Response of Thymus and other Human Lymphoid Tissues to PHA, PWM and Genetically Dissimilar Lymphoid Cells.* (32184)

M. Roy Schwarz (Introduced by R. J. Blandau)
University of Washington, Department of Biological Structure, Seattle

The ability of lymphoid tissues from various animals to respond to transforming or blastogenic agents is now generally established. One distinct exception to this generalization is the thymus. While lymphocytes from this organ in the rat and swine transformed in response to phytohemagglutinin (PHA 1,2), pokeweed mitogen (PWM 3) and genetically dissimilar cells(4), no response was observed in cultures of cells from the rabbit(5) and the guinea pig(6). Conflicting observations also exist concerning the ability of cells from human thymus to undergo transformation(7-11). The present study was undertaken, therefore, to determine if human thymus cells respond to PHA, PWM or genetically foreign cells and also to observe the effect of these agents on human cells from the tonsil and appendix.

Method. At the time of open-heart surgery, portions of thymus were obtained from 23 patients ranging in age from 3-16 years. Suspensions of cells were prepared by mincing the glands in Eagle's minimum essential medium without glutamine (MEM—Microbiological Associates, Bethesda, Md.) following which 15-40 $\times$ 10^6 cells were added to 5 cc cultures of MEM containing 25-35% fresh, human male or female serum and 500 units of potassium penicillin G (Squibb). Sixty-four of these cultures included 0.05 cc of stock PWM (Grand Island Biological, Grand Island, N. Y.) and 44 contained 0.05 cc of a 1:5 dilution of the stock PHA-P (Difco, Detroit, Mich.), while 46 (2 from each donor) without mitogen served as controls.

Suspensions of tonsillar cells were prepared as above from 31 donors ranging in age from 2 to 26 years. Sixty-two control and 128 experimental cultures were similarly prepared employing 3-8 $\times$ 10^6 cells/cc and PHA (64 cultures) or PWM (64 cultures) as the blastogenic stimulus. Ten to thirty million appendiceal cells obtained from 3 donors (age 8-40) at the time of non-appendiceal surgery, were added to 5 cc cultures containing either PHA (6 cultures), PWM (6 cultures) or no mitogen (6 cultures—controls).

For mixed lymphoid cultures, suspensions of tonsillar and thymic cells were prepared as above from 31 and 20 donors, respectively. Eighty-eight, 5 cc cultures containing 20 million tonsil cells from each of 2 donors or 20 million tonsil cells from one donor and 20 million thymus cells from another donor were then initiated in the usual manner. In one group of experiments (Table III) the percentage of tonsil and thymus cells contributed by each donor was varied from 25-75. In another instance, 0.6 $\times$ 10^6 appendiceal cells and 2.0 $\times$ 10^6 tonsillar cells/cc were cultured together.

After 48 or 72 hours at 37°C, the cells of the cultures were either smeared or they were pulsed for one-half hour with 4 μc/ml of H^3-thymidine (6.7 c/mM; thymidine-methyl-H^3, New England Nuclear). The cells from the pulsed cultures were then washed 3 times with Hanks' balanced salt solution and smeared. The percent large and medium cells (LMC)(13) was determined by counting 1000 cells on smears of all cultures and the percent labeled cells was defined by counting 1000 cells on radioautographs prepared in a standard manner (12). Any experimental culture which demonstrated either a higher percent LMC or a larger percent labeled cells than corresponding controls, was judged as having transformed.

Results. The results of these experiments may be seen in Fig. 1 and Tables I, II and III. In general, transformation occurred in the majority of cultures tested as judged by percent LMC and percent labeled cells (Table II). While Table I indicates that

* This investigation was supported by USPHS grant 07509.

the lowest value observed in experimental incubations was often exceeded by the highest value from control cultures, only incubations in which transformation had occurred (experimental values exceeded controls) were included in this Table. The percentages of all cultures which were judged to have transformed are indicated in Table II. In no instance was a positive response recorded by radioautography without a similar change in the LMC. In contrast, an increased percent LMC, including numerous typical transformed cells, was infrequently observed without H^3-thymidine incorporation by these cells. Because of these observations, all cultures were evaluated for the percent LMC while only a portion was subjected to the time consuming, radioautographic processing. While certain culture types demonstrated blastogenesis more frequently than others, the differences were not related to the sex of the donors. Moreover, in spite of variations in the transformed cells' basophilia and chromatin dispersion, no morphologically distinct and separate subpopulations of transformed cells were observed in any of the culture systems employed.

