Europ. J. Cancer Vol. 16, pp. 1047–1055
© Pergamon Press Ltd. 1980. Printed in Great Britain

0014-2964/80/0801-1047 $02.00/0

In Vitro Consequences of Sperm–Somatic Cell Interactions*

P. J. HIGGINS, E. BORENFREUND, M. Z. WAHRMAN and A. BENDICH†

Laboratory of Cell Biochemistry, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, New York 10021, U.S.A.

Abstract—*Following interaction with rat spermatozoa and subsequent proliferation* in vitro, *Chinese hamster lung fibroblasts synthesized fetal antigen and grew in semi-solid agar suspension culture but were not tumorigenic in nude mice. Control hamster cells were consistently negative for these properties even after long-term cultivation in liquid medium. Admixture with spermatozoa was also found to induce marked multinucleation and to enhance the cloning efficiency of human tumor (HeLa) cells in soft agar culture. Sperm-mediated induction of fetal antigen synthesis and anchorage-independent growth by cultured mammalian somatic cells may thus represent an early stage in the multi-step sequence leading to malignant transformation. Sperm–somatic cell culture systems may provide a convenient model system for the study of cell–cell interactions similar to those which may normally occur* in vivo.

INTRODUCTION

AFTER INCUBATION with heterologous sperm, cultured mammalian somatic cells acquire antigenic and morphologic characteristics reminiscent of those which accompany the process of oncogenic transformation [1–4]. Such alterations include the synthesis of fetal antigen by the progeny somatic cell population [1, 3–4], a property frequently associated with neoplastic transformation [5–7].

In this paper, we present observations on fetal antigen-positive hamster fibroblasts and their clonal derivatives. The availability of these cell lines afforded an opportunity to evaluate specific consequences of sperm–somatic cell interactions under controlled *in vitro* conditions as well as to study the relationship of fetal antigen expression to altered growth properties and cellular transformation. We furthermore examined the effects of homologous and heterologous spermatozoa on the growth pattern of human tumor cells after co-incubation *in vitro*. The use of such an *in vitro* system may provide an indication as to the potential consequences of sperm–somatic cell interactions which normally occur in the *in vivo* situation.

Accepted 15 January 1980.
*This work was supported by Public Health Service Grant CA08748 from the National Cancer Institute, the National Cancer Institute Contract NO1-CB-43904, the Ford Foundation Grant No. 770-0536, and the N.I.H. Biomedical Research Support Grant No. 5 SO7 RR 05534.
†Deceased.
Abbreviations: fetal bovine serum (FBS), Eagle's minimum essential medium (MEM), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), diethylaminoethyl (DEAE).

MATERIALS AND METHODS

Cell culture

Admixture of Chinese hamster lung fibroblast (DON) cells with rat sperm and culture methods have been described [4]. Briefly, spermatozoa freshly extruded from the vas deferens of adult Wistar rats were washed in Brackett's solution [8] containing 5% FBS and mixed with DON cells at a ratio of 15:1 in Ham's F-12 growth medium. After 2 hr at 37°C, the rat sperm–hamster fibroblast incubation mixture was pipetted into 60 mm Petri dishes for propagation in F-12 medium supplemented with 20% heat-inactivated FBS (complete F-12) in a moist atmosphere of 5% CO_2–95% air. Indirect immunofluorescence

test of the progeny population 28 days after admixture revealed a 3% incidence in the number of hamster cells which expressed cytoplasmic antigen reactive with antiserum to rat fetal tissue [4]. For cloning, a monolayer culture of hamster cells was suspended by trypsination, adjusted to a concentration of 10 cells/ml growth medium and 0.1 ml of cell suspension and 0.1 ml of growth medium pipetted into each of 96 wells in several Microtest II Tissue Culture Plates (Falcon Plastics, Oxnard, Ca.). Those wells containing a single cluster of about 100 cells after 7 days growth were marked as clones; 5 days later, the cells of each clone were dispersed and seeded to separate 35 mm Petri dishes for propagation. Two clones (A and B) were similarly isolated from the control DON culture at passage 30.

