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Cellular Proliferation in Relation to Antibody Synthesis.*†‡ (27083)

RICHARD N. BANEY,§ JACINTO J. VAZQUEZ,‖ AND FRANK J. DIXON‖
Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

This report will describe an attempt to determine the extent of mitotic activity of splenic cells of rabbits during a secondary immune response and its correlation with antibody synthesis by the combined use of immunohistochemical and autoradiographic technics. The latter technic employing tritiated thymidine has been widely used for precise studies of cellular proliferation in a variety of tissues(1). Additional studies to determine the mitotic potential of functionally differentiated antibody producing cells were made using the above mentioned procedures in conjunction with stathmokinetic technics employing colchicine(2,3). By these approaches, a marked proliferation of cells destined to produce antibody is demonstrable after antigenic stimulation, in which functionally differentiated, as well as immature precursors, participate.

Materials and methods. Two adult, 3 kg rabbits were given a primary course of immunization for a total of 150 mg of bovine gamma globulin (BGG) over 5 consecutive days according to the following schedule: 10 mg subcutaneously; 20 mg subcutaneously;

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§ U.S.P.H.S. Post-sophomoral Student Research Fellow.

‖ Present address: Division of Exp. Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif.

20 mg subcutaneously; and 30 mg/kg intravenously. Five days after completion of the course, the animals were bled and serum titers of 250 gamma of AbN were obtained. Four weeks later, the animals were given a single injection of 125 mg antigen intravenously. Simultaneously with the booster, and at 6 hour intervals thereafter until sacrifice at the end of 4 days, the animals received intraperitoneal injections of 0.25 μc/g body weight of tritiated thymidine for a total of 16 injections. Tritiated thymidine with specific activity of 1.9 C/mM was obtained from Schwarz Biochem. Inc., Mt. Vernon, N. Y. The tritium was localized in the methyl group of thymine of this commercially available preparation(4). At sacrifice, touch imprints and brush smears of spleen were prepared and stained for specific antibody (anti BGG) by the sandwich modification of the fluorescent antibody technic of Coons *et al.*(5) with technical procedures as described previously (6). Selected fields containing cells with specific antibody were photographed and vernier coordinates from the microscope stage were recorded for future relocation of the same fields (Fig. 1, top). Following this procedure, the slides were processed for autoradiography with Kodak NTB-2 liquid emulsion(7). Preparations were exposed for up to 4 weeks, developed and stained with Wright's stain. Previously photographed fields were then relocated and antibody containing cells as well as other mononuclear cells were observed for nuclear grains, indicative of thymidine incorporation (Fig. 1, bottom). Radiographic background in all preparations averaged less than one grain per 50 μ^2 (approximately the area presented by a nucleus, 8 μ in diameter). Due to the specificity of incorporation of labelled thymidine into the nucleus, cells showing more than 3 nuclear grains above background were considered labelled(1,8). Labelling indices or percentages, *i.e.*, number of labelled cells/total number of cells, were determined for each type of cell.

