hancement of the cellular defenses by certain microbial products. In some cases, as with laminarin, the activation of the prophenoloxidase system plays a vital role in the nonself recognition process; in other cases, as with endotoxin, although elevated phagocytosis was recorded no phenoloxidase was apparently generated. This may have resulted from the presence of a separate endotoxin-mediated prophenoloxidase activating system, as occurs in other arthropods (*18*). Unlike the laminarin-mediated complex, this system may be sensitive to the anticoagulant used in the enzyme assay or, alternatively, may be functional only in the presence of other factors such as agglutinins or specific ions that were not included in the assay. This point clearly merits further investigation, but even so the results represent the first step toward identifying recognition molecules in insects. These findings should provide a stimulus to research into vector species because it may now be possible to investigate ways by which parasites inhibit or manipulate the host defenses in order to survive in the hemocoel (*19*).

The data further support our earlier hypothesis (*2*) that in the cellular defenses of *Galleria* two cell types interact in a biphasic process. Cytochemical and ultrastructural studies indicate that the granular cells, but not plasmatocytes, contain phenoloxidase (*20*) and that these cells react by violently discharging their contents over nonself material when in contact with foreign surfaces (*2*).

This release corresponds to the activation and appearance of the phenoloxidase observed in this study and constitutes the first or recognition phase in the cellular reactions. The second phase results from the identification of the invading parasite as foreign by the coat formed from factors derived from activation of the prophenoloxidase system. This phase is mediated mainly by the plasmatocytes, which contain little or no phenoloxidase (*20*) but respond to coated surfaces either by forming multicellular sheaths around large parasites or by ingesting smaller ones (*2*) (Table 1). Specific receptor interactions may be part of the mechanism by which plasmatocytes react toward coated material, but this phenomenon has yet to be explored.

NORMAN A. RATCLIFFE
CATHERINE LEONARD
ANDREW F. ROWLEY
Department of Zoology, University College of Swansea, Singleton Park, SA2 8PP, United Kingdom

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Isolation and Culture of a Tetraploid Subpopulation of Smooth Muscle Cells from the Normal Rat Aorta

Abstract. *Smooth muscle cells with 4C (double diploid) DNA content have been found in major arteries. The proportion of 4C cells increases with normal aging and with hypertension. These cells may represent a state of arrest at the G_2 phase of the cell cycle or may be examples of true tetraploidy. Flow cytometric cell sorting was used to isolate 4C smooth muscle cells from the rat aorta, and the cells were cultured. Flow cytometry, Feulgen microdensitometry, and karyotyping of the progeny of the 4C cells established the presence of true tetraploid cells. These findings demonstrate the presence of reproductively viable tetraploid cells in a normal mammalian tissue.*

Smooth muscle cell proliferation may be central to atherogenesis (*1*), and therefore characterization of smooth muscle cell growth is essential for an understanding of vascular disease. During the process of growth and aging, an increasing frequency of smooth muscle cells with 4C (double diploid) DNA content has been observed both in humans (*2*) and in experimental animals (*3, 4*). Hypertension has also been found to be associated with an increased frequency of 4C nuclei (up to 40 percent) in the aortas of rats (*4*). Over the past 30 years, cytophotometric techniques for quantitating DNA content in situ (*5*) have shown that nuclei with polyploid DNA content are present in various plant and animal cells. However, the isolation of a reproductively viable population of tetraploid cells from normal mammalian tissues has not been reported. We now report the isolation and culture of a tetraploid subpopulation of smooth muscle cells from the normal rat aorta. This suggests the coexistence of heterogeneous populations of viable smooth muscle cells with 42 and 84 chromosomes.

Smooth muscle cells were isolated from the thoracic aortas of 3- to 4-month-old normotensive male Sprague-Dawley rats (Charles River) as described earlier (*4*). A suspension of cells (pooled from ten animals) was stained with Hoechst dye 33342 (8 μM weight to volume) for 30 minutes at 37°C. Cells were sorted with a flow cytometer–sorter (Coulter Epics V) and were processed at approximately 1000 cells per second (*6*). Flow cytometric analysis revealed that 90 percent of the cells had the normal diploid (2C) DNA content and 10 percent had 4C DNA content. This distribution is consistent with other data for animals of this age (*3, 4*). The cells corresponding to the 2C and 4C peaks were separated, and the cells were collected in culture medium [minimal Eagle's medium supplemented with 20 percent fetal calf serum, D-glucose (0.9 mg/ml), sodium pyruvate (6.6 μg/ml), and antibiotics]. The cells were then washed several times by centrifugation and were placed in culture dishes. During the initial growth period of the primary culture, cells were shielded from light to the maximum extent possible. The primary cultures were allowed to grow to near confluency in 35-mm petri dishes for several weeks, and the culture medium was replenished every 3 to 5

