OXYGEN FREE RADICAL ALTERED IMMUNOGLOBULIN G IN THE ETIOPATHOGENESIS OF RHEUMATOID ARTHRITIS

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DOOR VRIJE ZUURSTOFRADICALEN VERANDERD IMMUUNGLOBULINE G IN DE ETIOLOGIE EN PATHOGENESE VAN REUMATOIDE ARTRITIS

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"When you realize the value of all life, you dwell less on what is past and concentrate more on the preservation of the future."

Dian Fossey, *Woman in the mists.*

Voor mijn ouders
Voor Anne

ABBREVIATIONS

ANOVA	analysis of variance
BSA	bovine serum albumin
C1q-BT	C1q-binding test
CGD	chronic granulomatous disease
DEAE	diethylaminoethyl
2-DOG	2-deoxyglucose
EBV	Epstein-Barr virus
EC-SOD	extracellular SOD
Fab	antigen binding fragment
Fc	crystallin fragment
GSH-PX	glutathione peroxidase
HAG	heat aggregated gammaglobulin
HBSS	Hanks buffered salt solution
HNB-Br	2-hydroxy-5-nitrobenzyl bromide
HPLC	high performance liquid chromatography
HRP	horse-radish peroxidase
IC	immune-complex
IgG	immunoglobulin G
IgM	immunoglobulin M
JRA	juvenile rheumatoid arthritis
MHC	major histocompatibility complex
Mono(mer) IgG	monomer UV-irradiated IgG
MPO	myeloperoxidase
NADPH-oxidase	reduced nicotinamide adenine dinucleotide phosphate
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
PB	peripheral blood
PBS	phosphate buffered saline
PHGSH-PX	phospholipid hydroperoxide glutathione peroxidase
PIP	peroxidation inhibiting protein
PMA	4ß-phorbol-12-myristate-13-acetate
PMN	polymorhonuclear leucocytes
Poly(mer) IgG	polymer UV-irradiated IgG
RA	rheumatoid arthritis
RaIgG	rabbit IgG
RF(s)	rheumatoid factor(s)
RIA	radio immuno assay
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
SF	synovial fluid
SH	sulphydryl
SOD	superoxide dismutase
STZ	serum treated zymosan
TCA	trichloroacetic acid
UV	ultraviolet

CONTENTS

ONE

General introduction

CHAPTER I

GENERAL INTRODUCTION

I.1 Immunoglobulins

1.1 Immunoglobulin structure

Immunoglobulins are a family of structurally related glycoproteins which carry antibody activity, i.e., the property of specific combination with the substance which elicited their formation. Their production is induced when the host's immune system comes into contact with foreign substances (antigens) introduced into the body.
Immunoglobulins account for approximately 20% of the total plasma proteins. They can also be found in extravascular fluids (i.e. synovial fluid), in exocrine secretions and on the surface of some lymphocytes. In most mammals five distinct classes of immunoglobulins are recognized, namely immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin D (IgD) and immunoglobulin E (IgE)

Table I.1: *Nomenclature of the five classes of immunoglobulins. (Adapted from ref.1).*

Present nomenclature	Abbreviation	Old nomenclature
immunoglobulin G	IgG	gammaG-globulin 7S gamma-globulin
immunoglobulin A	IgA	gammaA-globulin β_2 A-globulin
immunoglobulin M	IgM	gammaM-globulin 19S gammaglobulin gamma-macroglobulin
immunoglobulin D	IgD	
immunoglobulin E	IgE	reagin

(table I.1). Structurally the antibody classes differ in size, charge, amino acid composition and carbohydrate content. Although the immunoglobulins possess the same basic molecular architecture the differences dictated by amino acid sequence of the polypeptide chains are reflected in a vast array of antigen-binding specificities and different biological activities. The structural heterogeneity within immunoglobulin classes has made it difficult to elucidate the exact structure and amino acid sequence of each immunoglobulin class. But in recent years the technique of somatic cell fusion (hybridomas) has produced homogeneous (monoclonal) antibodies which greatly facilitated this study.

All immunoglobulins have a basis 4-chain structure consisting of two identical heavy chains of molecular weight ± 50,000 and two identical light chains of molecular weight ± 23,000. The class and subclass of an immunoglobulin molecule is determined by its heavy chain type. IgG possesses γ-chains, IgA α-chains, IgM μ-chains, IgD δ-chains and IgE ϵ-chains. IgG, a monomeric protein, is the major immunoglobulin in normal human serum (± 70%). The heavy chains of IgG exist in four forms known as γ1, γ2, γ3 and γ4 which correspond to four subclasses of human IgG i.e. IgG1, IgG2, IgG3 and IgG4. Of IgA (15-20% of total serum immunoglobulin) two subclasses are known; IgA1 and IgA2 which possess the heavy chain types α1 and α2 respectively. Secretory IgA (sIgA) is the major antibody found in seromucous secretions (i.e. saliva) and exists as a dimeric complex. IgM (10% of total serum immunoglobulins) is a pentameric structure of total molecular weight of 970,000. It is the predominant antibody produced early in an immune response. IgD (< 1% of total serum immunoglobulins) is widely present on the surface of B-lymphocytes where it probably acts as an antigen receptor. Finally IgE, which constitutes only 0.004% of the total serum immunoglobulin pool, binds with high affinity to mast cells which they trigger upon recognition of specific antigen (allergens) in allergic reactions. The light chains exist in two forms known as kappa and lambda and are common to all classes of immunoglobulins. In any one immunoglobulin molecule both light chains and both heavy chains are of the same type and have the same amino acid sequence.

1.2 Structure of IgG

The IgG-molecule is a typical example of basic antibody structure. Figure I.1 shows the basic structure of IgG1.
A single disulphide bridge (S-S) between cysteine residues links light chains to heavy chains. The heavy chains are also linked together by disulphide bridges but the number varies with the subclass. Typically IgG1 contains two inter-heavy chain disulphide bonds; IgG3 may contain as much as 14 inter-heavy chain links. Each polypeptide chain is made up of a number of domains of rather constant size (100-110 amino acid residues) formed by intra-chain disulphide bridges. The N-terminal domains of all four polypeptide chains show much more variation than the others and are known as the variable regions (V_L: variable light chain; V_H: variable heavy chain). The carboxy-terminal ends of the chains are known as the constant domains. For the light chains this is the C_L-domain (constant light chain). The heavy chain of IgG1 contains three constant domains: C_H1, C_H2 and C_H3 (C_H: constant heavy chain).
Immunoglobulins are easily split by the enzyme papain in an area between the first and second constant region domains at the N-terminal side of the inter-heavy chain disulphide bonds. This results in the formation of two Fab-fragments and one Fc-fragment. Pepsin splits the molecule on the C-terminal side of the inter-heavy chain disulphide bonds, resulting in a large F(ab)'2-fragment (approximately 2 Fab fragments) and two pFc'-fragments. The region on the heavy chain susceptible to proteolytic cleavage is known as the hinge region.
Human immunoglobulin G contains basically an asparagine-linked sugar chain in the Fc-region of each heavy chain. The carbohydrate moiety of human IgG shows a higher structural multiplicity than other human serum glycoproteins (3). In addition to their Fc

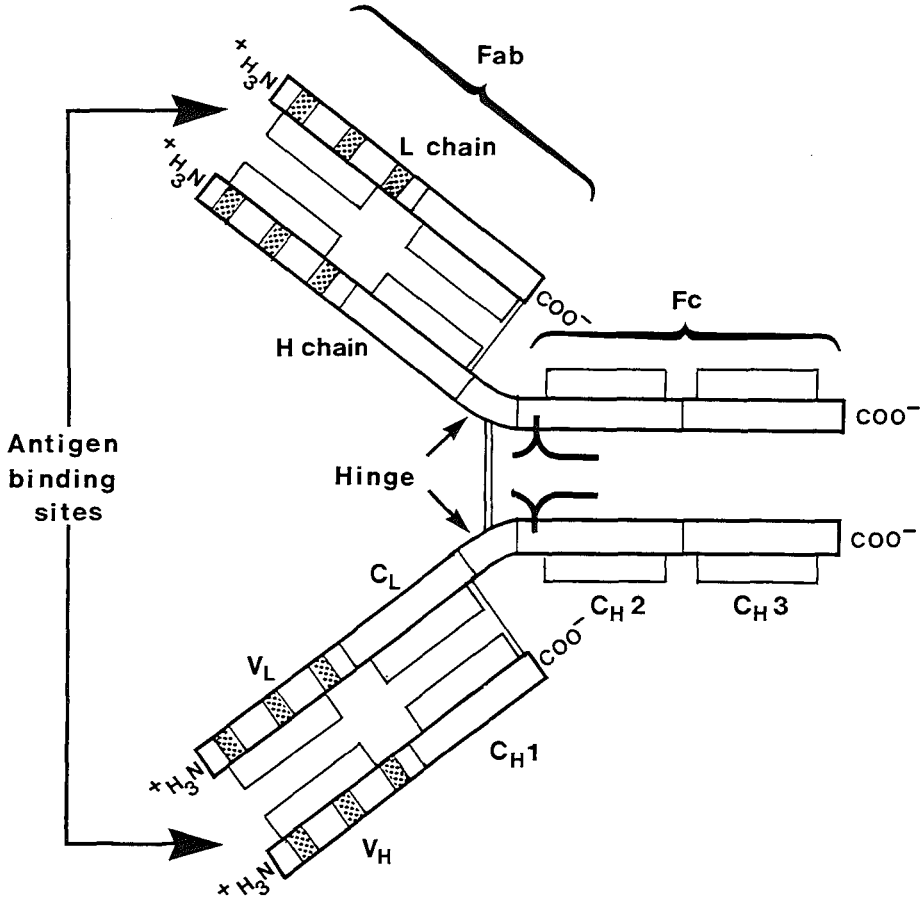

Figure I.1: *A simplified model for an IgG1 (kappa) human immunoglobulin molecule. The antibody molecule consists of two heavy (H) and two light (L) chains, linked by disulphide bridges (solid lines) and is divided into homologous regions of sequence (V_H, C_H1, C_H2 and C_H3), each of which has an intra-chain disulphide (the pattern of inter-chain disulphide bridging shown here is characteristic of human subclass IgG1). In V_H and V_L, the dotted segments represent the hypervariable regions of sequence that, in the three dimensional structure, together form the antigen binding site. The conserved asparagine-linked bi-antennary complex oligosaccharide chains are attached to Asn 297 in the C_H2 domains. Adapted from ref.2.*

oligosaccharides, some myeloma immunoglobulin molecules have oligosaccharides linked to the variable segments of heavy and light chains (4).

1.3 Functional properties of IgG

Immunoglobulin G-molecules consist functionally of two units. One unit in fact is formed by two identical subunits and is involved in binding to foreign material or antigen (antigen binding fragment = Fab). The V-regions on the light and heavy chains are primarily concerned with antigen binding and bare the unique antigen-specificity. V-regions are quite heterogenous but within the variable domains relatively short segments show extreme variation in sequence and are known as hypervariable regions. These regions have been shown to be the site most intimately involved in antigen-binding.
The other functional unit of immunoglobulin G (Fc = fragment crystallin) is involved in binding molecules generally related to antigen elimination, such as complement and receptors on cells. These molecules that interact with the Fc-part are known as effector molecules since they trigger effector functions. Complement subcomponent C1q interacts with the C_H2-domain of IgG. This triggers the classical pathway of the complement system. Fc-receptors are found on a number of cell types and are associated with a variety of functions including phagocytosis (monocytes, macrophages and neutrophils), antibody-dependent cellular cytotoxicity (monocytes and macrophages), transport (trophoblasts) and possibly immunomodulation (lymphocytes). The Fc-region of IgG also interacts with protein A, a major cell wall component of most strains of Staphylococcus aureus, and is the site which expresses the epitopes recognized by rheumatoid factors. The existence of rheumatoid factors which interact with the Fc-region of IgG will be discussed further below.

In conclusion, the structure of immunoglobulin molecules reflects their dual role in recognizing foreign material and triggering eliminating effector functions.

I.2 Rheumatoid arthritis

2.1 Clinical aspects of rheumatoid arthritis

The estimation of the prevalence of rheumatoid arthritis (RA) is, given the difficulty of definition, not easy. Probably the prevalence is between 0.3% and 1.5% (5). RA is two to three times more common in females than in males. The disease has an increasing prevalence with advancing age.
Rheumatoid arthritis can become manifest in various ways. Mostly it evolves in a relatively short period as an inflammation of one or several joints. The pain in the joint is often less endurable during the night. In the morning these joints are often stiff. An important feature is the inflammation of the synovial membrane. Usually the inflammation is symmetrically distributed, causing bone erosions and becoming chronic. The amount of synovial fluid increases, the synovial membrane thickens, leading to swelling of the joint and a reduction of the viscosity. Eventually material

present in the synovial cavity will destroy the cartilage and bone. Finally these processes can lead to a total destruction of the joint with complete functional loss. RA can develop in different ways: either very slowly and even or with exacerbations and remissions. Sometimes patients can feel well for a long period and then suddenly feel much worse.

RA is a systemic disease, i.e. not only joints but a variety of other organs can be affected. These extra-articular manifestations are listed below, usually they are the consequence of a vasculitis:

- rheumatic nodules
- pericarditis
- pleurisy, involvement of lung parenchyma
- keratoconjunctivitis sicca (secondary Sjögren's syndrome)
- neuropathy
- lymphadenopathy
- splenomegaly
- neutropenia

Almost all RA-patients have a more or less severe anemia. This anemia is not due to an iron deficiency, but results from the inflammation. Most likely this is caused by an inability to mobilize iron from its stores (6).

Typically, in the serum and synovial fluid of RA-patients autoantibodies directed against autologous IgG are often found. These autoantibodies are known as rheumatoid factors.

RA is diagnosed on the basis of several criteria (table I.2) drawn up by the American Rheumatism Association.

Table I.2: *1958 Diagnostic criteria for rheumatoid arthritis (7).*

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1. Morning stiffness.
2. Pain on motion or tenderness in at least one joint.
3. Swelling in at least one joint that represents soft tissue thickening or fluid, not bony overgrowth alone.
4. Swelling of at least one other joint. An interval free of joint symptoms between the two joint involvements may not be more than 3 months.
5. Symmetrical joint swelling with simultaneous involvement of midphalangeal, metacarpophalangeal, or metatarsophalangeal joints is acceptable without absolute symmetry. Terminal phalangeal joint involvement does not satisfy this criterion.
6. Subcutaneous nodules over bone prominences, on extensor surfaces or in juxtaarticular regions.

7. Radiographic changes typical of rheumatoid arthritis, which must include at least bony decalcification localized to or greatest around the involved joint and not just degenerative changes. Degenerative changes do not exclude patients from any group classified as rheumatoid arthritis.
8. Positive agglutination test: demonstration of the "rheumatoid factor" by any method that has been positive in not over 5% of normal controls in two laboratories, or from positive streptococcal agglutination test.
9. Poor mucin precipitate from synovial fluid with shreds and cloudy solution.
10. Characteristic histologic changes in synovial membrane with three or more of the following: marked villous hypertrophy; proliferation of superficial synovial cells, often with palisading; marked infiltration of chronic inflammatory cells, with tendency to form "lymphoid nodules"; deposition of compact fibrin, either on surface or interstitially; foci of necrosis.
11. Characteristic histological changes in nodules showing granulomatous foci with central zones of cell necrosis, surrounded by a palisade of proliferated mononuclear cells, peripheral fibrosis and chronic inflammatory cell infiltration.

The diagnosis of classic RA requires 7 of the above criteria. The diagnosis for definite RA requires 5 of the above criteria. Probable RA needs 3 criteria.

In 1988 a newly revised set of criteria has been published (8). This has replaced the earlier criteria, in which the distinction was made among "classic", "definite" and "probable" RA. In the 1988 criteria these distinctions are no longer made. Furthermore, the 1988 criteria are reduced to 7 points, therefore require less extensive or costly investigations.

Table I.3: *1988 Diagnostic criteria for rheumatoid arthirtis (8).*

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1. Morning stiffness.
2. Arthritis of three or more joint areas.
3. Arthritis of hand joints.
4. Symmetric arthritis.
5. Rheumatoid nodules.
6. Serum rheumatoid factor.
7. Radiographic changes.

The diagnosis RA requires 4 of the above 7 criteria. Designation as classic, definite or probable RA is not to be made.

2.2 Etiology of rheumatoid arthritis

The actual cause of rheumatoid arthritis is unknown. However, over the past decades much information has been gathered regarding the nature of the inflammatory process and the involvement of the immune system. Generally rheumatoid arthritis is considered to be an autoimmune disease, in which the immune system at a certain level fails to distinguish between "self" and "non-self". In the case of rheumatoid arthritis the structure seen as "non-self" is autologous immunoglobulin G. This is indicated by the presence of auto-antibodies known as rheumatoid factors which bind to IgG. It is not clear whether the existence of rheumatoid factors is a consequence of the acquirement of an antigen by external factors or that it is caused by a primary misregulation of the immune system. However, it must be clear that any hypothesis concerning the etiology of RA must take into account the existence and the production of rheumatoid factors.

2.2.a Genetic predisposition

Recently it became clear that RA was found with increased frequency in relatives of RA-patients. Studies showed a marked correlation between the occurrence of the MHC (major histocompatibility complex) antigen HLA-DR 4 and rheumatoid arthritis (9,10). The HLA-DR genes are analogous to the immune-response (Ir) genes in the murine MHC and code for some immune response antigens (11). The present notion is that these specific antigens code for an immunological response that leads to inflammation and continued disease activity as seen in RA.

2.2.b Initiating agent

The etiological factor that initiates an immune response in a host of appropriate genetic make up leading to RA is still unknown. A large number of candidates are suggested as initiating agent in RA. These can roughly be divided into two groups, consisting of exogenous factors and endogenous factors, respectively. Although not complete, the most prominent ones are listed below:

Exogenous factors

Bacteria: Of Streptococcus it is known that it can cause rheumatic fever (12). Furthermore it was demonstrated that intraperitoneally injected Streptococcal cell wall fragments in rats could induce arthritis (13). Antibodies against Streptococci appear to cross-react with IgG (and become rheumatoid factors) and with peptidoglycan, a major component of the extracellular matrix (14). However, there is no evidence that human RA is initiated by Streptococci.
Clostridium perfringens has also been implicated as initiating agent because high levels of this bacteria are frequently present in the faeces of RA-patients (15).

Other bacteria included as etiological agents are diphtheroid organisms (16). Of these bacteria higher than normal levels were reported in synovial membrane and synovial fluid suggesting that they play some role in RA.
Mycoplasmas have also been related to the etiology of RA (17). The most interesting aspect of mycoplasma biology is its ability to mimic the host cell surface, thereby escaping the immune system of the host and persisting chronically. This is particularly interesting while mycoplasmas are able to induce an RA-like disease in animals (18).

Viruses: The most prominent of the viral candidates implicated in the etiology of RA is the Epstein-Barr virus (EBV). Patients have large amounts of serum antibodies to Epstein-Barr virus antigens, as well as large amounts of antibodies in the synovial fluid (19). The virus is a potent and preferential inducer of rheumatoid factor production. Moreover, patients with RA produce more RF that is of higher avidity when their B cells are stimulated by EBV (20). It was also demonstrated that RA-patients had an abnormally elevated frequency of EBV-infected B cells in their blood (21). Recent studies in patients with early RA showed that titers of antibody to EBV were not elevated (22). These data suggest a role for EBV in the pathology rather than the etiology of RA.

Endogenous factors

Collagen: Ever since it was demonstrated that type II collagen can induce arthritis in rats (23) and mice (24) collagen has been implicated in the etiology of RA. In these rodents, it is clear that functional T cells are necessary to induce an arthritis (25). As it was the case with EBV, most data on collagen induced arthritis do not support the hypothesis that RA is caused by development of antibodies to type II collagen but rather that the inflammatory response is amplified by it. A study by Rowley *et al* (26) showed that RA was found to be associated with antibodies to denatured collagen rather than to native collagen, pointing to a secondary role in immune-mediated perpetuation of RA.

IgG: Most characteristic of RA are the anti-IgG antibodies or rheumatoid factors. It is assumed that they are produced as a result of autoimmunization to the patients' own IgG in altered form. The existence of rheumatoid factors points to a role of IgG in the etiology of RA. Especially since it has been shown that some patients have rheumatoid factor present in their sera prior to the onset of any symptoms of arthritis.

IgG as endogenous factor in the etiology and pathology of RA is the main focus point in this thesis.

2.3 Rheumatoid factors

Rheumatoid factors were first recognized by the ability of the sera of RA-patients to agglutinate sheep erythrocytes coated with rabbit IgG (27). This classic technique is still being used to detect RF in sera of RA-patients and is known as the Waaler-Rose test. Since then other methods have been developed of which the human IgG-latex fixation test is now the most widely used in clinical laboratories.
As mentioned before rheumatoid factors are auto-antibodies directed against antigenic determinants on the Fc-fragment of IgG-molecules. Over 70-90% of adult RA-patients produce the RF, the other patients are considered seronegative, i.e. they have RF titers falling in the normal range (28). The latter patients usually have milder synovitis than seropositive patients, and they seldomly develop extra-articular rheumatoid disease (29). Most RF are of the IgM isotype, but IgG (30), IgA (31), IgD (32) and IgE (33) rheumatoid factors have also been described. Although still unknown, several concepts have been postulated concerning the induction of RF-production in RA:

1. RF-production has been thought to be the result of polyclonal B-lymphocyte activation (34,35).
2. The production is triggered through stimulation by aggregated IgG, usually in the form of immune-complexes (36).
3. Induction of RF-production by exogenous antigens bearing cross-reactive determinants to human IgG (37,38).
4. RF-production is the result of a immune response to somehow altered IgG (39).

Several hypotheses have been put forward to explain how IgG could become immunogenic leading to RF-production and inflammation:

1. IgG of RA-patients is structurally different from IgG of normal subjects. This is either caused by an altered genetically controlled synthesis or this is the consequence of exposure to an endogenous/exogenous altering agent.
2. The development of a T cell mediated immune response against IgG. Possibly by a depletion of suppressor T lymphocytes, allowing B lymphocytes to produce auto-antibodies against certain determinants of IgG (40).
3. Rheumatoid factor production could represent idiotype-anti-idiotype interactions. The possibility that such idiotype-anti-idiotype interactions could be taking place in RA is suggested by the finding of anti-idiotypic antibodies directed against IgM-RF purified from a seropositive RA-patient (41). Moreover, these idiotypes are highly conserved on IgM-RF (42,43). In general, the idea is that RFs like other auto-antibodies are multispecific antibodies, namely, primarily specific for idiotypes and secondarily bind to human Fc-fragments.

2.4 Pathogenesis of rheumatoid arthritis

The pathogenesis of RA is complex and multifactorial. Important features of the pathogenesis are outlined below, the stages are chosen arbitrarily.

1. An inflammation is initiated by an unknown etiologic agent in a susceptible individual. The mode and intensity of this process is determined by a genetically controlled immune response.
2. In the synovial fluid the immune response catalyzes a number of processes which interact to produce a self-sustaining active inflammation. During this process polymorphonuclear leucocytes (PMN) are attracted to the synovial fluid, where they subsequently release proteolytic enzymes, prostaglandins and oxygen free radicals (44).
3. The synovial membrane is filled with mononuclear cells mostly of the helper T lymphocyte phenotype (45). These cells make contact with macrophages and B lymphocytes resulting in the activation of the B cells to plasma cells with resultant immunoglobulin production.
4. Formation of immune complexes composed of IgM and IgG rheumatoid factors within the joint (46).
5. Production of monokines and lymphokines that stimulate target cells (i.e. synovial cells, fibroblasts) to proliferate and produce, among others, prostaglandins, proteinases and connective tissue. Endothelial cells are activated to angionesis of capillaries. The produced monokines and/or lymphokines also induce formation of pannus (expanding synovial cells) tissue (47).
6. Finally, these processes lead to collagen degradation, depletion of proteoglycan from cartilage, resorption of demineralized bone.

I.3 Aim of the study

The particular association of RA with anti-IgG antibodies suggests an important role of IgG in the etiology and pathology of RA. One of the suggested mechanisms by which IgG could be altered is exposure to oxygen free radicals. During inflammation large amounts of oxygen free radicals are produced by PMN giving basis to this suggestion. In Chapter II of this thesis a general overview is presented on the role of oxygen free radicals in RA. Particular attention is given to the effects on proteins. From this it can be seen that oxygen free radicals play an important role in the inflammatory response and concomitant tissue damage in RA.

Briefly, the attention in this thesis is focussed on the following questions:

- Can IgG be altered by oxygen free radicals?
- What is the nature of these alterations?
- Does oxygen free radical altered IgG interact with rheumatoid factors and complement?
- What is the effect of oxygen free radical altered IgG on the non-specific immune system (granulocytes)?
- What is the effect of oxygen free radical altered IgG on the specific immune system (lymphocytes)?

TWO

Interactions between oxygen free radicals and proteins. Implications for rheumatoid arthritis. An Overview.

H.A. Kleinveld, A.J.G. Swaak, C.E. Hack and J.F. Koster.

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CHAPTER II

INTERACTIONS BETWEEN OXYGEN FREE RADICALS AND PROTEINS; IMPLICATIONS FOR RHEUMATOID ARTHRITIS. AN OVERVIEW

II.1 Introduction to oxygen free radicals

For a long time oxygen has only been regarded as beneficial and an absolute requirement for all respiring species. In aerobic organisms molecular oxygen (O_2) plays an important role as an electron-acceptor in mitochondrial electron-transport systems. However, in recent years increasing evidence has been gathered which points to a different role of O_2 in respiring species. The other side of cellular oxygen utilization encompasses the potential hazard of oxygen toxicity. The recognition of oxygen toxicity came with the notion that free radicals are the metabolites by which oxygen exerts its toxic effect. A free radical is by definition a molecule or atom that possesses an unpaired electron. In fact molecular oxygen itself is a biradical with two unpaired electrons with parallel spins each located in a different orbital. The formation of oxygen-derived free radicals can occur through the univalent reduction of molecular oxygen (fig.II.1). This leads successively to the formation of superoxide, $O_2^{\cdot-}$; hydrogen peroxide, H_2O_2; the hydroxyl radical, $OH^{\cdot}$; and H_2O. The superoxide radical and hydrogen peroxide, of which the latter has no unpaired electrons and is not a radical, are relatively unreactive and long-lived compounds in biologic systems. Their potential danger, however, lies in their ability to lead to the formation of the extremely reactive hydroxyl radical. The hydroxyl radical is a product of the classical Haber-Weiss reaction when H_2O_2 is directly reduced by superoxide, but this reaction only proceeds in the presence of transition metals. The role of these metals was first recognized by Fenton in 1894. He found that a mixture of H_2O_2 and an iron(II)-salt forms the hydroxyl radical. This reaction is called the Fenton reaction. In biologic systems under physiological conditions hydroxyl radicals are formed via the Fenton reaction. The Haber-Weiss reaction should be considered as a summation of the reduction of Fe^{3+} by $O_2^{\cdot-}$ and the Fenton reaction (fig.II.2).
Other oxygen metabolites in biologic systems are singlet oxygen ($^1O_2$), which is formed if one of the unpaired electrons of molecular oxygen moves to alleviate the spin restriction, and hypohalous acids including hypochlorous acid (HOCl) generated by the myeloperoxidase-H_2O_2-halide system.

II.2 Sources of oxygen free radicals

Oxygen free radicals are generated *in vivo* as byproducts of normal metabolism. Although diverse ways exist by which an individual can be exposed to free radicals other than through normal metabolism (including drug-metabolism, irradiation and

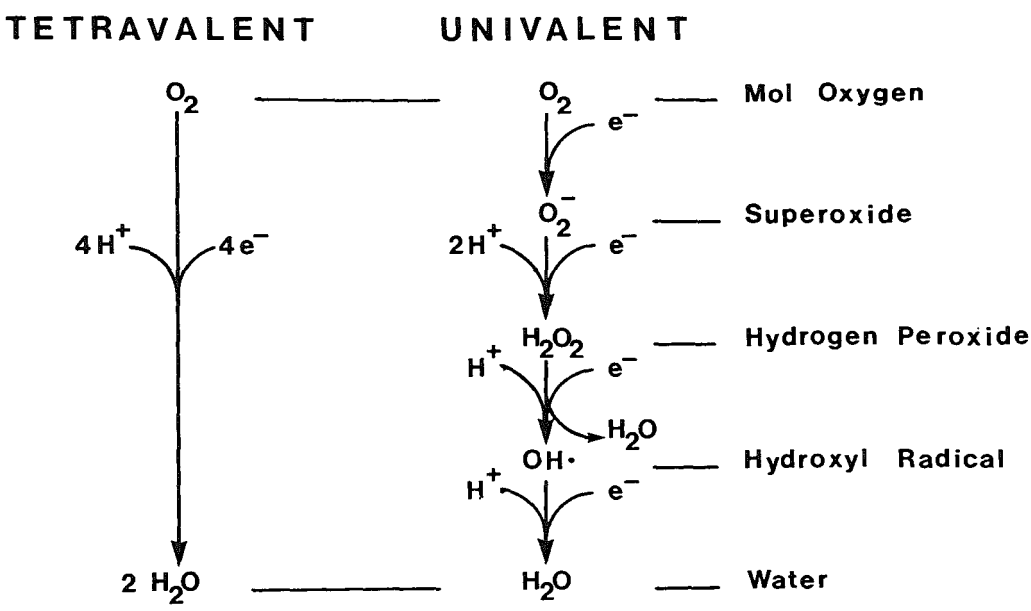

Figure II.1: *Univalent reduction of molecular oxygen yields oxygen free radicals.*

xenobiotics), we will focus on intra- and extra-cellular processes that generate oxygen free radicals:

A. Auto-oxidation of a wide variety of low molecular weight molecules, i.e. thiols (1), tetrahydropterins (2), catecholamines (3), flavins (4) and hydroquinones (5), generates superoxide as primary radical.

B. Numerous enzyme-substrate reactions generate superoxide during their catalytic cycling. Of these the reaction observed between xanthine and xanthine-oxidase is probably the best known and most frequently used *in vitro* system to study superoxide effects.

C. Mitochondrial electron transport systems were first reported to produce H_2O_2 (6), but later studies showed that most, if not all, mitochondrial H_2O_2 is derived from spontaneous or enzyme (SOD) catalyzed dismutation of superoxide (7).

