A New Approach to the Study of the Lymphocyte.* (26304)

WILLIAM J. BECKFIELD AND MOTOE HIRATA (Introduced by Joseph Seifter)
Wyeth Institute for Medical Research, Radnor, Pa., the Graduate School of Medicine, University of Pennsylvania, and Philadelphia General Hospital, Philadelphia

The lymphocyte is still an object of controversy. While some writers believe that it is an undifferentiated mesenchymal cell capable of developing into other cells(1,2,3) others feel that it is an end-form with a specific function and hence lacks prospective potencies(4,5,6). We have transferred large numbers of thoracic duct lymph cells from donor rats into granuloma pouches of recipient rats and studied these cells cytometrically and histologically. This new approach to the lymphocyte problem has been tested in over 100 recipient rats.

Method. Rats weighing 150 to 200 g were injected intraperitoneally or intravenously with 2.5 mg sodium pentobarbital/100 g of body weight, then anesthetized with sufficient ethyl ether to abolish the corneal reflex. Thoracic duct lymph cells were gathered through the left flank of the animals by a method similar to that described by Bollmann, Cain and Grindlay(7). After the peritoneal cavity was opened the dorsal peritoneum was incised transversely just below the diaphragm and the left kidney and adrenal were elevated and retracted medially. The need to strip the aorta and retract it separately was thus avoided and bleeding from its adventital vessels did not occur. The duct was opened just below the diaphragm by puncture with a 27 gauge hypodermic needle. Sharpened polyethylene tubing (#10 size) previously rinsed with 0.25% sodium heparin solution was inserted, tied in place with fine silk and held by ligature where it passed from the body wall. The wound was closed with clips and covered with gauze moistened in physiologic salt solution (PSS). The lymph was received in heparinized test tubes of 5 ml capacity fastened 10 to 15 cm below the level of the operating table. For the short time that lymph was collected the animal remained lightly anesthetized and tied to the operating platform. Hypodermoclycis with PSS was given during operation and collection.

Interscapular air pouches were prepared by the original method of Selye(8,9). For cytometric study, pouches of various ages were prepared by injecting 5 ml air into the interscapular region of 80 rats weighing 200 g. The age of the pouches ranged from less than 1 hour to 9 days. Pouches less than 1 day old received only 1 injection of air. One to 9 day old pouches were reinjected every 24 hours to maintain the same volume. Immediately after the last injection 1 ml of lymph, collected during the preceding hour, was introduced into the pouch. At various intervals from 15 minutes to 20 hours following injection of lymph, the rats were killed with ether. Five ml of saline were injected and the mixture was aspirated. Total and differential cell counts were done on the lymph before injection and on the saline-lymph mixture after withdrawal.

For histological study fresh pouches were similarly prepared by injection of approximately 3 cc of air in 34 rats weighing 150 g. Group A (7 rats) received air injection only. Group B (2 rats) received serum to which acridine orange had been added in a concentration of 1:5000 to test the affinity of tissue components for fluorochrome. In group C (7 rats), whole lymph was injected as soon as 0.5-1.0 cc had been collected. In group D (15 rats), cells to be identified in pouches by means of fluorescent microscopy were stained with acridine orange in concentrations from 1:10,000 to 1:100,000, washed with PSS using centrifugation at 1000 rpm, resuspended in PSS or dye-free lymph and injected into the pouch. Observations were made on motility, color of fluorescence and degenerative changes of the cells by means of blue light ultraviolet fluorescence using dark field illumination. Group E (3 rats) received cells stained with acridine orange and

* Presented in part at meeting of Fed. Am. Soc. Exp. Biol., Chicago, Ill., April 11, 1960.

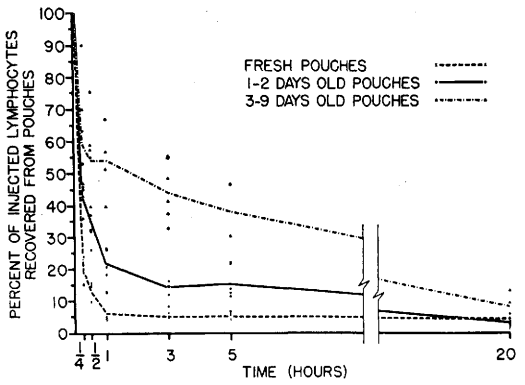

FIG. 1. Percent of inj. cells recovered by aspiration from pouches of various ages at various time intervals following inj. Lines shown on the graph are drawn through the means of groups of 3 to 6 rats.

killed by heat, ultraviolet irradiation or 1% ammonium molybdate solution. At intervals from ½ hour to 20 hours, the rats were killed with ether. Pouches dissected free of skin and most of the underlying muscle were fixed in Tellyesnicsky solution(10). Acridine orange stained pouches were placed first in freshly prepared 1% ammonium molybdate solution for 30 minutes following the principle of Baker(11). Tissues were imbedded in paraffin, semi-serial sectioned at 6 μ, deparaffinated, rapidly hydrated and mounted in glycerine. Luminous cells in the acridine orange groups were photographed by the light of a mercury vapor lamp filtered for blue light fluorescence, using a microscope equipped with Leitz UGI eyepiece filters. Photographic exposure was 5 to 10 minutes on 35 mm Kodak High Speed Ektachrome film (Daylight, ASA Index 160). The same section was then stained with hematoxylin and eosin for bright field examination and conventional photography.

