Neutrophils from systemic lupus erythematosus patients demonstrate increased nuclear DNA damage

J.R. McConnell[1], A.D. Crockard[2], A.P. Cairns[1], A.L. Bell[1]

[1]Queen's University Musculoskeletal Education and Research Unit, Department of Rheumatology, Musgrave Park Hospital, Belfast; [2]Regional Immunology Service, Royal Victoria Hospital, Belfast, Northern Ireland, UK.

Abstract

Objective

(i) To determine the levels of nuclear DNA damage in freshly isolated and cultured neutrophils from SLE patients (SLE), rheumatoid arthritis patients (RA) and healthy individuals and relate these to the percentage of apoptotic neutrophils. (ii) To assess rates of repair of neutrophil oxidative DNA damage.

Methods

The comet assay was used to quantify nuclear DNA damage in neutrophils from SLE patients (n=20), control subjects (n=15) and RA patients (n=15). Levels of DNA damage were related to apoptosis as assessed by annexin V binding and morphology. Rates of repair of neutrophil oxidative DNA damage was measured by incorporating formamidopyrimidine-DNA glycosylase (FPG) into the comet assay.

Results

Nuclear DNA damage in freshly isolated and cultured (20h) neutrophils was significantly greater in SLE patients (median = 12.5%, 27.3%; respectively) compared with RA patients (median = 9.4%, p=0.002; 19.3%, p=0.002; respectively) and control subjects (median = 8.2%, p=0.003; 18.7%, p=0.01, respectively). Significantly higher levels of circulating apoptotic neutrophils were demonstrated in SLE patients compared to RA and control subjects. Similar findings were observed following 20 h cultured neutrophil preparations. However, no significant direct correlation between neutrophil apoptosis and DNA damage was observed. Neutrophils from 3 of 5 SLE patients demonstrated an impaired ability to repair oxidatively modified DNA.

Conclusion

Neutrophils from SLE patients display increased DNA damage and, additionally, may demonstrate defective repair of oxidative DNA damage. These features, in addition to increased rates of neutrophil apoptosis, may act as contributing factors to autoantigen excess and immune activation.

Key words

Systemic lupus erythematosus, comet assay, neutrophil, DNA damage, apoptosis.

Jack R. McConnell, MSc; Alistair D. Crockard, PhD; Andrew P. Cairns, MD; and Aubrey L. Bell, MD.

Please address correspondence and reprint requests to: Aubrey L. Bell, MD, The Lupus Research Group, Queen's University Musculoskeletal Education and Research Unit, Department of Rheumatology, Musgrave Park Hospital, Stockman's Lane, Belfast BT9 7JB, Northern Ireland, UK.
E-mail: a.bell@qub.ac.uk

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Introduction

Native DNA is considered to be a poor immunogen (1). However, anti-DNA antibodies are present in sera of patients with systemic lupus erythematosus (SLE) (2, 3). These autoantibodies are relatively specific for SLE and the immune complexes they form are central to disease pathogenesis (4, 5). There is evidence of isotype switching, somatic mutation, affinity maturation, and epitope spreading suggesting a T-cell dependent antigen-driven response rather than non-specific polyclonal activation of B-cells. Analysis of the antibody response in SLE reveals clustering of specificities with particular focus on intracellular antigens derived from the cell nucleus including dsDNA and histone in the nucleosome. There is evidence that these antigens are exposed on the surface of apoptotic cells and therefore such cells may contribute to SLE pathogenesis (6).

It has been observed in SLE, that anti-DNA antibodies display preferential binding to oxidatively modified DNA when compared to native DNA (7-9). An oxidative attack on DNA will induce conformational change, single- and double-strand breaks and base modifications (10, 11) . The most common base modification is the formation of 8-hydrodeoxyguanosine (8-OH-dG) (12, 13). This moiety is generated by the interaction of a hydroxyl radical (·OH) at the C8 position of deoxyguanosine. DNA modified in this way exhibits enhanced immunogenic properties (14, 15) and has been detected in immune complexed DNA derived from sera of SLE patients (16).