D. The mixed function oxidases, cytochromes P450 and b5, of the microsomal system are also able to produce free radicals. These cytochromes, also located in nuclear membrane, are able to produce O_2^- and H_2O_2 depending on the nature of the available substrate.

E. Peroxisomes of eukaryotic cells have been shown to produce H_2O_2 directly without intermediate superoxide production (8). Part of the H_2O_2 produced by peroxisomal oxidases is scavenged by catalase, but some diffuses to the cytoplasma where it can

damage cytosolic components.
F. Plasma membranes of cells contain several potential sources of free radicals. The metabolism of arachidonic acid by cyclooxygenase and lipoxygenase involves free radicals intermediates. Not only intermediate carbon-centered free radical peroxides (lipoxygenase) are generated but also the highly reactive hydroxyl radical (cyclooxygenase). The presence of unsaturated fatty acids and transmembrane proteins makes the plasma membrane a critical site of free radical reaction. Lipid-peroxidation and protein-oxidation by autocatalytic processes in the membrane and by extra-cellularly generated free radicals can cause serious loss of membrane functions.

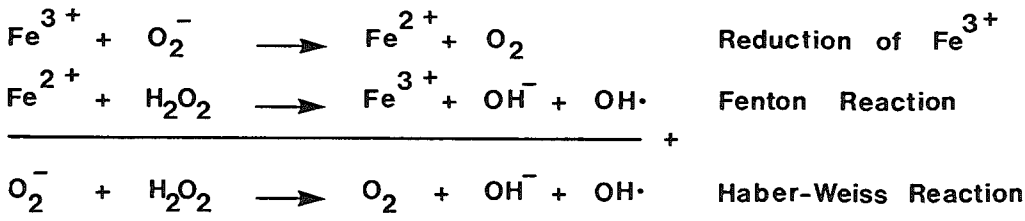

Figure II.2: *The Haber-Weiss reaction.*

G. Phagocytic cells (macrophages, monocytes and polymorphonuclear leucocytes) can produce enormous amounts of superoxide and hydrogen peroxide when stimulated. Upon recognition of a phagocytic or soluble stimulus neutrophils and macrophages experience a "respiratory burst" during which O_2 is reduced to superoxide and hydrogen peroxide. Also the hydroxyl radical has been identified as an oxygen metabolite generated during the respiratory burst, formed by the interaction of O_2^- and H_2O_2 in the presence of iron or copper (modified Haber-Weiss reaction). Activation of the membrane-bound NADPH-oxidase has been identified as the mechanism responsible for the observed free radical production by phagocytes. During phagocytosis this enzyme releases oxygen free radicals primarily into the phagocytic vacuole but a substantial part is released outside the phagocytic cell (9). The killing of phagocytosed microorganisms is largely dependent on the production of reactive oxygen metabolites. A deficiency in this system occurs in children suffering from chronic granulomatous disease (CGD).
Activated phagocytes release the enzyme myeloperoxidase (MPO) from their azurophilic granules during the respiratory burst. MPO activity generates hypochlorous acid (HOCl). Hypochlorous acid oxidizes a wide variety of biomolecules, but its most critical cellular target is probably the alpha-1-antitrypsin protein. This proteinase inhibitor is the major defense against elastase. Effects and implications of oxygen free radical damage to proteins, including alpha-1-antitrypsin, will be discussed below.

II.3 Effects on proteins

Our current knowledge on the involvement of oxygen free radicals in pathological conditions is largely based on studies of radical reactions with polyunsaturated lipids. The extreme high reactivity of free radicals implicates that upon their production *in vivo* they will react with any suitable (capable of electron-donation) biomolecule in their immediate vicinity. Understanding of this principle led to investigations of free radical damage to nucleic acids and carbohydrates. In recent years considerable evidence has also been gathered on free radical interaction with enzymes and other proteins. The structural effects of free radical damage on proteins can roughly be divided in three aspects:

1. modification of amino acids
2. fragmentation
3. aggregation or cross-linking

Clearly these effects are inter-linked as fragmentation cannot occur without peptide-bond breakage or amino acid destruction. Also aggregation of proteins is a secondary effect of amino acid alteration (fig.II.3).

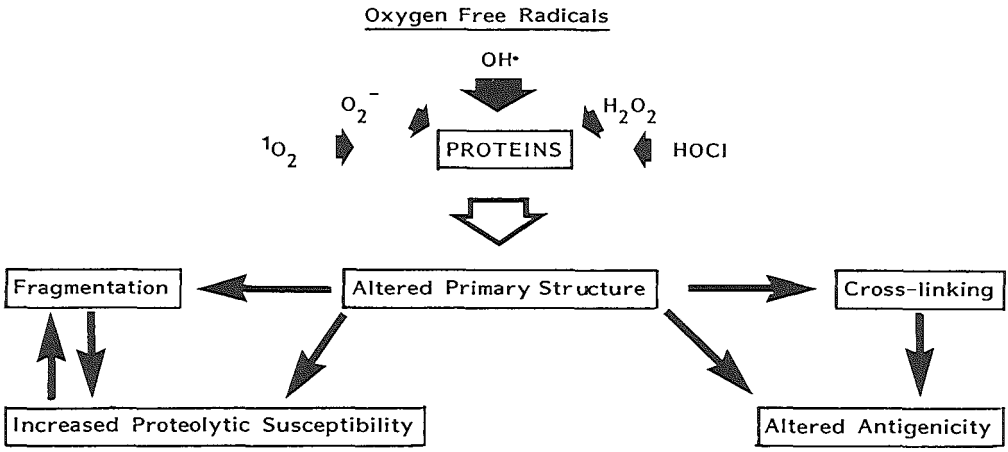

Figure II.3: *Effects of oxygen free radicals on proteins.*

It should be noted that free radical attack on proteins can include carbohydrate-modifications in the case of glycoproteins and that oxygen free radicals are also able to mobilize metals from metalloproteins. Both mechanisms can affect the secondary structure of proteins and lead to altered functions. Although carbohydrate-chain modifications can be of considerable physiological importance this is not a protein modification per se and therefore beyond the scope of this paper. Because metals play an important role in oxygen free radical chemistry the effects on metalloproteins, especially iron-containing proteins, will be discussed separately.

3.1 Structural effects

The primary structure of a protein defines its potential oxygen free radical attack sensitivity. Interaction of oxygen free radicals with amino acids leads to structural protein modifications. However, all four structural levels of protein architecture determine the accessibility of certain amino acids to free radicals and therefore the actual alterations that ensue. The modifications of a protein by oxygen free radicals are thus the result of general mechanisms, which can be applied to any protein but depend primarily on the structural entities of that protein, including possible carbohydrate chains and bounded metals. But, not only the structure of a protein determines its susceptibility, also the presence of other biomolecules such as lipids and carbohydrates can, during oxygen free radical attack, lead to specific interactions and alterations.

Considering oxidative damage to amino acids the most sensitive are the aromatic amino acids tryptophan, tyrosine and phenylalanine. These three amino acids contain unsaturated structures which are extremely sensitive to free radical induced oxidation. Especially the indol-ring of tryptophan is sensitive to photolysis, resulting in a loss of tryptophan fluorescence (Ex.max.295 nm, Em.max.348 nm). Indole-ring destruction products like N-formyl kynurenine (Ex.max.330 nm, Em.max.440 nm) and kynurenine (Ex.max.365 nm, Em.max.460 nm) are frequently observed after tryptophan photo-oxidation (10,11). Interaction of tryptophan with oxygen free radicals can also lead to deamination and decarboxylation (12). However, it seems unlikely that deamination and decarboxylation of tryptophan play an important role in the intact protein since the amino- and carboxyl-group are then part of peptide-bonds. The phenol- and phenyl-group are the free radical sensitive unsaturated compounds of resp. tyrosine and phenylalanine. The most frequently observed result of oxygen free radical attack on these amino acids is dityrosine formation (13-16), which can be detected by the generation of a specific fluorescence (Ex.max.330 nm, Em.max.410 nm). Dityrosine cross-linking of proteins, which in some cases occurs naturally (17,18), results from the phenolic coupling of two phenoxy radicals of tyrosine (19). Also the benzyl radical (of phenylalanine) can take part in or construct such cross-links. The involvement of phenylalanine in dityrosine formation can be directly by benzyl-radical participation or by a preceding $OH^{\bullet}$-radical addition which increases the tyrosine- and subsequently phenoxy radical concentration (15).

It should be noted that oxygen free radical induced changes in proteins containing tryptophan, tyrosine and phenylalanine mostly occur via the transient indolyl, phenoxy, and benzyl radicals. The possible secondary products formed depend on the immediate environment of these transient species.

A particular feature of native protein fluorescence is its domination by tryptophan fluorescence despite the presence of possible large amounts of tyrosine-residues. Partly this domination is caused by an energy transfer from tyrosine to tryptophan upon excitation. This phenomenon probably also determines, to a certain extent, the effects of oxygen free radicals on tryptophan-residues and tyrosine-residues in proteins.

The heterocyclic amino acid histidine is also susceptible to modification by oxygen free radicals. Several studies showed a decrease in histidine-content after free radical attack

on proteins. Exposure of bovine serum albumin (BSA) to the hydroxyl radical (10 nmol radicals/nmol BSA) led to a 24%-reduction in histidine-content (15). In this study Davies found a generalized loss of 9-10% of most amino acids. Mee and Adelstein (20), using the same free radical generating system as Davies (^{60}CO-radiation), only found losses of the aromatic amino acids and histidine when exposing the histone H2A to radicals. Other studies using H_2O_2 (21) showed a specific attack on histidine-residues of glycoproteins, with a simultaneous peptidolysis. The underlying mechanism here, and most probably in the majority of physiologically generated radical-induced damages, involves the liberation of $OH^{\bullet}$ by the metal (iron or copper) catalyzed Haber-Weiss reaction. Specificity of attack at histidine is due to the location of metals at this residue only. The mechanism of histidine destruction involves the addition of the $OH^{\bullet}$-radical to the C=C bond of the imidazole-ring thereby forming aspartic acid as an intermediate (22). This explains the often found increase in aspartic acid content of proteins after exposure to free radicals (20,21,23). Histidine residues can also contribute to protein cross-linking after exposure to oxygen free radicals (24). From photodynamic oxidation studies of model systems, spectrin and red cell ghosts it was shown that oxidation products of histidine contribute to cross-linking involving His-His, His-Trp and His-Tyr couplings.

Another amino acid which is extremely sensitive to oxygen free radicals is the sulfur-containing cysteine/cystine. Most reports on oxygen free radical damage to protein show that the cysteine/cystine content of the protein studied is altered. These alterations include the breaking of existing disulphide-bonds which lead to sulphydryl-group generation (25-27). Other studies have shown that disulphide-bridges are formed as a consequence of free radical action (28). Whether disulphide-bridges are formed or broken is probably time-dependent. Wickens (29) suggested that during free radical attack on IgG a balance exists between thiol-group generation and disulphide formation. This might well be a general mechanism applying to all cysteine/cystine containing proteins.

Several other amino acids have been included in oxygen free radical induced changes of protein structure. Among these are proline, lysine and methionine (21,23,30,31). However, in all studies it appears that these amino acids are altered/decreased to a lesser extend then the ones discussed above. It seems that oxygen free radical attack on other amino acids then tryptophan, tyrosine, phenylalanine, histidine or cysteine/cystine are less general and indicative of their minor sensitivity to oxygen radicals. However, in some cases oxidation of for example methionine residues may induce very relevant structural alterations in proteins. This in particular is illustrated with alpha-1-antitrypsin, the main inhibitor of neutrophilic elastase. Oxidation of alpha-1-antitrypsin involves oxidation of a methionine-residue at a critical position in the molecule (32). This process and its physiological implications are discussed below.

3.2 Proteolytic susceptibility

Modification of amino acids alters the primary structure of proteins. Alteration of the primary structure with subsequent conformational alterations undoubtedly underlies the increased susceptibility of proteins to proteolytic systems (proteinases, proteases and

peptidases) after oxygen free radical attack. It has been suggested (33) that intracellular proteolytic systems could in this way function as secondary antioxidant defense mechanisms. In preventing the accumulation of oxidatively damaged proteins as useless cellular debris this might well be the case, but this mechanism can also play another role. Proteins which are for instance present in (sub)cellular membranes can also be altered by oxygen-derived metabolites. Damage to these proteins by oxygen free radicals increases their proteolytic susceptibility and thus contributes synergistically to the ensuing tissue damage (34-36). One also has to keep in mind that these proteolytic enzymes are proteins as well, which are just as susceptible to oxygen free radical damage as other proteins. Oxygen free radical activity could in this way decrease the capacity of intracellular proteolytic systems by altering the hydrolytic enzymes themselves (37).

3.3 Oxygen free radicals and the proteinase-proteinase inhibitor balance

Neutrophils not only mediate inflammatory reactions by generating oxygen free radicals, but also by releasing proteolytic enzymes. These proteinases have a considerable tissue-destructive potential, in particular elastase can degrade a wide variety of proteins including complement proteins, clotting factors, and extracellular matrix components (38). However, the role of proteinases in inflammatory reactions *in vivo* have been questioned since in plasma and interstitial fluid large amounts of proteinase inhibitors are present (39), thus providing an effective antiproteinase shield. These inhibitors include alpha-1-antitrypsin, alpha-1-antichymotrypsin and alpha-2-macroglobulin. Alpha-1-antitrypsin, also called alpha-1-proteinase inhibitor, and alpha-1-antichymotrypsin both belong to the superfamily of the serine proteinase inhibitors (40). Elastase activity very effectively is controlled by alpha-1-antitrypsin, and to a lesser extend by alpha-2-macroglobulin (41,42). Because of this antiproteinase shield, which protects the tissues against deleterious effects of the neutrophilic proteinases, oxygen free radicals have been considered as the main mediators of tissue destruction by neutrophils *in vivo*. However in a fascinating review Weiss (43) recently pointed out that oxygen free radicals alone probably do not account for the tissue-destructive effects of neutrophils *in vivo*. He suggested that these effects are due to the concerted action of oxygen free radicals and proteinases. The role of the oxygen radicals in this process is to inactivate alpha-1-antitrypsin, allowing elastase unregulated in the neighborhood of the neutrophil. Inactivation of alpha-1-antitrypsin occurs by oxidation of a methionine-residue at a critical position, i.e. the P1-position, in the reactive center of the protein into methionine-sulphoxide (32). Oxidized alpha-1-antitrypsin virtually has lost its capacity to inactivate elastase, and instead is cleaved by this proteinase (44). In fact, the sensitivity of alpha-1-antitrypsin to oxidation has been used as evidence for a methionine-residue at its reactive center (45). There are indications that oxygen radicals also are involved in inactivating alpha-2-macroglobulin and antileukoprotease, the other inhibitors of elastase (42). In addition, neutrophils contain a metalloproteinase that also can inactivate alpha-1-antitrypsin, by catalytically cleaving a Phe-Leu peptidyl-bond in the reactive center (46). Interestingly, HOCl appeared to be involved in activating this metalloproteinase (46), suggesting a double role of this oxygen metabolite in the inactivation of alpha-1-antitrypsin. Except for alpha-1-antitrypsin, other

serine-proteinase inhibitors contain sequences in their reactive centers that are susceptible for catalytical cleavage by elastase (47). Thus, elastase not only mediates tissue destruction by degrading protein structures, but also inactivates proteinase inhibitors other than alpha-1-antitrypsin allowing unregulated action of other proteinases in the direct environment of the neutrophils. In rheumatoid arthritis the synergistic effect of free radicals on proteolytic activity may be very relevant to the destruction of collagen in the rheumatoid joint.

3.4 Metalloproteins

In biological systems hydroxyl radicals are formed through the Fenton reaction (fig.II.2). Metal-containing proteins could provide the catalyzing metal and in this way contribute to enhanced hydroxyl radical formation. However, these proteins could also have a completely different function by binding free metals. Whether a metal-containing protein stimulates the hydroxyl formation or provides protection probably depends on the metal-saturation and the nature of the metal-binding site. Although several other transition metal containing proteins have been studied most of the attention has been centered on iron-containing proteins.
The iron present in the human body is bound to several proteins, the most important being the haem-containing proteins (hemoglobin, myoglobin) and iron-binding proteins such as ferritin, transferrin and lactoferrin. Most of these proteins have been, in one way or another, related to aggravation of the inflammatory response by facilitating hydroxyl radical generation from activated oxygen species (48-55). The general underlying mechanism by which iron-containing proteins can potentiate the inflammatory response involves their action as a "Fenton-reagent". However with transferrin and lactoferrin there is some controversy as to whether they are promotors of hydroxyl radical generation by acting as "Fenton-reagent" or that they actually bind iron and minimize damage (56).
As oxygen free radicals were implicated in the pathogenesis of several human diseases the importance of iron-metabolism was soon recognized. A well known relationship exists between iron-concentration and the development of arthritis. Examples of such an interaction are hemochromatosis and hemophilia, diseases in which an arthritis is often found associated with iron accumulation in the joints. It has also been reported that treatment of RA-patients with intravenously administered iron leads to a worsening of the arthritis (57). Studies by Biemond *et al* (53,58,59) have shown that superoxide produced by stimulated PMN can release iron from ferritin, which is present in sufficient amounts in both synovial fluid and synovial membrane (fig.II.4). The oxygen radical species formed in the synovial fluid can cause destruction of cartilage (60,61), decreases the de novo synthesis of cartilage-proteoglycan (62), peroxidise cell-membranes (63,64), depolymerise hyaluronic acid (65) and attack proteins. As will be discussed below.

3.5 Proteins in rheumatoid arthritis

As mentioned above several studies revealed evidence for the involvement of oxygen free radicals in the pathogenesis of RA. Free radicals are thought to originate from

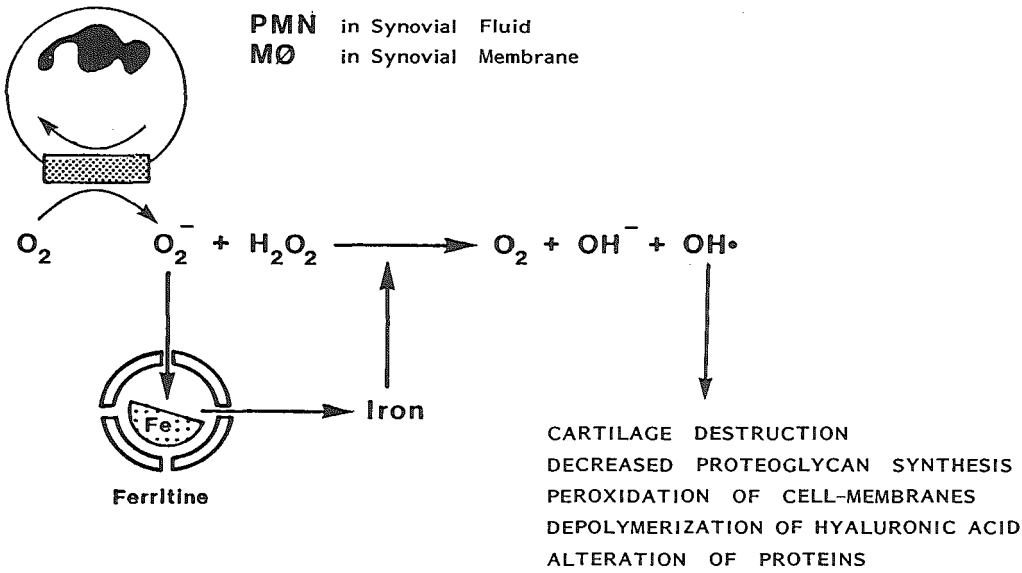

Figure II.4: *Superoxide produced by stimulated phagocytes releases iron from ferritin. This iron is able to convert superoxide and hydrogen peroxide to the more destructive hydroxyl radical (PMN: polymorphonuclear leucocytes; MØ: macrophages).*

activated phagocytes present in the inflamed joint. Apart from stimulation of phagocytes, hypoxic-reperfusion injury may also give rise to the generation of oxygen free radicals (66).

A number of studies implicated effects on proteins. For instance the polypeptide of proteoglycans in cartilage can efficiently be degraded by superoxide as well as hydroxyl radicals (60). Also collagen has been shown to be susceptible to oxygen free radical attack (67,68). Caeruloplasmin ,the blue copper oxidase, which has been shown to be elevated in RA synovial fluid (69) inhibits iron-catalyzed free radical reactions due to its ability to oxidize Fe^{2+} into Fe^{3+} (ferroxidase activity). Because of this activity caeruloplasmin is thought to function as an extracellular antioxidant inhibiting Fe(II)-catalyzed reactions such as lipid-peroxidation and the Fenton-reaction. Moreover caeruloplasmin has an additional antioxidant capacity that is to say it scavenges the superoxide anion radical stoichiometrically (70). A report that the caeruloplasmin ferroxidase activity is decreased in rheumatoid synovial fluid (71),possibly by previous exposure to oxygen radicals, was later shown to be due to a storage artifact (72). Although this lability of caeruloplasmin has hampered studies on its function and structure, several observations suggest that caeruloplasmin is susceptible to oxidative attack. Exposure of caeruloplasmin to models of oxidative stress (UV-irradiation and activated neutrophils) led to physicochemical modifications (73). An earlier report by

the same authors showed that exposure to oxygen free radical generating mechanisms (xanthine/hypoxanthine and xanthine-oxidase or to hydrogen peroxide/copper salt) could decrease the ferroxidase activity of caeruloplasmin (74).
In addition, oxidized alpha-1-antitrypsin has been isolated from synovial fluid from RA-patients supporting the influence of oxygen free radicals on the balance between proteinases and proteinase-inhibitors (75).

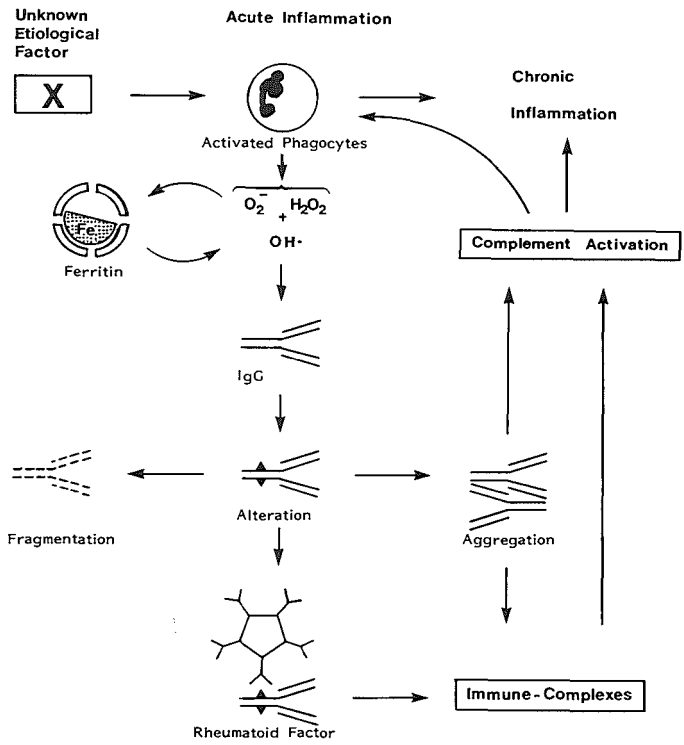

Figure II.5: *Proposed role of oxygen free radicals and immunoglobulin G (IgG) in the inflammatory response in rheumatoid arthritis.*

Protein modification by oxygen free radicals in RA may have particular implications since the majority of RA-patients is seropositive, i.e. they possess the rheumatoid factor. The rheumatoid factor is an antibody, mostly of the IgM class, directed against the patients own IgG suggesting it has been altered acquiring antigenicity. Studies by our group and others have shown that oxygen free radicals are able to modify and change IgG resulting in fluorescence changes (29), increased thiol-group content (25), altered amino acid content and the formation of reduction resistent intra and inter-molecular covalent cross-links (31,76,77). Furthermore oxygen free radical altered IgG showed an increased ability to bind RF, to bind the first component of the complement

system and was also able to activate the complement system (C3a generation) (78). The experimental data fit the current concept that oxygen free radical damage to IgG plays an essential role in the inflammatory reaction in RA (fig.II.5). As it is generally accepted that IgG is the primary antigen in immune complexes (IC) in RA the alterations on IgG caused by reactive oxygen species could well be the essential initiating factor in IC-formation. Not only through interaction with rheumatoid factors but also by direct formation of aggregated IgG-molecules. These immune complexes activate complement which results in a further stimulation of neutrophils and subsequent oxygen free radical production. In this way a vicious circle is established leading to chronic inflammation (31). Whether oxygen free radical altered IgG is indeed the main trigger for RF-production remains to be established.
Alteration of proteins by oxygen free radicals also includes oxidation of potentially protective enzymes such as SOD and catalase and comprises the modification of proteolytic enzymes responsible for fast protein degradation. It is important to take into account the inactivation of these enzymes by reactive oxygen species as well as the protective and proteolytic functions of these enzymes in moderating the impact of oxygen free radical attack when considering the overall effects of chronic inflammation. During ageing and chronic diseases the balance between these two counteracting mechanisms might well be disturbed.

II.4 Antioxidant defence mechanisms and therapeutic possibilities

If one considers the multiple ways by which oxygen free radicals can be generated protection against these reactive species is vitally important. Under normal physiological conditions a delicate balance exists between oxidants generating systems and antioxidant defense mechanisms. If in one way or another this balance is disturbed tissue and intracellular damage occurs which might result in pathology.
Various intra and extracellular antioxidants exist. Essential nutrients like vitamin E (α-tocopherol), vitamin C (ascorbic acid) and ß-carotene (major carotenoid precursor of vitamin A) have all been shown to exhibit oxygen free radical scavenging properties. Of these nutrients vitamin E has been known longest for its antioxidant ability and is the major lipid-soluble antioxidant present in all cellular membranes, where it protects against lipid-peroxidation (79,80). Vitamin C can function as an antioxidant in the aqueous phase of the cell, but it can also have a pro-oxidant effect under certain conditions, for instance in the presence of vitamin E (see also naturally occurring antioxidants as therapeutic drugs). The synergistic effect of vitamin E and vitamin C in preventing lipid-peroxidation was reported in a recent article by Wefers and Sies (81). They showed that in the presence of vitamin E ascorbate is switched from a potentially damaging agent to a protective agent. They also found that the chainbreaking activity of vitamin E was maintained primarily by vitamin C. Here, vitamin C acts as a second line antioxidant regenerating reduced vitamin E. Vitamin C has also been shown to act as a radical scavenger (82). In this study protection of alpha-1-antitrypsin against the myeloperoxidase derived oxidant hypochlorous acid was provided by ascorbic acid. Beta-carotene, another essential micronutrient, has recently been shown to be an important antioxidant (quenching of singlet oxygen) in cell-membranes (83). Vitamin

A itself however has only a very small capacity to scavenge free radicals (84).
Also caeruloplasmin, a copper transporting plasma-protein, has antioxidant capacities. This acute phase reactant has been shown to scavenge the superoxide anion radical (85,86). It can also inhibit iron-catalyzed free radical reactions due to its ability to oxidize Fe^{2+} into the less dangerous Fe^{3+} (ferroxidase activity). In this way caeruloplasmin functions as an extracellular antioxidant inhibiting Fe(II)-catalyzed reactions such as lipid-peroxidation and the Fenton-reaction. Several reports showed that ceruloplasmin is elevated in RA SF (69,87,88). Biemond *et al* (69) measured several protective factors in RA SF and concluded that ceruloplasmin is an important protector against oxygen free radicals, a conclusion supported by others (89,90).
Clearly iron-metabolism (as well as of other transition metals) plays an important role in free radical chemistry. The iron present in the human body is bound to several proteins, the most important being the haem containing proteins (hemoglobin, myoglobin) and iron-binding proteins such as ferritin, transferrin and lactoferrin. Particularly lactoferrin and transferrin could also protection against oxidative stress by binding free iron. In this way the catalytic action of iron in the Fenton-reaction is prevented.
Intracellular protection occurs mainly through a few antioxidant enzymes. Superoxide dismutase (SOD) is an important enzymatic defense against free radical formation (91). It catalyses the dismutation of the superoxide anion radical to hydrogenperoxide:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction occurs spontaneously but is greatly accelerated by SOD. Of SOD four separate isoenzymes have been characterized, one contains copper and zinc, another manganese and a third contains iron. The latter is only found in bacteria. The fourth isoenzyme (the third human form) is the extracellular SOD (EC-SOD). This enzyme first discovered by Marklund (92) also contains Cu and Zn.
Additional enzymatic defense is offered by the heme-containing catalase and the selenium dependent glutathione peroxidase (93,94). These enzymes are both capable of detoxifying hydrogenperoxide, catalase by a divalent reduction of H_2O_2 to H_2O and glutathione peroxidase by converting H_2O_2 to H_2O via oxidation of glutathione. Phospholipid hydroperoxide glutathione peroxidase (PHGSH-PX), originally named peroxidation inhibiting protein (PIP), is closely related to classical glutathione peroxidase (GSH-PX) with respect to amino acid composition, Se-content and kinetic mechanism (95). In contrast to classical GSH-PX it is able to reduce phospholipid hydroperoxides as well.
The various antioxidant defense systems are summarized in table II.1. Included are some less well established antioxidants that are not discussed in this paper.
It has been suggested that a correlation exists between a deficiency in the antioxidant status of RA patients and the destruction of joint tissues. However, several studies have been published of which some showed a decrease and others showed no change in antioxidant status in RA when compared to controls (69). It thus remains unclear if an imbalance in natural antioxidant mechanisms is of aetiopathological importance in RA.

