# Annexin V autoantibodies in rheumatoid arthritis

M I Rodríguez-García, J A Fernández, A Rodríguez, M P Fernández, C Gutierrez, J C Torre-Alonso

## Abstract

*Objective*—To investigate the occurrence of anti-annexin V autoantibodies in sera of patients with rheumatoid arthritis to assess involvement with the disease and any relation to glucocorticoid treatment.

*Methods*—Anti-annexin V antibodies were measured by an enzyme linked immunosorbent assay (ELISA) which used the purified human recombinant protein as antigen.

*Results*—Concentrations of anti-annexin V autoantibodies, predominantly of the IgG class, were significantly raised in sera from patients with rheumatoid arthritis compared to normal controls. This was not correlated with other indices of disease activity such as erythrocyte sedimentation rate or C reactive protein and was unrelated to glucocorticoid treatment.

*Conclusions*—Extracellular annexin V provides an antigenic stimulus for autoantibody production and its in vivo expression is independent of glucocorticoid control. Such autoantibodies may have a detrimental role in the arthritic condition by interfering with putative functions of annexin V, including collagen type II binding, inhibition of phospholipase $A_2$ activity, and Fc receptor activity.

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Rheumatoid arthritis is a chronic disorder of connective tissue marked by extensive synovitis. Synovia become infiltrated by activated T cells and by B cells which secrete IgM, IgG, and IgA autoantibodies to tissue and circulating proteins.[1] This autoimmune response appears to be triggered by an array of autoantigens that include human IgG-Fc fragments and extracellular cell matrix proteins such as collagens type I, II, IV, and V. Although these antibodies may have a physiological role in maintaining the integrity of self, their destructive potential in autoimmune disorders makes the identification of target antigens aetiologically important.[1] A corollary of this premise is that autoantibodies to proteins which have a protective role in joint maintenance might further contribute to the disruption of joint homeostasis and immune regulation.

The annexins are a family of calcium dependent, phospholipid binding proteins involved in membrane trafficking and signal transduction and with a potential role as anti-inflammatory and growth regulatory agents.[2] At least some of these properties may be due to the interaction of annexins with extracellular matrix proteins. Anchorin CII (chick annexin V) was first identified as a secreted, collagen type II binding protein[3] that appears to mediate the adhesion of matrix vesicles to calcifying cartilage.[4] Various annexins appear to be secreted by an exocytotic mechanism from cells in a collagen matrix and may thus play a role in remodelling the extracellular matrix.[5] Annexins also effectively inhibit types I, II, and III phospholipase $A_2$ by distinct mechanisms.[6]

The presence of raised concentrations of autoantibodies to human annexin I, V, and XI proteins (ANX1, ANX5, and ANX11) in various autoimmune diseases[7–13] has received attention because of the anti-inflammatory role of these proteins and their apparent inducibility by glucocorticoids used for treatment.[14] In rheumatoid arthritis, anti-ANX1 IgG was normal but IgM was stimulated following glucocorticoid treatment.[15] Anti-ANX11 IgG autoantibodies directed at epitopes in the unique N-terminus of this annexin were increased in rheumatoid arthritis and other autoimmune diseases.[16] Anti-ANX5 autoantibodies in systemic lupus erythematosus and pre-eclampsia may increase the risk of thrombosis,[11 12] but their status and possible role in rheumatoid arthritis have not yet been examined. Since autoantibodies to type II collagen in rheumatoid arthritis play a primary role in cartilage destruction,[17] the specific involvement of ANX5 and autoantibodies to it were considered relevant to the collagen immunity and inflammation in rheumatoid arthritis.