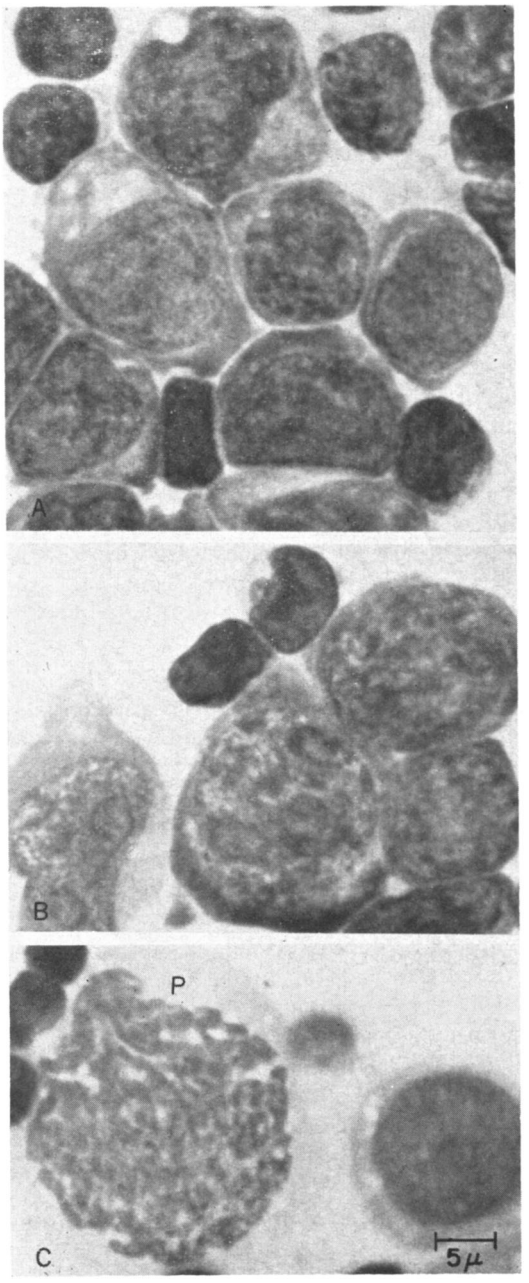

FIG. 1. Transformed or blast cells observed in 72-hr cultures containing A) human tonsil cells from 2 genetically dissimilar individuals, B) human appendix cells and PHA and C) human thymus cells and PWM.

Specifically, PHA and PWM induced the transformation of cells obtained from thymus glands, tonsils and appendices of human donors (Fig. 1B, 1C). In the case of the thymus, a 400-500% increase in the LMC and a 7-24-fold increase in the percent labeled cells was noted. In contrast, while unequivocal responses were noted in tonsil x PHA or PWM cultures, radioautographic values were lower than in thymus incubations. In addition, cells from the tonsils of 3 donors failed to transform as judged by the criteria employed in this study. In these cases, the average percent LMC in the inoculae of the cultures was 42 (range 37-51) as compared to 27 (range 22.0-34.5) in the cell suspensions which subsequently transformed. Three million cells per cc of medium were routinely employed in thymus and tonsil cultures, although transformation occurred at all concentrations tested. As compared to thymus glands and appendices, tonsils demonstrated a larger average percent LMC on smears of cells prepared either before or after incubation.

When tonsil cells from one individual were mixed with either tonsil, appendix, or thymus elements from a second person, transfor-

TABLE I. Results of Culturing Human Lymphoid Tissue with PWM, PHA or Genetically Dissimilar Lymphoid Cells. Average values and ranges (in parentheses) are indicated for all cultures in which experimental values exceeded control observations.

