

A broad screen for targets of immune complexes decorating arthritic joints highlights deposition of nucleosomes in rheumatoid arthritis

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Deposits of Ig and complement are abundant in affected joints of patients with rheumatoid arthritis (RA) and in animal models of RA in which antibodies are demonstrably pathogenic. To identify molecular targets of the Igs deposited in arthritic joints, which may activate local inflammation, we used a combination of mass spectrometry (MS) and protein microarrays. Immune complexes were affinity-purified from surgically removed joint tissues of 26 RA and osteoarthritis (OA) patients. Proteins complexed with IgG were identified by proteomic analysis using tandem MS. A striking diversity of components of the extracellular matrix, and some intracellular components, copurified specifically with IgG from RA and OA tissues. A smaller set of autoantigens was observed only in RA eluates. In complementary experiments, IgG fractions purified from joint immune complexes were tested on protein microarrays against a range of candidate autoantigens. These Igs bound a diverse subset of proteins and peptides from synovium and cartilage, different from that bound by normal serum Ig. One type of intracellular protein detected specifically in RA joints (histones H2A/B) was validated by immunohistology and found to be deposited on the cartilage surface of RA but not OA joints. Thus, autoantibodies to many determinants (whether deposited as "neoantigens" or normal constituents of the extracellular matrix) have the potential to contribute to arthritic inflammation.

autoantibodies | histones | mass spectrometry | proteomics

Rheumatoid arthritis (RA) has long been known to be associated with autoantibodies (autoAbs), most notably rheumatoid factor (RF). However, ascribing pathological relevance to RF has been problematic because of the poor specificity (only 80–90%) of this Ab for RA, as well as to the complexity of rheumatoid synovitis, which features numerous immune and nonimmune cell types interacting in a complex fashion. The venerable hypothesis that autoAbs play an important role in the pathogenesis of RA has been reinvigorated by several recent developments: (i) the identification of autoAbs to citrulline-containing proteins/peptides (anti-CCP), which have greater specificity for RA than does RF (1); (ii) the demonstration that the B-cell-depleting mAb rituximab is effective in treating established RA (2); and (iii) the development of several mouse models that, despite very different autoAb targets, nevertheless entail similar pathology resembling that of RA (3, 4).

Promotion of inflammation by autoAbs in RA is likely to extend well beyond RF and anti-CCP. The majority of RA patients have one or both of these Abs, but $\approx 20\%$ do not. More importantly, substantial minorities of RA patients have circulating Abs to type-II collagen (5), GPI (6–8), and cartilage glycoprotein Gp39 (9); the arthritogenic potential of autoimmunity to all of these antigens has been shown in animal models

(9–11). Furthermore, simultaneous assessment of many Ab specificities using autoantigen microarrays has revealed multiple reactivities in serum from most patients (12, 13).

Studies of the rheumatoid joint have suggested that Abs mediate pathology locally. IgG and complement component C3 are found together on the cartilage surface, in the synovium, and within phagocytes (14, 15). Complement activation occurs locally in inflamed joints in RA (16, 17). Little is known about whether the RA-associated autoAbs listed above are deposited in synovial tissues. IgG has been extracted from rheumatoid cartilage and been shown to retain RF activity as well as reactivity to type-II collagen (18). RF and anti-CCP Abs are both enriched in synovial fluid and synovium (19, 20) relative to serum, but whether this enrichment is a result of deposition or local synthesis is unclear, as evidence for the latter has been obtained for both of these Abs (19). Deposition of IgG, C3, and the target antigen (GPI) occur prominently on both the cartilage surface and in the synovium in the K/BxN mouse model, in which autoAbs are directly pathogenic (10, 21, 22). Likewise, in murine arthritis induced by injection of Abs to type-II collagen, IgG and C3 are deposited on the cartilage surface in affected joints (23).

The role of IgG, if any, in osteoarthritis (OA) is poorly understood. Although OA is not thought of as an autoimmune disease, it is now generally recognized to have an inflammatory component (24), as evidenced by synovitis and by inflammatory cytokines in the synovial fluid. The source of inflammation in OA remains unclear. IgG deposition has been detected in OA tissues, albeit at lower levels than in RA (18, 25), and with a linear rather than granular pattern of staining (15).