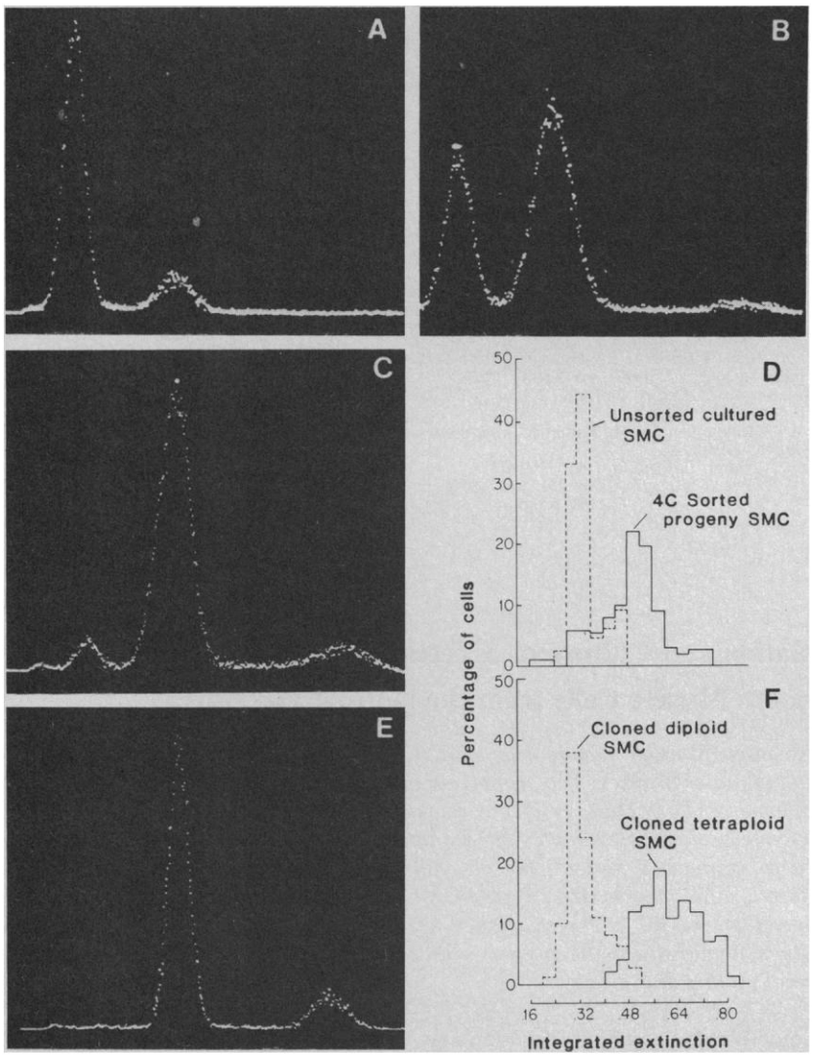

Fig. 1. (A) Flow cytometry of the progeny of the 2C subpopulation, showing 85 percent of cells with 2C DNA content. (B) Flow cytometry of the progeny of the 4C subpopulation (after seven population doublings in vitro), showing 70 percent 4C cells and 5 percent 8C cells. (C) Flow cytometry of the progeny of resorted 4C cells, showing 95 percent (4C plus 8C) cells. (D) Feulgen microdensitometry histogram of the progeny of the 4C cells (after seven population doublings), showing 71 percent 4C cells (solid line). Data for unsorted cultured smooth muscle cells (SMC) (showing 15 percent 4C cells) are presented for comparison (dashed line). (E) Flow cytometry of a clonal population of tetraploid cells showing the absence of diploid cells. (F) Feulgen microdensitometry histogram of diploid and tetraploid clonal cell populations.

