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CHARACTERIZATION OF TUMOUR NECROSIS FACTOR α RELEASE BY HUMAN GRANULOCYTES IN RESPONSE TO PROCAINAMIDE CHALLENGE

COLIN M. HILL, JOSEPH LUNEC, HELEN R. GRIFFITHS and KARL E. HERBERT*

Division of Chemical Pathology, Centre for Mechanisms of Human Toxicity, University of Leicester, Hodgkin Building, PO Box 138, Lancaster Road, Leicester LE1 9HN, U.K.; and Clinical Sciences, Glenfield Hospital (NHS) Trust, Groby Road, Leicester LE3 9QP, U.K.

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Abstract—The role of human granulocytes in the promotion of procainamide (PA) toxicity *in vitro* has been studied and one of the agents responsible for DNA strand scission and cell death in human target cells has been characterized. Crude peripheral blood mononuclear cells (cPBMNs) isolated by density centrifugation, and the lymphocyte cell lines—CCRF-HSB2 and WIL-2NS—were exposed to PA, and DNA strand breaks were quantified by fluorescent analysis of DNA unwinding. Therapeutic plasma concentrations of PA (0–50 μM) caused dose-dependent cytotoxicity, determined by dye exclusion, and strand breaks in cPBMNs incubated for 3 and 1.5 hr at 37°, respectively. Using 50 μM PA a five-fold increase in DNA strand breaks was observed after 1.5 hr, with significant induction of strand breaks also being observed for 10 and 25 μM concentrations. Toxicity was much reduced in lymphocyte cell lines (maximal killing = 3.0% at 50 μM PA compared with 13.2% in cPBMNs). A similar decrease in toxicity was observed where *N*-acetyl procainamide (NAPA) was substituted for PA (less than 50% of strand breaks at all concentrations). Further investigations showed that the presence of a contaminating granulocyte population in the cPBMN fraction was responsible for the induction of PA toxicity. Incubation of a highly enriched granulocyte population with PA for 1 hr prior to exposure to purified peripheral blood mononuclear cells (pPBMNs) led to the complete restoration of the toxic effects. The resulting cyto- and genotoxicity were not significantly different to levels observed in cPBMNs. Significantly, incubation of granulocytes with NAPA did not induce toxicity in target pPBMNs. Ultrafiltration of granulocyte supernatants led to the identification of two toxic fractions of < 3000 and > 30,000 Da. Temporal studies showed that the toxicity associated with the < 3000 Da fraction appeared during the first 10–15 min incubation with PA whereas the > 30,000 Da fraction did not display significant toxicity until the 40–60 min period. Further assessment of the nature of these agents indicated that the 30,000 Da fraction was a protein. SDS-PAGE analysis showed an inducible 17,800 Da species appearing in granulocyte supernatants after 40 min incubation with PA. Dot blot analysis indicated that tumour necrosis factor α (TNFα) was present in the > 30,000 Da fraction. Evidence that TNFα was the high-molecular weight species responsible for PA-induced toxicity was obtained from neutralization assays employing an anti-TNFα antibody. Treatment of granulocyte-derived supernatants with this antibody led to the specific removal of a significant portion of both the cyto- and genotoxicity (> 50% in both cases). Our data suggest a highly effective mechanism for the promotion of drug-induced lupus by PA. The specific induction of TNFα represents a highly effective mechanism for the induction of the inflammatory response commonly associated with this syndrome and provides a potential route for the specific induction of severe adverse drug reactions. Furthermore, the biochemical mechanisms of action of lupus-inducing drugs may provide insight into the molecular mechanisms leading to development of idiopathic lupus.

Key words: lupus erythematosus; drug-induced lupus; procainamide; granulocytes; DNA damage; tumour necrosis factor α

Drug-induced lupus DIL† is a clinical syndrome resulting from the prolonged exposure to a wide range of therapeutic agents and which resembles the auto-immune disorder SLE [1–3]. The drugs predominantly associated with DIL are hydralazine, procainamide, chlorpromazine, α-methyldopa and isoniazid. Although the precise aetiology of idiopathic SLE remains unclear, it is becoming apparent that specific environmental factors such as ultraviolet radiation, as well as genetic factors, may be involved [4–7]. An understanding of the mechanisms of action of the lupus-inducing drugs may serve to provide a fundamental mechanism for disease initiation and progression that may be applied to the development of idiopathic SLE.

The therapeutic agent most commonly associated with the development of DIL is the anti-arrhythmic drug PA, which results in 30% of all treated individuals developing a circulating antibody pool

* Corresponding author. Tel./FAX 0116 2525593.

† Abbreviations: DIL, drug-induced lupus; SLE, systemic lupus erythematosus; PA, procainamide; PAHA, procainamide hydroxylamine; LPS, lipopolysaccharide; TNFα, tumour necrosis factor α; cPBMN, crude peripheral blood mononuclear cells; pPBMN, purified peripheral blood mononuclear cells; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

that is comparable to that observed in SLE, with the exception of anti-double stranded DNA antibodies [8]. The precise mechanism of this adverse drug reaction is unknown, but previous findings have indicated that the *in vivo* effects of PA were attributable to the formation of the highly reactive metabolite, PAHA [9–13]. Investigations into the effects of procainamide have been predominantly concerned with the bioactivating potential of different cell types, and as such have failed to consider the specific immunotoxic effects and mechanisms.

Structurally, PA contains an aryl amine group that *in vivo* is primarily acetylated by hepatic acyl transferases [14]. Furthermore, individuals with a 'fast acetylator' phenotype were found to have a greater resistance to the development of DIL by PA [14]. These results indicated that acetylation represents the protective metabolic pathway, and further analysis has clearly implicated the formation of PAHA in the promotion and maintenance of DIL [10–13, 15]. The hypothesis is that where PA encounters circulating neutrophils and monocytes, that promote the oxidative metabolism, the net effect would be to accelerate the formation of PAHA, resulting in the onset of DIL. Thus, metabolism of PA by circulating immune cells is a potential mechanism for the specific promotion of DIL.

A role for myeloperoxidase in the metabolism of drugs causing the lupus-like syndrome has been proposed as the primary mechanism for the promotion of DIL [10–13, 15–17]. One potential effect of PA metabolism by peripheral blood cells would be the increased local availability of PAHA to circulating immune cells, with a subsequent increase in cyto- and genotoxicity. Such toxicity may promote immunomodulatory effects and thus increase immune cell recruitment. Specific recruitment of immune cells to combat PA-induced effects represents a potential mechanism for the development of localized inflammatory responses, and eventually the systemic involvement similar to that which has been associated with idiopathic SLE.

The current study is concerned with the investigation of the molecular mechanism of PA-dependent cytotoxicity and the role of granulocytes in accentuating, or inducing these effects. Furthermore, we are predominantly concerned with PA-dependent immune cell toxicity and the associated immunomodulatory effects.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (Poole, Dorset, U.K.).

It is important to note that all reagents to which cells were exposed were tissue culture grade, and had therefore been tested for the presence of LPS.

Cell lines and antibodies. The lymphocyte cell lines CCRF-HSB2 and WIL-2NS were purchased from ICN Flow (Thame, Oxfordshire, U.K.). These cell lines were maintained by regular passage in Gibco's RPMI 1640, supplemented with 1 mM L-glutamine and 10% foetal calf serum (cell culture reagents purchased from Life Technologies Ltd, Paisley, U.K.), and were cultured at 37° in a humidified, 5% CO_2 atmosphere.

A neutralizing rabbit anti-recombinant human TNFα antibody (IgG fraction: Cat. No. T0659) and peroxidase-labelled goat anti-rabbit IgG antibody (Cat. No. B9642) were purchased from Sigma.