## Methods

PATIENT DATA AND LABORATORY ANALYSES

Fifty one rheumatoid arthritis patients at the Hospital Central of Asturias fulfilled the American College of Rheumatology criteria [18] based on clinical and laboratory data (table) and were included in a prospective study after giving informed consent. Their mean age was 64 years, 65% were female and 35% male, and the mean duration of illness was at least 6 years. Healthy controls were matched in both age and sex to the patient groups. Patient records included identification and medical history, with clinical assessment of the onset, form, and duration of illness, morning stiffness, tender joint score, and swollen joint score. Laboratory analyses included measurements of blood count, erythrocyte sedimentation rate (ESR, Westergren), rheumatoid factor (RF) by nephelometry, and C reactive protein by nephelometry. Radiographs were taken of

Faculty of Medicine, University of Oviedo, E-33006 Oviedo, Spain: Department of Biochemistry and Molecular Biology
M I Rodríguez-García
M P Fernández

Hospital Central of Asturias, E-33006 Oviedo, Spain: Department of Rheumatology
J A Fernández
A Rodríguez
J C Torre-Alonso

Department of Immunology
C Gutierrez

Correspondence to:
J C Torre-Alonso,
Department of Biochemistry and Molecular Biology,
Faculty of Medicine,
University of Oviedo,
E-33006 Oviedo, Spain.

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*Patient data. Population characteristics, clinical symptoms and laboratory tests are given for 51 patients diagnosed with rheumatoid arthritis and subcategorised according to glucocorticoid treatment dosage*

| Feature | Description | Units | Treatment subgroups of rheumatoid arthritis patients | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | | Subgroup 1 | Subgroup 2 | Subgroup 3 | Combined total |
| Glucocorticoid | Dosage | mg d⁻¹ | 0 | < 7.5 | > 7.5 | Variable |
| Patients | Number | n | 17 | 19 | 26 | 51 |
| Age | Median (range) | years | 66 (24-78) | 64 (35-81) | 65 (36-79) | 64 (24-81) |
| Sex distribution | Male/female | n | 4/13 | 5/14 | 8/7 | 18/33 |
| Length of illness | Median (range) | years | 6 (1-18) | 6 (1-45) | 7 (1-14) | 6 (1-45) |
| Morning rigidity | Time > 1 h | n | 3 | 7 | 11 | 21 |
| Pain | Affected joints > 9 | n | 2 | 10 | 12 | 24 |
| Swelling | Affected joints > 6 | n | 2 | 6 | 10 | 18 |
| Rheumatoid factor | Mean (SD) (n > 30) | IU ml⁻¹ | 216 (374) (7) | 204 (198) (12) | 343 (381) (13) | 249 (321) (32) |
| Creactive protein | Mean (SD) | µg ml⁻¹ | 10 (16) | 19 (20) | 45 (48) | 24 (33) |
| ESR | Mean (SD) | mm/h | 23 (16) | 46 (31) | 53 (31) | 40 (29) |
| Anti-ANX5 titre* | Mean (SD) | ELISA | 243 (227) | 283 (299) | 278 (296) | 268 (271) |
| Anti-ANX5 titre* | Median (range) | ELISA | 145 (10-676) | 159 (38-1200) | 180 (11-1029) | 162 (10-1200) |

ESR, erythrocyte sedimentation rate; IU, international units; n, number; SD, standard deviation; *, total antibody levels.

the hands and feet. Active rheumatoid arthritis was defined by the presence of six or more swollen joints plus two of the following criteria: more than nine tender joints, morning stiffness longer than 1 h, or ESR greater than 30 mm/h.[19] Patients excluded were those with infection or serious cardiac, pulmonary, or renal disease.

### DATA ANALYSES

For the purposes of data analysis with respect to treatment, patients were categorised into three groups according to whether they received no glucocorticoid treatment, low dose prednisone (less than 7.5 mg per day), or high dose prednisone (more than 7.5 mg per day). The three rheumatoid arthritis patient groups were similar in age, sex, and duration of illness (table 1). The control donor group consisted of 49 healthy volunteers. Data comparisons used the SPSS and Microsoft EXCEL programs for microcomputers and included one way analysis of variance (ANOVA), Student's two tailed *t* test, $\chi^2$, and Wilcoxon rank sum test.

