indirect immunofluorescence is not recommended for some of these antibodies (e.g. certain RNA polymerases and myositis-associated autoantibodies) that are inconsistently expressed in the cell (Ceribelli et al. 2012).

For these analyses, ANA positive was strictly defined as sera exhibiting a nuclear staining pattern. However, the presence of cytoplasmic staining patterns was also determined. While cytoplasmic staining patterns with indirect immunofluorescence testing tend to be less valuable for diagnosis of some SAID such as myositis, they are valuable in other diseases such as SLE and systemic sclerosis. Studies of lung cancer detected autoantibodies that might produce cytoplasmic, rather than nuclear, staining patterns such as those common in myositis (Betteridge et al. 2018; Garcia-De La Torre 2015; Palterer et al. 2018). Anti-NXP autoantibodies are of particular interest since this antibody was elevated in patients exposed to Libby Amphibole (Pfau et al. 2019). However, only 4 patients in this study displayed cytoplasmic or mitotic staining, and including them in the analyses as ANA positive

did not markedly change the overall results. There were 3 with cytoplasmic staining, and only 1 had cancer (mesothelioma). Therefore, antibodies giving a cytoplasmic pattern were not predictive for cancer.

Because the ANA test is a general screening test that does not provide information regarding specific antigens, multiplexed ALBIA was also used to detect specific nuclear antigens. The current study found a higher mean level of two antibodies, namely antibodies to histone and Ro60, among lung cancer patients as compared to those without a cancer diagnosis. Anti-Ro60/SSA was reported to be associated with increased risk of cancer among lupus patients, particularly lymphomas and breast carcinoma (Bockle et al. 2012). One study demonstrated the expression of the antigen Ro60/SSA in pancreatic ductal adenocarcinoma tissue, and this expression enhanced cell proliferation in cancer cells (Liu et al. 2018). Because the Ro60/SSA protein is over-expressed in cancer cells, and that expression is involved in cancer aggression, Liu et al (2018) suggested that anti-Ro60/SSA autoantibodies may be driven by the cancer itself. To our knowledge, no study has specifically determined the expression of Ro60/SSA antigen in lung cancers or mesothelioma.

Anti-histone antibodies were also previously found in sera of patients with cancer (Abu-Shakra et al. 2001). In fact, in anti-histone H2B detected carcinomas, lung cancer at a rate of 37% and cervical cancer at 78% was reported (Kamei et al. 1992). A monoclonal antibody known to be specific to adenocarcinoma cells was shown to bind histone H2B (Kato et al. 1991). The test used in the current study did not specify which histone was detected, so further testing needs to determine whether the anti-histone antibody detected in the lung cancer patients was specific to H2B.

As mentioned above, autoantibodies can be studied as potential markers for cancer or for exposure to asbestos. Autoantibodies have long been associated with mesothelioma, with hopes that these would prove to be therapeutic or predictive biomarkers for this disease (Robinson et al. 2000; Zhang et al. 2013). However, none of these antibodies yielded specific or reliable staining on the standard indirect immunofluorescence HEp-2 cell (a laryngeal carcinoma cell line) testing used here.

Many of them are specific to the cancer, as upregulated proteins in, or expressed by, the cancer cells. Although mesothelioma was shown to be an immunogenic cancer, inducing an array of antibodies, to date none of them has been identified as a reliable marker for this cancer (Creaney et al. 2016). The presence of ANA in patients without cancer in the current study suggests that ANA may instead be primarily a marker of asbestos exposure. Autoantibodies are produced when proteins are modified by oxidative or enzymatic processes, leading to altered autoantigens to which the immune system responds (Borelli et al. 2018; Nagai et al. 2011; Rosen and Casciola-Rosen 1999). Indeed, Nagai et al (2011) reported that histones in particular are bound to asbestos fibers and undergo oxidative modifications. This suggests a mechanisms whereby histones may become immunogenic. Anti-histone antibodies have been shown to be markers for exposure to Libby Amphibole (Pfau et al. 2019), but an analysis of the relationship between autoantibodies and exposure for this cohort will require a different study design and is beyond the scope of this study. Further, the small sample size of the current study is acknowledged, and future research could explore the significant associations found in a larger sample.

Conclusions

Positive ANA tests were more frequent among individuals without cancer, opposing the hypothesis. However, this may be due to the fact that ANA test is a broad screening test for many autoantibodies, possibly masking the presence of a specific marker. There was an association between cancer and anti-histone autoantibodies. This autoantibody was previously shown to predict exposure to Libby Amphibole (Pfau et al. 2019), making this of particular interest for studies of asbestos exposure. There are several specific histone proteins, and antibodies to histone H2B (included in the ALBIA) are associated with some cancers, including lung cancer. Therefore, future studies are needed to determine the specific target histone in this population and follow up on its specificity for asbestos exposure and/or asbestos related cancers.

Funding: This work was supported by Cancer Council Western Australia.

Declaration of interest: The authors declare that there is no conflict of interest.

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Table 1. Demographic and exposure data by cancer diagnosis

	Non-malignant		Lung cancer		Mesothelioma		*p* value
N (females/males)	51 (15/36)		24 (8/16)		24 (6/18)		.817[a]
Mean age [b] (SD)	67.9 (8.3)		68.4 (7.4)		70.0 (7.9)		.603[c]
Females, mean age (SD)	63.4 (10.6)		63.7 (9.5)		67.8 (8.5)		.587[d]
Males, mean age (SD)	69.8 (6.3)		70.8 (5.0)		70.7 (7.8)		.608[c]
Smoking status [b]							
Ever smokers (n, %)	36 (70.6)		20 (83.3)		12 (50.0)		.041[a]
Current smokers (n, %)	2 (4.0)		6 (25.0)		0 (0.0)		.002[a]
Asbestos fibre type							.939[a]
Amphibole (crocidolite) (n, %)	32 (62.7)		16 (66.7)		15 (62.5)		
Mixed amphibole and chrysotile (n, %)	19 (37.3)		8 (33.3)		9 (37.5)		
	Mean	*Range*	*Mean*	*Range*	*Mean*	*Range*	
Total cumulative exposure (log (f/mL-years)) [f]	1.0	0.0-27.6	1.4	0.0-71.5	5.1	0.0-192.3	.002[g]
Females, total exposure	0.9	0.0-12.6	0.9	0.0-38.7	6.8	0.5-50.6	.173[g]
Males, total exposure	1.0	0.0-27.6	1.7	0.0-71.5	4.7	0.0-192.3	.017[g]
Time since first exposure [b] (years)	47.9	32-67	48.4	36-61	49.9	36-74	.851[g]

[a] Pearson's Chi-squared test
[b] At collection date
[c] One-way ANOVA
[d] Equality of medians test
[e] Fisher's Exact Test
[f] Log_{10} transformed; therefore anti-log results presented
[g] Kruskal-Wallis test

Figure 1. Levels of histone-H4 (left) and Ro60 (right) by cancer diagnosis. Each plot contains 6 box plots corresponding to the diagnoses by gender (from left, female lung cancer, male lung cancer, female mesothelioma (meso), male mesothelioma (meso), female no cancer diagnosis, male no cancer diagnosis). Dots represent outliers (values more than 1.5-fold the interquartile range).(Tukey 1977)