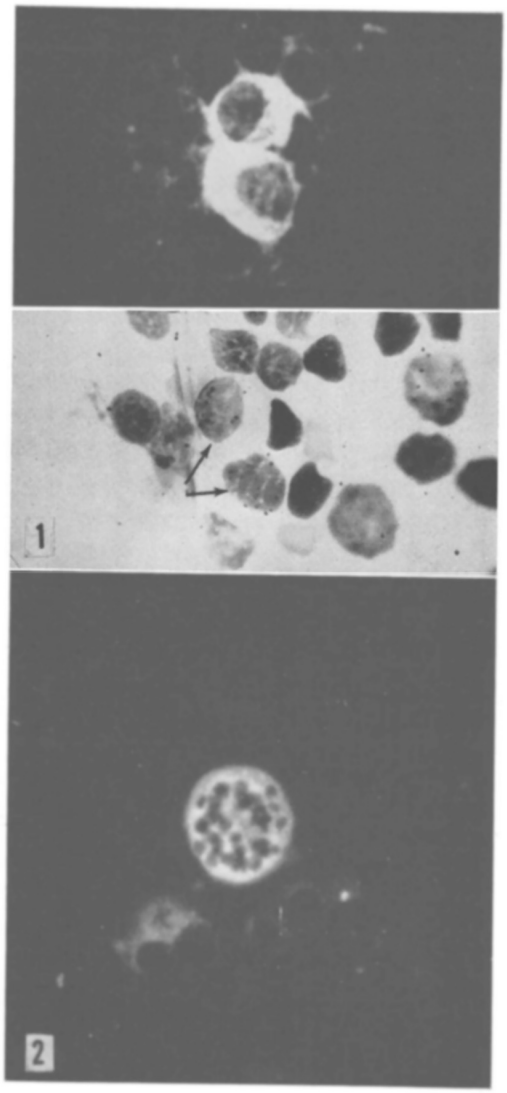

FIG. 1 (top). Fluorescent micrograph of spleen brush smear from a rabbit on day 4 of a secondary response to BGG. Note 2 cells showing bright specific cytoplasmic fluorescence indicating presence of anti BGG.

FIG. 1 (bottom). Wright's stain of the same field as described above after processing the preparation for autoradiography showing tritium grains on nuclei of the 2 antibody-containing cells (indicated by arrows). The cytoplasm of these cells is now poorly visualized as the result of the technical procedures. A non-fluorescent mononuclear cell with nuclear grains is seen to the right of picture. × 300.

FIG. 2. Fluorescence micrograph of antibody-containing cell in colchicine arrested mitosis. Note specific fluorescence of cytoplasm in contrast with non-fluorescent chromosome. × 300.

In vitro studies. Three adult rabbits received primary and secondary immunizations with BGG as described above. On the fourth day following secondary antigenic stimulation, the animals were sacrificed. Spleens were cut into small pieces, which were then

TABLE I. Splenic Mononuclear Cells (Day 4—Secondary response BGG).

Small lymphocytes	60%
Medium lymphocytes	20%
Plasma cells (all stages)	10%
Blast cells	10%

TABLE II. Antibody Containing Cells (Day 4—Secondary response BGG).

Small lymphocytes	3%
Immature plasma cells	40%*
Mature plasma cells	56%*
Plasma blast	1%

* Approximately 92% incorp. H^3 thymidine.

TABLE III. H^3 Thymidine in Splenic Mononuclear Cells (Day 4—Secondary response BGG).

Small lymphocytes	15%
Medium lymphocytes	50%
Plasma cells (all types)	92%
Blast cells	80%

H^3 thymidine q 6 hr × 16 after BGG

teased through a wire-mesh screen to obtain cell suspensions. Several 1 ml aliquots containing 200,000 to 300,000 cells/mm^3 were prepared from each animal in a medium containing 50% normal rabbit serum and 50% Hanks' balanced salt solution. Tritiated thymidine was added to each aliquot to a concentration of 1 μc/ml. Aliquots were then incubated at 37° for 15 to 45 minutes with frequent, gentle shaking to prevent sedimentation of cells and to insure adequate contact with the isotope. After incubation, excess thymidine was removed by gentle centrifugation, decantation and resuspension of cells in unlabelled medium. After 3 washings, the cells were smeared onto glass slides, stained for specific antibody, photographed, and processed for autoradiography as above.

Stathmokinetic studies. Six rabbits immunized as described above were given colchicine (Lilly, lot #2382) 1.5 mg/kg intraperitoneally, on day 4 of the secondary response and sacrificed 6 to 8 hours later. Smears of spleen were prepared and stained for specific antibody and with Wright's stain (Fig. 2). In appropriate dosage, colchicine is a specific mitotic poison, which acts on the spindle mechanism of dividing cells causing arrest of mitosis in the metaphase stage. In this manner, it was possible to observe whether a cell containing specific antibody might also undergo mitosis.

Results. Tables I and II show the differential mononuclear cell population and the differential population of antibody-containing cells in rabbits given multiple injections of thymidine during the course of a secondary response. As seen in Table III, 92% of all plasma cells incorporated tritiated thymidine, demonstrating that the majority of plasma cells present by the fourth day of a secondary response are newly formed cells arising from mitotic division of a precursor sometime after antigenic stimulation. No difference was noted in the labelling index of specific antibody containing plasma cells and non-fluorescent plasma cells. Among the antibody producing cell population, no difference in labelling index was noted between the immature plasma cells and the mature plasma cells, which together comprised 96% of all antibody producing cells. Small antibody containing lymphocytes and blast cells were also labelled, but were present in such small numbers that determination of an accurate labelling index was not possible.

