Transforming Growth Factor-α: A More Potent Angiogenic Mediator Than Epidermal Growth Factor

ALAIN B. SCHREIBER,* MARJORIE E. WINKLER, RIK DERYNCK

Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) are structurally related peptides. Purified human TGF-α produced in *Escherichia coli* and pure natural mouse EGF were compared for their ability to bind to target cells in vitro and to promote angiogenesis in the hamster cheek pouch bioassay. Both polypeptides were found to bind in vitro to several target cells, including endothelial cells, and to stimulate their DNA synthesis in an equipotent fashion. In vivo, however, TGF-α was more potent than EGF in promoting angiogenesis and, because TGF-α is known to be secreted by a variety of human tumors, it is suggested that this growth factor may contribute to tumor-induced angiogenesis.

TRANSFORMING GROWTH FACTORS (TGF's) are polypeptides that can confer phenotypic transformation to several normal cells (*1*). TGF-α binds to the receptor for epidermal growth factor (EGF) and has been isolated from a variety of tumor cells (*2*). TGF-β, which is not structurally related to TGF-α, binds to a distinct receptor and is synthesized by many normal and tumor cells (*3*, *4*). The polypeptide sequences of TGF-α and TGF-β and their precursors have been determined by complementary DNA (cDNA) cloning (*4*–*7*). When fully processed, TGF-α and EGF display a 35 percent homology with conservation of all six cysteine residues. This suggests that the three disulfide bridges are in the same positions in the two growth factors (*8*). The ability of EGF and TGF-α to bind to the same receptor (*9*, *10*) is presumably due to this similarity in conformation, especially to the structural homology in the third disulfide loop which may represent the receptor binding domain (*11*). Although both peptides trigger many biological effects in a similar manner, they also differ in some of their activities, for example, in the induction of cell ruffling (*12*) and in the promotion of calcium release from fetal rat long bones in vitro, with TGF-α being more potent than EGF (*13*, *14*). TGF-α is synthesized in embryos during early fetal development (*15*), in several virally transformed cells (*2*), and in a large variety of human tumors (*16*). TGF-α may play a role in neoplastic pathogenesis through an autocrine growth regulation mechanism (*17*).

The lack of TGF-α expression in hematopoietic cells and the presence of it in a variety of solid tumors, which depend on neovascularization for their development (*16*), prompted us to compare TGF-α and EGF for their ability to promote angiogenesis, that is, induce the formation of new blood vessels. We found that both polypeptides bound in vitro to target cells, including endothelial cells, and stimulated their DNA synthesis in an equipotent fashion. In vivo, however, TGF-α was a more potent angio-

A. B. Schreiber, Institute of Biological Sciences, Syntex Research, Palo Alto, CA 94304.
M. E. Winkler, Department of Protein Biochemistry, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080.
R. Derynck, Department of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080.

*Present address: Meloy Laboratories, Inc., Division of Immunology and Biologics, 6715 Electronic Drive, Springfield, VA 22151.

