Comparative Study of Cultured Burkitt Tumor Cells by Immunofluorescence, Autoradiography, and Electron Microscopy

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Cultured Burkitt cells were examined by immunofluorescence, autoradiography, and electron microscopy in an effort to identify the stainable cells with those harboring herpes-type virus particles. Immediately after a 2-hr pulse of ³H-thymidine, from 30 to 60% of the cells revealed heavy nuclear labeling. In most cases the grains were evenly dispersed, but in about 3 to 5% the grains showed a focal distribution and occasionally they extended into the cytoplasm. Such nuclear foci were rarely seen at 8 hr after the pulse. When the analysis was restricted to pre-selected immunofluorescent cells, up to 80% showed label at 8 hr and cytoplasmic grains were prominent. To reduce cellular deoxyribonucleic acid (DNA) synthesis, cells were X-irradiated with 3,000 to 6,000 R, and the isotope pulse was applied 1, 4, or 7 days later. Whereas the total number of labeled cells decreased in roughly twofold steps at the respective intervals (from 40 to 10%), the incorporation of ³H-thymidine into fluorescent cells was not affected by X irradiation. In each series, about 70% of the fluorescent cells contained label when they were examined at 24 and 48 hr after the pulse, whereas at 8 and 72 hr fewer were positive. At the earlier intervals, unlabeled fluorescent cells most likely represented cells which had completed viral DNA synthesis prior to the pulse; at the later intervals, unlabeled fluorescent cells were probably cells which commenced viral replication after the pulse. These data support the conclusion that the immunofluorescent cells are the ones which harbor virus, and also confirm the expectation that the virus is a DNA virus from a member of the herpes group. This conclusion was firmly established by sectioning and electron microscopic examination of individual fluorescent cells, all of which contained numerous virus particles, whereas the nonstained cells prepared in a similar manner were free of them.

The majority of continuous cultures derived from Burkitt lymphomas were found on electron microscopic examination to harbor herpeslike virus particles in a small proportion of the cells (3, 16, 20). It was further suggested that the virus-producing cells can also be detected by immunofluorescence techniques. Both the indirect technique with sera from African patients with Burkitt tumors, as well as sera from many African and American control individuals, and the direct technique with fluorescein-conjugated

human γ -globulins have been used (7, 11). Appropriate virus-specific immunofluorescence tests failed to identify the agent as herpes simplex, varicella, cytomegalo, or any one of eight different animal herpesviruses (7; G. Henle and W. Henle, *Trans. N.Y. Acad. Sci.*, *in press*). The suggestion that the immunofluorescence technique reveals those cells in Burkitt cultures which harbor the herpeslike agent is supported by the following facts (7; Henle and Henle, *in press*). (i) Estimates of the percentages of electron microscopically detectable virus-carrying cells and of fluorescent cells in given lines are nearly identical and ranged from none in the Raji line (2) to a maximum of about 10% in the Jiyoye line. (ii) Viral inhibitors or cultural manipulations

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which reduced or increased the percentage of fluorescent cells led to corresponding changes in virus-carrying cells. These observations were confirmed recently by Hinuma et al. (*personal communications*). (iii) Virus particles extracted and concentrated from cultured Burkitt cells became coated with antibodies and in part were agglutinated by immunofluorescence-positive but not -negative sera, indicating that the former contained antibodies to the capsid of the unidentified agent (9; Mayyasi et al., *personal communication*). Because the evidence is largely indirect, more direct proof for the identity of virus-carrying and fluorescent cells was sought by combination of immunofluorescence and autoradiographic procedures, and by selection and ultrathin sectioning of individual fluorescent cells for electron microscopic examinations.

