Nodular Disease in Rheumatoid Arthritis: Association with Cigarette Smoking and HLA-DRB1/TNF Gene Interaction

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ABSTRACT. Objective. To investigate the association of nodular disease in rheumatoid arthritis (RA) with smoking, seropositivity, and polymorphisms at HLA-DRB1 and TNF loci.

Methods. Consecutive patients with RA (n = 420) attending a hospital clinic were examined for the presence of subcutaneous nodules. Rheumatoid factor (RF) status and HLA-DRB1 genotype were determined on every patient, and their smoking history was recorded. TNFa microsatellite polymorphisms were examined in a subgroup of 144 patients. The relationships between smoking, RF status, HLA-DRB1 genotype, TNFa microsatellite polymorphism, and the presence of nodules were examined using chi-square tests and logistic regression analyses.

Results. Current smokers were more likely to have nodular disease than those who had never smoked (OR 1.8, 95% CI 1.0–2.9). An association was also found between RF positivity and nodular disease (OR 2.2, 95% CI 1.2–3.8) that remained significant after correction for current smoking. A combination of current smoking and seropositivity increased the risk of nodular disease (OR 3.9, 95% CI 1.7–9.1). Analysis of HLA-DRB1 genotypes in this RA population revealed that only DRB1*0401 homozygotes were associated with nodular disease, and that this was independent of the influence of smoking and seropositivity. Individual TNFa microsatellite alleles were not associated with the presence of nodules, but an interactive effect was found between the TNF a6 allele and homozygosity for DRB1*0401.

Conclusion. Our data indicate that nodular disease in RA is independently associated with current cigarette smoking, seropositivity, and homozygosity for HLA-DRB1*0401. The latter association involves a possible interaction with the TNF a6 microsatellite allele. (J Rheumatol 2002; 29:2313–8)

Key Indexing Terms:

RHEUMATOID ARTHRITIS	SMOKING	NODULES
RHEUMATOID FACTOR	HLA-DRB1	TNF

The development of subcutaneous nodules is the most common extraarticular manifestation (EAM) in rheumatoid arthritis (RA), found in roughly 20–25% of cases[1]. Rheumatoid nodules are usually associated with more severe joint damage and are frequently found in patients with other EAM such as vasculitis, pulmonary fibrosis, and serositis. The exact mechanisms involved in the pathogenesis of rheumatoid nodules are still uncertain, although it has been suggested that nodule formation originates from mechanically induced small blood vessel injury, leading to the accumulation of rheumatoid factor (RF) immune complexes within the injured vessels. Subsequent activation of macrophages leads to increased procoagulant activity, which catalyzes the polymerization of fibrinogen to form fibrin in the central zone of the nodule. Necrosis of the central core is believed to result from release of cytotoxic factors, collagenase, neutral proteases, and other enzymes from infiltrating mononuclear phagocytes[1-3].

Nodular disease has been associated with both RF positivity and cigarette smoking in patients with RA[4-8]. Production of RF has also been associated with cigarette smoking in patients with RA and healthy individuals[6-11]. The relationship between smoking, RF production, and nodular disease is unclear, although it seems reasonable to suggest that the promotion of RF production by smoking leads to increased deposition of RF complexes at nodule sites. Nevertheless, Wolfe showed that current or previous

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smoking was associated with the presence of rheumatoid nodules even after controlling for RF positivity[6].

Genetic factors are believed to play a role in the development of nodular disease and other EAM. Various studies have suggested an association between extraarticular disease in RA and certain HLA-DRB1 alleles, particularly DRB1*04 alleles, which encode the shared epitope (SE) sequence[12]. Ollier, *et al*[13] showed in a UK population that the frequency of HLA-DR4 was increased in nodular disease, and an HLA haplotype carrying DR4 (i.e., Cw3-Bw62-Dw4-DR4-DRw53) was associated with patient subsets displaying EAM. Weyand, *et al*[14] found that nodular RA was more frequent in seropositive patients with one or 2 DRB1*04 alleles. Other studies in US and French populations revealed that the HLA-DRB1*0401 allele in particular was associated with development of nodules and other EAM[15,16]. Homozygosity for the HLA-DRB1*0401 allele was associated with major organ involvement[15], while other genotypic combinations involving the DRB1*0401 allele were associated with rheumatoid nodules and vasculitis[15,16]. Conversely, the absence of EAM in patients with RA from Southern France has been attributed to the low frequency of DRB1*0401 in this population[17].

