Anal. Chem., 1957, v29, 144.

9. Atkins, G., Marotta, S. F., Proc. Soc. Exp. Biol. and Med., 1963, v113, 461.

10. Knigge, K. M., Hoar, R. M., ibid., 1963, v113, 623.

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Blastoid Transformation of Rabbit and Guinea Pig Peripheral Lymphocytes by Phytohemagglutinin.* (31132)

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It is now well established that small lymphocytes from human peripheral blood have a potentiality for growth and differentiation since they can be transformed into cytologically immature "blastoid" cells, capable of mitotic division, when grown in tissue culture with phytohemagglutinin (PHA)(1,2).

Despite the observations with human cells the possibility that a potentiality for blastoid transformation may be a biological characteristic of the mammalian small lymphocyte has heretofore not been studied extensively. Indeed the paucity of data in the published reports(3,4,5) and the conflict of opinions fail to provide convincing evidence that PHA can induce blastoid transformation in small lymphocytes from mammalian species other than man.

In view of the still largely unresolved questions concerning the potentialities of mammalian lymphocytes we have investigated the cytological and biochemical alterations elicited in rabbit and guinea pig peripheral lymphocytes when grown in tissue culture with PHA.

Materials and methods. 1. *Tissue culture.* Blood (30-50 cc) was obtained from rabbits and guinea pigs by cardiac puncture and collected inho heparinized syringes. The blood, from each species, was handled identically except for the method of erythrocyte sedimentation. The rabbit erythrocytes were sedimented with high molecular weight dextran (m.w. 250,000) added in half volume quantities to the heparinized blood whereas the guinea pig erythrocytes were more effectively sedimented by the addition of equal volumes of 3% gelatin. With both methods excellent erythrocyte sedimentation was obtained within 20-30 minutes at 37°C.

The supernatant leukocyte-rich plasma was then aspirated and the cells sedimented at 800 rev/min for 10 minutes. The cell button was subsequently washed 3 times with Eagle's minimal essential medium (Grand Island Biological, Grand Island, N. Y.), resuspended in complete medium (Eagle's MEM, supplemented with 20% fetal calf or rabbit serum and 1% L-glutamine 200 mM) and the total lymphocyte count determined. Replicate cultures each containing 3×10^6 lymphocytes in 4 ml complete medium were incubated in tightly sealed 16 × 150 mm culture tubes at 37°C. Replicate cultures were grown either with Phytohemagglutin (0.1 ml) Type M, Difco Labs, Detroit, Mich., or in its absence, and 2 or more culture tubes harvested at frequent intervals up to 72 hours. The cells were fixed in a mixture of absolute methanol and glacial acetic acid (3:1) and then stained with 1.0% acetic-orecein or Jenner-Giemsa. Most of the slides were examined by phase contrast microscopy which when combined with acetic-orecein staining provided excellent nuclear morphology. One thousand cells on each slide were examined and the percent of typical mature lymphocytes and cytologically transformed cells determined. The percent of transformed cells at each interval of culture was determined by averaging the differential counts from 2 or more replicate cultures. Total cell counts at each period of culture were not determined.

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2. *Autoradiography.* DNA and RNA synthesis were studied in the tissue cultures by autoradiography with H^3-thymidine and H^3-cytidine, respectively. Duplicate cultures at each time interval were gently agitated and then incubated for 2 hours with either H^3-thymidine 6.7 curies/mM or H^3-cytidine 2.68 curies/mM (New England Nuclear Corp., Boston, Mass.) added to the culture tubes at a final concentration of 1.25 μc/ml. At the end of the incubation the cells were washed 3 times with normal saline and then fixed for 10 minutes with absolute methanol and glacial acetic acid. Slides were prepared for autoradiography using Kodak NTB2 Nuclear Track Emulsion. The slides were stored for 14 days at 4°C and then developed in D-72 Kodak developer and stained with Jenner-Giemsa. One thousand cells on each slide were counted and the percent of labelled mononuclear cells determined.

Results. 1. Blastoid transformation of rabbit peripheral lymphocytes. The lymphoid cells in the initial culture inoculum consisted of typical small lymphocytes except for 0.5-2.4% of cells which in addition to larger size possessed a paler-staining and more delicate appearing nuclear chromatin.

When the lymphocytes were grown with PHA they were rapidly transformed into cytologically immature (blastoid) cells. The earliest cytological transformations were observed in small lymphoid aggregates. Indeed, throughout the entire culture period blast cell transformation was most prominent in lymphoid aggregates which tended to increase in size with prolongation of culture.