The human tumor HeLa cell line (generously provided by Dr. J. Fogh of this Institute) was grown in MEM supplemented with 10% FBS, penicillin, streptomycin and fungizone. Spermatozoa for admixture were obtained from human ejaculates or aseptically from the vas deferens of mice and washed by centrifugation in medium containing, in g/l: NaCl, 6.55; KCl, 0.30; $CaCl_2 \cdot 2H_2O$, 0.33; $NaH_2PO_4 \cdot H_2O$, 0.003; $MgCl_2 \cdot 6H_2O$, 0.11; $NaHCO_3$, 3.10; glucose, 2.50; crystalline bovine serum albumin, 3.01. In a typical experiment, 10^6 HeLa cells were suspended in 2.0 ml MEM containing 2% fetal bovine serum, penicillin, streptomycin and fungizone and mixed with 1.5×10^7 spermatozoa to yield a sperm:somatic cell ratio of 15:1. This mixture was incubated with gentle intermittant shaking for 2 hr at 37°C and pipetted into 60 mm plastic culture dishes which contained coverslips for further propagation. Scanning electron microscopy [9] provided evidence for the penetration or uptake of human sperm by HeLa cells [3].

Growth properties

Growth in low serum-containing medium [10, 11] was assayed in 60 mm dishes using 1% FBS-supplemented media; duplicate cultures initially containing 2×10^4 or 2×10^5 cells, were counted for each time point. Tests for anchorage-independent growth in semi-solid medium [12] employed complete F-12-supplemented Difco–Bacto Nutrient agar with a 0.5% underlay and a 0.36% Bacto–Agar overlay which held 5×10^3 or 1×10^4 cells in a typical experiment. Each cell line was plated at least in triplicate. Liquid media cultures were re-established from colonies picked from agar with a Pasteur pipette 34 days after initiation of growth, dispersed with trypsin, and seeded into Petri dishes containing complete F-12 medium. The tendency to form multinucleate cells after 5 days exposure to cytochalasin B [13–16] (final media concentrations: 2 μg cytochalasin B/ml; 0.2% DMSO) was examined essentially as previously described [15, 16]. A murine fibrosarcoma (QUA) induced with 3-methylcholantherene [17] and human HeLa cells served as malignant cell controls. Swiss or BALB/c nude mice were used to assay for tumorigenicity *in vivo*. Animals were examined for 16–20 weeks for evidence of tumor formation following subcutaneous injection of $0.2\text{–}1 \times 10^7$ cells. Labelling with ^{3}H-thymidine was used for the detection of mycoplasma in cell cultures by autoradiography [18].

DNA polymerase assays

Assays for extracellular reverse transcriptase utilized the procedure of Liebermann and Sachs [19]. A 10 μl sample of 10,000 *g* clarified tissue culture supernatant fluid was added to 50 μl of reaction mix containing; 2 μM Tris–HCl, pH 7.8; 6 μM NaCl; 0.1 μM $MnCl_2$; 0.4 μM DTT; 0.015% NP-40, 5 μg dT_{12-18}·poly rA (P–L Biochemicals, Milwaukee, Wisc.); 0.001 μM TTP and 6 μCi [^{3}H] TTP (New England Nuclear, Boston, Mass.). After 30–60 min at 37°C, the reactions were terminated by addition of a 100-fold excess of ice-cold 5% trichloroacetic acid containing 1% sodium pyrophosphate. Tubes were placed on ice for 15 min and acid insoluble radioactivity determined as described [20]. Control cells (generously supplied by Dr. S. Marcus of this Institute) included the non-virus-producing BALB/c bone marrow fibroblast line JLSV-9 and the RV-9 derivative which produces Rauscher leukemia virus [21, 22].

