

MOUSE-HUMAN HETEROKARYON ANALYSIS WITH A 33258 HOECHST-GIEMSA TECHNIQUE

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The ability to analyze for heterokaryon formation is of potential value in a wide variety of cell fusion experiments. Heterokaryon formation has traditionally been analyzed autoradiographically by isotopic labeling of one set of parental nuclei before fusion (Harris et al., 1966).

Hilwig and Gropp (1972) and Seth and Gropp (1973) have reported characteristic differences in the appearances of mouse and human interphase nuclei stained with the fluorochrome 33258 Hoechst, 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol-tri-hydrochloride. Exploiting these differences to monitor heterokaryon formation between established tissue culture lines of mouse and human origin, one obtains a rapid and simple replacement for autoradiography which requires no prior treatment of the parental cells. The fluorometrically differentiated parental nuclei found in heterokaryons may be subsequently irradiated. Giemsa stained, and distinguished by conventional bright-field transmission microscopy.

MATERIALS AND METHODS

Cells and Fusion

A9, a tissue culture line derived from mouse L cells (Earle, 1943), strain C3H, was grown in suspension in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Inc., Grand Island, N.Y.), with 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.). [³H]Thymidine (New England Nuclear, Boston, Mass.) at 0.05 μ Ci/ml was added to the medium 24 h before fusion. HeLa S-3 cells (Puck et al., 1956) were grown in monolayer in the same medium. Fusion was accomplished by a monolayer-suspension protocol (Giles and Ruddle, 1973) with Sendai virus at an approximate ratio

of one HeLa S-3 monolayer cell to two A9 cells in suspension.

Slide Preparation

The cells were harvested with Viokase (Grand Island Biological Co.) 20 h after fusion and suspended in phosphate-buffered saline. Cells were deposited on slides with a cytocentrifuge (Shandon Scientific Co. Ltd., London, England, catalog no. 0020) at 500 rpm for 8 min. In order to fix the cells, the slides were placed in 3:1 methanol-acetic acid for 5 min, in absolute methanol for 2 min, and then air dried.

Staining and Photography

Preparation 33258 Hoechst was a generous gift to this laboratory by Dr. A. Loew of Farbwerke Hoechst AG, Frankfurt. The slides were stained for 1 min in a 0.5 μ g/ml solution of 33258 Hoechst in 0.15 M NaCl-0.03 M KCl, 0.01 M phosphate (pH 7), rinsed twice in distilled water, and air dried. Cover slips were then mounted in 0.16 M sodium phosphate, 0.04 M sodium citrate (pH 7), and sealed with Kronig cement.

Fluorescence was observed under epi-illumination using a Zeiss UV microscope with an HBO 200 W mercury light source, a B6 38 excitation filter, an F1 450 chromatic beam splitter, an F1 436 interference line filter, and barrier filter 47. Fields were recorded and photographed with H. & W. control film (H. & W. Company, St. Johnsbury, Vt.) at ASA 400. The film was developed in D-19 (Eastman Kodak Co., Rochester, N.Y.) for 6 min.

Autoradiography

After fluorescence photography, the cover slips were removed and the slides dipped in Kodak NTB-2 (Eastman Kodak Co.) diluted one-to-one with distilled water. 7 days later, they were developed in D-19 for 4-5 min, and stained with a 4% Giemsa solution (Fisher Scientific

Co., Pittsburgh, Pa.) in 0.05 M Tris buffer (pH 7.5) for 15 min. The previously recorded fields were rephotographed with Kodak Plus-X at ASA 125 with a VG-9 green filter to enhance contrast.

RESULTS

Fig. 1 shows a photographic field of a HeLa S-3 and A9 cell fusion mixture containing one multinucleated heterokaryon along with several mouse and human parental cells. After fixation and treatment with preparation 33258 Hoechst (Fig. 1, a), the cell nuclei are stained to the virtual exclusion of cytoplasm and may be grouped into two classes: (a) nuclei with greater overall fluorescence including discrete bright chromocenters, and (b) nuclei exhibiting a generalized low level of fluorescence lacking discrete chromocenters. The bright staining pattern including discrete chromocenters is typical of the appearance of mouse interphase nuclei stained with preparation 33258 Hoechst (Hilwig and Gropp, 1972). The less intensely staining nuclei lacking chromocenters are human nuclei.

