Overgrowth Stimulating Factor Released from Rous Sarcoma Cells

Abstract. *An assay is described for a nonviral factor from the medium of Rous sarcoma cells which stimulates rapid and sustained cellular overgrawth in crowded chick embryo cultures. The factor is nondialyzable and thermolabile and is released in large amounts several days after the first visible transformation of newly infected chick embryo cells.*

When chick embryo cells in culture are exposed to high concentrations of the Bryan strain of Rous sarcoma virus (RSV), only a fraction of the cells are infected with RSV (*1*). The remaining cells are infected with the helper virus RAV which occurs in the stock and which interferes with RSV infection. Within a day, scattered cells undergo a malignant transformation which is characterized by rounding up of the cells, disorganization of the typical whorled pattern of fibroblastic growth, and persistent rapid multiplication of the cells under crowded conditions that would otherwise slow their growth (*2*). Most cells in the culture, necessarily including many not infected with RSV, take on the appearance and behavior of transformed cells by day 4 or 5 after infection. This suggests that the cells infected with RSV release material which stimulates continued multiplication ("overgrowth") among the crowded cells not infected by RSV as well as those infected.

A tissue culture assay was developed to detect the presence of such an "overgrowth stimulating factor" (OSF) based on its putative capacity to stimulate DNA synthesis in cultures of normal chick embryo cells whose growth rate has been inhibited by crowding (*2*). Primary cultures were prepared from the body walls of 10-day-old chick embryos (*3*). At 3 to 6 days after explantation, the cells were treated with trypsin and transferred (2×10^6 cells per dish) to 50-mm plastic petri dishes in 5 ml of medium 199 containing 2 percent tryptose phosphate broth, 1 percent calf serum, and 1 percent chicken serum. The convention used here to designate this mixture is 199 (2-1-1), the numerals in parentheses expressing the successive order of the constituents listed above. More consistent results are now obtained with 199 (2-0-2) during the first day of incubation. The day after transfer, when the cells were confluent, the fluid medium was removed, and the cells were overlaid with Scherer's medium containing 2 percent tryptose phosphate broth, 1 percent calf serum, 1 percent chicken serum, and 0.4 percent agar. Samples to be tested for their capacity to stimulate cellular overgrowth were incorporated in the agar medium. Agar was added because it allows density-dependent inhibition of cell growth (*4*) to occur even in the presence of fresh medium (*2*). At intervals after the cells were overlain, the agar medium was removed, and measurements were made of incorporation of [H³]thymidine into DNA, mitosis, and of cell number.

Medium removed from cultures infected with RSV was used as the potential source of OSF. These cultures were infected by transfer of 1×10^6 cells from primary cultures to 100-mm petri dishes in 10 ml of medium 199 (5-1-1) that contained 1×10^6 focus-forming units of the Bryan strain of RSV per milliliter. Beginning on day 3 after infection, the medium was collected and replaced daily.

Overgrowth stimulating activity became detectable in the medium 4 to 5 days after infection and increased greatly by 6 to 7 days after infection. Large volumes of the Rous-conditioned medium (RCM) were frequently cytotoxic (*5*), but the toxic substances could be removed by dialysis with no loss in overgrowth stimulating activity. Sedimentation of virus and other particulates at 75,000*g* for 2 hours had no effect on the overgrowth stimulating activity of the supernatant. Control cultures overlain with fresh medium underwent a decline in the rate of incorporation of [H³]thymidine from hour 6 onward (Fig. 1). Cultures with RCM included in the overlay underwent a marked increase of incorporation of [H³]thymidine by hour 12, and maintained much higher incorporation through the final measurement 65 hours after overlay. The rate of incorporation was three to six times higher in cultures treated with RCM than in the controls from hour 12 onward (Fig. 1). The number of cells in mitosis was also higher in the cultures treated with RCM, although the relative increase in mitoses was not as great as that of incorporation of [H³]thymidine (Table 1). This may be due in part to the differential detachment of cells in mitosis upon removal of the agar.

Despite the fact that the cultures were confluent when the experiment

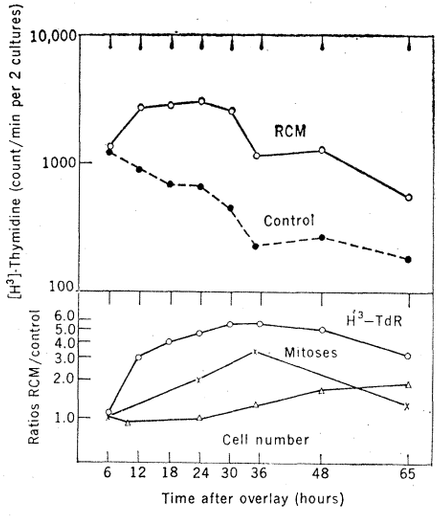

Fig. 1. DNA synthesis, mitoses, and cell numbers in chick embryo cell cultures after overlay with an agar-containing fresh medium only (*Control*), or fresh medium plus RCM (*RCM*). A potent working stock of OSF was prepared from the medium of cultures 6 days after infection with RSV. Rous sarcoma virus was removed by centrifugation, and toxic material was removed by dialysis. One milliliter of this stock was added to 4 ml of fresh medium in the agar overlay of a group of crowded cultures, and their capacity to incorporate [H³]thymidine, their mitotic index, and the total number of cells were determined at intervals after overlay. The same measurements were made on cultures overlaid with 5 ml of fresh medium in agar. The rate of DNA synthesis was estimated by adding [H³]-thymidine (0.1 μc/ml) to the cultures in 2.5 ml of medium 199 and incubating at 39°C for 1 hour. The cultures were washed repeatedly with tris-saline buffer, and the radioactive acid-soluble fraction was extracted with 5 percent trichloroacetic acid (4°C) for 5 minutes. Cellular DNA was hydrolyzed by heating the cultures in 10 percent trichloroacetic acid at 68°C for 2 hours. A sample was added to a mixture of Omnifluor, Triton X100, and toluene; the radioactivity was measured in a liquid scintillation counter. Mitotic activity was estimated after 6 hours of colcemide ($5 \times 10^{-8} M$) treatment (*2*), and total numbers of cells were determined in a Coulter electronic counter. Top panel, actual radioactivity counts of [H³]thymidine after 1 hour of labeling. Bottom panel, ratios of counts in RCM cultures: control cultures for [H³]thymidine radioactivity, percentage of mitoses, and total cell numbers.

