was observed when the antigen was histone H2B. Only three patients had detectable antibodies to both histones H3 and H4. With one exception (antibody for H4 in patient No. 18) the values were extremely low. Similarly, the response to HMG-1 and HMG-2 was also very infrequent. In contrast, 11 of the 15 patients had antibodies to chromosomal proteins HMG-17.

Grouping of the serums tested according to the clinical diagnosis (Table 2) reveals a correlation between detectable amounts of antibodies to chromosomal proteins and the diagnosis of SLE. None of the serums from the 14 patients with RA contained detectable amounts of antibodies against the seven nuclear proteins screened. Only two of the MCTD patients displayed antibody activity, and of these two, one (No. 13) had a strong response to HMG-17 and the other (No. 49) a moderate response to H2B and a weak response to H3. In contrast, 13 of the 29 SLE patients (45 percent) had antibodies against at least one of the chromosomal proteins tested, and 11 of these 13 displayed multiple specificities. Previous studies revealed that, in chromatin and nucleosomes, antigenic sites of histone H2B are more exposed to antibody binding than antigenic sites of histones H3 and H4 (*6*). Rekvig and Hannestad detected human autoantibodies that react with core mononucleosomes and provided evidence that the antigenic determinant resides in the trypsin-sensitive regions of histones H2B and H2A of the native histone octamer (*7*). Thus one of the immunogens in SLE may be the intact nucleosome. The weak response to HMG-1 and HMG-2, which presumably reside in the linker region between adjacent nucleosomes, further supports this possibility. A significant number of patients showed antibodies to HMG-17, which is found in chromatin in significantly lower amounts than HMG-1 and HMG-2 (*8*). It has been suggested that HMG-17 is associated with nucleosomes in the transcribable regions of the genome (*9*). Since HMG-17 is found only on a subset of the nucleosomes, one of the immunogens in SLE may be a subset of those nucleosomes that contain specialized DNA sequences.

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DNA Strand Breakage in Human Leukocytes Exposed to a Tumor Promoter, Phorbol Myristate Acetate

Abstract. *The phorbol myristate acetate–stimulated respiratory burst in human peripheral blood leukocytes is associated with extensive DNA strand breakage. Damage to DNA occurs before gross cellular damage is evident and may be related to the action of phorbol myristate acetate as a skin tumor promoter in animals.*

Phagocytic cells constitute a key element in the body's defense against infection (*1, 2*). In addition to the capacity for engulfing particles (phagocytosis), these cells have the capacity to produce potentially toxic active oxygen species (such as O_2^- and H_2O_2) in response to appropriate stimuli, a process termed the respiratory burst (*2, 3*). Active oxygen species kill microbes, but are also potential-

