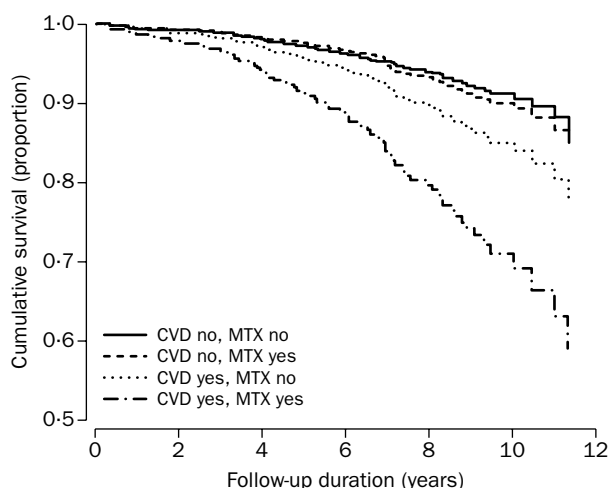




Cumulative survival in relation to the presence of cardiovascular diseases (CVD) and the start of methotrexate (MTX)

index DMARDs (2-6), which suggests a statistical interaction.

Cox's proportional hazard regression analysis with stepwise forward selection of variables was used to exclude differences in age, disease duration, severity of rheumatoid arthritis, and kinds and duration of DMARD treatment from the observed interaction. Variables (probability for entry 0.05; probability for removal 0.10) were: age at baseline; sex; measurements of rheumatoid-arthritis severity (disease duration, presence of rheumatoid factors, erosions or extra-articular manifestations or both, all at baseline); each kind of DMARD used prior to follow-up (seven different DMARDs [yes or no]); each kind of DMARD used at the start of follow-up (seven different index DMARDs); each kind of DMARD used in continuation of the index-DMARD (seven different DMARDs); duration of index-DMARD treatment; total number of DMARDs ever used (per patient); measurement reflecting chronic comorbidity (presence of chronic cardiovascular, pulmonary, gastrointestinal, and neurological diseases; diabetes mellitus; and co-occurrence of malignant disease); as well as the interaction variable *CVD*MTX-start*; the interaction variable measures the influence of the simultaneous presence of cardiovascular disease and MTX as index-DMARD on mortality. The results of the stepwise forward selection and the best-fit model are shown in the table. The interaction variable *MTX-start *CVD* (relative risk 3.40; $p=0.0054$) proved to be an independent predictor of mortality, indicating that, independent of other predictors, patients with CVD who had started MTX as index DMARD had on average a 3.4 times higher risk of death during follow-up than all other patients in the cohort (figure). Patients with rheumatoid arthritis and cardiovascular disease who had started MTX as index DMARD had a higher risk of death during follow-up than all other patients.

A second analysis, similar to the first analysis but without the patients who had used MTX before the start of follow-up ($n=560$), showed similar results: the interaction *MTX-start*CVD* was a significant prediction of mortality (relative risk 4.34; $p=0.0029$).

As a control for the specificity of the observation of

Risk factor	Relative risk	p
Age (per year)	1.10	0.00001
Co-occurrence of malignant disease (present or absent)	2.57	0.0004
Duration of index-DMARD treatment (per year)	1.22	0.00001
<i>MTX-start*CVD</i> (present*present)	3.40	0.0054
Diabetes mellitus (present or absent)	3.91	0.0002
Use of d-penicillamine after index-DMARD (yes or no)	0.38	0.0171

Independent relative risks for mortality of variables that add significantly to the prediction of mortality

increased mortality in patients with cardiovascular disease who had started with MTX, Cox regressions were run as in the first analysis, but now with the interactions of CVD with each of six separate index DMARDs (two index DMARDs were used more frequently than MTX) offered for selection. None of these interactions proved to be independent predictors of mortality in the stepwise forward selection process.

