Reports and Letters

Dissociation of Cultures from Picea glauca into Small Tissue Fragments and Single Cells

The separation of tissues of plants or animals into their constituent cells, and the establishment of viable cell clones therefrom has long been recognized as a potentially important method of approaching many problems in cellular biology. Dissociation of tissues cultivated *in vitro*—that is, their dissolution into cell groups and at times into separate cells—has recently been observed in cultures isolated from carrot, *Scorzonera*, and other plants that were maintained for long periods *in vitro* (*1*). Jablonski and Skoog (*2*) (compare also Newcomb *3*) have noted similar dissociation of pith parenchyma freshly isolated from the stem of tobacco. In these examples, dissolution was regularly accompanied by an enormous enlargement of the cells and led to necrosis of the cultures. Northcraft (*4*) reported the dissolution of carrot cultures by treatment with oxalate, but here also the cultures appear not to have been viable after treatment.

In the course of experiments on the *in vitro* cultivation of normal and tumor tissues of *Picea glauca* (*5, 6*) a number of strains were isolated in which normal tissue, following particular changes in the nutrient, showed a marked tendency to dissociate spontaneously and, in contrast to the cases cited above, continued to grow with undiminished vigor and without necrosis or other evidence of injury (*7*).

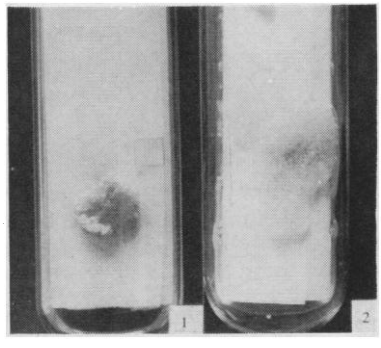

Fig. 1. (Left) Culture of normal tissue of *Picea glauca* grown on filter paper in a liquid nutrient containing 2,4-D, tyrosine, folic acid, and vitamin B_{12}. The culture is firm, crustose, and relatively slow growing (three-fourths original size). Fig. 2. (Right) Culture of normal tissue grown in a nutrient from which folic acid and vitamin B_{12} have been omitted. The culture has become gelatinous in texture. Note the small isolated nodules on the substratum above the main culture mass. These have arisen from groups of cells that have separated from the original culture (three-fourths original size).

The spruce tissues, after isolation on filter paper in a liquid nutrient, formed firm crustose calluses (Fig. 1) that did not change in character as long as the original nutrient formula was retained (December 1953 to August 1954). This medium contained a modified form of White's nutrient (*8*, p. 74) without glycine, but with a number of amino acids (compare White, *8*, p. 94), asparagine, glutamine, 2,4-dichlorophenoxyacetic acid (2,4-D) at 5×10^{-8} g/ml, and a series of vitamins including folic acid (pteroylglutamic acid) and vitamin B_{12}.

It was found in the course of further experiments that vitamin B_{12} (1.5×10^{-9} g/ml) was without effect on survival and growth of these cells and that folic acid (10^{-6} g/ml) was somewhat retarding. However, omission of these substances led, particularly in one strain of normal tissues, to a sharp change in growth pattern. During the first 4 to 6 weeks on a folic acid and B_{12}-deficient nutrient, small groups of cells split off from the culture (Fig. 2). After transplantation, these tissue fragments continued to grow with unusual rapidity. Later, after 8 to 12 weeks, the original firm crustose culture dissociated so extensively that mere immersion in the liquid medium was sufficient to separate it into few-celled groups or single cells (Fig. 3). Subcultures could then be made by taking up a drop of liquid containing small tissue fragments with a platinum loop or a pipette and transferring it to a fresh nutrient. By the spontaneous separation of single cells, these spruce cultures resemble animal tissues from which Sanford, Earle, and Likely (*9*) established single cell clones. They are clearly different from the plant tissue used for the same purpose by Muir, Hildebrandt, and Riker (*10*), which required mechanical disruption of the tissue. Besides the cultures that grew on the usual filter-paper substratum, others were maintained in a very shallow (1 to 2 mm depth) fluid layer in Carrel flasks (*11*). The dissociation of the spruce tissue appears to be irreversible, for cultures returned to nutrients containing folic acid and vitamin B_{12} do not revert to the crustose habit.