Oxidatively modified DNA has also been detected in nuclear DNA isolated from activated neutrophils (17). Furthermore, aberrant processing of oxidatively modified DNA leading to an accumulation of this molecule within the cell has been observed in lymphocytes isolated from patients with autoimmune disease (7).

We have recently reported increased numbers of circulating apoptotic neutrophils in the peripheral blood of patients with SLE, the levels of which were found to correlate with disease activity and anti-DNA antibody titre (18). To further explore this observation we have determined, by the comet assay, the levels of DNA damage and the rates of oxidative DNA repair in neutrophils isolated from patients with SLE, RA and healthy individuals and correlated this with the levels of circulating apoptotic neutrophils. Furthermore, we have determined the percentage of neutrophil apoptosis following 20 h culture to investigate whether increased numbers of apoptotic neutrophils in SLE are due to an augmented rate of apoptosis.

The comet assay or single cell gel electrophoresis assay is a sensitive method for the detection of DNA damage in single cells (19, 20). A small number of cells are embedded in agarose on a microscope slide and lysed. The application of an electric current pulls the charged DNA from the nucleus. The amount of DNA damage is quantified by measuring the amount of DNA released. The incorporation of the enzyme formamidopyrimidine-DNA glycosylase (FPG) into the assay allows the detection of oxidative damage. This enzyme has N-glycosylase and AP-lyase activity and will release damaged purine residues from DNA.

Employing this assay we have demonstrated that neutrophils from SLE patients displayed increased levels of neutrophil nuclear DNA fragmentation and an impaired capacity to repair oxidatively modified DNA.

Materials and methods

Patients and controls

SLE patients (n = 20) (17 female, 3 male, median age = 47.0 years) fulfilled Revised American Rheumatology Association (ARA) criteria (21) for diagnosis and were recruited from out-patients and rheumatology in-patient wards. Disease controls with rheumatoid arthritis (n = 15) (11 female, 4 male, median age = 50.5 years) were also recruited. Ethical approval was granted by the local medical ethical committee and written informed consent obtained from all patients. Normal healthy volunteers (n = 15) (11 female, 4 male, median age = 42.0 years) were recruited from hospital staff.

Disease activity
Disease activity was assessed using the Systemic Lupus Activity Measure (SLAM) and the British Isles Lupus Activity Group (BILAG) scores (22). Rheumatoid arthritis disease activity was assessed using the modified Disease activity score (DAS28) (23). Full blood picture, erythrocyte sedimentation rate, and C-reactive protein were measured.

Neutrophil isolation
Neutrophils were isolated from heparinised peripheral blood as previously described (24). Briefly, the erythrocytes were removed by sedimentation through a 0.75% dextran solution (Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 min. The neutrophils were then separated from the leukocyte pellet by centrifugation after laying over a discontinuous Percoll density gradient (Amersham Pharmacia). The neutrophil layer was aspirated and the cells washed once in phosphate buffered saline (PBS).

Neutrophil culture and treatment
Isolated neutrophils were resuspended (1×10^6/ml) in RPMI medium supplemented with 10% autologous serum, 100 U/ml penicillin (Sigma, Poole, Dorset, UK), 100 µg/ml streptomycin (Sigma), 2 mM L-glutamine (Life Technologies, Paisley, UK) and incubated at 37 ºC in a 5% CO_2 atmosphere for up to 20 h.
To induce DNA damage 1×10^6 neutrophils were resuspended in 200 µM H_2O_2 in PBS for 30 min at 37°C. Untreated neutrophils were incubated in PBS only. After exposure the cells were washed (x2) in PBS. For DNA repair the damaged cells were resuspended in RPMI as described above. At timed intervals (0.5 h, 1 h and 4 h after exposure) 1×10^6 neutrophils were removed from culture, washed and resuspended in chilled PBS (1×10^6/ml). Cells were stored on ice awaiting processing in the comet assay.

