Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2537–2540, April 1981
Medical Sciences

Mechanism of photosensitivity in systemic lupus erythematosus patients

(clastogenic agent/near-UV light/superoxide dismutase)

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Communicated by Pierre A. Joliot, December 3, 1980

ABSTRACT **Patients who have systemic lupus erythematosus have increased numbers of chromosome breaks and rearrangements correlated with a low molecular weight chromosome-damaging agent that is released from their lymphocytes into the serum. This clastogenic factor also produces chromosome breaks and sister chromatid exchanges in healthy persons' lymphocytes when they are incubated in the presence of lupus patients' serum or lymphocytes or purified factor. The lymphocytes from lupus patients are sensitive to near-UV (360- to 400-nm light. This sensitivity seems to be related to the presence of the clastogenic factor in these cells; lymphocytes of healthy persons exposed to the factor also become sensitive to light of the same wavelengths. A significant increase in nonviable cells (trypan blue exclusion test) was observed after 5 min of irradiation with 360- to 380-nm light in the presence of the factor. The number of chromosome aberrations observed after stimulation of the irradiated lymphocytes with phytohemagglutinin was also maximal after irradiation at 380 nm in presence of the factor. The combined action of near-UV light plus clastogenic factor was inhibited by superoxide dismutase if the enzyme were present during irradiation, suggesting that activation involves photoproduction of superoxide ions. Irradiation of the purified factor and immediate addition of it to lymphocytes gave the same results whereas preirradiation of cells or of medium was without effect. The presence of this photoactivated agent explains why patients who have lupus erythematosus show an aggravated condition after exposure to sunlight and the appearance of typical skin lesions.**

Genetic, immunological, and viral factors are involved in the origin of systemic lupus erythematosus (SLE), considered as the prototype of human autoimmune disease. Lupus patients are photosensitive and develop a facial rash, the typical "butterfly lesion," after exposure to sunshine. The systemic manifestations of the disease are also aggravated by sunlight. SLE has been claimed to be a DNA-repair-deficiency disease by Beighlie and Teplitz (1), but this is contested by others (2, 3). We here present data that could provide another explanation for the photosensitivity of lupus patients.

Previous work in our laboratory has shown that many human diseases involving autoimmune reactions and also the murine autoimmune disease of New Zealand black mice are accompanied by chromosomal instability (4–7). The increase in chromosome breaks and rearrangements in patients who have lupus erythematosus (8) and progressive systemic sclerosis (9), as well as in New Zealand black mice (10), is correlated with a <10,000-dalton chromosome-breaking agent. This clastogenic factor (CF) also produces chromosomal breakage and an increase in sister chromatid exchanges in healthy persons' lymphocytes when they are incubated in the presence of patients' serum or the purified clastogenic agent. The exact nature of the CF is not yet determined, but the breakage phenomenon seems to be related to the generation of activated oxygen species because superoxide dismutase prevents the production of chromosome damage (7–11). We have generated the superoxide anion radical $O_2^{\cdot-}$ photochemically in the culture medium by adding flavin mononucleotide and irradiating with 365-nm light. This method consistently gave an increase in chromosome breaks and rearrangements in blood cultures from healthy subjects, individual results being 20–25 aberrations per 100 mitotic figures.‡ When we attempted to use this method to study the influence of $O_2^{\cdot-}$ in blood cultures of lupus patients, the cultures did not grow. Light alone did not significantly increase the aberration rate in blood cultures from healthy persons at the doses used but inhibited growth of blood cultures from SLE patients. This observation prompted us to study in detail the photosensitivity of lymphocytes from SLE patients. The results confirm that, in contrast to normal lymphocytes, SLE lymphocytes are sensitive to 360- to 400-nm light. This sensitivity seems to be related to the presence of the CF in these cells; when exposed to the purified factor, lymphocytes of healthy subjects become sensitive to UV light of the same wavelengths.

MATERIAL AND METHODS

The criteria of the American Rheumatology Association were adopted for diagnosis in the seven SLE patients studied. Therapeutic agents that could produce chromosome damage had not been administered. The blood samples were obtained before x-ray investigations.

Healthy blood donors were recruited among the laboratory and hospital personnel.

