PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 156-159 (1977)

Functional Maturation of Mouse Thymus Cells (39626)

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Details of the functional development of each of the various lymphoid cells involved in immunological reactions is needed for understanding the events controlling the onset of immunological competence in an animal. It is only recently that immunologists have concentrated their efforts at establishing the ontogenic pattern of emergence of structural and functional parameters of immunocompetent lymphoid cell populations (1-7).

Regarding mouse thymus cell maturation, it has been shown that the maximal responsiveness of thymus cells to different mitogens arises at different stages of ontogeny (2, 8-10). Thymus-specific antigens can be detected on thymus cells beginning at Day 16 of development (11), i.e., as soon as there are detectable thymic small lymphocytes; on the other hand the ability of thymus cells to cause a graft-versus-host reaction arises several days later, around the time of birth (12, 13). Using an *in vivo* reconstitution technique, Chiscon and Golub (14) demonstrated thymus helper cell function in the immune response to sheep erythrocytes 24 hr prior to birth whereas Claman *et al.* (15) found such activity only 6 days after birth.

The present study was designed to determine the onset of the capacity of murine thymus cells to show helper function in the response to sheep erythrocytes (SRBC) using entirely *in vitro* methods. Thymus cells directly from thymus usually cannot provide the helper function *in vitro* unless activated with antigen in irradiated host (16) or mitogen *in vitro* (17). Treatment with mitogenic lectin concanavalin A (con A) was employed for our purpose. The study demonstrates that, as early as the sixteenth day of embryonic development, the thymus contains cells that can readily be activated *in vitro* to function as helper cells in the humoral immune response. The effect of con A on the expression of thymus cell surface antigens has also been reported in this paper.

Materials and methods. BDF_1 adult male mice and BDF_1 embryos (C57Bl/6 × DBA/2) from time pregnancies from our colony have been used in this study. Thymus cells, 3×10^6 per petri dish (No. 3001, Falcon Plastic, Calif.), were pretreated with 5 μg of concanavalin A per ml (3X crystallized; Miles Laboratories, Inc., Elkhart, Ind.) for 24 hr at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Minimum Essential Medium (Microbiological Associates, Bethesda, Md.), which had been supplemented with 10% heat-inactivated fetal bovine serum (Colorado Serum Co., Denver), glutamine, and antibiotics, was used for thymus cell culture. After two washes, 3×10^6 con A-treated or -nontreated thymus cells were combined with 5×10^6 bone marrow-derived B spleen cells. The B spleen cells were obtained from adult thymectomized mice X-irradiated with 850 R and reconstituted with syngeneic bone marrow. The combined cell suspension was cultured with about 1.2×10^7 SRBC (GIBCO, Madison, Wis.) as antigen for 4 days as described by Click and coworkers (18). Antibody-forming cells were assayed according to the method of Cunningham and Szenberg (19).

The relative difference in the expression of θ and H-2 antigens was measured by determining the amount of radioactivity released from Cr^{51}-labeled thymocytes exposed to anti-θ or anti-H-2 sera in the presence of complement; the dilution of anti-θ and anti-H-2 sera were such that the sera killed about 50% of adult thymus and bone marrow cells, respectively. The anti-θ serum was raised in AKR mice by repeated injections of thymus cells from C3H mice. To raise the anti-H-2 serum, CBA (H-2^k) mice were repeatedly injected with BDF_1 (H-

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ISSN 0037-9727

$2^{b,d}$) spleen cells. The percentage of cytotoxicity was calculated as:

$$\frac{\text{Mean counts per minute of experimental release} - \text{Mean counts per minute of spontaneous release}}{\text{Mean counts per minute of maximum release} - \text{Mean counts per minute of spontaneous release}} \times 100.$$

Spontaneous release was defined as the level of Cr^{51} released in the presence of normal mouse serum and complement. Maximum release was obtained by rapidly freeze–thawing the labeled cells three times.