This differentiation of the HeLa and A9 nuclei is confirmed after fluorescence photomicrography, autoradiography, and Giemsa staining of the same field (Fig. 1 b). The mouse cells that were [³H]thymidine labeled before fusion, as evidenced by the autoradiographic grains over their nuclei (Fig. 1 b), correspond to those cells whose nuclei exhibit bright overall staining and discrete chromocenters with the Hoechst dye (Fig. 1 a). The HeLa S-3 nuclei are those which do not carry any grains in Fig. 1 b and show a low level of fluorescence in Fig. 1 a.

It may also be noted that the intensity and distribution of Giemsa stain (Fig. 1 b), corresponds closely to the intensity of fluorescence emission (Fig. 1 a). The autoradiographically labeled mouse nuclei are seen to be stained heavily with Giemsa's. The human nuclei are only lightly Giemsa stained. This correspondence is sufficiently great so that the intense chromocenters characteristic of mouse nuclei stained with 33258 Hoechst are readily visualized in the subsequently Giemsa-stained nuclei. Thus, heterokaryon analysis can be performed either by fluorescence microscopy on the fluorochrome-stained slides or by conventional bright-field transmission microscopy after Giemsa staining.

The Giemsa stain differentiation of mouse and human nuclei reported here appears to be dependent upon prior staining with fluorochrome and

irradiation. Appropriate radiation may be obtained from brief (several minutes or less) exposure of a cover-slipped slide to either the combination of mercury light source, excitation filter, and glass optics used for fluorescence photomicrography (see Materials and Methods), or a longwave UV mineralogical lamp. Thus, the effective light is probably in the longwave UV (or the short visible) spectrum, but the optimum radiation flux or wavelength parameters have not yet been determined. Exposure to visible light during the routine handling of slides in the laboratory is not sufficient to generate differential staining of mouse and human nuclei with the Giemsa stain protocol we have employed.

We note that a considerable range of stain intensity may be encountered within nuclei of a single parental cell type, as seen in Fig. 1. Apparent stain intensity will depend upon the degree to which a cell is flattened on the microscope slide, or possibly upon other factors such as the metabolic state of the cell (G. Moser et al., 1975). Such variation, whatever the cause, may tend to obscure the parental origin of certain cells.

Fusion mixtures, in particular, may contain cells with unusual staining properties. For example, the nucleus of the large, well-spread cell pictured at the lower right corner of Fig. 1 exhibits the discrete chromocenters indicative of a mouse cell (Fig. 1 a, b). The presence of ³H-labeled mouse DNA within the nucleus is confirmed autoradiographically (Fig. 1 b). However, the overall stain intensity is low and reminiscent of that of a human nucleus. This leads us to suggest that this cell may be a recently fused hybrid cell, but cells of ambiguous parental origin appear infrequently. In a count of 11 photographed fields, 25 of 25 heterokaryon nuclei and 184 of 185 homokaryon nuclei were correctly assigned on the basis of 33258 Hoechst fluorescence, as confirmed by autoradiography.

DISCUSSION

Interspecific human-mouse cell fusion experiments have been primary vehicles in studies of genetic linkage and gene mapping (Ruddle, 1973). The procedure described in this report affords a quick and accurate alternative to autoradiography in cell fusion experiments. We have found that treatment with 33258 Hoechst is capable of distinguishing mouse and human nuclei from a wide range of established tissue culture lines or primary cultures. The differentiation in all cases relies upon the dual criteria of overall stain intensity and the presence