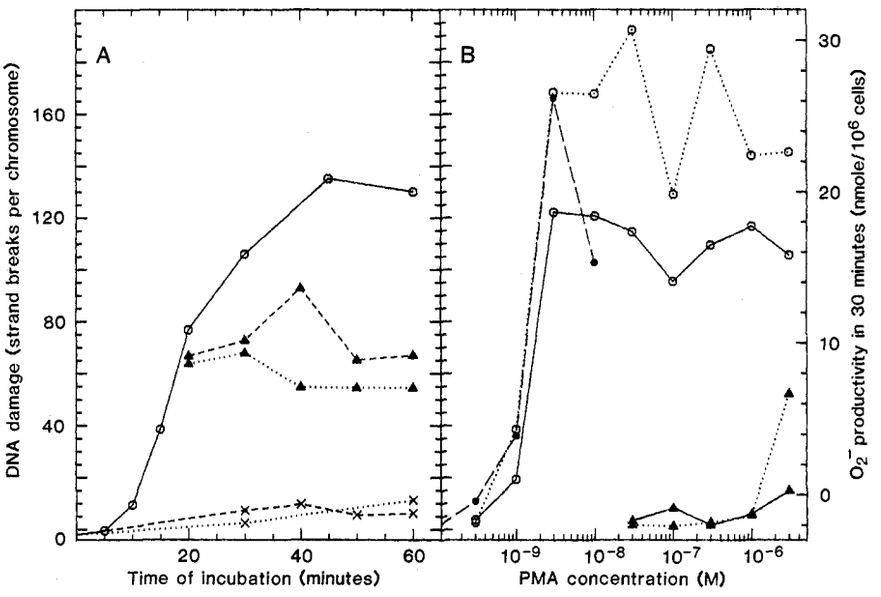

Fig. 1. (A) Time course of DNA damage after addition of PMA and effect of 2-deoxyglucose. Ten milliliters of a suspension of human leukocytes (2×10^6 per milliliter, rich in polymorphonuclear leukocytes) was incubated at 37°C with PMA (10^{-8} *M*). At the indicated times, samples were removed, chilled to 0°C, and analyzed for DNA strand break damage (○). Other samples were treated in a similar fashion, except that 2-deoxyglucose (1 m*M*) was added at 0 time (x) or at 20 minutes (▲), and fructose was substituted for glucose in the incubation medium. DNA strand break damage was determined as described in (*10*). The number of induced strand breaks was calculated by reference to the effect on DNA unwinding rate of ^{60}Co gamma rays and the assumption that 38 mGy causes 46 single-strand DNA breaks per cell or an average of one break per chromosome. (B) DNA damage with (○) PMA and (▲) PMA-ME, and (●) O_2^- production in human leukocytes exposed to PMA. Cells were incubated at 37°C with the indicated concentrations of PMA or PMA-ME for 40 minutes to assess DNA damage as in (A), and for 30 minutes to monitor O_2^- as the superoxide dismutase–inhibitable reduction of cytochrome C. The solid and broken lines indicate results with blood from two different donors; O_2^- production was measured in a third donor.

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ly autotoxic. For example, human polymorphonuclear leukocytes are killed when exposed to a particulate stimulus (*4*) or to the soluble agent phorbol myristate acetate (PMA) (*5*). Cell death, as judged by trypan blue staining or release of ^{51}Cr, occurred over a 15- to 30-hour period for the particulate stimulus or after 1 to 4 hours when PMA was used. High concentrations of catalase prevented death in both cases, but superoxide dismutase was protective only with the particulate stimulus. It is generally assumed that O_2^-, such as is generated by phagocytic cells, is an important effector of tissue damage, possibly because it can generate a reactive free radical, OH·. Biochemical studies with living cells have dealt primarily with damage to unsaturated fatty acids in cell membranes. We now report that active oxygen species generated during the respiratory burst can also rapidly lead to extensive DNA damage in situ.

Phorbol myristate acetate, in addition to being a stimulator of the respiratory burst in phagocytic cells, is a potent tumor promoter and an inflammatory agent when applied to skin. We and others have considered the possibility that these three actions of PMA may be connected (*6, 7*). Numerous other hypotheses have been proposed for the action of PMA because it causes a large number of effects on animals and cultured cells (*8*). One possible mechanism linking inflammation, phagocytic cells, and tumor promotion could be that application of PMA to skin that has been exposed to a carcinogen causes inflammation and then stimulation of the respiratory burst in phagocytic cells which migrate to the inflamed area. Active oxygen species generated by these cells could then act on the DNA of "initiated" cells, and this insult to the genome could cause expression of the tumor phenotype, perhaps by causing rearrangements of the genetic material (*9*). Such a hypothetical model requires a demonstration that DNA damage is produced in mammalian cells by products of the respiratory burst. The availability of a new, sensitive assay for detecting DNA strand break damage in leukocytes (*10*) allowed us to test this aspect of the model.

White blood cells enriched in polymorphonuclear leukocytes were isolated from EDTA-anticoagulated blood of healthy human volunteers (*11*). When these cells were incubated at 37°C with a low concentration of PMA ($10^{-8}M$), DNA strand breaks rapidly ensued (Fig. 1). Damage was detectable after a 5-minute lag and appeared to reach a maximum after 40 to 45 minutes. The level of damage was extensive, comparable in number of strand breaks to irradiation by 4 to 6 grays (400 to 600 rads) of ^{60}Co gamma rays. However, microscopic examination of cells for up to 60 minutes after PMA addition revealed no difference from control cells; the number staining with trypan blue was the same and ranged from 8 to 21 percent in different experiments. These values are similar to those of Tsan (*5*). Damage to DNA induced by PMA could be blocked by holding the temperature at 0°C (data not shown) or by addition of the inhibitor 2-deoxyglucose (in glucose-free medium) at the time of PMA addition (Fig. 1A). When 2-deoxyglucose was added 20 minutes after PMA addition, little or no further DNA damage occurred, and the previous damage remained constant or declined slightly. 2-Deoxyglucose inhibits O_2^- production in PMA-stimulated human polymorphonuclear leukocytes (*12*), presumably by affecting NADPH production (NADPH, reduced form of nicotinamide adenine dinucleotide phosphate) via the hexose monophosphate shunt. The relative stability in the number of induced breaks in cellular DNA observed after 2-deoxyglucose addition is consistent with the microscopic evidence that gross cellular disruption does not precede the observed DNA damage.

