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Permanent cell line expressing human factor VIII-related antigen established by hybridization

(endothelium/somatic cell genetics/differentiated cell lines/von Willebrand factor/hemostasis)

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ABSTRACT **A permanent human cell line, EA·hy 926, has been established that expresses at least one highly differentiated function of vascular endothelium, factor VIII-related antigen. This line was derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549. Hybrid cells that survived in selective medium had more chromosomes than either progenitor cell type and included a marker chromosome from the A549 line. Factor VIII-related antigen can be identified intracellularly in the hybrids by immunofluorescence and accumulates in the culture fluid. Expression of factor VIII-related antigen by these hybrid cells has been maintained for more than 100 cumulative population doublings, including more than 50 passages and three cloning steps. This is evidence that EA·hy 926 represents a permanent line.**

Differentiated functions of endothelium are critical for the vascularization process in normal and neoplastic tissue, for maintaining the blood–brain barrier, and for hemostasis. Factor VIII-related antigen (VIIIR:Ag) is an endothelial cell product (1) involved in the aggregation of platelets, and megakaryocytes are the only other cell type known to express this antigen (2).

VIIIR:Ag is present in normal human plasma at about 10 μg/ml, and decreased levels are found in classical von Willebrand disease, an autosomal dominantly inherited bleeding disorder in humans. VIIIR:Ag circulates as a large molecular complex and its platelet-aggregating activity is directly related to the size distribution of the complex (3). Factor VIII coagulant activity (antihemophilic factor) is also associated with the VIIIR:Ag complex in plasma. The detailed composition and stoichiometrics of the complex are still unclear. Heterologous antibodies produced in response to immunization with the plasma factor VIII complex have defined the major VIIIR:Ag and have demonstrated its presence in the cells that line vascular lumina. Such endothelial cells derived from human umbilical veins have been shown to synthesize VIIIR:Ag in culture (1). However, the survival of these differentiated cells in culture is quite limited and is dependent on special culture parameters and growth factors (4).

There are relatively few permanent cell lines that express facultative traits of tissues from which they were derived. Human differentiated cell lines are especially valuable because the types of experiments that can be conducted *in vivo* are further limited by ethical considerations. Permanent cell lines that express differentiated functions have perhaps most often been derived from tumors of differentiated cells, such as plasmacytomas. Somatic cell hybridization has also been used to immortalize certain facultative traits in culture, hybridomas (5) being the most renowned examples of this tactic. However, differentiated functions are most often extinguished in hybrid cells (6), and the principles that govern whether a function is extinguished or retained are not yet clear.

We have previously described interspecies hybrids between human endothelial cells and a number of rodent cell lines, in which VIIIR:Ag was not expressed (7). There has also been a preliminary report by others (8) of human–rodent hybrids that may express VIIIR:Ag, but the intracellular distribution of antigenicity that they detect is not similar to that of VIIIR:Ag in endothelial cells.

Here we report a human intraspecies hybrid, EA·hy 926, that expresses VIIIR:Ag with the same morphological distribution as in primary endothelial cells.

MATERIALS AND METHODS

Cell Culture. Cells were cultured on plastic ware at 37°C in a humid atmosphere containing 7% CO_2 in air. Culture medium was exchanged every 3–5 days. At confluence, 0.01% trypsin (TRL Worthington, no. LS00 044 52) in 8 mM phosphate-buffered saline (pH 7.4) with 0.54 mM EDTA and 5.5 mM glucose was used to detach the cells, which were then subcultured at lower cell density.

Human umbilical vein endothelial cells (HUV-EC) were isolated as described in detail by Gimbrone (9). The vein of an umbilical cord kept at 4°C for 4 hr postpartum was irrigated with phosphate-buffered saline. The endothelial cells were dissociated from the vessel wall with 0.1% collagenase (Sigma, no. C2139) in phosphate-buffered saline at 37°C for 20 min. The cells were separated from the collagenase solution by centrifugation and were distributed over 60 cm^2 in RPMI 1640 medium (GIBCO, no. 430-1800) with 15% heat-inactivated fetal bovine serum, 0.01 mM thymidine (10), penicillin at 100 units/ml, and streptomycin at 100 μg/ml. A portion of the medium (15%) had been preconditioned for 2 days by actively growing BALB/c 3T3 cells (11), followed by 0.45-μm filtration.