Isolation of cPBMNs, pPBMNs and granulocytes from fresh whole blood. cPBMNs were isolated from fresh heparinized blood by density gradient centrifugation, using Histopaque 1077 [18]. Isolation of pPBMN populations was achieved using a dual, discontinuous gradient of Histopaque 1077 and 1119, which also permitted the isolation of a highly enriched granulocyte population [18]. Purity of isolated granulocytes was assessed using light microscopy and a Leishman stain, and cells from five representative fields were examined. Granulocyte preparations were found to have less than 2.4% contamination with other cell types (at the upper confidence limit (99%)). Isolated pPBMNs were cleared of any residual adherent cells by incubating for 30 min in 5% CO_2 at 37°.

Granulocyte-mediated metabolism of PA and NAPA. Purified PA-hydrochloride was prepared fresh, at working concentraitons of between 0 and 50 μM, in RPMI 1640. Aliquots (1 mL) of PA were used to resuspend granulocyte pellets containing $1.5–2 \times 10^6$ freshly isolated granulocytes. The granulocytes and PA were incubated for 1 hr at 37°, in a humidified 5% CO_2 atmosphere. The granulocytes were then removed by centrifugation at 600 *g*, and the supernatants were used immediately. This procedure was also employed using NAPA as an alternative to PA.

Ultrafiltration of supernatants from granulocytes incubated with PA. Supernatants (1 mL) were obtained from granulocytes incubated with PA, and were serially centrifuged using Centricon (Amicon, Beverley, MA, U.S.A.) ultrafiltration devices with cut-off filters for 30,000, 10,000 and 3000 Da molecules. Centrifugation was at 800 *g* for 45 min at 4°. The fluid retained by the membrane at each stage was made up to 1 mL, and two further washing steps (800 *g*, 4°) were carried out using the same filter. The final samples obtained were all made up to 1 mL and used immediately to treat target cells.

Fluorimetric analysis of genotoxicity of PA, NAPA, and granulocyte-metabolized PA/NAPA. Aliquots (1.7 mL) of cells were obtained at a density of 2×10^6/mL in RPMI 1640, after treatment of the cells with the potentially genotoxic agents. Cells were pelleted by centrifugation at 300 *g* for 5 min. The pellets were resuspended in 1.7 mL each of isotonic solution (0.25 M mesoinositol, 10 mM sodium dihydrogen phosphate, 1 mM magnesium chloride) and lysis buffer (9 M urea, 10 mM sodium hydroxide, 25 mM CDTA, 0.1% (w/v) sodium dodecyl sulphate). Aliquots (0.4 mL) of cells were added to eight separate tubes, maintained on ice, and the DNA unwinding was induced by 0.1 mL each of alkali solutions 1 (45% lysis buffer in 0.2 M sodium hydroxide) and 2 (40% lysis buffer in 0.2 M sodium hydroxide). Two tubes were neutralized immediately (0.4 mL neutralizing solution—1 M glucose and 14 mM mercaptoethanol), two after

30 min on ice, and two more after a further 20 min incubation on ice. The remaining tubes were sonicated for 15 sec prior to neutralization. The levels of double-stranded DNA in each sample was measured using ethidium bromide fluorescence, following the protocol employed by Birnboim *et al.* [19] and incorporating the modifications described by Bhusate *et al.* [20].

Immuno-dot blotting. Aliquots (1 mL) of granulocyte-derived supernatants were vacuum blotted onto nitrocellulose filters using the Bio-Dot® apparatus (BioRad, Hemel Hempstead, Hertfordshire, U.K.). The filter was washed three times (under vacuum) using PBS containing 0.05% (v/v) Tween 20 and 2% (w/v) dried skimmed milk (PBST/milk). Seventy-five microlitres of 1:5000 rabbit anti-recombinant human TNFα (Cat. No. T0659) in PBST/milk was added to each well of the Bio-Dot® and incubated at 4° for 16 hr. The filter and well were washed three times in PBST/milk (under vacuum), before incubating with 75 μL of 1:10,000 peroxidase-labelled goat anti-rabbit IgG for 1 hr at 37°. The wells and filter were washed three times in PBST/milk prior to addition of substrate. The peroxidase substrate employed was freshly prepared diaminobenzidine (2 mg/mL) in PBS activated with 3% (v/v) H_2O_2. The substrate was incubated for 5–15 min with the filter prior to rinsing in water.

RESULTS

PA-induced strand breaks and cell death in cPBMNs

To determine whether PA was cytotoxic a preparation of cPBMNs was treated with a range of concentrations. A dose-dependent increase in cell death was observed with the most marked effect occurring at 50 μM (Fig. 1). At this concentration the maximal level of cell death was 12.0% (SD 0.9%) for a 3 hr incubation at 37°. Significant loss of cell viability was also observed for cells treated with either 10 or 25 μM PA (maximally 1.3 and 3.3%, respectively) (Fig. 1). In order to investigate the role of the aryl amine group in the observed toxicity, NAPA was incubated with cPBMNs. No significant cell death compared with the control was discernible, even at the maximum NAPA concentration (50 μM) (Fig. 1). Therefore at an equivalent dose PA was approximately 24-fold more cytotoxic than NAPA.

To determine the temporal relationship between cPBMN death and genotoxicity induced by PA we also measured the appearance of DNA strand breaks during the incubation. Comparison of the data obtained for the cytotoxicity of PA and NAPA with data relating to genotoxicity indicated that, whilst cell death was maximal after 3 hr, the induction of DNA damage (assessed as a function of the induction of strand breaks) reached a maximum 1.5 hr post-PA treatment (Fig. 2). The maximal genotoxic effect was calculated as a function of DNA unwinding, and attained a level of 4.30%/min for cells treated with 50 μM PA (Fig. 2). Furthermore, comparison of PA- and NAPA-induced genotoxicity indicated that, although both compounds were capable of inducing strand breaks, PA consistently induced twice the level observed for equivalent concentrations of NAPA (Fig. 2).

Procainamide toxicity in lymphocyte cell lines

To ascertain if the toxicity observed for cPBMNs was due to effects directly on lymphocytes and monocytes or was due to a small residual contamination with granulocytic cells, similar toxicity studies were undertaken using the lymphocyte cell lines CCRF-HSB2 and WIL-2NS. Cell death induced by PA (Fig. 3) was not significantly different from the control level (RPMI 1640 alone). However, PA maintained the ability to induce DNA strand breaks, although the level observed was significantly lower than that in cPBMNs ($P < 0.001$; data not shown). This genotoxicity was of the same order as that observed for NAPA, thus suggesting that the elevated levels of DNA strand breaks observed in PA-treated cPBMNs may be attributable to the specific *in vitro* production of a PA metabolite or the release of an immune cell derived clastogenic factor.

Genotoxicity and cytotoxicity of supernatants from granulocyte cultures incubated with PA

Given that single density gradient isolation of peripheral blood mononuclear cells leads to the co-isolation of a small population of granulocytes [18], we proposed that these cells may have been responsible for the observed toxicity in PA-treated cPBMNs. In order to investigate this, granulocytes were prepared and preincubated with PA for 1 hr. Resulting supernatants were employed to treat lymphocyte cell lines, and freshly isolated pPBMNs.

Preincubation of a freshly isolated granulocyte population (cell density 1.5–2.0 × 10^6/mL) with PA (50 μM) for one hour lead to the complete restoration of PA cytotoxicity in pPBMNs to the level observed for cPBMNs incubated with 50 μM PA (Fig. 3). Similar effects were observed for both 25 and 10 μM PA, with maximal toxicity being observed after 3 hr incubation with conditioned supernatants. Furthermore, preincubation in the presence of granulocytes led to the re-establishment of the genotoxic effects of PA, to a level comparable with that previously observed in cPBMN samples (Fig. 4), with a resultant nine-fold increase in the rate of DNA unwinding. The effect was dose dependent with proportional increases in DNA unwinding observed for 25 μM (4.6-fold) and 10 μM (1.9-fold) PA compared with untreated granulocyte supernatants.