The degree of chromosomal instability was studied on blood cultures from SLE patients, and the clastogenic activity of their sera was determined on blood culture preparations from healthy donors. The aberration rate in the cultures exposed to the CF was compared with that in simultaneous untreated cultures from the same person. All cultures were set up in duplicate.

The CF used was prepared as described (8): An ultrafiltrate of serum from SLE patients was obtained by filtration through Diaflo filter UM 10. Because the clastogenic agent is retained by filter UM 2, this filter was used for concentration of the UM 10 ultrafiltrate. The concentrated ultrafiltrates from several patients were pooled and further purifted by Sephadex gel filtration. The clastogenic effect of the purified fractions was tested on blood cultures from healthy donors. Because the activity varied among the patients, different quantities of the pooled CF

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Abbreviations: SLE, systemic lupus erythematosus; CF, clastogenic factor; P_i/NaCl, phosphate-buffered saline; SOD, superoxide dismutase.

‡ Emerit, I. & Michelson, A. M., Compte Rendu Sixth International Congress on Radiation Research, Tokyo, Japan, 1980.

samples were tested to determine the quantity that would produce a significant increase in aberrations but not be toxic enough to prevent culture growth. These quantities were 5–10% of the culture medium. For comparison of the effects of different wavelengths of light the same CF preparation was always used.

The influence of UV light on lymphocytes of SLE patients and lymphocytes of healthy persons exposed to the purified CF was studied in the following way.

1. Lymphocytes from SLE patients and from healthy persons were obtained by differential centrifugation on Ficoll–Isopaque (Nyegaard, Oslo). A total of 2×10^6 cells was suspended in 1 ml of phosphate-buffered saline (P_i/NaCl) (pH 7.2) and irradiated with light of various wavelengths with a monochromator. The duration of irradiation was 10 min in the absence of the CF and 5 min in its presence. The dose rates were approximately 1 $\mu W/cm^2$. The viability of the lymphocytes was examined before and 5 min and 2 hr after irradiation by using the trypan blue exclusion test. One drop of the lymphocyte suspension was mixed with one drop of a 0.1% trypan blue stain, and 200 lymphocytes were counted in a hemocytometer and those accepting the dye were scored.

2. After irradiation, the lymphocytes of three of the patients and the lymphocytes of the healthy persons were incubated for 3 days in tissue culture medium 199 (Flow Laboratories, McLean, VA) supplemented with 20% autologous serum or AB serum from the Blood Center. Cell division was obtained by addition of phytohemagglutinin M and P (Difco). The mitoses were blocked in metaphase by colchicine. After a hypotonic treatment (75 mM KCl) for 10 min, the cells were fixed in ethyl alcohol/glacial acetic acid (3:1). Iced wet slides were used for spreading of the mitoses, which were stained with Giemsa reagent. The mitoses were examined under the microscope on coded slides.

3. Whole blood (0.5 ml) or isolated lymphocytes suspended in 5 ml of culture medium was irradiated as a thin layer in 5-cm-diameter Petri dishes under a Mineralight 100 B lamp having an emission at 365 nm. The UV dose was measured with a Mineralight photometer and was 1000 $\mu W/cm^2$. The effect of the CF was compared with and without irradiation. In some experiments, it was added immediately before irradiation of the cells and, in others, it was added immediately or 30 min after. The anticlastogenic effect of superoxide dismutase (SOD) was studied by addition of bovine Cu–SOD (0.05 mg/ml) to the culture medium. The SOD was either present during irradiation or added 30 min after. In another approach, the purified CF was irradiated in the culture medium for 5 min and then immediately added to normal lymphocytes (precentrifuged to avoid dilution).

4. The purified CF was irradiated in P_i/NaCl in the absence or presence of SOD (50 μg/ml).

The cytogenetic procedures were the same as described for lymphocyte cultures. In all cases in which SOD was used, it was present at 50 μg/ml.

RESULTS

All seven SLE patients studied showed increased chromosome breakage, and the ultrafiltrates of their sera had clastogenic properties. The results of the trypan blue viability tests after irradiation of the patients' lymphocytes are shown in Table 1. In all seven patients, an increase in nonviable cells was observed after irradiation of the lymphocytes with monochromatic light for 10 min. The maximum number of stained cells was observed at 360–400 nm (peak ≈380 nm).

