Bacterial Biofilm Infection Detected in Breast Implant–Associated Anaplastic Large-Cell Lymphoma

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Background: A recent association between breast implants and the development of anaplastic large-cell lymphoma (ALCL) has been observed. The purpose of this study was to identify whether bacterial biofilm is present in breast implant–associated ALCL and, if so, to compare the bacterial microbiome to nontumor capsule samples from breast implants with contracture.

Methods: Twenty-six breast implant–associated ALCL samples were analyzed for the presence of biofilm by real-time quantitative polymerase chain reaction, next-generation sequencing, fluorescent in situ hybridization, and scanning electron microscopy, and compared to 62 nontumor capsule specimens.

Results: Both the breast implant–associated ALCL and nontumor capsule samples yielded high mean numbers of bacteria (breast implant–associated ALCL, 4.7 × 10^6 cells/mg of tissue; capsule, 4.9 × 10^6 cells/mg of tissue). Analysis of the microbiome in breast implant–associated ALCL specimens showed significant differences with species identified in nontumor capsule specimens. There was a significantly greater proportion of *Ralstonia* spp. present in ALCL specimens compared with nontumor capsule specimens (*p < 0.05*). In contrast, significantly more *Staphylococcus* spp. were found associated with nontumor capsule specimens compared with breast implant–associated ALCL specimens (*p < 0.001*). Bacterial biofilm was visualized both on scanning electron microscopy and fluorescent in situ hybridization.

Conclusions: This novel finding of bacterial biofilm and a distinct microbiome in breast implant–associated ALCL samples points to a possible infectious contributing cause. Breast implants are widely used in both reconstructive and aesthetic surgery, and strategies to reduce their contamination should be more widely studied and practiced. (Plast. Reconstr. Surg. 137: 1659, 2016.)

Clinical Question/Level of Evidence: Risk, V.

A recent association between breast implants and anaplastic large-cell lymphoma (ALCL), a subtype of CD4+, T-cell lymphoma, has been recently observed.1 An increasing number of reports have now suggested an etiologic relationship between ALCL and breast implants (termed breast implant–associated ALCL)2–5 (Fig. 1). Compared with classic systemic ALCL, breast implant–associated ALCL behaves in a more indolent manner and is divided into two pathologic varieties: the seroma-type, consisting of effusions with an encapsulating margin of CD30+ malignant lymphocytes; and the less common mass-type, with a more clinically aggressive infiltrating tumor.6–9 The U.S. Food and Drug Administration has
called for notification and further investigation of this phenomenon.\textsuperscript{10}

We have previously shown that contamination of breast implants with bacterial biofilm results in the development of capsular contracture, visible distortion, and pain, necessitating revision surgery.\textsuperscript{11} These findings have been further supported by clinical and experimental studies.\textsuperscript{12,13} Furthermore, we have shown that implants with a textured surface support a higher bacterial load\textsuperscript{14} and that there is a linear relationship between the number of bacteria and lymphocytic hyperplasia\textsuperscript{15} on infected breast implants. Implants with a textured outer shell were introduced in the late 1980s to improve tissue incorporation. Interestingly, textured implants are overrepresented in patients that develop breast implant-associated ALCL.\textsuperscript{3,7} We postulate that chronic bacterial biofilm infection around breast implants is an inflammatory trigger producing chronic lymphocyte activation, hyperplasia, and potential transformation into breast implant–associated ALCL. We aimed to analyze tissue from breast implant–associated ALCL for the presence of bacterial biofilm and, if detected, compare the bacterial species profile (microbiome) to that found in nontumor capsule specimens.

**PATIENTS AND METHODS**

Twenty-six breast implant–associated ALCL samples were gathered from four centers for this international collaborative study from a total of 22 patients (Table 1). In addition, three samples were obtained from the contralateral normal breast capsule in ALCL patients from one center (M. D. Anderson Cancer Center). Nontumor capsule specimens from 62 patients undergoing revision surgery for high-grade capsular contracture collected over a 5-year period from six centers were included for comparative analysis. The presence of bacterial biofilm was determined using a combination of methods outlined below.