A dose-response curve for DNA damage and for O_2^- production is shown in Fig. 1B. Cells were incubated with PMA or with a nonactive analog, phorbol myristate acetate methyl ether (PMA-ME) (*12*). After 40 minutes, DNA damage was assessed by the FADU procedure (fluorometric analysis of DNA unwinding) (*10*). The results of an experiment using two different donors are shown in Fig. 1B. In both cases, considerable DNA damage was observed. There was a fairly sharp threshold for PMA action between $1 \times 10^{-9}M$ and $3 \times 10^{-9}M$. Below this level, no strand breakage was detectable, and above this level, little additional breakage was observed. At concentrations up to $10^{-6}M$, PMA-ME had no effect. The respiratory burst, as judged by O_2^- production, exhibited a very similar threshold. When other cell types (mouse thymocytes or mouse erythroleukemia cells) were exposed to PMA ($10^{-8}M$ to $10^{-7}M$) under similar conditions, no DNA damage was observed (data not shown).

Table 1. Influence of various enzymes and free radical scavengers on PMA-induced damage in human peripheral blood leukocytes. Cells were isolated and incubated with PMA ($10^{-8}M$) at 37°C in the presence of the indicated agent. DNA damage due to PMA alone (expressed as the number of DNA strand breaks per chromosome) is shown in parentheses. Damage caused by the other agents alone was small (less than 10 breaks per chromosome) and was subtracted from each result. For the experiments with enzymes, cells were first incubated for 10 minutes with the enzyme and then for 40 minutes after PMA was added. For the experiments with scavengers, the preliminary incubation was for 20 minutes in experiments 1 and 3 and for 10 minutes in experiment 2. Incubation after addition of PMA was for 30 minutes in experiment 1 and for 40 minutes in experiments 2 and 3. In experiment 3, glucose was omitted from the incubation medium, and blood pooled from four donors was used for the preparation of leukocytes.

Agent added with PMA	Relative DNA damage (%)		
	Experiment 1	Experiment 2	Experiment 3
Enzymes			
None	100 (162)	100 (130)	
Catalase* (50 μg/ml)	62.4	53.6	
Catalase (250 μg/ml)	57.2	43.4	
Boiled catalase (250 μg/ml)	106.0	118.1	
Superoxide dismutase† (50 μg/ml)	66.7	82.9	
Superoxide dismutase (200 μg/ml)	38.8	48.2	
Boiled superoxide dismutase (200 μg/ml)	81.8		
Free radical scavengers			
None	100 (160)	100 (164)	100 (101)
Sodium benzoate (19 m*M*)	94.8		
Sodium benzoate (24 m*M*)		74.0	
Glycerol (70 m*M*)			89.7
Glycerol (106 m*M*)	98.7		
Sodium formate (50 m*M*)			117.0
Ethanol (85 m*M*)			111.0
m-Inositol (50 m*M*)			105.5
DMSO (70 m*M*)		82.8	
DABCO (10 m*M*)		80.8	
Tryptophan (10 m*M*)		114.0	

*Catalase was purchased from Sigma (17,000 U/mg). †Superoxide dismutase (Orgotein) was purchased from Diagnostic Data, Mountain View, California.

Evidence that products of the respiratory burst cause DNA damage is provided by the data of Table 1. The enzymes superoxide dismutase and catalase, which remove O_2^- and H_2O_2, respectively, had a pronounced but not complete inhibitory effect on PMA-induced DNA damage. Boiled enzymes did not protect the cells. High levels of enzyme were necessary to afford protection, as had been shown when cell damage was monitored by trypan blue staining (*4*). Although both O_2^- and H_2O_2 seem to be necessary for damage to occur (that is, protection is afforded by either superoxide dismutase or catalase), the mechanism of DNA damage has not been elucidated. Hydroxyl radicals are very reactive species and likely to be generated by O_2^- and H_2O_2, but OH· scavengers were ineffective in preventing damage. Similarly, the singlet oxygen scavengers diazabicyclooctane (DABCO) and tryptophan were ineffective. At high levels of dimethyl sulfoxide (DMSO) (0.5*M*), damage was decreased, but O_2^- production was also decreased, so that interpretation of the action of DMSO was uncertain (*7*). Thus, the actual sequence of events leading to DNA damage requires further investigation.