After irradiation with near-UV light, the lymphocytes of three patients were incubated for 3 days for cytogenetic analysis. Irradiation at 320 nm had no effect and the aberration rate was the same as in the nonirradiated culture; however, at longer wavelengths (360–400 nm), culture growth was poor and, at 360 nm, the breakage rate was increased significantly [e.g., from 24% to 34% (patient E) and from 26% to 42% (patient F)].

When lymphocytes from healthy persons were irradiated in the presence of the CF, an increase in nonviable cells was observed after 5 min of exposure, with a maximum at 360–400 nm. Two hours later, the trypan blue test showed a considerable increase in nonviable cells for all wavelengths compared with the lymphocytes irradiated in the absence of CF (Table 2). The increase was maximal at 380 nm. Exposure of the lymphocytes to the CF in the absence of UV light also resulted in an increase in the number of damaged cells (to 21.8%) that, 2 hr later, was comparable with that obtained by irradiation at wavelengths of <340 nm or >420 nm. Thus, irradiation at these wavelengths did not influence the damage produced by the CF. Clearly, irradiation at 254 nm did not increase the toxicity of the factor.

Analysis of the chromosome preparations obtained after incubation of the surviving and phytohemagglutinin-stimulated lymphocytes confirmed the observations of the trypan blue test: The number of chromosome aberrations in 100 mitotic figures was maximal after irradiation of the cells with 380-nm light in presence of the CF (Table 3). The aberration rate in these cul-

Table 1. Sensitivity of SLE and normal lymphocytes to near-UV light

Wavelength, nm	Viable cells, no.													
	SLE patient								Healthy persons					
	A	B	C	D	E	F	G	Mean	m-1	m-2	m-3	f-1	f-2	Mean
254	4	16					5							
300	4	10		6		3	6	5.8	4	3	3	2	5	3.4
320	6, 5	6	6	18	7	6	4	7.3	2	6	3	5	5	4.2
340	6	6		8				6.7	6	2	4	4	4	4.0
360	12, 22	26	10	36	13	10	6	16.9	7	5	4	1	4	4.2
380	8	44	22	14	16	6	14	17.7	6	3	3	4	10	5.2
400	5, 5	24		8	25		7	12.4	6	4	3	8	4	5.0
420	4	10	10	6	9	2	5	6.6	8	5	6	4	4	5.4
440	2	10		17			5	8.5	6	3	5	5	5	4.8
460	2	8	14	7				7.2	4	2	4	8	4	4.4
480	2	6		11				6.3	5	7	4	4	2	4.4
Control*	6, 4	1	6	3	2	2	3	3.4	3	2	3	3	2	2.6

Data represent numbers of stained lymphocytes among 200 cells counted 5 min after irradiation.
* Absence of light.

Table 2. Sensitivity of normal lymphocytes to near-UV light in the presence and absence of the CF

Wavelength, nm	Viable cells (after exposure), no. With CF 5 min	With CF 2 hr	No CF (2 hr)
254	6.0	20.0	6.2
300	6.2	23.0	4.6
320	3.8	16.3	6.4
340	4.8	34.6	5.8
360	7.4	23.5	5.0
380	11.4	49.5	6.2
400	13.5	28.5	6.6
420	8.2	24.5	5.3
440	3.2	15.5	4.5
Control*	7.0†	21.8	

* Absence of light.
† Thirty minutes after exposure.

Table 3. Chromosome aberrations after irradiation

Wavelength, nm	Aberrations, no. per 100 mitotic figures: Irradiation only	Irradiation + CF
254	4	19
300	8	13
320	8	11
340	12	16
360	5	20
380	7	32*
400	6	22
420	11	12
440	13	17

Lymphocytes (2×10^6) suspended in 1 ml of P_i/NaCl were irradiated for 5 min. The number of chromosome aberrations in 100 mitoses studied is maximal after irradiation by 380-nm light in the presence of the CF. Data are mean of two or three experiments. In the absence of UV, CF alone gave a mean aberration rate of 11.8 (eight experiments).
* Mean of eight experiments (comparison of 380 nm with other wavelengths).

tures was 16–18 per 100 mitotic figures. Since the CF was no longer present after transfer of the lymphocytes from the P_i/NaCl to the culture flasks, the number of aberrations in unirradiated lymphocytes exposed to the same quantity of CF in P_i/NaCl for 30 min was also determined. The aberration rate was 11.8 (mean of eight cultures), which is lower than that after exposure to the CF during the entire cultivation period but higher than that of control cultures. Irradiation of the lymphocytes at different wavelengths without addition of the CF resulted only in a slight increase of the aberration rate.