Results. The pattern of *in vitro* collaboration between B spleen cells and con A-treated and -nontreated thymus cells from adult and newborn mice is presented in Fig. 1. Only con A-treated thymus cells provided the helper function *in vitro;* other cell types, such as bone marrow (BM) cells, could not provide this function even after treated with con A. Using this *in vitro* technique of collaboration, it has been shown that mouse thymus cells, as early as the sixteenth day of embryonic life, develop the functional potential to collaborate with B spleen cells in mounting an antibody response against SRBC (Table 1). However, this potentiality of thymocytes could only be revealed by triggering them with con A. With an increase in age, the collaborating ability of embryonic thymus cells increases. The re-

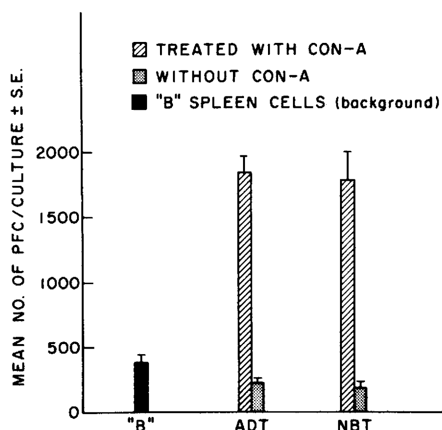

FIG. 1. *In vitro* collaboration between bone marrow-derived spleen (B) cells and con A-treated adult (ADT) or newborn (NBT) thymus cells, as measured by the number of plaque-forming cells (PFC) per culture. Each culture contains 5×10^6 B cells and 3×10^6 thymus or bone marrow (BM) cells with or without con A pretreatment for 24 hr, and each culture is immunized with about 1.2×10^7 SRBC. The PFC response of 5×10^6 B cells is the background response. Five experiments were performed with adult and newborn thymus cells and three experiments with bone marrow cells, each experiment consisting of five replicate cultures in each category; B-cell controls were maintained with each experiment.

TABLE I. ABILITY OF THYMOCYTES FROM DIFFERENT STAGES OF ONTOGENY TO COLLABORATE WITH ADULT B SPLEEN CELLS *in Vitro*.[a]

Types of T cells added to B spleen cells	Mean PFC/culture ± SE (Number of culture)		
	Without Con A	With Con A	P^b
Adult	612 ± 52 (15)	2735 ± 446 (14)	<0.01
Newborn	520 ± 133 (8)	1644 ± 354[c] (10)	<0.05
18-Day embryonic	96 ± 32 (13)	2466 ± 466 (15)	<0.01
17-Day embryonic	117 ± 34 (20)	1726 ± 212 (19)	<0.01
16-Day embryonic	170 ± 54 (8)	1087 ± 137 (14)	<0.01
15-Day embryonic	167 ± 88 (5)	584 ± 300 (5)	>0.01
B spleen cells (background)	(9) 703 ± 72	(14)	

sponse of 18-day-old embryonic thymus cells was almost identical with that of adult thymus cells.

After con A treatment of thymus cells, the percentage of cytotoxicity with anti-θ serum decreased and a corresponding increase in the percentage of cytotoxicity with anti-H-2 serum was observed; in contrast to its effect on thymus cells, con A caused no detectable change in surface antigens of bone marrow cells (Fig. 2).

Discussion. The present investigation demonstrates that thymocytes obtained from mouse embryos can exert demonstrable helper cell activity in an *in vitro* antibody response when they are activated by 24 hr of incubation with con A; indeed, as soon as small lymphocytes appear in the thymus, around the sixteenth day of gestation, they appear to have the potential for this immunological activity. The collaborative ability of embryonic thymocytes increased rapidly, reaching adult levels before birth. This increase appears correlated with the relative proportion of small lymphocytes found in the developing thymus (20) which also correlates with the relative efficiency with which con A and phytohemagglutinin induce thymidine incorporation by embryonic thymus cells (10).

