Mutagen-induced Disturbances in the DNA of Human Lymphocytes Detected by Antinucleoside Antibodies

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ABSTRACT

The alkylating mutagens N-methyl-N′-nitro-N-nitrosoguanidine, methyl methanesulfonate, and N-nitroso-methyleurea induced immunoreactivity to antinucleoside antibodies in human peripheral blood lymphocytes in vitro. This could also be detected in lymphocytes taken from a patient soon after i.v. administration of cyclophosphamide. The immunoreactivity response, which indicates denatured DNA or DNA single-strand breaks, was scored by immunofluorescent or immunoperoxidase techniques.

Examination of blood from 10 normal subjects showed that 32 ± 4% (S.E.) of resting peripheral blood lymphocytes were immunoreactive to antinucleoside antibodies. We have shown that these naturally occurring immunoreactive lymphocytes are largely accounted for by a subpopulation of thymus-derived lymphocytes bearing the Fc receptor for immunoglobulin M. The presence of these cells did not interfere with the use of peripheral blood lymphocytes for in vitro measurement of additional immunoreactivity caused by alkylating mutagens. The response proved to be dose dependent; up to 90% of lymphocytes could be rendered immunoreactive. Parallel studies with HeLa cells showed a similar dose-response relationship between mutagen action and immunoreactivity.

With some agents, the immunoreactivity technique detected effects at lower concentrations than could be detected by HeLa cell survival studies. With N-nitrosomethyleurea, measurement of DNA repair synthesis by [3H]thymidine autoradiography showed that in HeLa cells these two parameters of response to DNA damage increased in parallel. Our results provide a new basis for detecting the action of alkylating mutagens on human lymphocytes in vitro or in vivo.

INTRODUCTION

Detection of X-ray and chemical carcinogen damage to the DNA of HeLa cells using the antinucleoside antibody technique has been described previously (4–7, 18). These antibodies are directed against specific nucleosides and react only with single-stranded nucleic acids (11, 15). Normally, only S-phase cells are reactive when examined by immunofluorescent or immunoperoxidase staining of fixed cells (6, 17, 18), presumably because of the relative abundance of exposed single-stranded DNA during DNA replication. Cells in G1 normally show a low level of immunoreactivity (18). Agents known to damage DNA cause an increase in this response in a dose-dependent fashion, and in HeLa cells there is a parallel loss of cell viability (3, 4, 18). The immunoreactivity response may be caused by DNA strand scission and unwinding or by denaturation, coincident with DNA repair synthesis.

Highly purified antisera, such as those we used, were used by Schreck et al. (24) in determining the nucleoside sequence of repetitive DNA in the fixed chromosomes of the kangaroo rat. Denaturation of only 5 base pairs was sufficient to detect immunoreactivity, and a single specific exposed nucleoside could be detected (24).

Here we show that the normal level of immunoreactivity to antinucleoside antibodies in freshly isolated human peripheral blood lymphocytes is increased by exposure to mutagens. Naturally occurring immunoreactive lymphocytes were largely accounted for by a subset of thymus-derived lymphocytes bearing the receptor for the Fc portion of IgM (21).

Mutagens which induced immunoreactivity to antinucleoside antibodies in lymphocytes were tested for their ability to kill HeLa cells and induce immunoreactivity in these cells, because comparisons of both of these measures of mutagen action on cells could not be made with resting peripheral blood lymphocytes.

MATERIALS AND METHODS

Antinucleoside Antibodies. Specificity of antibodies against individual nucleosides was verified by agar diffusion and other tests previously described (8, 17).

Lymphocytes. Mononuclear cells were prepared from freshly drawn, citrated or heparinized human blood by the Ficoll-Hypaque method (12). Washed lymphocytes were briefly exposed to freshly prepared MNNG,3 MMS, or NMU in serum-free Eagle’s medium. The lymphocytes were washed, smeared on slides, air-dried, and fixed with acetone at −20° (17). Nuclear immunofluorescence was scored using fluorescein-labeled antiguanosine immunoglobulins at 1/50 (0.2 mg/ml) as described previously (6–8). A 15-min RNase pretreatment [pancreatic RNase (2 μg/ml), T-1 RNase (2 units/ml)] was done at room temperature to eliminate interference from RNA. RNase’s had been heated previously for 15 min at 85° to eliminate DNase.