A549 cells were originally derived by Lieber *et al.* (12) from a human lung carcinoma. Cells of a hypoxanthine phosphoribosyltransferase-deficient clone, A549/8 (13), were cultured in the presence of 0.1 μM 6-thioguanine in Dulbecco's modified Eagle medium (GIBCO, no. 430-1600) supplemented with 5% heat-inactivated fetal bovine serum. These cells died in HAT [100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine (14)].

Rhodamine 6G (Eastman Organic Chemicals, no. 10724) was added at 1 μg/ml to A549/8 culture 3 days before hybridization in an attempt to increase the yield of viable hybrids, as suggested by the work of Ziegler and Davidson (15).

Hybridization. A confluent 55-cm^2 dish of rhodamine 6G-treated A549/8 cells and a dish of first-passage HUV-EC were trypsinized and combined in used culture medium in a round-bottomed tube. The cells were pelleted together at 350 × *g* for

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Abbreviations: VIIIR:Ag, factor VIII-related antigen; HUV-EC, human umbilical vein endothelial cells; HAT, 100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine.

5 min, resuspended in serum-free medium, and centrifuged again. After all but a drop of the supernatant had been removed by aspiration, the cells were resuspended and 0.5 ml of 50% (wt/vol) polyethylene glycol of M_r 1,000 (Koch Light) was added. The suspension was centrifuged for 3 min at 125 × *g*; then 5 ml of serum-free medium was added gradually, and the cells were gently swirled for 3 min before centrifuging again at 350 × *g* for 5 min. The cell pellet was resuspended in endothelial cell medium and distributed to four dishes at varying densities. At the same time, rhodamine 6G-treated A549/8 cells alone were treated with polyethylene glycol in the same way and distributed to two dishes. Four days after fusion, the culture medium was changed to include HAT.

VIIIR:Ag Assays. Fluorescein-labeled goat antibody was used in a direct immunofluorescent assay to detect intracellular VIIIR:Ag. This antibody preparation has been shown to be specific for the component of normal human plasma that is missing in severe von Willebrand disease plasma (7). A mouse monoclonal antibody to VIIIR:Ag (16) was used in an indirect immunofluorescent assay. The primary monoclonal antibody was visualized by using a fluorescein-labeled affinity-purified goat antibody to mouse immunoglobulin (Bionetics, no. 8608-13). Cells to be assayed were grown on no. 1 glass coverslips, fixed in acetone, exposed to the antibodies, and washed in phosphate-buffered saline. Fluoresceinated antibodies that bound to cells were detected microscopically by incident light excitation and observation through a Leitz H2 filter system.

VIIIR:Ag in the medium removed from cell cultures was assayed by Laurell immunoelectrophoresis by using a rabbit antibody to VIIIR:Ag (17). The immunoprecipitates were visualized by using peroxidase-conjugated swine antibodies to rabbit immunoglobulins (Accurate Chemicals, no. P217) and 3-amino-9-ethylcarbazole as substrate.

von Willebrand factor activity was assayed as ristocetin-dependent platelet agglutination (18).

Chromosomes. Chromosomes were spread and stained as described by Hsu (19). For counting, spreads within disks of cytoplasm were selected, and care was taken to avoid counting a single spread more than once. Modal chromosome numbers were determined on the basis of chromosome counts of 50 cells.

RESULTS

When rhodamine 6G-treated A549/8 cells were mixed with primary endothelial cells and subjected to polyethylene glycol fusion and then plated in HAT-supplemented medium, all four subcultures continued to survive for many weeks after both of the control cultures of treated A549/8 cells had disintegrated. After 6 wk, each culture was replated at a density of 1:10 to eliminate the possibility that survival was dependent on cross feeding. The cultures continued to expand slowly but at a gradually increasing rate for the next few passages. Cellular morphology was varied and unlike either progenitor type.

Fusion-derived cells were first assayed for VIIIR:Ag after 14 wk and four passages. A large fraction of the cells in each of the four sublines was found to be VIIIR:Ag-positive. At this stage the cells were cloned and VIIIR:Ag-positive cells were found in eight of eight subclones. One of these subclones, EA·hy 926, was continuously expanded and additional subclones were isolated after 30 more passages. At least 16 of 17 clones had VIIIR:Ag-positive cells at this stage also.

Results of the direct immunofluorescent assay indicating VIIIR:Ag in fusion-derived cells are presented in Fig. 1 along with appropriate controls for specificity. VIIIR:Ag in the fusion-derived cells was found to be distributed extranuclearly in discrete bead formations, indistinguishable from its morphological distribution in primary endothelial cells. The other progenitor cell line, A549/8, was negative for VIIIR:Ag. Results of the direct immunofluorescent assay with a polyclonal goat antibody were confirmed by the indirect assay with monoclonal mouse antibody to VIIIR:Ag.