Other genes have been implicated in RA severity[18-24]. The tumor necrosis factor (TNF) locus has received particular attention, and a number of biallelic polymorphisms and microsatellite markers (a–e) have been identified in this region[25]. Several conserved HLA-DRB1/TNF microsatellite haplotypes have been found at higher frequencies in patients with RA compared with healthy controls[26], and there is evidence to suggest both independent and interactive effects of HLA-DRB1 and TNF polymorphisms on RA susceptibility and severity[20-22,27-29].

Since smoking, seropositivity, and HLA genotypes carrying DRB1*0401 have all been associated with nodular disease, we investigated whether these variables are independently associated with nodule development, and whether there is any additive or interactive effect between them. Due to the strong linkage disequilibrium between the HLA-DRB1 and TNF regions we also examined whether TNF microsatellite polymorphisms express any associations with nodular disease.

MATERIALS AND METHODS

The study was carried out on 420 patients with RA recruited consecutively in a clinic monitoring the effects of disease modifying antirheumatic drugs (DMARD). Demographic and clinical details are shown in Table 1. All patients were northern European Caucasians resident in north Staffordshire or Cheshire, and satisfied the American College of Rheumatology (formerly the American Rheumatism Association) 1987 ARA criteria for RA[30]. The majority of patients were being treated with one or more DMARD, which included hydroxychloroquine, sulfasalazine, gold, or methotrexate. A small number (~5%) of patients were being treated with corticosteroids. RF concentrations were measured using nephelometry and reported in international units (IU); a concentration > 60 IU/ml was considered positive[31].

Table 1. Demographic and clinical characteristics of patients with RA (n = 420).

Male:female	157:263
Median age, yrs (range)	59.0 (21–85)
Median age of onset, yrs (range)	48.0 (20–81.5)
Median disease duration, yrs (range)	9.3 (0.5–39)
Rheumatoid factor positive, %	62.1
Nodular disease, %	22.4
Erosive disease, %	85.0

Smoking history. A history of current and past smoking was obtained from each patient. Patients were classified by whether they had ever or never smoked, and whether they were currently smoking. Ever-smokers were those that had smoked at least one cigarette a day for one year or more. The frequency of cigarette smoking in the total RA population and in men and women separately is shown in Table 2.

HLA-DRB1 typing. DNA was extracted from EDTA treated blood using a phenol-chloroform extraction procedure. HLA-DRB1 phenotypes were determined using a commercial reverse dot blot method, (Inno-LiPA, Abbott Laboratories, Maidenhead, UK) and Inno-LiPA software. HLA-DRB1*04 subtypes were identified using either single strand conformational polymorphism (SSCP) after amplification with DR4-specific primers or DRB1*04-specific Inno-LiPA strips (Innogenetics, Belgium), which allowed intermediate to high resolution typing.

TNFa microsatellite typing. A subgroup of 144 patients were genotyped for TNFa microsatellite polymorphisms as described[26]. This subgroup did not differ significantly from the remainder of the patients in terms of demographics and disease characteristics (data not shown). Allele determination was performed using fragment analysis software (672 Genescan analysis) and Genotyper software (Perkin Elmer). DNA with known TNF genotypes, TUBO (a3/13), VAVY (a2/2), OMW (a7/7), and IBW9 (a4/4), were included on each gel as positive controls.

Statistical analysis. Associations between nodular disease, smoking, RF positivity, and HLA-DRB1/TNFa alleles were examined using chi-square analyses, or logistic regression analyses with correction for age, sex, and disease duration where appropriate. We also examined the effect of interaction between variables using multiple regression models that contained the interaction term as well as the corresponding main effects as independent variables. All analyses were carried out using the Number Cruncher statistical package for Windows (NCSS v. 6.0.4) or the PEPI software package (v. 2.0) for epidemiologic analysis[32].

RESULTS

Relationship between smoking and nodular disease. Patients with RA who were current smokers were significantly more likely to have nodules than those who had never smoked (28.1 vs 19.4%; OR 1.8, 95% CI 1.0–2.9, p = 0.04, after correction for age, sex, and disease duration). There was no increase in frequency of nodules in ex-smokers. These had a

Table 2. Frequency (%) of cigarette smoking in male and female patients with RA.