Table II.1: *Antioxidant defences.*

TYPE OF DEFENSE		REMARKS
A. **Enzymatic:**		
Superoxide Dismutase;	Cu^{2+}/Zn^{2+} SOD	Erythrocuprein, cytosol of eukaryotic cells
	Cu^{2+}/Zn^{2+} SOD	EC-SOD, extracellular fluids
	Mn^{2+} SOD	Mitochondria and prokaryotic cells
	(Fe^{3+} SOD	Bacteria)
Catalase		Heme-enzyme, cytoplasma
Glutathione Peroxidase		Se-dependent, cytoplasma
Phospholipid Hydroperoxide Glutathione Peroxidase		Se-dependent, membranes
B. **Non-enzymatic:**		
Vitamin E (tocopherol)		Lipid-soluble, membranes
Vitamin C (ascorbic acid)		Water-soluble, extra-cellular and cytosol
Beta-carotene		Lipid-soluble, membranes
Plasma-proteins;	Ceruloplasmin	Cu-transport
	Transferrin	Binds iron
	Lactoferrin	,,
	Albumin	Binds copper, inhibits Fenton-reaction
	Haptoglobin	Inhibits lipid-peroxidation
Uric acid		Radical scavenger, inhibits lipid-peroxidation

4.1 Naturally occurring antioxidants as therapeutic drugs

An important and obvious strategy in preventing and/or overcoming oxidant stress is the therapeutic administration of naturally occurring antioxidants. In recent years much work on this subject has been published evaluating the use of the above mentioned antioxidants as therapeutic drugs.
First clinical experience with SOD in the treatment of inflammatory disorders were reported some 15 years ago (96,97). These first trials encouraged further research on the effectiveness of SOD in inflammatory diseases. In studies on SOD-therapy of RA the drug was first given intramuscularly which gave only poor results (98). In later trials

SOD was given intraarticular which led to better results (99,100). In several experimental models of inflammation the effects of SOD-administration were studied. However, convincing evidence for its efficacy was not always shown (101,102). The treatment of RA with SOD theoretically appears to be promising but one of the problems with the use of enzymes is their relatively short physiological life. This also accounts for the therapeutic use of catalase of which only a limited number of clinical studies have been reported where it was shown to be effective (103,104). One way to overcome the limitations in the use of enzymes as a drug due to their protein character is to increase their half-life in the body. Cationization, as suggested by Schalkwijk *et al* (105), acylation (106) or coupling to Ficoll (107) of the antioxidant enzyme extends is half-life and thus increases its effectiveness. This approach might be an interesting option from a therapeutical point of view.
Vitamin E has also been tested for its anti-inflammatory properties. Although not well documented, occasional trials showed the effectiveness of high doses of vitamin E in some arthritic diseases (108-110). Most tests using animal model systems of human diseases gave disappointing results. Lipid-peroxidation, as Halliwell (111) has recently pointed out, occurs probably as a consequence of degenerative diseases (like RA) rather than being the cause of it. Lipid-peroxidation products can probably be found in any disease state as a result of cell destruction, not necessarily caused by free radicals. If lipid-peroxidation only occurs in a late stage of cellular destruction then vitamin E therapy as inhibitor of lipid-peroxidation is unlikely to be successful in protecting against oxidant stress. However, this needs further research as evidence is increasing which shows a correlation between lipid-peroxidation and atherosclerosis (112,113). The same consideration can be made on the dietary intake of selenium, an essential cofactor of glutathione peroxidase which is, just as vitamin E, supposed to decrease the generation of lipid-peroxidation products. Although depressed serum selenium in active RA was reported (114), trials with selenium supplementation in the diet did not show beneficial effects (115).
Another naturally occurring micronutrient which might contribute, when applied therapeutically, to the protection against reactive oxygen species is vitamin C. Probably vitamin C should only be used therapeutically in combination with vitamin E. In this way vitamin C does not act as a prooxidant but as an antioxidant sparing the available vitamin E. However, the question if vitamin C is a prooxidant (especially in association with iron) or an antioxidant (in combination with vitamin E) remains open; probably the answer depends partly on the intracellular and tissue concentration of this vitamin.

4.2 Synthetic antioxidant drugs

Initial therapy of rheumatoid arthritis consists usually of non-steroidal anti-inflammatory drugs (NSAIDs). These drugs are believed to exert their effect by the inhibition of prostaglandin-synthesis. However, in recent years it has become clear that NSAIDs have additional effects. It was shown that NSAIDs protect against oxygen free radical damage independent of their action on prostaglandin synthesis (116). NSAIDs have been shown to exhibit various effects including inhibition of superoxide-generation (117-119), protection against the hydroxyl-radical (120,121), decreasing lipid-peroxidation (64,122) and increasing intracellular glutathione (123). The latter function

of NSAIDs can be contributed to the fact that many of these anti-rheumatic drugs contain SH-groups or are converted to SH-reactive compounds *in vivo* (124). D-penicillamine, which is such an agent, is considered to elicit at least part of its beneficial effects in restoring the depressed thiol-levels in RA, thereby increasing protection against oxidative stress (125,126). Other sulphydryl containing or liberating drugs such as gold salts, levamisole and corticosteroids were shown to possess antioxidant properties which could be explained by their effect on SH-levels (127-130). Gold compounds have also been shown to have a direct inhibitory effect on the generation of reactive oxygen species by PMN (131).
Most of the first and second line drugs were not developed for their specific antioxidant properties. Most of the above mentioned effects of NSAIDs and gold compounds were discovered after their establishment as anti-rheumatic drugs. A directed search and development of specific antioxidant drugs for therapeutical use in inflammatory disorders like RA seems to be unavoidable. The use of iron-chelators like desferrioxamine, 2,3 dihydroxybenzoate and phenantroline as possible antioxidant therapy of RA looks promising in spite of recent drawbacks (132-134).

Although it is now quite evident that oxygen free radicals are involved in the pathogenesis of RA, it is still not clear what is the initiating process leading to free radical production. Not until this question has been answered an effective treatment and eventual prevention of RA seems possible.

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THREE

Oxygen free radical induced structural alterations on immunoglobulin G.

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CHAPTER III

OXYGEN FREE RADICAL INDUCED STRUCTURAL ALTERATIONS ON IMMUNOGLOBULIN G

Summary

Oxygen radicals can modify and change proteins. Studies on human IgG which had been exposed to an oxygen free radical generating system, i.e. ultraviolet irradiation, revealed that several protein modifications are induced. These modifications include an increase in SH-groups and a change in amino acid content and/or conformation which is reflected in a change of its fluorescence spectrum. Gelelectrophoresis of ultraviolet-irradiated IgG followed by densitometric analysis of the stained gels revealed a higher sensitivity for oxygen free radical damage of the heavy chains compared to the light chains.
Aggregation of IgG is another phenomenon occurring as a consequence of free radical activity. Analysis of IgG-aggregates by SDS-PAGE and autoradiography showed the formation of protein cross-links, resistant to reduction by ß-mercaptoethanol. These cross-links can be due to the formation of 0,0'-dityrosine. It is shown that ultraviolet-irradiation of tyrosine leads to the formation of a specific fluorescence. With other aromatic amino acids this phenomenon is not seen.
The finding of a reduction resistant cross-link between IgG-molecules as a consequence of oxygen free radical activity adds to the possible role oxygen free radicals have in the induction of rheumatoid factor production and immune complex formation in RA.

Introduction

Rheumatoid factors (RF) are a characteristic serological feature of definite or classical rheumatoid arthritis (RA). They are believed to evolve as antibodies, directed against the Fc-fragment of a patients own IgG. The antigenic determinants on the Fc-fragment with which rheumatoid factors react are located in the C_H2- and C_H3-domains (1). To cause the production of auto-antibodies it is necessary that these determinants are altered and thus seen as non-self. However the exact cause and nature of these alterations leading to RF production are up till now unknown. A possible causative agent of these alterations on IgG which renders it auto-antigenic are oxygen free radicals. Free radicals originate, among other possible sources, from activated polymorphonuclear leucocytes (PMN). In RA large infiltrations of PMN in the inflamed joint exist. Activated PMN produce various factors that modulate adjacent tissue and appear to produce the characteristic destructive features seen in RA (2-4). Oxygen free radicals produced by neutrophils can modify and change proteins. Studies on human IgG which had been exposed to an oxygen free radical generating system, i.e.

ultraviolet irradiation, revealed that several modifications were induced. These modifications include a change in amino acid content and protein conformation (5,6). Another phenomenon seen as a consequence of free radical activity is the aggregation of IgG (7). Aggregated IgG can, as well as immune-complexes, activate resting neutrophils to produce more free radicals. In this way a self-perpetuating mechanism of IgG-aggregation in rheumatoid inflammation may evolve (5).
In this study we tried to elucidate more about the role of oxygen free radicals in rheumatoid inflammation by investigating more extensively the structural modifications on IgG induced by free radicals.

Materials and methods

Chemicals

All chemicals were obtained from Merck AG, Darmstadt, Germany, unless otherwise indicated.

Preparations of immunoglobulins

Human IgG was isolated and purified from the sera of healthy normal volunteers by precipitation with 50% saturated ammonium sulphate in phosphate buffered saline (PBS) pH 7.4 followed after dissolution by a 33% saturated ammonium sulphate precipitation. The precipitate was dissolved in 50 mM Tris (pH 8.0) and extensively dialyzed to eliminate ammonium ions. IgG was finally purified by passage over a DEAE-cellulose column equilibrated with Tris-buffer. Human IgG-preparations were checked for purity in Ouchterlony immunodiffusion plates against rabbit anti-human IgG and rabbit anti-human serum specific antisera.
Rabbit IgG (RaIgG) was purchased from Sigma Chemical Co., St.Louis, MO., USA. Heat aggregated IgG (HAG) was obtained by heating at 63°C for 20 min.

Ultraviolet-irradiation

Human gammaglobulin solutions (± 5 mg/ml), L-tyrosine (2 mM), L-tryptophan (2 mM) and L-histidine (2 mM) were irradiated in matched quartz cuvettes (3 ml-volumes) at a distance of 6 cm from the light source. Ultraviolet irradiation was achieved using light sources at 254 nm and at 366 nm. Two hours UV-irradiated human IgG-samples were centrifuged (1 h; 100.000 g) in a Beckman L5-65 Ultracentrifuge to eliminate insoluble IgG-aggregates. Gelfiltration chromatography of the supernatant of ultraviolet irradiated IgG was performed on Biogel A 1.5 m agarose (Biorad Lab, Richmond CA, U.S.A.). The columnsize was 1.5 x 70 cm and the elution buffer used was 50 mM Tris-HCl buffer pH 8.0. The absorbance of the effluent was monitored at 280 nm. Elution-fractions containing the aggregated IgG were pooled. The IgG was labelled with ^{125}I using the chloramine T-method (8).

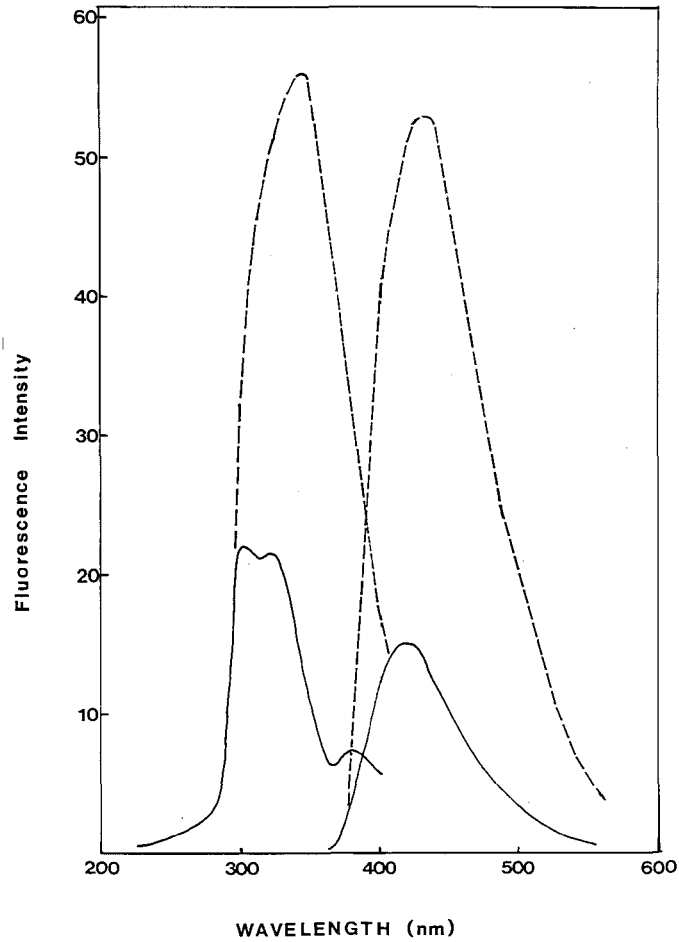

Figure III.1: *Fluorescence spectra of rabbit IgG (5 mg/ml) before (—) and after (---) ultraviolet irradiation for 60 min at 366 nm and 254 nm.*

Sucrose-gradients

Isokinetic 5-25 % ((W/N) sucrose gradients were made in PBS containing 10 mM EDTA and 0.5 % BSA. The supernatant (500 μl) of UV-irradiated human IgG and heat-aggregated (20 min., 63° C) human IgG were, after labelling with ^{125}I, layered on the gradient. After centrifugation for 15 h. at 30.000 r.p.m., 0.5 ml fractions were collected. In these fractions the ^{125}I-radioactivity was determined using a gamma-counter (LKB produkter AB, Stockholm, Sweden). Fractions containing monomer IgG and those containing the aggregated IgG were pooled separately.

Polyacrylamide gelelectrophoresis

Samples of ^{125}I-labelled monomer and polymer UV-irradiated as well as heat aggregated IgG were analyzed by Sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gelelectrophoresis was performed on a 5 % gel under non-reducing circumstances and on a 10 % gel under reducing circumstances using the system of Laemmli (9). SDS-PAGE was followed by autoradiography.
Native IgG and various samples of UV-irradiated IgG were also analyzed on 10 % gels under reducing circumstances. The gels were stained with Coomassie blue. Densitometric scans of the stained gels were recorded on a Vitatron TLD 100 universal densitometer (Vitatron Scientific B.V., Dieren, The Netherlands).

Biochemical determinations

The sulphydryl content of the immunoglobulin G-solutions was determined according the procedure of Ellman (10). Sialic acids (N-acetylneuraminic acid) were quantitated by the spectrophotometric method of Horgan (11). Protein concentrations were measured by the method of Lowry *et al.* (12).

Fluorescence measurements

Fluorescence measurements were performed as described by Lunec (5) on a Perkin Elmer MPF.3L scanning spectrofluorimeter (Perkin Elmer, Norwalk CO, U.S.A.).

Table III.1: *Rabbit IgG (5 mg/ml) was irradiated at 366 nm and 254 nm for 0, 10, 30, 60 and 120 min. The sulphydryl (SH) and sialic acid (SA)-concentration was determined in the total solution and in the supernatant after centrifugation (2 min, 8000 g). SH is expressed in μmol/mg protein and SA in μg/mg protein.*

Irradiation time (min)	Total		Supernatant	
	SH	SA	SH	SA
0	0.7	1.06	0.7	1.07
10	3.9	1.04	4.5	1.05
30	8.3	1.00	8.3	1.02
60	12.2	0.96	10.8	1.04
120	23.2	0.88	16.7	0.99

Results

Ultraviolet irradiation of rabbit IgG generated a typical fluorescence (fig.III.1). The excitation and emission maxima of irradiated RaIgG (respectively 350 nm and 425 nm) were clearly distinguishable from the native fluorescence of RaIgG.

The fluorescence changes of rabbit IgG induced by UV-irradiation were accompanied by an increase of sulphydryl groups with a factor 30 and a subsequent loss of sialic acids of 20 % (table III.1). However, not all batches of rabbit IgG showed a loss of sialic acids after irradiation. The results presented here are those measured in the total irradiated IgG-solution and those determined in the supernatant of the centrifuged (2 min, 8000 g in an Eppendorff bench centrifuge) irradiated IgG solution.

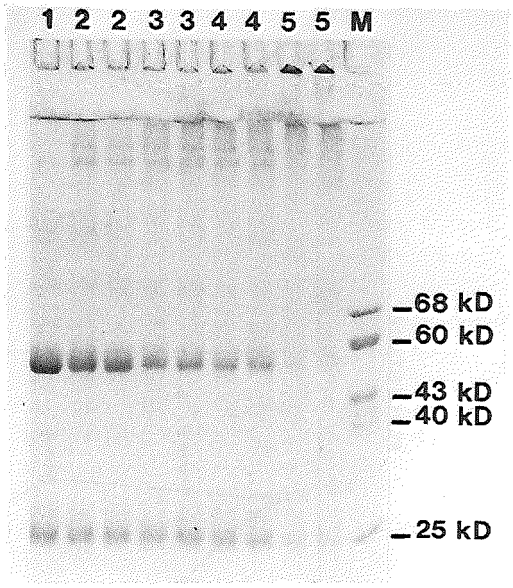

Figure III.2: *Analysis of human IgG preparations by SDS-PAGE. Samples were reduced and applied on a 10% polyacrylamide gel. 1. Native IgG; 2. 30 min UV-irradiated IgG; 3. 60 min UV-irradiated IgG; 4. 120 min UV-irradiated IgG; 5. 240 min UV-irradiated IgG; M, molecular weight marker proteins (albumin: 68,000; catalase: 60,000; ovalbumin: 43,000; aldolase: 40,000; chemotrypsin: 25,000).*

Samples of native and UV-irradiated IgG were analyzed on 10% polyacrylamide gels (fig.III.2). It is shown that ultraviolet-irradiation caused the formation of high molecular weight aggregations (visible as dark spots in the slots of the stacking gel). Densitometric scans of the electrophoresis patterns showed that the irradiation caused a decrease of heavy chain and light chain bands. The decrease of the heavy chain bands was greater and started earlier after the onset of irradiation than in the case of the light chain bands (fig.III.3).

Human gammaglobulin was ultraviolet irradiated for two hours, centrifuged (1 h; 100.000 g) and the supernatant was passed over a gelfiltration column. The elution-profile is shown in fig.III.4. Two peaks are revealed when the absorbance is measured at 280 nm. These peaks represent monomer and polymer (aggregated) IgG. The fractions containing the UV-aggregated IgG were pooled and the IgG was ^{125}I-labelled prior to analysis on SDS-PAGE. Gelelectrophoresis was performed on a 5 % non-reducing and a 10 % reducing gel (fig.III.5). Also analyzed on these gels were mono-

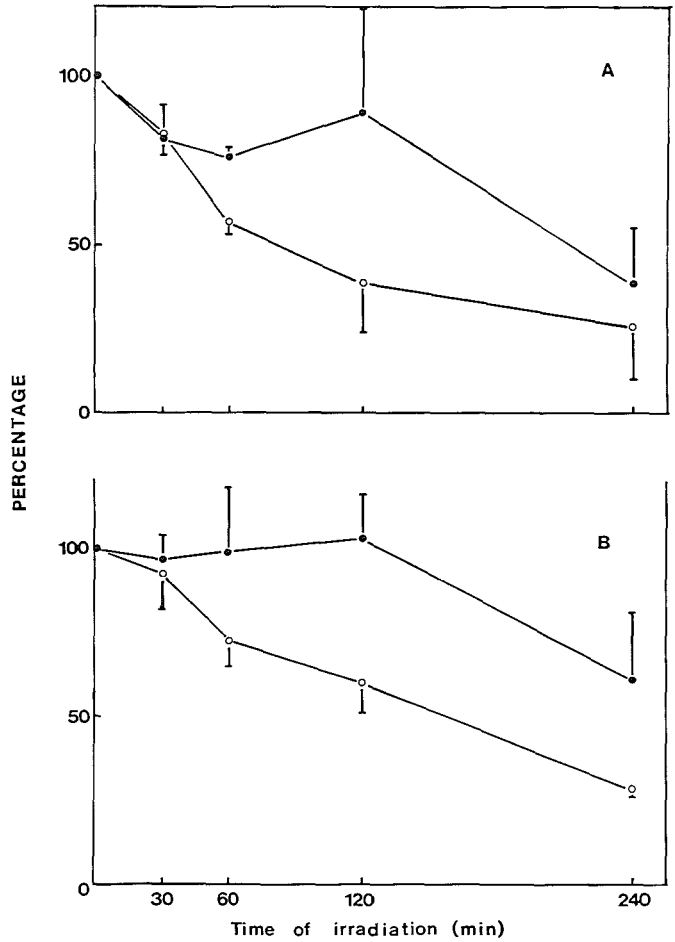

Figure III.3: *Relative peakheights in the densitometric scans of the electrophoretic bands of rabbit (A) and human (B) IgG heavy (o—o) and light (●—●) chains. Reduced samples of 30, 60, 120 and 240 min ultraviolet irradiated IgG were applied on a polyacrylamide gel. Reduced samples of untreated rabbit and human IgG were applied as controls. The peakheights are expressed as percentage of the control values. The results are the means (± SD) of three separate experiments.*

and polymer UV-irradiated and heat-aggregated IgG-fractions derived from sucrose density-gradient studies. Autoradiography of a 5 % gel showed monomer UV-irradiated IgG (lane 1) and monomer heat-aggregated IgG (lane 3) to be present as one band of approximately 150 kD.

Polymer UV-irradiated IgG (lane 5) and polymer heat-aggregated IgG (lane 4) were

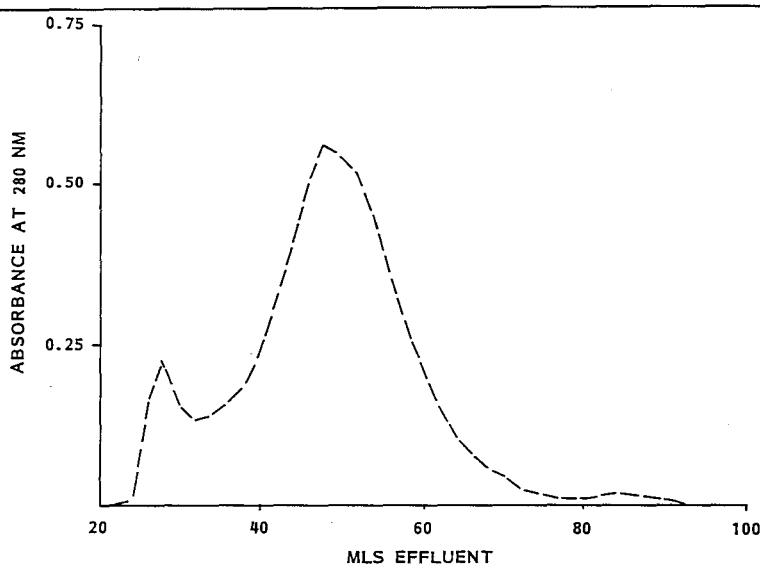

Figure III.4: *Elution-pattern of the supernatant of two hours UV-irradiated human immunoglobulin G. A 2 ml sample (5 mg/ml) was applied on a Biogel A 1.5 m gelfiltration column and eluted with a Tris-buffer (50 mM, pH 8.0).*

visible as dark spots in the slots of the gel. In lane 6 another polymer heat-aggregated ^{125}I-labelled IgG-fraction was applied revealed by a dark band in the slot of the gel. The quantity of polymer UV-irradiated ^{125}I-labelled IgG applied in lane 2 appeared to be to small to produce a dark band. Autoradiography of a 10 % SDS-polyacrylamidegel showed that electrophoresis under reducing circumstances of the monomer UV-irradiated IgG in lane 1 and the monomer heat-aggregated IgG in lane 3 resulted in both samples in two bands. The two bands revealed correspond to the heavy and light chains of the IgG molecule (resp. 50 kD and 23 kD). The polymer heat-aggregated IgG was also reduced in a heavy chain and a light chain band. The polymer UV-irradiated IgG however showed a completely different picture. Reduction of this sample by ß-mercaptoethanol led to the formation of two bands: one band in the slot of the gel and one band at the transition from stacking gel to running gel. No heavy and light chain bands were seen as a consequence of reduction of ultraviolet-aggregated IgG.

The results of the SDS-PAGE and autoradiography experiment with UV-irradiated and heat-aggregated IgG indicated oxygen free radical activity induces the formation of a reduction-resistant cross-link between human gammaglobulin-molecules. It might be that this was due to the formation of intermolecular dityrosine-cross-links. Therefore we investigated the effect of UV-irradiation on solutions of several amino acids. Ultraviolet irradiation of L-tyrosine resulted in a remarkable fluorescence change, giving rise to a fluorescence emission peak near 410 nm when excited at 340 nm for up to 10 min. (fig.III.6). L-Tryptophan showed an increase in fluorescence as well. The

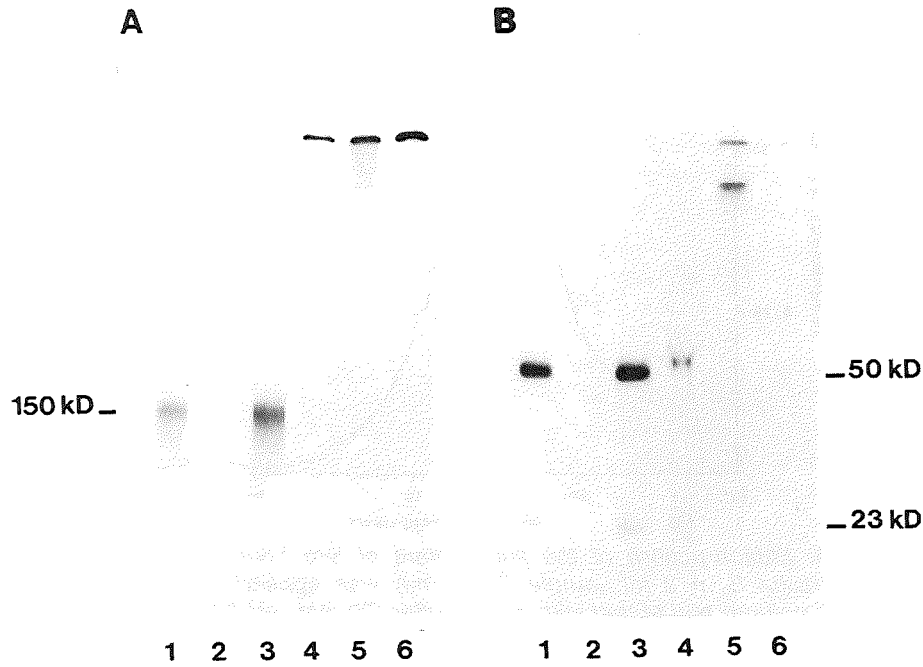

Figure III.5: *Autoradiographs of a 5% (A) and 10% (B) SDS-polyacrylamide gel that were electrophoresed under resp. non-reducing and reducing circumstances. Applied were monomer UV-irradiated human IgG (lane 1), monomer heat-aggregated human IgG (lane 3), polymer UV-irradiated IgG (lane 2 and 5) and polymer heat-aggregated human IgG (lane 4 and 6). All samples were ^{125}I-labelled. Samples applied on lane 1-4 were derived from sucrose-density gradient fractions. The samples applied on lane 5 and 6 were obtained by gelfiltration.*

emission peak (Ex 340 nm) of native tryptophan was moved from 400 nm to 450 nm of 10 min UV-irradiated tryptophan. L-Histidine did not show any fluorescence before or after ultraviolet irradiation at all.

Discussion

Immunoglobulin G is a divalent molecule of mol wt 150.000 which interacts with a large number of different molecular species. The Fab-part of the molecule reacts with antigen and on the Fc-part a number of molecules interact. Among them are C1q (a complement subcomponent), staphylococcal protein A and a number of Fc-receptor molecules of various cell-types (13). A special group of molecules interacting with IgG are rheumatoid factors (RF). RF are a predominant feature of RA. These auto-antibodies, recognized in all immunoglobulin classes (14-17), are directed against

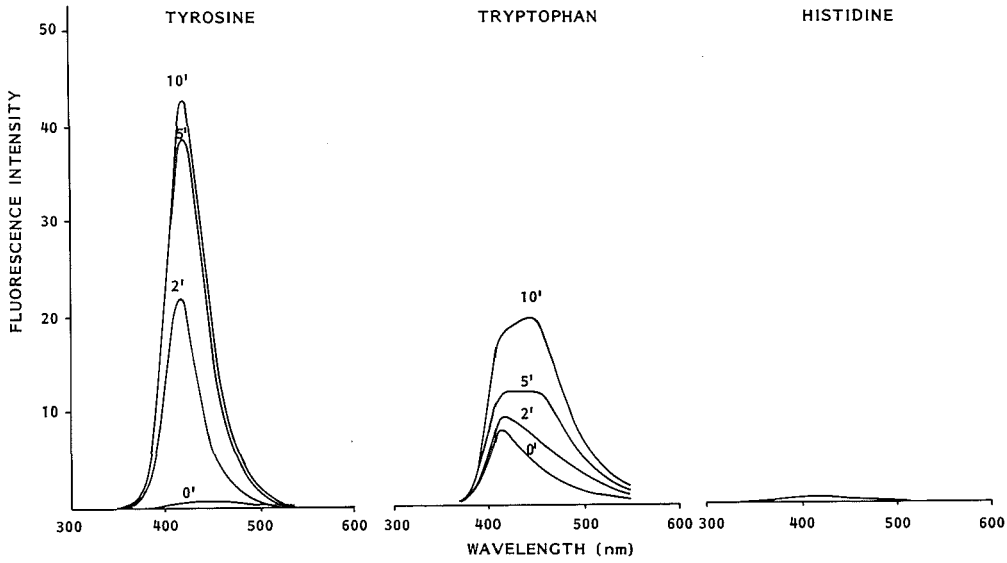

Figure III.6: *Fluorescence emission spectra (EX. 340 nm) of tyrosine, tryptophan and histidine, after 0 (controls), 2, 5 and 10 min UV-irradiation.*

epitopes on the Fc-region of IgG. To unravel the etiology and pathology of RA it is important to understand the immunological and biochemical mechanisms responsible for RF-production.

Because RF are directed against a constituent of the body itself, i.e. immunoglobulin G, this must be altered in such a way it is seen as "non-self". Oxygen free radicals are able to change and alter proteins, including IgG, and are produced in large quantities during acute and chronic inflammation as is seen in RA. Therefore they could have an important role in the pathogenesis of RA.

Fluorescence generation in rabbit IgG caused by ultraviolet irradiation can be attributed to free radical damage. Free radical damage occurs at the site of the aromatic amino acids and can be specifically characterized by fluorescence spectrophotometry (5). Fluorescence of proteins can be caused by three amino acid residues: tyrosine (tyr), tryptophan (trp) and phenylalanine (phe) (18). Tryptophan residues dominate the fluorescence of most proteins and investigations on fluorescence changes caused by free radicals have focussed on this amino acid (5,19).

The generation of sulphydryl groups illustrates the effect of free radicals on a non-aromatic amino acid: cysteine. Disulphide bonds are reduced and broken by oxygen radicals leading to the formation of thiol-groups (19). This formation of thiol groups is not only dependent on the number of cysteine residues, but also on the proximity of aromatic amino acid residues (20).