The blastoid cells, even at 18 hours, were readily distinguished from typical small lymphocytes by their prominent nucleoli and delicately reticulated pale-staining nuclear chromatin. Nuclear alterations were the principle criteria used to determine transformation since these changes were present in small cells, early in the culture, before an increase in overall size was evident. At 72 hours the cultures were heavily populated with blastoid cells in which vigorous mitotic activity was now evident (Fig. 1). The blastoid cells at 72 hours were 20-50 μ in diameter and possessed abundant cytoplasm which stained intensely

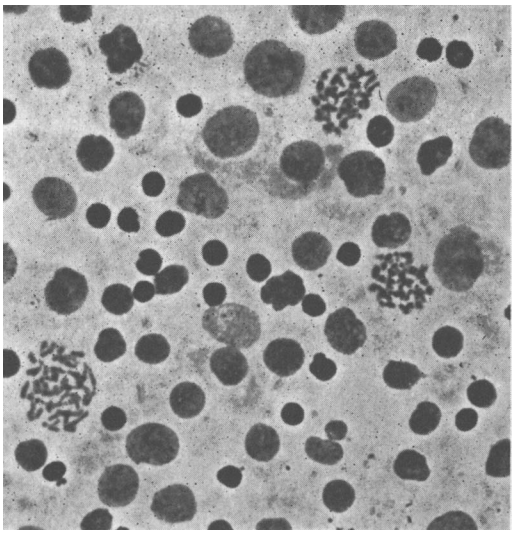
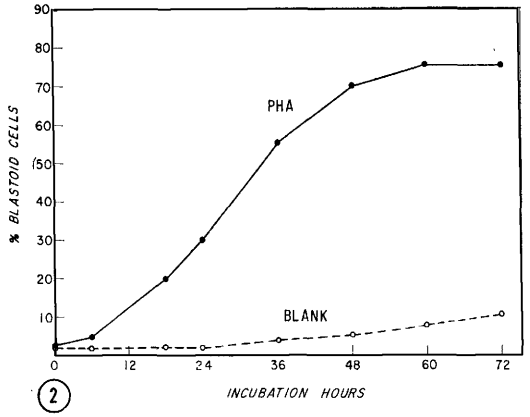


FIG. 1. Blastoid cells and mitoses in rabbit peripheral lymphocytes cultured for 72 hours with phytohemagglutinin.

with methyl green pyronin. The blastoid cells tended to localize in dense aggregates; however, numerous single cells with blastoid features were also present. The dense aggregation made accurate quantitation of the blastoid response difficult and it was only by counting numerous cells in each of replicate cultures that the degree of transformation could be enumerated. Many smaller cells with cytologically immature nuclei were also evident in the 72-hour cultures. They were the result presumably of mitotic division of the larger blastoid cells.

Quantitation of the blastoid response at frequent intervals throughout the 72 hours of culture disclosed significant blastoid transformation within the first 24 hours. Thus even as early as 18 hours about 20% of the cells were blastoid whereas less than 3% of the cells were so classified at the start of culture. The blastogenic response thenceforth proceeded rapidly and by 36 hours, 55% of the cells were cytologically immature (Fig. 2). After the second day of culture the percent of blastoid cells did not increase markedly; however, there was now a luxuriant growth in the cultures and when they were terminated at 72 hours, 75% of the cells were blastoid and mitotic figures were numerous. In contrast the cells grown without PHA re-



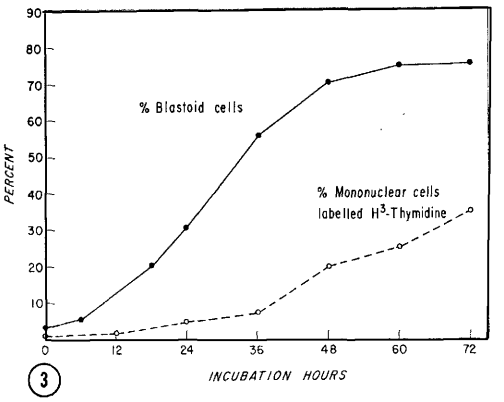


FIG. 2. Effect of phytohemagglutinin (PHA) on rabbit peripheral lymphocytes. The data in Fig. 2 and 3 are means of 2 or more replicate cultures from each of 10 rabbits.

FIG. 3. Effect of phytohemagglutinin on "blast" cell production and DNA synthesis in rabbit peripheral lymphocytes. Cultures were incubated for 2 hr with H^3-thymidine specific activity 6.7 curies/mM at a final concentration of 1.25 μc/ml. Autoradiographs were exposed for 14 days with Kodak NTB2 Nuclear Track emulsion.

mained as a relatively stable population of small lymphocytes; however, even in these cultures a 10% spontaneous blastoid transformation was observed at 72 hours.