Thus, because (i) RA is associated with immune complex (IC) deposition in the joint, (ii) similar complexes form in mouse models and appear enriched for the pathogenic autoAbs, and (iii) the autoAbs that actually deposit in the joint may not be accurately mirrored in the circulating serum pool, the composition of IC deposited in diseased joints remains an important and unresolved question, as it was when the problem was last approached 20 to 30 years ago (18, 26). Techniques for protein

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identification and analysis of Ab reactivity have advanced dramatically since then. We therefore applied these approaches to determine the molecular nature of IC in RA and OA joints, combining microarray profiling of joint Abs with a proteomic analysis of Ig-bound antigens.

Results

Analysis of Autoantigens Complexed to Ig in Joints. Our goal was to identify the targets of Ig in the joints of arthritic patients, pursuing the hypothesis that these autoAbs may partake in RA pathogenesis. We used an unbiased screening strategy, extracting IC (IgG and proteins bound to it) from joint tissues of RA patients and identifying these proteins by microcapillary HPLC linked to a tandem MS optimized for identification of peptides within complex mixtures. Because IC appear to be deposited in the synovium as well as in cartilage (14), both types of tissue were analyzed. These tissues were collected as anonymous surgical discards from patients undergoing joint arthroplasty or synovectomy [supporting information (SI) Table S1]. Because normal joint tissues are rarely removed surgically and were not available to us, we used tissues from patients with OA as comparators, with the caveat that OA often features some degree of local inflammation and that IgG has been extracted from OA cartilage, albeit at lower levels than from RA cartilage (18). All together, 23 samples from 18 RA patients and 13 samples from 8 OA patients were analyzed.

Qualitatively, immunofluorescence staining of the cartilage and synovial samples confirmed the surface deposition of IgG, C3, and IgM (see Table S1 and Fig. S1A), not only in all tested patients with RA but also in most with OA. To isolate bound IC, we extensively washed joint tissues to minimize contamination with uncomplexed IgG or loose IC. Tissues were then digested with collagenase to release Ig/antigen complexes, using a protocol gentle enough to preserve Ab reactivity and preformed complexes (18). These complexes were then affinity-purified on proteinG-Sepharose. As a control for nonspecific binding, an equal volume of extract was processed in parallel on nonderivatized Sepharose.

This protocol resulted in recovery of IgG in most cases (see Table S1 and Fig. S1B). One cartilage sample was frozen and sectioned after this digestion/elution process; qualitatively, no residual staining for IgG was seen, suggesting that the great majority of IgG had been successfully eluted. Although levels were quite variable, more IgG was obtained from RA (median 6.9 $\mu\text{g/ml}$) than OA (median 0.6 $\mu\text{g/ml}$) tissues ($P = 0.004$; Mann-Whitney U test). As expected, control columns yielded IgG levels below the assay detection limit in all cases; therefore, they were typically at least 40- to 1,000-fold lower than the yield from proteinG columns.

Eluates from proteinG and control columns were reduced and amidated, digested with trypsin, and fractionated on a microcapillary reverse-phase HPLC column linked to a tandem MS for determination of peptide mass and amino acid sequence. An example of the analytical strategy is shown for one RA sample in Fig. 1. Data on peptide masses and fragmentation patterns were used to interrogate a comprehensive database of predicted tryptic peptides using Mascot software. Peptides derived from IgG, IgM, IgA, complement, trypsin, keratins, and proteinG were ignored. Peptides not derived from these proteins (mean 28 ± 22.4 , median 24.5, per run) were studied in detail. Peptide assignments determined as likely to be correct based on a Mascot score >25 were confirmed by manual verification of fragmentation spectra.

The identities of the antigens found in joint IC are summarized in Table 1, with a full listing in Table S2, which illustrates the diversity of proteins detected. Overall, 43 known human proteins were identified, 24 of them in more than 1 independent sample. Known proteins were identified in 17 out of 23 RA samples and

9 out of 13 OA samples. As expected, significantly more proteins were detected in proteinG-purified samples (range 0–15 per sample, mean 3.7 ± 3.9 , median 2) than in controls (range 0–5, mean 0.9 ± 1.3 , median 0; $P < 0.0001$; Wilcoxon paired test). Those proteins found in both proteinG and control samples (see Table 1, *Bottom*) seemed likely to be nonspecific contaminants and were not studied further.