### EXPRESSION AND ISOLATION OF RECOMBINANT ANX5

Recombinant human annexin V was expressed in *Escherichia coli* strain RB791 (American Type Culture Collection No 53622) transformed with plasmid pKA5H. This plasmid contained the entire coding DNA sequence of the human annexin V gene[20] under the regulation of *trc* promoter.[21] The coding portion of human annexin V was obtained from placental RNA by reverse transcriptase polymerase chain reaction (RT-PCR) and subcloned into the *Nco*I and *Hind*III sites of plasmid pKK233-2 (Pharmacia). Recombinant protein was purified from bacterial cell lysates following published procedures.[22] Purity was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining, and by immunoblotting with specific polyclonal antisera (fig 1), kindly provided by R B Pepinsky (Biogen Inc, Cambridge, MA, USA).

### ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Microwell plates (Immuno Maxisorp, Nunc Intermed, Roskilde, Denmark) were coated overnight at 4°C with 0.5 µg per well recombinant human annexin V in 100 µl 50 mM carbonate/bicarbonate buffer, pH 9.6. After incubation, plates were washed four times with 200 µl phosphate buffered saline (PBS), pH 7.2, containing 0.05% Tween 20, and blocked with 5% milk powder in 200 µl PBS-Tween 20. Plates were then washed twice again and dilutions of sera in blocking solution (1:8 to 1:512) were applied to microtitre wells. Duplicate wells containing 100 µl of each dilution were incubated for 1 h at room temperature. Plates were washed three times with PBS-Tween 20 and wells were incubated for 1 h with 100 µl of a solution containing goat antihuman antibody (IgG, IgM or total) alkaline phosphatase conjugate (Sigma) diluted 1:5000 in PBS-Tween 20. Microtitre plates were again washed and 100 µl of 13.5 mM p-nitrophenolphosphate substrate added, with incubation in the dark for 15 min at room temperature. The reaction was terminated by addition of 100 µl 1N NaOH. Absorbence measurements at 405 nm were obtained with a
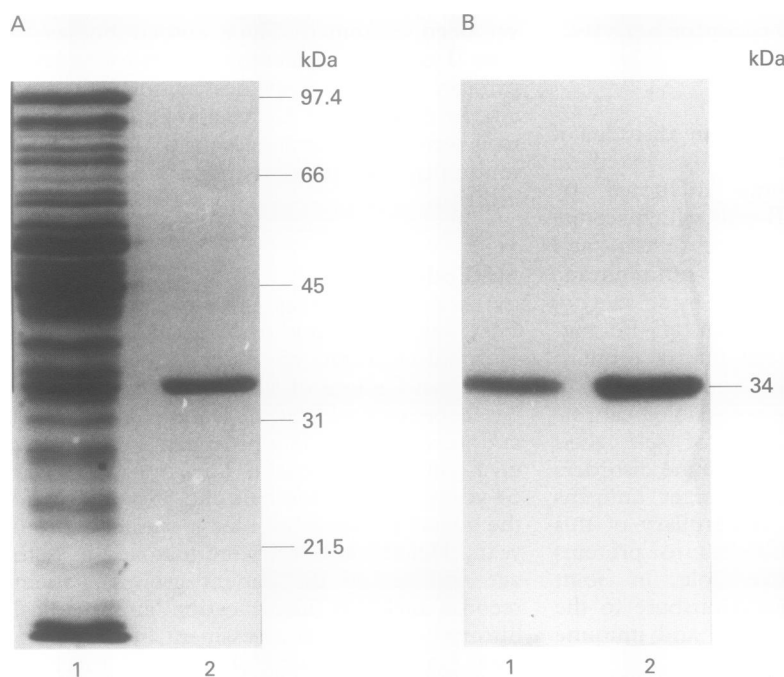