2. RNA and DNA synthesis in rabbit peripheral lymphocytes. Autoradiography with H^3-thymidine and H^3-cytidine disclosed that RNA synthesis preceded DNA synthesis by almost 24 hours in rabbit peripheral lymphocytes grown with PHA. Within the first few hours of culture 86-96% of the cells were labelled with H^3-cytidine whereas only 0.1-2.5% of cells were labelled with H^3-thymidine.

The kinetics of DNA synthesis in the PHA-stimulated cultures was studied to determine whether PHA induced a cytological transformation in the small lymphocytes prior to the onset of mitotic division. This was done in an attempt to identify the precursors of the blastoid cells since active replication of the few immature cells always present in the culture or of a small group of cells particularly stimulated by PHA might conceivably give rise to the entire blastoid population.

Fig. 3 compares the blastogenic response to PHA with the percent of cells labelled with H^3-thymidine at intervals up to 72 hours. Since small lymphocytes do not incorporate the label, it is possible to determine from the curves the percent of blastoid cells in DNA synthesis at each time interval. Thus, significant blastogenesis (30%) occurs within the first 24 hours of culture; however, at this time only 2% of cells were undergoing DNA synthesis. The lag in DNA synthesis was even more obvious at 36 hours when only 8% of the 55% blastoid cells were labelled with H^3-thymidine. By the third day of culture, when 75% of the cells were blastoid, there was a definite increase in DNA synthesis and now about 35% of the cells were labelled after a 2-hour incubation with H^3-thymidine. These data suggest that PHA-induced blastogenesis occurs in a population of small lymphocytes which are transformed into large cells prior to the advent of mitotic division. Active RNA synthesis proceeded throughout the entire culture period and at 72 hours between 92 and 98% of the cells were labelled with H^3-cytidine.

3. Extended cultures of rabbit lymphocytes. Cultures of peripheral lymphocytes with PHA were maintained for as long as 10 days in an effort to determine whether the blastoid cells were capable of further cytological differentiation. In some cultures there was a relative increase in the percent of small lymphocytes; however, this may have been due to a decrease in the population of large cells rather than to a transformation of large cells into small lymphocytes. Plasma cells were not ob-

served in any cultures even when maintained for 10 days. Cultures at 5, 8 and 10 days were incubated for 16 hours with H^3-thymidine (1.25 μc/ml) and autoradiographs prepared. Since H^3-thymidine is incorporated only into blastoid cells the appearance of labelled small lymphocytes might suggest their origin from the blastoid cells. Autoradiography subsequently showed that many smaller cells had incorporated H^3-thymidine. However, the intensity of nuclear labelling precluded the possibility of accurately assessing the cytological features of these cells.

4. Blastoid transformation of guinea pig peripheral lymphocytes. Guinea pig small lymphocytes also showed a profound blastogenic response when grown with phytohemagglutinin. Cytologically the transformed guinea pig cells were similar to those obtained in the rabbit. Using nuclear characteristics as well as size to judge immaturity, 0.8-2.5% of cells were considered immature at the start of culture, whereas at 72 hours a profound alteration in population had occurred and 74 to 92% of cells were blastoid in appearance.

The blastoid response was observed in cultures of guinea pig cells taken at frequent intervals after the start of culture. A modest blastogenic response was evident in cultures examined as early as 6 hours at which time about 4% of cells were transformed. The early transformation was localized exclusively in small lymphoid aggregates. At the end of 24 hours about 20% of the cells were blastoid and by 48 hours transformation was striking with almost 70% of the cells blastoid in appearance. RNA and DNA synthesis were active in the blastoid cells at 72 hours as determined by autoradiography with H^3-cytidine and H^3-thymidine.

Discussion. Our studies indicate that rabbit and guinea pig peripheral lymphocytes can transform into blastoid cells capable of RNA and DNA synthesis and mitotic division when grown in tissue culture with phytohemagglutinin. This cytological transformation occurs in a population consisting almost entirely of small lymphocytes and precedes active DNA synthesis by 36 hours. The cellular transformation is similar, both cytologically and morphologically, to that obtained in cultures of human peripheral lymphocytes and lends support to the concept that the small mammalian lymphocyte has a potentiality for growth and differentiation.