Morphology
Cytocentrifuge preparations of 3×10^5 cells in PBS (400 ml) were prepared using a cytospin (Shandon, London, UK). The cell suspension was centrifuged at 450 rpm for 5 min and air dried for 20 min. Once dry, the cells were stained using a Diff-Quik staining kit (Baxter Dade Diagnostics, Dudingen, Switzerland). Briefly, the slides were immersed in fixative solution (0.002 g/L fast green in methanol) for 60 seconds. Following fixation, excess fluid was drained from the slides and they were transferred to a solution of thiazine dye for 60 seconds, drained, and transferred to a solution of Eosin G for a further 60 seconds staining. The slides were then washed with water and air dried for morphological examination.
Morphological assessment was performed at x320 magnification and at least 200 cells were counted on each slide. Neutrophils with condensed nuclei were defined as apoptotic.

Annexin V staining
The percentage of annexin V positive neutrophils was determined by flow cytometery using fluorescein isothiocyanate (FITC) conjugated annexin V antibody (R&D Systems Europe Ltd, Abingdon, UK). 0.5×10^6 neutrophils were suspended in 100 ml of binding buffer (R&D Systems) and incubated with 1 µl of annexin V-FITC (25 µg/ml) (R&D Systems) and 10 µl propidium iodide (R&D Systems) for 15 min in the dark at room temperature. Immediate analysis was performed using a Coulter EPICS ELITE flow cytometer which was standardised for inter-run variability by calibration with Immunocheck fluorospheres (Coulter Corporation, Florida, USA). Cells were analysed on the basis of forward and side scatter signals and gated appropriately. For each assay 5000 cells were analysed.

Single cell gel electrophoresis (comet) assay
DNA damage. The extent of DNA damage in freshly isolated neutrophils and in neutrophils following 20 h culture was evaluated using the Trevigen comet assay kit (A.M.S Biotechnology, Oxon, UK) according to manufacturer's instructions.
Slides were examined using a Nikon epi-fluorescence microscope equipped with an excitation filter of 515-560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. Fifty images were analysed using the programme Komet 3.1 (Kinetic Imaging Ltd, Liverpool, UK).

Oxidative DNA damage
The extent of oxidative DNA damage in freshly isolated and damaged neutrophils was evaluated using the comet assay (25). A fully frosted microscope slide (Richardsons Supply Co. Ltd.; London) was covered with 100 ml of 1% normal melting point agarose (Sigma) in Ca^{2+} and Mg^{2+} free PBS and covered with a large coverslip. The slide was chilled at 4°C for 15 min to allow the agarose to solidify and the coverslip removed.
Approximately 10,000 neutrophils were resuspended in 85 µl of low melting point agarose at 37°C. This suspension was pipetted on top of the first layer, covered with a coverslip and allowed to solidify at 4°C for 10 min. After removal of the coverslip the slides were immersed in freshly prepared lysing solution (2.5 M $NaCl_2$, 0.1 M EDTA, 1 mM Tris with 1% (v/v) Triton X-100 added just before use) for 1h at 4°C.
The slides were then drained and washed (x3) for 5 minutes in enzyme reaction buffer (4 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, adjusted to pH 8.0 with KOH). After washing, 50 µl of formamidopyrimidine-DNA glycosylase (FPG) diluted 1/300 in enzyme reaction buffer was pipetted on to each slide, covered with a coverslip and incubated for 30 min at 37°C. Control slides received buffer only. Following enzyme treatment the coverslips were removed and the slides placed in an electrophoresis tank and covered with chilled alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA). The slides were left in this solution for 30 min before electrophoresis. Electrophoresis was conducted for 25 min at 25 V and 300 mA. Following electrophoresis the slides were washed (X3) in neutralising buffer (0.4 M Tris pH 7.5). Finally the slides were drained and stained with 50 µl of 20 µg/ml eth-

idium bromide. Slides were examined as described above.