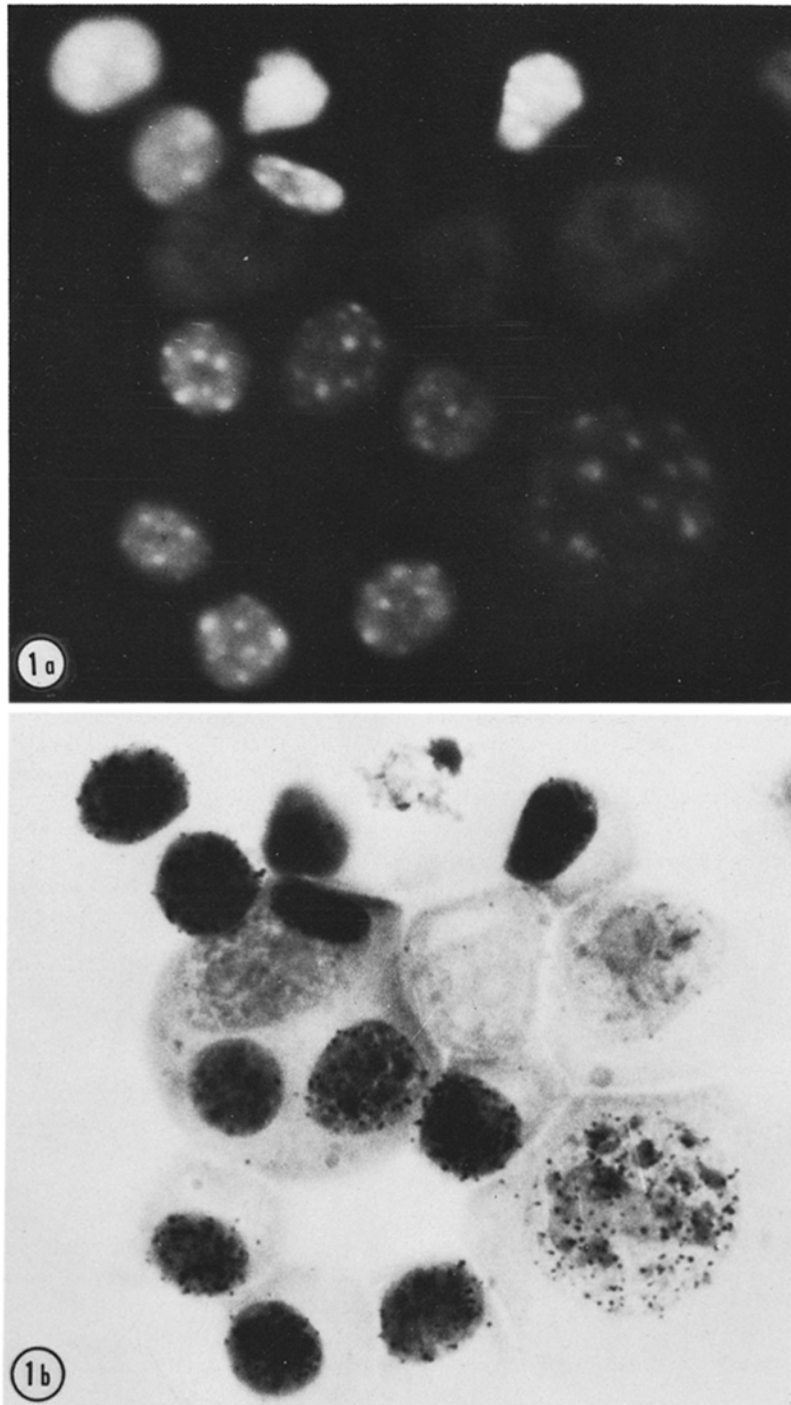


FIGURE 1 Photographic field of cells from a mouse A9-HeLa S-3 fusion mixture, cytocentrifuged onto a microscope slide and fixed as detailed in Materials and Methods. $\times 1,500$. (a) Fluorescence observed after staining with 33258 Hoechst. (b) Same field after Giemsa staining through developed autoradiographic emulsion. Mouse A9 cells were [^3H]thymidine labeled for 24 h before fusion.

in mouse nuclei or the absence in human nuclei of distinct bright chromocenters.

Mouse cell populations do contain some nuclei which lack bright chromocenters, due, we suspect, to an abnormal or unusual metabolic state of the cell. Human cell controls rarely or never include nuclei with brightly stained chromocenters that might be confused with a typical mouse nuclear staining pattern. Therefore, 33258 Hoechst treatment, optionally followed by light irradiation and Giemsa staining, can be expected to be of general use for heterokaryon analysis in man-mouse hybridizations.