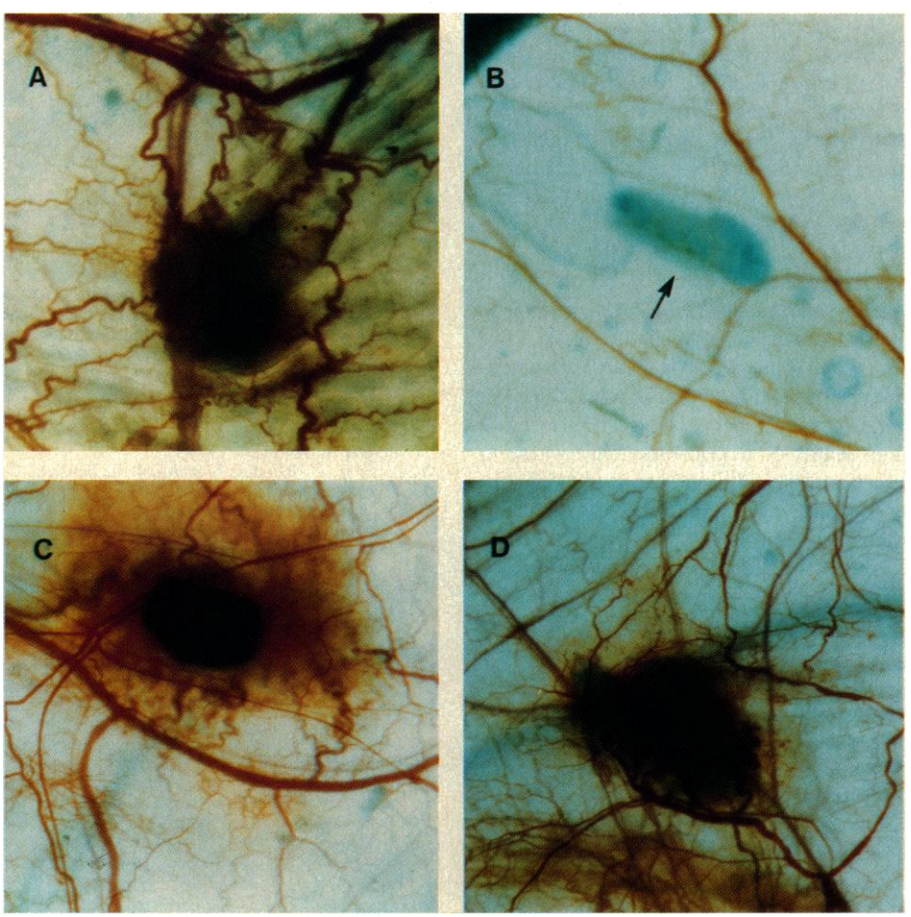

Fig. 1. Angiogenic stimulation by TGF-α and EGF in the hamster cheek pouch assay. Photomicrographs of the hamster cheek pouch in vivo 5 days after subcutaneous injection of 1 μg of TGF-α (A, score = 2), 1 μg of EGF (B, score = 0), 3 μg of TGF-α (C, score = 4), or 3 μg of EGF (D, score = 1) adsorbed on agarose beads (35-mm camera, Kodak Tungsten 50 films; magnification ×5). Note the absence of angiogenesis in (B) around the agarose beads (arrow) and the tortuosity of vessels in (A) and (C). In (C) there is also an extensive diffuse hemorrhage which gives it a score of 4. For the angiogenesis assay, male Syrian golden hamsters (120 to 150 g) of the same age (from Engle, Indiana) were anesthetized by intraperitoneal injection of a rodent anesthetic mixture (50 mg/kg Vetalar, 5 mg/kg Rompun, and 1 mg/kg acepromazine, Central City). The left cheek pouch was everted and observed through a Zeiss stereomicroscope. Mitogens were dissolved in 10 μl of sterile saline, mixed with 10 μl of Cibachrome blue agarose (Amicon), incubated at 37°C for 30 minutes, and injected subcutaneously. Five days later the animals were anesthetized as above and the pouch was again observed. The scoring was subjective: 0 = no new vessels; 1 = minimal ramification of vessels in the vicinity of injection site; 2 = new vessels reach injection site, area involved is <50% of periphery of injection site; 3 = many new tortuous vessels that reach and cross injection site in >50% of the periphery of injection site; 4 = many new tortuous vessels, hemorrhages, invasion vessels in areas removed from injection site. This scoring system was established on the basis of angiogenic responses observed with increasing concentrations of several different angiogenic factors (*37*). All results were scored in a blindly coded way by two different investigators whose results were in agreement.

genesis mediator than EGF in the hamster cheek pouch bioassay.

Human TGF-α was isolated from *Escherichia coli* containing a properly engineered expression plasmid (*5*). This purified, biologically active peptide was compared with pure natural mouse EGF for its ability to bind to the EGF receptors of four different cell types in culture. Binding was assessed by the competitive displacement at 4°C of radiolabeled EGF. The cell types tested included bovine pulmonary artery endothelial cells (BPEC) established from primary cultures, murine lung microvascular endothelial cells (LEII), the human A 431 epidermoid carcinoma cell line, and primary human foreskin fibroblasts (HFF). The A 431 and LEII cells contain an unusually high number of EGF receptors, while the two other cell types have a relatively low receptor number. EGF and TGF-α were equally potent (within experimental error) in displacing ^{125}I-labeled EGF from all four cell types (Table 1). Both peptides also stimulated DNA synthesis in quiescent HFF, BPEC, and LEII cells but not in A 431. There was no significant difference between the two growth factors either in the relative potency or in the maximal stimulation (Table 1).