Statistical analysis

The Mann-Whitney U test for unpaired samples was employed to determine the statistical significance of the results. Spearman's correlation coefficients were calculated to determine the relations of DNA damage and apoptosis. Statistical analysis was performed using SPSS for windows.

Results

Levels of DNA damage

DNA damage was determined by computerised assessment of the comet tail length (Fig. 1).

The percentage of DNA damage in freshly isolated neutrophils was significantly higher in SLE patients (median = 12.5, interquartile range (IQR)= 9.8 – 14.7) when compared with healthy controls (median= 8.2, IQR = 7.5–11.5; p = 0.003) and RA patients (median = 9.4, IQR = 7.5 – 11.5; p = 0.02). There was no significant difference between levels of DNA damage in RA patients when compared to controls (p = 0.11) (Fig. 2).

Following 20 h culture a significant increase in the percentage DNA damage was observed in neutrophils from SLE patients (median = 27.3, IQR = 19.7 – 41.0) compared with healthy controls (median = 18.7, IQR = 16.2-23.5; p = 0.01) and RA patients (median = 19.3, IQR = 15.4-22.8; p = 0.02). No statistically significant differences were observed between the RA patient and control subject groups (p = 0.71).

The gain in DNA damage above basal levels was significantly higher following culture in the SLE patient group (median = 16.2, IQR = 10.7-20.4) when compared with controls (median = 8.7, IQR=10.1 – 13.6; p = 0.03) and RA patients (median = 10.9, IQR = 6.0 – 14.1; p = 0.04). There was no significant difference between RA patients when compared to controls (p = 0.90).

Apoptosis measurements

Morphology. The percentage of circulating apoptotic neutrophils was higher in SLE patients (median = 4.0, IQR 2.0 – 5.0) compared to RA patients (median = 2.0, IQR 1-3; p = 0.01) and healthy controls (median = 1.0, IQR 1.0 – 2.0; p = 0.0001). The percentage apoptotic cells was higher in RA patients compared to controls but not statistically different (p = 0.06) (Table I).

Following culture a significant increase in the number of apoptotic neutrophils in the SLE patient group was observed (median = 61.0, IQR 52.0 – 74.0) when compared with healthy controls (median = 37, IQR 27.0 – 41.0; p = 0.0001) and RA patients (median = 38.5, IQR 29.2 – 54.7; p = 0.002). There was no significant difference in the percentage of apoptotic cells in cultured neutrophils from controls when compared to RA patients (p = 0.50).

Annexin V staining. The percentage of apoptotic neutrophils in freshly drawn blood, was significantly higher in SLE patients (median = 1.9, IQR1.4-2.5) compared with healthy controls (median = 1.1, IQR 0.45 – 1.8; p = 0.007) and RA patients (median = 1.1, IQR 0.45 – 2.2; p = 0.04) There was no significant difference in the percentage apoptotic cells between RA patients when compared to controls (p = 0.98) (Table I).

