0022-202X/78/7105-0334$02.00/0
The Journal of Investigative Dermatology, 71:334–339, 1978
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Vol. 71, No. 5
Printed in U.S.A.

Ultrastructural Study of the Nuclei in Premitotic and Repair DNA Synthesis Following UVB Injury

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Ultrastructural changes in nuclei synthesizing DNA were studied by cytochemical technique. Guinea pig ears were UVB irradiated and TdR-H^3 was injected intradermally into the irradiated sites 1 hr before biopsy. Areas of the epidermis containing more than 80% of cells in DNA (repair or premitotic) synthesis identified by light microscopic autoradiography were selected and cut at 600 Å. The glycolmethacrylate sections were stained with uranyl acetate and lead citrate, and consecutive sections were incubated with 0.01% pronase and 0.5% RNase before staining in order to observe DNA.

In cells undergoing DNA repair, the zone of DNA became discontinuous and DNA was scattered throughout the entire karyoplasm as small aggregates and fine filaments. Nuclei in S-phase showed essentially the same change, but quantitatively the disappearance of DNA from the nuclear membrane and distribution in the karyoplasm became much greater. These changes were not seen in specimens treated without cytochemical technique.

Biochemical and autoradiographic studies have demonstrated that 2 stages of metabolic change occur in epidermal cells after ultraviolet irradiation. The First, "injury stage," is seen as early as 1 to 3 hr postirradiation and is characterized by inhibition of mitosis, of premitotic DNA synthesis [1–5], and of synthesis of RNA and protein [1, 4, 6, 7]. In addition, in DNA, thymine dimers are formed and excised and DNA repair synthesis takes place in almost all nuclei of epidermal cells [1–3, 8, 9]. The second, "recovery stage," is noted by 24 to 48 hr after irradiation: large numbers of basal and suprabasal cells undergo premitotic DNA synthesis [4] and cell division. Consequently, the epidermis becomes much thicker [4, 6]. Syntheses of RNA and protein also increase during this phase [4].

Electron microscopy has been used to investigate morphological changes associated with these changes in cell functions. During the injury stage, Wier, Fukuyama, and Epstein [10] found the nucleolonemal pattern in approximately half of the nuclei to be fragmented or segregated and Wilgram et al [11] observed an increase in keratinosomes. In the recovery stage, Nix et al [12, 13] described enlargement of the nucleoli in spinous cells and the appearance of mucopolysaccharide bodies in granular cells. These studies, however, failed to correlate ultrastructural findings with changes of specific macromolecules, induced by ultraviolet light, because conventional electron microscopy does not selectively reveal chemical components in a cell.

Manuscript received March 15, 1978; accepted for publication May 23, 1978.

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This work was supported by Grants No. AM12433 and CA15605 from the National Institutes of Health, and in part by both a special appropriation from the State of California for the study of Psoriasis and funds donated by the Skin Disease Research Foundation, San Francisco.

The aim of the present study is to report the ultrastructure of nuclear DNA synthesis in epidermal cells during injury and recovery stages following ultraviolet irradiation. Light and electron microscopic autoradiography were used to detect nuclei undergoing repair and premitotic DNA synthesis, and a cytochemical technique with enzyme digestion was used to examine changes in distribution of DNA in these nuclei.

MATERIALS AND METHODS

A Hanovia air cooled hot quartz contact lamp which emits 14.96×10^5 ergs/cm^2/sec of ultraviolet light energy shorter than 320 nm at a distance of 3.4 cm was used as the light source. Nineteen sites on the back posterior aspects of adult albino guinea pig ears were irradiated with single doses of 3 MED as individually tested. Ten μC of TdR-H^3 (Schwartz Co., specific activity 13 c/mM) in 0.1 ml saline were injected intradermally into 7 exposed sites 15 min later, 4 exposed sites 2 hr later and 8 exposed sites 23 hr later after irradiation. Skin biopsies were taken 45 min after injection from the sites that had been injected at 15 min postirradiation. Other biopsies were taken from the irradiated sites at 1 hr after injection. Six nonirradiated sites on the posterior aspects of the ear skin were also injected intradermally with 10 μC of TdR-H^3 and specimens were taken 1 hr later as a control. The tissues were processed by the following techniques:

Light Microscopic Autoradiography

Specimens approximately 0.5 × 1.0 cm in size were immediately fixed in 4% buffered formalin, dehydrated, embedded in paraffin, and sectioned at 4 μ in thickness. Autoradiographs were made using Kodak nuclear track emulsion type NTB-2 and processed with D19 Developer followed by Edwal Quick Fixer after 3 and 6 weeks. The sections were stained with hematoxylin and eosin.