MATERIALS AND METHODS

Cells. For most of the studies, the Jiyoye (P3-J) line was used (Pulvertaft, *unpublished data*) because of its high percentage of fluorescent cells (5 to 10%). It was obtained from R. A. Manaker, National Cancer Institute, and was maintained in this laboratory on medium RPMI-1629 supplemented by 10% fetal calf serum (1629-10). The EB-3 (3) and Raji (15) lines were employed in a few tests. Both lines were routinely kept on 1629-10 medium, but for the experiments to be described the EB-3 cells were transferred to Basal Medium Eagle and 25% fetal calf serum which had been preincubated at 37 C for 1 week. This medium was found to increase the percentage of fluorescent cells in the EB-3 line and to reduce the cellular growth rate.

Immunofluorescence. The techniques employed have been fully described (7).

Autoradiography. Tritiated thymidine (specific activity 6 c/mmole) was added for 2 hr to cell cultures (10^6 cells/ml) at a final concentration of 1 μ c/ml. The cells were then washed three times and resuspended in fresh medium. Smears of cells were prepared either immediately or after varying periods of further incubation at 37 C. The cells were sedimented at $600 \times g$ for 10 min, the supernatant fluids drained off, and the sediments were resuspended in the remaining fluid. Small drops of these suspensions were spread on microscope slides for immunofluorescence tests by the established procedures (7), except that the cover slips were mounted with 50% glycerol. Between 15 and 30 well-isolated fluorescent and nonfluorescent cells were selected per slide, and their location was read from the scale of the mechanical microscope stage. After removal of the cover slips, the slides were rinsed in distilled water, dried, and coated with NTB-3 emulsion (Eastman Kodak Co., Rochester, N.Y.), diluted 1:3 in distilled water, and stored for 3 days in a refrigerator. The slides were developed with Kodak D-11 developer for 2 min, fixed with Kodak acid fixer, and rinsed for 20 min in tap water. After staining with modified Giemsa solution (19) for 10 min, the preselected cells were relocated under the microscope

and examined for presence of label and distribution of the grains.

X irradiation of cells. Volumes of 25 ml of cell suspension (4×10^6 cells/ml of medium) were irradiated with 3,000 or 6,000 R in 25-ml plastic flasks (Falcon Plastics, Inc., Los Angeles, Calif.). The source of X rays was a Keleket X-ray unit (200 kv and 20 ma); 0.25-mm copper and 1-mm aluminum filters were used and the distance was 30 cm. A Victoreen Radocon ratemeter was employed to calculate the dosage. After the exposure, the cell suspensions were diluted in medium to yield 5×10^5 cells/ml and were incubated at 37 C. At intervals thereafter, parts of the cultures were used for labeling with ^3H -thymidine; the remainder was kept for periodic cell counts and observation of the fate of the irradiated cultures.

Preparation of individual immunofluorescence cells for electron microscopy. Jiyoye cells were stained vitally in the following manner: 10^6 cells were sedimented, resuspended in 0.2 ml of human γ -globulin (diluted 1:40), and incubated for 1 hr at 37 C. After two washings in phosphate-buffered saline at pH 7.2 (PBS), the cells were suspended in 0.2 ml of appropriately diluted fluorescein isothiocyanate-conjugated goat anti-human γ -globulin and were incubated for 1 hr longer. The cells were washed twice again and then diluted in PBS to yield 10^3 cells per 0.5 ml. To this volume of cell suspension was added an equal volume of melted 1.2% agarose (L'Industrie Biologique Française S.A., Gennevilliers, France) in PBS, which had been kept at 50 C in a water bath. The mixture was poured immediately into a 60-mm plastic petri dish containing a solidified layer of 2.5 ml of agarose, distributed evenly by agitation, and allowed to harden. The petri dish was placed in a wooden holder, fitting the stage of a Zeiss Ultraphot microscope, and was examined at 250 times magnification with an ultraviolet phase-contrast condensor under ultraviolet illumination. The locations of well-separated fluorescent and nonfluorescent cells were noted on the stage scale. The preparations were then fixed with 3% glutaraldehyde for 20 min, washed twice with PBS, and postfixed for 10 min in 0.5% phosphate-buffered osmic acid. The osmic acid was removed by repeated washing with 70% ethyl alcohol, and the agar layers were further dehydrated in 80 and 95% ethyl alcohol. The petri plate was fixed again in the holder in the exact position, and the preselected fluorescent and nonfluorescent cells were relocated under the microscope by use of visible light. The objective was replaced by a Leitz slide marker, over which a brass tubing had been slipped with a diameter of 2 mm at its end. The stage was raised towards the tubing until a clear ring had been punched into the agar layer around the marked cells. Finally, the plate was covered with 100% ethyl alcohol, and the punched discs were floated into the solvent. They were further dehydrated and embedded in epoxy resin as previously described for individual antibody-producing cells (6).