The growth factors were then compared for their angiogenic effects in vivo. For this experiment we used a hamster cheek pouch bioassay because this allows the peptides to be prepared in physiological solvents for administration, in contrast to the rabbit corneal assay (*18*), and because we preferred to use a mammalian system rather than the chick chorioallantoic membrane assay (*19*). The growth factors were mixed at different doses (0.3, 1, 3, and 10 μg) with Cibachrome blue agarose beads under sterile conditions and injected subcutaneously in the pouch. These agarose beads serve as a nondenaturing form of a slow peptide release system and mark the site of injection. In separate experiments we mixed unlabeled TGF-α and EGF with ^{125}I-labeled growth factors and after injection we dissected the areas of the pouch that were affected and measured the radioactivity at different time points. The results indicated that the rates of release of the two growth factors were very similar (*20*).

The extent of angiogenesis in the hamster cheek pouch was assessed by subjective scoring on a scale from 0 (no new blood vessels) to 4 (many new tortuous vessels and hemorrhages in areas removed from the injection site) (Fig. 1). The scores indicated that the extent of angiogenesis was correlated with the dose of the growth factor (Fig. 2). At doses of 0.3 and 1 μg, EGF promoted little angiogenesis whereas TGF-α triggered extensive formation of new capillaries. At the 10-μg dose, EGF also induced extensive angiogenesis.

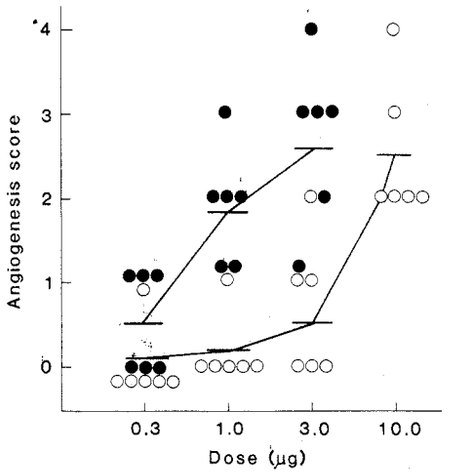

Fig. 2. Comparison of angiogenesis scores obtained with EGF and TGF-α in the hamster cheek pouch assay. The angiogenesis assay was performed and scored as described in Fig. 1. Increasing doses of either EGF (open circles) or TGF-α (closed circles) were used as stimulus. Each point represents the score of an individual animal. The curves were drawn through the median score values of each dose. Six control animals received only agarose beads. Five of these were scored as 0 and one hamster scored 1. In another control experiment six animals received agarose beads to which bovine serum albumin was adsorbed. In this group, four hamsters scored 0 and two scored 1.

The stimulation of capillary cell proliferation in vivo was also assessed by autoradiography of tissue sections of the cheek pouch after intraperitoneal injection of [*methyl*-^{3}H]thymidine. As shown in Fig. 3, there was no inflammatory response after the administration of either growth factor. Only a few polymorphonuclear leukocytes, mast cells, and macrophages were visible between the agarose beads, but these were present in similar numbers in untreated hamster tissue. In the animals studied by autoradiography, the nuclei of the endothelial cells that underwent active DNA synthesis were labeled with tritium. The ratio of the labeled capillary cells to the total number of endothelial cells correlated well with the subjective macroscopic angiogenesis score (Table 2). At the 1- and 3-μg doses, TGF-α was more efficacious than EGF in inducing angiogenesis.

Angiogenesis is a prominent feature of several physiologic and pathologic processes, including wound healing, luteinization, and solid tumor growth (*21*). The substances that promote angiogenesis can be roughly divided into two categories (*22*): (i) agents such as copper, heparin, and prostaglandins, mainly of the E type, that influence the migration of endothelial and other cells, particularly inflammatory cells; and (ii) polypeptide growth factors such as EGF, fibroblast growth factor, and endothelial cell growth factor, that presumably act on the capillary cells via their intrinsic mitogenic activity. Our histological results indicate that the angiogenic stimulation caused by TGF-α and EGF does not coincide with an inflammatory response.

