Volume 319, number 1,2, 66–70 FEBS 12210 March 1993

The effect of hydroxyl radical on the antigenicity of native DNA

Khurshid Alam, Asif Ali and Rashid Ali

Department of Biochemistry, Faculty of Medicine, A.M.U., Aligarh, India

Received 22 January 1993

Hydroxyl free-radical mediated in vitro modification of native calf thymus DNA showed single-strand breaks, decrease in melting temperature and structural alteration of purine and pyrimidine bases. Experimentally induced antibodies against modified DNA exhibited polyspecificity. Native DNA, RNA and synthetic polynucleotides in B-conformation were found to be an effective inhibitor of induced antibody–immunogen interaction. Naturally occurring human anti-DNA autoantibodies showed enhanced recognition of modified DNA as compared to native polymer. IgG isolated from anti-DNA antibody-positve SLE sera by Protein A-Sepharose 4B chromatography were affinity purified on nDNA-(polylysyl-Sepharose 4B) matrix. In competitive binding experiments, the affinity-isolated IgG showed almost equal recognition of both native DNA and hydroxyl radical modified DNA. The possible role of hydroxyl radicals in the etiopathogenesis of SLE has been discussed.

DNA; Hydroxyl radical; Antibody; SLE; B-conformation

1. INTRODUCTION

The monitoring of macromolecular damage to human tissues exposed to chemical substances that interfere with DNA is being pursued to explain the origin of antibodies in autoimmune disorders [1]. Oxygen free radicals have been known to cause damage to nucleic acids [2,3] and hydroxyl radicals have been implicated in the etiology of many human diseases [4]. These reactive oxygen species (ROS) are known to induce conformational changes in DNA [2] besides inducing single- and double-strand breaks [5,6]. Cellular metabolism has been known to generate reactive oxygen species like hydrogen peroxide, hydroxyl radical, singlet oxygen and hydroperoxy radicals [7]. It has been observed that reactive oxygen species-denatured DNA is a better antigen for anti-DNA antibodies found in SLE sera [8,9].

Trace metals such as copper and iron which are present in biological system might contribute to ROS-induced DNA damage [10–12]. In the presence of trace amounts of transition metal, hydrogen peroxide can participate readily in Fenton-like reactions resulting in the production of hydroxyl radicals [13].

In the present communication, the hydroxyl radical modified DNA was partially characterized and found to be highly immunogenic inducing antibodies which showed binding to native B-conformation. Moreover, naturally occurring human anti-DNA autoantibodies showed enhanced recognition of ROS-modified DNA in direct binding and inhibition ELISA.

Correspondence address: R. Ali, Department of Biochemistry, J.N. Medical College, A.M.U. Aligarh 202 002, India.

2. MATERIALS AND METHODS

2.1. *Materials*

Highly polymerized calf thymus DNA (Sigma, USA) was purified free of proteins, RNA, and single-stranded regions [14]. Normal human sera were obtained from healthy individuals. SLE sera were collected as described earlier [15].

2.2. *Modification of DNA*

The metal ions already present in DNA solution were chelated first by treatment with EDTA for 3 h at room temperature at a final concentration of 1 mmol·dm^{-3} and dialyzed extensively with 30 mmol·dm^{-3} phosphate buffer, pH 7.4 [16]. Assay tubes containing mixtures of DNA (1,515 μM bp), ferrous ions (525 μM) and hydrogen peroxide (3 030 μM) were incubated for 30 min at room temperature in 0.01 M phosphate buffer, pH 7.4. Deionized DNA with hydrogen peroxide and ferrous ions separately served as controls. At the end of incubation, reaction products were dialyzed exhaustively against PBS and their UV absorption characteristics were recorded on Shimadzu UV-240 spectrophotometer.

2.3. *Sucrose gradient centrifugation*

The damage to DNA by hydroxyl radicals was analyzed by alkaline sucrose gradient centrifugation. Native and modified DNA samples (0.1 ml each) were layered separately on top of a 4.8 ml linear 5–20% alkaline sucrose gradient containing 0.8 M NaCl, 0.2 M NaOH, 0.01 M EDTA and 0.015 M *p*-aminosalicylate at pH 12.5 [17]. The samples were centrifuged at 30 000 r.p.m. for 1 h at 20°C in an SW 50.1 rotor of a Beckman ultracentrifuge. After centrifugation, the bottom of the tubes were pierced with a needle and contents were fractionated by drop collection (0.7 ml each). The absorbance of collected fractions was monitored at 260 nm.