RESULTS

Autoradiography of cultured Burkitt cells. In the first set of experiments, Jiyoye cells were exposed to a 2-hr pulse of ^3H -thymidine and tested im-

mediately thereafter for immunofluorescence and presence of label by the methods described in the preceding section. Since, for ready removal, the cover slips were mounted with glycerol, the fluorescence observed tended to be weaker than that seen with the more commonly employed semipermanent mounting medium (18). The proportion of stained cells throughout was of the order of 7%, whereas the percentages of labeled cells varied from 30 to 60%, depending upon the interval after the last refeeding of the cultures. Under the experimental conditions employed, there were no significant differences between preselected fluorescent and nonfluorescent cells with respect to the percentages which were labeled, but the distribution of the grains differed markedly in the two groups. In labeled nonfluorescent cells, the grains were restricted to the nucleus and were usually evenly dispersed, but in about 3 to 5% they were limited to one or several foci. In labeled fluorescent cells, the grains, as a rule, showed an irregular nuclear distribution and often extended into the cytoplasm. Figure 1 presents examples of the types of labeling observed. Similar nuclear aggregates of grains were seen after a 2-hr exposure to ^3H -thymidine in the EB-3 line, which, like the Jiyoye cultures, harbors herpes-type virus. Since both the Jiyoye and EB-3 lines possess male karyotypes, a late replicating X-chromosome cannot account for this result. Furthermore, labeled Raji cells, representing another male Burkitt cell line but free of detectable herpes-type virus (2), failed to reveal focal nuclear distribution of grains. An early labeling of single chromosomes or chromosomal groups likewise can be excluded, since some of the cells with foci exhibited mitotic chromosomal condensations (Fig. 1a, d, g).

These results suggested that focal distribution of the grains is observed in those cells which at the time of labeling were synthesizing viral deoxyribonucleic acid (DNA) but in which the infectious process had not progressed as yet to production of detectable amounts of viral antigens. This suggestion is supported by the results of additional experiments in which cells were tested by autoradiography 8 hr or more after the isotope pulse. Under these conditions, nuclear foci of grains were no longer observed in nonfluorescent cells, and fluorescent cells were labeled in a higher percentage than nonfluorescent cells (80 and 33%, respectively, at 8 hr). The heavy cytoplasmic labeling at 8 hr, and even more so at 24 or 48 hr, after the pulse interfered with definite reidentification of preselected cells after development of the grains. Furthermore, some of the cells which were in the S period during labeling presumably be-

came engaged subsequently in synthesis of viral DNA and antigens, which would complicate interpretation of the results. Attempts were made therefore to reduce or prevent cellular replication by X irradiation.

Autoradiography of X-irradiated Burkitt cells. Suspensions of Jiyoye and Raji cells were exposed to 3,000 or 6,000 R and were divided into parallel cultures for pulse-labeling with ^3H -thymidine after 1, 4, and 7 days, respectively. At varying times thereafter, the cells were tested for immunofluorescence and presence of label as described. The total and viable (trypan blue exclusion) cell counts showed a slow, steady decline over the 10-day experimental period. Most of the cultures were ultimately lost, but in a few cellular replication became evident after long delays. As with other types of cells (15) the X irradiation led to the development of giant cells. In the case of the Jiyoye line, some of these showed brilliant immunofluorescence (Fig. 2a and b). The percentage of stainable cells tended to increase slightly on prolonged incubation after X irradiation, but, based on total cell counts, the actual number of fluorescent cells showed a decline. Raji cultures which were found to be free of detectable herpes-type virus failed to reveal immunofluorescent cells before as well as after X irradiation.