The effects of EGF and TGF-α in triggering several biological effects in vitro are indistinguishable, probably because both factors bind to the same receptor (*9*, *10*). Human TGF-α and human and murine

Table 1. Relative binding and mitogenic properties of EGF and TGF-α in vitro. The preparation of the growth factors and cell lines used are described in (*34*–*36*). For the binding assay, the cells were grown to confluency in 24-well dishes (Costar), washed twice with DMEM containing 0.1% bovine serum albumin (BSA), and incubated at 4°C for 60 minutes in DMEM, 0.1% BSA, 50 m*M* Hepes, *p*H 7.4, supplied with ^{125}I-EGF in the absence or presence of increasing concentrations of EGF or TGF-α. The cells were then washed three times with cold DMEM and 0.1% BSA and lysed with 0.1*N* NaOH, and the cell-associated radioactivity was determined. The nonspecific binding assessed in the presence of a thousand-fold excess of cold EGF did not exceed 10% of the total binding. In other experiments, cells grown to confluence in 48-well dishes (Costar) were brought to quiescence by incubation at 37°C for 48 hours with DMEM and 0.5% FBS. The cells were then incubated for 18 hours with the growth factor before being exposed for 4 hours to [*methyl*-^{3}H]thymidine (NEN) (1 μCi per well). The stimulation of DNA synthesis in these cells was determined by measuring the trichloroacetic acid precipitable radioactivity. Three different binding experiments and four mitogenesis experiments were performed to establish each mean value with standard deviation.

Cell line	K_{50} binding (n*M*)*		EGF receptor number†	EC_{50} mitogenesis (n*M*)		Maximal mitogenic response (percent enhancement)	
	EGF	TGF-α		EGF	TGF-α	EGF	TGF-α
A 431	1.0 ± 0.2	0.8 ± 0.1	1.8×10^6	NA‡	NA	NA	NA
HFF	1.1 ± 0.3	1.2 ± 0.2	1.2×10^5	0.2 ± 0.1	0.2 ± 0.1	420 ± 50	380 ± 40
LEII	0.9 ± 0.2	1.1 ± 0.3	5.0×10^5	0.5 ± 0.2	0.7 ± 0.1	650 ± 70	680 ± 30
BPEC	1.1 ± 0.5	1.4 ± 0.3	4.4×10^4	0.8 ± 0.2	0.6 ± 0.2	210 ± 40	240 ± 50

*The inhibitory concentration required to displace 50% of the binding of 1 n*M* ^{125}I-labeled EGF (IC_{50}) was derived from concentration plots in the range of 10^{-10} to 5×10^{-8} *M*. †The number of EGF binding sites per cell was calculated from Scatchard plots of saturation binding curves of ^{125}I-labeled EGF at 4°C. ‡Not applicable, A 431 cells are not mitogenically stimulated by either EGF or TGF-α.