We focused on the proteins detected in more than 1 experimental sample (the significance of an isolated finding being difficult to assess), which fell into 3 separate groups (see Table 1). Nine proteins were seen in both RA and OA IC and were detected relatively frequently (2–12 of 36 samples). Many of these proteins were abundant components of the extracellular matrix of cartilage. Five proteins were seen only in IC from RA samples, while only 1 was seen in OA eluates but not in RA eluates. In contrast to most of the proteins seen in both RA and OA samples, RA-specific and OA-specific proteins included only 1 matrix component (proteoglycan 4), instead including 3 types of protein that are normally intracellular: histones H2A and H2B, major vault protein, and pyruvate kinase. We found no evidence that either the RA-specific or other antigens represented citrullinated proteins (see *SI Text*). These results suggest that IgG is bound, specifically or nonspecifically, to a substantial fraction of the major components of extracellular matrix in both RA and OA, but that additional antigens are bound in RA of more diverse origin and more variability among patients.

Determining the statistical significance of such complex distributions poses a challenge, as the infrequent detection of any single peptide is not significant in itself. Thus, we applied a Monte Carlo resampling approach to computationally estimate the significance of the overall distribution of proteins identified, testing how frequently a bias of magnitude comparable to that of the actual distribution was observed among 1,000 simulated datasets produced by sampling randomly from a pool of proteins, with frequencies taken from the overall data. According to this simulation, the occurrence of 15 proteins identified in proteinG-purified but not control eluates was highly significant ($P < 0.004$) (Fig. S2A). Similarly, the identification of 5 proteins in RA but not OA eluates represented significant skewing ($P < 0.045$) (see Fig. S2A).

These results indicate that the proteins we detected were truly copurifying with IgG, suggesting that IC in arthritic joints involve a wide variety of proteins. We estimated the overall breadth of proteins involved in joint IC by using a statistical technique originally developed to calculate the species diversity in an ecosystem from limited data sampling, with a logarithmic fit of the number of unique species observed as a function of the number of samplings. As illustrated in Fig. S2B, the number of proteins identified among our 23 RA samples is far from reaching the asymptote, and extrapolating the fitted curve suggests that up to 100 species may be involved in joint IC among all RA patients considered as a group. Whether the variability seen among the samples in our study represents true biologic differences in IC composition among patients, as opposed to inefficient sampling and identification, cannot be distinguished.

In summary, the joints of human arthritic patients contain Ig complexed to a wide variety of proteins, with many ligands seen in both OA and RA and others apparently RA-specific.

Analysis of AutoAb Reactivities Present in Joints. The MS analysis identified some autoantigens bound to Abs in arthritic joints. As an independent and complementary approach, we asked the reciprocal question: what molecular targets could be specifically bound by Igs eluted from the joint? This question has been addressed in the past (18, 26), but the recent development of technology to simultaneously assess Ab binding to many antigens in a microarray format (12, 13) allowed us to measure the binding of joint-eluted IgG to a substantial number of proteins (and