Since no significant differences were noted in the responses of Jiyoye cultures to 3,000 and 6,000 R, the results of autoradiography were combined for presentation in Fig. 3. The percentages of labeled, preselected immunofluorescent cells revealed similar patterns, whether the 2-hr isotope pulse was applied 1, 4, or 7 days after irradiation. At 24 to 48 hr after the pulse, on the average 70% of the fluorescent cells were labeled. Significantly smaller percentages were recorded at 8 (in a preliminary experiment, not shown) and 72 hr. Most of the fluorescent cells showed cytoplasmic dispersal of the grains (Fig. 1h, 2c, d), but because of the large nucleus and the small rim of cytoplasm, it was often impossible to determine clearly the site of the label for accurate differential counts. In contrast to the results with fluorescent cells, the percentages of labeled preselected nonfluorescent cells decreased with prolongation of the interval between X irradiation and isotope pulse, but they tended to show random fluctuations during the respective observation periods, which probably were due to the small numbers sampled. When a total of 500 nonselected cells was checked for presence of label, the results within each group were constant at all time intervals tested; therefore, only these results are shown in Fig. 3. The percentages of total positive cells decreased from 42 to 19 to 10% when the isotope pulse was applied 1, 4, and 7 days after

FIG. 1. Examples of labeling patterns in Jiyoye (b, c, and f) and EB-3 (a, d, e, g, and h) cells after exposure to ^3H -thymidine for 2 hr. Note nuclear foci of grains (a-g), mitotic condensations (a, d, and g), and extensive cytoplasmic labeling in an apparently binucleated cell (h). Even dispersal of grains, as seen in the majority of labeled cells, is shown by some of the cells (d, g). All $\times 4,500$.

irradiation, respectively. Raji cells showed a lesser degree of labeling; i.e., 13 and 7% when exposed to the isotope 1 or 4 days after exposure to X rays.

Electron microscopic examination of individual fluorescent cells. Twelve fluorescent and non-

fluorescent cells each were selected and prepared for sectioning as described under Materials and Methods. Some of these were lost owing to technical difficulties, but five and six, respectively, yielded satisfactory preparations. Every section

FIG. 2. Immunofluorescence of Jiyoye cells before (a) and 5 days after X irradiation with 3,000 R (b) and staining (c) and autoradiography (d) of an individual cell 8 hr after a ^3H -thymidine pulse. (a and b) $\times 1,800$; (c and d) $\times 4,500$.

of the fluorescent cells revealed numerous virus particles with diameters from 95 to 100 μ (Fig. 4). The vast majority of these were devoid of nucleoids and lacked a second limiting membrane. Many were slightly distorted, being somewhat elongated. It was not possible to define their intracellular location because all the fluorescent cells studied were in advanced stages of degeneration. The nonstaining cells were in better condition, and none of the six examined contained virus particles.

DISCUSSION

The above studies have provided clear proof that the immunofluorescence techniques described previously (7) detect those cultured Burkitt cells which produce herpes-type virus particles. All the individual fluorescent cells

which were picked, sectioned, and examined electron microscopically contained numerous virus particles, whereas the unstained cells prepared in the same manner were free of them. The fluorescent cells had to be stained vitally for technical reasons. This particular procedure selects cells in the final phases of the infectious process when the reagents presumably are able to penetrate into the cytoplasm. The time required for staining and selection of individual cells under partly nonphysiological conditions may have had further detrimental effects upon the physical integrity of the cells. The selected nonstaining cells were, however, in relatively good condition. Because of the insensitivity of the vital staining method, it would not be surprising to find a few nonstaining cells with virus particles if a large number of them were examined.

