Table 1. Weights of brown (pooled from five bodily regions) and white (inguinal) fatty tissues of rats after 5 weeks at sea level or at 4350 m (simulated); prime figures are followed by standard errors. Percentage changes in body weight appear in parentheses.

Brown fat		White fat, absolute (g)
Absolute (g)	Relative to body wt. (%)	
A, *Seven rats at sea level (+35.8±4.2)*		
0.344±.041	0.085±.008	4.055±.286
B, *Five rats at 4350 m (−10.9±3.4)*		
0.500±.070	0.192±.022	2.250±.359
Difference, B–A (−46.6±5.3)		
+0.155±.081	+0.107±.023	−1.805±.459
Probability, Student t-test (<.001)		
<.10	<.001	<.001

data thus suggest that brown fat may be an important factor in acclimatization of the rat to high altitude.

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Lymphocytes of Small Mammals: Spontaneous Transformation in Culture to Blastoids

Abstract. *Small lymphocytes from rabbit and guinea pig, grown in tissue culture in the absence of phytohemagglutinin, can transform spontaneously into cytologically immature cells that are indistinguishable morphologically from blast cells. The transformation occurs in 38 to 75 percent of peripheral lymphocytes and 55 to 75 percent of splenic lymphocytes on the 5th day of culture. The altered cells can synthesize RNA and DNA and show mitotic division; synthesis of DNA occurs after the 5th day of culture, whereas RNA is synthesized throughout the duration of culture.*

Despite participation of lymphocytes in many immunologic processes and their apparent role as the precursors of antibody-forming cells, their potentials and the specific factors that may control their differentiation and function have not been fully elucidated. Several substances, particularly phytohemagglutinin (PHA), can transform human peripheral lymphocytes, when grown in tissue culture, into large, immature blastoid cells (*1*). In the presence of PHA, 70 to 90 percent of the lymphocytes are morphologically immature even after being cultured for 72 hours, whereas cells grown without PHA are essentially unchanged.

While culturing human peripheral lymphocytes in the absence of PHA, I observed a striking alteration in both size and cytological characteristics in some of the cells on the fifth day of culture: between 5 and 40 percent of the cells were transformed, apparently spontaneously, into blast-like cells and were indistinguishable from those stimulated with PHA. Lest the presence of polymorphonuclear leukocytes in the lymphocyte culture inoculum be a possible stimulatory factor, the original plasma-leukocyte suspension was purified by allowing the polymorphs to adhere to glass prior to culture.

Four of the 14 donors had a history of allergy to penicillin; their cells were cultured without addition of antibiotics. They were in a group of nine donors in whom a spontaneous transformation of 5 to 16 percent of lymphocytes occurred, whereas the highest rates of transformation (19, 26, 29, 34, and 40 percent) were in donors without a history of allergy to drugs. This observation led us to investigate further the potential of mammalian lymphocytes for spontaneous transformation to blastoids and to study the metabolic function of such cells as determined by their ability to synthesize DNA and RNA. We have now shown that small lymphocytes from either rabbit or guinea pig can transform spontaneously into large blastoid cells capable of synthesis of RNA and DNA and of mitotic division. This cytological differentiation occurs in both peripheral and splenic lymphocytes and is evident in about 38 to 75 percent of cells on the fifth day of culture.

Blood obtained from nonimmunized rabbits by cardiac puncture was collected in syringes containing heparin. The erythrocytes were sedimented by adding one-half volume of dextran of high molecular weight to the whole blood and incubating the mixture for 20 minutes at 37°C. The supernatant plasma, rich in leukocytes, was then

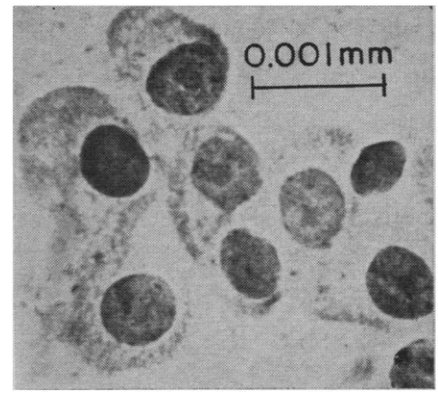

Fig. 1. Phase-contrast micrograph of rabbit peripheral lymphocytes grown in tissue culture for 5 days without phytohemagglutinin. The lymphocytes have transformed spontaneously into large blastoid cells possessing abundant cytoplasm, delicate nuclear chromatin, and prominent nucleoli.

aspirated and centrifuged at 800 rev/min for 5 minutes. The cells were washed twice with Eagle's minimal essential medium and the total lymphocytes were counted. Replicate cultures, each containing 3×10^6 lymphocytes in 4 ml of medium (minimal essential medium, modified for suspension culture, supplemented with 20 percent fetal calf serum and 1 percent L-glutamine), were incubated for 5 days at 37°C.