Therefore, the *in vitro* cytotoxicity and genotoxicity of PA are attributable, at least in part, to the specific actions of granulocytes. Substituting NAPA for PA indicated that N-acetylation blocked the toxicity of PA (see Tables 3 and 4 below); it was apparent that the toxicity associated with PA was not attributable to NAPA since both the cyto- and genotoxicity were significantly greater for granulocytes incubated with PA. The decrease in toxic effects was observed at all concentrations tested, with statistically significant decreases observed for all concentrations greater than 10 μM (Tables 1 and 2).

Temporal analysis of the granulocyte-dependent PA toxicity was undertaken via the assessment of

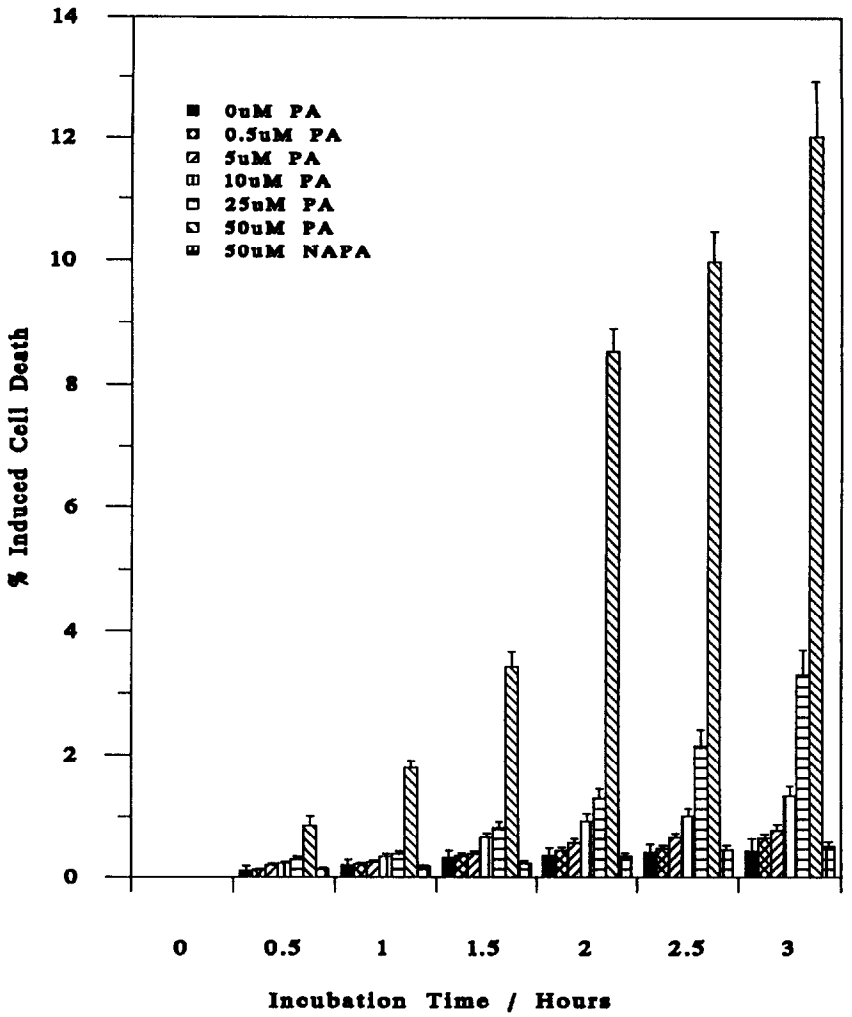

Fig. 1. Viability of cPBMNs treated with PA or NAPA. A suspension of 2×10^6/mL cPBMNs was treated with either PA (0–50 μM) or NAPA 50 μM for up to 3 hr. Samples were taken at timed intervals, mixed with an equal volume of trypan blue (0.4%) and counted in a haemocytometer. Cytotoxicity was assessed over a period of 3 hr as a function of the trypan blue inclusion. Five representative fields were counted at each time point for each sample and the percentage of cells not excluding trypan blue calculated. The results are expressed as percent increase in cell death with time. The data were obtained from 10 separate experiments, and the error bars represent a variation of 1 SD.

granulocyte supernatant toxicity at 5 min intervals post-addition of PA, for up to 60 min. There was a significant increase in supernatant cytotoxicity between 5 and 10 min, up to 3.0% cell death, followed by a gradual increase up to 40 min when cytotoxicity reached 4.0%. However a very significant increase in toxicity was observed at 40–45 min. Supernatants obtained from granulocytes incubated with 50 μM PA for 45–60 min induced up to 13.6% cell death (3 hr incubation with target cells). This data indicated a biphasic toxic effect, with initial toxicity developing during the first 10 min, and a secondary increase in toxicity presenting in the 40–60 min period (Fig. 5).

Given the biphasic temporal changes, it was postulated that at least two separate species were responsible for the total effect. Therefore, ultrafiltration techniques were employed in order to begin the biochemical characterization of the toxic agent(s).

Characterization of biochemical species responsible for granulocyte-procainamide toxicity

Initially, in order to characterize the biochemical species responsible for the toxicity of PA-treated granulocyte supernatants, samples were fractionated using molecular weight cut-off filters. Two separate fractions displayed both cyto- and genotoxic effects; one < 3000 Da fraction, and the other the $> 30{,}000$ Da fraction (Table 3). Analysis of these two fractions, obtained from granulocytes treated with 50 μM PA, showed that individually they are responsible for approx. 4.0% cell death over a 3 hr period in HSB-2 cells. However, when the two fractions were added simultaneously to target cells, the percentage cell killing increased to between

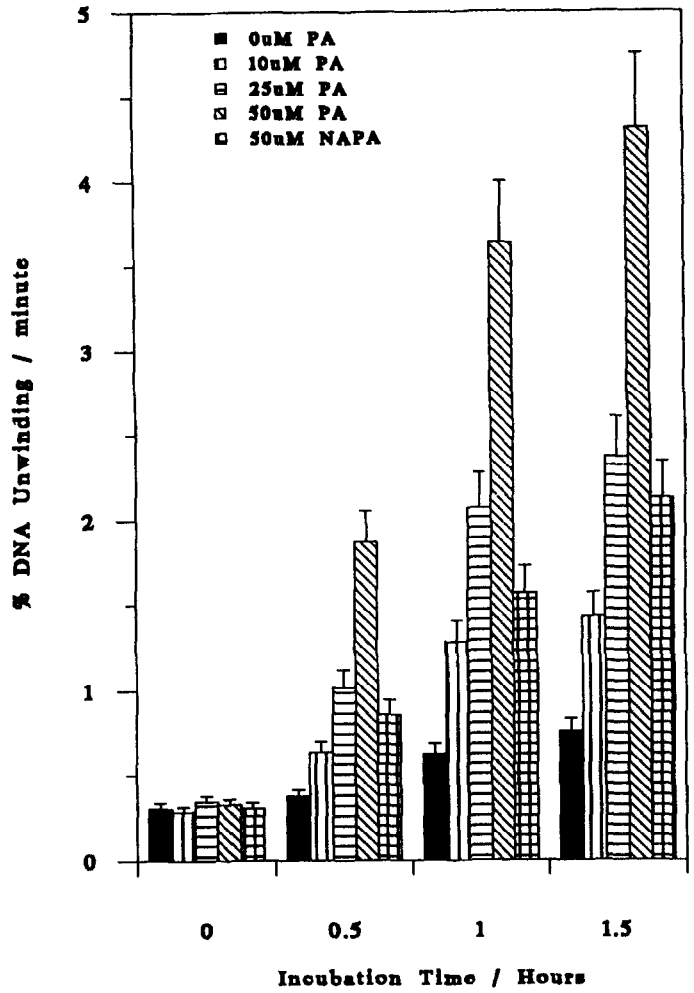

Fig. 2. Genotoxic effects in cPBMNs treated with PA or NAPA. cPBMNs were treated as described in the legend to Fig. 1 and DNA strand breaks measured as a function of the rate of DNA unwinding as detailed under Materials and Methods. The results illustrated were obtained from a series of 10 experiments undertaken in triplicate and the error bars represent a variation of 1 SD.