Another possible site of action of free radicals is the carbohydrate moiety of

immunoglobulin G. IgG contains different distributions of asparagine-linked bi-antennary oligosaccharide structures. It has been reported that RA-patients contain IgG with altered glycosylation patterns (21). In a first attempt to elucidate the effects of free radicals on the carbohydrate structures of IgG we determined the sialic content of rabbit IgG before and after ultraviolet irradiation. In some cases, as reported here, we could find a marked reduction of the sialic acid content after irradiation. This reduction was greater in the whole sample than in the supernatant of the centrifuged sample. These findings imply that UV-aggregated IgG contains less sialic acid. Such a loss of sialic acid can lead to aggregation by several ways. For instance by exposing a previously masked non-immunogenic determinant or by creating a lectin-like activity on the oligosaccharide binding sites resulting in a "sticky" IgG. However, analysis of other batches of rabbit IgG showed that irradiation did not cause a marked loss of sialic acid. These results probably reflect the great variance found in the glycosylation pattern of IgG.

The difference found between heavy and light chains in the densitometric scans of the electrophoresis gels points to a difference in sensitivity towards ultraviolet irradiation. The heavy chain seems more susceptible to free radical damage than the light chain. This is in agreement with the supposed auto-antigenic site of IgG, which has been localized in the constant region domains of the heavy chains (for review see 13).

In studying the effects of an oxygen free radical generating system on immunoglobulin G it was found that covalent cross-links between IgG-molecules were formed. In contrast the aggregated IgG formed as a consequence of heating could be reduced by ß-mercaptoethanol, thus suggesting two different aggregation mechanisms.

The formation of a reduction-resistant cross-link between IgG-molecules by an oxygen free radicals mediated action could *in vivo* be an important mechanism. The crucial feature of autosensitation is the self-associating and complex-formation capacity of IgG-molecules. Stabilization of these complexes comes from polyvalent IgG-Fc moiety-binders such as IgM-anti-IgG (the classical rheumatoid factor) and the complement subcomponent C1q. Oxygen free radical-induced covalent cross-links are another factor that could contribute to the stability of these immune-complexes *in vivo*. Pisko *et al* (22) showed that heat-aggregated IgG could induce rheumatoid factor producing cells from seropositive RA-patients *in vitro*. Possibly free radical mediated IgG aggregation may have a similar effect *in vivo*.

The finding that covalent cross-links are induced by oxygen free radical activity could be explained by the formation dityrosine. Dityrosine results form the oxidative phenolic coupling of two tyrosine residues (23,24). An interesting aspect of dityrosine formation is the concurrent increase in fluorescence. The typical fluorescence of dityrosine (Ex max 340 nm; Em. max 410 nm) could in part explain and contribute to the observed increase in fluorescence of IgG caused by oxygen free radical action. In this study it was shown that upon UV-irradiation of tyrosine a fluorescence is generated typical of dityrosine. Furthermore, UV-irradiation of tryptophan resulted in a fluorescence-change and increase which is largely responsible for the fluorescence seen upon oxygen free radical modification of IgG. As expected irradiation of histidine had no effect upon its fluorescence at all. These results support the hypothesis that oxygen free radicals can play an important role in the formation-induction of auto-antibodies in RA. The important finding is that two observed phenomena, i.e. aggregation and fluorescence

induction, that occur as a consequence of free radical activity can be explained through the mechanism of dityrosine-formation and tryptophan modification. The formation of dityrosine is dependent on the accessibility of the tyrosine residues in the IgG-molecules and thus on the tertiary structure. It is of interest therefore that Hunneyball and Stanworth (25) studying the effects of chemical modifications on the antigenicity of human gammaglobulin concluded that tyrosine-residues are directly involved in the auto-antigenic determinants on the C_H2-domain of human IgG reactive with rheumatoid factors. It is therefore highly plausible that these tyrosine-residues are freely accessible and thus susceptible to free radical attack. The observations of Hunneyball and Stanworth as well as our own results presented here emphasize the importance of tyrosine in the antigenicity of IgG and the effect free radicals have on these residues.

Further research, especially on the role of tryptophan, is currently in progress which could lead to a better understanding of the role of oxygen free radicals in the auto-antigenicity-induction on IgG and RF-production in RA-patients.

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FOUR

Covalent cross-links in oxygen free radical altered human immunoglobulin G.

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CHAPTER IV

COVALENT CROSS-LINKS IN OXYGEN FREE RADICAL ALTERED HUMAN IMMUNOGLOBULIN G

Summary

The damaging effect of an oxygen free radical generating system, i.e. ultraviolet irradiation, on human immunoglobulin G (IgG) was studied. The free radical altered IgG was analyzed by a high performance liquid chromatograph equipped with a TSK G 3000 SW-column. Gel filtration of 120 min UV-irradiated IgG resulted in three clearly distinguished peaks corresponding to polymer IgG (MW > 500 kD), dimer IgG (MW 300 kD) and monomer IgG (MW 150 kD). Analysis of oxygen free radical altered and aggregated IgG by SDS-PAGE and subsequent silver-staining revealed inter- and intra-molecular reduction-(by ß-mercaptoethanol)resistant cross-links between IgG-molecules were formed. Comparison of amino acid analyses of native IgG with oxygen free radical aggregated polymer IgG showed significant reductions in tyrosine- (7.0%) and histidine- (6.5%) content. These findings suggest that tyrosine and histidine are involved in covalent cross-linking between IgG-molecules caused by oxygen free radicals. These alterations on IgG induced by free radical-activity might render it antigenic, and could initiate the production of rheumatoid factors (RF).

Introduction

In recent years evidence has been gathered on the damaging effect that oxygen free radicals can execute on all kinds of biomolecules (1) and on the important role of free radicals in the pathogenesis of several diseases (2). In Rheumatoid Arthritis attention has been focused on the inflamed joint where, during the active stages of the disease, enormous amounts of oxygen free radicals are produced locally by polymorphonuclear leucocytes (PMN) (3). As a consequence of this radical-production hyaluronic acid is depolymerized (4), lipids are peroxydized (5,6) and cartilage is degraded (7). It has also been found that IgG can be changed by the action of oxygen free radicals (8,9). These changes include the generation of a specific new fluorescence, loss of the native fluorescence and an increase in sulphydryl-groups. Oxygen free radical action also causes the IgG-molecules to aggregate.
The finding that oxygen free radicals can modify and change IgG led to the hypothesis that "radical altered" IgG may *in vivo* induce the production of rheumatoid factors (RF). We have shown previously that free radical induced aggregation of IgG is established through reduction-resistant cross-links and we suggested that these cross-links might be caused by a oxidative phenolic coupling between tyrosine-residues (9). In further experiments described in this paper we show that both intra-and inter-molecular cross-linking occurs and we could confirm, by amino acid analysis, the suggestion that

dityrosine contributes substantially to reduction-resistant cross-linking. Furthermore these analyses led us to believe that also other couplings occur and contribute to the IgG-aggregation after oxygen free radical exposure.

Materials and methods

Purification of human IgG

Human IgG was isolated from the pooled sera of healthy donors by precipitation with 50% saturated ammonium sulphate in phosphate buffered saline (PBS) pH 7.4 followed, after dissolution in PBS, by another precipitation with 33% ammoniumsulphate. The precipitate was dissolved in 50 mM Tris-HCl pH 8.0 and dialyzed extensively. The IgG was finally purified by passage over a DEAE-cellulose column equilibrated with 50 mM Tris-HCl pH 8.0. The IgG was collected in the excluded fractions and extensively dialyzed against PBS.

Preparation of oxygen free radical altered IgG

Human IgG in a concentration of 5 mg/ml PBS pH 7.4 was exposed to oxygen free radicals by Ultra-Violet irradiation. Three ml-vol were irradiated (366 nm + 254 nm source) in matched quartz cuvettes, 1 cm^2 in cross section, at a distance of 6 cm from the light source.

High performance liquid chromatography

Gelfiltration of IgG-samples was carried out at room temperature on a commercial liquid chromatograph (LKB, Bromna, Sweden) equipped with a variable-wavelength UV-monitor. A TSK G 3000 SW column was used. Injection volumes were 100 µl, containing 0.25 mg protein. The proteins were eluted at 0.5 ml/h with PBS pH 7.4. Proteins were detected at 280 nm.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis

Electrophoretic separation of IgG samples were performed by the method of Laemmli (10) on slab gels containing 7.5% (w/v) acrylamide. The gels were silver stained using a silver staining kit from Bio-Rad Laboratories (Richmond, CA, USA).

Amino acid analysis

Amino acid analysis was done on 24 h 6 M HCl (110° C) hydrolysates, using a LKB 4151 alphaplus amino acid analyzer, equipped with a Resin 766 - LKB 732 column (200 x 4.6). A five-stepwise gradient of increasing pH and ionic strength was applied. The column eluent was mixed with o-phtaldehyde reacted at room temperature and detected with a Merck-Hitachi F1000 Variable Fluorescence Spectrophotometer at excitation wavelength 340 nm and emission wavelength 455 nm. Integration of

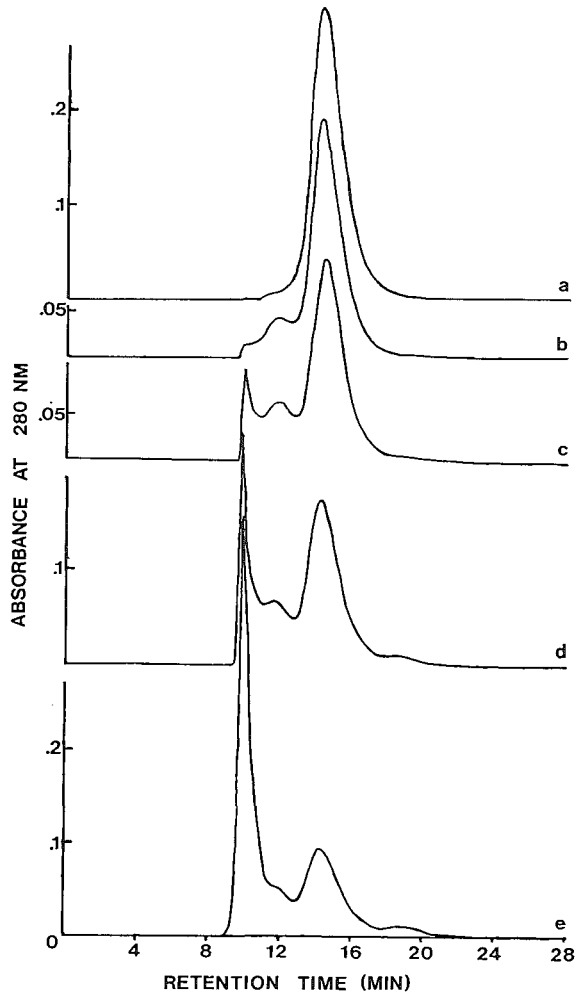

Figure IV.1: *HPLC-gelfiltration chromatograms of native IgG (a), 10 min UV-irradiated IgG (b), 30 min UV-irradiated IgG (c), 60 min UV-irradiated IgG (d) and 120 min UV-irradiated IgG (e). Peaks were identified as polymer IgG (RT: 9.9 min), dimer IgG (RT: 11.7 min), monomer IgG (RT: 14.2 min) and a low molecular weight piece (RT: 18.5 min).*

chromatogram peaks was carried out with a Merck-Hitachi D 2000 chromato-integrator.

Statistical methods

The student t-test (two-sided) was used to determine the statistical significance of differences observed.

Results

HPLC-gelfiltration of UV-irradiated IgG

Ultraviolet-irradiation of human IgG leads to aggregation of the immunoglobulin. Analysis of human IgG-samples irradiated for resp. 10, 30, 60 and 120 min on a HPLC-chromatograph equipped with a TSK G 3000 SW gelfiltration column, revealed an interesting pattern (figs.IV.1B-1E). Ten min irradiation already initiate aggregation of IgG as can be seen by the formation of dimer IgG. After 30 min irradiation the formation of dimer IgG still increases and the formation of larger aggregates (polymer IgG) has started. The gelfiltration pattern of IgG irradiated for 60 min revealed that dimer IgG-formation has reached a plateau, and that polymer IgG-aggregates were still increasing in number. The last figure (fig. IV.1E) gives the gelfiltration pattern of 120 min irradiated IgG and clearly shows the continued formation of polymer IgG, the steady turn-over of dimer IgG and the largely reduced monomer IgG peak. Interesting is the finding of a small "low" molecular weight peak (± 50 kD) which can already be found after 60 min irradiation and has increased after 120 min irradiation. For comparison the chromatogram of native IgG is also shown (fig.IV.1A). Table IV.1 lists the size of each peak expressed as percentage of the total for each chromatogram. This table illustrates clearly that the loss of the monomer IgG peak adds first to the dimer IgG peak and finally to the polymer IgG peak. Only a small portion of IgG is degraded.

Table IV.1: *Contribution of the polymer-, dimer-, monomer- and piece-peak in the total area of the HPLC-gelfiltration chromatograms of 10, 30, 60 and 120 min UV-irradiated human IgG and native (0 min) IgG. Values are expressed as percentages of the total.*

irradiation time (min)	polymer	dimer	monomer	piece
0	-	-	100	-
10	-	13	87	-
30	11	16	73	-
60	27	15	58	<0.1
120	48	14	32	5

- = not detectable.

SDS-PAGE

The monomer, dimer and polymer IgG fractions which were obtained after gelfiltration of 2 h UV-irradiated IgG were analyzed concomitantly with native IgG on 7.5% SDS-polyacrylamide gels. One gel was run under non-reducing circumstances (fig.IV.2A) and one under reducing circumstances (with ß-mercaptoethanol) (fig.IV.2B). Silver

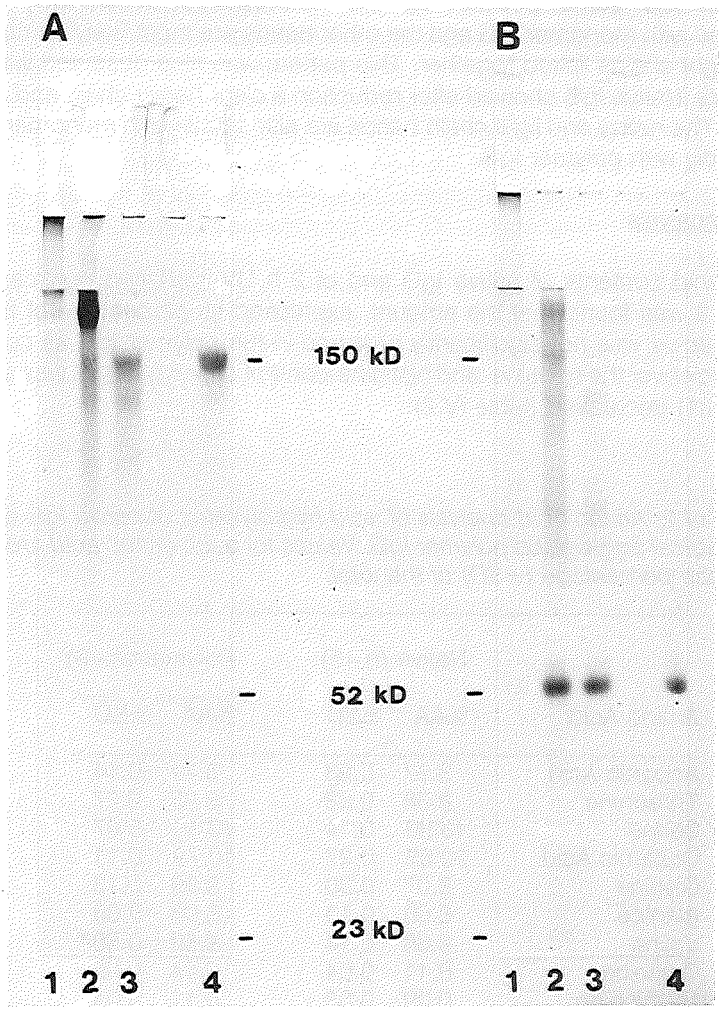

Figure IV.2: *Non-reducing (A) and reducing (B) 7.5% SDS-polyacrylamide gels of 120 min UV-irradiated IgG-samples: applied were polymer IgG (lane 1), dimer IgG (lane 2) and monomer IgG (lane 3). In lane 4 native IgG was applied. The gels were visualized with silver stain.*

staining of the non-reducing gel showed that polymer IgG remained partially in the slot of the stacking gel and partially on the border between stacking and running gel. Monomer IgG gave a dark band on the same level as the band of native IgG. Finally dimer IgG gave a band between monomer IgG and the polymer IgG band on the border between stacking and running gel. The reducing gel showed the following picture: reducing the polymer IgG did not result in complete dissociation into heavy and light chains, but instead it remained in the same positions as in the non-reducing

gel. Reducing dimer IgG gave rise to several bands. The greater part of dimer IgG remained on the same position as the non-reduced dimer IgG, one band corresponded with monomer IgG and the other bands are most likely combinations of heavy and light chains linked together. This pattern was also observed with reduced monomer IgG. Native IgG showed after reduction a clear heavy chain and a faint light chain band. The heavy and light chain bands are also visible with monomer and dimer IgG, but hardly with polymer IgG.

Amino acid analysis

The amino acid contents of native IgG and of 2 h UV-irradiated polymer IgG were determined. It was found that the amount, expressed as percentage of the total, of most amino acids was not significantly changed in the polymer IgG as compared to native IgG. However the tyrosine- and histidine-content of polymer IgG was significantly (both $p < 0.01$) decreased (table IV.2).

Table IV.2: *Amino acid analysis of acid hydrolysates of native IgG and oxygen free radical aggregated polymer IgG. Values for each amino acid are expressed as mean percentage (±SD) of the total.*

	Native (n=5)		Polymer (n=6)	
Amino Acid	%AA	±SD	%AA	±SD
Aspartic Acid	8.37	0.28	8.71	0.14
Threonine	8.38	0.58	8.93	0.27
Serine	13.96	0.14	13.90	0.07
Glutamic Acid	10.63	0.27	10.79	0.11
Glycine	9.07	0.30	8.99	0.13
Alanine	6.06	0.10	6.06	0.08
Valine	9.66	0.14	9.59	0.20
Cysteine	0.11	0.04	0.14	0.09
Methionine	0.09	0.03	0.11	0.04
Isoleucine	2.41	0.13	2.39	0.07
Leucine	8.54	0.19	8.44	0.09
Tyrosine	4.32	0.14	4.02	0.12*
Phenylalanine	4.05	0.11	3.89	0.09
Lysine	8.03	0.28	7.93	0.12
Histidine	2.45	0.10	2.29	0.03*
Arginine	3.88	0.08	3.80	0.06

** = two-sided t-test $p < 0.01$*

Discussion

Oxygen free radical attack on human IgG leads to aggregation of the protein. Separation of oxygen free radical altered IgG-aggregates by means of HPLC-gelfiltration revealed several peaks corresponding to monomer IgG, dimer IgG and polymer IgG which probably includes polymerization from trimer IgG to higher polymers. Besides this aggregation it was also observed that prolonged UV-irradiation (over 60 min) led to, but only to a very limited extend, the breaking-off of a "small" polypeptide (± 50 kD). From this it can be concluded that aggregation of IgG is the most pronounced, and probably most important, phenomenon occurring after exposure of human IgG to oxygen free radicals, and that degradation of the IgG-protein is a more or less minor side-effect.
The aggregation of IgG, as a consequence of free radical attack, is caused by the formation of reduction resistant cross-links. Reduction of the disulphide bridges of oxygen free radical altered IgG with ß-mercaptoethanol followed by analysis through SDS-PAGE revealed that IgG could no longer be completely reduced to heavy and light chains. Instead several extra bands appeared corresponding to various combinations of heavy and light chains tied together by apparently reduction-resistant cross-links. This clearly points to the formation of not only inter-molecular cross-links between IgG-molecules but also to intra-molecular cross-links connecting heavy and light chains. It should be stressed that aggregation of IgG caused by oxygen free radicals is of a entirely different nature than heat-aggregation of IgG. In an earlier paper (9) we showed that heat-aggregated polymer IgG could be completely dissociated by reduction with ß-mercaptoethanol into heavy and light chains.
About the identity of the oxygen free radical induced cross-links we have suggested (9) that these could be due to the formation of o,o'-dityrosine cross-links. To test this hypothesis we conducted amino acid analyses on native and polymer IgG. The results show that a decrease of tyrosine-residues occurs upon aggregation which can be explained by the fact that dityrosine cross-links are resistant to acid hydrolysis (11) and could thus account for a decreased quantity of tyrosine-residues in the amino acid analyses of oxygen free radical aggregated polymer IgG hydrolysates. The decrease in tyrosine-residues with 7% means that in each IgG-molecule 3-4 of these amino acids are involved in cross-linking. Quantitatively this dityrosine concentration is sufficient to explain the observed cross-linking. However it should be pointed out that other possible cross-linking mechanisms are not strictly ruled out. Oxidative phenolic coupling between phenylalanine-residues and tyrosine-residues is a possible cross-linking which, considering the apparent (but not significant) decrease in the amount of phenylalanine, could occur to a very limited extend. Furthermore, the results show that histidine is also reduced in aggregated IgG. This could point to a role for histidine in the aggregation-process as well. A role in protein cross-linking which has been described for histidine in photo-oxidized protein molecules by Dubbelman *et al* (12). From this it was shown that (photo-)oxidizing histidine could lead to protein cross-linking reactions, involving, among others, His-His, His-Trp and His-Tyr couplings. Our findings suggest that as a consequence of oxygen free radical action on human IgG the same cross-linking mechanisms occur.
It should at this point be emphasized that, since we applied an acid hydrolysis, we are

not able to give any data on changes in the tryptophan content of IgG after oxygen free radical attack. However earlier studies (8, 9) suggested an important role for tryptophan. These studies and the results presented here, prompted us to conduct at present further research upon the role of tryptophan.
Finally, the results described in this paper give support to the idea that oxygen free radicals cause the formation of covalent cross-links between IgG-molecules. In physiological conditions covalent cross-linking could be caused by oxygen free radicals released by activated neutrophils. In RA-patients these oxygen free radical-altered and -covalent cross-linked IgG aggregates may act as persisting antigens and induce RF-production.

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FIVE

On the role of tryptophan in the oxygen free radical induced covalent cross-links in human immunoglobulin G.

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CHAPTER V

ON THE ROLE OF TRYPTOPHAN IN THE OXYGEN FREE RADICAL INDUCED COVALENT CROSS-LINKS IN HUMAN IMMUNOGLOBULIN G

Summary

The purpose of the present study was to determine the nature of the changes in amino acid composition of immunoglobulin G, and its relation to the observed covalent cross-linking, induced by the activity of oxygen free radicals. Special attention was focused on the effect on tryptophan. Aqueous solutions of IgG were exposed to an oxygen free radical generating mechanism (ultraviolet-irradiation at 254 nm and 366 nm). Amino acid analyses of native IgG and 2 hours ultraviolet-irradiated IgG revealed that as a consequence of oxygen free radical action the contents of the aromatic amino acids tryptophan and tyrosine, the heterocyclic amino acid histidine and the sulfur-containing amino acid cystine were significantly reduced. Chemical determination in the intact protein confirmed the specific attack on tryptophan. Furthermore, it was shown that indole-ring destruction is the main effect of tryptophan-photooxidation in the intact protein. The specific attack of oxygen free radicals on the aromatic amino acids and their involvement in the formation of covalent cross-links in oxygen free radical altered IgG was demonstrated by comparative tryptic peptide-mapping of both native IgG and oxygen free radical altered polymer IgG.
The observed effects of oxygen free radicals on IgG show their potential antigenicity-enhancing abilities, thereby providing a possible explanation for the observed existence of rheumatoid factors and circulating immune-complexes in rheumatoid arthritis.

Introduction

One of the main systems by which phagocytes (neutrophils, eosinophils, monocytes and macrophages) kill invading organisms is the generation of oxygen derived free radicals such as superoxide anion (O_2^-), the hydroxyl radical ($OH^\bullet$) and hydrogen peroxide (H_2O_2). The formation of these intermediates of oxygen reduction is catalyzed by a membrane bound NADPH-oxidase which is dormant in resting cells and becomes activated during phagocytosis or following interaction of the cells with suitable soluble or particulate stimuli (1).
In rheumatoid arthritis (RA), which could be described as a chronic form of synovitis, large numbers of stimulated polymorphonuclear leucocytes (PMN) and macrophages accumulate in the synovium during the active stages of the disease. These cells release large amounts of reactive oxygen species. Involvement of oxygen free radicals in the pathogenesis of RA is suggested by the following observations: the finding of

degradation products of lipid peroxidation in serum and synovial fluid (2-6), depolymerization of hyaluronic acid accompanied with a reduced viscosity of the synovial fluid (7) and degradation of cartilage (8). RA is characterized by the occurrence of rheumatoid factors (RF), auto-antibodies specifically directed against the Fc-portion of IgG. Structural alteration of IgG by oxygen-derived free radicals has been implicated as a possible pathway by which IgG can become auto-antigenic and can induce the production of RF. Studies on the effect of free radicals on IgG showed the loss of native fluorescence (Ex.298 nm, Em.336 nm) and the subsequent generation of a new specific fluorescence (Ex.360 nm, Em.454 nm) (9), thiol-group formation and aggregation (10-12). Changes in amino acid composition were also reported (13,14). These oxidative modifications are not limited to IgG alone but are common to a wide variety of proteins, including: bovine serum albumin, transferrin, hemoglobin etc. (15-19).
Of special interest is the observed aggregation of IgG when exposed to free radical generating mechanisms (i.e. ultraviolet-irradiation, peroxidizing lipids and activated PMN). These free radical altered IgG-aggregates may not only explain the induction of RF-production but also the occurrence of circulating immune-complex-like material that is frequently detected in RA. Therefore it is of particular importance that we were able to show, by competitive radio immuno assay, that oxygen free radical altered IgG binds with higher affinity to rheumatoid factors than native unaltered IgG (20).
The liberation of a variety of cellular products upon activation of PMN, among which several proteolytic enzymes, makes it difficult, if not impossible, to determine the sole effects of oxygen free radicals. Therefore, we used low-intensity ultraviolet-irradiation (254-366 nm) to generate oxygen free radicals. This well established system (10,13,21) is highly reproducible and it has the advantage over the use of activated PMN that only free radicals are generated.
In a previous article (22) we have shown that the IgG-molecules which constitute the free radical aggregated complexes are linked together by reduction-resistent (i.e. not dissociated by ß-mercaptoethanol) covalent cross-links. Moreover also intra-molecular reduction-resistent covalent cross-links are formed between the 4 polypeptides (2 light and 2 heavy chains) that form an IgG molecule (14).
The present study was undertaken to investigate further the nature of these covalent cross-links.

Materials and methods

Chemicals

All chemicals were obtained, unless otherwise indicated, from Merck AG, Darmstadt, Federal Republic of Germany.

Purification of human IgG

Human IgG was isolated from the pooled sera of healthy donors by precipitation with 50% saturated ammonium sulphate in 10.5 mM phosphate buffered saline pH 7.4

(PBS) followed, after dissolution in PBS, by another precipitation with 33% saturated ammonium sulphate. The precipitate was dissolved in 50 mM Tris-HCl pH 8.0 and dialyzed extensively against the same buffer. The IgG was finally purified by passage over a DEAE-cellulose (Whatman, Maidstone, England) column equilibrated with 50 mM Tris-HCl pH 8.0. The IgG was collected in the excluded fractions and extensively dialyzed against PBS.

Preparation of oxygen free radical altered IgG/tryptophan

Human IgG at a concentration of 5 mg/ml PBS was exposed to oxygen free radicals by ultraviolet-irradiation. Three ml-vol were irradiated for 2 hours in matched quartz cuvettes, 1 cm^2 in cross section, at a distance of 6 cm from the light source. The light source at 254 nm was a sterilAir G-9 bulb and at 366 nm a S.Y.L.V.A.NIA F8T5/BLB. L-Tryptophan was irradiated in a concentration of 1 mM in PBS.

Gelfiltration of UV-irradiated IgG

Gelfiltration chromatography of ultraviolet irradiated IgG was performed on a Sepharose S300 (Pharmacia, Uppsala, Sweden)-column (70 x 1.5 cm) at a flow rate of 20 ml/hour. A 6 ml sample of 2 h ultraviolet-irradiated IgG was applied onto the column. PBS was used as elution buffer. Fractions of 4 min were collected. The absorbance of the effluent was monitored at 280 nm. The volume at which monomer IgG eluted from the column was determined in separate experiments. Fractions containing the aggregated IgG, i.e. fractions that showed absorbance at 280 nm and eluted in front of the monomer IgG peak, were pooled.

Amino acid analysis

Amino acid analysis was done on 24 h (under vacuum) 3 M para-toluene sulphonic acid (110°C) hydrolysates, using a LKB 4151 alphaplus amino acid analyzer (LKB, Bromma, Sweden), equipped with a Resin 766-LKB 732 column (200 x 4.6 mm). A five-stepwise gradient of increasing pH and ionic strength was applied. The column eluent was mixed with o-phtaldehyde reacted at room temperature and detected with a Merck-Hitachi F1000 Variable Fluorescence Spectrophotometer at excitation wavelength 340 nm and emission wavelength 455 nm. Integration of chromatogram peaks was carried out with a Merck-Hitachi D 2000 chromato-integrator.

Tryptophan determination in the intact protein

Tryptophan in the intact protein was determined with 2-hydroxy-5-nitrobenzyl bromide (HNB-Br; Sigma,St.Louis, MO, U.S.A.) using the method of Barman and Koshland (23). Briefly, proteins were denatured at 37°C for 16-20 h in 1 ml 10 M urea, 5 mg HNB-Br in 0.1 ml acetone was added at room temperature. The labelled protein was passed down a Sephadex G-25 (Pharmacia)-column (230 x 11 cm) previously equilibrated with 0.18 M acetic acid (pH 2.7) to keep the protein in solution. The protein was precipitated with 50% TCA (overnight at 4°C). After washing (2x) with 5 ml of an

ethanol-HCl solution (2 ml conc.HCl + 98 ml 95% ethanol), the precipitate was dissolved in 1 ml conc. HCl. A portion of this solution (0.1 ml) was adjusted to pH > 12 by addition of 2.5 M NaOH to a final volume of 2.5 ml. The concentration of HNB groups in this solution was determined by spectrophotometric assay at 410 nm with the use of an extinction coefficient of 18,000 M^{-1} cm^{-1}.