In order to study the distribution of cells in which incorporation of TdR-H^3 was seen, sections approximately 1 cm in length were divided into 280 μ length segments and labeled and nonlabeled nuclei in each segment were counted. In the epidermis taken at 1 to 3 hr after irradiation, the percent of sparsely labeled nuclei (3 to 15 grains) of basal, spinous and granular cells, considered to be in DNA repair synthesis [1] was computed. In tissue taken 24 hr postirradiation showing DNA premitotic synthesis, the percent of heavily labeled nuclei (more than 16 grains) of basal and suprabasal cells was determined.

Electron Microscopy

Skin minced into small pieces was fixed in 3% glutaraldehyde buffered with 0.067 M cacodylate (pH 7.3) for 30 min, then washed in the same buffer for 15 hr. They were dehydrated and embedded in GMA as previously reported [16]. Sections 1 μ thick were cut using 3 to 4 different blocks prepared from the same skin sites. They were processed for light microscopic autoradiography. Blocks in which either repair or premitotic DNA synthesis was demonstrated in at least 80% of cells were selected. Thin sections were cut from the well-labeled blocks and picked up on stainless steel grids coated with formvar. They were digested with 0.01% Pronase E (Kaken Chemical) solution, pH 7.3 at 37°C for 5 min, to remove the protein in nuclei, and washed with distilled water. They were then incubated in 0.5% RNase (Worthington) solution, pH 6.4 at 37°C for 1 to 2 hr. The sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1A. For comparison, thin sections without the enzyme treatment were stained and examined.

In order to quantify the ultrastructural findings of nuclei, the following morphometric analyses were used: All nuclei observed in 5 grids from each skin block were photographed at machine magnification of

5000 ×. The microscope was calibrated by using a carbon grating replica (Fullam). We chose randomly 15 nuclei containing the nucleoli from each of 3 hr (spinous cells) and 24 hr (basal cells) postirradiation group and their nonirradiated control groups. The electron micrographs were enlarged to 24,000 ×. We counted the number of clear spaces along the nuclear membrane. Length of the nuclear membrane was measured by using a tape measure and average number of clear spaces which occur in 10 μ nuclear outline was calculated. Length of each clear space was also measured and percent of clear space in different sizes was computed. In addition, length of the nuclear membrane of basal cells was measured in 35 nonirradiated cells and 24 cells of 24 hr postirradiation. The number of small aggregates in the karyoplasm was counted by a cytomorphometric method of Meyer and Schroeder, employing a regularly spaced lattice superimposed over the micrographs [15].

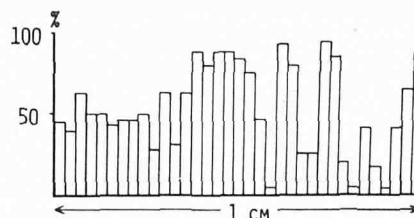

Fig 2. Percent of heavily labeled nuclei of basal and suprabasal cells counted in a representative section at 24 hr after irradiation. Each *bar* indicates the counts made in 280 μ length of the skin segment.

RESULTS

Light Microscopic Autoradiography

1. Paraffin embedded section: In nonirradiated skin, no nuclei showed sparse labeling and about 5% of basal or suprabasal cells demonstrated heavy labeling. Sparse labeling was observed in skin specimens taken at 1 to 3 hr postirradiation. However, labeling occurred in groups of basal, spinous and granular cells of the epidermis, while groups of cells adjacent to those areas were much less intensely labeled. Figure 1 demonstrates this distribution of labeled cells in a representative section. In one area almost all nuclei are labeled, whereas nearby less than 40% of the nuclei are labeled. A few basal cells showed heavy labeling.

At 24 hr after irradiation, many of the basal and suprabasal cells showed heavy labeling. The labeling was again spotty and groups of labeled cells appeared in-between groups of nonlabeled cells. Labeled cell counts of a tissue section at this stage (Fig 2) revealed 90% of the nuclei labeled in some areas, while in other areas less than 40% of them were labeled.