RESULTS

The DON mass culture and clones A and B consisted primarily (95%) of mononucleate cells which grew as monolayers in typical swirling fibroblastic fashion; 90% of the metaphase plates examined (a minimum of 300 were counted) had the normal diploid (2n = 22) chromosome complement of the male Chinese hamster. These cells were anchorage-dependent for growth and did not give rise to tumors upon s.c inoculation into nude mice. Approximately 50% of the target DON population had taken up or were penetrated by at least one spermatozoan 48 hr after incubation

with 15 times as many rat sperm [4]. By 96 hr, 14% of the somatic cells were multinucleate and often displayed extensive cytoplasmic vacuolation as compared to the <4% binucleate and <0.3% multinucleate cell incidence in control cultures. The tendency to form giant multinucleate cells appears to be a common feature of *in vitro* propagated somatic cells after their exposure to homologous or heterologous spermatozoa (Fig. 1) and has

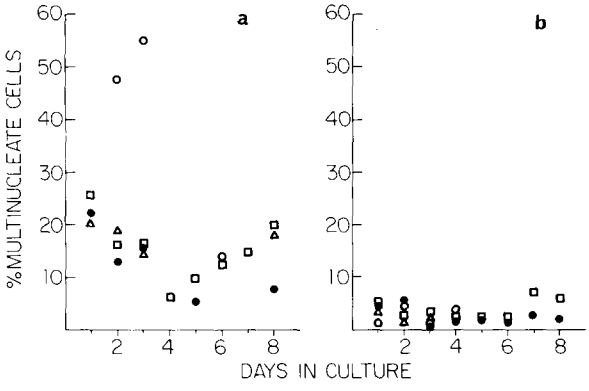

Fig. 1. General tendency of somatic cells to form multinucleate cells after exposure to homologous and heterlogous spermatozoa in vitro. *Admixture of systems were as follows: (a)* ○*—mouse bone marrow fibroblasts/mouse/sperm;* □*—Chinese hamster lung fibroblasts/Chinese hamster sperm;* △*—Chinese hamster lung fibroblasts/mouse sperm;* ●*—Chinese hamster lung fibroblasts/mouse sperm. (b) Corresponding cell lines maintained under identical* in vitro *conditions as in (a) but without the addition of spermatozoa. Admixture systems and sperm preparations were as described in "Materials and Methods".*

been observed previously [1, 3, 4]. This phenomenon is not unique to normal cells since the proportion of multinucleate cells in HeLa cultures 24–72 hr after admixture with human or mouse spermatozoa was 13–15% compared to the 2–3% seen in control HeLa populations. By 2 weeks after initial admixture, grossly multinucleate cells appear to die off and the percentage of polynucleate cells in sperm-treated cultures approached that in control populations.

Indirect immunofluorescence test of 50 individual clones derived from the sperm-treated DON mass culture showed one, clone 47, to be synthesizing antigen reactive with antiserum to rat fetal tissue (Fig. 2). Parallel analyses of DON cultures not exposed to sperm consistently yielded negative indirect immunofluorescence tests with this same antiserum. To ascertain whether the expression of fetal antigen by sperm-treated somatic cells correlated with alterations in their growth properties, control cells and two clones isolated from the sperm-treated DON population, clone 47 (fetal antigen-positive) and clone 17 (fetal antigen-negative), were used in subsequent tests.

Agar culture plating efficiencies (P.E.) of clones 47 and 17 obtained from rat sperm-treated DON cells were 0.4 and 1.2%, respectively. The ager P.E. for control DON cells which had not been exposed to sperm never exceeded 0.009%. A similar increase in agar suspension culture P.E. was noted in HeLa cells upon co-cultivation with either human or mouse spermatozoa. HeLa cells, 2–4 weeks after initial admixture with sperm, exhibited agar P.E. 60–80% higher than that observed in untreated control cultures. Exposure of HeLa cultures to DNA from calf thymus or human spleen was without effect (unpublished observations).

When examined for the tendancy to form multinucleate cells upon exposure to cytochalasin B, a technique employed to distinguish normal from neoplastic cells [13–16], DON cells isolated from sperm-treated or control cultures, as well as several agar-derived lines obtained from the sperm-treated population, failed to yield cells with more than 4 nuclei/cell after treatment with the drug. Under identical conditions, 6% of murine fibrosarcoma (QUA) and 20% of human tumor (HeLa) cells were found to contain 5–9 nuclei/cell after exposure to cytochalasin B, a shift characteristic of transformed cell lines. Unlike QUA tumor cells, all of the DON lines studied were incapable of growth in 1% FBS-supplemented media (Fig. 3). The growth properties of the various DON cell lines are summarized in Table 1. In order to determine whether viral production accompanied fetal antigen expression, analysis of DON cell tissue culture fluid aliquots for retroviral reverse transcriptase was carried out using Mn^{2+} as the divalent cation [19]. Under the conditions of the assay used, extracellular RNA-dependent DNA polymerase activity was demonstrable only in the culture fluids of RV-9 cells (Table 2) which produces Rauscher leukemia virus.