Smoking Status	All Patients	Women	Men
Never	38.3	51.7	15.9
Past	29.3	20.2	44.7
Current	32.4	28.1	39.4

similar frequency of nodular disease (21.3%) to the RA group that had never smoked. In all smokers the number of years smoked (and pack-years) was significantly associated (p = 0.03) with the presence of rheumatoid nodules.

Relationship between nodules, RF status, and smoking. The frequency of nodular disease was significantly higher in RF positive than RF negative patients (26.8 vs 15.1%; OR 2.2, 95% CI 1.2–3.8, p = 0.002). Correction for age, sex, and disease duration using logistic regression analysis made little difference to the significance level (p = 0.006). The association between nodular disease and seropositivity remained significant (p = 0.015) after correcting for current smoking in the regression model. Seropositive patients who were also currently smoking had a significantly increased risk of having nodular disease compared with nonsmoking seronegative patients (29.5 vs 9.6%; OR 3.9, 95% CI 1.7–9.1, p = 0.0002).

Relationship between nodular disease and HLA-DRB1. No association was found between presence of the HLA-DRB1 SE and nodular disease (p = 0.6). To determine whether any particular SE phenotype(s) were associated with nodular disease we carried out multivariate logistic regression analysis that included each of the major SE phenotypes in our population (i.e., HLA-DRB1*0401, *0404 or *0408, and *0101) as independent variables. After correction for age, sex, and disease duration the model revealed no significant association between nodular disease and any SE phenotype (Table 3).

We next examined whether different HLA-DRB1 genotypes carrying 2 SE alleles were associated with nodule development (Table 4). The highest frequency of nodular disease (36.6%) was found in patients homozygous for DRB1*0401. This was the only combination significantly different from SE–/SE– patients (OR 2.4, 95% CI 1.0–6.1, p = 0.04), although significance was lost after correction for multiple comparisons. Patients homozygous for DRB1*0404 also had an increased frequency of rheumatoid nodules (33.3%), but the significance of this was uncertain because of the small number (n = 6) with this genotype. Of the heterozygous combinations, only DRB1*0401/*0101 patients showed an increased frequency of nodular disease (30.9%), but this was not statistically significant. Over 50% (7/13) of the patients with this genotype and rheumatoid nodules were current smokers. Genotypes carrying DRB1*0401 in combination with any allele lacking the SE (DRB1*0401/*X) showed no increase in the frequency of nodules. The association of DRB1*0401 homozygotes with nodular disease remained significant after adjustment for current smoking and RF status in a logistic regression model that also included correction for age, sex, and disease duration (Table 5).

Table 4. Frequency of nodular disease in patients with different HLA–DRB1 genotypes.

SE Genotypes	n	Nodular Disease n	%	OR (95%CI)
*0401/*0401	41	15	36.6	2.4 (1.0–6.1)
*0401/*0101	42	13	30.9	1.9 (0.7–4.8)
*0401/*0404	33	3	9.1	0.4 (0.2–1.7)
*0401/*0408	13	2	15.4	0.8 (0.1–4.2)
*0401/*X[†]	115	24	20.9	1.1 (0.5–2.4)
*0404/*0101	5	1	20.0	1.1 (0.04–11.3)
*0404/*0404	6	2	33.3	2.1 (0.2–15.2)
*0101/*0101	5	0	0	—
SE–/SE–	83	16	19.3	1.0 (reference)

[†] In the DRB1*0401/*X genotype, X represents any allele without the shared epitope.

Relationship between nodular disease and TNFa microsatellite alleles. Global chi-square analyses revealed no differences in the overall frequencies of individual TNFa microsatellite alleles in patients with and without nodular disease (Table 6).