Analysis of tryptophan photoproducts

Tryptophan photoproducts formed by 2 h ultraviolet-irradiation were analyzed by reversed-phase chromatography on a Waters high performance liquid chromatograph equipped with a Novapack C-18 (Waters, Milford, MA, U.S.A.)-column (3.9 mm x 15 cm). A 50 µl sample (1 mM tryptophan in PBS) was applied onto the column. A mixture of tryptophan and tryptophan-metabolites, at a concentration of ca. 50 µM each, served as standard. The photoproducts were eluted by a linear gradient of methanol from 0 to 40% in 10 mM potassium phosphate, pH 3.6 (gradient volume: 20 ml), at a flow rate of 0.5 ml per min. Column eluates were monitored concomitantly for absorbance at 254 nm and indol-fluorescence (Ex 280 nm, Em 340 nm).

Peptide mapping

The IgG-samples (resp. native monomer IgG and oxygen free radical altered polymer IgG) were denatured in 6 M guanidine-HCl pH 8.6 for 50 min at 50 °C (under N_2), reduced with 5 µM dithiothreitol (Sigma) for 30 min at 50 °C (under N_2) and alkylated using 25 µM iodo-acetic acid for 50 min at 20 °C in darkness (under N_2). After desalting over a G-25 column (Pharmacia) TPCK-trypsin (Worthington, Freehold, NJ, U.S.A.) was added (1%, w/w). The hydrolysis was carried out for 20 h at 37°C in 0.1 M $NaHCO_3$, pH 8.0. The reaction mixture was lyophilized and dissolved in 0.1% trifluoroacetic acid. Fractionation of the resultant peptides was accomplished by reversed-phase high performance liquid chromatography (HPLC) using a Lichrosorb RP-18 (Merck, Darmstadt, Germany) column (250 x 4 mm). As solvents A and B 0.1% (v/v) trifluoroacetic acid in water and 0.1% (v/v) trifluoroacetic acid in 60% (v/v) acetonitrile (FSA, Loughbourough, England), respectively, were used. The separation was carried out with a linear gradient ranging from 100% (v/v) solvent A (0% solvent B) to 10% (v/v) solvent A (90% solvent B) at a flow rate of 1 ml per min (gradient volume: 90 ml). Peptide elution was monitored by absorbance at 214 nm. Aromatic amino acids containing peptides were monitored by absorbance at 280 nm.

Statistical methods

The students t-test (two-tailed) was used to determine the statistical significance of differences observed.

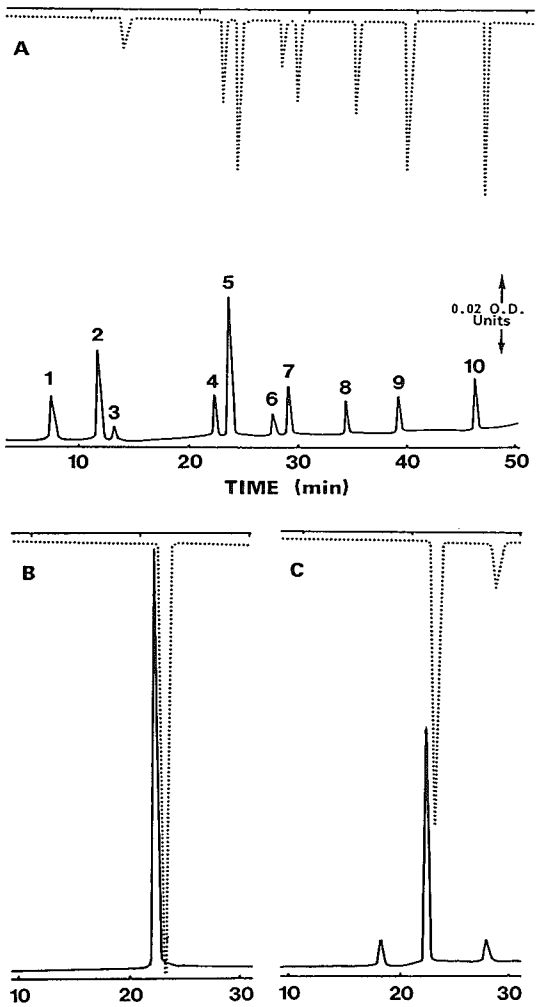

Figure V.1: *Fractionation of tryptophan and tryptophan-metabolites by reversed phase HPLC.*
The separation was achieved on a Novapak C-18 column using 10 mM potassium phosphate pH 3.6 with a gradient from 0 to 40 % methanol. The effluent was monitored for absorbance at 254 nm (—) and indol-fluorescence (....). A. Standard mixture of tryptophan and tryptophan-metabolites: 1. 3-hydroxy-kynunerine, 2. kynunerine, 3. 5-hydroxy-tryptophan, 4. tryptophan, 5. 5-hydroxy-indol, 6. tryptamine, 7. 5-hydroxy-indol acetic acid, 8. indol acetamide, 9. indol lactic acid, 10. indol acetic acid. B. Native tryptophan. C. Two hours ultraviolet-irradiated tryptophan.

Results

Amino acid analysis of native monomer IgG and oxygen free radical altered polymer IgG

Table IV.2 showed the amino acid composition of acid hydrolysates of native and free radical aggregated IgG. The latter was obtained by gelfiltration of 2 hours ultraviolet-irradiated native IgG as described in materials and methods. As 6 M HCl-hydrolysis destroys all tryptophan residues an additional milder hydrolysis method was also used. Also with milder hydrolysis a higher yield of cystine is obtained giving a more reliable value for this amino acid. In table V.1 the results of the additional experiments on the tryptophan- and cystine-content in both IgG preparations are given. For comparison, the values of tyrosine and histidine, as determined by this method, are also presented. The values for the other amino acids obtained by the latter method were comparable to the values presented in table IV.2. The results clearly indicate that the aromatic amino acids, tyrosine and tryptophan, were significantly reduced in oxygen free radical attacked IgG. Also the heterocyclic residue histidine, and the sulfur-containing residue cystine were significantly reduced. The values of all other amino acids were not significantly altered by two hours ultraviolet-irradiation.
The reduction in tryptophan content of free radical altered polymer IgG by comparison with native IgG was confirmed by chemical estimation of tryptophan-residues in the intact protein with HNB-Br. The results showed a decrease of 11,8 ± 1,4 % (n=3) in tryptophan content, representing a decrease from ca. 16 to ca. 14 residues, during 2 hours ultraviolet-irradiation.

Table V.1: *Amino acid analysis of mild acid hydrolysates of native IgG and oxygen free radical altered polymer IgG. Hydrolysates of native IgG (8 experiments) and of oxygen free radical altered IgG (5 experiments) were prepared in 3 M p-toluene sulphonic acid and analyzed as described under materials and methods. Values are expressed as mean moles (±SD) per 100 mol of all amino acids recovered.*

Amino Acid	Amino acid composition in mol %	
	Native IgG	Polymer IgG
Cystine	0.88 ± 0.01	0.53 ± 0.04[a]
Tyrosine	4.56 ± 0.15	4.17 ± 0.08[a]
Histidine	2.03 ± 0.08	1.89 ± 0.01[b]
Tryptophan	0.70 ± 0.07	0.62 ± 0.02[c]

[a] indicates a significant difference at $p<0.001$
[b] indicates a significant difference at $p<0.01$
[c] indicates a significant difference at $p<0.05$

Analysis of tryptophan photoproducts

Tryptophan photoproducts were analyzed by a reversed phase HPLC-method. The column eluates were monitored for absorbance at 254 nm and indol-fluorescence, which are both specific for tryptophan. A mixture of tryptophan and known tryptophan-metabolites was used as a standard (fig.V.1A). Analysis of L-tryptophan and 2 hours ultraviolet-irradiated L-tryptophan revealed that two additional peaks appeared in the absorption (254 nm)-chromatogram (figs.V.1B and V.1C). One peak with a retention-time of 27,8 min also showed indol-fluorescence. This peak could positively be identified as tryptamine, the decarboxylated form of tryptophan. Another A254-peak (retention-time 18.2 min) did not show any fluorescence at emission wavelength 340 nm when excited at 280 nm, this peak represents a tryptophan-photoproduct in which the indol-structure has been destroyed by the action of oxygen free radicals. Most likely this peak represents N'-formyl-kynunerine.

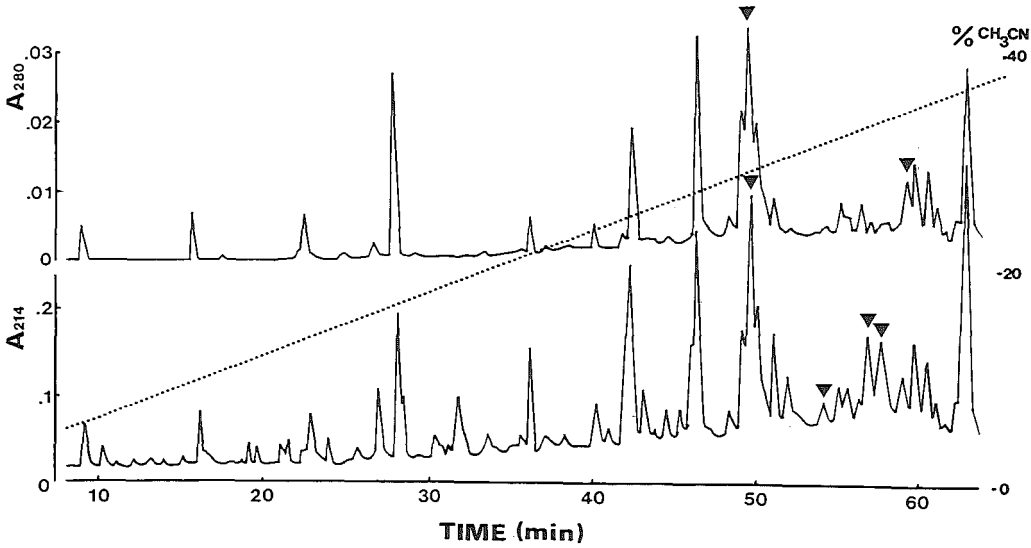

Figure V.2: *Fractionation of tryptic peptides of native IgG by reversed phase HPLC. The separation was achieved on a Lichrosorb RP-18 column using 0.1 % trifluoroacetic acid with a gradient from 0 to 54 % acetonitrile (indicated by dotted line). The absorbance of the effluent was monitored at 214 nm and at 280 nm. Peptides which are absent or significantly smaller in the peptide-map of oxygen free radical altered polymer IgG (fig.V.3) are indicated (▼).*

Comparative tryptic peptide-mapping

Tryptic peptides of alkylated native IgG and alkylated oxygen free radical altered polymer IgG were subjected to reversed-phase HPLC. The peptides were eluted with

a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. So far, over 20 peptide mappings of both native and oxygen free radical damaged IgG were performed. Not withstanding their complexity the maps appeared to be highly reproducible. Representative examples of the resultant peptide maps of native IgG and of oxygen free radical damaged IgG are shown in fig.V.2 and fig.V.3 respectively. In both figures the peptide maps as obtained by monitoring the column eluates for absorbance at 214 nm as well as at 280 nm are presented. The peptide maps of native IgG and oxygen free radical altered polymer IgG showed many structural similarities, although some differences existed. Several peptides (as indicated in fig.V.2) present in the "native" A214-peptide map were absent or significantly reduced in the "free radical altered" A214-peptide map. Interestingly A214-peptide mapping of oxygen free radical altered polymer IgG revealed an additional peptide (as indicated in fig.V.3) that was significantly smaller in the A214-peptide maps of native IgG. Of particular interest were the peptide maps as visualized by monitoring for UV-absorbance at 280 nm. From this it can be seen that in the peptide map of oxygen free radical altered polymer IgG some additional aromatic amino acids containing peptides (as indicated in fig.V.3) appeared as compared to the native IgG peptide maps.

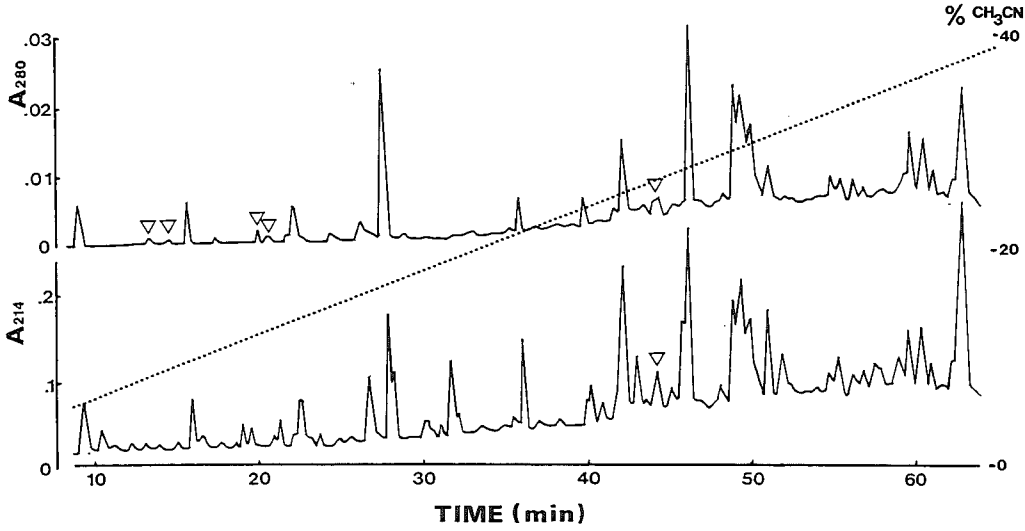

Figure V.3: *Fractionation of tryptic peptides of oxygen free radical altered polymer IgG by reversed phase HPLC. The separation was achieved on a Lichrosorb RP-18 column using 0.1 % trifluoroacetic acid with a gradient from 0 to 54 % acetonitrile (indicated by dotted line). The absorbance of the effluent was monitored at 214 nm and 280 nm. Peptides which are absent or significantly smaller in the peptide-map of native IgG (fig.V.2) are indicated (▽).*

Discussion

Rheumatoid factors play a major role in the pathogenesis of RA. Identification of the events that lead to the production of these auto-antibodies may yield clues to the etiology of this disease.
There are indications that oxygen free radicals can induce changes in the IgG-molecule that lead to aggregation, auto-antigenicity and subsequent RF-production (13, 24-27). In order to evaluate the role of oxygen free radical damaged IgG in RA, we undertook studies to analyze the changes that occur in IgG upon exposure to oxygen free radicals.
Recent work from our group (22) showed that the aggregation of IgG as a consequence of oxygen free radical activity is caused by the formation of reduction resistant cross-links. Here we present evidence that tyrosine and histidine, and particularly tryptophan are involved in this covalent cross-linking.
Tryptophan residues can, as a result of photo-oxidation, bind to other amino acids, leading to protein cross-linking and aggregation. Ichijima and Iwata (28) showed that in proteins (crystallins) cross-linking occurs as a consequence of ultraviolet-irradiation. Incubation of crystallins with tryptophan metabolites increases the ultraviolet-irradiation induced cross-linking. Moreover, Dilley (29) found that S-carboxymethyl-lysozyme which contains tryptophan forms cross-links upon photo-oxidation, while S-carboxymethyl-ribonuclease which contains no tryptophan, did not form cross-links. This was also observed in histone H2A, a nucleosome protein which lacks tryptophan. The hydroxyl radical and superoxide anion are not able to induce cross-linking in this protein (30). Model studies on photo-oxidation by Verwey *et al* (31) showed that tryptophan is able to bind to histidine residues after exposure to ozone. They also found that photo-oxidized histidine is able to bind to other histidine-residues and to tyrosine. Thus there are several ways by which photo-oxidation and more specific oxygen free radicals can cause protein cross-linking. Our results suggest that in the case of ultraviolet-irradiation of IgG the same cross-linking mechanisms occur.
The decrease of the aromatic amino acids, tryptophan and tyrosine, and of cystine and histidine, as shown in the amino acid analyses of free radical altered IgG are either caused by a total breakdown of these amino acids or by the formation of photoproducts which are no longer detected as the original amino acid. We favor the latter explanation since hardly any breakdown products were detected in oxygen free radical attacked IgG-preparations (14). It is therefore more likely that several photoproducts are formed, which create and contribute to covalent cross-linking and altered fluorescence. To study this in more detail, we irradiated an aqueous solution of L-tryptophan and identified its photoproducts. From this it could be seen that only two major photo-oxidation products of tryptophan are formed after exposure to UV-irradiation. One of these products was identified as tryptamine which points to an effective decarboxylation of tryptophan-molecules. Irradiation of tryptophan at 254 nm alone also yields this photoproduct (32). However,it seems unlikely that decarboxylation plays an important role in the intact protein, since the carboxyl-group is then part of a peptide bond. Therefore the other photoproduct observed upon irradiation seems to be more important in clarifying the free radical induced changes in tryptophan-content of IgG. This photoproduct is most probably N-formyl-kynunerine

since it lacks indol-fluorescence and because of its position on the HPLC-profile (fig.V.1C). N-formyl-kynunerine is also known to be one of the initial products of tryptophan indole-ring photolysis (33,34). Other experiments performed by us (unpublished data) with a different HPLC-solvent system (acetonitrile) revealed a limited number of quantitatively small tryptophan photoproducts additional to the two discussed above. One of these very small fractions could be identified as kynunerine, representing a further photo-oxidizing of N-formyl-kynunerine. Prolonged ultraviolet-irradiation (18 h) did not lead to an increase of photoproducts-diversity but only to a total destruction of tryptophan and tryptophan-metabolites (unpublished data). It follows from these data that indol-ring destruction is the most important mechanism of tryptophan-photolysis in the intact protein. This, together with photo-oxidation of tyrosine- and histidine-residues, causes protein cross-linking.

Another possible mechanism of protein cross-linking is the formation of dityrosine. Dityrosine cross-links result from the phenolic coupling of two phenoxy-radicals of tyrosine (35). The generation of dityrosine during ultraviolet irradiation of poly-L-tyrosine, copolymers and the dipeptide Tyr-Tyr was demonstrated by Lehrer and Fasman (36). We have shown that UV-irradiation of an aqueous solution of L-tyrosine generated the typical specific fluorescence of dityrosine (22). The results of the amino acid analysis of oxygen free radical altered polymer IgG are consistent with a molecular cross-link between tyrosine-residues.

The changes induced by the action of oxygen free radicals on IgG includes an increase in sulphydryl-groups (12). The finding that the thiol-containing amino acid cystine is the most susceptible amino acid to oxygen free radical attack is therefore not surprising and was reported earlier by Lunec *et al* (13). It is consistent with the oxygen radical dependent reduction and breaking of disulphide bonds and the formation of thiol-groups. It has been suggested by Wickens *et al* (10) that during free radical attack on IgG a balance exists between the intra-molecular thiol-group generation and the formation of inter-molecular disulphide bridges. However, we showed that the intra- and inter-molecular cross-links formed in IgG are resistent to reduction (14), making it highly unlikely that disulphide bonds play an important role in this covalent cross-linking.

Comparison of the tryptic peptide-maps of native- and oxygen free radical altered polymer IgG revealed the disappearance of several peptides and the generation of others as a consequence of radical action. The maps also showed the presence of aromatic amino acids in the newly formed peptides, suggesting an important role of these residues in the formation of such peptides. These findings support the conclusions drawn from the amino acid analyses and tryptophan-irradiation experiments.

In summary we have shown that exposure of IgG to an oxygen free radical generating system (i.e. UV-irradiation) results in a decrease of the aromatic amino acids, tryptophan and tyrosine, the heterocyclic amino acid histidine and the sulfur-containing amino acid cystine. We also showed a different tryptic peptide-mapping of IgG after exposure to oxygen free radicals. Furthermore we could confirm earlier findings, that as a consequence of this exposure intra- and inter-molecular cross-links are formed. The results also point to an important role of the aromatic amino acids in the formation of these cross-links.

The results presented here support the hypothesis that oxygen free radicals are involved in the aetiopathogenesis of RA. As shown IgG can be modified by oxygen free radicals. Although the alterations are often relatively small, the implications for antigenic and immunological behavior can be of significant importance. Recently, we reported that ultraviolet-irradiated IgG has an increased affinity for binding to the rheumatoid factor as compared to native IgG (20). This observation is of particular importance as interaction between altered IgG and rheumatoid factor might well form the essence of rheumatoid pathology. The results presented here add biochemical background to the observed immunological behavior. Future studies will reveal whether the observed effects can be extended to the *in vivo* situation.

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SIX

Possible role of free radical altered immunoglobulin G in the etiopathogenesis of rheumatoid arthritis.

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Rheumatol.Int. 9, 1-6, 1989.

CHAPTER VI

THE POSSIBLE ROLE OF FREE RADICAL ALTERED IGG IN THE ETIOPATHOGENESIS OF RHEUMATOID ARTHRITIS

Summary

Alteration of IgG by oxygen-derived free radicals have been implicated in the possible process *in vivo* which can render IgG to become autoantigenic and leads to the production of rheumatoid factor (RF) and perpetuation of inflammation as in rheumatoid arthritis (RA). In this study the impact of UV-irradiation on IgG was investigated and the ability of RF for binding the UV-altered gammaglobulin. Inhibition studies of the binding of 125I heat aggregated human gammaglobulin (HAG) for binding to RF coated sepharose beads shows that UV-irradiated IgG was able to bind RF to the same extent as HAG. Binding studies to 125I-C1q proved that UV-irradiated IgG could bind the first complement component, but also that the complement system could be activated illustrated by the C3a generation.
These results sustain the hypothesis that free radical damage of gammaglobulins plays a role in the chronicity of the inflammatory reaction in RA.

Introduction

Evidence exists that free radicals and free radical secretion products are important mediators in the inflammatory reactions (1-5). In different studies the possibility is claimed that for example free radical attack on immunoglobulins might be responsible for changes in their biochemical and thereby in their antigenic behavior. Gamma globulin fluorescence, indistinguishable in its spectral characteristics from that induced by UV-irradiation, could also be induced by other oxygen free radical generating systems (5). These alterations in the gammaglobulin are consistent with observations made on (autologous) gammaglobulins isolated from the synovial fluid of patients with rheumatoid arthritis (6). In a rat allergic air pouch model (7,8), a part of the hypothesis was confirmed that autologous free radical altered IgG is able to converse the transient into a persistent chronic inflammation. Free radical altered gamma globulins will aggregate. Generally aggregated gamma globulin complexes are thought to play a part in the chronic inflammation associated with rheumatoid arthritis, and also may perpetuate its course (9). The mentioned alterations mediated by free radical attack on the gamma globulins - their tendency to aggregate and the changes in the antigenic behavior - could play a role in the pathogenesis of rheumatoid arthritis.
The present work was undertaken to extent these findings and explore their possible biological implications. One part of the study is focused on the effects of *in vitro* UV-altered gamma globulin on the complement system. UV-irradiation is a well established oxygen free radical generating system. The question raised whether UV-irradiated IgG

is capable to bind complement components and thereby activating the complement system. If this would be the case than further evidence is obtained about the pathway how these products can play a role in the self-perpetuating mechanisms of the inflammatory reaction as occurring in rheumatoid arthritis (RA). Another question was related to the antigenic behavior of the UV-altered gamma globulins. When these products play an etiopathogenic role in rheumatoid factor production than rheumatoid factors might recognize UV-altered gamma globulins.

Materials and methods

C1q binding test (C1q-BT)

The C1q-BT was performed as described by Zubler (10). IgG was purified from a Cohn fraction II by chromatography on a DEAE Sephacel column (Pharmacia) of a sera pool of healthy normal volunteers. Heat aggregated human IgG (HAG) was obtained by incubating a solution of 7 mg/ml of this IgG for 20 min at 63 °C.

Preparation of free radical altered IgG

Ultraviolet irradiations were performed as described by Lunec (11). IgG solutions were irradiated in matched quartz cuvettes (3 ml volumes) at a distance of 6 cm from the light source. Ultraviolet irradiation was achieved using light sources of 254 nm and 366 nm. Two hours UV-irradiated IgG samples were centrifuged (1 h, 100,000 g) in a Beckman L5-65 ultracentrifuge to eliminate insoluble IgG aggregates. Gelfiltration chromatography of the supernatant of UV-irradiated IgG was performed on a Biogel A 1.5 m agarose (Biorad Lab, Richmond CA, USA). The column size was 1.5 x 70 cm and the elution buffer used was 50 mM Tris-HCL buffer pH 8.0. The absorbance of the effluent was monitored at 280 nm. Elution fractions containing the aggregated (polymer) IgG and monomer IgG were pooled separately. ^{125}I-labeled IgG was obtained by radioiodination with ^{125}I using the chloramine T method.

Inhibition of rheumatoid factor (RF) binding

Competition of binding ^{125}I HAG to RF coated sepharose beads by UV-irradiated IgG was determined in a radioimmunoassay. Rheumatoid factors (RF) were isolated from the heat-inactivated (50 °C, 30 min) sera of three latex positive RA patients by 50% ammonium sulphate precipitation. RF was coupled to CNBr-activated sepharose 4B (Pharmacia). In a polystyrene tube (12 x 45 mm) 125 µl of sepharose in phosphate buffered saline (PBS, pH 7.4) containing 10 mM EDTA and 0,1% Tween 20 was added and incubated with 125 µl of a dilution of UV-irradiated IgG for 2 hours at room temperature under constant mixing. Then 10 µl ^{125}I HAG (10^4 cpm/tube) was added and incubated overnight at room temperature under constant mixing. As control 125 µl of sepharose coupled RF was incubated with 125 µl buffer (PBS) and 10 µl ^{125}I HAG. The tubes were washed 5 times with physiological salt and counted for bound ^{125}I radioactivity in a gamma counter (LKB Products AB, Stockholm, Sweden). The results,

Table VI.1: *Competition of binding ^{125}I-labelled heat-aggregated human IgG to rheumatoid factor coated sepharose beads. The competition was assayed with rheumatoid factors of three RA-patients. The results of the three experiments are given in the mean value and range of the obtained inhibition by the different competitors (native IgG, 30 min UV-irradiated IgG and 120 min UV-irradiated IgG).*

Competitor	Concentration IgG (mg/ml)	Percentage inhibition	
		Mean	Range
Native IgG	0.1	1	(0-2)
	0.5	12	(0-25)
	2.5	44	(25-55)
30 min UV-irradiated IgG	0.1	8	(0-8)
	0.5	27	(0-40)
	2.5	49	(40-55)
120 min UV-irradiated IgG	0.1	25	(0-35)
	0.5	47	(25-60)
	2.5	100	-

expressed as percentage binding, were calculated from the ratio of cpm, bound by the RF in the presence of ^{125}I HAG and competitor native or UV-irradiated IgG over the cpm bound in the absence of competitor.

Inhibition C1q-BT

Human C1q was isolated according to the method of Yonemasu (12). The C1q was radiolabeled with ^{125}I as described by Sobel (13), except that after dialysis the ^{125}I-C1q was layered on an isokinetic 5-25% (w/w) sucrose gradient. The specific activity was 0,2-0,3 μCi/μg C1q. A total of 250 ng ^{125}I-C1q was added to each sample of IgG or UV-irradiated IgG in a 50 μl volume to 50 μl serum. This mixture was then treated with 50 μl 0,4 M EDTA, pH 7.5 instead of 100 μl 0,2 M EDTA. PEG precipitates were made in the same manner as in the C1q-BT (10). After centrifugation the precipitates were dissolved in PBS and the radioactivity was measured.

C3a generation

C3a was measured by a radioimmunoassay (14), a modification of the procedure described by Hugli (15). Briefly the binding of ^{125}I labeled purified C3 to anti-C3a antibodies immobilized onto a solid phase (sepharose) is inhibited by C3a present in

the investigated sample. To prevent interference by native C3, the samples are first incubated with 11% polyethylene glycol and the supernatant is tested in the assay. Results are expressed as nmol C3a per L. Fresh serum (50 μl) was incubated with HAG or with equivalent concentrations UV irradiated IgG (50 μl volume) for 1 hr at room temperature.

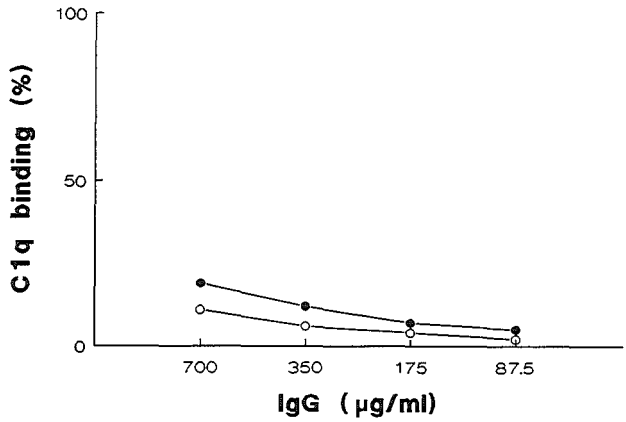

Figure VI.1: *The binding of ^{125}I-C1q to native IgG (o–o) and to monomer UV-irradiated IgG (●–●). Differences are not significant.*

Results

Binding of UV-irradiated IgG by rheumatoid factors

Ultraviolet-irradiated human IgG was tested in a competitive radioimmunoassay with ^{125}I labeled heat aggregated human IgG for binding to RF-coated sepharose beads. The binding (in cpm) of ^{125}I HAG in the absence of competition with UV-irradiated IgG was considered 100%. The effect of native IgG and UV-irradiated IgG on the binding of ^{125}I HAG to RF-coated sepharose beads is illustrated in table VI.1. The results of the experiments show that 0,5 mg of 30 min irradiated IgG had an inhibitory effect on the binding of ^{125}I HAG to RF. The effect of 120 min UV-irradiated human IgG was even greater.