*Figure 1 Purity of human annexin V recombinant protein used for ELISA assays. The protein was produced and purified as described under Methods. Coomassie stained SDS-PAGE gels (A, left) show the resolution of proteins in whole homogenates following induction of plasmid containing bacterial cultures (lane 1) and of the liposome purified product (lane 2). The corresponding immunoblot (B, right) employed specific polyclonal antisera to identify the 34 kDa bands in whole homogenate (lane 1) and purified protein (lane 2) as human annexin V.*

Titertek Multiskan (EFLAB, Finland) ELISA plate reader. Blank values were subtracted from sample readings and results were expressed in ELISA units, defined as $AU_{405}$(sample) × dilution factor ÷ reaction volume (ml). Consistency was maintained in the ELISA by including two identical serum samples of high and low anti-annexin V titre in each plate and assay. Specificity was also tested by preincubating patient sera with fixed quantities of soluble annexin V in the range 0.5-10 µg to block binding by 25-75%, when compared to the control absorbence without competition.

## Results

### RAISED ANTI-ANX5 AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

The mean anti-ANX5 autoantibody titre was measured by ELISA in sera from 51 rheumatoid arthritis patients compared to the 49 control subjects (fig 2). ELISA readings, expressed as the mean, were higher for rheumatoid arthritis patients than control subjects, at 268 (SD 271) *v* 156 (161) arbitrary units. The increase in anti-ANX5 autoantibodies in rheumatoid arthritis patients was significant (P = 0.016) by a two tailed *t* test assuming unequal population variances.

### ROLE OF GLUCOCORTICOID TREATMENT

The anti-ANX5 data from rheumatoid arthritis were reanalysed according to treatment subgroups in order to determine whether the increase in autoantibody was related to glucocorticoid treatment (fig 3). The mean (SD) values for the three subgroups of rheumatoid arthritis patients were 243 (227) units for untreated patients, 283 (299) for those receiving low dose glucocorticoid (less than 7.5 mg per day), and 278 (296) for those receiving high dose glucocorticoid (more than 7.5 mg per day). No significant difference was observed between the three subgroups using one way analysis of variance or a two tailed *t* test, even when the two groups receiving glucocorticoid were compared as a single sample population to the untreated patient group.

### ANTIBODY TYPING

Isotyping was performed to determine the class of antibody present in the sera of rheumatoid arthritis patients. The results (fig 4) showed a clear predominance of IgG compared to IgM, which remained in the normal range. This contrasted with previous findings of elevated IgM class autoantibodies to ANX1 in rheumatoid arthritis patients receiving high-dose glucocorticoid treatment [15].

### CORRELATION WITH DISEASE INDICES

The finding that glucocorticoid treatment was not a determinant of anti-ANX5 levels indicated a direct relation between ANX5 and disease status. Various indices were therefore measured to assess their correlation with anti-ANX5 titres in all 51 rheumatoid arthritis
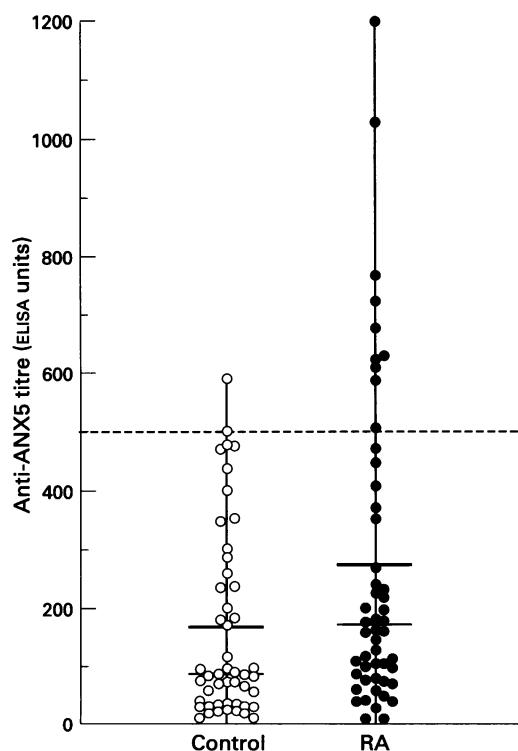