In some experiments, an immunoperoxidase technique was used as an alternate to immunofluorescent staining of lymphocytes (8, 17). Smears were air-dried and fixed for 5 min in acetone at −20° (0.37% formaldehyde in phosphate-buffered

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3 The abbreviations used are: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MMS, methyl methanesulfonate; NMU, N-nitroso-methyleurea; Tc, T-cells with receptors for IgG; Tm, T-cells with receptors for IgM.
saline [NaCl (137 mM)-Na₂HPO₄ (8 mM)-KCl (2.7 mM)-KH₂PO₄ (1.5 mM)] without calcium and magnesium (10) for 10 min at 20° also was satisfactory, but acetone fixation was found preferable. RNase digestion preceded the 2-layer bridge immunoperoxidase staining. Rabbit antiguanosine or anticytidine was applied. After one rinse, approximately 0.1 ml of goat anti-rabbit immunoglobulin, precoated with horseradish peroxidase, 0.14 mg/ml (Miles Research Products, Elkhart, Ind.), was placed on the smear for 30 min. A dark brown color was developed by finally incubating the slides in diaminobenzidine and hydrogen peroxide.

Both preexisting as well as mutagen-induced specific nuclear immunoreactivity were abolished by preincubation of cells with DNase, but not with RNase as used previously (7). Only diffuse weak membrane fluorescence was observed when normal or mutagen-treated lymphocytes were stained with fluorescein-labeled preimmune globulins. The antinucleoside antibodies used here are specific for individual nucleosides as verified in immunodiffusion tests. The antibodies are raised in rabbits given injections of bovine serum albumin nucleoside conjugates; after exhaustive absorptions, they do not react with bovine serum albumin. The weak diffuse immunoreactivity observed in cells scored as negative could be due to some residual immunoreactivity against bovine albumin adsorbed to cells in culture.

**Cell Killing by Mutagens.** To measure the influence of the mutagens on HeLa cell viability, the colony formation assay was used (3). Approximately 200 single HeLa cells were plated in each 60-mm plastic Petri dish. After 2 hr of incubation at 37° in a CO₂ incubator to allow for attachment, the cells were exposed to mutagens in serum-free medium for up to 60 min. After the mutagens were removed, the cells were overlaid with growth medium with 10% fetal bovine serum and incubated for 10 days for colony formation and counting. The plating efficiency of untreated HeLa cells was about 50%.

**Repair Synthesis Measured by [³H]Thymidine Autoradiography.** Logarithmically growing HeLa cells were incubated with [³H]thymidine as previously described (5). After 4 weeks exposure of labeled cells to photographic emulsion, 2 classes of cells were found, heavily labeled cells (S-phase cells) and lightly labeled non-S-phase cells which had incorporated [³H]thymidine during repair synthesis.

**Nylon Column Fractionation of Human Lymphocytes.** Mononuclear cells prepared from heparinized venous blood by Ficoll-Hypaque sedimentation (25) were washed and resuspended in Eagle’s minimal essential medium containing 10% fetal calf serum at a concentration of 1 x 10⁶ cells/ml. Nylon columns were prepared by filling 10-ml disposable syringes with loosely compressed scrubbed nylon fiber (Fenwal Laboratories, Marion Grove, Ill.) to the 5-ml mark. The cell suspension was then layered onto nylon wool columns and incubated at 37° for 30 min. Nonadherent cells were gently washed from the column with additional medium, pelleted, and smeared on glass slides. Macrophages and B-lymphocytes are removed by adherence to the column; T-lymphocytes pass through and are concentrated.