Tissue & mitogen	% LMC* No. experiments	% LMC* Control	% LMC* Experimental	% Labeled cells (H^3-thymidine) No. experiments	% Labeled cells (H^3-thymidine) Control	% Labeled cells (H^3-thymidine) Experimental
Thy† × PHA	22	9.7 (1.6–31.0)	50.3 (10.1–79.0)	15	1.0 (.02– 2.0)	15.6 (6.6–43.0)
Thy × PWM	23	8.5 (.6–31.0)	35.1 (20.8–76.7)	15	1.0 (.02– 2.0)	23.5 (7.0–42.6)
Ton‡ × PHA	31	15.4 (2.9–28.0)	50.6 (6.6–88.0)	13	4.4 (.8 –12.0)	17.7 (2.9–45.5)
Ton × PWM	31	13.4 (2.6–28.0)	48.1 (6.2–65.0)	16	5.2 (1.0 –12.0)	26.4 (8.1–88.0)
App§ × PHA	3	2.0	50.0	—	—	—
App × PWM	3	3.5 (2.0– 5.0)	54.5 (14.5–79.5)	3	1.0	19.5
Ton × Ton‖	31	17.6 (2.3–42.0)	35.1 (5.6–63.5)	20	7.8 (2.5 –14.0)	14.6 (10.0–38.5)
Ton × Thy‖	20	8.7 (1.6–24.0)	34.5 (7.0–71.9)	11	1.0 (.2 –15.0)	9.76(3.6–26.0)
Ton × App‖	1	6.0	22.0	1	5.0	20.5

* LMC = large and medium cells; nuclear diameters greater than 7 μ on smears.
† Thy = Thymus. ‡ Ton = Tonsil. § App = Appendix.
‖ Each of the two donors supplied one-half of the cells in the culture inoculum.

mation was observed irrespective of the cell concentrations contributed by each of these donors (Tables I, III). If a constant percentage of the cells in the culture inoculum were capable of transforming, this result suggests that cells from both donors were responding. It is to be noted that in these mixtures, the differences between experimentals and controls were not as large as those observed when tonsil, thymus or appendix cells alone were exposed to PHA or PWM. This was especially true of tonsil x tonsil and tonsil x appendix mixtures.

TABLE II. Summary of Lymphocyte Transformation in Human Lymphoid Cultures. Percentage of experimental cultures is indicated which, after 48-72 hours, demonstrated a greater percentage of LMC and a higher percent labeled cells.* The absolute values obtained for each of these parameters are indicated in Table I.

Type culture	LMC	Labeled cells
	%	%
Thymus × PHA	100 (18)	100 (8)
Thymus × PWM	100 (15)	100 (4)
Tonsil × PHA	94 (30)	93 (8)
Tonsil × PWM	93 (31)	94 (9)
Appendix × PHA	100 (1)	—
Appendix × PWM	100 (3)	100 (1)
Tonsil × Tonsil	77 (21)	75 (4)
Tonsil × Thymus	85 (9)	66 (2)
Tonsil × Appendix	100 (1)	100 (1)

* Figures in parentheses indicate number of individuals tested.

Discussion. In contrast to previous reports(8,10,11) this study demonstrated that when thymic cells were exposed to non-cellular or cellular mitogens, blastogenesis resulted. The discrepancy with McIntyre and Segel(10) may be due to the lower cell concentration (1×10^6/cc) and/or the longer periods of the incubation (7 days) employed

TABLE III. Results of Varying the Number of Cells from Each Donor in Incubations of Genetically Dissimilar Lymphoid Cells. Average values and range of values (parentheses) observed are indicated.