11.8–13.0% (Table 4). These findings suggest that a synergistic mechanism of cell killing may be occurring. Furthermore, the toxic effects were dependent on the dose of PA (Table 4). A similar pattern was observed for the genotoxic effect, with the level of DNA damage induced by a single agent being significantly less than half the total effect for dual application of the agents ($P < 0.02$). The level of DNA damage observed for the different fractions eluted was compared with control levels for untreated cells, and it was observed that in the > 30,000 Da fraction, the rate of DNA unwinding was 2.38%/min, compared with a control level of 0.36%/min (Table 3). This level was comparable to that observed for the < 3000 Da fraction, and both active fractions were significantly higher than control levels ($P < 0.001$) (Table 3). Active ultrafiltration fractions were invariably both cytotoxic and genotoxic suggesting that there was a relationship between these two processes.

Given that at least two separate agents were isolated, further characterization was required. Therefore, work was undertaken in order to establish the molecular nature of the high-molecular-weight agent. Boiling of the high molecular weight fraction, or exposure to non-specific protease activity (pronase E), led to the removal of that toxicity occurring after 40 min incubation with granulocytes (Fig. 5). Thus, we postulated that the agent(s) responsible for this portion of the cytotoxicity were proteinaceous. Therefore, investigations by denaturing SDS-PAGE and native-PAGE were undertaken in order to assess more accurately the molecular weight(s) of the agent(s). Although a number of unidentified protein species were induced in the supernatant by exposure to 50 μM PA, the predominant species, appearing 45–60 min post-exposure, had a molecular weight of 17,800 Da (SD 1000 Da) on SDS-PAGE (Fig. 6). Subsequent dot blots probed with a rabbit anti-recombinant human TNFα antibody showed the presence of TNFα at low levels from 30 min after PA was administered, and that a significant rise in

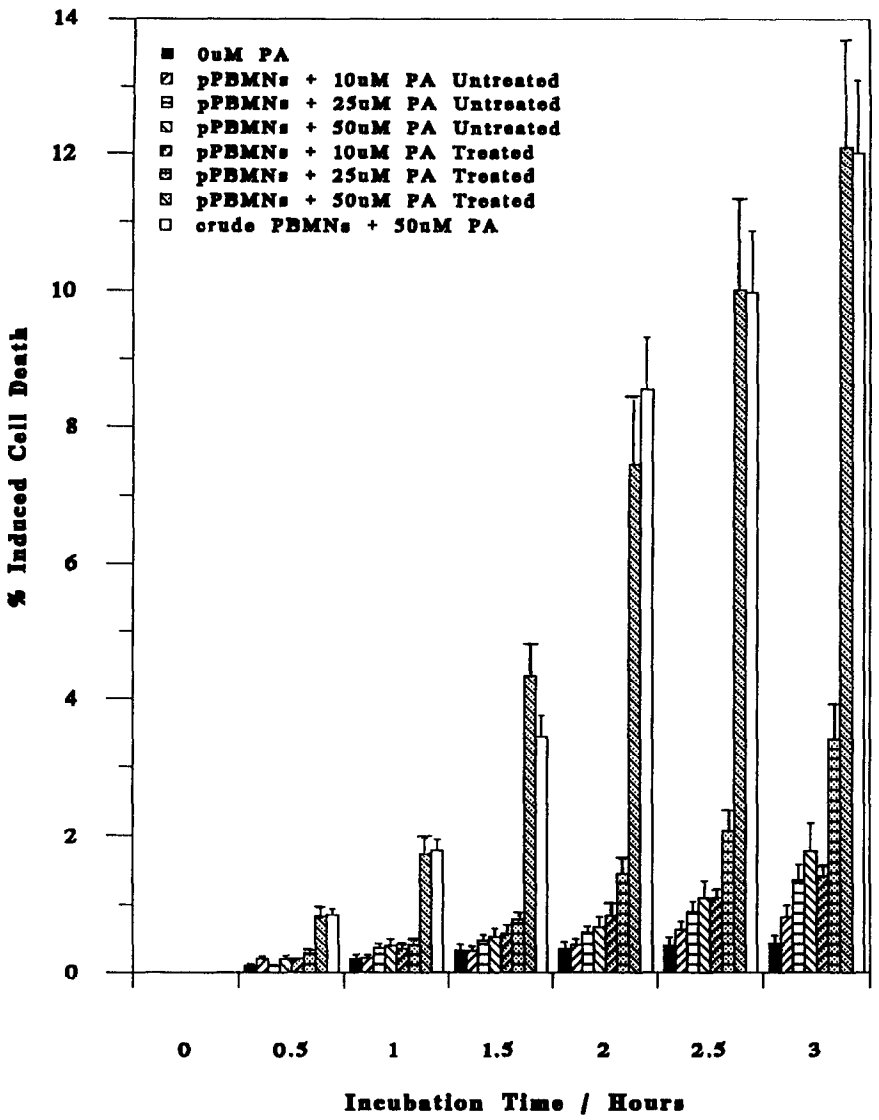

Fig. 3. Effects of pre-incubation with granulocytes on the cytotoxicity of PA and NAPA. Purified PBMNs were incubated with supernatants from PA-treated granulocytes and cytotoxicity was assessed as described in Fig. 1. Data from untreated cells and a population of crude PBMNs treated with PA is also shown. The results represent the mean of 10 experiments undertaken in triplicate. The error bars represent a variation of 1 SD.

the TNFα content of the supernatant occurred 45 min post-PA administration (Fig. 7). A dose–response for PA-induced TNFα was also discerned by dot blotting, indicating that even at 5 μM, PA is capable of inducing TNFα release (data not shown).

In order to establish that TNFα was the agent responsible for the PA-inducible toxicity, granulocyte supernatants were treated with a rabbit anti-recombinant human TNFα (IgG fraction) prior to exposure to target cells. Using this antibody in granulocyte supernatants treated with 50 μM PA resulted in partial detoxification with the level of cell death at 3 hr decreasing to 5.1% compared to 12.6% without anti-TNFα or when using a non-specific rabbit IgG as control (Fig. 8). In a similar manner anti-TNFα treatment specifically eliminated the genotoxic effects (Fig. 9; $P < 0.01$ for all concentrations).

DISCUSSION

Current understanding of the molecular mechanisms leading to the development of SLE is limited by the absence of clear data relating to the nature of the aetiologic agents responsible for its propagation. By using DIL as a model, we propose that it may be possible to determine common, specific molecular changes which precede the development of the idiopathic disease. In order to investigate this hypothesis we have studied the cyto-,