**Fractionation of Human Peripheral Blood Lymphocytes by Rosetting Techniques.** The methods have been described in detail elsewhere (23). Briefly, lymphocytes were separated by Ficoll-Hypaque density gradients and then rosetted with sheep erythrocytes which had been pretreated with isoethyliouronium bromide, to ensure stability of T-cell rosettes. Rossetted T-cells were then separated from other cellular elements of the blood by Ficoll-Hypaque density gradients. The pelleted rosetted T-cells were then treated with 0.87% NH₄Cl to lyse the sheep erythrocytes (over 98% of these T-cells could be rosetted with sheep erythrocytes). After extensive washing, the harvested T-cells were divided into 3 aliquots for study. The first aliquot was stained with fluorescein-labeled rabbit antiguanosine without further treatment (unfractionated T-cells). The other 2 aliquots were used to isolate T-cell subsets with FC receptors for IgG or IgM as previously reported (23). By using trypsinized and isoethyliouronium-pretreated sheep RBC, rosettes were obtained comparable to the ox RBC used by Moretta et al. (21). One aliquot was rerosetted using such pretreated sheep RBC coated with purified rabbit IgG directed against sheep RBC. The residual aliquot of T-cells was rosetted with sheep RBC coated with rabbit IgM. These rosettes led to the isolation T-cell subsets with receptors for the FC of IgG (Tγ) and IgM (Tμ), respectively. The Tγ and Tμ lymphocytes were freed of sheep RBC by ammonium chloride lysis before staining with fluorescein-labeled antiguanosine. Lymphocytes from nylon columns and rosetting were coded before examination.

**RESULTS**

**Interaction of Lymphocytes and Alkylating Agents.** Fig. 1a shows the typical appearance of human lymphocytes stained with fluorescein-labeled antiguanosine antibodies. These lymphocytes were isolated from a cancer patient just before i.v. administration of 750 mg of the alkylating agent, cyclophosphamide, as part of his treatment. The pretreatment lymphocytes were indistinguishable from those of normal subjects. Seventy% of these cells showed nonspecific diffuse homogeneous nuclear fluorescence which is considered negative. The appearance of these nuclei was similar to those of G1 HeLa cells. Unexpectedly, 30% of the cells showed multiple areas of intense specific nuclear fluorescence similar to that found with S-phase HeLa cells or X-irradiated or mutagen-treated G1 HeLa cells (6, 7, 18). Fig. 1b shows the appearance of lymphocytes from the patient 30 min after administration of cyclophosphamide; 87% of the cells were now positive.

Virtually every lymphocyte in Fig. 1b exhibits discrete focal areas of intense specific nuclear fluorescence similar to that observed in HeLa cells after X-ray or carcinogen exposure (18).

The intensity of fluorescence observed after cyclophosphamide exposure was greater than with unexposed cells, but this is not readily appreciated from photographs. Results with immunoperoxidase staining of these 2 lymphocyte preparations confirm the above results (not shown). Immunopositive and negative lymphocytes from a normal subject are shown in Fig. 1, c and d.

**Origin of Immunoreactivity to Antinucleoside Antibodies in Normal Human Peripheral Lymphocytes.** A naturally occurring level of 32 ± 4% (S.E.) immunoreactive cells was observed in experiments with unfractionated peripheral lymphocytes from 10 normal subjects. This finding was unexpected, since our own studies and those of many other laboratories indicate that there is little, if any, DNA synthesis in resting human peripheral blood lymphocytes. Since these cells...
were a constant feature of the mutagen experiments, it was of interest to identify the subpopulation of lymphocytes responsible for it.

We found that the proportion of immunoreactive lymphocytes was increased by passage through nylon columns which largely eliminates B-lymphocytes and macrophages (Table 1). Results of experiments shown in Table 1 suggest that the biggest contribution to immunoreactive lymphocytes came from T-lymphocytes which are enriched by nylon column filtration. This was confirmed by preparative rosetting of T-lymphocytes with sheep erythrocytes (Table 2). Seventy-five % of E rosetting lymphocytes (total T-cells) were immunoreactive. Further fractionation into Tγ and Tδ subsets showed that the Tδ lymphocytes were responsible for most of the immunoreactivity.

In another experiment, similar to that of Table 2, 83% of total T-cells were scored as positive and only 19% of nonrosetting cells were immunoreactive.