Cultures	No. of cells	% of cells contributed by Donor 1	% of cells contributed by Donor 2	% LMC	% Labeled cells
Tonsil × Tonsil	2.6×10^6/cc	100	—	5.4 (3.2– 8.5)	5.3 (2.3 –12.0)
		—	100		
		50	50	11.6 (5.6–16.5)	13.8 (10.0 –19.0)
		75	25	11.9 (3.4–34.6)	13.8 (8.0 –22.5)
		25	75	11.8 (3.6–35.2)	13.0 (9.0 –23.0)
Tonsil × Thymus*	5.0×10^6/cc	100	—	11.3 (2.9–24.0)	2.0 (1.34– 2.9)
		—	100	6.6 (1.6–11.8)	1.0 (.02– 2.0)
		50	50	33.6 (1.6–71.9)	11.9 (3.6 –25.9)
		25	75	31.4 (1.0–63.9)	22.7 (8.5 –43.6)
		75	25	37.8 (2.4–72.4)	15.6 (4.6 –23.3)

* Tonsil from donor 1; thymus from donor 2.

by these authors. The importance of incubation time is reflected in the observation that rat thymic cultures which were cultured for 14 days demonstrated cellular degeneration and failure of H^3-thymidine uptake after 6 days(13). A similar reduction in cell number (2×10^6/cc) may account for the lack of transformation in Stroselli's *et al*(11) cultures. While Winkelstein and Craddock(8) initially were unable to obtain transformation of thymic cells, in later studies, results identical to those reported here were observed (9).

The response of tonsillar cells to PHA supports the studies of Oettgen *et al*(14). In addition, these cells were transformed under the influence of PWM and foreign lymphoid cells. The elevated percentage of LMC and percent labeled cells in both control cultures and in the original cell suspensions suggest that immune or inflammatory responses were occurring in these organs at the time of surgery. This may have accounted for the reduced percentage of cultures containing tonsil cells that evidenced transformation.

The presence of transformed cells in appendiceal cultures indicated that this organ does not differ from the thymus or tonsil in this regard. While it has been suggested that the appendix of mammals is homologous to the Bursa of Fabricius in the chick and as such, differs from the thymus gland in function(15), no basis for this proposal was found in the transformability of the contained cells.

In certain cultures, an unequivocal increase in LMC and an occasional mitotic figure was observed without uptake of H^3-thymidine on radioautographs. This depression of H^3-thymidine incorporation has also been observed infrequently in this author's cultures of rat lymphoid cells. The LMC and occasional mitotic figures suggests that division and possibly DNA synthesis were occurring, although the latter has not been demonstrated. IF DNA synthesis was occurring, a possible explanation is that anabolites from the nucleic acids of dying cells had depressed the biosynthetic pathways of thymidine incorporation but not DNA synthesis itself. Alternately, DNA synthesis itself may have been depressed by anabolites with subsequent blockage of proliferation, a phenomenon known to occur in other mammalian culture systems(16).

While not reported here, a small number of the described cultures were pulsed with H^3-thymidine and a portion of the contained cells was assayed for total radioactivity by a Packard Tri-Carb Liquid Scintillator(17). In these instances, a larger amount of H^3 activity was noted in experimental incubations than was observed in controls. However, the differences were not as great, a result observed also by others(9). At the present time, no satisfactory explanation for this observation is available, although a partial block of H^3-thymidine incorporation in experimental cultures and not in controls may have played a role.

Summary. When human thymus, tonsil and appendix cells were exposed *in vitro* to PHA, PWM or allogeneic lymphoid cells for 2 to 3 days, blast cells were observed. Studies suggest that in mixed lymphoid cultures, the blast cells may have originated from both donors. Infrequently, in cultures containing transformed cells, an absence of H^3-thymidine incorporation was observed by radioautography.

The author wishes to thank Miss Nancy Edson for technical assistance and Drs. Earl Lasher, Edgar Swartz, Loren Winterscheid and Eugene Strandness for obtaining the specimens.

1. Rieke, W. O., Schwarz, M. R., Anat. Rec., 1964, v150, 383.

2. Weber, W. T., J. Cell Physiol., 1966, v67, 285.

3. Schwarz, M. R., in preparation.

4. ———, Immunology, 1966, v10, 281.