Investigation of HLA-DRB1 and TNF interactions in nodular disease. We have shown previously that radiographic severity of RA is associated with an interaction between the SE and the TNF a6 microsatellite polymorphism[21], while Mu, *et al* found an interaction between the SE and TNF a11 in relation to RA severity[20]. Due to the possible association of HLA-DRB1*0401 homozygotes with nodular disease in this study we examined whether there was any evidence for interaction between HLA-DRB1*0401 homozygotes and TNF a6 or TNF a11 microsatellite alleles. Investigation of the patients genotyped for TNFa microsatellites revealed that 7/37 (18.9%) patients with nodules were homozygous for DRB1*0401 and carried a TNF a6 allele, compared with only 6/107 (5.6%) of those without nodules (OR 3.93, 95% CI

Table 3. Multivariate logistic regression analysis to investigate the association of SE+ phenotypes with nodular RA. Logistic regression analysis with presence or absence of nodules as the dependent variable. The model was corrected for age, sex, and disease duration by including these covariates with the independent variables (DRB1*0401, DRB1*0404 or *0408, DRB1*0101).

Variable	Regression Coefficient	Standard Error	OR (95% CI)	p
DRB1*0401	0.271	0.251	1.31 (0.8–2.1)	0.28
DRB1*0404 or *0408	–0.712	0.396	0.49 (0.2–1.1)	0.07
DRB1*0101	0.130	0.286	1.14 (0.6–2.0)	0.65

Table 5. Multivariate logistic regression analysis to investigate the associations of HLA–DRB1*0401 homozygosity, current smoking, and RF status with nodular disease. Logistic regression analysis with presence or absence of nodules as the dependent variable. All independent variables were adjusted for each other in the model, and included correction for age, sex, and disease duration. The p values for DRB1*0401/*0401, current smoker, RF, and female sex represent the significance of each variable compared with individuals negative for that variable. The OR and 95% CI for the continuous variables age and disease duration are calculated per year. Chi–square for the model with 6 degrees of freedom = 30.9 ($p < 0.0001$).

Variable	Regression Coefficient	Standard Error	OR (95% CI)	p
DRB1*0401/*0401	0.815	0.399	2.26 (1.03–4.9)	0.040
Current smoker	0.657	0.277	1.92 (1.1–3.3)	0.018
RF	0.739	0.305	2.08 (1.2–3.7)	0.015
Sex (female)	0.428	0.288	1.53 (0.9–2.7)	0.137
Age	–0.011	0.013	0.99 (0.97–1.01)	0.383
Disease duration	0.066	0.020	1.07 (1.03–1.11	0.001

Table 6. Allele frequencies of TNFa microsatellite markers in RA patients with or without nodules.

	Allele Frequencies	
	With Nodules	Without Nodules
TNFa	(2N = 74)	(2N = 214)
1	0.014	0.005
2	0.364	0.362
3	0.0	0.009
4	0.095	0.061
5	0.014	0.019
6	0.230	0.220
7	0.027	0.056
8	0.014	0.005
10	0.081	0.061
11	0.122	0.187
12	0.014	0.0
13	0.027	0.023
14	0.0	0.0

1.1–14.5, p = 0.015). Using a multivariate logistic regression model, we found that TNF a6 showed a significant interaction (p = 0.036) with DRB1*0401 homozygosity in nodular disease, after correction for current smoking and RF (Table 7). No interaction was found between TNF a6 and DRB1*0401 heterozygosity or between TNF a6 and the SE (data not shown). TNF a11 was found associated with DRB1*0401 homozygosity in only one patient with nodules and 2 without, so it is unlikely to be making an important contribution to nodule development.

DISCUSSION

We have confirmed that the presence of subcutaneous nodules in RA is associated with current cigarette smoking. This is independent of the association of nodules with RF positivity, although a combination of current smoking and seropositivity appears to have the highest risk of nodular disease. HLA-DRB1*0401 homozygosity was associated with rheumatoid nodules independently of both current smoking and RF, but an interactive effect was found with the TNF a6 microsatellite allele. No TNF microsatellite allele alone was associated with nodular disease.

The role of smoking in the development and/or severity of RA is still uncertain, although a number of studies suggest that it is important[5-7,33-39]. It is clear that smoking is closely associated with 2 of the main features of rheumatoid disease, i.e., RF production and development of rheumatoid nodules. Nonetheless, these features also occur in patients with RA who have never smoked, so other, unidentified

Table 7. Multivariate logistic regression analysis to investigate the interaction between HLA-DRB1*0401 homozygosity and the TNF a6 microsatellite allele in nodular disease. Logistic regression analysis with presence or absence of nodules as the dependent variable. The model contains the interaction term between HLA–DRB1*0401 homozygosity and the TNF a6 allele as well as the corresponding individual effects. The analysis was corrected for current smoking, RF, age, sex, and disease duration by including these covariates with the independent variables. Chi-square for the model with 8 degrees of freedom = 34.1 ($p < 0.0001$).