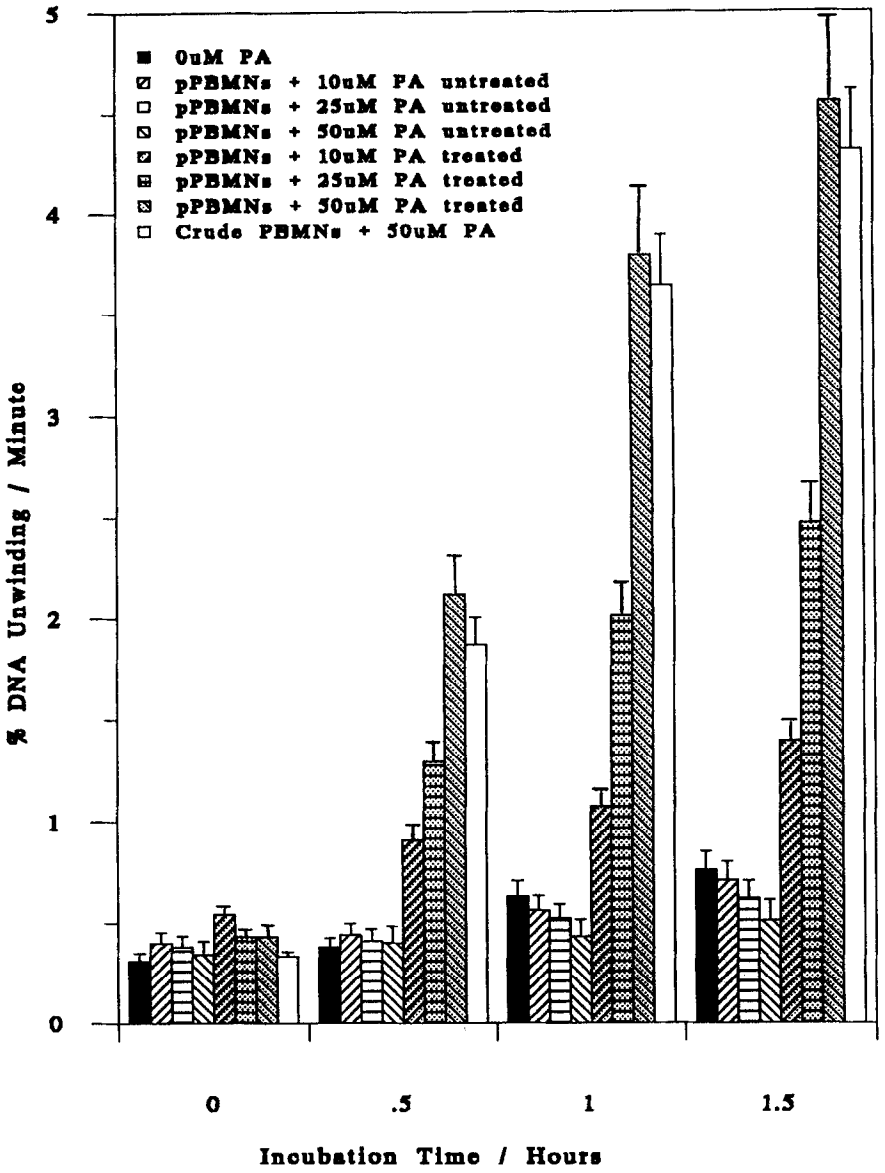

Fig. 4. Assessment of DNA strand breaks induced by PA pre- and post-incubation with isolated granulocytes. Purified PBMNs were incubated with supernatants from PA-treated granulocytes and DNA strand breaks measured according to the procedure described in Materials and Methods. Data from untreated cells and a population of crude PBMNs treated with PA is also shown. The results illustrated were obtained from a series of eight experiments undertaken in triplicate, using granulocytes isolated from five healthy volunteers. The error bars represent a variation of 1 SD.

geno-, and immunotoxic effects of PA in a range of freshly isolated human leukocyte populations and in established lymphocyte cell lines. Our studies describe the specific induction of TNFα release, from freshly isolated human granulocytes, in response to PA treatment *in vitro*. We propose this cytokine accounts for, in part, the toxic effects of PA on target lymphocytes *in vitro*.

Data on the cyto- and genotoxicity of ultrafiltration fractions indicated that although a low-molecular weight toxic agent was present (approx. < 3000 Da), a significant portion of the toxicity was attributable to a high molecular weight agent, tentatively designated to be > 30,000 Da. Further characterization of this activity led to the conclusion that 45–60 min post-PA exposure, granulocytes release the inflammatory mediator TNFα. The level detected was related to the initial concentration of PA (data not shown). Although the denaturing PAGE data implicated a 17,800 Da protein, this finding is not contradictory to the activity being present in the > 30,000 Da fraction, as TNFα activity is attributable to the activity of homodimers and homotrimers of a 17,000 Da subunit [22–24].

Table 1. Comparative cytotoxicity of PA and its metabolite NAPA following pre-incubation with granulocytes

Concentration (μM)	Cytotoxicity (% non-viable cells after 3 hr incubation) PA	NAPA	P value
0	0.7 (0.2)	0.7 (0.2)	Not significant
5	0.8 (0.2)	0.8 (0.1)	Not significant
10	1.9 (0.4)	1.2 (0.2)	<0.01
25	3.9 (0.7)	1.8 (0.3)	<0.002
50	12.0 (0.9)	2.1 (0.5)	0.0015

The data in the table represent the means of four experiments undertaken in triplicate, using 0, 5, 10, 25, and 50 μM PA and NAPA pre-incubated for 1 hr with 2×10^6 granulocytes at 37° with 5% CO_2. The table shows the mean cytotoxic effects of the two agents (SD) on cPBMNs treated for 3 hr at 37°, and shows the P values obtained for analysis of variance tests (ANOVA) using the Null hypothesis that there was no difference in cytotoxicity.

Table 2. Comparative effects on DNA strand scission of PA and NAPA following pre-incubation with granulocytes

Concentration (μM)	DNA strand breaks (% DNA unwinding min after 1.5 hr incubation) PA	NAPA	P value
0	0.43 (0.08)	0.38 (0.06)	Not significant
5	0.58 (0.14)	0.42 (0.08)	Not significant
10	1.38 (0.10)	0.92 (0.11)	<0.02
25	2.47 (0.07)	1.24 (0.13)	<0.01
50	4.38 (0.15)	2.13 (0.18)	<0.005

The data in the table represent the means of four experiments undertaken in triplicate, using 0, 5, 10, 25, and 50 μM PA and NAPA pre-incubated for 1 hr with 2×10^6 granulocytes at 37° under 5% CO_2. The table shows the mean strand breakage effects of the two agents (SD) on PBMNs treated for 3 hr at 37°, and shows the P values obtained for analysis of variance tests (ANOVA) using the null hypothesis that there was no difference in genotoxicity.

The data obtained on the *in vitro* activity of granulocytes treated with PA implicated at least two separate agents as mediators of both the cyto-, and genotoxic effects. Although we have not yet characterized the agent responsible for toxicity within the <3000 Da fraction it has been established that granulocytes metabolize PA to PAHA [9–12] which induces strand breaks in DNA. Our finding that substitution of NAPA for PA resulted in loss of toxicity suggested the effects of PA are due, at least in part, to the specific modification of the aryl amine group. This is further supported by clinical evidence which indicates that NAPA is pharmacologically active, without any of the adverse drug reaction associated with the parent compound [25]. Therefore, it is possible that the toxicity observed in the low molecular weight fraction is due to the formation of the highly reactive hydroxylamine. However, all previous studies required specific cell activation signals prior to demonstrating an ability to transform the relatively non-toxic PA into a more toxic form, and attributed all the activity observed to the generation of the reactive hydroxylamine (PAHA) metabolite. Our studies have shown that freshly isolated, non-activated granulocytes enhance PA toxicity. It is clear that a certain proportion of freshly isolated cells are activated due to the isolation procedures, and that despite all precautions, contaminating monocytes may be co-isolated. Therefore, it is possible that the effects observed are due to activated monocytes or granulocytes. However, previous studies demonstrated that despite the activating potential of Ficoll, freshly isolated monocyte and granulocyte populations exhibit only a very low level of metabolism of PA [9–12]. Furthermore, the levels of cyto- and genotoxicity observed in our study were 70% lower than those previously obtained, possibly since our studies have employed only granulocyte-derived material as opposed to purified PAHA. The administration of complete granulocyte supernatants, although less specific than single metabolites, may be more