Results. Placement of the tube in the thoracic duct was usually completed within 30 minutes after the rat was anesthesized. One ml or more of lymph containing 30 to 60 million cells was obtained in the first hour of collection. These figures are similar to those reported by Mann and Higgins(12). The lymph cells at time of injection showed no degenerative changes by dark field or phase contrast microscopy and when stained with acridine orange exhibited bright yellow fluorescence under the mercury vapor lamp.

The percentage of lymphocytes recovered by aspiration 15 minutes to 20 hours after injection into granuloma pouches of various ages is shown in Fig. 1. Sections of the walls of fresh and 7-day-old pouches are shown in Fig. 2A and B. In fresh pouches only 20% injected cells were recovered in 15 minutes, 5% in 3 hours and 4% in 20 hours. In 3-9 day old pouches cells disappeared much less rapidly. In 1-2 day old pouches the figures were intermediate.

While the lymphocytes disappeared from the lumen, polymorphonuclear leukocytes migrated into it. In pouches of all ages total number of polymorphonuclear leucocytes found in the saline-lymph mixture rose from an average of 1.06 million after 15 minutes to 3.25 million after 3 hours and to 5.32 million after 20 hours. Monocytes on the other hand did not change significantly. It is true that after 5 hours the saline-lymph mixture contained an average of 10% and after 20 hours 17% monocytes as compared to 6% after 15 minutes. The total numbers, however, were 1.23 million after 15 minutes, 1.08 million after 5 hours and 1.12 million after 20 hours.

Only freshly prepared pouches similar to Fig. 2A were used for histologic studies. No leukocytic infiltration was seen in pouches injected with air only (Group A). Following injection of serum, whole lymph or lymph cell suspensions both polymorphonuclear leukocytes and monocytes appeared in the wall. Very few lymphoid cells were found within 20 hours after serum injection. Injected lymph cells (Groups C and D) occupied the lumen and inner portion of the pouch wall (Fig. 2C). They were readily distinguished from the host's polymorphonuclear and monocytic reaction cells. Lymph cells stained with acridine orange (Group D) appeared brilliant yellow in fluorescence microscopy of the sections in contrast to the faint autofluorescence of the tissue (Fig. 2D). Injected cells which had adhered to and entered the wall of the pouch were distributed irregularly. They were seen chiefly in the mesenchyme between the lobules of

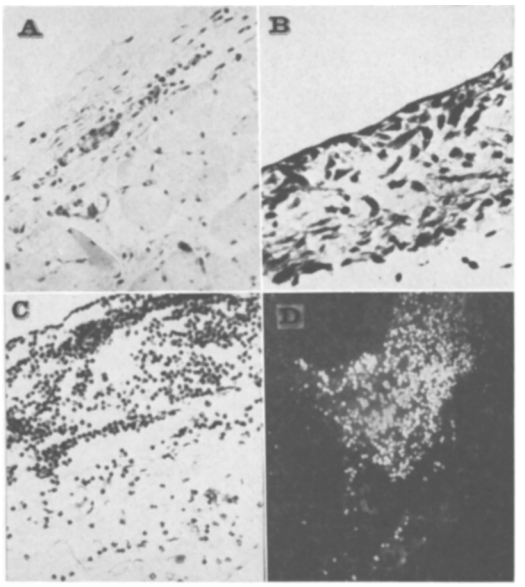

FIG. 2. A. Granuloma pouch 24 hr after one inj. of air, hematoxylin and eosin (H & E). B. Granuloma pouch after 7 daily inj. of air (H & E). C. Lymph cells 4 hr after inj. into air pouch prepared 10 min. previously (H & E). D. Fluorescent acridine orange stained lymph cells 1 hr after inj. into air pouch. Photomicrograph by blue light fluorescence with UGI eyepiece filter and 8 min. exposure on High Speed Ektachrome (copy from 35 mm slide). Lumen of pouch seen in upper left corner.

fatty tisue. Depth of penetration and structure of acridine orange stained cells was indistinguishable from that of the unstained Group C cells but because of the fluorescence in Group D these were more easily found deep in the pouch wall. At ½ hour, only a few of the injected cells appeared in section to have degenerative changes. After 6 hours, considerable nuclear fragmentation and lysis were seen in those cells which remained adherent to the inner surfaces of pouches. Group E cells killed with heat or ultraviolet light liberated dye which transferred to other tissue elements. Fluorescent serum (Group B) produced similar tissue fluorescence. Nuclei of ammonium molybdate killed cells, on the other hand, were identified within the pouch 18 hours after injection, were not seen in the pouch wall and did not impart appreciable fluorescence to other structures. Orange fluorescing granules were found in germinal centers of the axillary nodes of some Group D animals. Whether this represented nuclear material from degenerated injected cells or dye transfer is not known.