2.4. *Absorption-temperature scan*

Native DNA and hydroxyl radicals modified DNA were subjected to heat denaturation under identical conditions. The cuvette temperature was raised form 30°C to 95°C at a rate of 1°C/min after incubation for 10 min at 30°C. The change in absorbance at 260 nm was recorded and per cent denaturation of both the samples were evaluated [18].

Published by Elsevier Science Publishers B.V.

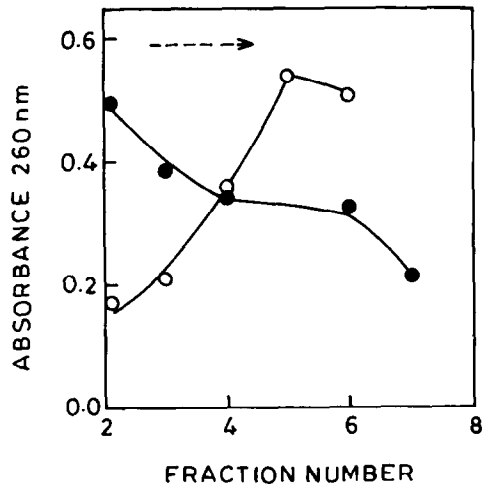

Fig.1. Profile showing sedimentation of native (-○-) and modified DNA (-●-) duplex through alkaline sucrose density gradient. The arrow on top shows the direction of sedimentation.

2.5. *DEAE Sephadex A-50 column chromatography*

The hydrolyzed native and modified DNA samples were separated on DEAE Sephadex A-50 column as described earlier [18]. Native and modified bases were identified on the basis of their unique absorption spectrum. The extent of base modification was calculated from the elution profile by measuring the peak area.

2.6. *Immunological procedures*

Female rabbits were immunized intramuscularly with 100 μg of modified DNA complexed with an equal amount of methylated BSA (MBSA) and emulsified in complete Freunds' adjuvant [19]. ELISA, on polystyrene plates, and competition–inhibition experiments were carried out as detailed elsewhere [20].

2.7. *Immunoaffinity purification of SLE anti-DNA antibodies*

The IgG isolated from SLE sera by Protein A-Sepharose 4B chromatography were affinity purified on an nDNA-(polylysyl- Sepharose 4B) column as described earlier [23]. Affinity isolated anti-nDNA IgG was used in competitive binding experiments.

3. RESULTS

It is an established fact that Fenton's reagent (hydrogen peroxide plus ferrous ions) results in the production of hydroxyl radical [11]. In the present studies, radical formation was evident by its ability to cause single strand breaks in supercoiled DNA (pSK 3 kb). As a result, the nicked circular form showed slow electrophoretic migration as compared to fast migrating supercoiled form in agarose gel electrophoresis (data not given).

Native DNA sedimentation in alkaline sucrose gradient showed a single, sharp boundary, indicating a high molecular weight species. Treatment of hydroxyl radical modified DNA under identical conditions was found to loose the sharp boundary pattern, and instead it revealed a sedimentation profile quite distinct from the one observed for native DNA (Fig. 1). The altered sedimentation profile of modified DNA indicates that the single-strand breaks are randomly situated which appeared as DNA fragments of varying size that sediment more slowly than the intact single strands of alkali-denatured native DNA.

The ultraviolet absorption scan of modified DNA showed hypochromicity at 260 nm. Temperature controlled denaturation of native and modified DNA showed a significant decrease in melting temperature (T_m) (Fig. 2). A 6.5°C decrease in melting temperature could be attributed to the local helix distortion caused by base modification and partial unstacking. The physico-chemical characteristics of native and modified duplex, has been presented in Table I.

Native and modified DNA were analyzed on DEAE Sephadex A-50 matrix after acid hydrolysis to separate and quantitate the modified bases. Column chromatography of modified DNA hydrolysate indicated ROS-induced base modification (Table II). The relative sus-

Table I

Ultraviolet and thermal denaturation characteristics of native and modified DNA under identical experimental conditions

Parameter	Native DNA	Modified DNA
Absorbance ratio (260/280)	2.20	1.95
Per cent hyperchromicity at 95°C	36.03	25.10
Melting temperature (T_m), °C	89.0	82.5
Onset of duplex melting, °C	80.0	74.0