2. GMA or Epoxy Resin Embedded Section: The chances of finding skin blocks which contain more than 80% of labeled cells differed from one biopsy to another because of spotty appearance of the labeled cells. However, we were able to obtain at least 2 pieces of such specimens for each biopsy and only adjacent sections from those blocks were used for electron microscopy (Fig 3*a* and Fig 4*a*).

Electron Microscopy

1. Nuclei in GMA embedded epidermal cells: At 1 and 3 hr after irradiation, the karyoplasm of spinous cells showed very slight changes compared to nuclei of nonirradiated skin (Fig 3*b* and 3*c*). Electron dense material appeared along the nuclear membrane with the exception of the nuclear pores. The karyoplasm was filled with electron dense aggregates and a filamentous meshwork which became somewhat denser in the irradiated epidermis. The only difference noted was in nucleoli which became condensed and sometimes segregated as described by Wier, Fukuyama, and Epstein [10]. Similarly, the ultrastructure on nonirradiated basal cells was not easily distinguishable from that of basal cells at 24 hr postirradiation, even though at least 80% of the cells were shown to be in the process of DNA synthesis by their heavy labeling (Fig 4*a*, *b* and *c*). An electron dense zone was seen at the nuclear membrane while aggregates of various sizes and interconnected filaments filled other parts of the karyoplasm.

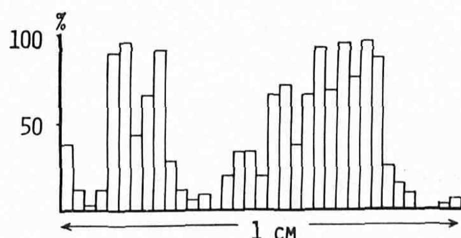

Fig 1. Percent of sparsely labeled nuclei of basal, spinous and granular cells counted in a representative section at 1 hr after irradiation. Each *bar* indicates the counts made in 280 μ length of the skin segment.

2. Ultrastructure of nuclear DNA after enzymatic digestion: The distribution of DNA in basal and spinous cells of nonirradiated control epidermis has been previously described [16]. The same results were observed in the present study. Briefly, DNA was condensed along the nuclear membrane and formed dense clumps within the karyoplasm of spinous and basal cells (Fig 3*d* and 4*d*). At 1 and 3 hr after irradiation, the nuclei undergoing DNA repair showed a number of clear spaces by the nuclear membrane which caused discontinuation of the DNA zone (Fig 3*e* and 3*f*).

The total number of clear spaces and the lengths of each space were measured in each of 15 nuclei of nonirradiated and 1 hr postirradiated spinous cells. The length of the total nuclear outline studied was 415.9 μ and 491.5 μ in nonirradiated and irradiated spinous cells, respectively. The number of clear spaces which appeared in nonirradiated cells was 252 compared with 305 in irradiated cells. Average of the frequency of spaces occurring was computed as 5.9 spaces/10 μ in nonirradiated and 6.2 spaces/10 μ in irradiated cells, and there were no significant changes induced by DNA repair synthesis (Table I). However, when each space was measured and their size variation compared, significant changes appeared (Table II). In nonirradiated cells, 40% of the spaces fell in the range of 440-1380A; 50.4% were between 1380–2750 Å and 8.1% between 2750 and 4130 Å. Only 1.5% were larger than 4130 Å. In contrast, in irradiated spinous cells, only 2.5% of spaces showed the 440–1380 Å size; 66.1% ranged from 1380–2750 Å; and 16.6% were from 2750–4130 Å. About 15% of the clear spaces were larger than 4130 Å.

Another ultrastructural change was observed in the karyoplasm in the early postirradiation period. DNA aggregates of large and irregular shape in nonirradiated spinous cells, were seen as small and uniform shaped filamentous aggregates in irradiated "injury stage" cells. The number of small DNA aggregates in the nuclei as determined by the lattice technique increased postirradiation from 0–2 in controls to 6–8 in most nuclei, with a few ranging as many as 9–11 aggregates after UV injury. The relationship between the small aggregated DNA and the clear spaces by the membrane is not known, but in some areas, the size of aggregates matches areas of nearly clear spaces, suggesting the possibility that DNA relocated.