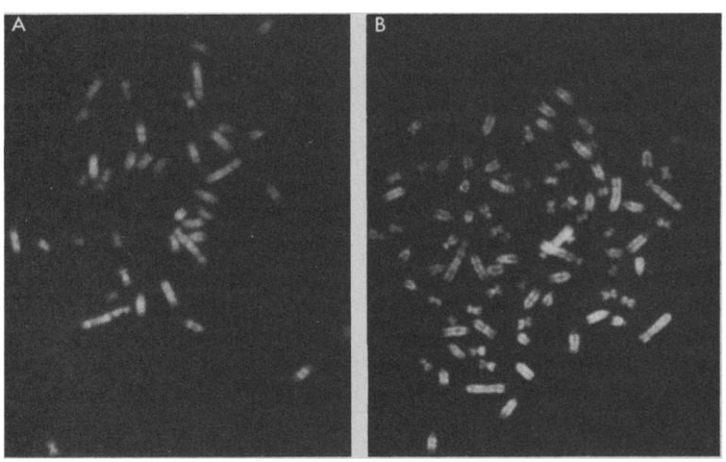

Fig. 2. Photographs of (A) diploid and (B) tetraploid quinacrine-banded metaphases from 4C sorted progeny cells. The diploid metaphase contains 42 chromosomes and the tetraploid metaphase contains 84 chromosomes.

days. After that, the cells were routinely subcultured at weekly intervals by harvesting with trypsinization (*7*).

Flow cytometry (*8*) and Feulgen microdensitometry (*9*) were performed on the progeny of the sorted 2C and 4C subpopulations of cells after seven population doublings had elapsed in vitro. Analysis of the progeny of the 2C cells showed only a small 4C peak (15 percent) and no 8C peak (Fig. 1A), suggesting that these cells were mostly diploid. Analysis of the progeny of the 4C cells showed that about 70 percent of the cells had 4C DNA content, about 5 percent had 8C DNA content (Fig. 1, B and D), and the rest were diploid. One possible explanation for the diploid contamination is imperfect separation of cells in the process of sorting. An alternative explanation is the possible presence of G_2 diploid cells in the animal at the initial isolation. The large 4C peak and the presence of an 8C peak suggest that most of the cells in this subpopulation were true tetraploid cells with 8C content at the G_2 state. A second cell sorting of the 4C progeny increased the purity of this subpopulation to more than 90 percent (Fig. 1C).

In order to establish that the sorted progeny of the 4C cells (70 percent 4C peak) contained true tetraploid cells, we performed karyologic analyses. Cells were prepared for karyotyping by standard techniques and were stained with quinacrine mustard (*10*). Twenty-eight well-banded metaphases were selected at random and were photographed. Examination revealed that 19 cells (68 percent) were tetraploid with 80 to 84 chromosomes, and 9 cells (32 percent) were diploid. Representative tetraploid and diploid metaphases are shown in Fig. 2. These data demonstrate the existence of a population of true tetraploid smooth muscle cells in the normal rat aorta.

Serial subcultivation of the 4C progeny cells resulted in a gradual loss of the tetraploid subpopulation. Flow cytometric analysis revealed 70, 48, 40, and 27 percent 4C cells after 7, 10, 11, and 13 population doublings in vitro, respectively. Studies of the proliferative response of hepatocytes after partial hepatectomy in mice have shown decreased [^{3}H]thymidine labeling with increasing polyploidization in vivo (*5*). Studies of atherosclerotic plaques in humans have shown a decreased proportion of 4C cells in the plaques when compared with surrounding tissue (*2*). These data suggest that the diploid cells have a relative growth advantage compared with tetraploid cells under conditions of an unusual growth stimulus.

In order to determine the stability of the tetraploid state in culture, we established single cell clones of tetraploid cells. Flow cytometry of serially subcultivated tetraploid clones demonstrated that the cells remained tetraploid without reversion to the diploid state [Fig. 1, E and F].

A diploid set of chromosomes is the usual composition of the eukaryotic genome. The diploid state is maintained by the reproduction of DNA and separation of chromosomes during the mitotic cycle. The emergence of an increasing percentage of nuclei with 4C DNA content in association with normal aging and with hypertension may be due either to arrest at the G_2 stage of the mitotic cycle or to the development of true tetraploidy. The presence of reproductively viable tetraploid cells in the normal rat aorta could represent a stem cell population that proliferates preferentially during normal aging and that can be significantly expanded by hypertension. Alternatively, the increased frequency of these cells may be due to continuous conversion of diploid cells with an abnormal mitotic mechanism to the state of tetraploidy (*5*). The role of tetraploid smooth muscle cells in normal growth, aging, and disease is still unknown. Further characterization of the tetraploid cell population including its growth kinetics and interaction with diploid cells may increase our understanding of cellular polyploidy and of vascular physiology.