Discussion. Of the possible sites in which the lymphocyte may change into another cell or perform a specific function, tissue seems more likely than either blood or lymph. The wide dispersion of tissue lymphoid cells and the problem of positive identification has prevented effective observation or counting. The method which we have described provides a high concentration of thoracic duct lymph cells in the lumen and wall of a reproducible, dissectable connective tissue pouch. It is generally accepted that normal thoracic duct lymph contains over 90% small lymphocytes while the remainder are almost entirely intermediate and large lymphoid cells which may be safely counted as lymphocytes(2). The simple air pouch used here is an air bubble within loose interscapular connective tissue. The air in the pouch is moist, near body temperature and in gas equilibrium with the tissue of the host. At least in the fresh pouch, there is no cellular barrier between injected lymph cells and connective tissue.

Collection of thoracic duct lymph causes little or no cell damage. The preserved structure, motility, and color of cell fluorescence just before injection indicate that the procedures used have not killed many of the cells. Rate of disappearance from the lumen and depth to which cells have penetrated the pouch wall within the first 4 hours also suggest actively motile cells, particularly so because molybdate killed, thoroughly washed cells remained in the lumen after 18 hours. In one animal the polyethylene tubing was led from the thoracic duct directly into an interscapular pouch through a needle. Tissue sections obtained from this preparation were very similar to those from pouches injected with washed acridine orange stained lymph cells. Many abnormalities were seen when cells aspirated from pouches were smeared and stained. Although this may be partly the effect of the recovery procedure and smear technic, it also seems likely to us that the cells which failed to enter the pouch wall were the less actively motile cells. These may represent the 5% which could be recovered after 1 hour from fresh pouches or at

20 hours from pouches of various ages.

About 40% of the residual fluorescent cells aspirated ½ hour after injection into pouches similar to but not included in Group D showed fading of stain, cytoplasmic and nuclear alteration and orange fluorescing granules resembling Gall bodies. In Wright-stain smears, these specimens resembled non-fluorescent aspirates. Because of these considerations and the apparently identical behavior of unstained and acridine orange stained cells in tissue, we have not carried out a complete series of aspiration studies with vitally stained cells. Chemical fixation (trapping) of acridine orange within the vitally stained cells is essential to subsequent histologic study. As the fluorochrome gradually leaves the living cell and ammonium molybdate is toxic, other methods of labeling may prove preferable for either histologic or cytologic studies of long duration.

The transfer of thoracic duct lymph cells into granuloma pouches as described permits simultaneous injection of antigen, fatty substances and other materials which are known to stimulate development of plasma cells, macrophages, and other cells. Also either donor or pouch animal can be pre-treated to alter the lymph cells or their tissue environment.

The fate of transferred lymphocytes has not yet been determined. Some of the cells disintegrated. The difference in disappearance rates between fresh and old pouches (Fig. 1) however, indicates that disintegration was no major event. Many injected lymphocytes migrated through the connective tissue surrounding the pouches. This was seen especially in fresh pouches surrounded by loose connective tissue. There was no evidence of transformation of lymphocytes into monocytes in these short-term experiments or those previously reported by us (13).

Summary. A new approach to the study of the small lymphocyte is presented. Thoracic duct lymph cells from donor rats are injected into granuloma pouches in recipient rats and the transferred cells are studied both cytologically and histologically. Advantages and limitations of the new method are mentioned.

1. Rebuck, J. W., Crowley, J. H., *Ann. N. Y. Acad. Sci.,* 1955, v55, 757.

2. Yoffey, J. M., Courtice, F. C., *Lymphatics, Lymph and Lymphoid Tissue,* Harvard Univ. Press, Cambridge, 1956.

3. Trowell, O. A., *Internat. Rev. Cytology,* Academic Press, N. Y., 1958, v7, 235.

4. Ehrich, W. E., *Entzuendung, Handb. Allgem. Pathol.,* Springer, Berlin, 1956, v7, 1.

5. Amano, S., *Ann. Report, Inst. Virus Res.,* Kyoto Univ., Series A., 1958, v1, 1.

6. Low, F. N., *Electron Microscopy of the Lymphocyte, in the Lymphocyte and Lymphocytic Tissue* (ed. by J. W. Rebuck), Hoeber, New York, 1960, p. 54.

7. Bollmann, J. L., Cain, J. C., Grindlay, J. H., *J. Lab. Clin. Med.,* 1948, v33, 1349.

8. Selye, H., *J.A.M.A.,* 1953, v152, 1207.

9. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 328.

10. Coles, C. H., *Project Mouse,* New Hampshire Chapt. Jackson Lab. Assn., (ed. by Clarence C. Little), 1952, p53.

11. Baker, J. R., *Principles of Biological Microtechniques,* Methuen and Co., London, 1958, pp294, 295.

12. Mann, J. D., Higgins, G. M., *Blood,* 1950, v5, 177.

13. Beckfield, W. J., Hirata, M., Seifter, J., Ehrich, W. E., *Fed. Proc.,* 1960, v19, 241.

Received September 26, 1960. P.S.E.B.M., 1961, v106.