From the values of Table 2, the final percentage of immunoreactive cells to be expected among unfractonated lymphocytes might be approximated. Since in our experiments B-cells compose about 12%, Tγ about 20%, and Tδ about 45% of total lymphocytes, 43% of unfractonated lymphocytes would be expected to be immunoreactive from the following calculation: 12% x 0.3 + 20% x 0.3 + 45% x 0.75 = 43% (probably a minimum value, since not all cells were included in the calculation). In fact, 34 ± 4% was found; this discrepancy could be due to differences in recovery for individual lymphocyte subsets or damage in processing.

Dose-dependent Increase in Immunoreactivity to Antinucleoside Antibodies in Lymphocytes and HeLa cells by MNNG, MMS, and NMU; Comparison with HeLa Cell Viability. In vitro exposure of freshly isolated human lymphocytes to increasing doses of MNNG increased the proportion of immunoreactive lymphocytes (Chart 1). The concentrations of MNNG used were similar to those studied previously with G1 lymphocytes (Chart 1). The 10 to 15% of reactive cells is largely due to the contribution of contaminating S-phase cells. The influence of MNNG treatment on the colony-forming ability of HeLa cells was determined in triplicate. The mean and S.E.’s (bars) relative to control values are shown. ---, immunoreactive HeLa nuclei; ○, survival of HeLa cells.

Table 1

<table>
<thead>
<tr>
<th>% of immunoreactive lymphocytes</th>
<th>Donor</th>
<th>Before column</th>
<th>After column</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>83</td>
<td></td>
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(62% E-rosetting⁸ cells) (79% E-rosetting cells)

⁸ Sheep erythrocyte rosettes.

Table 2

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<tr>
<th>Lymphocyte population</th>
<th>positive (%)</th>
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<tbody>
<tr>
<td>Unfractionated lymphocytes</td>
<td>38</td>
</tr>
<tr>
<td>Non-E-rosetting⁹ cells</td>
<td>30</td>
</tr>
<tr>
<td>Total E-rosetting cells</td>
<td>75</td>
</tr>
<tr>
<td>E-IgM-rosetting cells (Tδ)</td>
<td>76</td>
</tr>
<tr>
<td>E-IgG-rosetting cells (Tγ)</td>
<td>30</td>
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⁹ Sheep erythrocyte rosettes.

Table 1. a, immunoreactivity to fluorescein-labeled antiguanosine antibodies of freshly isolated human lymphocytes induced by exposure to MNNG for 10 min at 37°. Variation in the proportion of immunoreactive cells among untreated lymphocytes from 4 individuals in separate experiments is shown as the S.E. (bars) (28 ± 7.3%). Variation of percentage of positive cells found after MNNG treatments is shown as the mean, and the range was observed with 2 of these lymphocyte preparations. ●, immunoreactive lymphocyte nuclei. b, immunoreactivity in G1, HeLa cells (enriched by selective detachment) after MNNG treatment (redrawn from Ref. 7). The 10 to 15% of reactive cells is largely due to the contribution of contaminating S-phase cells. The influence of MNNG treatment on the colony-forming ability of HeLa cells was determined in triplicate. The mean and S.E.’s (bars) relative to control values are shown. – – –, immunoreactive HeLa nuclei; ○, survival of HeLa cells.

were detectable at a concentration of 6.7 x 10⁻⁶ M. By contrast, this concentration reduced HeLa cell viability very little.

Results of the experiments of Chart 2 show a dose-dependent increase in lymphocyte immunoreactivity with MMS. The HeLa cell killing assay of MMS under the same concentration range and time of exposure was made to provide a comparison of immunoreactivity induction and cell killing with a human cell line which has a high plating efficiency. The HeLa cell killing assay was no more sensitive but less convenient than the immunoreactivity induction assay using lymphocytes, but with HeLa cells the immunoreactivity response was more sensitive. This will be discussed.

The increase in lymphocyte immunoreactivity on exposure to NMU (Chart 3) was readily detectable at doses too low for detection by HeLa cell killing. The sensitivity of the lymphocyte immunoreactivity to NMU can be appreciated by comparing it with the HeLa cell killing test (Chart 3, NMU concentration 5 x 10⁻⁶ M and 5 x 10⁻⁷ M). Chart 3 also shows that, with 5 x 10⁻⁵ M NMU, the HeLa cell immunoreactivity response, like the lymphocyte assay, was more sensitive than the HeLa cell killing assay.