A number of different donors were examined to assess variability in the level of PMA-induced DNA damage in leukocytes. After cells were incubated for 40 minutes at 37°C with 10^{-8}*M* PMA, the measured amount of damage (expressed as induced DNA strand breaks per chromosome) was 148.7 ± 24.4 percent (average ± standard deviation) for 16 blood samples from 12 donors over a 2-month period. Variation between duplicate analyses of the same sample was on average only 4.3 percent, indicating that differences seen between individuals was considerably greater than experimental variability.

These results show that exposure of phagocytic cells to PMA can lead to extensive DNA damage through active oxygen species and also indicate that phagocytic cells may play a role in PMA-induced skin tumor promotion. According to this model, a tumor initiator such as 7,12-dimethylbenz[*a*]anthracene is responsible for a mutation in a critical gene of a skin cell. Subsequent repeated applications of PMA lead to inflammation, migration of phagocytic cells to the area, and then triggering of these cells to produce DNA-damaging oxygen radicals. A combination of mutational damage by the initiator and strand break damage by the promoter may be necessary for the eventual development of a malignant tumor. Benzoyl peroxide, recently shown to be a potent tumor promoter (*13*), can directly cause DNA strand break damage in human leukocytes (*7*).

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11. Red blood cells were lysed with ammonium chloride (*10*) and white blood cells were collected by centrifugation at 0°C. The cell pellet was washed twice with cold balanced salt solution and suspended at a final concentration of about 2×10^6 cells per milliliter at 0°C. The balanced salt solution contained (in m*M*) NaCl, 137; KCl, 5; $MgSO_4$, 0.8; Na_2HPO_4, 8.5; and glucose, 5 (at a final *p*H of 7.40). The cell suspension was rich in polymorphonuclear leukocytes and depleted in lymphocytes, relative to the starting blood sample. The range in cell composition in isolated white blood cells from four donors was 65 to 79 percent polymorphonuclear leukocytes, 14 to 25 percent lymphocytes, less than 10 percent monocytes and eosinophils, and less than 30 red blood cells per 100 white blood cells.
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Norethisterone, a Major Ingredient of Contraceptive Pills, Is a Suicide Inhibitor of Estrogen Biosynthesis

Abstract. *Norethisterone (17α-ethynyl-19-nortestosterone) is an effective irreversible inhibitor of estrogen synthetase (aromatase), the enzyme responsible for the conversion of androgens to estrogens, even at a* 2×10^{-6} *molar concentration. This irreversible inactivation, which is directed toward the active site of aromatase and requires the cofactor-reduced nicotinamide adenine dinucleotide phosphate, is both time- and concentration-dependent. Ethisterone (17α-ethynyltestosterone), in contrast, is not a suicide inhibitor of aromatase even at concentrations of* 10^{-4} *molar.*

Norethisterone (NET) is widely used as the active ingredient in oral contraceptives (*1*). Even though the compound is taken by humans over prolonged periods, little information is available on the mechanism of its activity. In particular, there is no information on the irreversible inactivation of aromatase by NET. A high concentration (1 m*M*) of NET covalently modifies rat liver cytochrome P-450 (*2, 3*), but shows no competitive inhibitory action on microsomal aromatase at 780 μ*M* (*4*).

Development of an effective irreversible inhibitor of estrogen biosynthesis is the goal of many researchers. Such an inhibitor is sought for possible chemotherapeutic use in estrogen-dependent mammary cancers and also to aid in elucidating the mechanism of the aromatase reaction (*5–11*).

We have studied the effect of NET on the aromatase activity of human placental microsomes using the following procedure. A known amount of the inhibitory compound was incubated with lyophilized human placental microsomes (4 mg/ml) (*12*) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (0.5 mg/ml) in 0.067*M* phosphate buffer (*p*H 7.49) in a total volume of 10 ml, the mixture being placed in a Dubunoff shaker at 37°C. At specific time intervals a 0.5-ml portion was assayed for aromatase activity. The activity was determined by means of a radiometric assay in which we used [1β-^{3}H,4-^{14}C]androstenedione as substrate and measured the ^3H_2O produced (*5, 12*). A portion of the mixture together with [1β-^{3}H,4-^{14}C]androstenedione (10.5 μ*M*, 1.33×10^7 disintegrations per minute per micromole for ^{3}H and 2.05×10^5 dis/min-μmole for ^{14}C) was incubated with 0.5 mg of NADPH in 0.067*M* phosphate buffer at a total volume of 2.0 ml for 10 minutes. The logarithmic percentage of aromatase activity was plotted against time for each run (Fig. 1) (*13*).

We found that NET caused a time- and dose-dependent irreversible inhibition of aromatase (Fig. 1B) at concentrations of

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