Following culture a significant in-

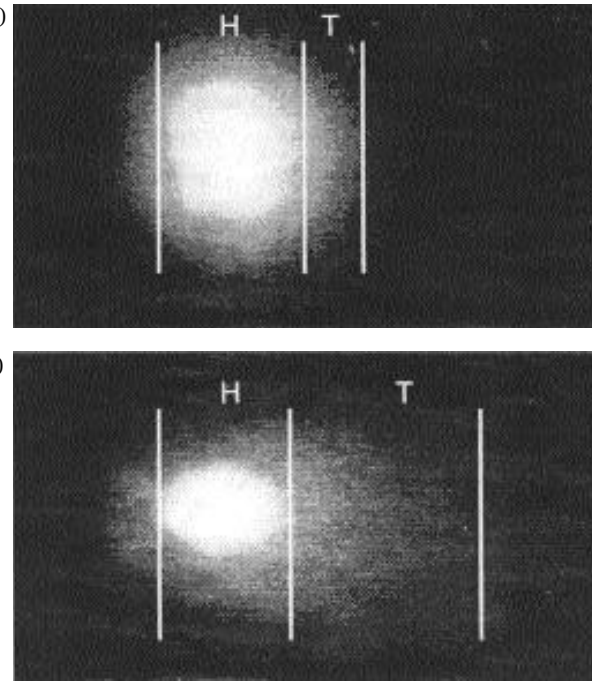

Fig. 1. Representative comet assay profiles for (**a**) freshly isolated neutrophil, demonstrating that the majority of DNA is intact and remaining within the comet head (H); (**b**) neutrophil following 20 h culture, showing comet tail (**T**) formation which is indicative of DNA damage (magnification X40).

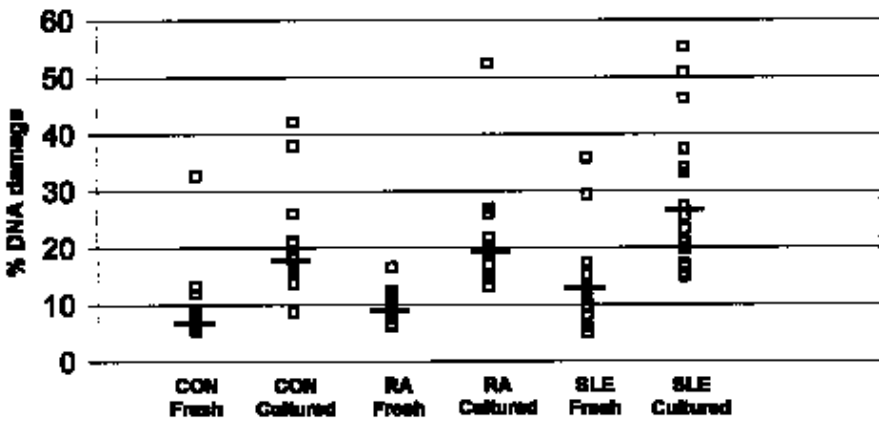

Fig. 2. Comparison of levels of DNA damage in freshly isolated and cultured neutrophils from systemic lupus erythematosus patients (SLE) healthy controls (CON) and rheumatoid arthritis patients (RA) measured using the comet assay. Each point represents one individual and medians are indicated for each group.

Table I. Percentage apoptotic neutrophils (median; IQR) determined in fresh and cultured cells (20 h) by annexin V staining and morphological analysis.

	Morphologically Apoptotic (%)				Annexin V positivity (%)			
	Fresh		Cultured		Fresh		Cultured	
SLE	4.0	(2.0-5.0)	61.0	(52.0-74.0)	1.9	(1.4-2.5)	38.8	(30.8-45.0)
CON	1.0	(1.0-2.0)	37.0	(27.0-41.0)	1.1	(0.4-1.8)	22.5	(17.0-31.2)
RA	2.0	(1.0-3.0)	38.5	(29.2-54.7)	1.1	(0.4-2.2)	26.1	(11.0-35.6)

crease in the number of apoptotic neutrophils in the SLE patient group (median = 38.8, IQR 30.8 – 45.0) was observed compared with healthy controls (median = 22.5, IQR 17.0 – 31.2; p = 0.0001) and RA patients (median = 26.1, IQR 11.0 – 35.6; p = 0.02) (Table I). There was no significant difference in the percentage apoptotic cells between RA patients when compared to controls (p = 0.75) (Table I).

On each occasion not more than 2% if the cell population stained for PI.

Correlation of DNA damage and apoptosis

The correlation of DNA damage and levels of apoptosis measured using morphology in freshly isolated cells was not statistically significant for SLE patients (r = -0.27; p = 0.24), RA patients (r = -0.44; p = 0.13) and controls (r = 0.029; p = 0.91). A similar trend was observed following culture. No significant correlation between apoptosis determined by morphology and DNA damage was observed for SLE patients (r = -0.29; p = 0.2), RA patients (r = 0.02; p = 0.63) and controls (r = 0.075; p = 0.79).