Repair Synthesis. Induction of immunoreactivity to antinucleoside antibodies by mutagens might be associated with DNA strand breaks which uncover single-strand regions or DNA denaturation, coincident with DNA repair.

We detected repair synthesis by [³H]thymidine autoradiography following 30 min NMU exposure of randomly growing HeLa cells. Procedures similar to those of Bases et al. (5) were followed. Comparison of Chart 3 and Table 3 shows that with increasing NMU concentrations DNA repair processes in HeLa
At least 500 cells from each treatment group were scored. NMU heavily labeled cells (S-phase) were 5.2 x 10^{-6} M 27.9, 6.2 x 10^{-5} M 3.7, None 25.8. Lightly labeled cells (repair synthesis) were 2 x 10^{-7} M 1.0, 10^{-6} M 0.8, 10^{-5} M 6.6. Immunoreactivity to antinucleoside antibodies in lymphocytes was not accompanied by \(^{3}H\)thymidine autoradiographic evidence for DNA repair synthesis.

The focal distribution of lymphocyte immunofluorescence after mutagen action (Fig. 1b) could well reflect condensation of chromatin in addition to exposure of single-stranded DNA regions.

Two kinds of DNA repair synthesis have been described, a nucleotide excision repair (large patch) and base excision repair (small patch (22)). The intensity of the immunoreactivity response in individual cells might indicate which kind of repair was associated with different agents.

Excision repair seems to be most active in proliferating cells, a topic recently reviewed by Cleaver (9). As shown in Chart 2, the immunoreactivity response was less active in resting lymphocytes than in growing HeLa cells exposed to MMS. By contrast, with MNNG and NMU the immunoreactivity dose responses of both cell types were virtually identical (Charts 1 and 3). This difference might be due to the greater responsive-

cells increased in parallel with induction of immunoreactivity. With resting lymphocytes, we failed to find autoradiographic evidence for repair synthesis, with or without exposure to NMU, despite prolonged photographic exposures. This was in contrast to results with HeLa cells (Chart 3; Table 3).

**DISCUSSION**

These results demonstrate that the antinucleoside antibody method for studying the action of alkylating mutagens on the DNA of human cells can be applied to human peripheral blood lymphocytes. The mutagen effect was scored by relatively simple, rapid procedures. The sensitivity of lymphocyte immunoreactivity for detecting the action of alkylating mutagens is as great as or greater than the HeLa cell killing assay method with agents such as NMU or MNNG. The speed and potential application of this new technique to examination of cells from individuals exposed to alkylating mutagens are of special interest. Examination of lymphocytes which are immunoreactive to antinucleoside antibodies by flow cytometry (1) appears to be a probable future application of these results. Thus far, we have been unable to measure the intensity of fluorescence from individual cells. Flow cytometry has shown some promise for this and could be useful, especially since the intensity of fluorescence might reflect the kind of DNA repair activity induced by different agents.

It is not yet known to what extent the immunoreactivity response to alkylating agents in HeLa cells or lymphocytes reflects DNA damage alone and how much is due to denaturation associated with DNA repair. With lymphocytes, the immunoreactivity response to NMU was not accompanied by \(^{3}H\)thymidine autoradiographic evidence for DNA repair synthesis.

**Chart 2.** a, immunoreactivity induction by MMS in human lymphocytes. For the MMS-treated lymphocytes the S.E. (bars) was estimated from

\[
\text{S.E.} = 1.96 \sqrt{\frac{pq}{n}}
\]

where \(p\) is the fraction positive, \(q\) is the fraction negative, and \(n\) is the number of cells counted. ○, immunoreactive lymphocyte nuclei. b, survival of HeLa cells exposed to MMS at 37° for 20 min. ●, immunoreactive HeLa nuclei; ○, survival of HeLa cells. Error terms are as in Chart 1.