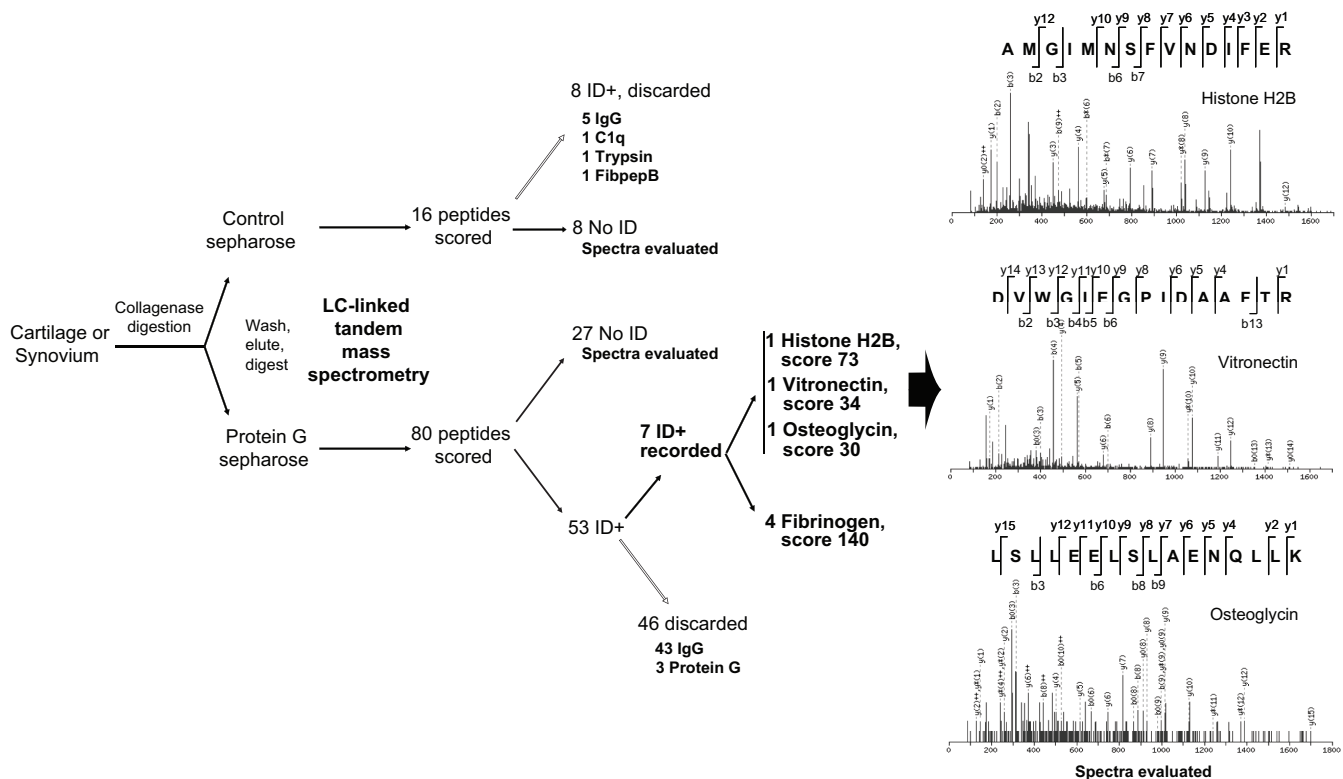


Fig. 1. A typical example of MS analysis of a synovial extract. Synovium from a RA patient was surgically excised, digested with collagenase, and the supernatant divided in half to incubate with proteinG Sepharose or control Sepharose. After extensive washing of the beads, bound material was eluted, reduced and digested exhaustively with trypsin, and loaded onto a microcapillary HPLC system linked to a Q-TOF tandem MS. The remaining peptides detected in the mixtures were analyzed for mass, fragmented, and the ions resulting from fragmentation detected. Data on the masses of each tryptic peptide and of its fragmentation ions were compared to those predicted for all tryptic peptides from all proteins in the NCBI nr database. Among peptides showing reasonable similarity to those in the database (80 in the proteinG eluate and 16 in the control eluate), approximately half were derived from IgG or predictable contaminants, such as keratin, proteinG, or trypsin (ID+, discarded), and many additional peptides did not closely match any known human or human pathogen sequence (no ID). Seven peptides (ID+, recorded) from the proteinG eluate-matched human sequences: 4 derived from fibrinogen and 1 each from histone H2B, vitronectin, and osteoglycin. Visual inspection of the fragmentation spectra for the latter 3 peptides confirmed the assignments, as most masses corresponded closely ($\delta < 0.02$) to masses predicted to be formed by the predominant mode of fragmentation ("b" and especially "y" series).

epitopes derived from them). A summary of the 503 proteins and peptides tested on these microarrays can be found in [Table S3](#). Included were 307 previously reported autoantigens (12 chosen on the basis of binding by serum autoAbs in RA (e.g., fibrinogen, vimentin, and gp39) and 196 antigens added because of their detection in the MS analysis reported above [e.g., histones H2A and H2B, cartilage oligomeric matrix protein (COMP), and osteoglycin]. Overlapping peptides were synthesized with and without substitution of citrulline for arginine. For the selection of peptides derived from large proteins, we focused on regions containing the peptides identified by MS, on the rationale that Ab-bound epitopes would be more likely to have been protected from proteolysis *in vivo* (27) and might thus be relatively enriched in tissue eluates. The arrays were probed with a subset of the IgG-enriched eluates that had been used for MS analysis, limited to those samples that had yielded sufficient IgG to allow probing with 1- μ g IgG per array: 11 RA samples, 3 OA samples, and 2 samples from a patient with psoriatic arthritis (see [Table S1](#)). To distinguish Ig specificities unique to the joint, as opposed to those naturally present in normal Ig, we compared the results with those obtained using Ig from 22 serum samples from healthy volunteers.