No significant correlations were observed for neutrophil DNA damage and levels of apoptosis measured using annexin V staining in freshly isolated cells from SLE patients (r = 0.325, p = 0.16), RA patients (r = -0.32; p = 0.92) and controls (r = -0.24; p = 0.37). Similarly, following culture no significant correlation was observed between the levels of apoptosis measured by annexin V staining and DNA damage for SLE patients (r = 0.23; p = 0.31), RA patients (r = 0.23; p = 0.41) or controls (r = -0.07; p = 0.77).

Disease activity, DNA damage and apoptosis

For SLE patients the median SLAM score was 9 (IQR 5–13) and the median BILAG score was 11 (IQR 8–15). There were no significant correlations between DNA damage and ESR, CRP, SLAM or BILAG. No significant correlations were observed between apoptotic neutrophil counts and ESR, CRP, SLAM or BILAG.

For RA patients the median DAS 28 score was 9 (IQR 3.5–14). No significant correlations were observed between DNA damage and DAS 28 score, ESR, and CRP. No correlation was observed between apoptotic neutrophils and DAS 28 score, ESR, and CRP.

Oxidatively damaged DNA

There was no significant difference in the basal levels of FPG labile sites measured in freshly isolated neutrophils from SLE patients (5) (median = 9.1, IQR = 5.6-13.0) when compared to healthy controls (5) (mean = 8.1, IQR = 6.4-17.6; p = 0.75) and RA patients (5)