*Figure 2    Anti-annexin V autoantibody levels in rheumatoid arthritis patients and normal controls. ELISA readings were taken from sera of 49 healthy volunteers (empty circles) and 51 patients diagnosed with rheumatoid arthritis (filled circles) and expressed on the ordinate scale as arbitrary ELISA units. Solid horizontal lines mark the mean (bold) and median (thin) values for each group and the dashed line signifies the level corresponding to two standard deviations above the control mean. Vertical lines through the points span the full range of values.*
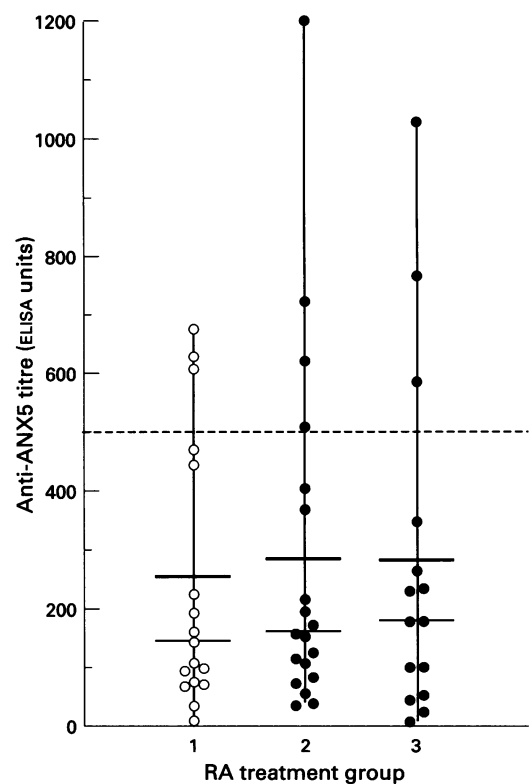


*Figure 3    Anti-annexin V autoantibody levels in rheumatoid arthritis patients grouped according to glucocorticoid treatment regimen. Group 1 (n = 17) received no steroid treatment, group 2 (n = 19) received prednisone at less than 7.5 mg/d, and group 3 (n = 15) received prednisone greater than 7.5 mg/d. Solid horizontal lines mark the mean (bold) and median (thin) values for each group while vertical lines span the full range of values. The dashed line marks a threshold corresponding to two standard deviations above the control mean in fig 2.*
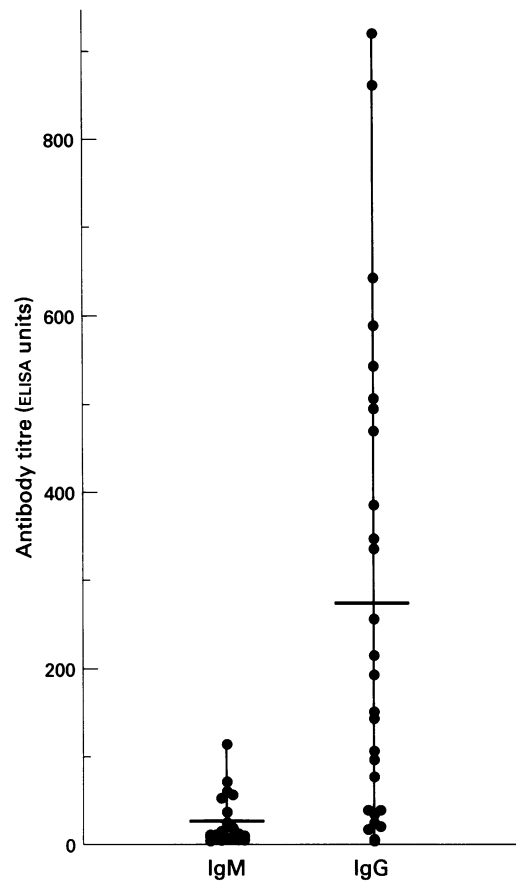
Figure 4   Classification of anti-ANX5 autoantibody
isotype in rheumatoid arthritis. ELISA units are represented
by filled circles to denote IgG or IgM class antibody
observed in 27 rheumatoid arthritis patients. Horizontal
solid lines mark the mean value for each group and vertical
lines indicate the range of values.