Binding of UV-irradiated IgG by ^{125}I-C1q

The binding of ^{125}I-C1q to native IgG, heat aggregated IgG or UV-irradiated IgG fractions are illustrated in figs.VI.1 and VI.2. Both the monomer UV-irradiated IgG fraction as well as the aggregated (polymer) UV-irradiated IgG fraction compared with native (monomer) IgG and the heat aggregated IgG preparation, respectively, showed

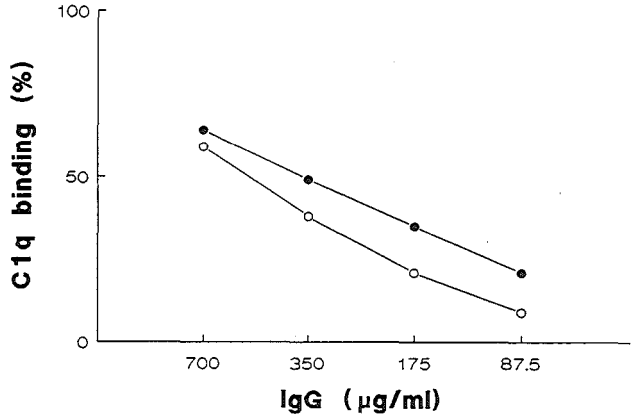

Figure VI.2: *The binding of ^{125}I-C1q to heat-aggregated IgG (o–o) and to aggregated (polymer) UV-irradiated IgG (●–●). No significant difference.*

a comparative or slightly higher tendency to bind the ^{125}I-C1q.

C3a generation by UV-irradiated IgG

Also in these experiments the behavior of UV-irradiated IgG was studied by comparing the monomer UV-irradiated IgG fraction with native IgG, and UV-irradiated aggregated IgG with heat aggregated IgG. The results are illustrated in figs.VI.3 and VI.4. No

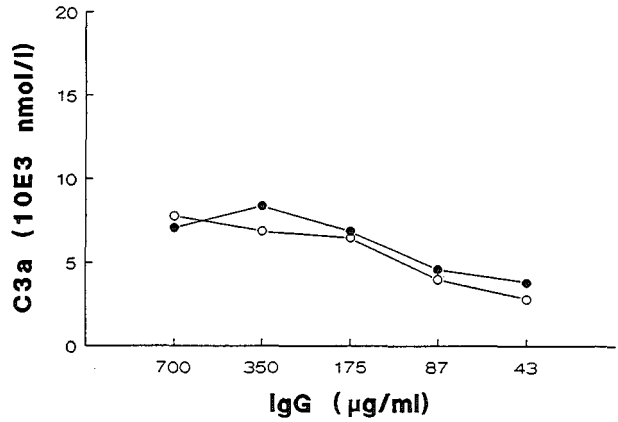

Figure VI.3: *C3a generation in normal fresh serum caused by incubation with monomer UV-irradiated IgG (●–●) and native IgG (o–o). No significant difference between C3a generation caused by UV-irradiated IgG and native IgG.*

obvious differences existed in the ability to generate C3a, neither between native IgG and monomer UV-irradiated IgG, nor between HAG and aggregated (polymer) UV-irradiated IgG. In the absence of addition of IgG no C3a generation will take place (upper limit after 1 h incubation with PBS in 190 nmol/L). To exclude the possibility of differences in the kinetics of C3a generation caused by the different IgG preparations, the incubation time was varied (0, 5, 10 and 30 min) as well as the concentration. Between the monomeric fractions no differences existed in the ability to generate C3a. The C3a generation caused by HAG reached the plateau value in a much faster way than that caused by the aggregated UV-irradiated IgG fraction (fig.VI.5).

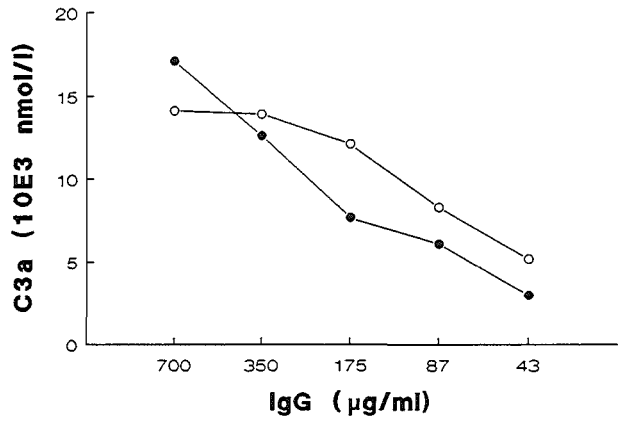

Figure VI.4: *C3a generation in normal fresh serum caused by incubation of aggregated (polymer) UV-irradiated IgG (●–●) and HAG (○–○). No significant difference.*

Discussion

By observing that free radical derived products can be generated *ex vivo* and are demonstrable in synovial effusions, the question arises if these free radical mediated changes play a role in the acquisition of auto-antigenic properties of gamma globulins (rheumatoid factor production?) or perpetuation of the inflammatory reactions (synovitis). That in the inflammatory reaction the production of free radicals play an important role is beyond the question (1-5). Degradation of the hyaluronic acid, and thereby reducing the viscosity of synovial fluid (17), and peroxidation of the phospholipid membranes (18) are thought the most important joint damaging mechanisms. In the past, suggestions were made that the chronicity of the inflammatory reaction in rheumatoid arthritis is caused by an deficient scavenger mechanism. However, real evidence is lacking, only a more relative deficient scavenger mechanism could be demonstrated (17).
The presence of increased numbers of polymorphonuclear neutrophils (PMN), the

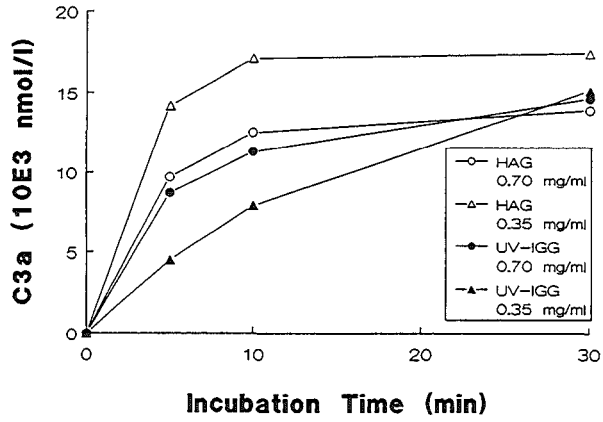

Figure VI.5: *Effect of different incubation times and protein concentrations on the C3a generation caused by HAG and aggregated (polymer) UV-irradiated IgG.*

presence of immune complexes (IC), rheumatoid factors (RF) and the evidence of complement consumption (19,20) are the main characteristics of the joint fluids from patients with rheumatoid arthritis. In *ex vivo* studies a direct activation of PMN could be obtained by incubation with sera and synovial fluid from patients with RA (21,22). This response could be modulated by the presence of RF. In the absence of fresh serum the level of enhancement of PMN-activation was minimal, both observations suggest that in activation of PMN caused by synovial fluid of RA patients the amount or presence of IC, complement components and RF play an important role.
Overall the concept is maintained that IgG is the primary antigen in IC in RA and that RF can act as an antibody (23,24). In the studies of Hewitt (8) it was clearly shown that free radical altered IgG has two effects in a model of inflammation (the rat allergic air pouch). First a rise in the production of gamma globulins, accompanied by an increase of the characteristic fluorescence associated with free radical damage and secondly, evidence was obtained that free radical altered IgG could convert an inflammatory insult to a more persistent stimulus. The possible biological importance of free radical altered gamma globulins is earlier demonstrated by the fact that in synovial fluids gamma globulins could be detected with a fluorescence spectrum indistinguishable from that caused by ultraviolet irradiation of IgG (25). These fluorescence changes were the same as those induced by free radical generating PMN. In our study (16) on the effects of free radicals on IgG it was found that covalent cross-links between IgG molecules were formed, in contrast to cross-links in aggregated IgG formed as a consequence of heating (20 min, 63 °C) which could be reduced by ß-mercaptoethanol. This fact demonstrates that free radicals could be held responsible for the formation of stable IC.
The results of the competitive radioimmunoassay described in this report showed that UV-irradiated IgG binds with high affinity to rheumatoid factor. Comparison with native IgG revealed that UV-altered IgG was more competitive with heat aggregated IgG for

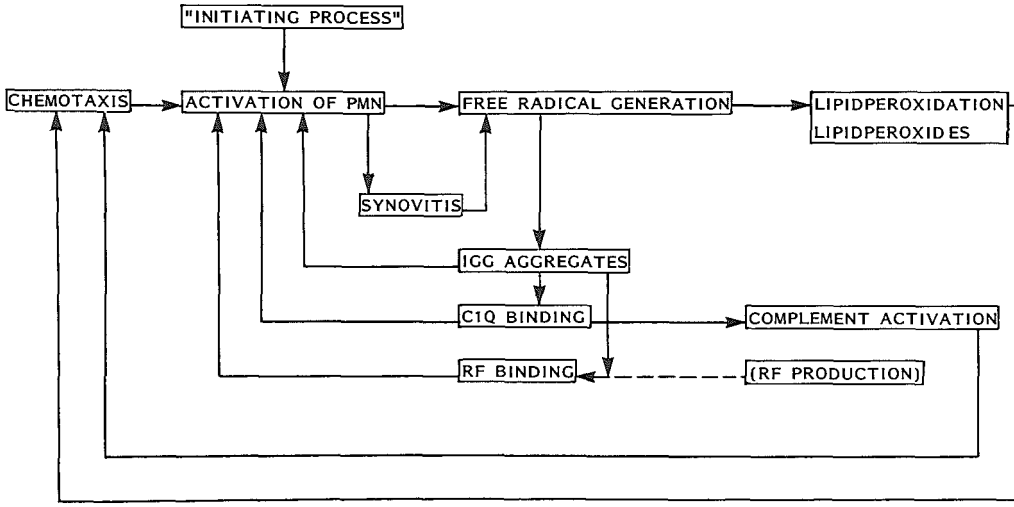

Figure VI.6: *Hypothesis of the etiopathogenesis of rheumatoid arthritis: suggested scheme for the involvement of free radicals.*

binding to rheumatoid factor. These findings suggest that the rheumatoid factor can react specifically with gamma globulins modified by oxygen free radicals.
We observed that the UV-altered IgG could bind the first component of the complement system, but also that the complement system could be activated (C3a generation). Our results added more evidence to the hypothesis that the free radical damage of gamma globulins play an essential role in the chronicity of the inflammatory reaction of RA (fig.VI.6). The generation of oxygen free radicals by PMNs leads to IgG modification and aggregation. These IgG aggregates will cause a perpetuation of PMN-activation and induce gamma globulin production. This in turn leads to a further formation of IgG aggregates which, sustained by complement activation, will increase the phagocytosis of gamma globulin complexes as well as the liberation of chemotactic stimuli (C3a generation, our results). Whether these free radical-altered gamma globulins are responsible for the RF production is still the question. As can be seen from our results and results from others (21,22) once RF is present a further stimulation of the inflammatory reaction can take place.

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SEVEN

Differential activation of superoxide- and hydrogenperoxide-production by oxygen free radical altered immunoglobulin G.

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Submitted for publication.

CHAPTER VII

DIFFERENTIAL ACTIVATION OF SUPEROXIDE- AND HYDROGENPEROXIDE-PRODUCTION INDUCED BY OXYGEN FREE RADICAL ALTERED IMMUNOGLOBULIN G

Summary

The production of superoxide anion and hydrogenperoxide by neutrophils from normal human peripheral blood and from rheumatoid arthritis patients' peripheral blood and synovial fluid was measured. The stimuli used in this study were native IgG, heat-aggregated IgG, monomer (oxygen free radical altered) IgG and polymer (oxygen free radical altered) IgG. Superoxide radical production by neutrophils from normal human subjects was not significantly stimulated by any form of IgG. In contrast, the hydrogenperoxide-production by normal human neutrophils and by rheumatoid arthritis patients' neutrophils was significantly enhanced if stimulated with monomer IgG or polymer IgG. These results indicate that hydrogenperoxide-production by neutrophils could be stimulated independently from superoxide-production.
With rheumatoid arthritis neutrophils a significant increase in superoxide-production was observed when stimulated with polymer IgG. Other IgG-stimuli did not enhance superoxide-production significantly. Both superoxide- and hydrogenperoxide-production by rheumatoid arthritis neutrophils were significantly higher than the production by normal neutrophils when stimulated with serum treated zymosan. The latter results suggests that neutrophils of rheumatoid patients have been potentiated ("primed") in the *in vivo* rheumatoid situation.
Thus, our results indicate that hydrogenperoxide can be produced independently from superoxide. Furthermore, they show that free radical altered IgG stimulates superoxide- and hydrogen-peroxide-production by rheumatoid arthritis neutrophils, confirming the reported self-perpetuating mechanism of free radical altered IgG damage in rheumatoid inflammation.
The possible mechanisms responsible for the observed phenomena are discussed.

Introduction

The release of reactive oxygen species (oxygen free radicals) represents one of the main systems by which neutrophils kill invading organisms. However, oxygen free radicals can also be responsible for the damage to the tissues (1-3). Therefore, oxygen free radical damage has been implicated in the etiology and pathology of several diseases (4), including rheumatoid arthritis (RA). The mechanism by which neutrophils are triggered to produce oxygen free radicals is not well understood. Many soluble and particulate stimuli able to produce some level of response *in vitro* are non-physiologic (i.e. PMA). Physiologic secretagogues such as immune-complexes (5-7), complement

factors (8,9), N-formylated peptides (10-11), leukotrienes (12), platelet activating factor (13), arachidonic acid (14) and cytokines (15,16) are able to trigger the respiratory burst to some extend. However, most of these studies only recorded the superoxide-production and disregarded the hydrogenperoxide-production. Moreover, some of these stimuli were used in concentrations not likely to occur *in vivo*, or were shown to have a "priming" effect leading to increased neutrophil radical production when subsequently given a second stimulus like PMA (8,16).
One of the physiologic stimuli implicated in the etiopathology of RA is complexed immunoglobulin G. Exposure of IgG to oxygen free radical generating mechanisms led to subsequent induction of a new specific protein fluorescence (17), generated SH-groups (18-20), altered the amino acid composition and induced the formation of reduction resistent covalent cross-links causing aggregation (21).
The purpose of this study was to explore the effects of oxygen free radical altered IgG on the superoxide- and hydrogenperoxide-production by neutrophils. Lunec *et al* (22) described the self-perpetuating mechanisms of oxygen free radical damage to IgG in rheumatoid inflammation. In their report these investigators delineated the stimulatory effect of monomer oxygen free radical altered IgG and polymer oxygen free radical altered IgG on neutrophil superoxide-production. Because this report is rather confusing concerning the exact IgG-concentration used (0.625 mg/ml according to material and methods, 0.2 mg/ml according to the results) these experiments were repeated using various IgG-concentrations. Concomitantly the hydrogenperoxide production was measured. Furthermore, we extended our investigation to rheumatoid arthritis patients. Neutrophils were isolated from the peripheral blood and synovial fluid of rheumatoid arthritis patients. The results were compared with experiments conducted with neutrophils isolated from normal healthy persons.

Materials and methods

Subjects

Thirteen patients with classical or definite rheumatoid arthritis were studied. Their diagnoses were made according to the American Rheumatism Association criteria (23). (Age: mean 52, range 21-79; Sex: 7 ♀♀ and 6 ♂♂). Controls were 14 healthy volunteers matched for age and sex.

Chemicals

All chemicals were obtained, unless otherwise indicated, from Merck AG, Darmstadt, Federal Republic of Germany.

Immunoglobulin-preparations

Human IgG was isolated from the pooled sera of healthy donors by precipitation with 50 % saturated ammonium sulphate in phosphate buffered saline pH 7.4 (PBS) followed, after dissolution in PBS, by another precipitation with 33% saturated

ammonium sulphate. The precipitate was dissolved in 50 mM Tris-HCl pH 8.0 and dialyzed extensively against the same buffer. The IgG was finally purified by passage over a DEAE-cellulose (Whatman, Maidstone, England) column equilibrated with 50 mM Tris-HCl pH 8.0. The IgG was collected in the excluded fractions and extensively dialyzed against Hanks' balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM $Na_2HPO_4.2H_2O$, 0.8 mM $MgSO_4.7H_2O$, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$ and 5.5 mM glucose, pH 7.4). Heat-aggregated IgG (HAG) was obtained by heating at 63 °C for 20 min. To remove insoluble heat-aggregates the preparation was centrifuged at 1500 g for 15 min.
Human IgG at a concentration of 5 mg/ml was exposed to oxygen free radicals by ultraviolet-irradiation. Three ml-vol were irradiated for 2 hours in matched quartz cuvettes, 1 cm^2 in cross section, at a distance of 6 cm from the light source. The light source at 254 nm was a sterilAir G-9 bulb and at 366 nm a S.Y.L.V.A.NIA F8T5/BLB. Monomer UV-irradiated IgG (monomer IgG) and polymer UV-irradiated IgG (polymer IgG) were obtained by gelfiltration. Gelfiltration chromatography of ultraviolet irradiated IgG was performed on a Sepharose S300 (Pharmacia, Uppsala, Sweden)-column (70 x 1.5 cm) at a flow rate of 20 ml/hour. A 6 ml sample of 2 hours ultraviolet-irradiated IgG was applied onto the column. HBSS was used as elution buffer. Fractions of 4 min were collected. The absorbance of the effluent was monitored at 280 nm. The volume at which monomer IgG eluted from the column was determined in separate experiments. The fractions containing monomer IgG were pooled. Fractions containing the aggregated IgG, i.e. fractions that showed absorbance at 280 nm and eluted in front of the monomer IgG peak, were also pooled.

PMN-isolation

Heparinized blood and synovial fluid, were diluted 1:1 with PBS, layered on a cushion of Lymphoprep (Nycomed AS, Torshov, Norway) and centrifuged (30 min, 400 g) to separate neutrophils and erythrocytes from mononuclear leucocytes and platelets. Erythrocytes were lysed with 0.155 M NH_4Cl in 0.01 M $NaHCO_3$ and 0.1 mM EDTA, pH 7.4. If required synovial fluid cell-suspensions were filtered through two layers of gauze to remove unwanted debris. The cells were subsequently washed twice with HBSS. Final cell suspensions contained >95% viable cells and were adjusted to 1.0 x 10^7 granulocytes/ml and kept at 4 °C until use. The relative numbers of monocytes, lymphocytes and granulocytes were calculated from the total numbers of leucocytes and differential counts in May-Grünwald Giemsa-stained smears of the isolated PMN-fractions. PMN-fractions isolated from peripheral blood of normal healthy controls contained 92.7 ± 4.5 % neutrophils, 1.2 ± 0.8 % lymphocytes, 0.1 ± 0.3 % monocytes and 6.0 ± 4.2 % eosinophils; PMN-fractions from rheumatoid patients peripheral blood contained 94.5 ± 2.6 % neutrophils, 2.8 ± 1.8 % lymphocytes, 0.5 ± 0.8 % monocytes and 2.2 ± 1.4 eosinophils; PMN-fractions from synovial fluid typically consisted of 85.9 ± 14.3 % neutrophils, 13.1 ± 14.1 % lymphocytes, 0.4 ± 0.9 % monocytes and 0.6 ± 1.0 % eosinophils. Percentages are expressed as means ± SD of 9 controls and 13 RA-patients.

Superoxide anion assay

Generation of superoxide by neutrophils was determined by measuring the SOD-inhibitable reduction of ferricytochrome C (Janssen Chimica, Beerse, Belgium). The incubation mixtures (total volume 1 ml), in 16.0 x 100 mm test tubes, containing in final concentration 10^6 PMN/ml and 150 μM cytochrome C were preincubated at 37 °C under constant stirring. After 5 min the stimulus in 200 μl HBSS was added. As stimuli were used 10 ng/ml phorbol myristate acetate (PMA), 1.25 mg/ml serum treated zymosan (STZ), native IgG, heat aggregated IgG (HAG), monomer UV-irradiated IgG (monomer IgG), polymer UV-irradiated IgG (polymer IgG) and bovine serum albumin (BSA). All stimuli were used in the assay at a final concentration of 0.2 mg/ml unless indicated otherwise. As control 200 μl HBSS was added as stimulus. At 0, 5, 10, 15 and 30 min after addition of the stimulus 125 μl aliquots were taken and diluted in 0.5 ml PBS placed on ice. The samples were then centrifuged for 5 min at 500 g and the absorption at 550 nm was measured. The superoxide-production was calculated from the increase of absorption by using an extinction-coefficient of 21,000 litre mol^{-1} cm^{-1} for cytochrome C. Control experiments showed that cytochrome C reduction was inhibited (>90%) by the addition of 10 μg/ml superoxide dismutase (SOD; Boehringer Mannheim, Federal Republic of Germany), indicating that the reaction was specific for superoxide anion. Cytochrome C reduction in reaction mixtures without cells was also measured and found not to be significantly occurring with any of the reagents.

Hydrogenperoxide assay

Production of H_2O_2 by neutrophils was determined, parallel with the superoxide assay, by measuring the oxidation of the fluorescent scopoletin to a non-fluorescent product. The final concentrations used were 10^6 PMN/ml, 15 μM scopoletin (Fluka AG, Buchs, Switzerland) and 2500 Units/ml horse radish peroxidase (HRP; Boehringer Mannheim, Federal Republic of Germany). Preparations were incubated at 37 °C in 16.0 x 100 mm test tubes under constant stirring. The stimuli used were the same as described under the superoxide anion assay. At 0, 5, 10, 15 and 30 min after addition of the stimulus 125 μl aliquots were taken and diluted in 0.375 ml PBS placed on ice. The samples were then centrifuged at 500 g for 5 min. The extinction of scopoletin fluorescence in the supernatant was measured with the excitation wavelength of 350 nm and the emission wavelength of 460 nm. The amount of H_2O_2-produced was standardized by the use of known amounts of H_2O_2. Control experiments indicated that >90% of the loss in fluorescence observed in response to monomer IgG and polymer IgG was abolished by addition of 30,000 units of catalase and 100% of the loss in fluorescence was abolished by omission of HRP, indicating that the reaction was specific for hydrogenperoxide.

Effect of deoxyglucose on superoxide- and hydrogenperoxide-production

The glycolysis inhibitor 2-deoxyglucose (2-DOG; Sigma Chemical Co., St.Louis, USA) was added to PMN-suspensions of normal healthy controls in a final concentration of

25 mM. The cell-suspensions were then incubated at 37 °C for 5 min before stimulus addition

Statistical Analysis

Data are presented as the means ± SEM.
The data obtained in the dose-response experiments were tested for statistical differences using the paired t-test.
All other data were statistically evaluated by analysis of variance (ANOVA). Because the data were not always normally distributed (as determined by Bartlett's test for equal variances) a non-parametric rank Bonferroni t-test was used to test for significant differences. Significance was considered at $p<0.05$.

Results

Dose-response effect of IgG-stimuli on oxygen radical production

The effect of the various IgG stimuli (i.e. native IgG, monomer IgG, polymer IgG and HAG) on superoxide- and hydrogenperoxide-production by PMN was obtained from healthy subjects. The superoxide- and hydrogenperoxide-production as relative to dose of IgG-stimuli are shown in fig.VII.1. From this it can be seen that after 30 min incubation there is no statistically significant effect on superoxide-production by any of the used stimuli as compared to non-stimulated controls. Neither is there any difference in response on the stimuli used, nor is there any effect of increasing the stimulus concentration from 0.1 mg/ml to 0.6 mg/ml. The results of the hydrogenperoxide determinations show a totally different pattern. Evidently each IgG-stimulus gives a significant increase of the hydrogenperoxide-production by PMN as compared to non-stimulated controls. Moreover, the oxygen free radical altered IgG-stimuli (i.e. monomer IgG and polymer IgG) induce an even greater H_2O_2-production than native IgG and HAG. Also there is a marked dose-response effect on H_2O_2-production when PMN are stimulated with monomer IgG or polymer IgG. The highest concentration of monomer IgG (0.6 mg/ml) gave a six-fold (significant) increase in H_2O_2-production compared to control incubations. The same concentration polymer IgG induced a five-fold (significant) increase. Native IgG and HAG induced respectively a approximately two-fold and three-fold increase, independent of the concentration used. From these data it can be seen that 0.2 mg/ml of all IgG-stimuli is sufficient to induce a significant hydrogenperoxide-peroxide production clearly distinguishable from control levels. None of the concentrations used had an effect on superoxide-production. Therefore, 0.2 mg/ml of the various IgG-stimuli seemed appropriate to test for differences in H_2O_2-production by PMN isolated from normal subjects and RA-patients. The superoxide-production by PMN from these groups was also determined using this concentration.

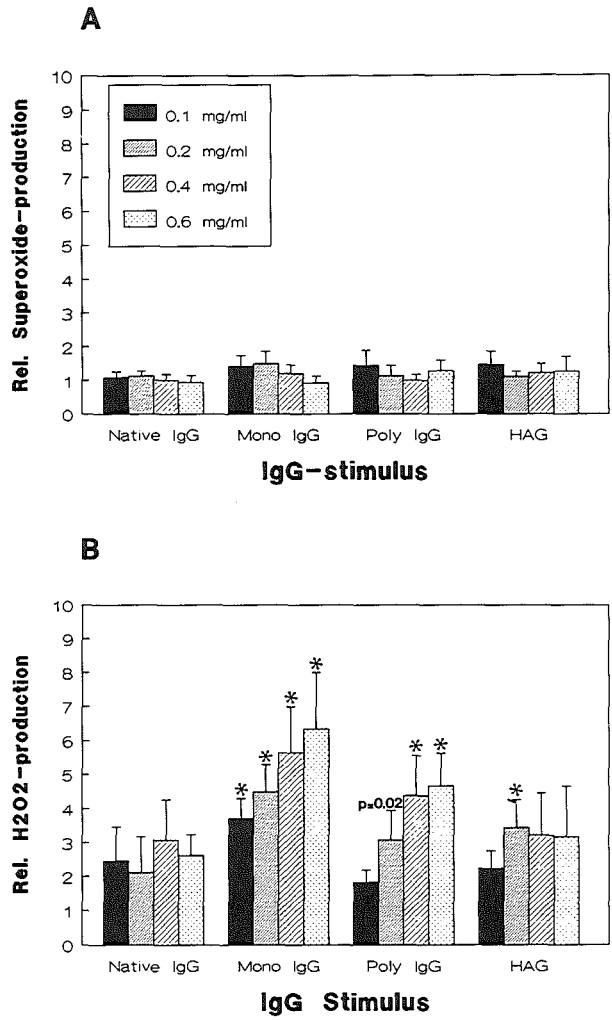

Figure VII.1: *The relative superoxide-production (A) and hydrogenperoxide-production (B) by polymorphonuclear leucocytes (PMN). Cells from normal human subjects were stimulated either with native IgG, monomer UV-irradiated IgG (Mono IgG), polymer UV-irradiated IgG (Poly IgG) or heat-aggregated IgG (HAG).*
The results are presented as the mean ratio (± SEM) over the non-stimulated control values. The height of the bars depict the means of at least 4 experiments in which cytochrome C reduction and scopoletin-fluorescence extinction were determined as described in materials and methods.
Significant differences with non-stimulated cells as determined by the paired t-test are indicated.
**P<0.01*

Effect of various stimuli on superoxide-production by PMN from normal healthy controls and from rheumatoid arthritis patients

PMN were isolated from the peripheral blood of normal healthy volunteers and from the peripheral blood and synovial fluid of rheumatoid arthritis patients. The stimuli used were BSA, native IgG, HAG, monomer IgG and polymer IgG. The results indicate that no significant difference in O_2^--production is induced by any of the stimuli when compared to the superoxide-production by non-stimulated PMN (after 30 min 10^6 PMN had produced 13.5 ± 2.7 nmol O_2^-, n=14). Largely the same results were obtained with PMN isolated from the peripheral blood and synovial fluid of RA-patients. However there is one distinct difference, stimulation with polymer IgG induces a significant stimulation of superoxide-production by PMN from RA-patients as compared to polymer IgG-stimulated PMN from normal healthy controls. The effect being even greater with synovial fluid PMN than with peripheral blood PMN of RA-patients. These results are clearly shown in fig.VII.2.

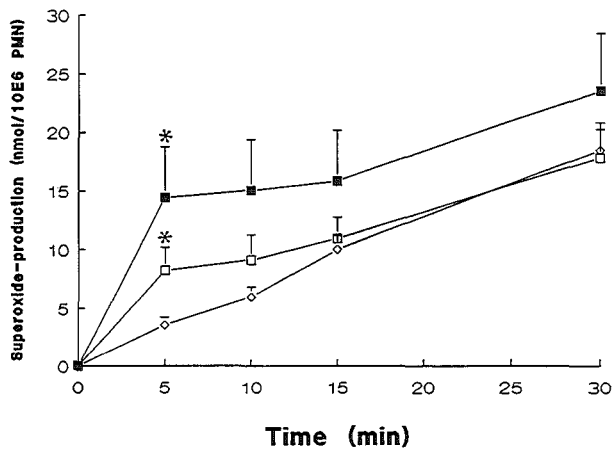

Figure VII.2: *Time course of superoxide-production by polymorphonuclear leucocytes (PMN) stimulated with 0.2 mg/ml polymer UV-irradiated IgG. Cells were isolated from normal peripheral blood (◇) and from the peripheral blood (□) and synovial fluid (■) of rheumatoid arthritis patients.*
*The results are the means (± SEM) of experiments with cells from 14 normal human subjects and 13 rheumatoid arthritis patients. Statistically significant differences between normal cells and rheumatoid arthritis patients' cells are indicated. *P<0.05*

Effect of various stimuli on hydrogenperoxide-production by PMN from normal healthy controls and from rheumatoid arthritis patients

PMN isolated from the peripheral blood of healthy controls and from the peripheral blood and synovial fluid of rheumatoid arthritis patients were assayed for there

hydrogenperoxide-production capacity. Essentially the same stimuli were used as with the superoxide-production determinations. The effects of the various stimuli on H_2O_2-production by normal PMN are shown in fig.VII.3. Basically the outcome of the dose-response experiments is confirmed by these results. Again the effect of the various IgG-stimuli on hydrogenperoxide-production is entirely different from the effects on superoxide-production. Considering the effects on H_2O_2-production when compared to non-stimulated PMN the stimuli can be divided in three: 1. BSA which induces essentially the same response as non-stimulated PMN. 2. Native IgG and HAG that induce a 2-3 fold increase in H_2O_2-production. 3. monomer IgG and polymer IgG that induce a 5-6 fold increase in H_2O_2-production. This division in stimuli can be made for each of the three groups of PMN studied. The experiments with peripheral blood neutrophils and synovial fluid neutrophils of rheumatoid arthritis patients showed identical results. From statistical analysis (ANOVA) it was found that no differences between the three groups was observed in response to any of these stimuli.