5. Chapman, N. D., Dutton, R. W., J. Exp. Med., 1965, v121, 85.

6. Oppenheim, J. J., personal communication.

7. Claman, H. N., Proc. Soc. Exp. Biol. and Med., 1966, v121, 236.

8. Winkelstein, A., Craddock, C. G., Blood, 1965, v24, 876.

9. Craddock, C. G., personal communication.

10. McIntyre, R. O., Segel, W. D., Lancet, 1966, I, 1265.

11. Stroselli, E., Maccani, U., Longo, A., Haemat. Arch., 1965, vL, 220.

12. Everett, N. B., Rieke, W. O., Reinhardt, W. O.,

Yoffey, J. M., in Ciba Symposium on Haempoiesis, J. & A. Churchill, Ltd., 1960, 43.

13. Schwarz, M. R., Rieke, W. O., Anat. Rec., 1966, v155, 493.

14. Oettgen, H. F., Silber, R., Niescher, P. A., Hirschhorn, K., Clin. & Exp. Immunol., 1966, v1, 77.

15. Good, R. A., Cooper, M. D., Peterson, R. D. A., Kellum, M. J., Sutherland, D. E. R., Gabrielsen, A. E., Ann. N. Y. Acad. Sci., 1966, v135, 451.

16. Cooper, R. A., Perry, S., Brietman, T. R., Cancer Res., 1966, v26, 2267.

17. Caffrey, R. W., Everett, N. B., Rieke, W. O., Anat. Rec., 1966, v155, 41.

Received February 10, 1967. P.S.E.B.M., 1967, v125.

A Sensitive Bioassay for Prolactin Based on H^3-Methyl-Thymidine Uptake by the Pigeon-Crop Mucous Epithelium. (32185)

M. BEN-DAVID (Introduced by W. E. Heston)

Laboratory of Biology, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Most of the many possible methods for prolactin detection(1-6) are of limited value because they are either semiquantitative, subjective, or highly insensitive. The pigeon-crop sac method is the most widely employed because of its simplicity and relative sensitivity. Nevertheless, it is still not completely objective and not sensitive enough to detect small quantities of prolactin. Many efforts were made in order to decrease its subjectivity and to increase its sensitivity and hence many modifications of the original method(7) were suggested, such as local administration of the prolactin over the crop gland rather than the original systemic application(8,9) or graphically measuring the area of the local crop-sac(10,11) or P^{32} uptake(12).

A considerable step to improve its objectivity was recently made by Nicoll who designed a special apparatus which permits removal of a standardized 4 cm diameter disc of the crop-sac mucosal epithelium from the local injection(13). However, the mentioned modifications still do not show significant reactions to doses as low as 1-10 mU of prolactin.

It has been shown that prolactin given either locally to the pigeon-crop or systemically, increases mitotic activity and hence induces proliferative activity of the crop sac (14,15). It has been shown also that the proliferation of the crop-sac is accompanied by increased synthesis of nucleic acids(16, 17). It is expected, therefore, that an increased uptake of labelled thymidine by the pigeon-crop should occur after prolactin administration, a matter which could be used as a new approach for prolactin assay with the pigeon crop-sac. An improvement of such a method could be achieved by a combination of three ways: 1) preventing of fast absorption of the hormone, a matter which could be achieved by dissolving the hormone in agar solution, making a gel to be injected subsequently intradermally over the crop-sac; 2) extracting only the reactive epithelial tissue of the crop-sac, and not the whole gland, for final estimation of the reaction; 3) exposing both sides of the reactive crop-sac tissue to the same amount and to an immediate supply of the radioactive material to be incorporated, *i.e.*, intravenous administration of the labelled thymidine.

A sensitive and objective method for prolactin assay that included these three procedures was developed.

Materials and methods. Fifty-four Silver King pigeons, 6-8 weeks old, were used for experimentation. The ovine prolactin used was the generous gift of the Endocrine Study Section, National Institutes of Health, batch #NIH-P-S-7, with mean potency of 24.3 IU/mg. For convenience, a potency of 24 IU/mg was employed in the calculations. The total amount of prolactin to be injected was dissolved in 0.4 ml of 0.5% agar, making a gel working solution. An injection of 0.1 ml of this solution was given intradermally upon the left side of the pigeon crop-sac and repeated three more times according to a schedule sum-