patients. The mean values for rheumatoid fac-
tor [RF, 249 (321) IU ml⁻¹], C reactive protein
[24 (33) µg ml⁻¹] and ESR [40 (29) mm/h]
were characterised by a modest rise of
anti-ANX5 titres (fig 5). However, none of
these laboratory tests showed any significant
positive correlation with anti-ANX5: $r < 0.25$
with standard errors over 265 $OD_{405}$ units in

predicting anti-ANX5 from RF, C reactive
protein, or ESR values. Anti-ANX5 titres were
also grouped according to indices of clinical
severity but there was no apparent correlation
with the disease activity indices of morning
rigidity, pain, or swelling.

## Discussion

The prevalence of anti-ANX5 autoantibodies
in autoimmune disease was first reported by
Matsuda et al [11][12] who associated it with
thrombotic risk in pre-eclampsia and systemic
lupus erythematosus. The present finding of
significantly raised anti-ANX5 IgG autoanti-
body titres in rheumatoid arthritis sera extends
our knowledge of the immunochemical
variables of this disease and may ultimately
shed light on its aetiology and effective
treatment. Since antibody levels were not
correlated with any of the specific disease indi-
ces measured and did not appear to be a
consequence of glucocorticoid treatment, their
cause and effect remain to be clarified.

A perceived role for annexins as anti-
inflammatory[14] and immunosuppressive[23]
agents implies that they may serve as normal
protective regulators in the extracellular
environment. The specific involvement of
ANX5 in collagen type II binding[3] and bone
calcification[4] further suggests that its neutral-
isation by autoantibodies could have direct
pathological consequences in rheumatoid
arthritis. To the extent that ANX5 may
mediate the interaction of collagen II with
matrix vesicles,[4] its neutralisation by autoanti-
bodies might be expected to expose collagen II
epitopes and interfere with bone calcification.
Reduced ANX5 levels would also be expected
to disinhibit the actions of known inflamma-
tory mediators such as secretory phospholipase
$A_2$ and interleukin 1.[14]

The association of anti-ANX5 antibodies
with rheumatoid arthritis was independent of
glucocorticoid treatment, in contrast to
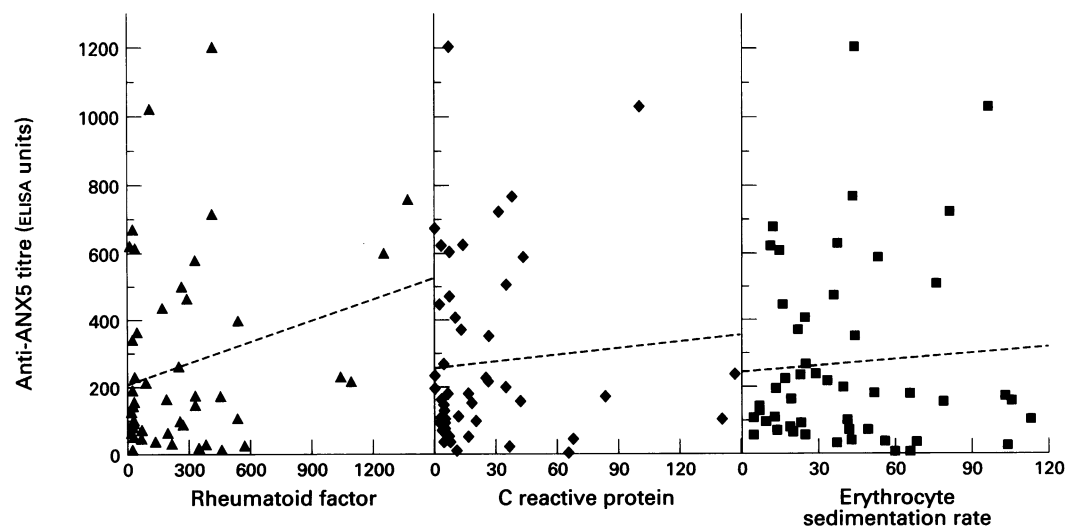previous findings for ANX1.[15] Cell culture