Variable	Regression Coefficient	Standard Error	OR (95% CI)	p
DRB1*0401/*0401/TNF a6	3.145	1.502	23.2 (1.2–445)	0.036
DRB1*0401/*0401	–0.550	1.237	0.58 (0.1–6.5)	0.657
TNF a6	–0.718	0.561	0.48 (0.2–1.5)	0.200
Current smoker	1.251	0.468	3.49 (1.4–8.7)	0.007
RF	1.107	0.516	1.12 (1.1–8.3)	0.016

environmental factors may be important. It is also possible that many of these patients will have partners who smoke, or they may have been exposed to cigarette smoke in work and/or social environments. Further studies are needed to investigate whether passive smoking could have an effect in such patients. Data currently available suggest that seropositivity and development of nodular disease in nonsmokers are dependent on genetic factors. We reported that there is a relationship between carriage of the DRB1*0401 allele and RF production that is independent of smoking[40]. However, unlike nodular disease the association with seropositivity was primarily with a single copy of DRB1*0401, and no additional risk was conferred by homozygosity for this allele. Moreover, the level of RF was not associated with the number of DRB1*0401 alleles and no interactions with TNF a6 or other TNF alleles were found (unpublished observations).

Since development of nodules appears to be associated with DRB1*0401 and seropositivity, it could be argued that nodular disease is dependent upon an HLA-DR-restricted immune response. Such a suggestion has been made for RF production[41]. However, the development of nodules may require additional (or alternative) trigger mechanisms to that required for RF production, since nodule formation shows a stronger association with 2, rather than one DRB1*0401 allele, and the association appears to involve an interaction with the TNF a6 allele. Nevertheless, our study indicates that carriage of 2 DRB1*0401 alleles and a TNF a6 allele is not an essential requirement for nodule development, since patients carrying different HLA-DRB1 and TNF allele combinations show increased risk of developing nodular disease if they smoke.

Investigations of the relationship between DRB1 genotypes and nodular disease in other studies have produced conflicting results. Weyand, *et al*[15] found that nodular RA was associated with genotypes carrying heterozygous combinations of 2 shared epitope alleles. A later study from France found that the strongest association with nodules was found in genotypes carrying a single DRB1*0401 allele (i.e., DRB1*0401/X — where X represents all DRB1 alleles without the SE)[16]. Recently, a study from the USA found no relationship between nodular disease and number of SE alleles or particular DRB1 genotypes[42]. Similarly, we found no DRB1 genotype associations with rheumatoid nodules in a group of patients from northwest Spain[43]. We also found no evidence of DRB1/TNF interactions in that population. Some of these differences may be explained by different distributions of the DRB1 genotypes and TNF microsatellite alleles in different populations. For example, the frequency of DRB1*0401 homozygotes in the French and Spanish studies (3% and 1.5%, respectively) was much lower than in our study, where nearly 10% of patients carry this genotype. It is also worth noting that none of the previous analyses were corrected for the effect of smoking. In this regard it is interesting that one study found an increased frequency of nodules in male patients[42]. We did not find this, but such a finding would be consistent with the generally higher frequency of smoking among men (for the age group involved).

Unlike production of RF, the development of subcutaneous nodules does not occur in healthy individuals who smoke. This suggests that smoking and RF production per se do not induce development of nodules. However, these factors may exacerbate the process in individuals with RA by promoting further damage to small blood vessels initially injured by the disease process. The initial trigger for this injury remains unclear, but the common occurrence of nodules over pressure sites suggests that trauma plays a role[1].

Our results extend those of previous studies by indicating that RF, smoking, and HLA-DRB1/TNF gene interaction may have linked but separate roles in the development of rheumatoid nodules. We postulate that smoking may exacerbate the development of nodules through injurious effects on blood vessels in susceptible areas, but may also act indirectly through stimulation of RF production and subsequent deposition of RF complexes at such sites. Although the production of RF may also be increased in individuals with a single HLA-DRB1*0401 allele, the carriage of 2 DRB1*0401 alleles with a TNF a6 allele may provide an additional or alternative immune mediated pathway for promotion of nodule development.

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