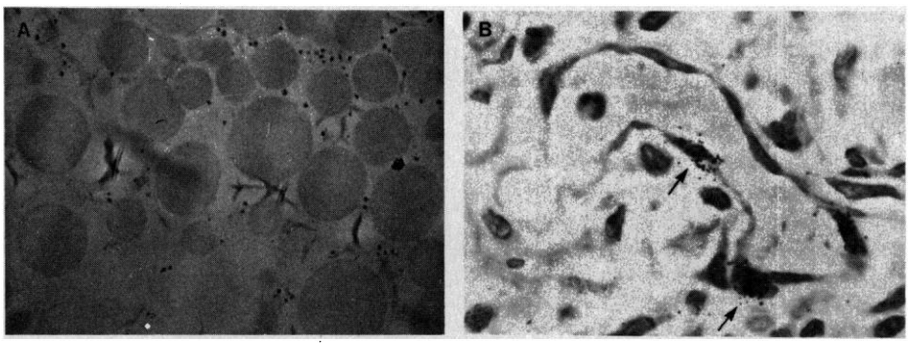

Fig. 3. Histological evaluation of the hamster cheek pouch angiogenesis assay. (A) Toluidine blue staining of formalin-fixed, paraffin-embedded, 6-μm perpendicular section of cheek pouch in the area injected with 3 μg of TGF-α and agarose beads. Magnification ×125. Note the absence of inflammatory infiltrate around the agarose beads; the result was similar with 3 μg of EGF. (B) Toluidine blue staining of glutaraldehyde-fixed, glycol methacrylate–embedded 3-μm perpendicular section of cheek pouch injected with 3 μg of TGF-α and exposed for autoradiography for 3 weeks. Magnification ×500. Note the numerous grains in the capillary endothelial cell nucleus (arrow). For histology, the animals were killed and 6-μm sections of the formalin-fixed pouch embedded in paraffin were stained with 1% toluidine blue in sodium benzoate. For autoradiography, the animals were injected intraperitoneally with 1 mCi of [*methyl*-^{3}H]thymidine 8 hours before they were killed. Sections (3 μm) of the glutaraldehyde-fixed pouch embedded in glycol methacrylate were dipped in NTB-2 autoradiographic emulsion (Kodak), kept in slide boxes for 3 weeks, and stained with 1% toluidine blue in sodium benzoate.

EGF are quantitatively equally effective in vivo in inducing precocious eyelid opening in newborn mice (*23*). We show here that TGF-α and EGF bind equally effectively to the EGF receptors on several cell types, including endothelial cells, and appear to be equally potent mitogens in vitro for endothelial cells. It was reported previously that EGF has angiogenic activity (*24*) and can induce a mitogenic response in microvascular endothelial cells (*25*). Our data indicate that, in vivo, TGF-α is more effective than EGF in promoting angiogenesis. A similar difference in potency between these growth factors was recently observed in their ability to induce bone resorption (*13*, *14*).

These quantitative differences in activity could conceivably be due to a lower sensitivity of TGF-α to protease digestion in vivo, to differences in clearance ratios, or to other factors that would result in a higher bioavailability of TGF-α. However, the two peptides had similar kinetics of release, and they were previously found to be equipotent in an eyelid opening assay in newborn mice, suggesting that they have similar pharmacological properties (*23*). It is also possible that, in addition to binding to the EGF receptor, TGF-α binds to another receptor that plays a role in the promotion of angiogenesis. It has been reported that TGF-α interacts with a separate receptor on rat kidney cells (*26*), but an antibody to the EGF receptor prevents the mitogenic activity of both TGF-α and EGF (*27*). There could be high- and low-affinity EGF receptors, as suggested, for example, by Gregoriou and Rees (*28*), and these might display different binding parameters for EGF and TGF-α. Even if one assumes that there is only one type of EGF receptor, one might postulate that TGF-α and EGF interact differently with the receptor at the cell surface or during internalization of the ligand-receptor complex. Another possibility is that, in vivo, both growth factors induce the production of angiogenic mediators from another cell type, for example, fibroblasts, in addition to the endothelial cells. If this were the case, these cells would have to display a differential response to TGF-α and EGF. The recent development of in vitro models of capillary tube formation (*29*, *30*) and isolated organ angiogenesis may facilitate studies of the mechanistic basis for the angiogenic response to both growth factors.

Table 2. Comparison of the mitotic activity of the capillary endothelial cells in vivo with the extent of angiogenesis caused by EGF or TGF-α. The hamster cheek pouch assay and [*methyl*-^{3}H]thymidine labeling were performed and scored as for Figs. 1 and 3. After toluidine blue staining the ratio of nuclear grain-stained capillary endothelial cells over the total number of endothelial cells was determined. About 150 endothelial cells were examined per animal. The endothelial cells were distinguished from the other cells on the basis of anatomical localization and histological morphology. This ratio for the mitotic activity of the endothelial cells correlates well with the macroscopic angiogenesis score. Each value corresponds to one animal.