began, cell number in the cultures treated with RCM doubled by 65 hours, while that of control cultures remained constant. This caused multilayered growth in the RCM-treated cultures. The first sign of extensive overlapping appeared at 36 hours, when cells had become slightly rounded, and the whorled pattern typical of fibroblastic cell populations had been disrupted. At this time, the treated cells were more basophilic than the controls when stained with Harris' hematoxylin (*2*) and the *p*H of the medium was lowered. In these characteristics, the cells treated with RCM took on some of the features of Rous sarcoma cells. Unlike cells directly and irreversibly transformed by infection with RSV, however, they resumed their normal fibroblastic morphology and intercellular arrangement by 100 hours after overlay.

I chose 18 hours as the standard interval between overlay and incorporation of [H³]thymidine for the assay of activity of OSF, because this is the shortest time in which treatment reliably produces a much faster rate of DNA synthesis than that found in untreated cultures. It has the additional advantage of convenience and elimination of complications due to the presence of RSV, since the process of virus infection does not produce a comparable effect until 36 hours after infection (*2*).

In the absence of RCM the rate of incorporation of [H³]thymidine per cell is ten times higher in sparse, rapidly growing cultures than it is in crowded, slowly growing cultures and about two times higher than in cultures of intermediate density (Fig. 2). The addition of RCM to the sparse cultures had no significant effect on their rate of thymidine incorporation, but the addition of as little as 0.01 ml to the 5-ml agar overlay of crowded cultures increased the incorporation rate significantly. The addition of larger amounts of RCM to cultures with 1×10^6 cells stimulated their incorporation to a plateau slightly higher than that of the sparse cultures. The addition of 0.2 ml of RCM to the overlay of 2×10^6 cells stimulated a sixfold increase in incorporation, which was close to the maximum regularly attainable in this system.

The OSF is quantitatively recovered from RCM by precipitation with 60 percent saturated ammonium sulfate. The half-life of OSF at 60°C is 5 minutes, although about 5 percent of the stimulatory activity survives heating at 90°C for 30 minutes, which suggests that minor components that can stimulate overgrowth are present. The thermal lability of OSF distinguishes it from the factor in serum which exerts a similar effect on the growth of crowded cultures (*6*) and from the hyaluronic acid of Rous sarcoma cells which reportedly stimulates the growth of human fibroblasts (*7*). Neutralizing antibody to the virus has no effect on the activity of OSF, which indicates the strain-specific envelope antigen of the virus is not the active component of OSF.

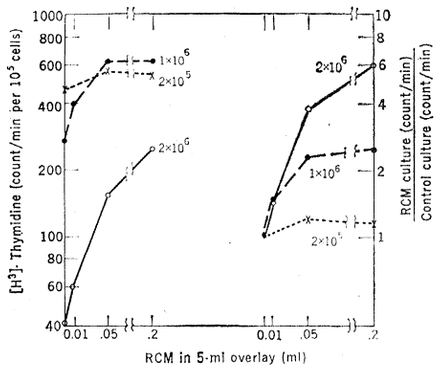

Fig. 2. Effect of various amounts of RCM on DNA synthesis in sparse and crowded cultures of chick embryo cells 18 hours after agar overlay. Numerals on graph indicate the numbers of cells plated. Left panel, actual radioactive counts of [H³]-thymidine after one hour of labeling. Right panel, ratios of [H³]thymidine in RCM cultures to [H³]thymidine in control cultures.

Under the cultural conditions described here, OSF is usually not detectable in the medium until 5 days after infection, but the amount may then increase as much as 100-fold during the following day, and this high amount is maintained for at least 3 days with daily medium change. No activity is detected for at least 6 days in the medium of uninfected cultures treated identically. Some activity may be detected when uninfected cultures become older and very crowded, but even then the amount is much less than that found in the medium of cultures infected with RSV at the same time.

Recently I have found overgrowth stimulating activity in normal chick embryo cells disrupted by sonic oscillation. Within 24 hours after infection by RVS, the intracellular activity per milligram of protein has increased by half, and by 3 days after infection it is three to four times higher than in the uninfected cultures. The intracellular increase of activity is therefore contemporaneous with the malignant transformation of the cells, whereas the release into the medium seems to be a later consequence related to the increased leakiness of the Rous sarcoma cells. The OSF could not be detected in the medium of cells infected with the Schmidt-Ruppin strain of RSV, but the kinetics of its increase within cells was similar to that of the Bryan strain. The OSF could therefore be the agent which effects the release of RSV-infected cells from density-dependent inhibition (*2*).

The overgrowth stimulating effect can be mimicked with as little as 3 μg of crystalline trypsin per milliliter or with pronase. Since peptidases undergo marked increases in many tumors (*8*) and since trypsin unmasks tumor-type agglutinins in normal cells (*9*), it is possible that OSF is a peptidase or protease. In this regard, it is of interest that the mesenchymal growth factor isolated from mouse submaxillary glands has both peptidase and esterase activities (*10*) and the nerve growth factor isolated from the same source has similar enzymatic activities (*11*).

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References and Notes

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