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Reactive oxygen species induce antigenic changes in DNA

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Reactive oxygen species (ROS) are released at sites of inflammation during the respiratory burst which accompanies the phagocytic process. Using an in vitro system to simulate this process we have shown that ROS induce antigenic changes in DNA. More specifically, results of experiments using ROS scavengers have shown that hydroxyl radicals produced in close proximity to DNA-bound metal ions play a predominant role. ROS-mediated attack resulted in increased binding of anti-DNA antibodies to the denatured DNA. These changes were detected using IgG, IgA and IgM isotype binding to antibodies in systemic lupus erythematosus sera. Of these the IgA isotype was most discriminating in its detection of hydroxyl radical-induced damage.

Reactive oxygen species; DNA antibody, Anti-; Systemic lupus erythematosus

1. INTRODUCTION

Chronic inflammation is a pathophysiological process characterised by infiltration of activated macrophages and polymorphonuclear leukocytes into the site of injury [1]. These cells can release potentially damaging reactive oxygen species (ROS) into the extracellular fluid during the respiratory burst and accompanying phagocytic process. Human IgG, when exposed to ROS, becomes antigenic and produces characteristic autofluorescence [2,3]. Similarly, reactivity of ROS towards DNA induces conformational change [4], base damage [5], both single- and double-strand breaks [5–7] and often the subsequent production of clastogenic factors [8]. In addition, antigenic and mutagenic changes may occur which could become incorporated into the germline genes. ROS must permeate both cellular and nuclear membranes in order to gain access to organelle-bound DNA; hydrogen peroxide is sufficiently lipophilic to do this [4].

In systemic lupus erythematosus (SLE), a chronic inflammatory disorder, the presence of antibodies to native DNA can be detected in serum samples [9]. Whilst antibodies to single-stranded DNA are found in several inflammatory conditions, including rheumatoid arthritis, antibodies to double-stranded DNA are rarely present in clinical conditions and serve as an immunochemical marker in the diagnosis of SLE [10].

We propose that the failure to recognise DNA as 'self' and the consequent production of autoantibodies in SLE may be a result of ROS attack on DNA causing changes in structure at the macromolecular level. In order to investigate this hypothesis, a series of in vitro studies was carried out to determine if ROS-damaged molecules behaved as better antigens for anti-DNA antibodies found in SLE sera.

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2. MATERIALS AND METHODS

2.1. *Materials*

DNA (type XIII, from human placenta), SOD (EC 1.15.1.1, from bovine erythrocytes), hydrogen peroxide, ascorbic acid, bovine serum albumin, lyophilised serum – positive and negative for anti-DNA antibodies, peroxidase-conjugated goat anti-human IgG, IgA and IgM, cysteine, mannitol and thiourea were all obtained from Sigma (Poole, England). Desferrioxamine was a generous gift from Ciba Pharmaceuticals (Hor-

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sham, England), microtitre plates were from Gibco (Denmark) and all buffer reagents were from BDH and of Analar grade. Distilled water was flushed slowly through a 25 cm column containing chelex resin (BDH) before use to remove contaminating metal ions from the system and all solutions were made up in this unless otherwise stated.

2.2. *Denaturation of DNA*

Double-stranded human DNA was dissolved in chelex-treated water at 2.5 mg/ml. Single-stranded DNA was prepared by immersing a sample of this stock solution in a boiling water bath for 15 min then rapidly cooling on ice. Both double- and single-stranded DNAs (0.5 mg/ml) were incubated in separate experiments with hydrogen peroxide (200 μM) and ascorbic acid (10 mM) at 37°C for 1 h. Control experiments had shown there to be sufficient transition metal ions bound to the DNA macromolecule to catalyse ROS production and no further metal ions were added to the system.

2.3. *ELISA assay*

The ELISA procedure used was baded on that described by Stokes et al. [11]. Briefly, untreated and treated DNA was bound to Nunc 96 microtitre plates at 100 μg/ml in carbonate buffer (pH 9.6). All incubation mixtures were left for 1 h at 37°C in a humidified chamber. Following 3 washes in PBS containing 0.05% Tween 20, albumin was used to block any unoccupied sites. Sera of seropositive and seronegative anti-DNA antibody controls were diluted 1:100 and added to the washed plates. Peroxidase-conjugated goat anti-human IgG, IgA and IgM were used to label IgG, IgA and IgM autoantibodies, respectively. The reaction of peroxidase with hydrogen peroxide and *o*-phenylenediamine at pH 5 was monitored spectrophotometrically at 492 nm.