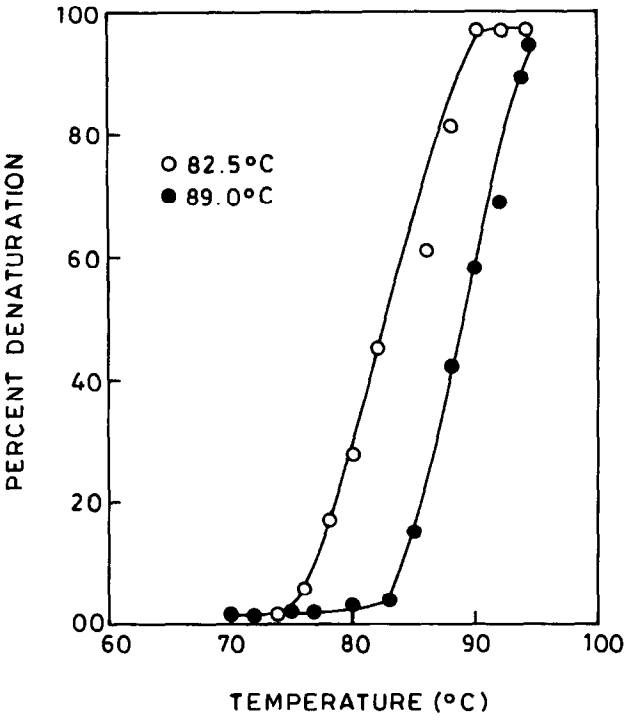

Fig.2. Effect of temperature on the melting of native (-○-) and modified DNA (-●-). The T_m values of the duplex are shown top left.

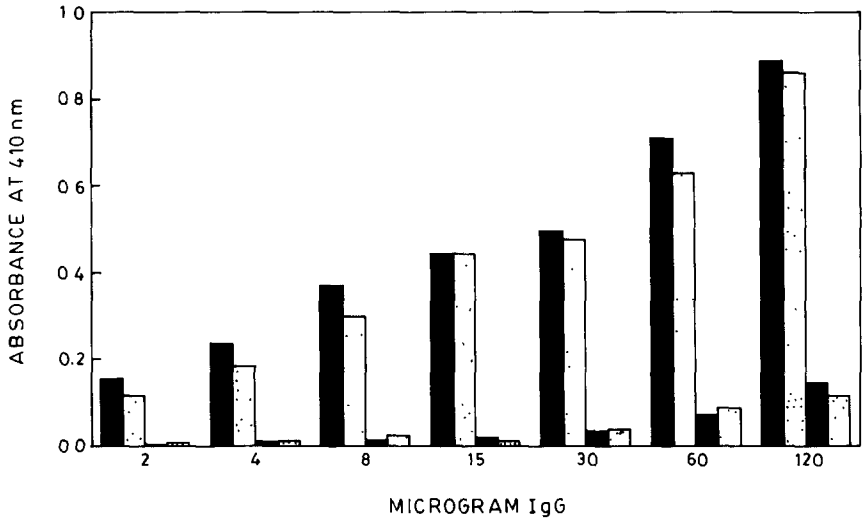

Fig.3. Binding of anti-ROS-DNA IgG with native DNA (stippled bars) and ROS-DNA (filled bars). The corresponding small bars represent premmune IgG binding with native DNA and immunogen, respectively.

ceptibility of DNA bases to modification by hydroxyl radical was as follows:

Thymine> Cytosine> Adenine = Guanine

Unlike native DNA, hydroxyl radical-modified calf thymus DNA induced high titer antibodies. DEAE Sephacel-purified immune IgG showed a high degree of binding with native DNA as well as immunogen (Fig. 3). Preimmune IgG as control showed negligible binding to these antigens. To ascertain the antibody specificity, competition ELISA with immunogen and other double-stranded nucleic acids was carried out. In competition–inhibition assay, the induced antibody binding was fifty per cent inhibited by the immunogen at a concentration of 0.32 μg/ml (Fig. 4a). The cross reactivity of induced antibodies with B-conformations like native DNA, poly (dA-dG)·poly(dC-dT), poly(dA-dU)·poly(dA-dU) and poly (dI-dC)·poly(dIdC) was of varying degree (Fig. 4b).