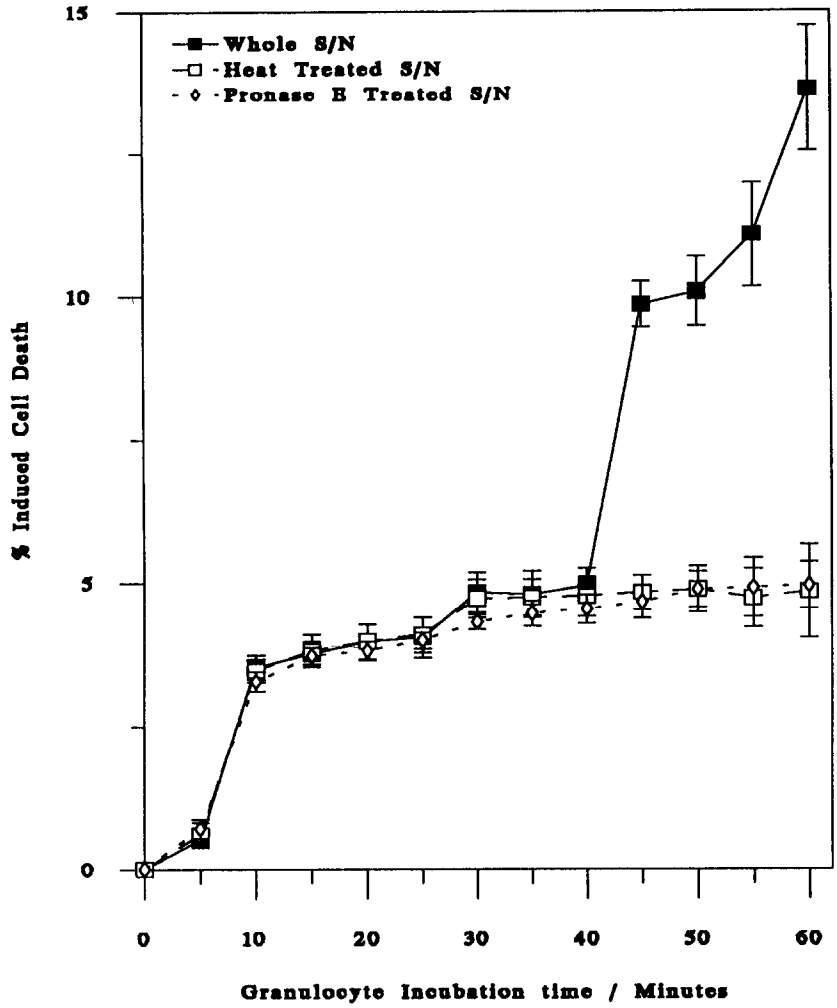

Fig. 5. Time course of granulocyte-induced PA toxicity. Aliquots of granulocyte supernatant were obtained at 5 min intervals during PA treatment and were applied to CCRF-HSB2 cells (2×10^6/mL). The induction of cell death was assessed as described in Fig. 1. The effects of heat (boiling for 10 min) and protease (Pronase E—1 mg/mL for 1 hr) treatment of granulocyte supernatants on subsequent cytotoxicity are also shown. The results illustrated represent the means of five experiments undertaken in triplicate using granulocytes isolated from five different healthy human volunteers. The error bars represent a variation of 1 SD.

Table 3. Cytotoxicity and DNA strand scission of ultrafiltered fractions from PA-treated granulocyte supernatants

Treatment	% Non-viable cells (after 3 hr)	% DNA unwinding/min (after 1.5 hr)
Control (untreated)	0.40 (0.2)	0.36 (0.03)
<3000 Da fraction	4.00 (0.2)	2.29 (0.08)
3000–10,000 Da fraction	0.45 (0.1)	0.41 (0.06)
10,000–30,000 Da fraction	0.39 (0.1)	0.39 (0.07)
>30,000 Da fraction	4.39 (0.3)	2.38 (0.09)

The table shows the data obtained from a triplicate assessment of the toxicity of the four fractions obtained by centrifugal ultrafiltration of supernatants from granulocytes (2×10^6/mL) incubated with 50 μM PA for 1 hr at 37°. The results refer to the cytotoxicity and DNA strand scission of these fractions after treatment of HSB-2 cells for 3 hr and 1 hr, respectively. A control level of background cytotoxicity has also been included. Statistical analysis was undertaken using ANOVA, and the null hypothesis that the toxicity of the fractions was the same.

Table 4. Cytotoxicity towards pPBMNs of fractionated supernatants from granulocytes treated with different doses of PA

Concentration of PA (μM)	Cytotoxicity (% cell death after 3 hr incubation)			
	<3,000 Da fraction	>30,000 Da fraction	Control (untreated cells)	PA treated cells (positive control)
0	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.2)
5	0.5 (0.2)	0.5 (0.2)	—	0.9 (0.2)
10	0.9 (0.2)	0.9 (0.3)	—	1.4 (0.2)
25	1.4 (0.2)	1.6 (0.3)	—	4.0 (0.3)
50	3.7 (0.4)*	4.3 (0.3)*	—	12.4 (0.6)

The data shown in this table were obtained from the triplicate analysis of the > 30,000 Da and < 3000 Da fractions obtained from granulocyte/PA supernatants. The cytotoxicity was analysed after a 3 hr incubation with pPBMNs at 37°, 5% CO_2 using the trypan blue exclusion assay. Also included are a control (untreated) population and a cPBMN population treated with 0, 5, 10, 25, and 50 μM PA.

* Statistical analysis of the combined effects of these fractions, compared to the positive control, indicates that the combined effect is significantly different from the sum of the effects of individual supernatants ($P < 0.002$).

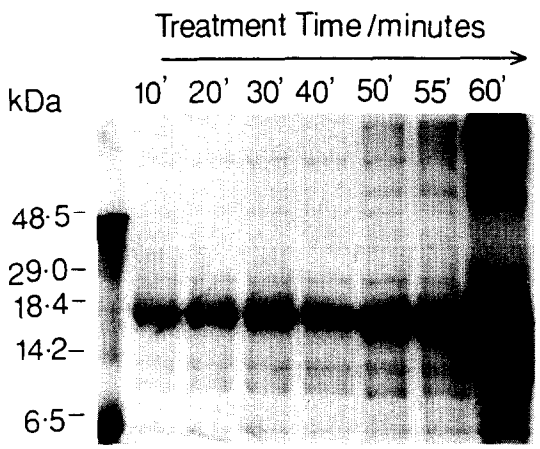

Fig. 6. SDS-PAGE analysis of proteins in granulocyte/PA supernatants. SDS-PAGE was undertaken using 9% polyacrylamide from a Protogel® stock (National Diagnostics) and following the protocol of Hames *et al.* [21]. The gel was stained using 0.5% (w/v) Coomassie blue G (Fisons, Loughborough, U.K.). The samples were derived from 50 μM PA-treated granulocytes (1.5–2.0 × 10^6/mL) at the time (min) indicated above each lane.

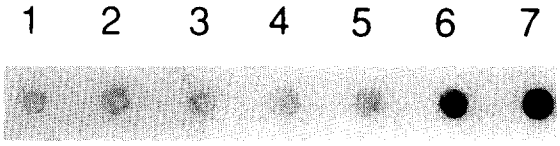

Fig. 7. Production of TNFα in granulocyte supernatants treated with PA. PA-treated granulocyte supernatants (50 μM PA, 1.5–2.0 × 10^6 cells/mL) were dot-blotted onto nitrocellulose filters and probed with an anti TNFα antibody as described under Materials and Methods. The wells 1–7 represent the time in minutes after addition of PA at which the supernatant was removed (wells 1–5 represent 10 min intervals from 10–50 min and wells 6 and 7 represent 55 and 60 min after PA addition, respectively).

representative of the situation *in vivo*, and as such may relate more closely to the immunopathological changes occurring during DIL development. These data also suggest that specific activation of granulocytes is not a prerequisite for enhancement of PA toxicity. Further work is required to determine the temporal relationship between the appearance of hypothesized PA metabolites and release of TNFα and whether PA or one of its metabolites is responsible for the TNFα release.