ITZHAK D. GOLDBERG
ELIOT M. ROSEN
Joint Center for Radiation Therapy, Harvard Medical School, Boston, Massachusetts 02115
HOWARD M. SHAPIRO
Center for Blood Research, Harvard Medical School
LAWRENCE C. ZOLLER
Department of Anatomy, Boston University School of Medicine, Boston 02118
KYL MYRICK
STEPHEN E. LEVENSON
Joint Center for Radiation Therapy, Harvard Medical School
LISA CHRISTENSON
Center for Blood Research, Harvard Medical School

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7. The cells were subcultured every 6 to 8 days in standard fashion. The medium was removed, and the dish was trypsinized [1:250 trypsin EDTA in buffered saline (Gibco)]. The trypsinization was stopped by addition of fresh medium, and the cell suspension was counted. Cells were plated in fresh medium at an inoculation density of 1×10^4 cells per square centimeter. Cultures were incubated at 37°C with 5 percent CO_2 and 95 percent humidified air.
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10. Subconfluent cultures were incubated for 30 minutes in the presence of Colcemid (Gibco) at a final concentration of 0.1 μg/ml. Cells were dislodged from the flasks by treating with 0.25 percent trypsin-EDTA and then centrifuged at 150*g* for 7 minutes. The supernatant was discarded, and the cell pellet was suspended in 75 m*M* KCl solution and allowed to stand at room temperature for 10 minutes. The cells were centrifuged and then suspended in a 3:1 (by volume) methanol–acetic acid fixative. After 1 hour, two changes of fixative were made. Air-dried slides were prepared and were stained for 7 minutes in a solution (50 μg/ml) of quinacrine mustard (Sigma). Slides were mounted in tris-maleate buffer (*p*H 5.6) and were observed under a Leitz orthoplan fluorescence microscope equipped with an orthomat camera. Well banded (Q bands) metaphases were photographed.
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Serotonin Selectively Inhibits Growth Cone Motility and Synaptogenesis of Specific Identified Neurons

Abstract. *The motile activity of growth cones of specific identified neurons is inhibited by the neurotransmitter serotonin, although other identified neurons are unaffected. As a consequence, affected neurons are unable to form electrical synapses, whereas other neurons whose growth is unaffected can still interconnect. This result demonstrates that neurotransmitters can play a prominent role in regulating neuronal architecture and connectivity in addition to their classical role in neurotransmission.*

The characteristic morphology and resultant connectivity of adult neurons is due to the combined action of precisely timed intrinsic and extrinsic signals on individual neurons (*1*). Extrinsic signals arising from neighboring neurons can regulate neuronal architecture (*2*), although proximate regulatory agents are not yet defined. One suggestion is that "trophic" substances released from some nerve terminals can control the growth of adjacent neurons (*3*). In light of the demonstration that neurotransmitter can be released from growth cones of growing neurons in culture (*4*), a candidate for such a regulatory agent is the classical chemical transmitter itself (*5*). We now report that the neurotransmitter serotonin can inhibit neurite outgrowth. We demonstrate a growth inhibition specific to individual growth cones by a time-lapse study of the large identified neurons of the snail *Helisoma*. We also demonstrate that this inhibitory action of serotonin prevents the formation of electrical synapses between specific identified neurons with overlapping outgrowth, while connections between neurons whose growth cones are unresponsive to serotonin continue to form (*6*).

These experiments were performed on buccal ganglion neurons 5 and 19 and on pedal ganglion neuron P5, all of which have been studied in terms of growth and connectivity (*6, 7*). Individual neurons were removed from ganglia of adult snails and plated in cell culture (*8, 9*), where neurons undergo a characteristic sequence of outgrowth. Growth cones arise from the cell body and elaborate an extensive network of neurites for 3 to 4 days until a morphological steady state is attained (*6*).

The behavior of growth cones of individual identified neurons is readily analyzed by time-lapse low-light video microscopy (*10*). The activity of growth cones from *Helisoma* neurons characteristically consists of a probing of the environment by filopodia and a ruffling action of lamellipodia. Concurrently, the neurite extends continuously at a nearly