To probe the possible role of hydroxyl radical (or ROS in general) in the pathogenesis of SLE, four sera having different levels of anti-DNA autoantibodies were tested. Binding of these antibodies (at 1:100 dilution of serum) with native and modified DNA has been depicted in Fig. 5a. The binding pattern of some of these autoantibodies up to 1:6400 dilution of serum with either of the antigens were similar to those observed at lower dilution. It was found that SLE autoantibody recognition of modified DNA was better than native DNA under identical conditions. However, one serum sample (No. 1) showed slightly increased binding of native DNA as compared to modified DNA preparation. Competition–inhibition assays have shown that modified DNA is a better competitor of anti-DNA antibody-nDNA interaction in both double- and single-stranded forms (Fig. 5b). The amount of competitor required to block fifty percent of autoantibodies binding was found to be 0.03 μg/ml and 0.038 μg/ml for modified DNA in double- and single-stranded form. The fifty percent inhibition of autoantibodies binding with native DNA duplex DNA and its heat denatured form was observed at 0.13 μg/ml and 0.20 μg/ml, respectively.

Table II

Modification of bases in DNA exposed to hydroxyl radicals

Base	Modification (%)
Adenine	30.05
Guanine	29.20
Cytosine	38.80
Thymine	49.25

In competition ELISA, the anti-native DNA IgG affinity-purified from two different SLE sera were found to be recognized by both native DNA and its hydroxyl radical-modified form. More than 90 percent of affinity IgG binding was eliminated by 5 μg/ml of either native or modified DNA used as competitors.

4. DISCUSSION

The stimulus that causes the production of anti-DNA antibodies in SLE is obscure. Native double-stranded DNA (B-conformation) is unlikely to be involved in view of its poor immunogenicity [21,22]. The possibile participation of some unusual forms of DNA in disease pathogenesis can not be ruled out [23]. There has been considerable interest in recent years on the damaging potential of reactive oxygen species (ROS) on living system [24,25]. These radicals modify DNA at various sites including base damage and cross linking between cytosine and tyrosine in nucleohistone [26,27].

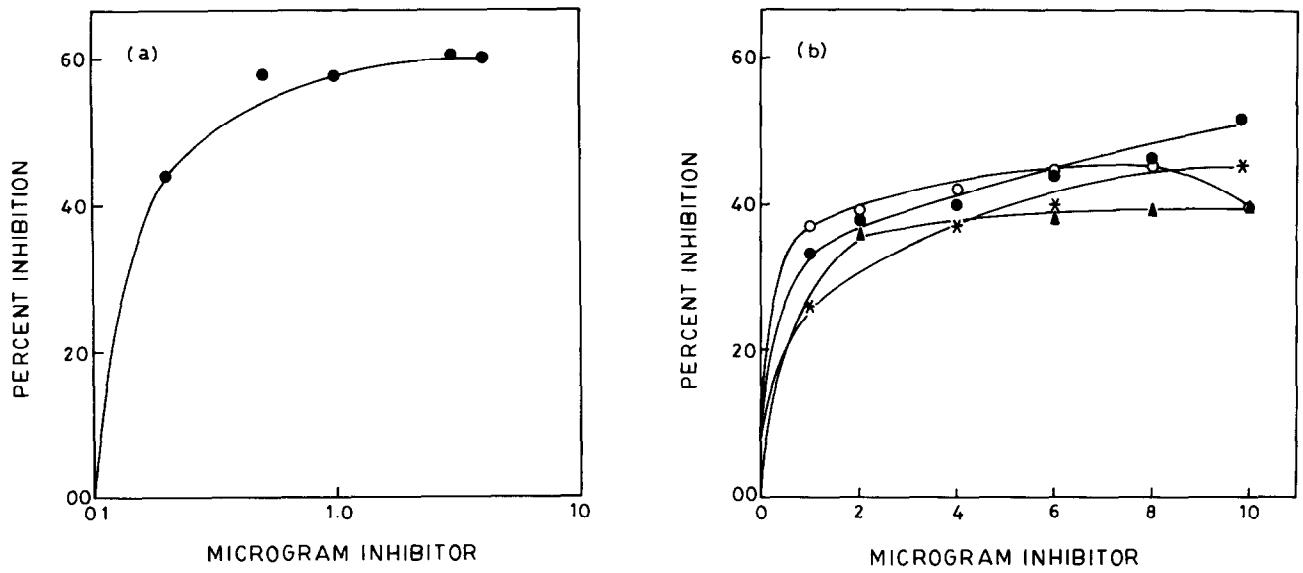

Fig.4. (a) Inhibition of immunogen-antibody interaction by ROS- DNA. (b) Inhibition of immunogen–antibody interaction by native DNA (-★-); poly(dA-dG)·poly(dC-dT) (-○-); double-stranded poly(dI-dC) (-●-) and poly(dA-dU) (-△-).