2.4. *Scavenger and chelator studies*

The following free radical scavengers: cysteine (50 mM),

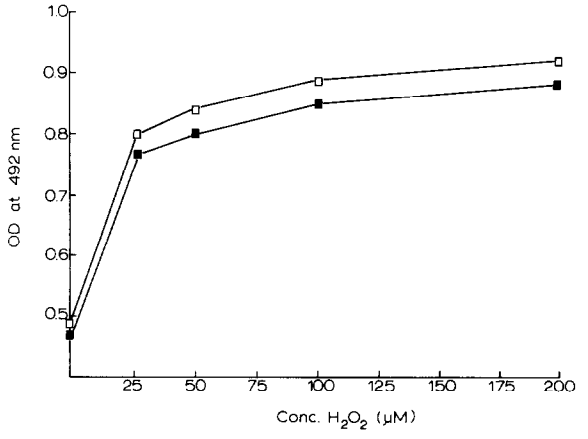

Fig.1. Dose-dependent increases in anti-DNA antibody binding to single- and double-stranded DNA. Anti-DNA antibody binding to double (■) and single (□) stranded DNA, both incubated in the presence of 10 mM ascorbic acid, was measured as a function of increasing hydrogen peroxide concentration.

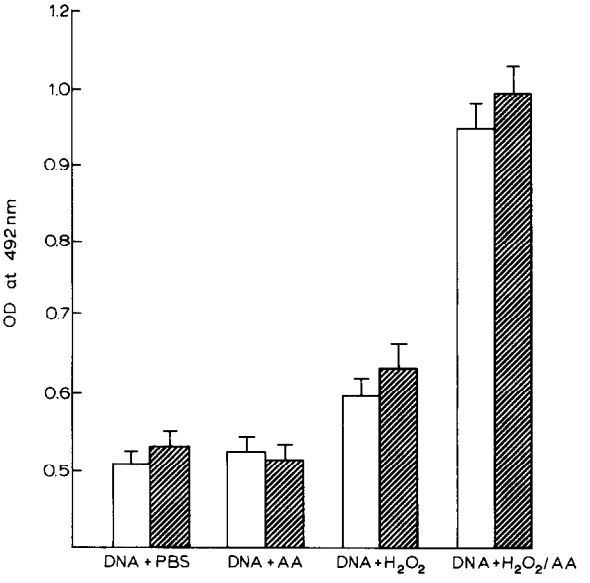

Fig.2. Effect of hydrogen peroxide and ascorbic acid on anti-DNA antibody binding. Double (□) and single (■) stranded DNAs were incubated with 10 mM ascorbic acid (AA), 200 μM hydrogen peroxide (H_2O_2) and phosphate-buffered saline (PBS). Results are means ± 1 SD for four separate experiments.

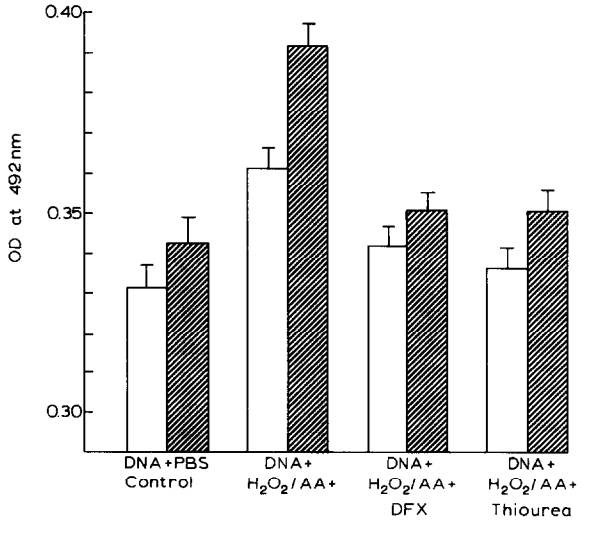

Fig.3. Effect of radical scavengers on anti-DNA antibody binding. Results are means ± 1 SD (n = 4) showing the inhibition by desferrioxamine (DFX) (0.5 mM) and thiourea (50 mM) of the ROS-induced increase in anti-DNA antibody binding to double (□) and single (■) stranded DNA.

histidine (50 mM), SOD (100 μg/ml), mannitol (50 mM) and thiourea (50 mM), and the iron chelator desferrioxamine (0.5 mM) were included to define the nature of the ROS responsible for changes induced in human DNA following its incubation with hydrogen peroxide and ascorbic acid as above.