**Bacterial Load**

Samples (50 to 100 mg) from breast implant–associated ALCL \((n = 26)\) and nontumor capsules \((n = 62)\) were digested using a combination of proteinase K and lysozyme, and the genomic DNA was extracted using phenol/chloroform extraction followed by ethanol precipitation as described previously.\textsuperscript{16} Total bacterial DNA in each sample was determined by real-time quantitative polymerase chain reaction of the 16S rRNA gene using eubacterial universal primers.\textsuperscript{16}

The 18S rRNA gene (GenBank NR_003286.2) was used as a reference to normalize the amount of ALCL/capsule tissue used in DNA extraction.\textsuperscript{16} The total number of bacteria was expressed per milligram of tissue based on the average number of copies of the 18S rRNA gene in human tissue. The calculation of total bacteria number in each sample was based on an average of five copies of 16S rRNA gene per bacterial cell.

The presence of *Ralstonia* spp. was confirmed independently by real-time quantitative polymerase chain reaction using *Ralstonia* spp.–specific primers *Ralstonia*\_16sF (5′-GGCCTCATGCTATAGGAGC-3′) and *Ralstonia*\_16sR (5′-TACTGATCGTCGCTTGGTG-3′) designed by 16S rRNA gene sequence alignment using Clustal Omega service in the European Bioinformatics Institute\textsuperscript{17} and SeqMatch service in Ribosomal Database Project II.\textsuperscript{18}

Real-time quantitative polymerase chain reaction was carried out in 25 μl of reaction mix containing 1× Brilliant II Sybr Green QPCR Master mix (Agilent Technologies, Inc., Santa Clara, Calif.), 400 nM forward and reverse primer, and 100 ng of DNA template with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds as described previously.\textsuperscript{16} Each real-time quantitative polymerase chain reaction was run with standard samples of known concentrations ranging from $10^2$ to $10^8$ copies/μl.

**Bacterial Community Profiling**

Bacterial tag-encoded FLX amplicon pyrosequencing was used to determine all of the bacterial species present in samples from 19 breast implant–associated ALCL samples, 12 nontumor capsule...
samples, and three samples taken from capsules of the contralateral breast of women with breast implant–associated ALCL. Pyrosequencing of the V1 to V3 regions of the 16S rRNA gene was commercially performed by Molecular Research (MR DNA, Shallowater, Texas) laboratory using the titanium platform (Roche, Basel, Switzerland) as described previously. Pyrosequencing data were analyzed by QIIME software (Werner Lab, Cortland, N.Y.) and bacterial species identified using the Ribosomal Database Project II database.

The composition and relative abundance of bacterial species (microbiome) for specimens were compared, and statistical differences between the relative abundance of each bacterial species were determined using one-way analysis of variance.

### Scanning Electron Microscopy

Nontumor capsule specimens were fixed in 3% glutaraldehyde. Breast implant–associated...
ALCL samples were cut from formalin-fixed, paraffin-embedded blocks and deparaffinized by immersion in xylene. All samples were dehydrated in ethanol, followed by immersion in hexamethyldisilazane (Polysciences, Inc., Warrington, Pa.) as described previously. Dried samples were coated with 20-nm gold film in a sputter coater and examined in a scanning electron microscope.

**Fluorescent In Situ Hybridization**

Bacterial aggregates in breast implant–associated ALCL samples and nontumor capsule specimens were detected and located by fluorescence in situ hybridization using Cy3 fluorescent dye–labeled bacterial universal probes EUB338 (5′-GCTGCCTCCGTAGGAGT-3′) \(^{21}\) and FAM fluorescent dye–labeled Ralstonia spp.–specific probe Ralstonia 185 (5′-GGCCTATGCTATAG-3′) (Life Technologies, Carlsbad, Calif.). The Ralstonia spp.–specific probe was designed by 16S rRNA gene sequence alignment using the Clustal Omega service in the European Bioinformatics Institute\(^{17}\) and the SeqMatch service in the Ribosomal Database Project II.\(^{18}\) Fluorescent in situ hybridization was performed essentially according to the method described by Thurnheer et al. with 5 ng/μl Cy3 EUB338 probe, 20 ng/μl FAM Ralstonia spp.–specific probe, and 10% formamide concentration in hybridization buffer. Fluorescent in situ hybridization images were examined under confocal laser scanning microscopy.\(^{14}\)

**Statistical Analysis**

The \(t\) test was used to compare the number of bacteria by real-time quantitative polymerase chain reaction in breast implant–associated ALCL versus nontumor capsule or the contralateral breast tissue, and the Mann-Whitney rank sum test was used to compare the age of patients and the time since implantation using Sigma-Plot11 statistical program (Systat Software, Inc., San Jose, Calif.). Samples where human 18S rRNA gene did not augment were excluded. The statistical analysis of the 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing data was performed by QIIME scripts,\(^{20}\) Calypso software (http://bioinfo.qimr.edu.au/). Alpha diversity was calculated using the Shannon index and OUT Richness in QIIME. The default number of Monte Carlo permutations was used to calculate the \(p\) values, and the significance threshold was \(p < 0.05\).