Thymidine labelling experiments showed all cultures to be free of mycoplasma contamination during the course of this study.

DISCUSSION

The data reported here suggest that somatic cells may acquire one or more subsets of the *in vitro* properties exhibited by transformed cells. Interaction with spermatozoa resulted in the acquisition by the progeny of the target cells of at least some of these changes (ex-

pression of fetal-type antigen and capacity for growth in agar culture) but not others (ability to grow in 1% serum-containing medium or

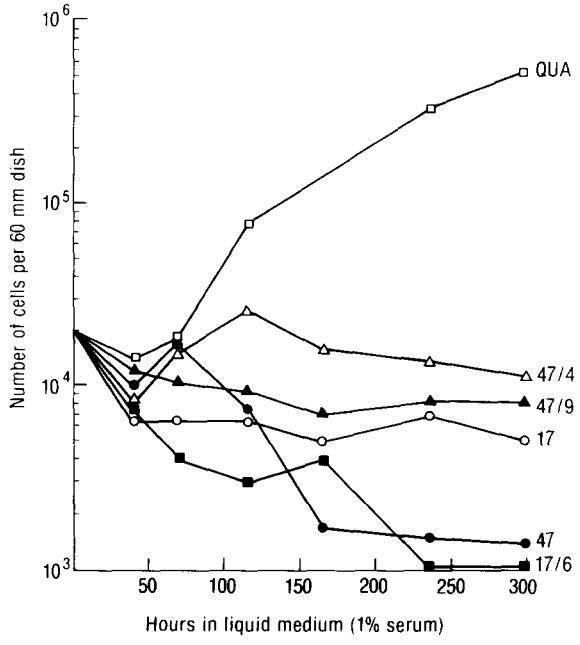

Fig. 3. Inability of agar-derived DON sub-lines and their parent clonal isolates to proliferate in Ham's F-12 medium containing 1% FBS. Designations of the individual cell lines are given in legend to Table 1. Growth of murine fibrosarcoma line (QUA) is included as positive control; cultures initially contained 2×10^4 cells from each cell line. Similar results were observed in cultures to which 2×10^5 cells were initially plated indicating that the DON lines used here were incapable of growth in 1% serum and that this inability to grow was not merely a function of low initial plating density [42].

as tumors in nude mice and the tendency to exhibit a cytochalasin B-induced shift to multinucleate cell formation). Of particular interest is the fact that these target cell changes were induced after only a single exposure of relatively short duration to spermatozoa, unlike the *in vivo* situation where multiple exposure of female genital tissue to sperm naturally occurs over long periods of time.

Seminal components have recently been implicated as contributing factors in the etiology of gynecological malignancies, particularly cancers of the uterine cervix [23–24]. Although a definite cervical tissue 'oncogen' remains to be identified, spermatozoa and seminal fluid viruses, acting independently or synergistically as initiators or promotors, are regarded as potential causative agents [23, 25–27]. Spermatozoa of several mammalian species, including the human, can penetrate or be taken up by various tissue-cultured somatic cells [1, 3, 9, 28] including those derived from cervical epithelium [26] and induce morphological and karyological alterations in somatic cells [1–4, 29] that are similar to those induced by carcinogens [1, 3]. Since a single incubation with sperm can initiate specific transformation-associated events within a given somatic cell population *in vitro*, it may be reasonable to assume that such events may also occur *in vivo*. Formation of giant multinucleate and fetal antigen-synthesizing cells, observed in somatic cell populations after admixture with sperma-

Table 1. Growth properties of hamster lung fibroblasts after admixture with rat spermatozoa compared to control normal and tumorigenic cell lines