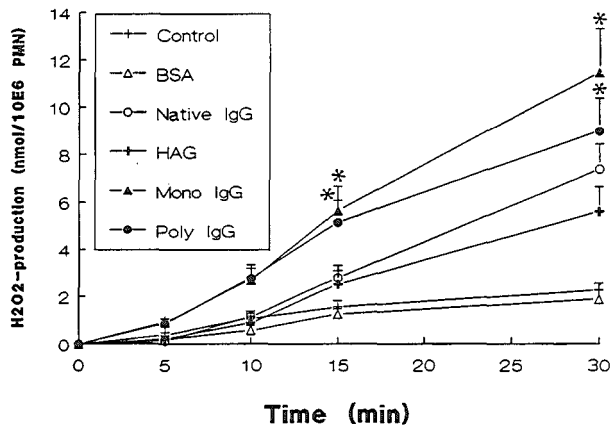

Figure VII.3: *Time course of hydrogenperoxide-production by normal human polymorphonuclear leucocytes (PMN). Cells were incubated either with native IgG, monomer UV-irradiated IgG (Mono IgG), polymer UV-irradiated IgG (Poly IgG), heat-aggregated IgG (IgG) or bovine serum albumin (BSA). The concentration of each stimulus was 0.2 mg/ml. Non-stimulated cells served as controls.*
*The results are expressed as the means (± SEM) of 14 separate experiments. Significant differences with non-stimulated controls are indicated. *P<0.05*

Effects of PMA and STZ

We also determined the effect of phorbol myristate acetate (PMA) and serum treated zymosan (STZ) on the superoxide- and hydrogenperoxide-production by PMN isolated from normal peripheral blood and from peripheral blood and synovial fluid of RA-

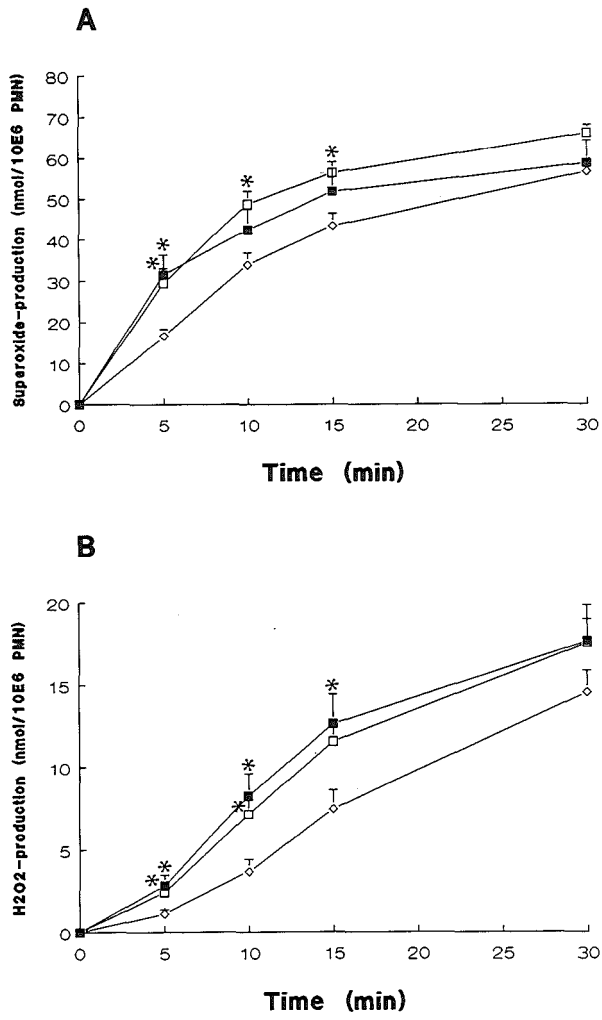

Figure VII.4: *Time course of superoxide-production (A) and hydrogenperoxide-production (B) of polymorphonuclear leucocytes (PMN) stimulated with serum treated zymosan (STZ). PMN were isolated from normal peripheral blood (◇) and from the peripheral blood (□) and synovial fluid (■) of rheumatoid arthritis patients.*
The results are expressed as the means (± SEM) of experiments with PMN from 14 healthy human volunteers and 13 rheumatoid arthritis patients. Statistically significant differences between rheumatoid arthritis patients' cells and normal cells are indicated.
$*P < 0.05$

patients. No difference between the three groups was found in response to stimulation by PMA neither in superoxide-production nor in hydrogenperoxide-production. Interestingly, a significant stimulation of oxygen radical-production was observed with STZ-stimulated PMN from RA-patients as compared to STZ-stimulated normal PMN (fig.VII.4). This was particularly evident during the first 15 min of incubation.

Effect of 2-DOG on the oxygen radical production by normal PMN

Deoxyglucose is a well known inhibitor of neutrophil glycolysis and depresses the respiratory burst (24). Incubation of PMN from normal subjects with 25 mM 2-DOG resulted in a complete inhibition of superoxide- and hydrogenperoxide production. None of the stimuli used, including PMA and STZ, was able to overcome this inhibition. A similar effect was obtained when PMN were incubated in HBSS without glucose. These results indicate that superoxide- and hydrogenperoxide-production are glucose-dependent.

Discussion

Oxygen free radical production by PMN is an important defence against microbial infections, these reduction products of molecular oxygen are also thought to play key roles in some pathological conditions including rheumatoid arthritis. Modification of immunoglobulin G by these reactive oxygen metabolites is the possible etiological cause for rheumatoid factor production. To assess the role of immunoglobulin G modification in the activation of the respiratory burst, normal human neutrophils were incubated with several forms of IgG and the oxygen free radical production was measured and compared with the effects on PMN from rheumatoid arthritis patients. All forms of IgG tested, i.e. native IgG, heat aggregated IgG (HAG), monomer oxygen free radical altered IgG (monomer IgG) and polymer oxygen free radical altered IgG (polymer IgG) had no effect on the superoxide-production by normal human PMN. However, they significantly activated the hydrogenperoxide-production. Moreover, the oxygen free radical altered forms did so in a dose-dependent fashion. These observations indicated that hydrogenperoxide-production by PMN can be stimulated independently of superoxide-production. These findings are of considerable importance since until now the general consensus was that all hydrogenperoxide produced by neutrophils was derived from the superoxide anion. Either by spontaneous dismutation or by enzyme-catalyzed (SOD) dismutation. Here it was found that hydrogenperoxide-production was independent of superoxide-production. One explanation could be that IgG acts as a dismutase, but this must be rejected since no decrease in superoxide-production was observed in IgG-stimulated PMN as compared to non-stimulated controls. Neither did we found a decrease in superoxide-production when PMN were stimulated concurrent with IgG (any form) and PMA compared to stimulation with PMA alone (results not shown). These latter experiments point to a direct production of hydrogenperoxide without intermediate superoxide production. Direct divalent reduction of molecular oxygen to hydrogenperoxide is very well possible as can be seen with glucose-oxidase and other enzymes. Direct production of hydrogenperoxide is of

pathophysiological importance, since superoxide scavenging mechanisms are bypassed in this way and while hydrogenperoxide is able to modify proteins in the presence of transition metals (25,26). Hydroxyl radicals, generated by the so-called Fenton-reaction (1), are in this respect the modifying agents.
To compare the effects of the different IgG-stimuli on PMN from normal human subjects with the effects on PMN isolated from RA-patients, one concentration of stimuli was used (0.2 mg/ml). Basically the same results were obtained with each of the three groups. Hardly any effect of IgG-stimulation on superoxide-production was found, and again, confirming the earlier findings, monomer IgG and polymer IgG stimulated the hydrogenperoxide-production of PMN (in all three groups). Native IgG and HAG did so too, but to a lesser extent. These results, together with the findings of the dose-response experiments, strongly point to an independent mechanism by which superoxide and hydrogenperoxide are produced by PMN.
Experiments with 2-DOG and experiments without glucose designed to block the NADPH-supply showed a complete inhibition of oxygen free radical production. Therefore it seems likely that both superoxide and hydrogenperoxide are formed through the NADPH-dependent oxidase-system, which could be stimulated to produce either superoxide or hydrogenperoxide separately. The NADPH-oxidase system is a membrane-associated enzyme complex that is perceived to participate directly in the production of oxygen free radicals during the respiratory burst upon activation by an adequate stimulus. The complete structure of this oxidase has yet to be completely clarified. The exact mechanism by which the NADPH-oxidase comes in the activated state is also unknown. Most evidence (however indirect) support the idea that activation of the respiratory burst is mediated through protein-kinase C (stimulated by diacylglycerol and Ca^{2+}) mediated phosphorylation of a 47-kDa protein (25). However, it was found that the stimulation of neutrophils with STZ is not completely blocked by a protein kinase C-inhibitor (26). This suggests an alternative activation pathway, distinct from the pathway involving protein kinase C, possibly this occurs via arachidonic acid formation (27). Although in the present study no conclusive evidence is presented concerning the identity of the activating mechanism(s) involved, the results clearly show distinct effects of oxygen free radical altered IgG on free radical production by PMN strongly suggesting differential activation, which could explain the finding of superoxide-independent production of hydrogenperoxide.
Apart from the mechanistic differences found in response to stimulation with the various IgG-preparations, interesting differences between PMN from normal and RA-patients were observed. Most pronounced was the difference in effect of polymer IgG-stimulation on the superoxide-production by PMN of each group. RA peripheral blood PMN produced larger amounts of superoxide then did peripheral blood PMN from normal human subjects. The stimulatory effect on PMN from synovial fluid was even greater. This difference in response to polymer IgG probably reflects a difference in "priming" between the PMN. Enhancement of neutrophils' response known as "priming" is observed when cells are pretreated *in vitro* for a short period with substimulatory doses of FMLP (10,28), PMA (29), LPS (30) or with other more physiological stimuli (8,17). This "priming" could have taken place in the RA *in vivo* situation, leading to an increased *in vitro* response to polymer IgG. From the (May-Grünwald Giemsa stained) smears of the isolated PMN fractions it was concluded that isolated RA synovial fluid

cell-preparations contained significant higher amounts lymphocytes than the other isolated PMN fractions. During the isolation procedure these lymphocytes obviously were spun down together with the neutrophils. Their apparent higher than usual weight is probably a consequence of their activated state (31). It therefore seems plausible that products (lymphokines) shed by activated lymphocytes are responsible for the "priming" of synovial fluid neutrophils. It was not attempted to characterize the factors responsible for this phenomenon. The effect of polymer IgG on the H_2O_2-production of RA peripheral blood PMN and synovial fluid PMN is not different from the effect on PMN from normal human subjects. This again points to an independent triggering of superoxide- and hydrogenperoxide-production.
The enhanced oxygen free radical production (both superoxide and hydrogenperoxide) by PMN from RA-patients upon stimulation with STZ is further evidence of increased responsiveness of RA-PMN confirming the "primed" status of these neutrophils. The finding that STZ-stimulation of both peripheral blood PMN and synovial fluid PMN from RA-patients show enhanced radical production as compared to normal PMN, points to a pathogenetic rather than an inflammatory causative mechanism.
As assessed by gelfiltration chromatography the soluble aggregates derived by UV-irradiation (polymer IgG) and by heat aggregation (HAG) are approximately of the same size ($\geq 10^6$ dalton). Therefore the observed difference in capacity between polymer IgG and HAG to induce superoxide- and hydrogenperoxide-production can be attributed solely to structural differences between these protein aggregates. This also accounts for the differential effects of native IgG and monomer IgG. The finding that monomer IgG is as good as (or even better) a stimulator as polymer IgG stresses the stimulatory capacity of oxygen free radical altered IgG in addition to the effect of aggregated IgG. This phenomenon extends the findings of Lunec *et al* (23) who pointed to the self-perpetuating mechanism of oxygen free radical damage to IgG. These researchers found that monomer UV-irradiated IgG and polymer UV-irradiated IgG stimulated the superoxide-production by normal human PMN, they did not measure the hydrogen-peroxide-production. In our study no stimulatory effect was found on the superoxide-production, probably because lower IgG-concentrations were used, but more important these low concentrations were enough to promote hydrogenperoxide-production. At this point it seems appropriate to adopt the suggestion made by Lunec *et al* (23) as to the mode of induction of the respiratory burst by neutrophils by these forms of IgG. This states that in the case of monomer IgG the binding to the cell occurs probably through Fc-receptors on the cell-membrane. For this to happen efficiently the IgG-molecule needs to denature or unfold. Aggregation could enhance this reaction, at least until either the aggregation is such as to conformationally restrict Fc-binding, or, cause saturation of receptors that in turn reduces the availability of the receptors as a trigger for free radical production. It has recently been shown that the 40 kD Fc-receptor (FcRII) on human neutrophils is essential for the IgG induced activation of the NADPH-system, and that the other of the two Fc-receptors on human neutrophils, the FcRIII, has a function in the binding (32). Especially binding of small IgG-complexes is FcRIII-dependent, which concomittantly increases the affinity for FcRII. Here we postulate that the increased stimulatory effect of free radical altered IgG on hydrogenperoxide-production is caused by increased binding to FcRIII and subsequent increased affinity for FcRII, which finally through

specific signal transduction leads to increased H_2O_2-production.
In conclusion the data presented here reveal that a stimulation of hydrogenperoxide-production can be established without stimulation of superoxide-production. This can be achieved by activating PMN with low concentrations of oxygen free radical altered IgG. The molecular basis of the effects of oxygen free radical altered IgG on the PMN respiratory burst can be at present only be a matter of speculation. Modifications of receptors, alterations of transducing system(s) involved in stimulus-response coupling or the kinetic properties of the NADPH-oxidase system can all be involved. Furthermore it was found that PMN isolated from peripheral blood of RA-patients and from synovial fluid of RA-patients showed an higher response to polymer IgG (only superoxide) and STZ compared to PMN from normal human subjects. These differences are probably due to altered activation states of PMN derived from RA-patients.
These results show the modulation capacities of oxygen free radical altered IgG, which could induce clearly distinguished responses in rheumatoid arthritis patients. Further experimentation should provide a clearer insight of the mechanisms behind these phenomena.

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EIGHT

Proliferative responses of rheumatoid arthritis synovial fluid lymphocytes to oxygen free radical altered immunoglobulin G.

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Submitted for publication.

CHAPTER VIII

PROLIFERATIVE RESPONSES OF RHEUMATOID ARTHRITIS SYNOVIAL FLUID LYMPHOCYTES TO OXYGEN FREE RADICAL ALTERED IMMUNOGLOBULIN G

Summary

Immune responses to various antigens were assessed in eight rheumatoid arthritis patients, three juvenile rheumatoid arthritis patients, five osteoarthritis patient, one septic arthritis and a normal healthy control. The antigens used were native IgG, monomer oxygen free radical altered IgG, polymer oxygen free radical altered IgG and heat aggregated IgG. The synovial fluid lymphocytes of rheumatoid arthritis patients differed from the other groups in having a significantly higher response to polymer oxygen free radical altered IgG. The observation that the groups did not differ in their responses to the other antigenic stimuli tested indicates that reactivity of the rheumatoid arthritis synovial fluid lymphocytes was specific.
These results strengthen the hypothesis that oxygen free radical altered and aggregated IgG is relevant to the etiopathogenesis of rheumatoid arthritis.

Introduction

Rheumatoid factor (RF) production is an essential feature of rheumatoid arthritis. RFs are auto-antibodies directed to antigenic determinants of the Fc region of IgG and are found in the majority of adult patients. Any hypothesis concerning the etiology of RA must take into account the production of RFs. In order to explain this phenomenon, it has been suggested that the auto-antigen must have been altered in such a way that it is no longer seen as a "self"-structure. It has been shown by our group and others that oxygen free radicals could alter and modify IgG. The changes induced on immunoglobulin G include sulphydryl-group formation (1), amino acid alterations (2,3), generation of a new specific fluorescence (4) and lead to aggregation (3). The hypothesis that oxygen free radical altered IgG could be the auto-antigen in rheumatoid arthritis was strengthened by our observation that oxygen free radical altered IgG binds with high affinity to the rheumatoid factor (5). Further studies showed that oxygen free radical altered IgG stimulates the hydrogenperoxide-production by PMN. Moreover, oxygen free radical aggregated IgG specifically stimulates the superoxide-production by PMN isolated from rheumatoid arthritis patients (6).
The results of the studies on the interactions of oxygen free radical altered IgG with rheumatoid factors and the non-specific defence-system led us to extend our studies to interactions with the immune-system. Therefore we investigated the reactivity of RA-lymphocytes to oxygen free radical altered IgG. Peripheral blood and synovial fluid lymphocytes of patients with RA or other joint diseases (osteoarthritis, juvenile RA and

septic arthritis) and peripheral blood lymphocytes of controls were tested in an *in vitro* proliferation assay for their response to native IgG, monomer oxygen free radical altered IgG, polymer oxygen free radical altered IgG and heat aggregated IgG.

Materials and methods

Subjects

Seven patients (4 females/3 males) with RA defined by the American Rheumatism Association criteria (7) were studied. They were 51.1 ± 14.1 years (mean ± SD) of age. Six patients were treated with non-steroidal anti-inflammatory drugs; two were treated with D-penicillamine; and three had received injections with gold. Patients who used prednisolone and/or methotrexate were excluded.
Ten patients (5 females/5 males) with other conditions were also studied. They were 39.5 ± 20.9 years (mean ± SD) of age. They served as controls and included 3 patients with juvenile RA (JRA), 5 patients with osteoarthritis (OA), one had a septic arthritis (TBC) and one was a normal control (of this subject only peripheral blood mononuclear cells were tested).

Immunoglobulin isolation and purification

Human IgG was isolated from the pooled sera of healthy donors by precipitation with 50 % saturated ammonium sulphate in phosphate buffered saline pH 7.4 (PBS) followed, after dissolution in PBS, by another precipitation with 33 % saturated ammonium sulphate. The precipitate was dissolved in 50 mM Tris-HCl pH 8.0 The IgG was collected in the excluded fractions and extensively dialyzed against Hanks' balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM $Na_2HPO_4 \cdot 2H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$ and 5.5 mM glucose, pH 7.4). Heat-aggregated IgG (HAG) was obtained by heating at 63 °C for 20 min. To remove insoluble heat-aggregates the preparation was centrifuged at 1500 G for 15 min. Human IgG at a concentration of 5 mg/ml was exposed to oxygen free radicals by ultraviolet-irradiation. Three ml-vols were irradiated for 2 hours in matched quartz cuvettes, 1 cm^2 in cross section, at a distance of 6 cm from the light source. The light source at 254 nm was a steriLair G-9 bulb and at 366 nm a S.Y.L.V.A.NIA F8T5/BLB. Monomer UV-irradiated IgG (monomer IgG) and polymer UV-irradiated IgG (polymer IgG) were obtained by gelfiltration. Gelfiltration chromatography of ultraviolet irradiated IgG was performed on a Sepharose S300 (Pharmacia, Uppsala, Sweden)-column (70 x 1.5 cm) at a flow rate of 20 ml/hour. A 6 ml sample of 2 hours ultraviolet-irradiated IgG was applied onto the column. HBSS was used as elution buffer. Fractions of 4 min were collected. The absorbance of the effluent was monitored at 280 nm. The volume at which monomer IgG eluted from the column was determined in separate experiments. The fractions containing the aggregated IgG, i.e. fractions that showed absorbance at 280 nm and eluted in front of the monomer peak, were also pooled.

Proliferation assay

Peripheral blood or synovial fluid mononuclear cells were separated from fresh (heparinized) blood or synovial fluid on Lymphoprep gradients (Nycomed, Pharmachemie, Haarlem, The Netherlands). Proliferative responses of these cells were measured in round-bottomed microtitre plates (Costar, Cambridge, UK) in triplicate wells. Each well contained 1 x 10^5 mononuclear cells suspended in 0.2 ml RPMI-1640 medium (Gibco, Breda, The Netherlands) supplemented with 15 % normal human AB-serum obtained from a single pool of healthy blood donors, 10 mmol Hepes buffer (Sigma), 2 mmol glutamine, penicillin (100 U/ml) and streptomycin (10 μg/ml) in the presence or absence of antigens (native IgG, monomer IgG, polymer IgG and HAG at 10 μg/ml). Cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air for 6 days to test proliferative response to antigens. For the last 16 h of incubation each well was pulsed with 0.2 μCi of {^{3}H}-thymidine (specific activity 2 Ci/mmol; Amersham, Buckinghamshire, UK). Cultures were harvested on fiberglass filters and thymidine incorporation was measured in a liquid scintillation counter (Packard, Brussels, Belgium). Results were expressed as a stimulation index (ratio of mean cpm with antigen/mean cpm without antigen).

Statistical analysis

Statistical calculations were performed on a IBM-compatible personal computer with the use of a standard statistical package (Stata 2.0). Comparisons between groups were made with the Wilcoxon Rank-sum test (parameters did not appear to be normally distributed). The effects of stimuli within groups were compared using the Wilcoxon Signed-Ranks test. P values $\leq$0.05 were considered significant. Results are presented as mean ± SEM.

Results

Proliferative responses of control lymphocytes

Figure VIII.1A illustrates the individual stimulation indices of peripheral blood lymphocytes of the controls to the four forms of IgG. The controls are presented in three groups: a juvenile rheumatoid arthritis group of 3 patients (of one patient only synovial fluid lymphocytes were tested), an osteoarthritis group of 5 patients and the last group is formed by a septic arthritis patient and a normal healthy volunteer. The responses to each form of IgG was generally the same, and did not differ significantly from the response to medium alone. The mean stimulation indices (± SEM) for native IgG, monomer IgG, polymer IgG and HAG were, respectively: 1.45 ± 0.27, 1.36 ± 0.24, 1.20 ± 0.25 and 1.14 ± 0.16.
Figure VIII.1B shows the individual stimulation indices of synovial fluid lymphocytes of the control patients group. From 6 controls (3 JRA, 2 OA and 1 septic arthritis) the synovial fluid lymphocytes were tested. The responses to four forms of IgG are presented. No significant differences between the responses to the four IgG forms

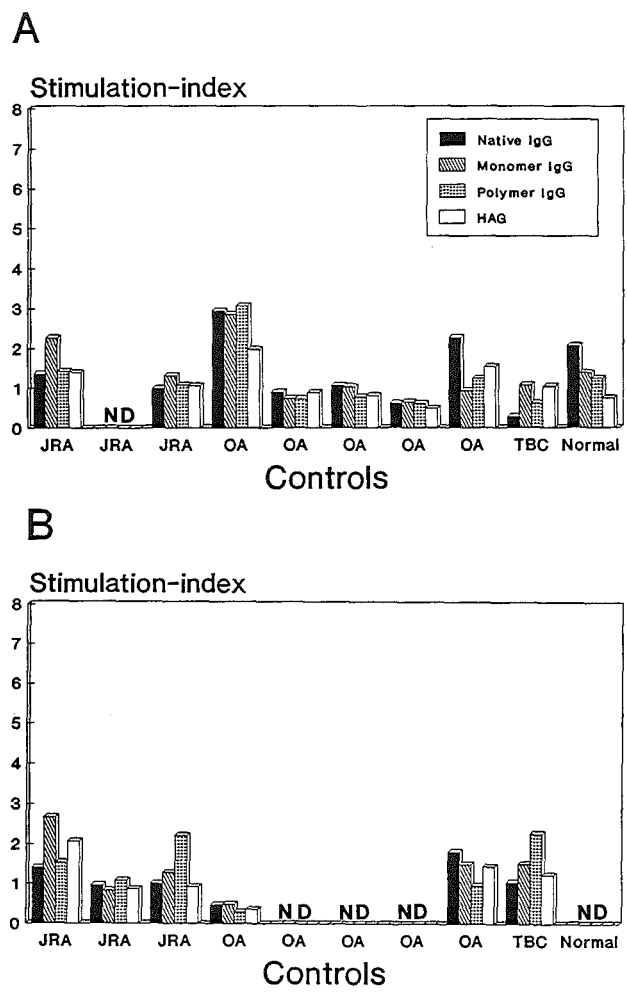

Figure VIII.1: *Proliferative responses of control peripheral blood (A) and synovial fluid (B) lymphocytes to various antigenic stimuli. The antigens used were native IgG, monomer oxygen free radical altered IgG (monomer IgG), polymer oxygen free radical altered IgG (polymer IgG) and heat aggregated IgG (HAG). The controls were divided into three groups: 1. three juvenile rheumatoid arthritis patients (JRA), 2. five osteoarthritis patients (OA) and 3. a septic arthritis patient (TBC) plus a normal healthy control. Results are expressed as a stimulation index: ratio of mean cpm with antigen over mean cpm without antigen (ND = not done).*

was observed. The mean stimulation indices (±SEM) were for native IgG: 1.12 ± 0.19, for monomer IgG: 1.38 ± 0.31, for polymer IgG: 1.39 ± 0.32 and for HAG this was 1.15 ± 0.24.

Proliferative responses of rheumatoid arthritis lymphocytes

The proliferative responses of peripheral blood lymphocytes of RA-patients to the four forms of IgG are shown in fig.VIII.2A. The lymphocytes of 7 RA-patients were studied. The responses were not strikingly different from those of the control peripheral blood lymphocytes. No statistically significant differences in responses to any of the antigenic stimuli were observed. The mean stimulation indices (± SEM) for native IgG, monomer IgG, polymer IgG and HAG were: 1.07 ± 0.11, 1.03 ± 0.14, 1.09 ± 0.17 and 1.15 ± 0.21, respectively.
The proliferative responses of synovial fluid lymphocytes from 5 RA-patients are illustrated in fig.VIII.2B. Again the same four IgG stimuli were used. The mean stimulation indices (± SEM) were, for native IgG: 0.97 ± 0.20, for monomer IgG: 1.44 ± 0.28, for polymer IgG: 3.17 ± 1.15 and for HAG: 1.00 ± 0.11.
The response of RA synovial fluid lymphocytes to polymer IgG was significantly higher than to native IgG ($P=0.04$). The response to polymer IgG was also higher than to monomer IgG and HAG, but in both cases this was not statistically significant (both: $P=0.08$).

Responses to polymer oxygen free radical altered IgG

In fig.VIII.3 the proliferative responses of peripheral blood lymphocytes and synovial fluid lymphocytes from controls and rheumatoid arthritis patients to polymer oxygen free radical altered IgG are shown. It appeared that the proliferative response of RA synovial fluid lymphocytes was strikingly higher than the response of RA peripheral blood lymphocytes ($P=0.03$) and the response of control peripheral blood lymphocytes ($P=0.03$). The response of control synovial fluid lymphocytes was lower than the response of RA synovial fluid lymphocytes although not statistically significant ($P=0.10$).

Ratio of response of synovial fluid lymphocytes/peripheral blood lymphocytes

To assess differences between peripheral blood lymphocytes and synovial fluid lymphocytes within each patient, the mean ratio of responses of synovial fluid lymphocytes and the corresponding peripheral blood lymphocytes from controls and rheumatoid arthritis patients were calculated. The ratio of responses to stimulation with the 4 forms of IgG are listed in table VIII.1. Stimulation of RA-lymphocytes with polymer oxygen free radical altered IgG resulted in a significantly higher ratio than when RA-lymphocytes were stimulated with native IgG ($P=0.04$). Furthermore, the ratio of polymer IgG stimulated RA lymphocytes was also significantly higher than the corresponding ratio of HAG stimulation ($P=0.04$).
Moreover, when compared to control lymphocytes, stimulation of RA lymphocytes with polymer IgG gave a significantly higher ratio than stimulation with native IgG ($P=0.02$),

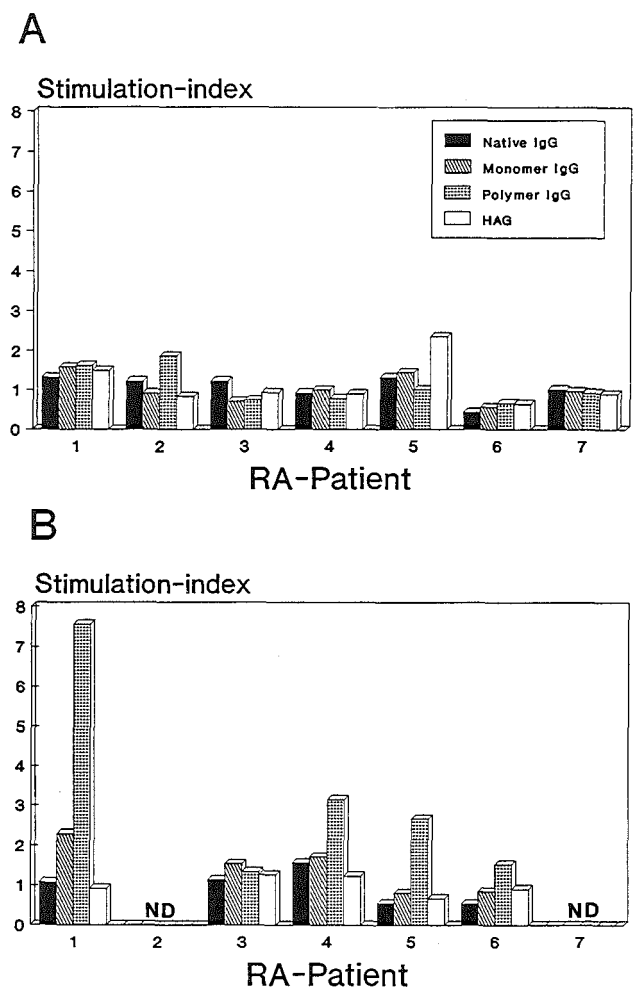

Figure VIII.2: *Proliferative responses of rheumatoid arthritis peripheral blood (A) and synovial fluid (B) lymphocytes to various antigenic stimuli. The antigens used were native IgG, monomer oxygen free radical altered IgG (monomer IgG), polymer oxygen free radical altered IgG (polymer IgG) and heat aggregated IgG (HAG). Eight rheumatoid arthritis patients were studied, of two patients no synovial fluid lymphocytes could be tested. Results are expressed as a stimulation index: ratio of mean cpm with antigen over mean cpm without antigen (ND = not done).*

monomer IgG (P=0.05) and HAG (P=0.02). The ratio of polymer IgG stimulation of control lymphocytes was not significantly lower (P=0.11).