The results of these analyses provide epidemiological support for the hypothesis that MTX may promote atherosclerosis in patients who have already signs of atherosclerotic vascular disease. The observation is specific for MTX, because no link between any of the other index DMARDs and the presence of cardiovascular disease with regard to mortality was found. The increased mortality in this group of patients may be attributed to a MTX-induced rise in homocysteine.

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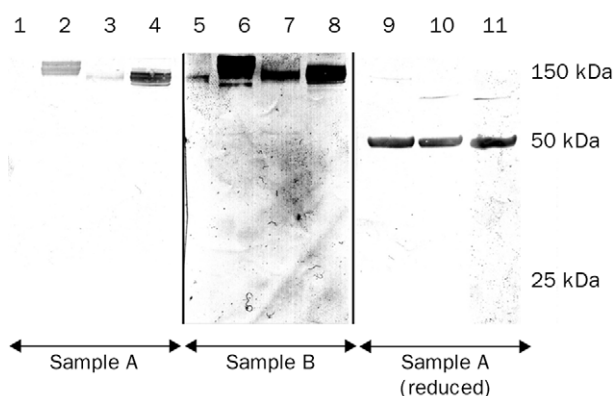
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Evidence for presence of IgG4 anti-immunoglobulin autoantibodies in all human beings

C Hennig, L Rink, H Kirchner

IgG4 anti-IgG autoantibodies were found to be present in all serum samples from a group of healthy people; IgG4 anti-IgA and anti-IgE were present in only 13 of 218 (6%) and 8 of 218 (3.5%) samples, respectively. These antibodies also showed affinities towards animal immunoglobulins. We suggest that IgG4 anti-IgA antibodies (the strongest found heterophilic immunoglobulins) are responsible for most artifacts obtained with commercial ELISA systems.

There is a rising number of reports about naturally occurring autoantibodies in healthy individuals. These immunoglobulins are directed against various self-antigens such as serum proteins, intracellular structures,¹ and even autologous ABO blood-group antigens. Since the 1960s, antibodies themselves have been known to bear antigenic determinants (idiotopes) which define the so-called idio type of an antibody and which are located in the variable regions of the molecules. Antibodies against these idiotypes can be raised in experimental systems. In 1974, Jerne formulated his hypothesis of a network of immunoglobulins and anti-immunoglobulins (anti-idiotypes) regulating tolerance, autoimmunity, and immunological memory.² The existence of such a network would require the continuous presence of anti-immunoglobulin autoantibodies



Western blot showing binding of serum IgG4 to immunoglobulins

Lanes 1 and 5=IgA; lanes 2, 6, and 10=IgG3; lanes 3, 7, and 11=IgG2; lanes 4, 8, and 9=IgG1. Sample A=representative serum sample; sample B additionally contained IgG4 anti-IgA. Reduction of the disulphide bonds of the blotted immunoglobulins showed that the binding site of the IgG4 anti-immunoglobulins is located on the heavy chain.

in the circulation. However, most evidence to support this theory has arisen from experimental approaches with heterologous systems and altered immunoglobulins for immunisation.³ We aimed to confirm whether anti-immunoglobulin autoantibodies are really detectable in the human circulation.

The major problem in the detection of IgG antibodies directed against other IgG antibodies with immunological methods is that the antigen and antibody have almost the same structure. Therefore, in the past, IgG from animals was used as the antigen. Because of altered affinities and only partial homologies, these systems are not appropriate for studying naturally occurring anti-immunoglobulin autoantibodies. We used human myeloma IgG subclasses (IgG 1–4, κ and λ ; Sigma, Deisenhofen, Germany) as antigen, and subclass-specific detection antibodies (set 1: Pharmingen, San Diego, USA [monoclonal]; set 2: the Binding Site, Birmingham, UK [polyclonal]). The detection system was adjusted to give similar results between the single-subclass antibodies. With logical exceptions (ie, IgG anti-IgG1, IgG2 anti-IgG2, and so on), this system allowed us to measure IgG anti-IgG interactions directly. In addition, human IgA (human serum; Dianova, Hamburg, Germany), IgM (myeloma; Sigma), and IgE (myeloma; ICN, Costa Mesa, USA) were used as antigens. Samples included plasma from healthy blood donors (aged 17–64 years; $n=218$), and cord blood ($n=14$). For the screening experiments (by ELISA), antigens were coated onto microtitre plates at equal concentrations (1.5 $\mu\text{g/mL}$), blocked with 3% bovine serum albumin in phosphate-buffered saline, and incubated with serum samples diluted 1:2. Bound immunoglobulins were visualised by means of detection antibodies conjugated with horseradish peroxidase.