**RESULTS**

**Clinical Features**

Table 1 lists the clinical summary data from each of the 22 patients with breast implant–associated ALCL included in the study. The mean patient age was 52.7 years (range, 29 to 77 years) and the mean duration of time between insertion of implants and diagnosis of ALCL was 8.8 years (range, 3 to 25 years).

In two breast implant–associated ALCL patients (University of Southern California), multiple samples were obtained from varying locations within the tumor for analysis. The total number of samples available for analysis was 26. In three patients, some of the corresponding clinical and operative data were not available. Of the 20 patients with a known indication, 10 had implants for postmastectomy reconstruction, whereas the remaining 10 had implants for cosmetic augmentation. Twelve patients (63 percent) presented with a unilateral malignant effusion, whereas four (21 percent) presented with a tumor mass. One patient presented with a combination of tumor and effusion and one patient presented with disseminated subcutaneous disease. In one patient the diagnosis of breast implant–associated ALCL was an incidental finding while undergoing implant exchange for capsular contracture. In 13 patients, the type of implant being removed was recorded. All of these implants had a textured outer shell (Table 1).

Information on treatment of breast implant–associated ALCL was available in 19 patients. Six patients (32 percent) were treated with capsulectomy and removal of implants. One patient (BRCA-positive) chose additional prophylactic mastectomies and one patient chose replacement of her breast implants at the time of capsulectomy and implant removal. Eleven patients (57 percent) received adjuvant therapy (Table 1). All patients remain disease free at a median of 2.2 years (range, 0.7 to 8.1 years) after primary treatment.

Patients with nontumor capsule had a mean age of 43.8 years (range, 33 to 72 years) and a mean implantation time of 7.4 years (range, 2 to 28 years). The nontumor capsule patients were significantly younger than ALCL patients (\(p < 0.01\)). There was no significant difference in duration of implantation between the two groups (\(p = 0.1\)).

**Bacterial Load**

Five of the breast implant–associated ALCL samples were negative for bacteria by real-time...
quantitative polymerase chain reaction. In all five samples, the real-time quantitative polymerase chain reaction of the human 18S rRNA gene was inhibited by a factor of at least 100, suggesting that polymerase chain reaction inhibitors existed in these samples. One negative sample was one of two samples obtained from different areas of the same tumor. In the second sample, both the human 18S rRNA gene and the bacterial 16S rRNA gene augmented normally. The remaining 21 breast implant–associated ALCL samples and 62 nontumor capsule specimens yielded large quantities of bacterial 16S rRNA gene, normalized to human 18S rRNA. The mean number of bacteria detected was $4.7 \times 10^6$ bacteria/mg of tissue for breast implant–associated ALCL ($n = 21$) and $4.9 \times 10^6$ bacteria/mg of tissue for nontumor capsule specimens ($n = 62$). Three samples taken from the contralateral breast in patients with breast implant–associated ALCL yielded $7.6 \times 10^5$ bacteria/mg of tissue, which was significantly lower than detected in their breast implant–associated ALCL tissue ($p = 0.035$).

**Scanning Electron Microscopy**

Seven breast implant–associated ALCL and three contralateral samples with adequate material were subjected to scanning electron microscopy. All samples demonstrated transformed lymphocytes and bacterial biofilm (Fig. 2).

Eighteen capsular samples with adequate material were subjected to scanning electron microscopy. All samples demonstrated bacterial biofilm (Fig. 3).

**Bacterial Community Profile**

The microbiome could be determined for 19 breast implant–associated ALCL samples and was compared to 12 nontumor capsule specimens and three capsule samples taken from patients with ALCL on the contralateral breast. Figure 4 summarizes the bacterial species diversity (microbiome) and its differences among the three groups.

In both breast implant–associated ALCL and contralateral breast specimens, a significant predominance of *Ralstonia* spp. was seen ($p < 0.05$). By contrast, *Staphylococcus* spp. was seen in significant predominance in nontumor capsule specimens ($p < 0.001$) (Fig. 5).