Table VIII.1:*Ratio of responses of synovial fluid lymphocytes over peripheral blood lymphocytes isolated from the same patient. Five RA-patients and 5 controls were studied. The responses to native IgG, monomer IgG, polymer IgG and HAG are shown. Results are expressed as the means ± SEM. Significant statistical differences are reported in the text.*

Stimulus	Patients	
	Controls	RA
Native IgG	1.00 ± 0.32	1.99 ± 0.69
Mono IgG	1.46 ± 0.71	3.49 ± 1.74
Poly IgG	1.62 ± 0.62	6.09 ± 2.33
HAG	1.03 ± 0.37	2.27 ± 1.11

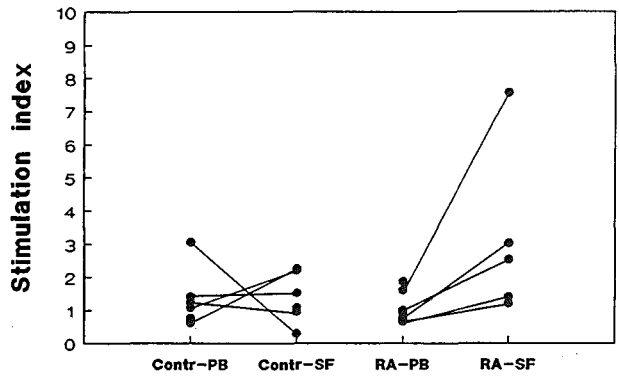

Figure VIII.3: *Stimulation index of proliferative responses of lymphocytes to polymer oxygen free radical altered IgG. Lymphocytes of four groups were studied: 1. control peripheral blood lymphocytes (Contr-PB), 2. control synovial fluid lymphocytes (Contr-SF), 3. rheumatoid arthritis peripheral blood lymphocytes (RA-PB) and 4. rheumatoid arthritis synovial fluid lymphocytes (RA-SF). Lines connect results obtained with patients whose peripheral blood and synovial fluid lymphocytes were tested simultaneously.*

Discussion

Rheumatoid arthritis is a systemic autoimmune disease that is characterized by genetic predisposition, unknown etiology, production of auto-antibodies and chronic joint inflammation. The most prominent immunologic features of RA are RF - immune-complexes related phenomena and proliferative immune-responses. Identification of the factors that induce disregulation of immune responses leading to auto-immune reactions gives important clues to the etiology of RA.
Immune responses are mediated by lymphocytes. The proliferative responses of these cells upon antigenic stimulation were studied. As mentioned earlier the occurrence of antiglobulins (RF) should be accounted for in any etiological mechanism for RA, therefore altered IgG was used as stimulus. The results of the proliferative assays showed a specific response of RA synovial fluid lymphocytes to polymer free radical altered IgG. In contrast, lymphocytes derived from RA peripheral blood and controls showed no significant proliferation to any of the stimuli used. Although the degree of stimulation within the RA-group varies, the observations clearly indicate that synovial fluid of the RA-patients studied contains a subset of T lymphocytes which is absent in peripheral blood and controls.
It is feasible that the presence of such altered IgG-specific T lymphocytes in the synovial fluid are indicative for the actual *in vivo* antigenic stimulus. As can be seen from the experiments with HAG and monomer oxygen free radical altered IgG, respectively, neither aggregation nor oxygen free radical induced alteration alone is capable to cause proliferation. The combination of aggregation and alteration seems to be an absolute requirement. Polymer oxygen free radical altered IgG could therefore be a candidate for the antigenic stimulus in the RA-synovial fluid. But also immune-complexes containing monomer altered IgG meet these requirements and should therefore too be included in the possible *in vivo* antigenic stimuli candidates.
The existence of altered IgG-specific T-lymphocytes in the synovial fluid of RA-patients offers interesting possibilities. Culturing of T-lymphocytes isolated from RA-synovial fluid in the presence of altered IgG specifically triggers certain T-lymphocytes. The relevant T-cell clone could then be isolated and further characterized. It would also be possible to employ this clone in identifying the *in vivo* antigen itself.
The concept that oxygen free radical altered IgG could be the primary antigen in RA was demonstrated by the fact that in synovial fluid immunoglobulin G could be detected with a specific fluorescence (8). This fluorescence was different from the native fluorescence and indistinguishable from that caused by UV-irradiation or free radical producing PMN. The results presented here are not only consistent with this finding but also point to the crucial role oxygen free radical altered IgG plays in the rheumatoid immune response.

Acknowledgements

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NINE

General discussion.

CHAPTER IX

GENERAL DISCUSSION

IX.1 Effect of oxygen free radicals on IgG-structure

In this study low-intensity ultraviolet irradiation (at 254 nm and 366 nm) was used to generate oxygen free radicals. This well established system (48-50) is highly reproducible and has the advantage over the use of activated PMN that only free radicals are generated.
Exposure of human immunoglobulin G to UV-irradiation resulted in several structural modifications. Typically it leads to the generation of a new specific fluorescence and the concomitant loss of the native fluorescence (Chapter III). This phenomenon is not specific for IgG but is also seen with other proteins exposed to oxygen free radical generating mechanisms (51,52).
It was also demonstrated that after oxygen free radical attack sulphydryl-groups on IgG had increased. This reflects the extreme sensitivity of the sulfur containing amino acid cysteine/cystine to oxygen free radical damage, usually leading to a destruction of the disulphide-bridges (53,54).
Because of the reported lower sialic acid (and galactose) content of IgG in patients with rheumatoid arthritis when compared to osteoarthritis patients and normal healthy controls (55), it was interesting to see whether this could be due to oxygen free radical activity. This was of particular interest since it has been reported that autologous asialo-IgG is arthritogenic when injected intra-articular in the rabbit (56). However, the effect of oxygen free radicals on the carbohydrate-chain of IgG was not uniform. The determination of terminal sialic acids on the carbohydrate chain of oxygen free radical altered IgG gave varying results. Sometimes a decrease was observed but with other IgG-badges no effect on sialic acid content was measured. Probably this reflects the variation in the glycosylation pattern of IgG.
Interesting was the observation that the heavy chains of IgG were more susceptible to oxygen free radical attack than the light chains. Particularly because the heavy chains contain the sites (C_H2- and C_H3-domain) that interact with rheumatoid factors (57). The results suggest a possible oxygen free radical mediated alteration on IgG, which subsequently could induce rheumatoid factor production. Whether oxygen free radical altered IgG interacts with rheumatoid factors will be discussed below (Chapter VI).
Another effect of UV-irradiation is aggregation of the IgG-molecules. Gelfiltration of two hours UV-irradiated IgG was used to separate the monomeric IgG from the aggregated polymeric IgG. As can be seen from the analysis of the aggregated IgG complexes, the IgG-molecules are bound together by reduction-resistent covalent cross-links. These covalent bonds not only link the polypeptide IgG-molecules but also the constituting heavy and light chains within one IgG-molecule (Chapter IV). Clearly this aggregation mechanism is different from heat-aggregation as heat-aggregated IgG is completely reduced by a reducing agent (ß-mercaptoethanol) (Chapter III). The

formation of oxygen free radical induced covalently cross-linked IgG *in vivo* could lead to the production of stable complexes consisting of aggregated IgG in association with IgM-rheumatoid factors. This mechanism may also play a role in the self-associating complexes of IgG and IgG-rheumatoid factors.
The amino acid determinations performed on HCl-hydrolysed polymer oxygen free radical altered IgG revealed a decrease of the tyrosine- and histidine-content as compared to native IgG (Chapter IV). This was confirmed by further analyses of mild-acid hydrolysates of polymer oxygen free radical altered IgG (Chapter V). The latter method gave additional information on the effect of oxygen free radical attack on the tryptophan-content of IgG, as this aromatic amino acid is destroyed by HCl-hydrolysis. Furthermore, mild acid hydrolysis gave more reliable results on the cystine-content. In summary, the analyses showed that oxygen free radical attack on IgG resulted in a decrease in the content of the aromatic amino acids tyrosine and tryptophan, of the heterocyclic amino acid histidine and of the sulfur-containing amino acid cystine. These oxidative modifications are not limited to IgG, but have been observed in a variety of other proteins, including bovine serum albumin, transferrin and hemoglobin, although the amino acids affected and the extent of damage varied (58-62). These processes are discussed more thoroughly in Chapter II.
Especially the decrease of the tryptophan-content in oxygen free radical attacked IgG is interesting, since this aromatic amino acid dominates the native fluorescence of proteins. Therefore, the loss of native fluorescence and the generation of a new specific fluorescence after exposure of IgG to oxygen free radicals is a direct consequence of the modifications induced on tryptophan. That tryptophan plays an important role in oxygen free radical induced damage was confirmed by the tryptic peptide-maps of altered IgG. When compared to the peptide-maps of native IgG it was evident that because of oxygen free radical activity a different peptide-mapping resulted. Moreover, the newly formed peptides contained aromatic amino acids. These findings suggest an important role of these amino acid residues in the formation of these peptides. Taken together, the results of the amino acid analyses and the peptide-mappings support the idea that tryptophan, tyrosine and histidine are involved in the formation of covalent cross-links. Possible cross-linking mechanisms are dityrosine formation (63,64) and couplings involving tryptophan (Trp-Trp, Trp-Tyr and Trp-His) and histidine (His-His and His-Tyr) (65,66).

IX.2 Effect of oxygen free radicals on IgG-function

2.1 Rheumatoid factor

In Chapter VI the effect of oxygen free radicals on some functional properties of IgG were determined. The ability of rheumatoid factors to bind to oxygen free radical altered IgG was investigated. Inhibition studies, using a competitive radio immuno assay, of the binding of ^{125}I-heat aggregated IgG to rheumatoid factor-coated sepharose beads showed that oxygen free radical altered IgG binds with high affinity to rheumatoid factor. Comparison with native monomeric IgG revealed that oxygen free radical altered IgG was more competitive with heat aggregated IgG for binding to

rheumatoid factor. These findings suggest that rheumatoid factor can react specifically with IgG modified by oxygen free radicals.

2.2 Complement

Another function of IgG is the binding of complement components and subsequent activation of the complement system. The question was raised whether oxygen free radical altered IgG has this capability. Binding studies to ^{125}I-C1q proved that oxygen free radical altered IgG could bind the first component of the complement system. It was also shown by determining the C3a-generation that oxygen free radical altered IgG could activate the complement system.
The presence of large numbers of PMN, immune complexes, rheumatoid factors and depressed levels of complement components (sign of increased consumption) are the main characteristics of inflamed rheumatoid joints (67,68). The results presented in Chapter VI add more evidence to the hypothesis that oxygen free radical damage to IgG plays an essential role in the chronicity of this inflammatory reaction in RA.

IX.3 Interaction with the non-specific defence system

Oxygen free radical production by PMN and macrophages has a physiological function in destroying invading microorganisms as part of the non-specific defence. Excessive free radical production by activated PMN, resulting in tissue damage has been implicated in the etiopathogenesis of RA (69). How PMN are triggered *in vivo* is not well understood. Lunec *et al* (49) suggested a self-perpetuating mechanism involving oxygen free radical altered IgG. To study this in more detail we determined the superoxide- and hydrogenperoxide-production of PMN isolated from normal subjects and RA-patients using oxygen free radical altered IgG as stimulus (Chapter VII). RA-PMN showed a significant increase in superoxide-production when stimulated by polymer oxygen free radical altered IgG when compared to PMN from normal subjects. Furthermore, stimulation of RA-PMN with serum treated zymosan enhanced the superoxide- as well as the hydrogenperoxide-production to a larger extent than it did with the radical-production of PMN from normal subjects. The latter suggests that PMN isolated from peripheral blood and synovial fluid of RA-patients have been potentiated ("primed") in the *in vivo* situation.
The studies also showed that hydrogenperoxide-production by neutrophils could be stimulated independently from superoxide-production with oxygen free radical altered IgG. The possible mechanism behind this phenomenon could involve receptor-modifications, alterations in the transducing system(s) or the kinetics of the NADPH-oxidase system. The direct stimulation of hydrogenperoxide-production is of pathophysiological importance, since mechanisms scavenging superoxide are bypassed in this manner and while hydrogenperoxide, through metal-catalyzed production of hydroxyl radicals (70), is able to modify proteins (59,71).

IX.4 Interactions with the immune system

The former studies led us to extend our investigations to the interactions with the specific defence system. The reactivity of oxygen free radical altered IgG with lymphocytes isolated from RA-patients and appropriate controls was determined (Chapter VIII). Peripheral blood and synovial fluid lymphocytes of patients with RA and other joint diseases were tested in an *in vitro* proliferation assay for their response to oxygen free radical altered IgG. The lymphocytes isolated from the synovial fluid of RA-patients showed significantly higher proliferative responses to polymer oxygen free radical altered IgG than lymphocytes from the other groups. Other antigenic stimuli, including heat aggregated IgG, gave no differences in response (usually no response at all) between the groups, indicating that reactivity of the RA synovial fluid lymphocytes to polymer oxygen free radical altered IgG was specific. Moreover, the presence of an oxygen free radical altered IgG-specific T lymphocyte-subset in the synovial fluid of RA patients not only indicates the presence of oxygen free radical altered IgG *in situ*, but also points to the crucial role it plays in the rheumatoid immune response.

IX.5 Concluding remarks

Several studies have shown proof for the involvement of oxygen free radicals in the etiopathogenesis of RA. Lipid peroxidation products like malondialdehyde, typical of free radical attack on membranes, have been found in the sera and synovial fluid of RA-patients (72,73). Also lipid peroxidation products of higher molecular weight were detected in synovial fluid of RA-patients (74,75). The presence of depolymerized low molecular weight hyaluronic acid in RA showed oxygen free radical caused degradation of hyaluronic acid and consequently reduced viscosity (76). Further evidence comes from the observations that in RA and in experimentally-induced arthritis in animals, superoxide dismutase (SOD), a superoxide destroying enzyme, given systemically or locally, induces a decrease of inflammation (77,78).
In RA synovial fluid, activated PMN are the source of oxygen free radicals. It has been shown *in vitro* that exposure of IgG to oxygen free radicals generated by activated PMN induces the formation of a specific fluorescence. It was also shown that an IgG-containing gelfiltration fraction of RA synovial fluid possessed the same specific fluorescence (79). Earlier studies had already reported, using circular dichroism spectral analyses, that IgG from patients with RA differ in conformation from normal IgG (80). These findings support the hypothesis that oxygen free radicals could induce neo-antigens on IgG, promoting rheumatoid factor production, and show the presence of altered IgG in RA. The results presented in this thesis give additional information on the nature of the oxygen free radical induced modifications of IgG and extend the evidence on the involvement of oxygen free radical altered IgG in the inflammatory and immune response in RA.

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SUMMARY

Oxygen free radicals

Oxygen has always been considered beneficial and an absolute requirement for all respiring species. However, in recent years increasing evidence has been gathered which points to a different role of oxygen. The recognition of oxygen toxicity came with the notion that oxygen free radicals are potential hazardous oxygen metabolites, which play a role in a variety of pathological conditions.
Oxygen free radicals are also involved in the pathogenesis of rheumatoid arthritis (RA). RA is characterized by chronic synovitis, during which polymorphonuclear leucocytes (PMN) are attracted to the joints, thereby producing large amounts of oxygen free radicals. This can lead to tissue damage and destruction of the joint.
The etiological factor responsible for the onset of RA is still unknown. Oxygen free radicals production has been implicated as one of the possible etiological factors. This thesis focusses on the possible role of oxygen free radicals in the etiopathogenesis of RA.

Rheumatoid factors

Most RA-patients have rheumatoid factors (RF) in their sera and synovial fluid. RF are antibodies directed against a patients own immunoglobulin G (IgG). Therefore, they are called auto-antibodies, and RA is known as an auto-immune disease. The existence of RF means that IgG has been altered in such a way that it is no longer recognized by the immune-system as "self". The hypothesis postulated in this thesis says that oxygen free radicals modify IgG to such an extend that it is no longer recognized as "self" and induces RF-production.

IgG-alteration

Chapters I and II deal with the central focus points of this thesis: immunoglobulin G, rheumatoid arthritis and oxygen free radicals. In Chapter II extra attention is given to oxygen free radical damage to proteins with special reference to proteins in RA.
Characteristics of oxygen free radical damage to IgG-structure are described in Chapter III. We used ultraviolet-irradiation as an oxygen free radical generating system. The modifications induced on IgG encompassed an increase in SH-groups, induction of a new specific fluorescence and aggregation of IgG-molecules. Furthermore, it was shown that the IgG heavy chains were more susceptible to oxygen free radical damage than the light chains. This is of particular importance since rheumatoid factor reactivity is located on the constant domains of the IgG heavy chains.
In Chapter IV the oxygen free radical induced IgG-aggregates were studied. It was found that the IgG-molecules were connected by inter- and intra-molecular covalent

cross-links. Amino acid analyses showed significantly decreased amounts of tyrosine- and histidine-residues in oxygen free radical altered IgG. The results clearly point to the involvement of tyrosine and histidine in the formation of covalent cross-links. In Chapter III it is indicated that possibly dityrosine-formation takes place as a consequence of free radical activity.
The role of tryptophan in oxygen free radical altered IgG has also been studied (Chapter V). As tryptophan dominates protein-fluorescence it was clear that the induction of a new fluorescence, after free radical attack on IgG, indicated an important role of tryptophan. Model-studies in which tryptophan was irradiated with UV-light revealed the destruction of the indole-ring resulting in the formation of 'N-formyl-kynunerine en kynunerine. These results confirmed the fluorescence- and structure-modifying capacity of oxygen free radicals. Amino acid analysis of oxygen free radical altered IgG after mild-acid hydrolysis ascertained the sensitivity of tryptophan. The analyses showed a significant decrease in tryptophan-, tyrosine-, histidine- and cysteine/cystine-content when compared to native IgG. Tryptic peptide-mapping of oxygen free radical altered IgG showed and confirmed the involvement of the aromatic amino acids in the formation of covalent cross-links.

IgG-function

The effects of oxygen free radicals on the functional properties of IgG were also studied, the results of this study are presented in Chapter VI. The complement-binding and complement-activating effector function of IgG as well as the RF-binding capacity were studied. It was found that oxygen free radical altered IgG binds the first complement component (C1q-binding) and that the complement system could be activated (C3a-generation). The study also showed that altered IgG binds to RF with higher affinity than native IgG.

Interaction with PMN

Chapter VII deals with the interaction between oxygen free radical altered IgG and polymorphonuclear leucocytes (PMN). This was of importance since, as mentioned before, stimulated PMN produce large amounts of oxygen free radicals. Incubation of aggregated oxygen free radical altered IgG with RA-PMN leads to a stimulation of the superoxide(O_2^-)- and hydrogenperoxide(H_2O_2)-production. Furthermore, it was shown that H_2O_2-production by PMN could be stimulated by oxygen free radical altered IgG, independent from O_2^--production. These results show a self-perpetuating mechanism of free radical production and IgG-modification, in which superoxide scavenging systems are bypassed.

Interaction with lymphocytes

Immune responses to various antigens were assessed, the results are described in

Chapter VIII. Immune responses are mediated by B-lymphocytes (humoral response) and T-lymphocytes (cellular response). After stimulation with specific antigen these cells will proliferate. Lymphocytes from RA-patients and controls were studied for their proliferative responses to, among others, oxygen free radical altered IgG. RA synovial fluid lymphocytes showed a clearly higher response after antigenic stimulation with aggregated oxygen free radical altered IgG than lymphocytes from other groups. The groups did not differ in their responses to the other stimuli, indicating that the response of synovial fluid lymphocytes to altered IgG was specific.

Conclusions

The studies described in this thesis show that oxygen free radicals can alter IgG and they indicate the nature of these alterations. It was shown that oxygen free radical altered IgG binds and activates complement, and that it binds RF with high affinity. A self-perpetuating mechanism of oxygen free radical damage, bypassing superoxide scavenging systems, was reported. Together these results indicate that oxygen free radical damage to IgG plays an essential role in the chronicity of the inflammatory response in RA. The generation of oxygen free radicals by PMN leads to IgG modification and aggregation. This leads to perpetuation of PMN-activation and immunoglobulin-production. This in turn will, sustained by complement activation, increase phagocytosis and liberation of chemotactic stimuli. Whether oxygen free radical altered IgG is responsible for RF-production is still the question.
The results presented in Chapter VIII indicate the presence of oxygen free radical altered IgG-specific T-lymphocytes in the synovial fluid of RA-patients. These results point to a crucial role of oxygen free radical altered IgG in the immune response. Further studies are necessary to elucidate more about this role. It would be interesting to isolate and culture this specific T cell-clone and employ it to identify the *in vivo* antigen itself.

SAMENVATTING

Vrije zuurstofradicalen

Naast de een ieder bekende noodzakelijkheid van zuurstof (O_2) voor de ademhaling is in de laatste jaren meer en meer onderkend dat O_2 ook toxisch kan zijn en een rol speelt in de pathogenese van diverse ziektebeelden. De toxiciteit van zuurstof is gerelateerd aan de vorming van vrije zuurstofradicalen.
Ook in reumatoïde artritis (RA) is aangetoond dat zuurstofradicalen een rol spelen in de pathogenese. RA wordt gekenmerkt door langdurige ontstekingen van de gewrichten. In de ontstoken gewrichten produceren polymorphnucleaire leukocyten (PMN) grote hoeveelheden zuurstofradicalen, die het gewricht en in de gewrichtsholte grote schade kunnen berokkenen.
De factor(en) die RA veroorzaakt is nog steeds niet bekend. Een van de mogelijke factoren die genoemd worden is de vorming van vrije zuurstofradicalen. Het onderzoek beschreven in dit proefschrift richt zich met name op de rol van zuurstofradicalen in RA.

Reumafactoren

RA wordt serologisch gekenmerkt door de aanwezigheid van reumafactoren (RF). Dit zijn antilichamen gericht tegen het eigen antilichaam immuunglobuline G (IgG), ze worden daarom ook auto-antilichamen genoemd. RA staat derhalve bekend als een auto-immuunziekte. Het herkennen van een lichaamseigen stof als lichaamsvreemd houdt in dat er een verandering aan die stof moet zijn opgetreden. De hypothese beschreven in dit proefschrift stelt dat vrije zuurstofradicalen het lichaamseigen IgG zodanig veranderen dat het niet langer als "eigen" wordt gezien en RF-productie wordt geïnduceerd.

Veranderingen aan IgG

De inleidende Hoofdstukken I en II beschrijven uitvoerig de centrale onderwerpen van dit proefschrift: het immuunglobuline G, reumatoïde artritis en zuurstofradicalen. In het tweede hoofdstuk wordt tevens nader ingegaan op het effect van vrije zuurstofradicalen op eiwit-structuur en -functie, waarbij de nadruk ligt op eiwitten in RA. Karakteristieken van zuurstofradicaal schade aan de structuur van IgG worden in Hoofdstuk III behandeld. Als zuurstofradicaal-genererend systeem werd bestraling met ultraviolet licht gebruikt. De modificaties aan IgG, geïnduceerd door O_2-radicalen, omvatten een toename van de SH-groepen, inductie van een specifieke fluorescentie en aggregatie van IgG-moleculen. Tevens werd aangetoond dat de zware ketens van IgG gevoeliger zijn voor zuurstofradicaal schade dan de lichte ketens. Dit laatste is met name van belang aangezien RF-activiteit is gelocaliseerd op gedeeltes van de zware

ketens van het IgG-molecuul.
In Hoofdstuk IV wordt beschreven dat de door zuurstofradicalen activiteit gevormde IgG-aggregaten samengesteld zijn uit covalent verbonden IgG-moleculen. Zowel binnen het IgG-molecuul zelf als tussen IgG-moleculen onderling werden verbindingen aangetoond. Aminozuur analyses toonden dat het door O_2-radicalen veranderde IgG significant verminderde hoeveelheden van de aminozuren tyrosine en histidine bevat. Deze resultaten duiden erop dat tyrosine en histidine betrokken zijn bij de vorming van de covalente bindingen. In Hoofdstuk III worden aanwijzingen gegeven dat mogelijk dityrosine-vorming hierbij een rol speelt.
Het belang van tryptofaan in het door zuurstofradicalen veranderde IgG werd nader onderzocht. De resultaten hiervan staan beschreven in Hoofdstuk V. Dat tryptofaan een belangrijke rol speelt is af te leiden uit de vorming van de nieuwe specifieke fluorescentie. Eiwit-fluorescentie wordt namelijk gedomineerd door tryptofaan-fluorescentie, derhalve duidt verandering van eiwit-fluorescentie op een verandering in tryptofaan.
Model-studies waarbij tryptofaan werd blootgesteld aan UV-bestraling, resulteerde in destructie van de indol-ring leidend tot de vorming van N-formyl-kynunerine en kynunerine. Dit geeft de fluorescentie- en structuur-veranderende capaciteit van O_2-radicalen duidelijk aan. Aminozuur analyses van mildzure hydrolysaten van het door O_2-radicalen veranderde IgG bevestigden het effect op tryptofaan. De analyses toonden een significante afname in zowel tryptofaan-inhoud als in tyrosine-, histidine- en cysteine/cystine-inhoud ten opzichte van natief IgG. De analyses van de peptiden verkregen door trypsinisaties lieten zien dat de aromatische aminozuren betrokken zijn bij de door zuurstofradicalen geïnduceerde veranderingen. Deze resultaten duiden eveneens op een rol van tryptofaan en tyrosine in de vorming van covalente bindingen in het door O_2-radicalen veranderde IgG.

IgG-functie

In dit stadium was het van belang te onderzoeken wat de implicaties voor de functionele eigenschappen van IgG waren. Eén van de effector functies van IgG is de interactie met complement; dit is een (cascade-)systeem van serumeiwitten dat na activatie lijdt tot de lysis van de (vreemde of geinfecteerde) cel, waaraan complement is gebonden. Hoofdstuk VI beschrijft de effecten van O_2-radicalen op de complement-bindings en -activatie functie van IgG, ook het effect op de RF-binding wordt hier beschreven. Het door zuurstofradicalen veranderde IgG bleek zowel complement te binden (C1q-binding) als te activeren (C3a-generatie). Er werd een radio-immuno assay (RIA) ontwikkeld waarmee de reactiviteit van veranderd IgG met RF kon worden getest. Uit proeven met RF van verschillende RA-patiënten bleek dat het veranderde IgG een hogere affiniteit voor RF heeft dan natief IgG.

Interactie met PMN

In Hoofdstuk VII wordt ingegaan op de interactie tussen veranderd IgG en PMN. Dit

is van belang omdat, zoals reeds eerder gezegd, PMN grote hoeveelheden O_2^--radicalen produceren als zij gestimuleerd worden. Incubatie van RA-PMN met geaggregeerd door O_2^--radicalen veranderd IgG leidt tot een stimulering van de superoxide (O_2^-) en waterstofperoxide (H_2O_2) productie. Tevens werd aangetoond dat H_2O_2-productie door PMN kon worden gestimuleerd met door O_2^--radicalen veranderd IgG, onafhankelijk van de O_2^--productie. Deze bevindingen tonen een zichzelf instandhoudend mechanisme van radicaal-productie en IgG-verandering aan, waarbij superoxide neutraliserende mechanismen worden omzeild.

Interactie met lymfocyten

Het achtste hoofdstuk handelt over immune responsen tegen verschillende antigenen. Immuun responsen worden gereguleerd door B-lymfocyten (antilichaam-productie) en T-lymfocyten (cellulaire respons), zij vermenigvuldigen zich na herkenning van specifiek antigeen. Lymfocyten van RA-patiënten en controle patiënten werden bestudeerd op hun proliferatieve responsen tegen o.a. zuurstofradicaal veranderd IgG. Lymfocyten uit de synoviale vloeistof van RA-patiënten vertoonden een duidelijk hogere respons na antigene stimulatie met geaggregeerd door O_2^--radicalen veranderd IgG dan lymfocyten uit de andere groepen. Het feit dat de groepen niet verschilden in hun responsen op de andere stimuli duidt erop dat we hier te maken hebben met een specifieke reactie van RA-synoviaal vloeistof lymfocyten.

Conclusies

De studies beschreven in dit proefschrift tonen dat zuurstof-radicalen IgG veranderen en geven aan wat die veranderingen zijn. Het door zuurstofradicalen veranderde IgG bindt en activeert complement en vertoont ten opzichte van natief IgG een verhoogde affiniteit voor RF. Er werd aangetoond dat in RA-patiënten aanwijzingen voor een zichzelf instandhoudend mechanisme van zuurstofradicaal schade aanwezig zijn, daarmee een mogelijke verklaring gevend voor de chroniciteit van RA. De resultaten geven aan dat door zuurstof radicalen veranderd IgG een essentiele rol speelt bij de onstekingsreactie zoals we die zien in RA. De productie van vrije zuurstof radicalen door PMN leidt tot de verandering en aggregatie van IgG. Dit leidt weer tot activering van PMN en stimulering van IgG-productie. Tenslotte neemt, gesteund door complement activatie, de fagocytose toe en komen ontstekings-mediatoren vrij. Of het door zuurstof-radicalen veranderde IgG verantwoordelijk is voor RF-productie blijft (nog) de vraag.
De resultaten beschreven in Hoofdstuk VIII duiden op de aanwezigheid van veranderd IgG specifieke T-lymfocyten in de synoviaal vloeistof van RA-patiënten. Dit wijst op een cruciale rol van het door zuurstof-radicalen veranderde IgG in de immuun respons. Voortgaand onderzoek is nodig om deze rol te verduidelijken. Het zou interessant zijn om deze specifieke T cel-kloon te isoleren en te kweken, waarna deze gebruikt zou kunnen worden om het *in vivo* antigeen te identificeren.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 25 oktober 1957 te Amersfoort. In 1976 behaalde hij het diploma Atheneum ß aan het Christelijk Lyceum te Alphen aan de Rijn. In 1978 werd begonnen met de studie Biologie aan de Rijksuniversiteit te Utrecht, waar hij in juni 1981 het kandidaatsexamen B1 behaalde. Hierna werd de studie voortgezet met het hoofdvak Vergelijkende Endocrinologie (Prof. Dr. P.G.W.M. van Oort), waaraan o.a. gedurende 8 maanden in Israel werd gewerkt. Vervolgens werd een hoofdvak Scheikundige Dierfysiologie (Prof. Dr. D.I. Zandee) bewerkt, op deze afdeling werd tevens een studenten-assistentschap vervuld.. Na het volgen van een bijvak Klinische Immunologie (Prof. Dr. R.E. Ballieux) werd in augustus 1985 het doctoraalexamen afgelegd. In oktober 1985 trad schrijver in dienst van de Erasmus Universiteit Rotterdam alwaar tot eind 1989 op de afdeling Biochemie I (Prof. Dr. J.F. Koster) dit proefschrift werd bewerkt.

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Antioxidant defence systems in rheumatoid arthritis.
Submitted for publication.