The results obtained by irradiation of normal whole blood at 365 nm (Mineralight) are shown in Table 4. When the CF was added to the culture medium without irradiation, it produced 12–16 aberrations per 100 mitotic figures; In experiment 6, a double dose produced 26 aberrations per 100 mitotic figures. Again, irradiation at 360–380 nm increased the number of aberrations but only when the CF was present during irradiation and not when it was added 30 min later. Irradiation in the absence of CF did not increase the aberration rate. The combined action of UV light plus CF was inhibited by addition of SOD. However, the exogenous SOD must be present during irradiation; the protective effect is not observed if it is added 30 min after exposure. The same results were obtained with isolated lymphocytes. Irradiation of lymphocytes alone for 5 min at 365 nm (1000 $\mu W/cm^2$) caused no significant damage and addition of the CF to nonirradiated normal lymphocytes caused an average of 20.4% breakages (14.3, 22.9, 24.1) in three experiments, whereas irradiation of lymphocytes in the presence of the CF increased this figure to 32.5%. Again, this increase occurred only if the CF was present *during* irradiation and not if it was added immediately after or 30 min later. Because irradiation of the lymphocytes (or of the medium) followed by addition of the CF did not show this increase in the number of aberrations, the CF was irradiated in culture medium under the same conditions (5 min) and immediately added to the packed lymphocytes. The increase in chromosome breaks (average of six experiments) was 33.95% (27.3, 28.6, 33.3, 18.8, 50.0, 45.7).

Similar results were obtained when purified CF was irradiated in P_i/NaCl and then added to the cells in culture (Table 5, cultures 1 and 2) where, again, the presence of SOD in the culture medium was strongly inhibitory (17.1 → 3.1, 11.4 → 0 aberrations per 100 mitotic figures). The increase in activity of the CF by irradiation in P_i/NaCl is also shown for these cultures; activation by irradiation at 380 nm is clearly inhibited by SOD (cultures 1, 2, 3, and 4, in which the SOD present during the irradiation was removed by filtration through UM 10; see control). Doubling the quantity of CF increased the number of aberrations in the absence of irradiation but was lethal after 5 min of irradiation at 380 nm. Inhibition of the unirradiated factor by SOD by using twice as much factor per culture is shown in cultures 5 and 6.

DISCUSSION

The results of our study, in which monochromatic light at different wavelengths was used, confirmed that lymphocytes from SLE patients are sensitive to 360- to 400-nm light. The next step was to study the influence of monochromatic light on normal lymphocytes in the presence of the CF from lupus patients. Again, the dye-exclusion test showed a maximum of nonviable

Table 4. Effect of CF and SOD on aberration rate in irradiated whole blood

Experiment	Chromosome aberrations, no. per 100 mitotic figures: 1	2	3	4	5	6	7	Mean
Blood irradiated at 0 hr of culture	8	6	4	6	5	7	12	6.9
Blood cultured with CF, no irradiation	16	16	16	12	14	26	15	16.4
Blood irradiated at 0 hr in presence of CF	34	24	34	32	25	40	26	30.9
Blood irradiated at 0 hr, CF added 30 min later					18	20	16	18.0
Blood irradiated at 0 hr in presence of CF and SOD					4	16	10	10.0
Blood irradiated at 0 hr in presence of CF, SOD added 30 min later					19	46	20	28.4

Blood was irradiated for 5 min with near-UV light (Mineralight B 100 lamp). Addition of CF after irradiation results in the same breakage rate as CF alone.