**Fluorescent In Situ Hybridization**

Eleven breast implant–associated ALCL samples were subjected to fluorescent in situ hybridization analysis to demonstrate the presence of *Ralstonia* spp. Fluorescent in situ hybridization was able to visualize bacteria in 10 of 11
samples. The single sample that was negative was also negative for bacteria by real-time quantitative polymerase chain reaction. *Ralstonia* spp. was confirmed in five of these 10 samples (Fig. 6). *Ralstonia* spp. was also identified using pyrosequencing in all of these samples.

**Exclusion of Environmental Contamination**

Individual breast implant–associated ALCL samples were fixed and paraffin embedded with or without sectioning at separate time points and at separate locations, limiting the possibility of cross-contamination. In addition, the Macquarie University laboratory environment was sampled and excluded potential contamination of specimens with *Ralstonia* spp. from an external source.

**DISCUSSION**

The so-called infectious hypothesis to explain the genesis of capsular contracture is now widely accepted and has been corroborated by animal and human studies. The concept of chronic viral and bacterial infections resulting in other lymphomas is well established. The finding of high bacterial load, present as a biofilm, in breast implant–associated ALCL samples is novel. The high numbers of bacteria in breast implant–associated ALCL are comparable to the numbers found around nontumor capsule samples in this study and reported previously around high-grade capsular contracture. We are aware of some anecdotal reports suggesting that bacteria have not been detected in both breast implant–associated ALCL and nontumor capsule specimens. A recent article by Poppler et al. reported a paucity of bacteria in tissue expanders removed before implant placement for breast reconstruction. In a commentary on this article, Deva has outlined the many reasons why false-negative results occur when trying to detect biofilm around implants.

The microbiome in the breast implant–associated ALCL samples is significantly different from the microbiome of the nontumor capsule samples tested here and different from previously identified bacteria present in breast implant contracture. The presence of *Ralstonia* spp. in these samples is an unexpected finding. *Ralstonia* spp. are nonfermenting Gram-negative bacilli found in soil and water. *Ralstonia* spp. have been reported in nosocomial infections resulting from contamination of medical solutions (e.g., water for injections, aqueous chlorhexidine solution) and are being increasingly recognized as a pathogen causing serious soft-tissue and implant-related infections. *Helicobacter pylori* is also classed as a nonfermenting Gram-negative bacillus. *H. pylori* has been implicated in causing gastric lymphoma and produces a number of virulence factors that have relevance to lymphomagenesis. In *H. pylori* infection, chronic inflammation of the gastric mucosa secondary to infection has been cited as a major contributor to the pathogenesis of gastric marginal zone lymphoma. More recently, virulence factors from *H. pylori* such as the cytoxin-associated gene A protein have been shown to deregulate intracellular signaling pathways and promote lymphomagenesis. The lymphoma arising from *H. pylori* infection is of B-cell origin, and it is interesting to speculate why breast implant–associated ALCL is of T-cell origin. The underlying mechanism could be related to chronic bacterial antigen stimulation, and preliminary results have shown similar activation of T-cell oncogenes (*JunB*, *SATB1*, and *SOCCS3*) in both breast implant–associated
ALCL and activated lymphocytes around capsular contracture.\textsuperscript{38} It is thus biologically plausible that chronic antigenic stimulation from bacteria coating breast implants may lead to development of a T-cell lymphoma.

The development of breast implant–associated ALCL is likely to be a complex process resulting from interplay of host, implant, and microbial factors, including bacterial phenotype, immune response, the patient’s genetic background, and the textured implant surface. This may explain why some patients with biofilm infection around breast implants proceed to contracture and why others (far less commonly) proceed to lymphocytic hyperplasia and breast implant–associated ALCL. It may also account for the variation in the clinical behavior of patients with breast implant–associated ALCL who have other phenotypic or immunologic risk factors for the development of malignancy. In our series, the mean age of presentation with ALCL was older than patients with nontumor capsules and comparable to

Fig. 4. (Above) Microbiome analysis from breast implant–associated ALCL, contralateral breast capsule from breast implant–associated (BIA) ALCL, and capsular contracture showing significant species differences in bacteria identified between capsular specimens and specimens taken from breast implant–associated ALCL patients (including contralateral breast samples). (Below) Scatter analysis of main species identified showing significant differences in prevalence of \textit{Staphylococcus} spp. versus \textit{Ralstonia} spp. among breast implant–associated ALCL, contralateral breast samples, and capsular contracture samples. The size of the plot represents the percentage abundance of specific species in each sample.
previous reported series. Age-related immune changes might also play a role in genesis of breast implant–associated ALCL. There are a number of other observations regarding the epidemiology of breast implant–associated ALCL that could be explained by bacterial infection. The paucity of cases in Europe may relate to differing rates of biofilm contamination, local variations in the microbiome, and/or effective antiinfective strategies practiced by European surgeons. In Australia and New Zealand, by comparison, there are a higher number of breast implant–associated ALCL cases being reported, with a similar preference for textured implants. We are in the process of examining these cases.