Fig. 2 graphically illustrates the results of these microarray experiments, which are further detailed in [Dataset S1](#). The normalized signal intensity for each probe is shown in a pseudocolor representation, with the probes ordered according to their differential intensities comparing joint-derived IgG to normal

circulating Ig (differential estimated by a Wilcoxon rank sum test). Statistical analysis using the Significance Analysis of Microarrays (SAM) algorithm (28) gave similar results (see [Dataset S1](#)). This representation clearly delineated a spectrum of antigens that were preferential targets for IgG eluted from joints relative to normal serum IgG. These preferential reactivities were highly significant (55 peptides at $P < 10^{-5}$, equivalent to $P < 0.02$ after a conservative Bonferroni correction for multiple sampling). Thus, a broad array of targets was bound by joint IgG, consistent with the wide representation of antigens uncovered by the MS analysis.

The second conclusion that stands out from the representation of Fig. 2 is that, as a whole, the reactivities that most distinguish joint IgG from normal serum IgG are shared between different arthritis states, whether RA or non-RA. At first glance, the spectra are indistinguishable. This observation was consistent with the results of the previous MS screen, in which antigens found in both RA and OA IC were more numerous than the RA-specific ones. Importantly, however, reactivities preferentially found in IgG eluted from joints were significantly skewed toward those protein antigens specific for RA joint IC. For example, 13 of the top 50 and 28 of the top 100 reactivities distinguishing joint IgG (considering all eluates or only RA eluates) from normal serum IgG represented antigens detected only in RA joint IC by MS (mostly histone-derived peptides: 13 and 26, respectively) compared with 0 of the bottom 50 and 5 of the bottom 100 reactivities ($P < 0.0001$), or compared with 65

Table 1. Summary of proteins copurifying with IgG extracted from joint tissues

Group*	Protein [†]	Eluate [‡]								
		RA cartilage		RA synovium		OA cartilage		OA synovium		Control
		+/total	# pep	+/total	# pep	+/total	# pep	+/total	# pep	+/total
RA and OA	Osteoglycin	1/11	1	6/12	1	3/8	1–2	2/5	1–2	0/36
	COMP	4/11	1–3	0/12		5/8	1–6	1/5	1	0/36
	Fibrinogen	0/11		4/12	1–11	2/8	1–4	1/5	2	0/36
	Clusterin	3/11	1–5	1/12	2	3/8	2–3	1/5	2	0/36
	Vitronectin	3/11	1–4	2/12	1	2/8	1–2	0/5		0/36
	Lumican	0/11		3/12	1–3	1/8	2	3/5	1–3	0/36
	Biglycan	3/11	1	0/12		1/8	1	0/5		0/36
	Tenascin-C	2/11	2	0/12		3/8	1–3	0/5		0/36
	Prolargin	1/11	2	0/12		1/8	2	0/5		0/36
RA only	Histone H2A or H2B	0/11		3/12	1–2	0/8		0/5		0/36
	Proteoglycan 4	1/11	3	1/12	1	0/8		0/5		0/36
	Major vault protein	0/11		2/12	1	0/8		0/5		0/36
	Pyruvate kinase	2/11	1–2	0/12		0/8		0/5		0/36
	Ferritin	1/11	2	1/12	1	0/8		0/5		0/36
OA only	Apolipoprotein E	0/11		0/12		2/8	2	1/5	2	0/36
Found in control	Fibromodulin	4/11	1–3	0/12		5/8	4–6	0/5		1/36
	Fibronectin	8/11	1–18	2/12	3–4	5/8	3–15	1/5	1	5/36
	Collagen VI	0/11		2/12	1	0/8		0/5		1/36
	Serine protease 11	1/11	1	0/12		2/8	1	1/5	2	1/36
	CILP	1/11	3	2/12	1	0/8		1/5	1	2/36
	Actin	0/11		1/12	1	0/8		0/5		1/36
	Aspartate transaminase	1/11	3	0/12		0/8		0/5		1/36
	Albumin	1/11	1	4/12	1–7	1/8	1	1/5	5	11/36
	Cathepsin B	0/11		0/12		0/8		0/5		2/36

*All proteins detected in 2 or more samples are shown (after exclusion of Ig, complement proteins, and keratins), grouped on the basis of their detection in proteinG-purified extracts of RA and OA joint tissues, or of their detection in any sample prepared using control beads.