Table 5. Effect of purified CF

Experiment	Chromosome aberrations, no. per 100 mitotic figures					
	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6
Unirradiated CF	5.6	11.4	14.3	16.7	18.4	23.5
Unirradiated CF added to cells + SOD (50 μg/ml)					5.4	8.4
Irradiated CF (5 min at 380 nm)	17.1	22.3	No growth	No growth	No growth	No growth
CF irradiated in presence of SOD and then filtered to remove SOD	5.3	6.5	14.3	16.0		
Irradiated CF added to cells + SOD (50 μg/ml)	3.1	0			4.0	
SOD irradiated and removed by filtration, filtrate added to cells + irradiated CF	17.1	22.9	No growth	No growth		

Cultures of stock solution diluted 1:3. Cultures 1–3, 50 μl; cultures 4–6, 100 μl.

cells at 360–380 nm. Simultaneously, cytogenetic analysis of the surviving lymphocytes showed a considerable increase in the activity of the CF. We may assume that the CF is responsible for the photosensitivity of SLE lymphocytes containing and releasing this agent. The effects of UV light and the CF were not additive; a considerable increase in chromosome damage beyond that produced by the CF alone was observed only if the CF was present during irradiation. Preirradiation of the medium or the cells had no enhancement effect when the CF was added later. However, the increase in clastogenic activity shown by cells both irradiated and exposed to the CF was also obtained if the CF was preirradiated and immediately added to the cells. This shows unequivocally that the increased activity is due to photoactivation of the CF rather than to action by the CF as a photosensitizer for energy transfer to the cell.

The protector effect of SOD is indirect evidence that the specific substrate for this enzyme, the $O_2^{\cdot-}$ radical, plays a role. SOD prevented chromosome damage only if it was present during exposure and not if it was added 30 min later. This shows that protection is due to scavenging of the toxic radicals, *not* to repair of the damage.

It appears that the CF itself is photosensitive and gives rise to the production of $O_2^{\cdot-}$, which then reacts with and activates the factor. Photoactivation of the factor via $O_2^{\cdot-}$ is strongly inhibited by SOD (see Table 5). The clastogenic action of the activated factor is also inhibited by SOD. In the absence of irradiation, SOD can also protect the cells against higher doses of the CF, probably because some activation occurs by normal cellular production of $O_2^{\cdot-}$. It is thus clear that extracellular SOD protects by inhibition of the activation of the CF and also by inhibition of the clastogenic process, which presumably involves production of $O_2^{\cdot-}$ (or derived HO· radicals) by the activated factor. Given that exogenous (probably membrane-bound) SOD can inhibit perinuclear halo formation (12), it appears that extracellular (or membrane-bound) SOD can influence intracellular events.

We have found that the CF is a small molecule (M_r 1000–10,000) and resistant to proteases. Its activity is destroyed by heating to 50°C but conserved over long periods at −30°C. The CF does not inhibit SOD; indeed, the oxidation of luminol by the hypoxanthine-xanthine oxidase system is doubled in the presence of the CF, suggesting an increased production of $O_2^{\cdot-}$ (or other reactive species). It is possible that the photoactivated form is an unstable hydroperoxide formed *in vivo* in the absence of irradiation by the action of cellular-produced $O_2^{\cdot-}$; clastogenic activity is inhibited in both cases by SOD. The presence of an activated oxygen species producing DNA damage is also suggested by the fact that perinuclear halo formation in human fibroblasts resulting from irradiation at 365 nm is completely inhibited if the cells are pretreated with SOD (12).

Beighlie and Teplitz (1) have claimed that SLE might be a DNA-repair-deficiency disease. They observed a decreased unscheduled DNA synthesis in lymphocytes of SLE patients after exposure to 254-nm light, but repair synthesis in SLE fibroblasts was normal. Cleaver (2) concluded that SLE patients are not deficient in DNA repair mechanisms. This study suggests that the DNA lesions in SLE patients are not due to deficient repair but rather are the result of excessive damage induced by a diffusable clastogenic and sister chromatid-inducing agent, the action of which is increased by light at 360–380 nm.

Most of the solar radiation reaching the earth is in the IR, visible, and near-UV regions; far-UV wavelengths (200–300 nm) are almost completely absorbed by the ozone layer in the stratosphere. Ozone concentration and other atmospheric conditions, the sun's zenith angle, the altitude above sea level, and other factors make the destructive effects of sunlight highly variable. The presence of a photoactivated clastogenic agent in SLE patients provides an explanation of why these subjects are sensitive to sunlight and show an aggravation of their condition after exposure and appearance of typical skin lesions for which the use of sunscreens has been recommended.

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