Phytohemagglutinin type M (0.1 ml) was added to some of the tubes at the beginning of culture, and these cells were grown for 72 hours. Duplicate cultures were harvested within one hour, and then two or more cell cultures from each animal were harvested 3 and 5 days later. The cells were examined by phase-contrast microscopy after being fixed in a mixture of absolute methanol and glacial acetic acid (3:1) and being stained with 0.5 percent acetic-orcein. The cells were also examined by light microscopy after being stained with Jenner-Giemsa; however, nuclear detail was considerably enhanced by phase-contrast microscopy, which was used for most of the counts. One thousand cells on each slide were counted, and the percentages of typical small lymphocytes and of altered lymphocytes were determined.

The cytological criteria for transformation were based both on the size of the cells and on the appearance of the nuclear chromatin. Thus cells with prominent nucleoli and a pale-staining, delicately reticulated nuclear chromatin were considered transformed even if they were of about the same size as

Table 1. Percentages of rabbit peripheral lymphocytes spontaneously transformed in culture to blastoids at intervals up to 120 hours. Lymphocytes grown in suspension culture with Eagle's minimal essential medium modified for suspension culture (Grand Island Biological); values are means of two or more replicate cultures.

Initial*	72 hours*	120 hours*	120 hours†
0.8	8.6	72	74
1.8	12	75	60
2.4	12	63	64
2.2	6.5	50	43
2.5	9	48	54
1.6	8	38	56
1.8	4.5	67	64
2.0	4	74	75
2.6	9	66	66
1.4	8.6	71	70

* Medium supplemented with 20 percent fetal calf serum. † Medium supplemented with 20 percent autologous rabbit serum.

small lymphocytes. In order to study synthesis of DNA and RNA, duplicate cultures at the start of the experiment, at 3 days, and at 5 days were gently agitated and then incubated for 2 hours with 1.25 μc of tritiated thymidine or tritiated cytidine for each milliliter of culture medium. After incubation, the cells were washed three times with normal saline and then fixed for 10 minutes with a mixture of absolute methanol and glacial acetic acid. Slides were prepared for autoradiography by use of NTB-2 Kodak Nuclear Track Emulsion. The slides were stored in lightproof boxes for 14 to 21 days at 4°C, and were then developed in Kodak D-72 and stained with Jenner-Giemsa. One thousand cells on each slide were counted, and the percentage of the total mononuclear cells containing six or more grains were determined.

At the beginning of culture, lymphoid cells in the inoculum consisted of typical small lymphocytes, except for 0.8 to 2.6 percent medium or large lymphocytes (Table 1) which, in addition to being larger, possessed a reticulated nuclear chromatin. The small lymphocytes did not incorporate tritiated thymidine into DNA, but 0.1 to 2.5 percent of the lymphocytes that appeared immature were labeled with tritiated thymidine (Table 2). In contrast with the absence of DNA synthesis, between 86 and 96 percent of the blastoid cells were undergoing synthesis of RNA, as evidenced by labeling with tritiated cytidine (Table 2); about the same percentage of small lymphocytes had also incorporated this label. By the 3rd day of culture the cells grown in the presence of PHA had undergone a cytological transformation that was similar to that observed in cultures of human lymphocytes. Thus, after 72 hours of culture, 75 to 86 percent of the cells were morphologically immature, and mitoses were frequent.

In the cultures grown without PHA, 4 to 12 percent of the cells were blastoid in appearance at 72 hours (Table 1). By the 5th day of culture, however, significant alteration in size and cytological characteristics was evident in the lymphocytes grown in the absence of PHA; 38 to 75 percent of the cells had transformed, apparently spontaneously, into immature cells having prominent nucleoli, finely reticulated nuclear chromatin, and abundant, pale-staining cytoplasm (Fig. 1).