The excellent blastogenic response described in this report is different from that obtained previously by Marshall and Roberts (4) who failed to stimulate the peripheral leukocytes of guinea pigs, rabbits, mice and rats with PHA. However, methodological factors might have been responsible for the discrepant findings. We have always added L-glutamine to our media since the addition of this growth factor has been used efficaciously by us in cultures of human peripheral blood. No mention of L-glutamine was made by Marshall and Roberts who failed to obtain adequate growths with PHA nor by a number of authors who were able to induce a blastoid response and mitoses with PHA in guinea pig, rabbit, rat and mouse(3,5,6). The most successful rate of transformation of up to 90% of rabbit lymphocytes was apparently obtained without glutamine(7).

Limited blastoid transformation was noted in our cultures when lymphocytes were grown without PHA for 3 days. We have previously shown that as lymphocyte cultures are maintained without PHA there is a progressive increase in the percentage of blastoid cells and ultimately these cells undergo DNA synthesis(8). The basis for spontaneous transformation is not understood; however, there are no obvious cytological differences between the spontaneous blastoid cells and those produced with PHA. Blastoid transformation in the absence of phytohemagglutinin has also been noted in cultures of human peripheral blood(9); however, in this situation it is possible that the lymphocytes were responding to antigens in the fetal calf sera employed in the culture media.

The occurrence of blastoid transformation, in the absence of PHA, suggests that the small mammalian lymphocyte is inherently capable of differentiation and that this potential is exquisitely stimulated by phytohemagglutinin.

Summary. Rabbit and guinea pig peripheral lymphocytes were grown in suspension culture with minimum essential medium sup-

plemented with fetal calf or rabbit serum and 1% L-glutamine, 200 mM. The addition of phytohemagglutinin transformed the lymphocytes into cytologically immature blastoid cells capable of RNA and DNA synthesis and mitotic division. The response began within the first 24 hours of culture and by 72 hours about 75% of the surviving cells were transformed into blastoid cells. Autoradiography with H^3-thymidine disclosed that phytohemagglutinin induced blastogenesis occurs in a population of small lymphocytes which are transformed into large cells prior to the advent of mitotic division.

1. MacKinney, A. A., Jr., Stohlman, F., Jr., Brecher, G., Blood, 1962, v19, 349.

2. Marshall, W. H., Roberts, K. B., Quart. J. Exp. Physiol., 1963, v48, 146.

3. Lycette, R. R., Pearmain, G. E., Lancet, 1963, vII, 386.

4. Marshall, W. H., Roberts, K. B., ibid., 1963, vI, 773.

5. Nichols, W. W., Levan, A., Blood, 1962, v20, 106.

6. Schwarz, M. R., Anat. Rec., 1964, v148, 333.

7. Sell, S., Gell, P. G. H., J. Exp. Med., 1965, v122, 423.

8. Sabesin, S. M., Science, 1965, v149, 1385.

9. ———, Lancet, 1965, vII, 294.

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Gamma M Synthesis During the Secondary Antibody Response In Mice.* (31133)

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Several studies using rabbits(1-5) and rats (6), have demonstrated the transient appearance of only small amounts of gamma M antibody following secondary antigenic stimulation. The characteristic features of the typical secondary response (*i.e.,* a higher titer of antibody in a shorter period) were not fulfilled with respect to gamma M synthesis, in that titers of macroglobulin appeared at levels only equal to or lower than those demonstrated initially. In one instance in rabbits (3), a single small dose of polio virus stimulated only gamma M synthesis. Reinjection of such rabbits at a critical time not exceeding 3 days after cessation of gamma M synthesis, resulted in a typical secondary response with the prompt appearance of gamma M globulin in higher titers than had been seen initially. This communication reports that gamma M synthesis in the inbred Balb mouse following 2 injections of a purified protein antigen has the characteristics of a typical secondary response.

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Materials and methods. 4-8-week-old male, inbred mice, Balb strain, were injected intraperitoneally with 1.0 mg antigen, human gamma globulin (HGG), lot #7286, Calif. Biochem. Corp., further purified by DEAE cellulose chromatography. The secondary response was induced in the same manner 36 days after the initial exposure. Using high titer rabbit antihuman plasma, only a single band of precipitate was demonstrated on reaction with the purified antigen at a concentration of 5 mg/ml in the Ouchterlony gel diffusion test and by immunoelectrophoresis.

Mice were anesthetized with ether and bled by severing the axillary vessels. Equal volumes of blood were pooled from 3 or 4 mice and the serum separated and kept frozen until antibody titers were determined by the passive tanned cell hemagglutination technique. The red cells were sensitized with 1:20,000 w/v tannic acid in 0.15 M NaCl. After washing and centrifugation the suspension of 2.5% v/v tanned cells was exposed to 250 μg/ml of antigen per ml red cell suspension for 15 minutes at room temperature. After centrifugation, the cells were washed and a