At 24 hr after irradiation, basal cells in "recovery stage," in which premitotic DNA synthesis was detected by autoradiography showed ultrastructural changes very similar to those described in "injury stage" spinous cells, above. At this time, TdR-H^3 was no longer incorporating into spinous cells and their ultrastructure was not distinguishable from the nonirradiated spinous cells. A discontinuation of the DNA zone along the nuclear membrane was more readily seen in basal cells during premitotic DNA synthesis than in spinous cells in DNA repair synthesis (Fig 4*e* and 4*f*). This observation was confirmed by counting the clear spaces in 15 randomly selected basal cells (Table I). The length of the total nuclear outline examined was 432.8 μ and 538.5 μ in nonirradiated and in 24 hr postirradiated basal cells respectively. Since these nuclei showed 214 and 563

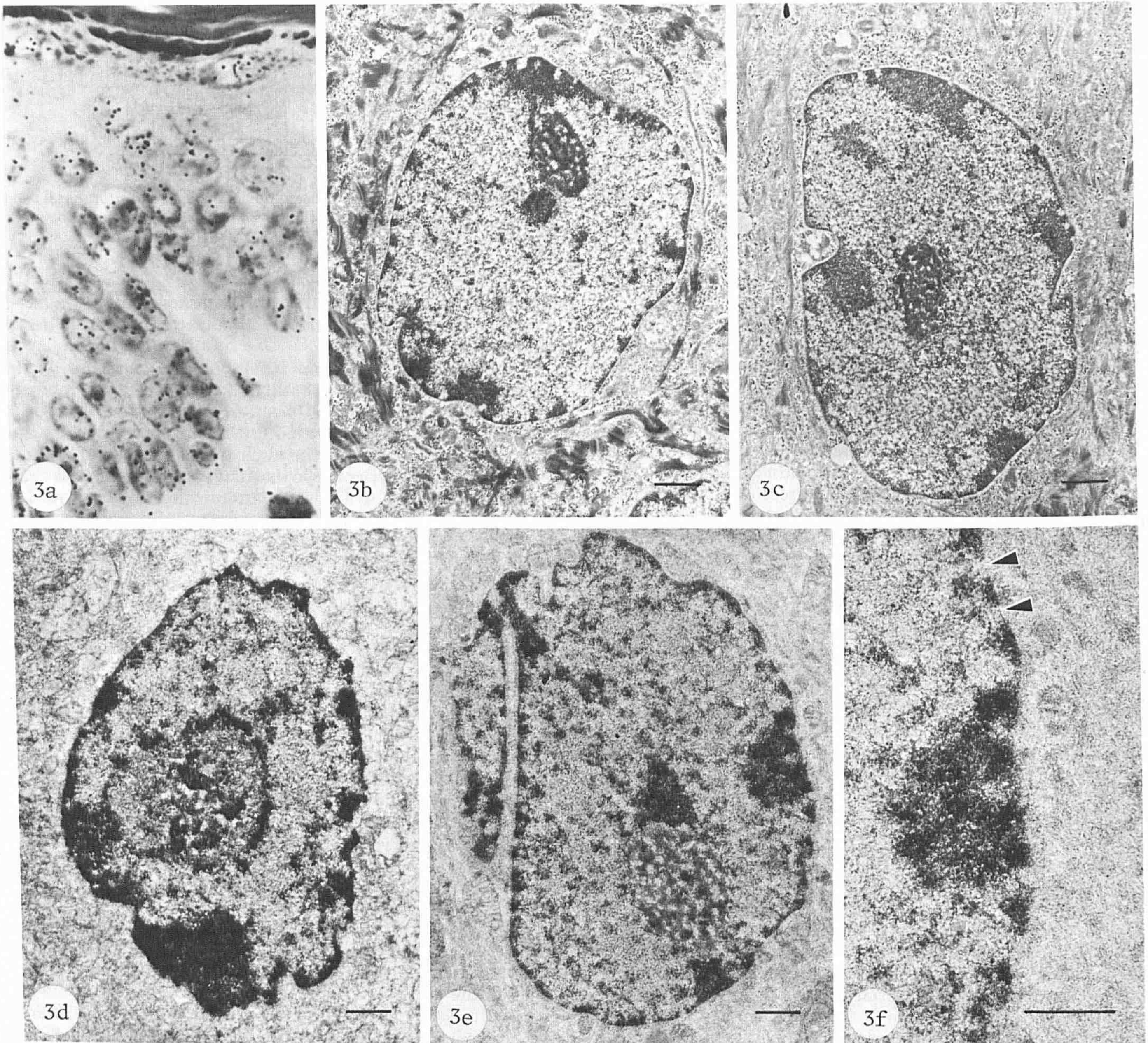

FIG 3.*a*, Light microscopy autoradiography of guinea pig epidermis at 3 hr after irradiation (1 hr after injection of TdR-H^3). A high percent of nuclei show the sparse labeling. *b* and *c*, Ultrastructure of nonirradiated (*b*) and irradiated (*c*) spinous nuclei. The 1 hr postirradiated nucleolus shows a slight condensation, GMA embedded and stained with uranyl acetate and lead citrate (no enzyme used).—1μ. *d*, A nucleus of nonirradiated spinous cell. GMA embedded and treated with pronase and RNase prior to the staining.—1μ. *e* and *f*, A nucleus of spinous cell demonstrating clear spaces in the electron dense zone by the nuclear membrane and diffusely scattered small aggregates at 1 hr after irradiation. *Arrows* indicate the locations showing a possibility of relocation of DNA from the membrane zone to the karyoplasm as small aggregates.—1μ.

clear spaces, average of the frequency of spaces occurring in irradiated cells was twice that of control cells. In nonirradiated basal cells, 17.3% of the spaces were between 440–1380 Å, and 66.5% ranged from 1380–2750 Å. Other spaces were larger but none were larger than 5000 Å. In contrast, in irradiated basal cells, about 9% of the clear spaces attained a space larger than 5500 Å. In addition, the percentage of clear spaces between 2750–4130 Å increased, while the proportion of those smaller than 2750 Å decreased (Table III). These increases in the clear spaces resulted not from extension of the nuclear surface area, but from a decrease in the DNA zone along the nuclear membrane, because length of the nuclear membrane did not significantly change after irradiation (Table IV).