The transformed cells varied between 10 and 50 μ in diameter. The smaller cells (10 to 20 μ) were thought to be either in the process of enlargement or to have resulted from mitotic division of the large blastoid cells; however, even the smallest of the transformed cells could be readily distinguished from small lymphocytes by characteristics of their nuclei. As with PHA present, the transformed cells tended to grow in clusters; mitoses were frequent, and the cytoplasm stained intensely with methyl-green pyronin. Despite the obvious mitotic activity the cultures were never as heavily populated as those grown with PHA present. Synthesis of DNA and RNA was active in the transformed cells, as determined by autoradiography with tritiated thymidine and tritiated cytidine. After 2-hour incubation 12 to 30 percent of the blastoid cells were labeled with tritiated thymidine and 95 to 98 percent of the blastoid cells had incorporated tritiated cytidine into RNA (Table 2).

Since the original culture inoculum consisted almost entirely of small lymphocytes, these cells had probably transformed into the blastoid cells seen 5 days later. Although unlikely from a quantitative standpoint, the blastoid cells may conceivably have derived from rapid proliferation of the 0.8 to 2.5 percent of immature lymphocytes present in the inoculum at the beginning of culture. However, active synthesis of DNA, as measured by incorporation of tritiated thymidine, did not occur until the 5th day of culture, when 12 to 30 percent of the cells were labeled after 2-hour incubation. By contrast, on the 3rd day of culture the cell population consisted of 4 to 12 percent of blastoid cells of which only 0.2 to 2.4 percent were labeled with tritiated thymidine (Table 2). On the following day there was an abrupt increase in the percentage of blastoid cells (30 to 40 percent) and a concomitant decrease in small lymphocytes. These results suggest that the blastoid cells derived from a progressive cytological transformation of the small lymphocytes present in the original culture inoculum, and that cell replication was vigorous only after the spontaneous change had taken place.

Table 2. Synthesis of DNA and RNA in cells spontaneously transformed to blastoids; rabbit peripheral lymphocytes were grown in suspension culture and incubated for 2 hours with 1.25 μc tritiated thymidine or tritiated cytidine before harvesting and autoradiography. Values show the range of labeled cells in cultures from ten animals after 0, 72, and 120 hours.

Time (hr)	Blastoid cells labeled (%)	
	Tritiated thymidine	Tritiated cytidine
0	0.1 to 2.5	86 to 96
72	0.2 to 2.4	92 to 98
120	12 to 30	95 to 98

The unexpected observation of extensive cell transformation to blastoids led us to search for factors that might conceivably have elicited such a change. Three possible stimulatory factors were considered: (i) foreign proteins in the tissue-culture media (for example, fetal calf serum), (ii) penicillin or streptomycin, which are routinely added to the media, and (iii) polymorphonuclear leukocytes in the original cellular inoculum or their degradative products.

Lymphocytes from the ten rabbits listed in Table 1 were therefore cultured in media containing 20 percent of autologous serum rather than fetal calf serum, and cultures were also grown without penicillin or streptomycin. The use of rabbit serum did not reduce the number of transformed cells (Table 1); in fact, addition of autologous serum enhanced the growth of the cells in that there was more luxuriant growth of the cultures and an apparent increase in mitotic figures. Furthermore, there was no diminution in the percentage of blastoid cells when the cells were grown without either penicillin or streptomycin or without both.

The lymphocytes were purified before culture by allowing the polymorphonuclear leukocytes in the original plasma-leukocyte supernatant to adhere to glass. The plasma, rich in leukocytes, was first mixed with an equal volume of minimum essential medium before

10-ml portions were placed in 120-ml, flat-sided, glass prescription bottles. The bottles were incubated on their sides for 90 minutes at 37°C. The lymphocytes and any nonadherent polymorphs were gently decanted, centrifuged, washed twice in medium, and cultured as described. This procedure effectively reduced the number of contaminating polymorphs and provided a reasonably pure culture inoculum of small lymphocytes.

However, the presence of polymorphonuclear leukocytes in the cultures had no obvious stimulatory effect on cellular transformation, since their removal did not reduce the percentage of blastoid cells after 5 days of culture. Indeed, rather than provoke stimulation of blastoids, the presence of granulocytes may inhibit the growth and differentiation of lymphocytes in vitro (*3*).

Spontaneous transformation to blastoid cells was also studied in cultures of guinea pig peripheral lymphocytes. Erythrocytes from nonimmunized guinea pigs were sedimented by the addition of an equal volume of 3 percent gelatin to heparinized blood, and the cells were cultured in 20 percent rabbit or fetal calf serum. By the 5th day of culture, between 58 and 72 percent of the cells were blastoid in appearance, whereas only 0.8 to 2.5 percent were blastoid before culture.