Stimulus	Dose (μg)	Angiogenesis score	Index $\left(\frac{\text{labeled cells}}{\text{total cells}} \times 100\right)$
None		0	1
EGF	1	0	3
EGF	3	2	8
EGF	10	3	17
TGF-α	1	2	9
TGF-α	3	3	19

The relative potency of TGF-α as an angiogenic mediator suggests that, in addition to its potential importance as an autocrine growth regulator, it may play a role in malignancy-associated neovascularization, contributing to the generation of a local microenvironment that is favorable for solid tumor growth. This possibility is supported by the observation that TGF-α messenger RNA cannot be detected in hematopoietic tumor cell lines but is expressed in many solid tumors that undergo neovascularization (*16*). Immunohistochemical analysis with an antibody to TGF-α revealed prominent staining in the cells bordering the capillaries of an experimentally induced solid tumor (*31*). Such a role for TGF-α in angiogenesis does not exclude the likelihood of other angiogenic mediators secreted by the tumor cells, for example, the chondrosarcoma-derived growth factor (*32*) or angiogenin (*33*), also participating in the tumor-induced neovascularization.

REFERENCES AND NOTES

1. J. E. De Larco and G. J. Todaro, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4001 (1978).
2. G. J. Todaro, D. C. Lee, N. R. Webb, T. M. Rose, J. P. Brown, in *Cancer Cells 3*, J. Feramisco, B. Ozanne, C. Stiles, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1985), vol. 3, pp. 51–58.
3. M. A. Anzano *et al.*, *Cancer Res.* **42**, 4776 (1982).
4. R. Derynck *et al.*, *Nature (London)* **316**, 701 (1985).
5. R. Derynck, A. B. Roberts, M. E. Winkler, E. Y. Chen, D. Goeddel, *Cell* **38**, 287 (1984).
6. D. C. Lee, T. M. Rose, N. R. Webb, G. J. Todaro, *Nature (London)* **313**, 489 (1985).
7. R. Derynck, J. A. Jarrett, E. Y. Chen, D. V. Goeddel, *J. Biol. Chem.*, in press.
8. H. Marquardt, M. W. Hunkapiller, L. E. Hood, G. J. Todaro, *Science* **223**, 1079 (1984).
9. J. Massagué, *J. Biol. Chem.* **258**, 13614 (1983).
10. J. P. Tam, H. Marquardt, D. Rosberger, N. Y. Heath, G. J. Todaro, *Nature (London)* **309**, 376 (1984).
11. J. J. Nestor, Jr., S. R. Newman, B. De Lustro, G. J. Todaro, A. B. Schreiber, *Biochem. Biophys. Res. Commun.* **129**, 226 (1985).
12. S. Myrdal, *J. Cell Biol.* **101**, 2440 (1985).
13. P. H. Stern *et al.*, *J. Clin. Invest.* **76**, 2016 (1985).
14. K. J. Ibbotson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2228 (1986).
15. D. C. Lee, R. M. Rochford, G. J. Todaro, L. P. Villareal, *Mol. Cell. Biol.* **5**, 3644 (1985).
16. R. Derynck *et al.*, in preparation.
17. M. B. Sporn and G. J. Todaro, *N. Engl. J. Med.* **303**, 878 (1980).
18. M. A. Gimbrone, Jr., R. S. Cotran, S. B. Leapman, J. B. Folkman, *J. Natl. Cancer Inst.* **52**, 413 (1974).
19. R. Auerbach, L. Kuba, E. Knighton, J. Folkman, *Dev. Biol.* **41**, 391 (1974).
20. The ^{25}I-labeled EGF or TGF-α (150,000 count/min) were mixed with 3 μg of EGF or TGF-α and adsorbed to Cibachrome blue agarose (138,000 and 142,000 count/min were bound in the case of EGF and TGF-α, respectively). The beads were injected subcutaneously in the cheek pouch as described in Fig. 1. For each peptide and each time point, one hamster was killed, the area of the pouch containing the agarose beads was dissected, and the radioactivity was measured. At 12 hours, and at 1, 2, and 5 days after injection, the agarose beads had retained 32, 20, 5, and 0.6% of the injected radioactive EGF, and 28, 17, 6, and 0.8% of the labeled TGF-α.
21. J. Folkman, *Ann. N.Y. Acad. Sci.* **401**, 212 (1983).
22. D. Ben Ezra, *Am. J. Ophthalmol.* **86**, 455 (1978).