[†]COMP, cartilage oligomeric matrix protein; CILP, cartilage intermediate layer protein

[‡]Results are expressed as the number of samples in which a given protein was detected, over the total number of samples of that type (RA or OA, cartilage or synovium, or control sepharose eluates); # pep, number of different peptides detected from a given protein (range).

out of 503 antigens on the array ($P \leq 0.007$ for each pairwise comparison). Several antigens appeared to show preferential reactivity to RA compared with non-RA eluates (see [Dataset S1](#)), but these differences did not reach statistical significance after conservative adjustment for multiple comparisons; power to detect such differences, however, was quite limited by the

availability of only 5 non-RA eluates (3 OA and 2 psoriatic arthritis) with sufficient IgG for testing.

Thus, the microarray analysis of IgG reactivities in joint eluates confirmed the results obtained by MS of IC: a wide array of antigens was bound by IgG from inflamed joints, and this finding was true of both RA and OA joints. This pattern of broad reactivities shared between different arthritis groups may also be complemented by less frequent reactivities more specific to RA, in particular against histones.

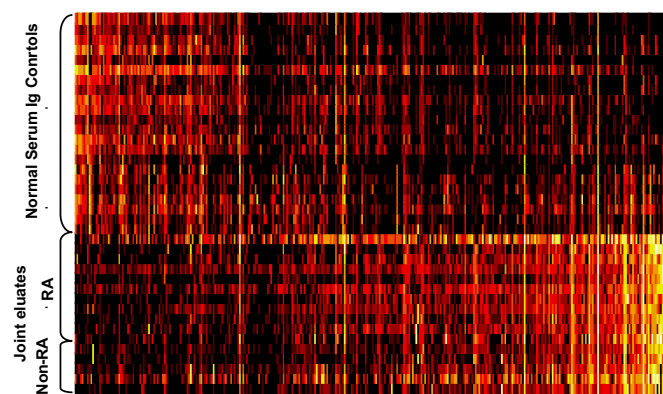


Fig. 2. Binding of joint-eluted IgG vs. normal serum Ig to antigens on a microarray. IgG purified from 16 individual joint extracts from RA and non-RA patients was evaluated for binding to 503 antigens (listed in [Table S3](#)) in a microarray format as in [Materials and Methods](#). Comparison was made with 22 normal sera. Colors denote amount of binding: undetectable (black); progressively greater binding (red, yellow, white); full range of scale represents approximately a 1,000-fold difference. Complete numerical data are shown in [Dataset S1](#). Antigens are ordered by difference (fold change) between mean binding by joint-eluted vs. control Ig. Increased binding to 55 antigens was apparent at a level of $P < 10^{-5}$.

Validation of AutoAb Reactivities Uncovered in Joints. To validate at least some of the Ab reactivities uncovered in joint IC, we performed immunofluorescent staining on sections of joint tissues, attempting to detect antigens in extracellular deposits. Among the antigens detected by MS of joint-eluted IC, histones H2A and H2B drew our attention because of their apparent specificity for RA joints and the binding of several of their peptides by joint-eluted Ig. Because these histones form the core of the nucleosome, it was also of interest to determine whether these higher-order structures were deposited. In a preliminary screen, cartilage surfaces from two RA patients stained not only with polyclonal Abs and mAbs recognizing separate histones or DNA, but also with an mAb (PL2–6) directed against an intact H2A/H2B/DNA nucleosomal complex (29). Therefore, a larger-scale immunohistological analysis of human arthritic cartilage was undertaken using this mAb. H2A/H2B/DNA was readily detected on 8 out of 9 RA cartilages, compared with 0 out of 19 OA or posttrauma controls (Fig. 3, [Table S4](#)). H2A/H2B/DNA material was found on a substantial fraction of the cartilage surface, as was IgG; colocalization was not absolute, as expected given the diversity of IC in the joints that we had found previously. Thus, because histones can be found deposited in