In the *in vitro* studies, under the conditions of this experiment, one to two per cent of the entire mononuclear population incorporated tritiated thymidine. Small lymphocytes, comprising approximately 70% of the mononuclear population were uniformly unlabelled. The tritium label was confined to large and medium sized mononuclear cells, a few of which were antibody containing cells. After administration of colchicine most mitoses were observed in large and medium sized mononuclear cells. Approximately one per cent of antibody containing cells in the preparations examined were seen in mitosis.

Comments. The duration of the "S" phase, or period of DNA synthesis which immediately precedes mitosis, has been variously reported as lasting from 6½ to 8 hours in different mammalian cell systems(1,9). Based on the premises that thymidine is a specific precursor for DNA and is incorporated into DNA only at the time of synthesis in preparation for cell division and that the activity

of incorporated tritium labelled thymidine is distributed to the daughter cell at mitosis(1) one can expect that by administering tritium labelled thymidine at 6 hour intervals, virtually all cells dividing during the period between booster injection of antigen and sacrifice would be exposed to, and incorporate labelled thymidine. The value of 92% stated as the labelling index of the plasma cell series here is probably conservative and may actually approach 100%. This low value might result from the initiation and completion of DNA synthesis in some cells between exposures to isotope or from technical limitations in the sensitization of the radiographic emulsion by the label in some cells. These factors, alone or in combination, would tend to lower the observed incidence of labelling. The results presented are in general agreement with those of other investigators employing similar technics(8,10,11) who have implicated a rapidly proliferating population of immature lymphoblasts and plasmablasts as the chief source of plasma cells arising after antigenic stimulation. As a corollary, the large percentage of labelled antibody containing cells found in these experiments indicate that most plasma cells do not arise by direct, non-mitotic differentiation from small lymphocytes. The present studies could not offer quantitative data concerning the relative mitotic activity of the various stages leading to development of the mature plasma cell. However, by the use of colchicine and by short *in vitro* incubation of cells with thymidine it has been shown that functionally differentiated antibody producing cells can divide, indicating that mitotic activity within the family of antibody producing cells is not an exclusive property of the immature or precursor forms.

Summary. A technical approach for the study of the kinetics of antibody producing cells is possible by combination of autoradiography and the fluorescent antibody technic. Results show that: 1. Almost all antibody containing cells present by day 4 of a secondary response are newly formed cells arising from mitotic division of a precursor sometime after antigenic stimulation; 2. Most plasma cells do not arise by direct, non mitotic differentiation from lymphocytes; and 3. Functionally differentiated antibody containing cells can divide.

1. Hughes, W. L., Bond, V. P., Brecher, G., Cronkite, E. P., Painter, R. B., Quastler, H., Sherman, F. G., *Proc. Nat. Acad. Sci.,* 1958, v44, 476.

2. Allen, E., Smith, M., Gardener, W. U., *Am. J. Anat.,* 1937, v61, 321.

3. Leblond, C. P., Allen, E., *Endocrinol.,* 1937, v21, 455.

4. Friedkin, M., *Fed. Proc.,* 1960, v19, 312.

5. Coons, A. H., Leduc, E. H., Connolly, J. M., *J. Exp. Med.,* 1955, v102, 49.

6. Vazquez, J., *J. Lab. and Clin. Med.,* 1958, v51, 271.

7. Messier, B., Leblond, C. P., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 7.

8. Schooley, J. C., *J. Immunol.,* 1961, v86, 331.

9. Lajtha, L. G., Oliver, R., Kumatori, T., Ellis, F., *Rad. Res.,* 1958, v8, 1.

10. Nossal, G., Makela, O., *Lab. Invest.,* 1961, in press.

11. Urso, P., Makinodan, T., *Fed. Proc.,* 1961, v20, 25.

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Connective Tissue VI: Synthesis of Collagen by Rat Uterine Slices.* (27084)

KUNG-YING TANG KAO, SHIH-DZUNG CHEN LU† WILLIAM HITT AND THOMAS H. MCGAVACK

Geriatrics Research Labs., Vet. Admin. Center, Martinsburg, W. Va., and Dept. of Medicine, George Washington University School of Medicine, Washington, D. C.

One of us(1) reported *in vitro* synthesis of collagen by sponge biopsy connective tissue. A year later, Green and Lowther(2) ob-

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† Present address: 2015 Polk St., Corvallis, Oregon.