All samples contained substantial amounts of IgG4 antibodies against the three tested IgG subclasses (table). Almost all samples also contained IgG2 anti-IgG1 (although concentrations were not significantly higher than those of the blank [3% bovine serum albumin in phosphate-buffered saline]). We found no anti-IgM, and only some samples which contained IgG4 anti-IgE (3.5%) or anti-IgA (6%). All results were confirmed with the second set of subtype-specific antibodies, and by running of denatured immunoblot experiments (figure). This process ruled out the possibility of interference by the antibody-binding activities of the coated myeloma proteins themselves. Although all of these auto-antibodies also had affinities for animal immunoglobins (tested with mouse IgG, data not shown), anti-IgG and anti-IgE generated no artificial sandwich-formation leading to

	IgG1 (n=218)	IgG2 (n=218)	IgG3 (n=218)	IgG4 (n=218)
Anti-IgG1	..	122 (56%)	3 (1.5%)	218 (100%)*
Anti-IgG2	0	..	0	218 (100%)*
Anti-IgG3	0	6 (2.7%)	..	218 (100%)*
Anti-IgG4	5 (2.4%)	5 (2.3%)	0	..

* $p<0.005$, Mann-Whitney U test.

Number of serum or cord-blood samples with extinction values at least five times higher than those of the blank

false-positive results in our assays. This finding was confirmed by exchanging the detection antibodies with antibodies of a different antigenic specificity from the same species as a negative control.⁴ Only IgG4 anti-IgA displayed a sandwich-forming activity with other commercial ELISA systems; furthermore, it was identical to the major group (85%) of sandwich-forming heterophilic anti-immunoglobulin antibodies recently described by our group.⁴ Although the minor group (15%) consisted of IgG4 antibodies, we have not yet identified a common auto-antigen for this group.

IgG4 seems to be the most frequent subclass of anti-immunoglobulin autoantibodies in healthy people. Such antibodies are present in cord blood and persist throughout life. The results imply that IgG4 antibodies, and probably also IgG2 antibodies, may play an important, if not crucial, role in an immunoglobulin network. We also found that IgG4 anti-immunoglobulins are also present in high-dose immunoglobulin preparations (IVIGs, data not shown), which are used for treating autoimmune disorders such as Guillain-Barré syndrome, idiopathic thrombocytopenic purpura, Kawasaki's syndrome, and dermatomyositis. Although the mechanism of action of IVIGs is still incompletely understood, antibodies within these preparations are known to interact with the disease-mediating autoantibodies of the patient. Anti-idiotypic interactions and down-regulation of complement deposition have been described as modes of action. Since IgG4 is itself a non-complement-activating immunoglobulin, an infusion of a surplus of regulatory IgG4 anti-immunoglobulins could be responsible for both these effects. If this is the case, enrichment of IVIGs with IgG4 could be of great benefit. Furthermore, in allergic diseases, continuous exposure to low doses of antigen leads to a class switch from harmful IgE to harmless IgG4.⁵ An induced switch from Th1-mediated, inflammation-causing IgG1/3 production (commonly found in systemic autoimmune disorders) to IgG4 antibodies could thus be used to treat various autoimmune diseases.

Our results also show that naturally occurring heterophilic anti-immunoglobulin antibodies occur in all healthy people and are not, as was believed for a long time, induced by contact with animal proteins. These molecules are autoantibodies which only crossreact with other species, and their potency to interfere in ELISAs depends on their ability to crosslink the assay antibodies; IgG4 anti-human IgA is the strongest interfering immunoglobulin.

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