VIIIR:Ag was found to accumulate in the culture medium of fusion-derived cells as shown in Fig. 2. von Willebrand factor activity was also detected in the culture medium from these cells at a level similar to that in primary HUV-EC culture medium. For both types of cells, the sizes of the platelet aggregates formed were rather small.

Despite the expression of this highly differentiated function, the fusion-derived cells were not thought to be descended from the endothelial cells alone, because they continued to expand far beyond the survival time of any of the other hundreds of endothelial cell cultures established in this laboratory. To demonstrate that the cells were indeed hybrids, the chromosomes were examined and counted. Shortly after fusion, at a cumulative population doubling level of 10, the modal chromosome number of hybrid line EA·hy 920 was 100, a number close to the sum of 61 chromosomes from an A549/8 cell and 46 chromosomes from a normal human diploid endothelial cell. Subsequently, the modal chromosome number of subclone EA·hy 926 stabilized around 80, as shown in Fig. 3. At each stage, a large dicentric-like marker chromosome originating in A549 cells was observed in most spreads.

These hybrid cells have quite vigorous growth properties. They have been in culture for many population doublings beyond the level at which nonpermanent cell lines generally succumb to senescence. Population doubling level here refers to the logarithms of the relative expansions in area of confluent cells at each passage. It has not been corrected for <100% attachment efficiency and is therefore an underestimate. When the cumulative population doubling level was 52, EA·hy 926 cultures were determined to have a doubling time of 12 hr and to attain a density of 10^5 cells per cm^2. The cloning efficiency was 21% at a cumulative population doubling level of 109, and cells reached confluency again within a week when 1/16th of a confluent culture was passed to a new dish. The Hoechst 33258 test (20) for mycoplasma contamination of these cultures was negative.

CONCLUSIONS AND DISCUSSION

We have described sustained expression of VIIIR:Ag, the product of a highly differentiated tissue, in a continuous cell line. There has been no persistent change in the fraction of antigen-positive cells during >100 population doublings since this cell line was constructed by hybridization. Because most chromosome segregation from hybrid cells occurs soon after fusion, and intraspecies hybrids are known to be relatively stable (21), we expect expression of VIIIR:Ag to persist indefinitely in these hybrid cells.

Absolute criteria for predicting the immortality of a cell line are not established. Clonal endothelial lines isolated from fetal bovine aorta have been shown (22) to have a life span limited to about 80 population doublings under normal culture conditions similar to those described here. The survival of human endothelial cells in culture has generally been found to be much more limited than that of bovine cells. However, the hybrid human cells described here do not appear to have the limited expansion potential characteristic of normal diploid cells because they have already reached >100 population doublings. The fact that the growth rate of these cells has gradually increased ever since their isolation is further reason to believe that the hybrids represent a permanent line.

There is precedent for immortalizing genetic traits in culture by hybridization of normal cells with cells of permanent lines. However, most often, only constitutive ("housekeeping") functions continue to be expressed in hybrid cells (6). VIIIR:Ag is

a facultative ("differentiated") function normally expressed only in endothelial cells and megakaryocytes. We previously fused human vascular endothelial cells to cells of 10 different rodent lines and isolated hundreds of interspecies hybrid clones, none of which sustained the expression of human VIIIR:Ag (7). The VIIIR:Ag-positive hybrids described here are intraspecies hy-