Interestingly, analysis of the capsules from the contralateral breast in patients with breast implant–associated ALCL in this study revealed a similar microbiome to that surrounding the tumor samples but with significantly fewer bacteria. This is consistent with our previous finding of a linear correlation between the number of activated lymphocytes and the number of bacteria. It is likely that the threshold for inflammation and/or lymphocyte proliferation would be determined not just by number of infecting biofilm bacteria but also by the species of biofilm bacteria. There are likely to be two pathways of inflammation: for Gram-positive biofilm, a pathway toward inflammation and fibrosis leading to capsular contracture; and for Gram-negative (e.g., *Ralstonia* spp.), a pathway toward lymphocyte stimulation and/or transformation. More study of the relationship between bacterial biofilm, lymphocytes, and the local breast environment is needed to clearly elucidate possible pathways from biofilm infection to the development of malignancy.

The detection of bacteria in our breast implant–associated ALCL samples, however, was subject to a number of limitations. The tumor material sent for analysis was preserved in formalin and/or mounted on slides following formalin fixation. The detection and identification of bacteria in fixed tissue is less sensitive than analysis of fresh tissue. In addition, a limited portion of the tumor was subjected to biofilm analysis, increasing the risk of sampling error. The finding of nearly ubiquitous bacteria in these breast implant–associated ALCL samples could therefore be an underrepresentation of the true numbers of bacteria in and around the tumor. The detection of polymerase chain reaction inhibitors was also factored into our analysis.

The use of a combination of detection techniques has reduced the likelihood that these findings were to the result of sample contamination.
from an external source. Furthermore, we have shown good correlation among \textit{Ralstonia} spp. identified in pyrosequencing, polymerase chain reaction, and fluorescent in situ hybridization with visual confirmation of bacteria on scanning electron microscopy.

A number of observations about breast implants, biofilm, and breast implant–associated ALCL now point to an underlying infective stimulus. The observation that textured implants are associated with the majority of breast implant–associated ALCL cases\textsuperscript{3,7} is consistent with our findings that textured implants support a higher biofilm load\textsuperscript{14} and produce a significant T-cell hyperplasia directly in proportion to that load.\textsuperscript{15} Where recorded, textured implants were used in all of our breast implant–associated ALCL patients. In addition, as textured implants were first introduced in the late 1980s and popularized in the 1990s, the emergence of breast implant–associated ALCL some 10 years after this is consistent with the time frame for chronic biofilm infection to produce inflammation, immune activation, and subsequent transformation. Further evidence to support the underlying inflammatory origin includes the dependence of breast implant–associated ALCL cell culture lines on interleukin 2.\textsuperscript{2} Our finding that these breast implant–associated ALCL samples have significant contamination with bacterial biofilm is further evidence supporting an infective etiologic contribution. The differences in microbiome detected between breast implant–associated ALCL and contracted specimens suggest that some bacterial species (e.g., \textit{Ralstonia}, \textit{Helicobacter}) may be more likely to trigger lymphocytic proliferation and transformation in susceptible hosts. It is important, however, to acknowledge that these data only show an association of bacterial biofilm in breast implant–associated ALCL samples and a significant difference in microbiome between breast implant–associated ALCL and nontumor capsule specimens. To prove that bacteria are causative for breast implant–associated ALCL will be difficult in view of the rarity of the transformative event. We plan to further study the potential of \textit{Ralstonia} spp. to activate lymphocytes using our established laboratory models and build the case for an infective trigger.

**CONCLUSIONS**

More importantly, the potential for bacteria to trigger lymphocyte proliferation and/or transformation does reinforce the need to develop and disseminate strategies for the prevention of bacterial infection of breast implants. We have previously published a 14-point intraoperative plan that aims to reduce breast implant contamination at the time of insertion\textsuperscript{12} with the aim of ultimately reducing
contracture. All surgeons should adopt these strategies into their routine practice as a matter of priority. Furthermore, all patients with breast implants should be enlisted into a program of ongoing surveillance, and new technologies that protect and prevent breast (and other) implants from infection should be the focus of ongoing research.

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