3. RESULTS

In order to determine the effect of ROS on DNA, seropositive and seronegative serum binding to native and ROS-denatured DNA was measured. Fig.1 shows a dose-dependent increase in anti-DNA antibody binding to both double- and single-stranded DNA mediated by the combination of hydrogen peroxide and ascorbic acid: hydrogen peroxide alone was able to induce a 5-times smaller increase in binding to DNA (see fig.2). As expected, there was no significant increase in the binding of seronegative sera to single- or double-stranded DNA following denaturation by hydrogen peroxide plus ascorbic acid (not shown).

The effect of various ROS scavengers on the induction of antigenic changes to both single- and double-stranded DNA is shown in fig.3. Thiourea, a hydroxyl radical scavenger and Cu^{2+} chelator, caused inhibition in serum binding of 71% ($p < 0.001$) and 62% ($p < 0.001$) for single- and double-stranded DNA, respectively, when compared to ROS-treated DNA. Similarly, the iron chelator desferrioxamine inhibited autoantibody binding by 69% ($p < 0.001$) for single-stranded DNA and

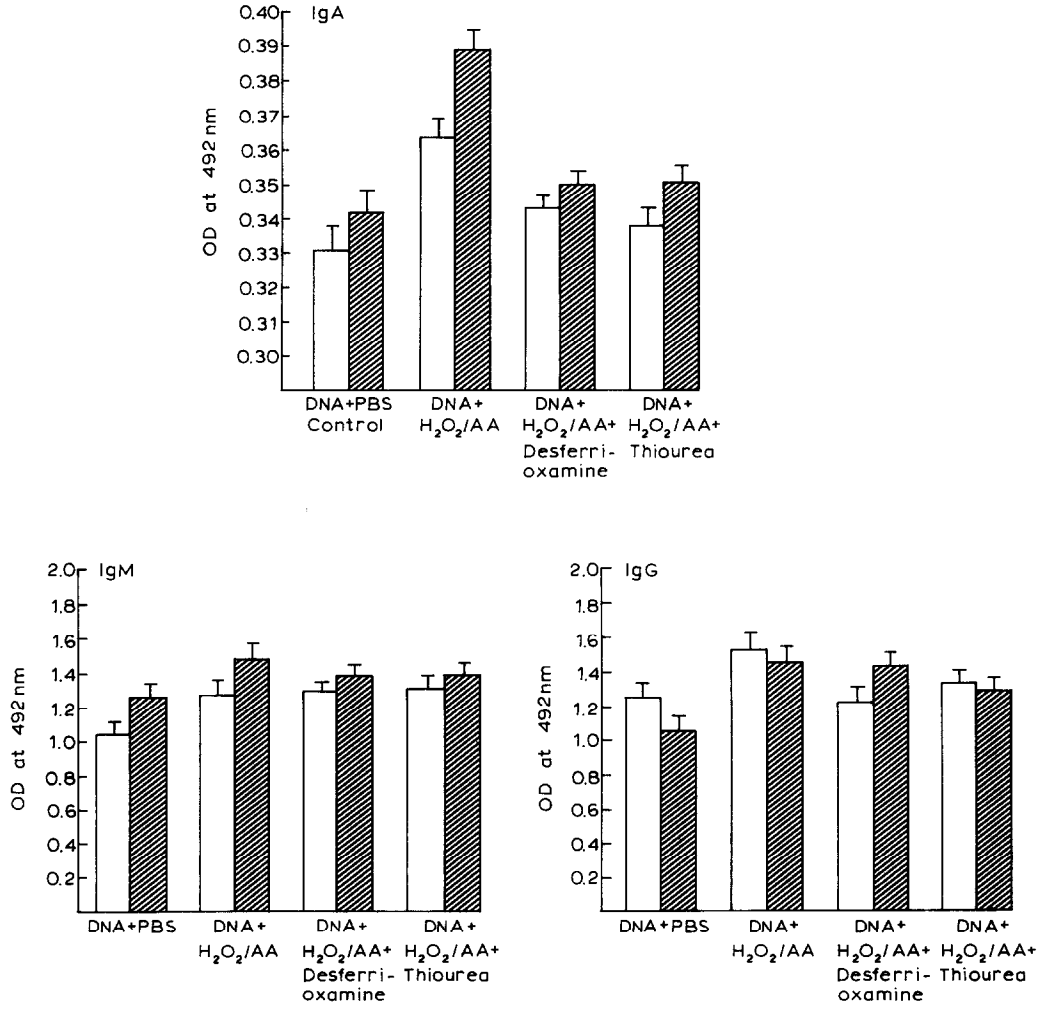

Fig.4. Differential binding of anti-DNA isotypes against reactive oxygen species denatured DNA. Binding of anti-IgG, IgM and IgA isotypes to double (□) and single (■) stranded DNA.

78% ($p < 0.01$) for double-stranded DNA. Mannitol, histidine, cysteine and SOD were unable to offer significant protection against ROS-induced damage.

Fig.4 shows the differential binding of the major isotypes of anti-DNA antibodies to ROS-denatured DNA. The IgG, IgA and IgM antibody isotypes all individually detected the increase in seropositive serum binding to ROS-denatured DNA compared to the control. The inhibition induced by the presence of desferrioxamine and thiourea, however, varied between the isotypes. For example, desferrioxamine inhibited binding of serum anti-DNA antibodies to double-stranded DNA by 20, 10 and 8% for IgG, IgA and IgM, respectively, and thiourea similarly inhibited by 14% for IgG, 10% for IgA and 3% for IgM. The decreases in absorbance for the IgM isotype were not significant for all inhibitors used; the IgG isotype was only able to detect a significant decrease for double-stranded DNA incubated with desferrioxamine. However, binding of the IgA isotype to both double- and single-stranded DNA is significantly inhibited in the presence of thiourea and desferrioxamine although the actual absorbance measurement for the IgA isotype is 4-times smaller than that for IgG.

4. DISCUSSION