Figure 5   Correlation analysis between anti-ANX5 autoantibody titres and rheumatoid factor (RF, IU ml⁻¹), C reactive
protein (µg ml⁻¹), and ESR (mm/h). Paired values of anti-ANX5 (ordinate scale) with either RF (triangles), C reactive
protein (diamonds), or ESR (squares) for all 51 rheumatoid arthritis patients are grouped into separate panels. Dots
represent the calculated linear regression lines, for which the respective correlation coefficients (r) were 0.25 for RF, 0.08 for
C reactive protein, and 0.07 for ESR.

studies have shown the glucocorticoid inducibility of ANX1 and possibly ANX2, but not ANX5.[24] [25] Differential regulation of ANX5 and ANX1 is consistent with the observation that annexin V genes lack the classical glucocorticoid regulatory elements[26] [20] that exist in annexin I genes.[27] However, since the responses of isolated cells to glucocorticoids have limited relevance for whole organisms, our observations on ANX5 during human glucocorticoid treatment provide important corroboration of the tissue culture findings. Thus the phenomenon of glucocorticoid resistance is more likely to be related to impaired induction of ANX1 than ANX5 as a mediator,[28] and to the reduction of ANX1 levels by specific autoantibodies.[29]

The anti-ANX5 autoantibodies detected in rheumatoid arthritis were predominantly of the IgG class, consistent with an antigen driven mechanism of antibody formation. Since ANX5 has only six amino acids in its non-homologous N-terminus, epitopes in the annexin core region are probable targets. This contrasts with normal levels of anti-ANX1 IgG in rheumatoid arthritis and a raised IgM in response to prednisone.[15] It also differs from ANX11[16] where IgG antibodies predominated in rheumatoid arthritis but were directed against the unique amino-terminal portion of the molecule. Regardless of the type of antibody or its precise antigenic epitopes, the extracellular levels of various annexins are likely to be compromised in rheumatoid arthritis.

The role of annexins in immune processes[14] [23] may be multifaceted as they have recently been found to act as Fc receptors, possibly involved in immunoglobulin binding to phagocytic cells.[30] [31] Thus, in addition to binding their own specific autoantibodies at antigen affinity sites, they may also clear body fluids of other antibody complexes by binding their Fc regions to the surface of macrophages and leucocytes. In effect, the elimination of circulating annexins by their own autoantibodies might be expected to aggravate the autoimmune condition by impeding the clearance of other autoantibody complexes, including the Fc fragments comprising RF. However, that possibility does not seem to be consistent with the observed relation between anti-ANX5 and RF levels (fig 5).

Another recent study also observed a significant elevation of IgG-specific autoantibodies to both ANX5 and ANX6 in a smaller sample group of rheumatoid arthritis patients.[32] The authors noted a similar lack of correlation in antibody levels with disease activity, RF level, or glucocorticoid treatment. However, negative correlations between IgG concentrations and patient age or glucocorticoid dosage were not confirmed by the present study, and may simply be related to the apparent decline of disease activity with age in their patient group.

Raised concentrations of autoantibodies to ANX5, ANX1, and ANX11 in rheumatoid arthritis may reflect damage to collagen containing tissues such as synovial joints. The consequences for inflammatory responses involving secretory phospholipase $A_2$, collagen metabolism, and antibody regulation would be expected to be detrimental, but assessment will require a better understanding of the physiological role of annexins. The identification of annexin V as an autoantigen in rheumatoid arthritis expands the scope of autoimmune processes in this disease and may ultimately lead to the development of techniques for controlling them.

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