23. M. Smith *et al.*, *Nature (London)* **315**, 515 (1985).
24. D. Gospodarowicz, H. Bialecki, G. K. Thakral, *Exp. Eye Res.* **28**, 501 (1979).
25. B. R. McAuslan, V. Bender, W. Riley, B. A. Moss, *Cell. Biol. Int. Rep.* **9**, 175 (1985).
26. J. Massagué, M. P. Czech, K. Iwata, J. E. De Larco, G. J. Todaro, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6822 (1982).
27. G. Carpenter, C. M. Stoscheck, Y. A. Preston, J. E. De Larco, *ibid.* **80**, 5627 (1983).
28. M. Gregoriou and A. R. Rees, *EMBO J.* **3**, 929 (1984).
29. J. Folkman and C. Haudenshild, *Nature (London)* **288**, 55 (1980).
30. T. Maciag, J. Kadish, L. Wilkins, M. B. Stemerman, R. Weinstein, *J. Cell Biol.* **94**, 511 (1982).
31. J. Wilcox, personal communication.
32. Y. Shing *et al.*, *Science* **223**, 1296 (1984).
33. J. N. Fett *et al.*, *Biochemistry* **24**, 5480 (1985).
34. The EGF was isolated from mouse submaxillary glands by extraction with 1*M* HCl containing 1% trifluoroacetic acid, 5% formic acid, and 1% NaCl, and concentrated on a Sep-Pak column (Waters) and by reversed-phase high-pressure liquid chromatography (HPLC) on a C18 Bondapak column (Waters) according to Elson *et al.* (*35*). The EGF was labeled with Na ^{125}I (New England Nuclear) using Enzymobeads (immobilized glucose-oxidase, lactoperoxidase system, Bio-Rad) to a specific activity of 120,000 count/min per nanogram of protein. Human TGF-α was isolated from *E. coli* W3110 containing the plasmid pTE5 as described (*5*). The correctly folded TGF-α was further purified by HPLC. Both TGF-α and EGF were more than 99% homogeneous as assessed by HPLC and by amino acid compostion. The A 431 human epidermoid carcinoma cells were from G. Todaro. The LEII murine lung microvascular endothelial cells (from A. Curtis) were characterized by Fajardo *et al.* (*36*), who demonstrated the presence of angiotensin converting enzyme and receptors for modified low-density lipoprotein on their surface. The human foreskin fibroblasts (HFF) and bovine pulmonary artery endothelial cells (BPEC) were established from primary cultures. All cells were grown in Dulbecco's minimal essential medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Hyclone).
35. S. D. Elson, C. A. Brown, G. D. Thorburn, *Biochem. Int.* **8**, 427 (1984).
36. L. Fajardo, A. B. Schreiber, N. Kelly, G. Hahn, *Radiat. Res.* **103**, 276 (1985).
37. A. B. Schreiber, in preparation.
38. We thank G. Todaro for the A 341 cells and A. Curtis for the LEII cells, A. C. Allison for review of the manuscript, L. Fajardo for help with the autoradiography, W. Kowalski for assistance, and J. Arch for preparation of the manuscript.

9 December 1985; accepted 15 April 1986

Cultivation of the *Drosophila* Sex-Ratio Spiroplasma

KEVIN J. HACKETT, DWIGHT E. LYNN, DAVID L. WILLIAMSON, ANNETTE S. GINSBERG, ROBERT F. WHITCOMB

Uncultivable for more than 25 years, the sex-ratio spiroplasma of *Drosophila willistoni* grew in a tissue culture medium (H-2) containing an embryo-derived lepidopteran cell line (IPLB-TN-R^2). After adaptation, it grew in a cell-free H-2 medium. This success demonstrates the usefulness of cell culture systems for cultivation of fastidious microorganisms and facilitates study of the sex-ratio trait in *Drosophila*.

SEX-RATIO DISTORTION CAUSED BY microorganisms and chromosomal and extrachromosomal factors has been documented for a variety of plants and animals (*1*). In 1961 Poulson and Sakaguchi correlated the appearance of "small spirochetes, presumably treponemata" in the hemolymph of adult female *Drosophila* flies to the expression of a sex-ratio trait in the progeny of the flies (*2*). Eventually it was realized (*3*) that the organisms were not spirochetes but belonged instead to a newly recognized microbial group of wall-less prokaryotes (Mollicutes, Spiroplasmataceae, *Spiroplasma*). These spiroplasmas, known as sex-ratio organisms (SRO's), selectively kill the male progeny of infected females of four Neotropical *Drosophila* species—*D. equinoxialis*, *D. nebulosa*, *D. paulistorum*, and *D. willistoni* (*3*, *4*).