The cells of this jellylike callus tissue are considerably larger than those of the firm, crustose callus culture from which it was derived. The hard callus consists chiefly of round cells averaging about 15 μ in diameter, with occasional long cells 40 μ in length. The corresponding measurements for dissociated spruce tissue are 40 μ and 140 μ. Cell divisions have been observed only in the smaller cells (Fig. 4). Extremely large cells (up to 920 μ) such as those observed in dissociated cultures of Japanese ivy (*Parthenocissus tricuspidata*) by Gautheret (*1*) did not occur in cultures from *Picea glauca*.

The examples of plant tissue dissociation previously noted have been obtained in a number of ways: by use of high auxin concentration (indoleacetic acid, 10^{-5} g/ml) or change in osmotic value of the nutrient (*1*); or by prevention of formation of the middle lamella by binding of calcium with oxalate (*4*). Jablonski and Skoog (*2*) have shown that, in their studies, dissociation occurred when auxin was present and the nutrient lacked a particular component from malt extract or other sources supposed to be essential for cell division. None of these explanations suffices for the spruce cultures described here. The growth-substance concentration used (2,4-D, 5×10^{-8} g/ml) was not changed during the period in which the change from crustose to gelatinous cultures occurred. Oxalate or other calcium-binding materials were not used at any time. Omission of folic acid and vitamin B_{12}

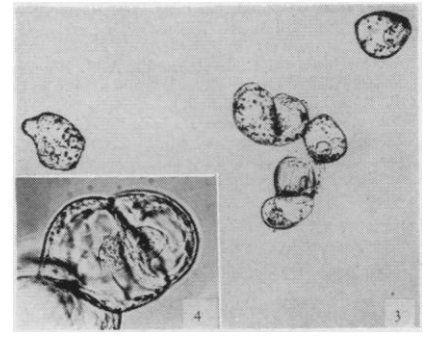

Fig. 3. Small cell groups and single cells from a culture that has been submerged in the nutrient (×100). Fig. 4. (Lower left) Cell division in dissociating spruce tissue. The cell has just completed mitotic division; the two daughter nuclei are still paired next to the newly formed transverse wall (×200).

could not have significantly affected the osmotic value of the nutrient, and cell division was obviously not suppressed. Another possibility, suggested by Newcomb (*3*), that lack of ascorbic acid might be important for the breakdown of the middle lamella, is likewise not a sufficient explanation here, for other cultures of normal tissue on nutrients with widely varied concentrations of vitamin C (10^{-7} to 10^{-4} g/ml), and even without this compound, formed hard, crustose calluses.

It seems probable that three factors may be involved in the transformation of spruce cultures: (i) some as yet undefined peculiarity in certain tissues; (ii) a change in the physiological characteristics (probably in the protein and carbohydrate metabolism) of the dissociating conifer tissues; and (iii) a selection of particularly fast-growing cells during the long period (December 1953 to August 1954) during which the cultures were maintained on a nutrient containing a growth-restricting component (folic acid). These possibilities will have to be explored. Whatever the explanation, the possibility of establishing at will large numbers of cultures derived from single cells or small groups of cells can be important for an analysis of various cellular changes.

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References and Notes

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7. This work was aided by grants-in-aid from the American Cancer Society (CP-47) made on recommendation of the Committee on Growth of the National Research Council, and from the National Cancer Institute (C-2061). I should like to thank Philip R. White, who initiated the original investigation, for supplying the necessary facilities and for his cooperation throughout.
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Experimental Monocytic Leukemia

It is now generally recognized that the essence of leukemia consists in the inability of the immature leucocyte to respond to the forces that normally regulate its maturation and proliferation. This occurs because the matrix of the specific tissue produces pathological leucocytes. But we know neither the factors that regulate the production, nor those that regulate the maturation of the white cells in the places of origin, nor, finally, those that regulate the level of blood leucocytes, under normal and pathological conditions.

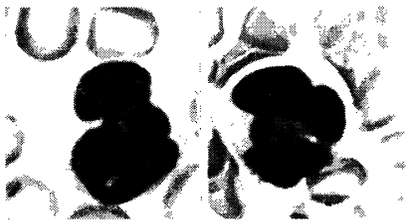

Fig. 1. Rabbit blood: two pathological cells.