We have established that granulocyte-induced PA toxicity is mediated at least in part by TNFα, with the appearance of putative PA metabolite(s) being equally dependent on the presence of granulocytes. Both TNFα and PAHA induce fragmentation of cellular DNA [9, 26]. The nature of the cell death induced by supernatants from granulocytes exposed to PA is currently being examined by flow cytometry and using our newly developed determination of DNA ladder fragments [27] which is applicable to lysates of < 10^3 cells and so could be used on small numbers of lymphocytes isolated from human subjects. Although our current methodology did not permit the detection of apoptosis, it is probable that this form of cell death would occur in the presence of TNFα [28]. This is in part supported by the results obtained using the DNA strand break assay employed here, by which it was possible to detect a very high level of DNA unwinding, indicating substantial DNA strand scission, often a hallmark of apoptosis. Therefore, we speculate that, due to PA effects, excessive apoptosis results in the release of nucleosomes, with a concomitant induction of an inflammatory response to the nucleosome-DNA units. Recent studies have characterized the release of interleukin-1 and interleukin-6 as a nucleosome/

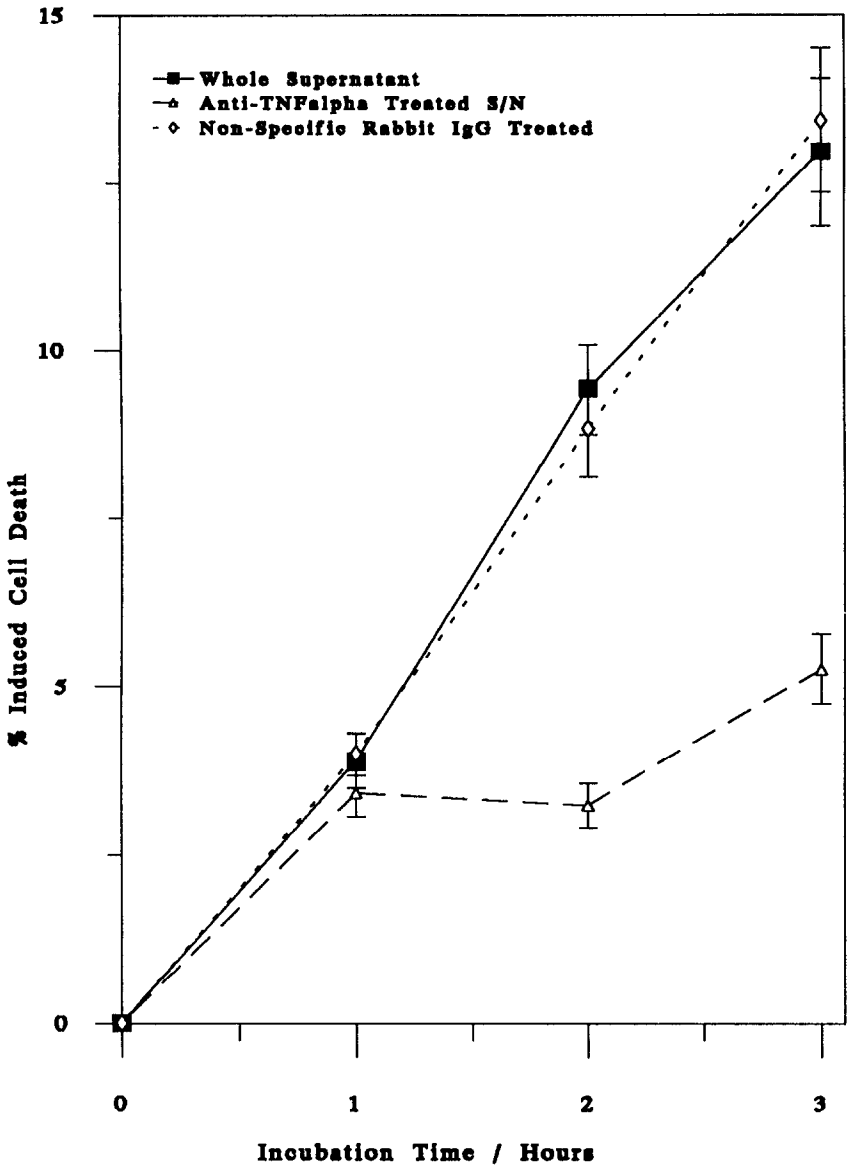

Fig. 8. Effect of treatment with anti-TNFα on cytotoxicity of granulocyte supernatants. The effect of treatment (1 hr at 37°) of granulocyte supernatants with anti-recombinant human TNFα antibody (1:500 in RPMI 1640) on cytotoxicity was determined in pPBMNs as described in Materials and Methods. The results illustrated represent the mean of five experiments performed in triplicate. The error bars represent a variation of 1 SD. A control using a rabbit IgG fraction (1:500 in RPMI 1640) is also shown.

DNA-inducible response [29]. Induction of cytokine production by these apoptotic products is feasible in SLE, as a pool of circulating nucleosomal-sized DNA fragments has been described [30]. The induction of interleukin-1 and interleukin-6 release is also attributed to TNFα; thus, the specific induction of excessive apoptosis, in association with TNFα release represents a potential mechanism for the promotion of DIL and idiopathic SLE. In addition, TNFα acts as a specific proinflammatory mediator, with the ultimate effects of inducing inflammatory infiltrates at the site of its production [31]. Thus the production of TNFα promotes the classical immunopathological changes associated with acute inflammation. Therefore, although TNFα may be an important promoting factor in the onset of drug-induced lupus, the development of a chronic inflammatory state associated with the idiopathic disease would require the sustained production of further mediators.

In summary, PA toxicity *in vitro* is dependent on the presence of a granulocyte population and the availability of the aryl amine group of the compound. The observed toxicity *in vitro* is attributable to TNFα