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*chart 3.* a, immunoreactivity induction by NMU in human lymphocytes. ○, immunoreactive lymphocytes. b, survival of HeLa cells exposed to NMU for 30 min. ●, immunoreactive HeLa cell nuclei; ○, survival of HeLa cells. The top and bottom data points for HeLa immunoreactivity represent results obtained after 30 and 60 min, respectively, and are not significantly different; error terms are omitted for clarity. HeLa cell survival values were obtained in triplicate or quadruplicate in each experiment and are expressed as a fraction of control values. Error terms with bars, S.E. of the mean values obtained from 3 separate experiments; error terms without bars, mean values and range in 2 separate experiments. The 2 HeLa cell survival values without error terms are from single experiments.

**Table 3**

<table>
<thead>
<tr>
<th>NMU</th>
<th>Heavily labeled cells (S-phase) (%)</th>
<th>Lightly labeled cells (repair synthesis) (%)</th>
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<tbody>
<tr>
<td>5 x 10^{-4} M</td>
<td>27.9</td>
<td>62.3</td>
</tr>
<tr>
<td>5 x 10^{-3} M</td>
<td>27.3</td>
<td>23.7</td>
</tr>
<tr>
<td>None</td>
<td>25</td>
<td>8.3</td>
</tr>
</tbody>
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* Fifty grains per cell.
* Four to 50 grains per cell. Emulsion was exposed for 4 weeks. The background was 0 to 3 grains per cell.
ness of HeLa cells to small patch repair induction (MMS). Similarly, HeLa cells were more responsive to 1000 rads of X-irradiation, a small patch-inducing agent, than lymphocytes.\(^4\)

Human lymphocytes that were stimulated to divide by phytohemagglutinin exhibited a 20-fold increase in DNA repair synthesis capacity following ionizing radiation (16). These considerations suggest that the immunoreactivity response of cells following damage to DNA reflects uncovering of single-strand DNA regions due to DNA strand breaks as well as DNA denaturation coincident with repair.

A level of 32 ± 4.4% immunoreactive nuclei was consistently found when human peripheral blood lymphocytes were studied. This value is largely the contribution of a subset of thymus-derived lymphocytes bearing the Fc receptor for IgM (T\(_M\) cells). With G\(_1\) cells, the 10 to 15% background of immunoreactive cells (18) represents contamination of G\(_1\) cells by cells in S phase. The background immunoreactivity in peripheral blood lymphocytes strongly indicates the presence in T\(_M\) subset lymphocytes of exposed DNA single strands. A small fraction of animal cell DNA is consistently isolated in single-strand form (19).

Data consistent with the presence of single-stranded DNA in human peripheral blood lymphocytes has been noted previously in studies using acridine orange staining and flow cytometry (2, 26).

Lymphocytes of the T\(_M\) subset are known to differ from T lymphocytes by their greater responsiveness to phytohemagglutinin (20) and resistance to certain agents, including ionizing radiation (14). T\(_M\) lymphocytes are less abundant in the blood of patients with certain immunodeficiencies (13) and appear to exert a helper function in relation to immunoreponses of the lymphocytes (21). The immunoreactive DNA in T\(_M\) lymphocytes described here may indicate that in these lymphocytes more unwound DNA is present than in other lymphocytes. This would be consistent with a more advanced stage in their program of readiness for DNA synthesis and their more active DNA repair capacity (14).

REFERENCES

Immunoreactivity to Antinucleoside Antibodies in Lymphocytes

Fig. 1. Immunoreactivity of lymphocytes from a patient receiving cyclophosphamide. a, lymphocytes taken before treatment and stained with fluoresceinated antiguanosine (0.2 mg/ml); 32% were positive (long arrow shows very intense discrete focal intranuclear staining). Diffuse homogeneous nuclear fluorescence is weak and nonspecific (short arrow). b, lymphocytes taken 30 min after treatment; 87% were positive (long arrows). The smaller fluorescent objects in the background are platelets. Original magnification, × 200. Differences in intensity of fluorescence are not as well represented in photographs as under direct observation. Lymphocytes from a normal subject (c and d) include immunoreactive (long arrows) and negatively staining ones (short arrows).
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