Although many attempts have been made to cultivate SRO's, including trials that involved tissue culture media and insect cell or organ culture systems, none were successful (*5*). The media used in these tests contained different types and amounts of serum and serum substitutes, vitamins and cofactors, amino acids and peptones, nucleic acids, hormones, membrane-interacting substances, and fly homogenates. Also, many different temperature and oxygen conditions were used. None of the tested media or culture systems sustained cell division; the best formulations permitted maintenance of motile, helical organisms for no more than 60 days. These failures impeded research on the SRO's and prevented identification of the male-lethal factor and fulfillment of Koch's postulates.

In 1985 a previously uncultivable spiroplasma (LD-1) from the Colorado potato beetle was cultivated in the presence of actively growing insect cells (*6*). We report here that insect cell cultures have also been successfully used for primary isolation of the *D. willistoni* SRO (WSRO) and that cultured WSRO's have been adapted to a cell-free medium.

All *Drosophila* stocks were maintained as described by Williamson *et al.* (*3*). To obtain spiroplasmas, 2.5 μl of hemolymph was collected at Stony Brook by glass needle (*7*) from a total of 100 *D. pseudoobscura* (Piñon Normal strain) females transmitting the B3 strain of WSRO. The hemolymph was transferred to 0.5 ml of M1D medium (*8*) and shipped at ambient temperature to our laboratory. Five days after the hemolymph was collected, 0.2 ml of the suspension was placed in 4 ml of a medium containing equal parts of DCCM (*9*) and M1D media and filtered through a membrane having a pore diameter of 0.45 μm. The filtrate was then distributed in 0.1-ml aliquots to each well of a 24-well microtiter plate (2.5-ml capacity per well) containing either an embryo-derived *Trichoplusia ni* insect cell line (IPLB-TN-R^2) (*10*) cultured in 0.4 ml of modified Grace's (TNM-FH) medium (*11*) or one of six previously described (*6*) lepidopteran or coleopteran cell culture systems (three wells per system). Primary isolation was also attempted (i) in several cell-free tissue culture media [DCCM medium, modified Goodwin's medium (*12*), and TNM-FH medium]; (ii) in commonly used spiroplasma media [SP-4 (*13*) and M1D media]; or (iii) in several "insect cell–conditioned" media [DCCM, modified Goodwin's, or TNM-FH media that had been conditioned for 1 or 3 days by the growth of the respective insect cell lines (three tubes per medium)]. The cell lines were subcultured weekly and maintained at 23°C until used; all WSRO-containing cell cultures were kept at 26°C. Attempts were made to adapt the first, third, fifth, and ninth passages of the cell-cultured WSRO isolate to cell-free media. We removed insect cells by passing the culture through a membrane filter (pore diameter, 0.45 μm), and the WSRO-containing filtrate was transferred at passage ratios of 1:1 to 1:4 into DCCM, modified Goodwin's, TNM-FH, or H-2 (*14*) media. We estimated SRO titers in the wells and tubes by counting the number of organisms in 3-μl samples by dark-field microscopy (*6*, *15*).

We found that the best results in primary isolation of WSRO were obtained by using the *T. ni*–derived cell lines. Primary isolation was accomplished only in the IPLB-TN-R^2 cell line cultured in TNM-FH medium. The other *T. ni* cell line, IAL-TND1 (*16*), cultured in modified Goodwin's medium, supported WSRO growth and multiplication for about 1 month; continuously cultivable strains were not obtained in this cell line. In the previously described formulations, the organisms became elongate and

K. J. Hackett, D. E. Lynn, A. S. Ginsberg, R. F. Whitcomb, Insect Pathology Laboratory, U. S. Department of Agriculture, Beltsville, MD 20705.
D. L. Williamson, Department of Anatomical Sciences, State University of New York, Stony Brook, NY 11794.