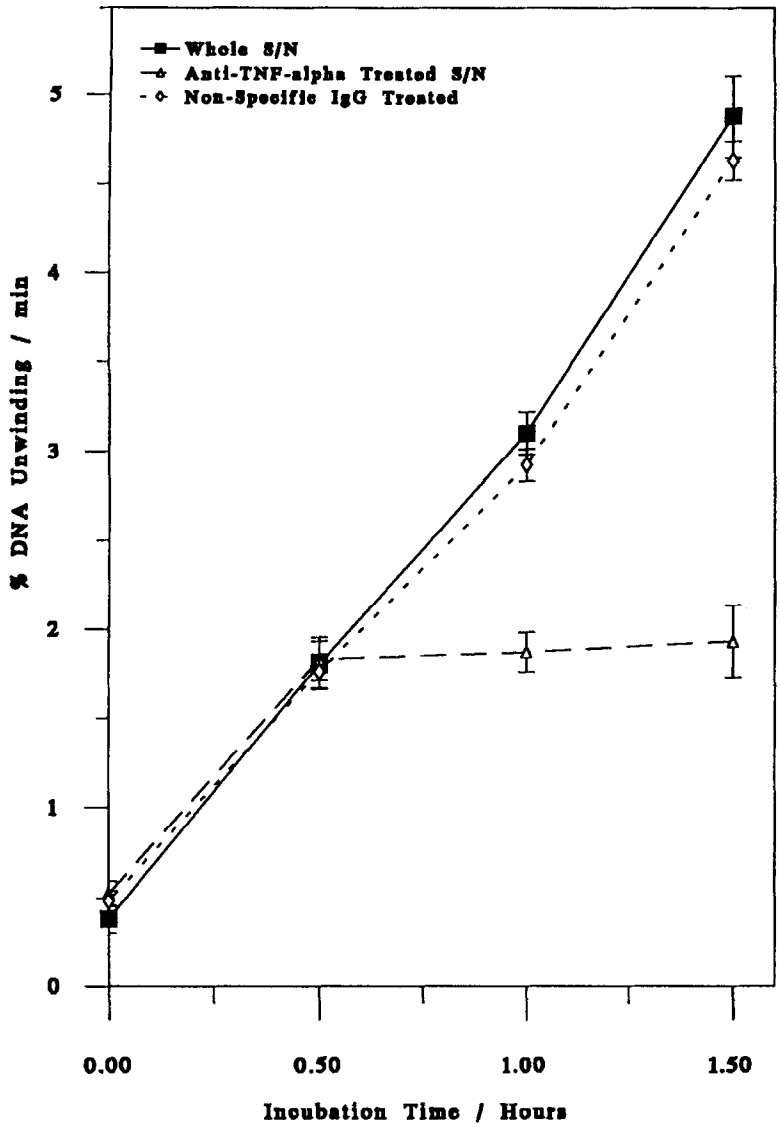

Fig. 9. Effect of treatment with anti-TNFα on DNA strand scission induced by granulocyte supernatants. The effect of treatment (1 hr at 37°) of granulocyte supernatants with anti-recombinant human TNFα antibody (1:500 in RPMI 1640) on the induction of DNA strand breaks in pPBMNs was determined as described in Materials and Methods. The results illustrated represent the mean of three experiments performed in triplicate. The error bars represent a variation of 1 SD. A control using a rabbit IgG fraction (1:500 in RPMI 1640) is also shown.

and a low-molecular-weight species (< 3000 Da). Although we have not characterized the low-molecular-weight agent, it is apparent from previous studies, that this fraction probably contains procainamide hydroxylamine produced by granulocyte-mediated metabolism [9–13]. Therefore, the existence of a bioactivating pathway, dependent on the circulating cell pool, indicates that PA toxicity *in vivo* may be due to the extra-hepatic metabolism of the drug [9–13]. Furthermore, our data implicating a PA metabolite other than NAPA in TNFα release, suggest a potential mechanism for specific drug metabolites in the promotion of DIL. Possibly, TNFα and the low-molecular weight fraction from PA-treated granulocytes exert their effects by promoting apoptosis-like DNA fragmentation.

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REFERENCES

1. Lee SL and Chase PH, Drug-induced lupus erythematosus: A critical review. *Semin Arthritis Rheum* **5**: 83–103, 1975.
2. Harmon CH and Portanova JP, Drug-induced lupus: Clinical and serological studies. *Clin Rheum Dis* **8**: 121–135, 1982.
3. Bigazzi PE, Autoimmunity induced by chemicals. *Clin Toxicol* **26**: 125–156, 1988.
4. Tan EM and Stoughton RB, Ultraviolet light alteration of cellular DNA *in vivo*. *Proc Natl Acad Sci USA* **62**: 708–712, 1969.
5. Davis P and Percy JS, Role of ultraviolet light and UV DNA in the induction of skin lesions in experimental animals. *Br J Dermatol* **99**: 201–209, 1978.
6. Landry M and Sams WM Jr, Systemic lupus erythematosus. Studies of antibodies bound to skin. *J Clin Invest* **52**: 1871–1876, 1973.
7. Alvarellos A, Ahearn JM and Provost TT, Human leukocyte antigen associations in subacute cutaneous lupus erythematosus. *J Clin Invest* **67**: 312–318, 1981.
8. Henningsen NC, Cederberg A, Hanson A and Johanson BW, Effects of long term treatment with procainamide. *Acta Med Scand* **198**: 475–482, 1975.
9. Rubin RL, Uetrecht JP and Jones JE, Cytotoxicity of oxidative metabolites of procainamide. *J Pharmacol Exp Ther* **242**: 833–841, 1987.
10. Uetrecht JP, Zahid N and Rubin RL, Metabolism of procainamide to a hydroxylamine by human neutrophils and mononuclear leukocytes. *Chem Res Toxicol* **1**: 74–78, 1988.
11. Rubin RL and Curnutte JT, Metabolism of procainamide to a hydroxylamine by neutrophils activated *in vitro*. *J Clin Invest* **83**: 1336–1343, 1989.
12. Uetrecht JP and Sokoluk B, Comparative metabolism and covalent binding of procainamide by human leukocytes. *Drug Metab Dispos* **20**: 120–123, 1992.
13. Uetrecht JP and Zahid N, *N*-Chlorination and oxidation of procainamide by myeloperoxidase: toxicological implications. *Chem Res Toxicol* **4**: 218–222, 1991.
14. Woolsey RL, Drayer DE, Reidberg MM, Carr K and Oates JA, Effect of acetylator phenotype on the rate at which procainamide induces anti-nuclear antibodies and the lupus syndrome. *New Engl J Med* **298**: 1157–1159, 1978.
15. Uetrecht JP, Reactivity and possible significance of hydroxylamine and nitroso metabolites of procainamide. *J Pharmacol Exp Ther* **232**: 420–425, 1985.
16. Uetrecht JP and Zahid N, *N*-Chlorination of phenytoin by myeloperoxidase to a reactive metabolite. *Chem Res Toxicol* **1**: 148–151, 1988.
17. Furst SM and Uetrecht JP, Carbamezapine metabolism to a reactive intermediate by the myeloperoxidase system of activated neutrophils. *Biochem Pharmacol* **45**: 1267–1275, 1993.
18. English D and Andersen BR, Single step separation of red blood cells, granulocytes, and mononuclear leukocytes on a discontinuous density gradient of Ficoll-Hipaque. *J Immunol Methods* **5**: 249–254, 1974.
19. Birnboim HC and Jevack JJ, Fluorimetric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation. *Cancer Res* **41**: 1889–1892, 1989.
20. Bhusate LL, Herbert KE, Scott DL and Perrett D, Increased DNA strand breaks from patients with rheumatoid arthritis. *Ann Rheum Dis* **51**: 8–12, 1992.
21. Hames BD and Rickwood D, In *Gel Electrophoresis of Proteins* IRL Press, Oxford, U.K., 1981.
22. Arai K, Lee F, Miyajima A, Miyatake S, Arai N and Yokota T, Cytokines: Co-ordinators of immune and inflammatory responses. *Annu Rev Biochem* **59**: 783–836, 1990.
23. Smith RA and Baglioni C, The active form of tumor necrosis factor is a trimer. *J Biol Chem* **262**: 6951–6954, 1987.
24. Eck MJ, Beutler B, Kuo G, Merryweather JP and Sprang SR, Crystallization of trimeric recombinant human tumor necrosis factor (cachectin). *J Biol Chem* **263**: 12816–12819, 1988.
25. Kluger J, Drayer DE, Reidenberg MM and Lahita R, Acetyl-procainamide therapy in patients with previous procainamide-induced lupus syndrome. *Ann Int Med* **88**: 650–658, 1981.
26. Fehsel K, Kolb-Bachofen V and Kolb H, Analysis of TNFα-induced DNA strand breaks at the single cell level. *Am J Pathol* **139**: 251–254, 1991.
27. Evans MD, Wolfe J, Lunec J, Perrett D and Herbert KE, The analysis of internucleosomal DNA fragmentation in apoptotic thymocytes by dynamic sieving capillary electrophoresis. *J Chromatogr B (Biomed Appl)* (in press): 1995.
28. Dao T, Ariyasu T, Micallef M and Minowada J, Tumour necrosis factor-α induces apoptosis in human leukaemia T cell line MOLT-16: Its relation to bcl-2 and c-myc expression. *Int J Oncol* **4**: 1371–1375, 1994.
29. Hefeneider SH, Cornell KA, Brown LE, Bakke AC, McCoy SL and Bennett RM, Nucleosomes and DNA bind specific cell surface molecules on murine cells and induce cytokine production. *Clin Immunol Immunopathol* **63**: 245–251, 1992.
30. Rumore PM and Steinman CR, Endogenous circulating DNA in systemis lupus erythematosus. *J Clin Invest* **86**: 69–74, 1992.
31. Beutler B and Cerami A, The biology of cachectin/TNF—a primary mediator of the host response. *Ann Rev Immunol* **7**: 625–655, 1989.