The increased number of small filamentous aggregates of DNA, observed in spinous cells, was also seen in basal cells by 24 hr after irradiation. The lattice technique showed 3–5 or less aggregates in control nuclei, whereas most nuclei of basal cells in the recovery stage had 9–11 aggregates. The degree of increase was much larger in cells during premitotic DNA synthesis than in spinous cells during DNA repair synthesis.

DISCUSSION

Ultraviolet light induced 2 types of DNA synthesis in epidermal cells of guinea pig ear. DNA repair synthesis was seen in groups of granular, spinous and basal cells at 1 to 3 hr and premitotic DNA synthesis occurred in clusters in basal and suprabasal cells 24 hr after injury. It was difficult, however, to distinguish between the ultrastructure of karyoplasm in irradi-

ated and nonirradiated cells by conventional electron microscopy, the only difference seen being a slightly denser filamentous network in irradiated cells. Recently, we have shown that nuclear DNA can be selectively demonstrable at the ultrastructural level [16]. From among the several techniques available for observation of DNA [17], we selected the enzyme digestion technique of Monneron [18] and the Schiff-thallium reaction of Moyne [19, 20]. We found both techniques to be applicable for investigation of epidermal cells.

In the present study with the enzyme digestion technique, the ultrastrucutral changes found in cells synthesizing DNA were: (a) the formation of clear spaces in the DNA zone along the nuclear membrane, and (b) the appearance of small aggregates scattered diffusely in the karyoplasm.

In lymphocytes and HeLa S-3 cells, Maul et al [21] found that the total number of nuclear pores doubles in 2 phases, one of which corresponds to DNA synthesis stimulated by phytohemagglutinin. However, in spinous cells undergoing DNA repair synthesis, the total number of clear spaces remained approximately the same as the nonirradiated control, and each space was too large to be the nuclear pores. These findings indicate that the nuclear pores do not increase durring DNA repair, but suggest the possibility that "dispersion" of DNA may first occur from the nuclear pore area, although a direct visualization of the pore was not included in this study. In contrast, the number and size of spaces increased in basal cells during premitotic DNA synthesis. In this instance, the nuclear pores may have increased in the epidermal cells as seen in