Cells	Morphology	Agar growth*	Population doubling time (hr)† 20% FBS	1% FBS	Tumorigenicity‡
Clone 17	triangular, vacuolated	1.2	12–15	NG •	0/2
Clone 47	large, sprawling	0.4	12–15	NG	0/3
17/6§	triangular, vacuolated	agar-derived	12–15	NG	0/3
47/4‖	spindle-shaped	agar-derived	12–15	NG	0/2
47/9‖	triangular	agar-derived	12–15	NG	0/2
DON Mass	fibroblastic	0			0/2
Clone A	fibroblastic	0.009	12–15	NG	0/2
Clone B	fibroblastic	0			0/1
QUA**	spindle-shaped	9.1	21–23	40–42	7/7

*Colonies formed in semi-solid agar medium expressed as percentage of initial plating density.
†In F-12 medium supplemented with either 20% or 1% FBS (v/v); 2×10^4 and 2×10^5 cells plated in duplicate for each cell line.
‡Subcutaneous inoculation into nude mice ($0.2–1 \times 10^7$ cells per inoculum).
§Agar-derived subline of DON clone 17.
‖Agar-derived subline of DON clone 47.
•No growth.
**Mouse fibrosarcoma cells.

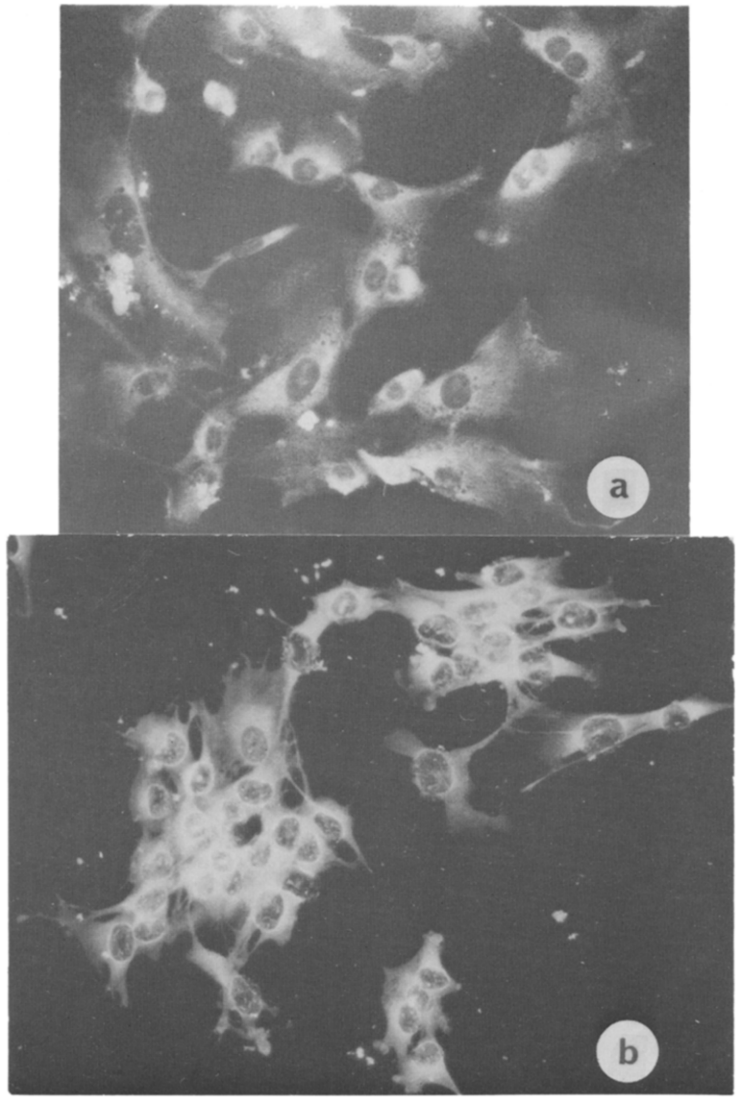

Fig. 2. Positive indirect immunofluorescence test for the presence of fetal antigen(s) in a group of methanol-fixed DON cells 48 days after initial admixture with rat spermatozoa (a) and in clone 47 cells (b) which were subsequently isolated from the DON/rat sperm parent culture; u.v. microscopy, ×330.