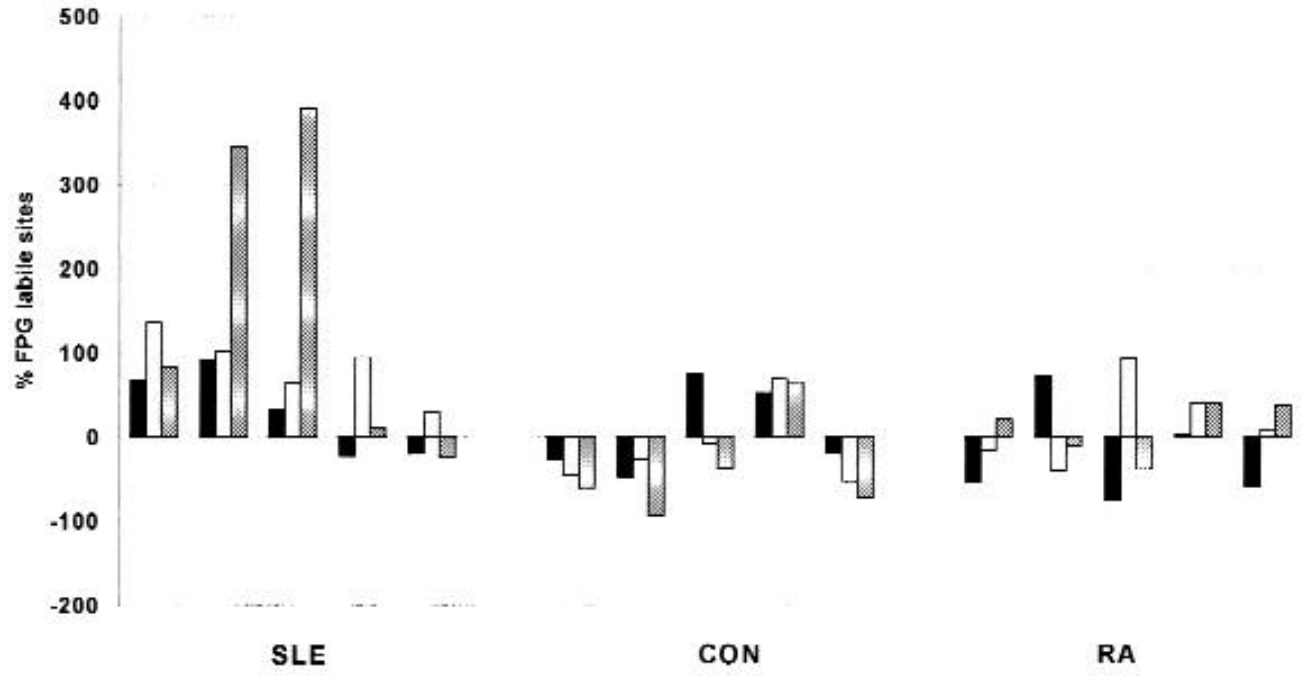

Fig. 3. The percentage of FPG labile sites in neutrophils following hydrogen peroxide treatment from systemic lupus erythematosus patients (SLE),healthy controls (CON) and rheumatoid arthritis patients (RA) measured using the comet assay. Values represent the percentage FPG labile sites expressed as a percentage of the basal level for each individual studied following 0.5 h (black bars); 1 h (white bars); and 4 h (gray bars) repair time.

(median = 9.2, IQR = 6.2 – 24.3; p = 0.97). Similarly, the percentage of FPG labile sites recorded for the RA patient and control groups were not significantly different (p = 0.91).

Following hydrogen peroxide treatment an accumulation of FPG labile sites was observed in neutrophils isolated from three of the SLE patients studied. No such increase was observed in cells isolated from RA patients or controls (Fig. 3).

Discussion

Apoptosis is a highly regulated process that brings about cell death without the release of inflammatory mediators. It is orchestrated, at least in part, by the caspase group of enzymes (26). These proteases produce the morphological features of apoptosis such as cell shrinkage, membrane blebbing and nuclear condensation by the direct disassembly of cell structures. They also mediate nuclear DNA fragmentation by triggering a caspase activated DNase (CAD) by proteolysis of its inhibitor (ICAD) (27).

The percentage DNA damage within an individual cell can be determined using the comet assay (19, 20). The comet assay has recently received much attention and reports of a dose dependent increase in the level of nuclear DNA damage after exposure of cells to UV irradiation (28), ionising radiation (29) or triggers of apoptosis (30) has been documented.

We employed this technique to investigate the levels of nuclear DNA fragmentation in neutrophils from SLE patients, RA patients and healthy individuals. We found significantly higher levels of DNA damage in both freshly isolated and cultured neutrophils from SLE patients compared to RA patients and controls.

We did not include a positive control for each comet assay and we acknowledge that this is a limitation of our study. There are many agents or environmental triggers such as diet, underlying infection and air pollution that are potentially capable of inducing nuclear DNA damage and we can only speculate as to which factors are responsible for mediating DNA damage in our study. It is therefore not possible to control for them all. However, we can exclude the age and sex of the patients studied as no significant difference was observed between study groups.

Furthermore, it is recognised that increased levels of oxidative stress can lead to nuclear DNA damage and that SLE and RA are inflammatory diseases that are intimately associated with oxidative stress. Therefore, it is possible that the increased levels of DNA damage detected in neutrophils from SLE patients are a consequence of an inflammatory environment. However, no such increase above control levels was observed in neutrophils from RA patients. This finding may suggest that neutrophils from SLE patients are more susceptible to ROS mediated DNA damage or have an impaired ability to repair such damage. Evidence for this view comes from work on lymphocytes in SLE. Harris *et al.* (31) have demonstrated defective repair of O6-methylguanine, a DNA alkylation product in SLE lymphocytes. Bashir *et al*. (7) have reported an accumulation of 8-oxo7-hydrodeoxyguanosine (8-oxo-dG), a major product of DNA oxidation, in lymphocytes of patients with autoimmune disease. 8-oxo-dG is known to be immunogenic and has been detected in immune complexes derived from SLE patients (16). Also, it has been demonstrated that in vitro activation of neutrophils can lead to the formation of 8-hydroxydeoxyguanosine within the cells own DNA (17, 32). Oxidative DNA damage pathways have been previously examined (33, 34). However, in this work we are focusing on oxidative pathways in neutrophil apoptosis which have received little attention. To further explore neutrophil processing of oxidatively modified DNA damage we used a modification of the comet assay. To enable the detection of specific types of oxidative DNA damage an additional enzyme digestion step was incorporated into the assay. The repair endonuclease (FPG) (25) was used to release oxidatively damaged bases from DNA, release of a damaged base would insert a strand break. By subtracting the level of damage detected in untreated cells from enzyme treated cells the percentage of oxidatively modified DNA could be estimated.