FIG. 3. Preferential labeling of fluorescent (FA+) cells as compared with that of the total cell population by 2 hr ^3H -thymidine pulses 1, 4, or 7 days after X irradiation. The decline in viable cells in the irradiated culture is shown in the upper curve

The results of autoradiography also denoted the identity of immunofluorescent and virus-containing cells. In addition, they agree with the earlier conclusion, based on negative-contrast electron microscopy, that the Burkitt-associated virus is most likely a member of the herpes group; i.e., a DNA virus (3, 10, 21). With DNA viruses, which replicate in the nucleus, it is not readily possible to distinguish between labeled cellular and viral DNA, but an unusual nuclear distribution of the grains, and especially their extension into the cytoplasm, combined with virus-specific immunofluorescence may permit some degree of differentiation (1, 4, 5, 12, 13). This was achieved also in the present study in that labeling of viral DNA may be surmised from the following observations. (i) Immediately after the isotope pulse, some nonfluorescent cells revealed nuclear foci of grains which were no longer evident 8 hr later. Such foci were observed in cell lines harboring herpes-type virus but not in a line free of this agent. (ii) Extension of the label from the nucleus into the cytoplasm was the rule in fluorescent cells, whereas in nonstaining cells the grains were restricted to the nucleus. (iii) Fluorescent cells were generally labeled in a significantly higher percentage than nonfluorescent cells, especially when the cultures were exposed to ^3H -thymidine several days after X irradiation.

It has not been possible to inhibit cellular DNA synthesis completely by exposure of the cells to 3,000 or 6,000 R, but its extent decreased substantially as time progressed. Cellular DNA

replication apparently continued in some of the cells for a limited period after the lethal dose of X rays, and thymidine was presumably used in diminishing amounts for repair of damaged chromatid material (17). The striking fact remains that the percentages of labeled immunofluorescent cells did not decrease with a delay in the ^3H -thymidine pulse for at least 7 days after irradiation. The percentages of labeled fluorescent cells at the various intervals after pulse-labeling were of the same order, whether the cells were exposed to the isotope 1, 4, or 7 days after X irradiation. Although it is possible that the infection stimulated some cellular DNA synthesis (4, 9), the peculiar nuclear distribution of the label and the spread of the grains into the cytoplasm suggest, as already discussed, that a substantial fraction, if not all, of the labeled DNA was of viral origin. It thus appears that viral replication was not significantly affected by X irradiation.

The fact that the percentages of labeled fluorescent cells showed peaks at 24 to 48 hr and were lower at 8 and 72 hr implies that a regular turnover of virus-producing cells occurred, i.e., that the life span of such cells was limited and that new cells started successively to synthesize viral components. Accordingly, cultures at the time of the isotope pulse may be taken to be composed of (i) cells which had terminated production of all viral components [these registered as fluorescent, unlabeled cells ($\text{F}+^3\text{H}-$) as long as they survived]; (ii) cells which were producing viral antigen while viral DNA synthesis was still

FIG. 4. Section of a fluorescent cell. The fine structure was not maintained. Virus particles (VP) in proximity to condensed chromatin indicate nuclear localization. $\times 73,500$.

in progress (these were scored $F+^3H+$ throughout their remaining life span); (iii) cells which synthesized viral DNA, but not yet viral antigen [these were $F-^3H+$ early after the pulse (8 hr) and showed initially nuclear foci of grains, but in time (24 hr) turned $F+^3H+$]; and, finally, (iv) cells (the majority) which did not produce viral components, but a few of these were slated to do so after the isotope pulse. Once these cells reached the stage of antigen production, they registered as $F+^3H-$, unless they belonged to the few which synthesized cellular DNA at the time of labeling.

The available data correspond with the assumption that the cellular life span from initiation of viral DNA synthesis to ultimate degeneration amounts to about 4 days. Under this condition, no more than about 70% of the fluorescent cells should reveal label at 24 to 48 hr, and substantially less at 8 or 72 hr. The nonlabeled fluorescent cells observed soon after the pulse would belong to group (i), and these, as they gradually succumb to the infection, are replaced by steadily increasing numbers of group (iv) cells.

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