The potential for spontaneous transformation was not confined to circulating lymphoid cells; a similar change was also observed in 5-day cultures of splenic lymphocytes of guinea pig. The splenic tissue was teased apart in minimal essential medium, filtered through mesh, washed three times with medium, and then cultured for 5 days at 37°C in minimal essential medium supplemented with fetal calf serum (20 percent). By contrast with the peripheral lymphocytes, the splenic cells were more heterogeneous in appearance at the beginning of culture. Many of the splenic lymphocytes were larger than the circulating lymphocytes; most of them had a dense, deeply basophilic, nuclear chromatin, while approximately 10 to 16 percent had reticulated nuclei similar to those of the blastoid cells. By the 3rd day of culture with PHA present, 75 to 85 percent of the splenic cells were blastoid in appearance, and by the 5th day 55 to 75 percent of the cells grown without PHA had transformed spontaneously into blastoid cells. Both the PHA-promoted and spontaneously transformed splenic and peripheral blastoid cells were capable of synthesis of RNA and DNA, as evidenced by labeling with tritiated cytidine and tritiated thymidine, respectively.

The stimulus for transformation to blastoids is not known. The immature cells that developed spontaneously or with PHA present tend to appear in aggregates. It has been suggested that the blastogenic properties of PHA may be related to cell-membrane contacts caused by agglutination of leukocytes. Thus one may speculate that spontaneous transformation to blastoids is also related to the lymphocytic aggregation that always occurs to some extent, even when cells are cultured in the absence of PHA. Possibly the transformation reflects a response by the small lymphocytes to antigen or antigens in the culture media. I tried to eliminate certain obvious antigens, such as foreign proteins and penicillin, but cell-bound antigens or antigens resulting from cell degeneration conceivably may have elicited such response.

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Kinetin-Induced Chloroplast Maturation in Cultures of Tobacco Tissue

Abstract. *Cultured tobacco tissue possesses proplastids capable of differentiating into mature chloroplasts. Kinetin (6-furfurylaminopurine) is a specific requirement for this differentiation; the absence of this compound results in a blockage of the formation of grana. The possibility that kinetin exerts a direct effect upon chloroplast differentiation is considered.*

We describe here the effect of the plant growth regulator, kinetin (6-furfurylaminopurine), on chloroplast maturation and chlorophyll synthesis in cultured tobacco tissue. The function and fine structure of developing chloroplasts in this system has already been reported (*1*). Kinetin has been implicated in many aspects of plant metabolism, and it is required for the growth of many cultured tissues (*2*).

The tissue used in these experiments was originally isolated from pith of *Nicotiana tabacum* L. var. Maryland Mammoth, and it had been subcultured for a period of 2 years. This strain of tobacco tissue will grow in the absence of kinetin, but complete chloroplast development occurs only in the presence of this compound. A modified Murashige and Skoog medium (*3*), which was used as the basic culture medium, contains the auxin, naphthalene acetic acid, at a concentration of 0.5 mg/liter. Explants from tissue grown without light (dark-grown) were grown in continuous light (light-grown) on media with or without kinetin. Light was supplied by a bank of warm-white fluorescent tubes providing an intensity of 500 ft-c (45 lu/m^2) at the level of the cultures. Both light- and dark-grown tissues were cultured at 25°C. Tissue samples were taken from both light- and dark-grown cultures at either 28 or 45 days for chlorophyll determinations (*4*) and for fine-structure examination by electron microscopy. The tissue was fixed in 1.5 percent $KMnO_4$, dehydrated in a graded water-acetone series, and stained in 1.0 percent uranyl nitrate. The fixed and stained material was embedded in epoxy resin (*5*) and sectioned with a diamond knife. Sections were mounted on uncoated grids and viewed in the Siemens Elmiskop I.

The proplastid illustrated in Fig. 1*A* is typical of those found in tissue grown in the dark for 28 days on a medium containing kinetin (0.5 mg/liter). Lamellar formation appears to occur by vesiculation and elaboration of the inner proplastid membrane. Lamellar sheets, as seen by serial sections, are formed in the proplastids, but there is no fusion of lamellae to form grana. Prolamellar bodies have not been observed in the proplastids of dark-grown cultured tissue. Cultured tissue growing in the light in