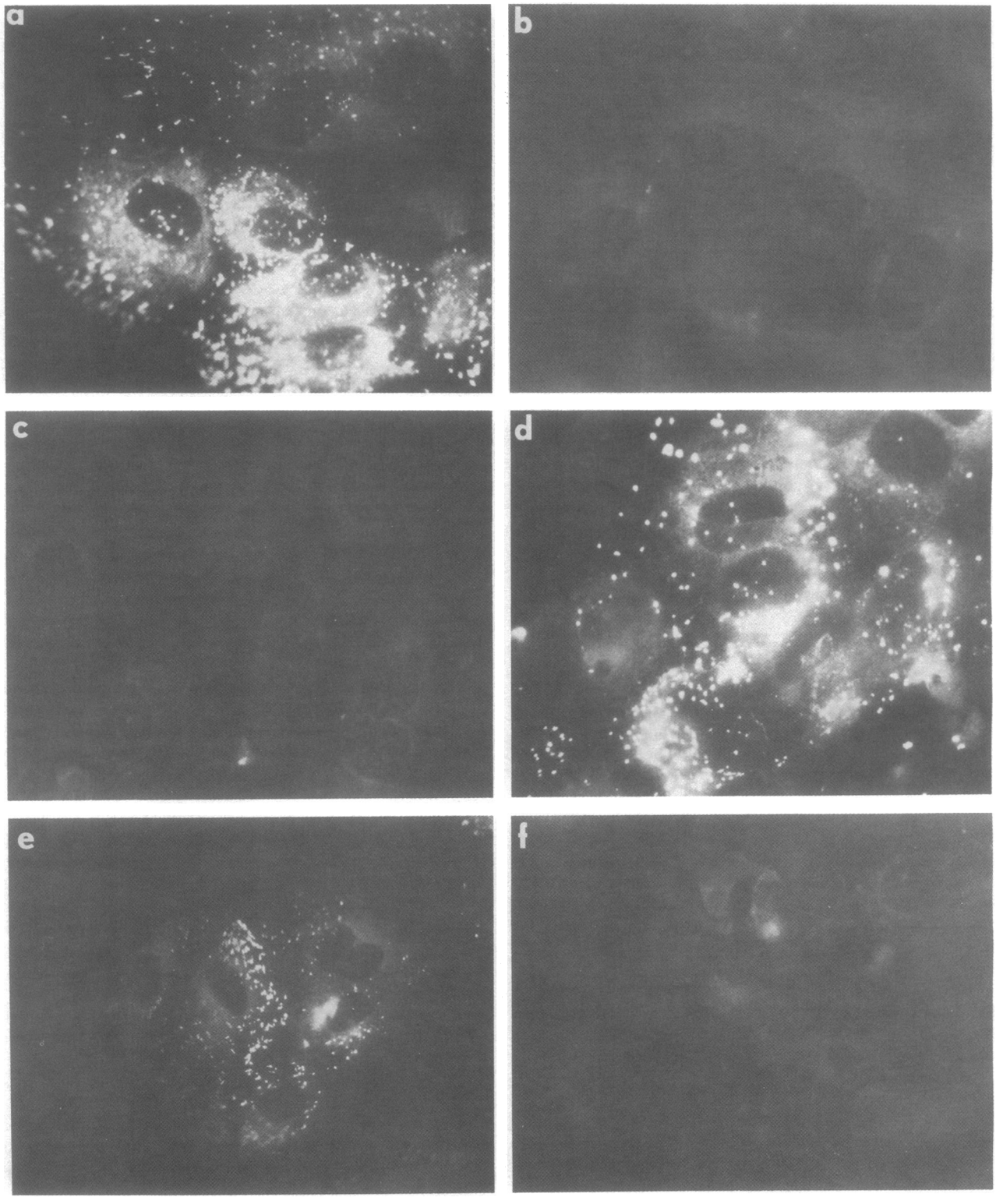

FIG. 1. Cells stained for VIIIR:Ag with fluoresceinated antibodies. (*a*) Primary HUV-EC were stained directly with fluoresceinated goat antibody to VIIIR:Ag preabsorbed with severe von Willebrand plasma, which does not remove the antibodies to VIIIR:Ag. (*b*) Primary HUV-EC were treated similarly with fluoresceinated goat antibody to VIIIR:Ag but preabsorbed with normal plasma, which removed the antibodies to VIIIR:Ag. (*c*) A549/8 cells treated with fluoresceinated goat antibody to VIIIR:Ag do not bind the antibody. (*d*) Hybrid cells, EA·hy 926, were treated similarly with fluoresceinated goat antibody to VIIIR:Ag preabsorbed only with severe von Willebrand plasma. (*e*) Hybrid cells, EA·hy 926, were stained indirectly by using monoclonal antibody to VIIIR:Ag as the primary reagent and fluoresceinated goat antibody to mouse immunoglobulin as the secondary reagent. (*f*) Hybrid cells, EA·hy 926, were treated similarly but with normal mouse serum as the primary reagent and the same fluoresceinated goat antibody to mouse immunoglobulin as the secondary reagent.