The characteristic bright chromocenters of mouse nuclei stained with 33258 Hoechst were first noted by Gropp and his co-workers who reported that the number of chromocenters varied with the species of mouse and that some chromocenters were associated with nucleoli (Hilwig and Gropp, 1972; Seth and Gropp, 1973). 33258 Hoechst has been shown to stain preferentially the constitutive heterochromatin of mouse metaphase chromosomes (Hilwig and Gropp, 1972; Kucherlapati et al., 1975) and to interfere specifically with normal condensation of mouse pericentric heterochromatin during *in vitro* treatment of mouse cells (Hilwig and Gropp, 1973) or man-mouse hybrids (Kim and Grzeschik, 1974; Kucherlapati et al., 1975). 33258 Hoechst has been shown to fluoresce with greater relative intensity in the presence of AT-rich DNA (Weisblum and Haenssler, 1974), and the largest class of AT-rich DNA ($G + C = 34.2\%$) in mouse is the $\rho = 1.691$ density satellite (Flamm et al., 1967). This satellite has been localized in the mouse centromeric constitutive heterochromatin (Pardue and Gall, 1971). The arrangement of constitutive heterochromatin in interphase mouse germ cells has been studied (Hsu et al., 1971) and is very similar, in terms of the number and arrangement of chromocenters, to the patterns obtained in nuclei stained with 33258 Hoechst. In view of these observations, it should be readily accepted that the characteristic chromocenters of 33258 Hoechst-stained mouse nuclei arise due to preferential fluorescence from loci of AT-rich constitutive heterochromatin in the nuclei (Hilwig and Gropp, 1973; Seth and Gropp, 1973). Consequently, the 33258 Hoechst differentiation should be useful for heterokaryon analysis in any interspecific cross with distinguishable amounts or chromosomal distributions of AT-rich (hetero)chromatin.

Conversion of 33258 Hoechst staining to an

analogous Giemsa stain distribution is a separate matter for consideration. This conversion phenomenon has been reported previously in studies of sister chromatid exchanges in Chinese hamster ovary cells. These studies employ a technique (Latt, 1973) involving bromodeoxyuridine (BrdU) labeling for two cell divisions, which yields one bright and one dim chromatid upon staining with 33258 Hoechst. The chromatid differentiation is retained after Giemsa treatment (Perry and Wolff, 1974; Wolff and Perry, 1974). However, related studies have reported similarly differentiated Giemsa-stained chromatids in BrdU-labeled cells in the absence of prior treatment with a fluorochrome. The Giemsa differentiation has been achieved with no preliminary treatment (Zakharov and Egolina, 1972; Ikushima and Wolff, 1974) and with 10 min of incubation at 87–89°C in 1.0 M NaH_2PO_4 , pH 8.0 (Korenberg and Freedlender, 1974).

Analogously, high-contrast differentiation of the heterochromatin of interphase mouse nuclei has been obtained using Wright's stain with no pretreatment (Yasmineh and Yunis, 1970). It has been possible to visualize specific features of human interphase heterochromatin by using either fluorescent quinacrine mustard dihydrochloride or Giemsa stain (Kim, 1974). Therefore, pretreatment with 33258 Hoechst is certainly not an explicit prerequisite for differentiation by Giemsa stain.

One might be tempted to postulate that differential staining is due to related intrinsic binding affinities for the fluorochrome and for a component of Giemsa stain. However, it has been persuasively shown that the difference in 33258 Hoechst fluorescence in BrdU-labeled cells is not due to differential binding but to a BrdU-quenching effect (Latt, 1973; Latt, 1974 *a*). The quenching is abolished when the mounting medium is changed from pH 7.0 to pH 4.1 (Latt, 1974 *b*).

Most importantly, for certain stain protocols, such as that reported here, irradiation affords one means to generate differential Giemsa staining once a slide has been stained with fluorochrome. Similar observations have been made with acridine orange (Perry and Wolff, 1974) and by using 33258 Hoechst to differentiate chromatids (K. M. Huttner and F. H. Ruddle, unpublished observations). In these instances, we may infer that differential Giemsa staining is enhanced by a photo-reaction of the fluorochrome-chromatin complex. Such a reaction might facilitate Giemsa binding

in proportion to the amount of fluorochrome present in a favorable chemical environment or binding mode.

Whether or not this is a valid inference, many investigators will find it useful to convert a specimen differentially stained with 33258 Hoechst to a differentially stained Giemsa rendition. Promising applications of this technique are currently under investigation.

SUMMARY

The bibenzimidazol derivative 33258 Hoechst can be used to distinguish microfluorometrically between mouse and human nuclei in heterokaryons. This affords a quick and accurate alternative to autoradiography in the analysis of such heterokaryons. The 33258 Hoechst fluorescence patterns can be converted after irradiation to a Giemsa rendition of the differential staining.

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