In the present studies, hydroxyl radical was found to alter the topological state of native DNA and rendering it immunogenic. The polyspecificity exhibited by immune IgG was evident from inhibition ELISA results. Native DNA, RNA and synthetic polynucleotides in B-conformation besides immunizing antigen were effective inhibitors. In an earlier study, native DNA on ultraviolet irradiation in the presence of hydrogen peroxide-induced antibodies which showed recognition for B-conformation [28].

The results presented in this communication may provide a basis for the production of naturally occurring anti-nucleic acid antibodies in autoimmune disorders. The spontaneous production of anti-DNA antibodies in SLE might arise as a consequence of conformational changes in DNA due to damage caused by hydroxyl radicals. The augmented level of ROS in tissues is well related to many pathological conditions [29]. Recent studies have suggested that active SLE patients contain markedly decreased superoxide dismutase (SOD) activity and therefore the free radical level in these patients is expected to be much higher [30]. The strong cross reactive binding potential of anti-nDNA IgG towards ROS-modified DNA demonstrates the possible participation of a modified DNA antigen in SLE pathogenesis, since dsDNA of the B-conformation

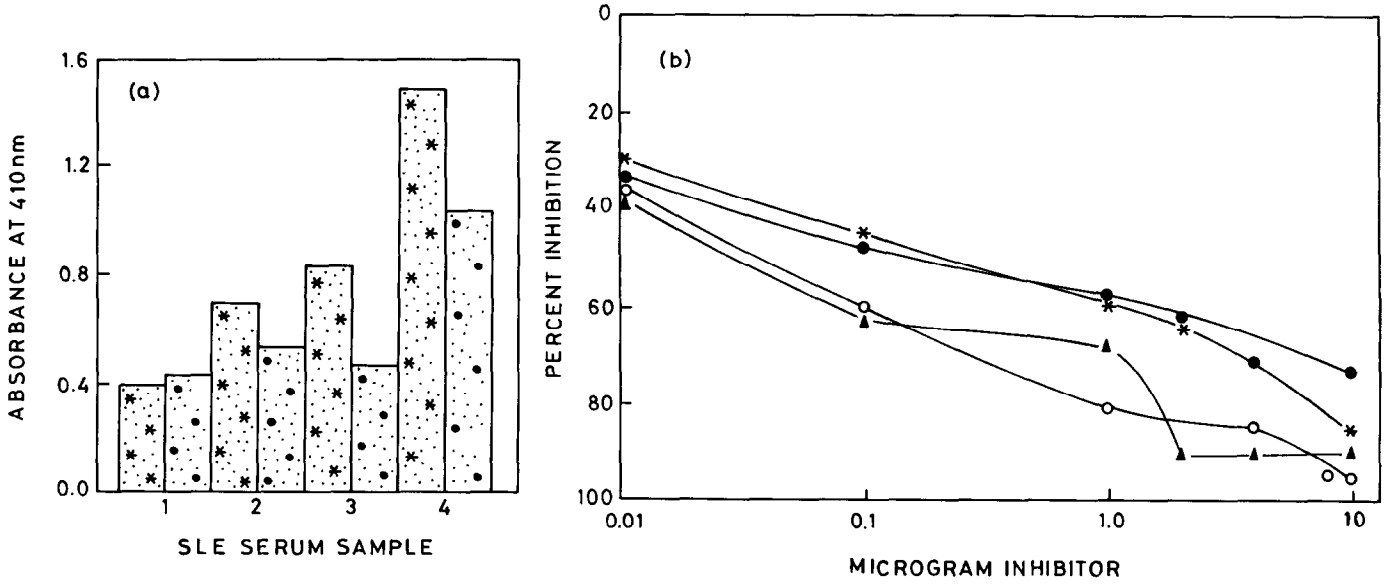

Fig.5. (a) Binding of human anti-DNA antibodies to ROS-DNA (stippled bars with stars) and native DNA (stippled bars with dots). (b) Inhibition of autoantibody-nDNA interaction by native DNA (-●-), ROS-DNA (-▲-) and heat denatured DNA (-★-) and ROS-DNA (-○-).

is a poor immunogen [22]. Hydrogen peroxide on diffusion to a vulnerable part of the cell might generate hydroxyl radicals by reaction with ferrous ion, often bound to the phosphates of the DNA backbone and to various proteins including those associated with chromatin, which in turn could modify DNA [11,12].