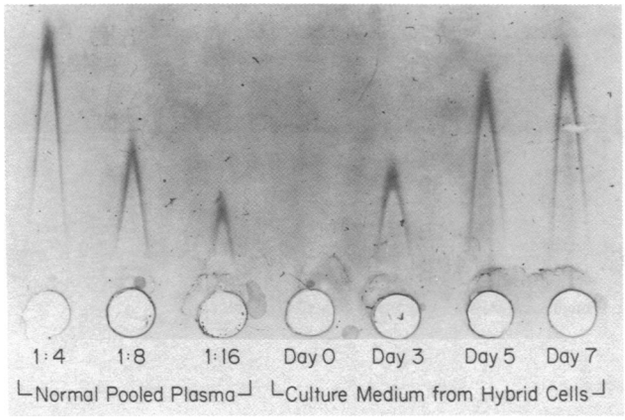

FIG. 2. Rockets of immunoprecipitates were formed by electrophoresis of 20-μl samples through an agarose gel containing 0.25% antiserum to VIIIR:Ag and were visualized by using a peroxidase-conjugated secondary antibody. Dilutions of pooled normal plasma are shown as standards on the left. Culture medium samples are shown on the right after various intervals of exposure to confluent EA·hy 926 cultures at 0.1 ml/cm^2.

brids, which may be significant in the sustained expression of this differentiated function.

Another feature of this cell fusion that may have been significant to the sustained expression of VIIIR:Ag was the pretreatment of the A549/8 cells with rhodamine 6G. Ziegler and Davidson (15) have shown that rhodamine 6G could favorably influence the yield of viable hybrids from certain cell lines and that it eliminated mitochondrial genetic determinants. This agent may also influence the direction of chromosomal segregation in hybrid cells (23). It is conceivable that rhodamine 6G pretreatment can effect the sustained expression of a number of differentiated functions in hybrid cells.

To test whether the expression of VIIIR:Ag in hybrids of primary endothelial cells and A549/8 cells is affected by pretreatment of the A549/8 cells with rhodamine 6G, equal portions of an endothelial cell preparation were fused with rhodamine 6G-treated and untreated cultures of the permanent cell line. No viable hybrids were detected. The hybrid yield from fusions involving primary cell lines depends, of course, upon the vigor of the particular cell preparation used. The substantial yield of hybrids described in this report resulted from a fusion involving a primary endothelial cell preparation and only rhodamine 6G-treated A549/8 cells. In eight previous attempts to fuse primary endothelial cells and A549/8 cells without rhodamine 6G pretreatment, no viable hybrids had been recognized. This suggests that rhodamine 6G may enhance the

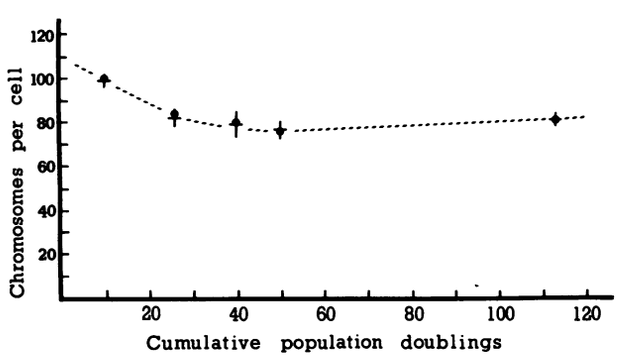

FIG. 3. The modal chromosome number in EA·hy 926 is shown at various intervals after fusion. The first point represents EA·hy 920 before its subclone EA·hy 926 was isolated. The mean chromosome numbers are indicated by the positions of the horizontal bars, and the vertical lines represent 2 SDs about the means.

hybrid yield for this combination of cell types. However, each attempt involved different endothelial cell preparations of unspecified vigor.

Intraspecific hybrids cannot be used for chromosome mapping in the usual way, because a chromosome originating in one progenitor cannot usually be distinguished from its counterpart originating in the other progenitor cell type of the same species. However, these intraspecific hybrids demonstrate that VIIIR:Ag expression can be sustained in hybrid cells and suggest that permissive interspecific hybrids may be feasible with the right combination of progenitor cells.

The morphology and distribution of VIIIR:Ag in EA·hy 926 cells and in primary endothelial cells are indistinguishable. Therefore, it can be expected that the process by which this antigen is accumulated intracellularly, the process by which it is released, and perhaps even the manner in which its release is regulated will be faithfully reflected in the continuously proliferating hybrid cells. Permanent cell lines that express differentiated functions like VIIIR:Ag are valuable because they can be manipulated *in vitro* in ways that are impossible or impractical or unethical *in vivo*, they can be cryologically preserved, and they represent an economical continuous supply of homogenous cellular material for structural, functional, and regulatory studies.

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