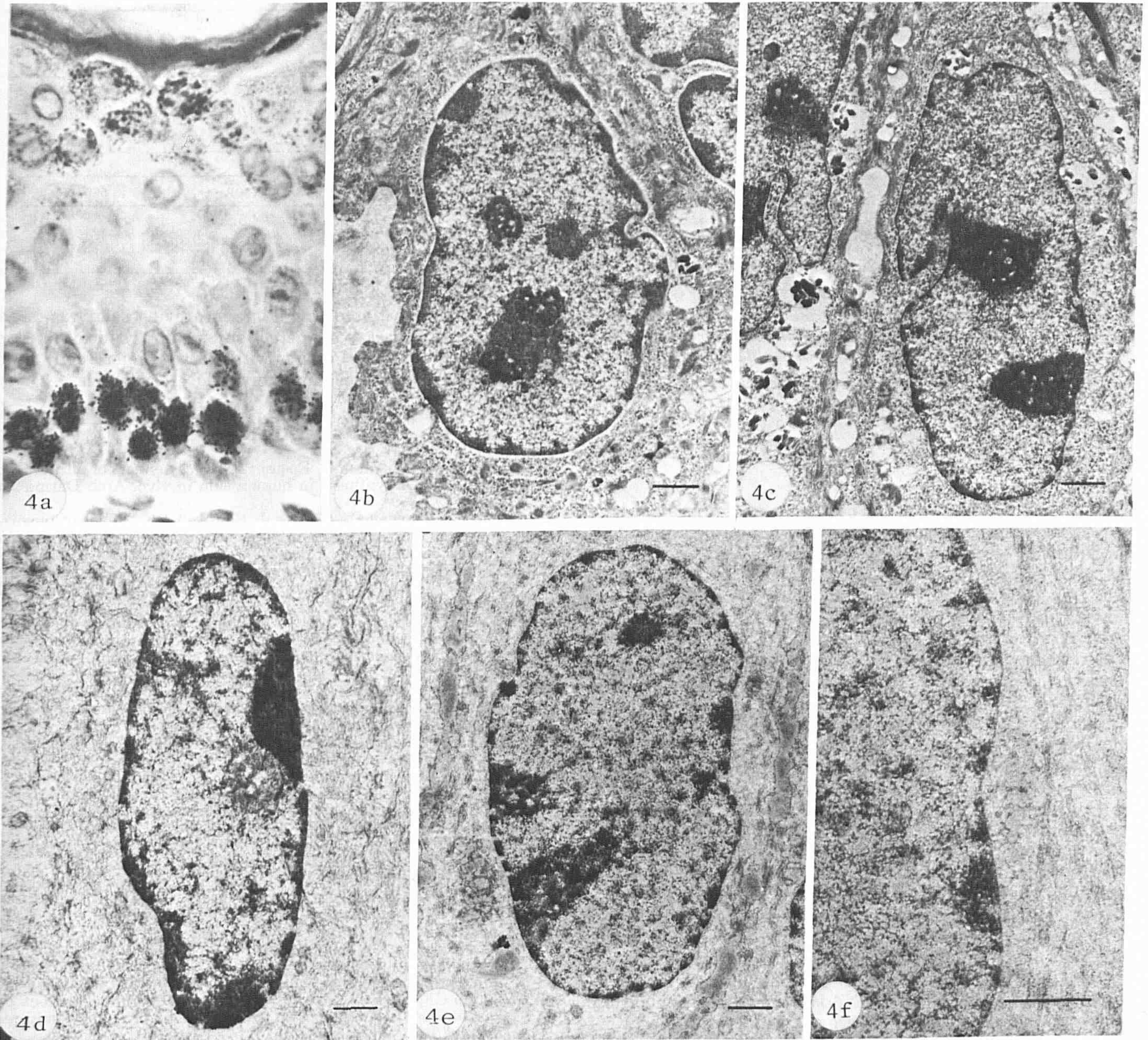

FIG 4.*a*, Light microscopic autoradiography of guinea pig epidermis at 24 hr after irradiation (1 hr after injection of TdR-H^3). The heavy labeling is seen in basal and suprabasal cells. *b* and *c*, Ultrastructure of nonirradiated (*b*) and irradiated basal nuclei (*c*). The electron dense zone along the nuclear membrane is somewhat less in the 24 hr postirradiated cell. GMA embedded and stained with uranyl acetate and lead citrate (no enzyme used).—1μ *d*, A nucleus of nonirradiated basal cell in GMA embedded tissues. Pronase and RNase were used before uranyl acetate and lead citrate staining.—1μ. *e* and *f*, A nucleus of basal cell shows clear spaces in the electron dense zone by the nuclear membrane and small aggregates in the karyoplasm at 24 hr postirradiation.—1μ.