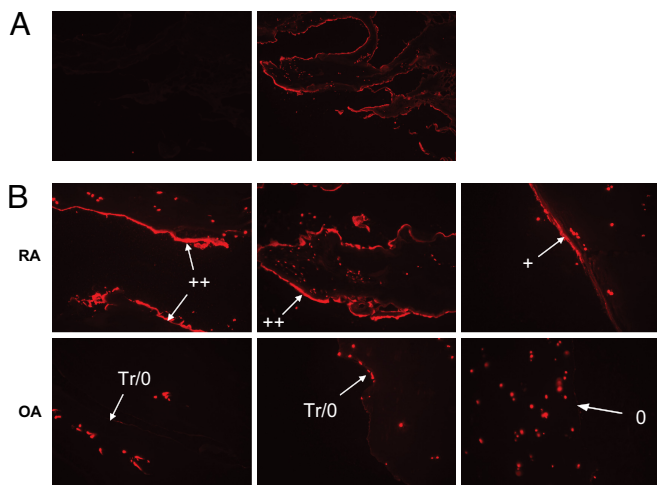


Fig. 3. Immunofluorescence staining of human cartilage samples with a mAb recognizing a complex of H2A/H2B/DNA. Frozen sections were prepared and stained as in *Materials and Methods*. (A) Staining of a RA cartilage sample with an isotype control mAb (Left) or anti-H2A/H2B/DNA (Right) is shown (10 \times objective) followed by Texas-red-conjugated goat anti-mouse-IgG. Staining with this control mAb was done in parallel for all samples and was undetectable in all cases. (B) Three examples each of RA (Top) and OA (Bottom) cartilage samples stained with anti-H2A/H2B/DNA are shown (20 \times objective). Arrows denote the cartilage surface and mAb staining is apparent in red. Examples include the full range of intensities seen, using qualitative descriptions (+++, +, Tr/0, 0) as summarized in Table S4. Additional punctate staining seen in all samples represents staining of chondrocyte nuclei, which served as an internal technical positive control. All images have been modified equally, to offset the dimming effect of shrinking them.

immune complexes in the joints of RA but not OA patients, immunohistological analysis validated, for histone antigens at least, the conclusion of the MS and microarray analyses.

Discussion

Broad methods of protein identification and quantifying Ab reactivities have allowed an unprecedented analysis of IC extracted from cartilage and synovial surfaces of human arthritic joints. Our results revealed unexpected profiles of antigens participating in IC. Most such antigens were abundant extracellular proteins and were associated with IgG in both RA and OA joints. IgG eluted from RA and OA joints contained highly overlapping reactivities that differed greatly from that of normal circulating IgG. Analyses of both IC and the reactivities of joint-eluted IgG suggested that a smaller subset of antigens was RA-specific and included histones H2A and H2B. In support of this notion, deposition of nucleosomal material in RA but not OA joints was verified by histology.

The detection of IC in OA joints and the sharing of self-antigen targets with RA might seem surprising if one were to consider OA as a purely mechanical disorder. Yet, IgG is clearly deposited in OA joints, as observed here and in previous studies (15, 18, 25), and secondary inflammation is common in OA (24). Less IgG was extracted from OA than RA joints, correlating with the very different degrees of inflammation and joint destruction in these two diseases. Of course, a causal relationship between these broad-spectrum autoAbs and joint inflammation remains conjectural. However, given the evidence from animal models that Abs to various joint components (GPI, type-II collagen, COMP) are sufficient to elicit destructive joint inflammation (22, 23, 30), it is reasonable to speculate that the Abs observed here do contribute to inflammation in different arthropathies. Thus, one can imagine a scenario where different root causes (autoimmune in RA, mechanical in OA) lead to an exposure of joint macromolecules, which

results in local accumulation of IC derived from the pool of natural autoAbs or from induced B-cell responses. These IC would then promote local inflammation through the Fc segments of Igs, which might be further enhanced by the deposition of RF. In this view, the deposition of joint-binding Abs and the proinflammatory feed-forward loop it entails would be a generic component of human arthritic diseases.

Although we have not distinguished natural from induced autoAbs in IC, diversification of induced autoAb responses over time in chronic autoimmune inflammatory diseases has strong precedent. Epitope spreading has been documented at the T-cell and B-cell levels in mouse models of multiple sclerosis, type-I diabetes, RA, and myasthenia gravis (31), with support for an analogous process occurring in human diseases provided by accumulation of circulating autoAb specificities over time in human type-I diabetes and systemic lupus (31, 32).

The broad and largely shared reactivities of joint Ig contrast with the serology of human arthropathies, where few disease-associated reactivities such as RF and anti-CCP have been identified. The explanation is likely two-fold. First, serology has focused on identifying reactivities unique to one or a few diseases rather than on shared reactivities. Second, diverse Ab reactivities, such as those found in joint IC, would likely elude serum-based screens, as each individual specificity is probably present in low concentration and may be of low affinity. Thus, it is not surprising that analysis of serum specimens using the same autoantigen microarrays has yielded different information (33).