We found similar basal levels of oxidatively damaged DNA in freshly isolated neutrophils from both the patient and control groups. However, following the induction of oxidative damage by hydrogen peroxide treatment, neutrophils from three of five SLE patients failed to repair the oxidatively modified DNA.

All five patients examined for oxidative DNA damage repair fulfilled ARA criteria for SLE. The three patients who failed to repair DNA demonstrated a significantly different clinical picture to the other two. All three were sampled in the early stages of a major flare in their lupus, before changes were made to their maintenance medication. Two of the patients were on low dose oral steroid, the dose having been constant for at least six months; one developed an aggressive vasculitis and arthritis, the second, a cerebral vasculitis presenting with seizures and headaches, and became gravely ill. The third patient was not on oral steroid but was maintained on hydroxychloroquine and NSAIDs – she developed a severe neutropenia with subsequent sepsis. All three required immunosuppressive treatment and parenteral steroid (following appropriate antibiotics in the final case) to induce remission.

The two patients who demonstrated repair were both in remission, on low dose oral steroid, one having received intravenous steroid the previous month for a flare of lupus arthritis, but having achieved both clinical and biochemical remission by the time of sampling.

Considering these findings it is possible that neutrophils from active SLE patients may have an impaired ability to repair oxidatively modified DNA, although investigation of a larger series is required.

Increased DNA damage may be associated with increased rates of apoptosis. We demonstrated significantly higher levels of circulating apoptotic neutrophils by morphology and annexin V binding in the peripheral blood of SLE

patients when compared to RA patients and healthy individuals. We also demonstrated significantly higher levels of apoptotic neutrophils from SLE patients following culture. These data confirm and extend our previous findings of increased numbers of circulating apoptotic neutrophils in SLE which could be explained by increased rates of production and/or defective clearance of apoptotic cells (18). The significantly greater number of apoptotic neutrophils after 20 h culture provides evidence for an increased rate of neutrophil apoptosis in SLE patients and is concordant with our investigations of the kinetics of SLE neutrophil apoptosis. We demonstrated, by determining the levels of apoptosis every 4-6 h over a 24 h period an increased rate of SLE neutrophil apoptosis on comparison with RA patients and controls (unpublished observations). These data are also in agreement with the studies of others that have shown augmented lymphocyte apoptosis from SLE patients (35, 36).

There was no significant correlation between the levels of nuclear DNA damage and apoptosis measured by morphology or annexin V staining. As previously discussed there are many confounders that can influence DNA damage which maybe responsible for the poor correlation observed between DNA fragmentation and apoptosis. However, possible explanations for our observation include, that the morphologic events used to define apoptosis in this study may occur at different timepoints from DNA fragmentation. Alternatively, as well as detecting double strand DNA breaks that are indicative of apoptosis, the comet assay will also detect other forms of DNA damage such as alkali labile sites and single strand breaks (19, 20). The measurement of these alternative forms of 'non-apoptotic' DNA damage and the sequential events of apoptosis may explain the poor correlation observed.

It is not clear from this work whether neutrophil DNA damage is specifically linked to apoptosis. However, we have demonstrated increased rates of spontaneous neutrophil apoptosis in SLE. Therefore, increased rates of spontaneous apoptosis are not confined solely to lymphocytes. Either or both cell types may be relevant to disease pathogenesis.

Taken with the impaired ability of lupus monocyte-derived macrophages to remove apoptotic debris (37) these findings support the hypothesis that if apoptotic SLE neutrophils with increased amounts of oxidised DNA remain in circulation they may contribute to immune activation and ultimately autoantibody production.

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