TABLE I. *Frequency of clear spaces per 10 micron length of nuclear membrane observed in spinous and basal cells of irradiated and nonirradiated epidermis*

	Spinous cell nuclei			Basal cell nuclei		
	No. of nuclei	No. of clear spaces/10 μ of membrane	P value	No. of nuclei	No. of clear spaces/10 μ of membrane	P value
Nonirradiated cells	15	5.9 ± 2.7	—	15	5.0 ± 2.0	—
Irradiated cells	15	6.2 ± 1.5[a]	NS[b]	15	10.1 ± 2.5[c]	<0.000002

[a] At 1 hr post-UV irradiation.
[b] Not significant.
[c] At 24 hr post-UV irradiation.

TABLE II. *Size variation of clear spaces along the nuclear membrane measured in spinous cells at 1 hr after ultraviolet light irradiation as compared with nonirradiated control spinous cells*

	No. of nuclei	Percent of clear spaces in each size range				
		440Å	1380Å	2750Å	4130Å	5500Å
Nonirradiated cells	15	40.0 ± 25.7	50.4 ± 21.4	8.1 ± 13.6	1.2 ± 3.2	0.3 ± 1.0
Irradiated cells	15	2.5 ± 3.5	66.1 ± 13.8	16.6 ± 9.7	11.7 ± 7.8	3.7 ± 5.2
P value		<0.00002	<0.03	<0.06	<0.000002	<0.02

TABLE III. *Size variation of clear spaces along the nuclear membrane measured in basal cells at 24 hr after ultraviolet light irradiation as compared with nonirradiated control basal cells*

	No. of nuclei	Percent of Clear Spaces in each Size Range				
		440Å	1380Å	2750Å	4130Å	5500Å
Non-irradiated cells	15	17.3 ± 8.9	66.5 ± 13.5	11.7 ± 11.0	4.5 ± 6.0	0
Irradiated cells	15	10.5 ± 7.8	41.2 ± 19.5	25.5 ± 16.3	14.1 ± 6.9	8.7 ± 9.5
P value		<0.04	<0.0004	<0.03	<0.0004	<0.002

TABLE IV. *Length of nuclear membrane measured in randomly selected basal cells at 24 hr after ultraviolet light irradiation as compared with nonirradiated control basal cells*

	No. of nuclei	Length of nuclear membrane (μ)	P value
Nonirradiated cells	35	33.9 ± 6.0	—
Irradiated cells	24	35.0 ± 9.5	NS

lymphocytes and HeLa cells. In phytohemagglutinin stimulated cells the increase in nuclear pores was found to correspond to the number of "simultaneous replicating replication sites" [18]. The lack of increase in the total number of clear spaces in spinous cells in the early injury stage may reflect the fact that repair synthesis does not involve an increase in the net amount of DNA in the nuclei.

Large aggregates of DNA in the karyoplasm also disappeared. Instead, there were small filamentous aggregates of DNA scattered throughout the fine mesh of DNA in the karyoplasm. Circumstantial evidence that small aggregates by the nuclear membrane matched areas of nearly clear spaces suggests that DNA adjacent to the membrane may contribute to the formation of aggregates. Using autoradiography and electron microscopy, Blondel [22] found that TdR-H^3 incorporation was in cells almost wholly deprived of areas of condensed chromatin. The appearance of the clear spaces described in the present study may represent one stage in the reorganization of chromatin. Transformation of "condensed" chromatin to "dispersed" chromatin appears to occur in both DNA repair and premitotic synthesis but to a different degree. Current concepts about how DNA is synthesized generated by Okazaki et al [23], Painter and Schaefer [24] and Sato, Tanaka, and Sugimura [25], indicate that DNA is formed as short nucleotide chains, "Okazaki Patches." These chains join with longer ones and finally become bulk DNA. The small filamentous aggregates may correspond to the bulk DNA which has been produced.

The present study demonstrated that distribution of DNA changes during DNA synthesis and that the ultrastructure patterns are common to both types of DNA synthesis. Although incorporation of tritiated thymidine into living cells was used exclusively as a marker for detection of DNA replication, the use of cytochemical techniques with electron microscopy also provides an ultrastructural marker for DNA synthesis.

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