More specific, however, appear to be the histone and anti-histone autoAbs in IC of RA joints, as suggested by the MS and microarray analyses and confirmed by immunostaining of cartilage for histone-containing structures. H2A/H2B/DNA complexes might deposit already in the form of IC or independently of IgG, as chromatin is released from dying cells, and its adherence to joint surfaces might be promoted by its characteristic charge distribution. Bound nucleosome components could then contribute to inflammation by attracting circulating autoAbs to the joint surfaces, much as GPI or collagen act in the serum-transfer models of arthritis (23, 34). In such a scenario, circulating antinucleosome/histone Abs would only contribute to arthritis if inflammation had been initiated in a joint by other means—a mechanism similar to that proposed for anti-CCP immunity in RA (4) and also a plausible explanation for the absence of arthritis in many persons who have detectable antihistone autoAbs. The notion of anti-nucleosome/histone Abs being arthritogenic is plausible in RA based on detection of such Abs in variable percentages of RA patients in several studies (35, 36), and is even more plausible in other diseases featuring inflammatory arthritis (e.g., systemic lupus and oligoarticular juvenile inflammatory arthritis) that are more strongly associated with Abs to nucleosomal components (37, 38). Alternatively, because chromatin has the potential of activating cells of the innate and adaptive immune systems by triggering the TLR9 receptor (39), nucleosome-containing material could deliver activating signals to infiltrating cells in the joint as well.

In summary, the picture of Ab specificities revealed by this analysis of Igs in arthritic joints is somewhat different from what might have been expected based on screens of circulating reactivities, providing a unique and potentially functionally relevant insight into the immunology of inflammatory arthritis.

Materials and Methods

Human Cartilage and Synovium and Collagenase Extracts Thereof. Using a modification of a published protocol known to preserve Ab reactivity (18), cartilage and synovium collected and discarded during total joint replacement or synovectomy operations were (separately) rinsed with several changes of PBS at 4 $^{\circ}$ C with gentle rotation over 3 to 4 days, then digested with collagenase (Worthington) for 16 to 24 h at 37 $^{\circ}$ C. Digestion was stopped by adding EDTA to 10 mM, and the liquid fraction was recovered by decanting and centrifugation.

Purification and Digestion of IgG and Associated Proteins. Control Sepharose was made by incubating cyanogen-bromide activated Sepharose (Amersham Pharmacia) with Tris-HCl. Each collagenase extract was precleared with control Sepharose, then half was incubated with proteinG Sepharose (Amersham) and half with control Sepharose, 25 to 300 μ l packed beads based on the volume of the lysate, at 4 °C for 1 h with rotation. The beads were then washed 6 to 7 times with PBS, and then bound material was eluted with 2 volumes of 0.1 M glycine, pH 2.7, with pH subsequently adjusted to between 8.0 and 8.5. A 50- μ l aliquot was processed for MS by treatment with DTT, then iodoacetamide, then Trypsin Gold (Promega) 30 μ g/ml at 37 °C for 16 to 24 h, as described previously (40). Digested samples were stored at -20 °C. The concentration of IgG in each undigested eluate was determined by ELISA and used to calculate the approximate concentration in the original extract: $([IgG] \times \text{eluate vol})/(\text{vol of extract incubated with proteinG})$.

Separation and Sequencing of Peptides. The samples were separated using a nanoflow liquid chromatography system (Waters Cap LC) equipped with a picofrit column (75 micron ID, 10 cm, NewObjective) at a flow rate of \approx 150 nL/min using a nanotee (Waters) 16/1 split (initial flow rate 5.5 μ l/min). The LC system was directly coupled to a tandem MS (Q-TOF micro, Waters). Analysis was performed in data dependent analysis mode and parent ions with intensity greater than 6 were sequenced in MS/MS mode. Data were analyzed using Mascot software to interrogate the NCBI nr database (release 20070810) as detailed in the *SI Text*. Statistical analysis of the distribution of proteins identified by tandem MS is described in detail in *Results*.

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Screening for Antibody Binding to Proteins and Synthetic Peptides by Microarray. Twenty-residue peptides containing either arginine or citrulline were synthesized commercially (Sigma GenoSys). Human cartilage oligomeric matrix protein was purified according to a published protocol (41). Other proteins and proteoglycans were purchased (Sigma). Generation, probing, and analysis of protein microarrays were performed as described previously (12, 13) (see also *SI Text*).

Staining of Cartilage Sections. Human cartilage samples were frozen in OTC medium shortly after recovery. After frozen sectioning at 6 μ m thickness, sections were stained with fluorescently labeled antibodies and analyzed by fluorescence microscopy using conventional techniques.

See *SI Text* for complete methods.

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