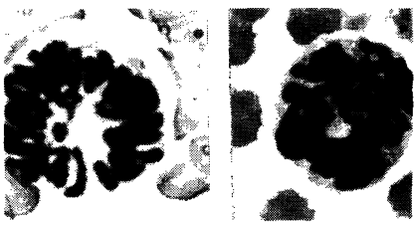

Fig. 2. Rabbit blood: two atypical mitoses.

Researches on the pathogenesis of human leukemia must obtain these results: (i) proliferation of the reticuloendothelial system in a specific way, with production of pathological leucocytes; (ii) immaturity in these leucocytes; and (iii) passage of these cells into the circulating blood, demonstrating that they no longer respond to the forces that regulate their maturation and proliferation. These results were obtained in the experiments reported here. Rabbits of average weight 2 kg were repeatedly injected with different proteins. The most efficacious proved to be lactoglobulin, lactoalbumin, egg albumin, and several others. Whole cow's milk, inoculated as soon as it had been milked, always gave excellent results. The blood alterations relate principally to leucopoiesis, whereas the erythropoiesis is altered only in a terminal stage, when the animal reaches a stage of cachexia.

The increase in the number of leucocytes is considerable. Instead of 8000/mm³, as is average for the normal rabbit, the number of 30,000 to 40,000 is reached after a prolonged treatment, for example, with milk or with lactoglobulins. The smears of blood indicated leukemia. Monocytes and monoblasts predominated, appearing often with a monstrous nucleus and giving the cells a neoplastic aspect (Fig. 1). Of great importance was the presence of numerous cells with direct and indirect division, either bipolar or multipolar, typical or atypical, symmetrical or asymmetrical (Fig. 2). The most important alterations were observed in the spleen (Fig. 3), in the liver (Fig. 4), in the bone marrow, and in the lymph nodes; but thymus, kidneys, adrenals, and lungs were also involved.

The principal alterations occurred in the reticuloendothelial system. In fact, the reticuloendothelial cells of the sinusoids of the lymph nodes, of the spleen, and of the liver, and the living cells of the capillaries of the bone marrow and of the macrophages all are influenced by the treatment. From these cells originate cells of the monocytic type and their pathological forms just described, which multiply luxuriantly in the organs and in the blood.

The findings are those characteristic of human leukemia: (i) the production of pathological leucocytes in the organs of animals treated with heterologous proteins, in which an extended metaplasia is found, especially in the spleen, liver, bone marrow, kidneys, lymph nodes, and wherever active mesenchymal tissue is present; (ii) the permanent increase of white cells, especially of pathological type, highly immature, and sometimes abnormal. These elements continue to multiply through a direct division and through mitosis in the circulation. Therefore, these cells do not respond to the factors that under normal conditions regulate their maturation and their proliferation.

On the basis of my experiments, I can conclude that the heterologous proteins influence the monocytic potentiality of

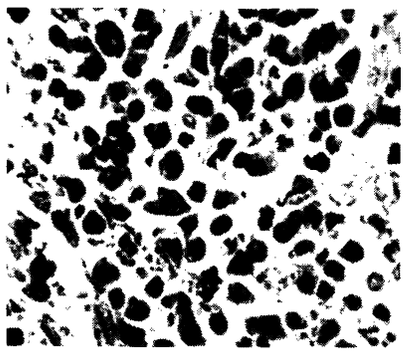

Fig. 3. Spleen of rabbit treated with lactoalbumin.

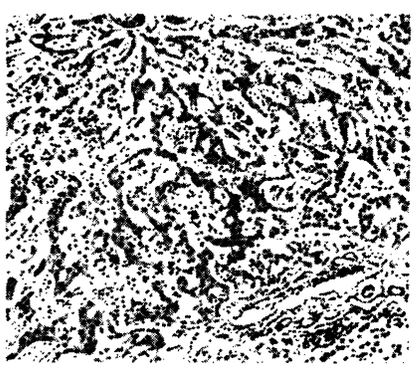

Fig. 4. Liver of rabbit treated with whole cow's milk.