Acknowledgements: This work was supported in part by a research grant (4/5/88-G) to R.A. from the Department of Atomic Energy, Government of India. The financial support to K.A. in the form of research fellowship from U.G.C. New Delhi, is gratefully acknowledged.

REFERENCES

[1] Wick, G., Kromer, G., Neu, N., Fassler, R., Ziemiecki, A., Muller, R.G., Ginzel, M., Beladi, I., Kuhr, T. and Hala, K. (1987) Immunol. Lett. 16, 249–258.
[2] Allan, I.M., Vaughan, A.T.M., Milner, A.E., Lunec, J. and Bacon, P.A. (1988) Brit. J. Cancer 58, 34–37.
[3] Schneider, J.E., Browning, M.M., Zhu, X., Eneff, K.L. and Floyd, R.A. (1989) Mutation Res. 214, 23–31.
[4] Halliwell, B. and Gutteridge, J.M.C. (1990) Methods Enzymol. 186, 1–85.
[5] Halliwell, B. and Aruoma, O.I. (1991) FEBS Lett. 281, 9–19.
[6] Schneider, J.E., Browning, M.M. and Floyd, R.A. (1988) J. Free Radical Biol. Med. 5, 287–295.
[7] Babior, B.M. (1984) Blood 64, 959–966.
[8] Blount, S., Griffiths, H.R. and Lunec, J. (1989) FEBS Lett. 245, 100–104.
[9] Blount, S., Griffiths, H., Emery, P. and Lunec, J. (1990) Clin. Exp. Immunol. 81, 384–389.
[10] Stoewe, R. and Prutz, W.A. (1987) J. Free Radical Biol. Med. 3, 97–105.
[11] Floyd, R.A. (1981) Biochem. Biophys. Res. Commun. 99, 1209–1215.
[12] Floyd, R.A. (1983) Arch. Biochem. Biophys. 225, 263–270.
[13] Imlay, J.A., Chin, S.M. and Linn, S. (1988) Science 240, 640–642.
[14] Ali, R., Dersimonian, H. and Stollar, B.D. (1985) Mol. Immunol. 22, 1415–1422.
[15] Tan, E.M., Cohen, A.S., Fries, J.F., Masi, A.T., McShane, D.J., Rothfield, N.F., Schaller, J.G., Talal, N. and Winchester, R.J. (1982) Arth. Rheum. 25, 1271–1277.
[16] Blakely, W.F., Fuciarelli, A.F., Wegher, B.J. and Dizdaroglu, M. (1990) Radiat. Res. 121, 338–343.
[17] Fujiwara, Y., Tatsumi, M. and Sasaki, M.S. (1977) J. Mol. Biol. 113, 635–649.
[18] Hasan, R. and Ali, R. (1990) Biochem. Int. 20, 1077–1088.
[19] Ishaq, M. and Ali, R. (1984) Immunol. Commun. 13, 447–455.
[20] Alam, K. and Ali, R. (1992) Biochem. Int. 26, 597–603.
[21] Pisetsky, D.S., Grudier, J.P. and Gilkeson, G.S. (1990) Arth. Rheum. 33, 153–159.
[22] Madaio, M.P., Hodder, S., Schwartz, R.S. and Stollar, B.D. (1984) J. Immunol. 132, 872–876.
[23] Alam, K., Islam, N., Hasan, R., Ali, A. and Ali, R. (1992) Microbiol. Immunol. 36, 1003–1007.
[24] Bulkley, G.B. (1983) Surgery 94, 407–411.
[25] Halliwell, B. (1987) FASEB J. 1, 358–364.
[26] Gajewski, E. and Dizdaroglu, M. (1990) Biochemistry 29, 977–980.
[27] Dizdaroglu, M., Rao, G., Halliwell, B. and Gajewski, E. (1991) Arch. Biochem. Biophys. 285, 317–324.
[28] Ara, J., Ali, A. and Ali, R. (1992) Immunol. Invest. 21, 553–563.
[29] Willson, R.L. (1979) Oxygen free radicals and tissue damage, Ciba Foundation Symp. volume 65 (new series) p 19.
[30] Jiang, X. and Cheng, F. (1992) Clin. Immunol. Immunopathol. 63, 39–44.