Anti-DNA antibodies, such as those found typically in SLE, have a greater capacity to bind to ROS-denatured DNA than to untreated control samples. This denaturation, induced by ROS, is highly dependent on metal ions and hydrogen peroxide, since desferrioxamine and thiourea both inhibit the oxidative increase in seropositive autoantibody binding. Hydrogen peroxide reacts in the presence of a reducing agent, such as ascorbic acid, and metal ions to produce the highly reactive hydroxyl radical:

$$M^{n+} + H_2O_2 \longrightarrow M^{n+1} + OH^- + OH^{\cdot}$$

(Fenton reaction)

Substantial experimental evidence suggests that metal ions, notably copper, are bound to DNA [12–14] and stabilize the association between DNA supercoils and the non-histone matrix [15]. Copper ions, however, are susceptible to chelation by thiourea (as are iron ions) thereby preventing hydroxyl radical formation at specific sites along the macromolecule.

The chelation of metal ions from our assay necessitates that DNA-bound metal ions catalyse the Fenton reaction and thus produce hydroxyl radicals in close proximity to DNA. Scavenging of this reactive radical species will only occur if the scavenger can diffuse within 15 Å of the DNA – the distance across which a hydroxyl radical will remain active [7]. The accessibility of the metal ions on the DNA macromolecule to potential scavengers is therefore important. The failure of one hydroxyl radical scavenger, mannitol, to prevent markedly oxidative changes to DNA compared with another, thiourea, may arise because thiourea is more able to diffuse close to the DNA compared to mannitol coupled with the fact that the relative rate constants for scavenging hydroxyl radicals is 4.7-times greater for thiourea than for mannitol [16].

In conclusion, DNA denatured by ROS is a better antigen for antibodies found in SLE sera than native DNA. It is known that treatment of double-stranded DNA with the superoxide-generating system xanthine-xanthine oxidase transforms the DNA into an immunogenic state [17]. Similarly, ROS appear to increase the antigenicity of DNA. Using polyclonal antibody binding we cannot at present determine whether increased binding is related to an alteration in the base or sugar moieties individually or if antibodies are raised to a gross structural change. The use of monoclonal antibodies may reveal more information on the type of denaturation induced. However, we have shown that free radicals produced in close proximity to DNA-bound metal ions induce changes in the antigenic structure. In SLE, chronic inflammation may generate sufficient ROS to denature DNA such that an immune response is induced and autoantibodies to a 'self-DNA' are produced.

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