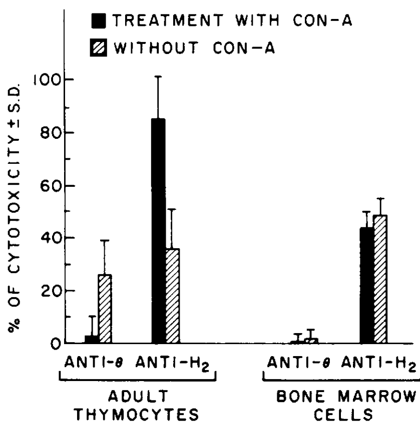

FIG. 2. Effect of con A on θ and H-2 antigen expression on adult thymocytes and bone marrow cells, as measured in terms of the susceptibility of con A-treated and -nontreated cells to the specific antiserum. This figure is representative of one experiment consisting of triplicate values per point. Similar results were obtained in four other experiments using the same type of antisera and in three experiments using antisera raised in congenic mice (latter experiments will be reported somewhere else).

There may be several reasons for our ability to demonstrate immunocompetence of thymus cells during embryonic life. An *in vitro* test system, like ours, in contrast to the studies cited above (14, 15), does not encounter some of the difficulties of the *in vivo* reconstitution technique, such as the problem of the seeding efficiency of injected cells or the possibility of the functional potency of the cells being masked *in vivo* by one or more host factors.

In the present investigation, thymus was used instead of spleen as the source of thymocytes. Thus, our results are not in contrast with those of Spear and Edelman (5) who have shown that con A-responsive T cells and T-cell helper function could not be detected in the spleen cells from 1-day-old mice.

That the nature of con A activation is meaningful in terms of the development of immunocompetence of thymus cells is suggested by several ancillary findings. (i) Con A bound to bone marrow cells rather than to thymus cells cannot substitute for thymus cells in an antibody response (see Fig. 1). (ii) Con A bound to thymus cells does not immediately activate those cells; rather the activation occurs over a 24-hr period (17) (unpublished observation). (iii) It is considered that con A-mediated stimulation of T lymphocytes closely resembles lymphocyte activation by specific antigen in morphologic as well as in certain functional aspects (21). (iv) The shift in the percentage of cytotoxicity with a specific antiserum (see Fig. 2) may reflect on the different degree of expression of the specific antigen on the con A-treated T-cell surface. The possibility of the selection of T cells with a lower amount of θ and a higher amount of H-2 by cell death seems unlikely. Even if con A were to select the variety of cortisone resistant functional thymocytes having a lower amount of θ and a higher amount of H-2, these normally constitute only about 5% of the total thymocytes in a thymus, and, hence, do not account for 30% to more than 60% recovery of thymus cells in different experiments after 24 hr of con A treatment (unpublished observation). It is then possible that incuba-

tion with con A caused a decrease in the expression of θ antigen and a corresponding increase in the expression of H-2 antigen. This type of shift in surface antigens (high to low θ; low to high H-2) is known to occur during the final steps of thymus cell maturation (22). (v) As soon as the embryonic thymus cells can be activated for helper function *in vitro* (see Table 1), the con A-induced shift in the expression of these cell surface antigens can also be detected (to be published).

As helper function of thymus cells treated with con A is the main concern of this study, we are not going into a detailed discussion of con A-induced inhibitory T cells.

The early detection of mouse thymus cell helper function in an antibody response tends to argue against a requirement for an extended period in ontogeny during which a somatic process of generation of immunological diversity takes place. On the other hand, the results presented in this paper possibly provide some support for the discovery that antigen-binding cells can be detected early in the development of mouse thymus (3). However, as there are evidences of cross-specificities of T-cel humoral factor, only further experimentation showing the antigen specificity of con A-treated helper T cells will provide clarity in the argument against somatic process of generation of immunological diversity.

Taken together with the finding of early differentiation of cells from the embryonic liver and yolk sac to perform various functions associated with cell mediated immunity (23, 24), it seems clear that we can no longer think of young mammalian embryos as immunologically inert.

Summary. Ontogeny of thymus cell function in humoral antibody response was studied *in vitro* using cells from different embryonic stages and neonates of BDF_1 mice. As early as the sixteenth day of embryonic development, the thymus contained cells that were readily activated *in vitro* by con A to function as helper cells in an antibody response against sheep erythrocytes. The helper function of embryonic thymocytes increased rapidly, reaching adult levels before birth. It has also been shown that con A-treated thymus cells are less susceptible to anti-θ serum and correspondingly more susceptible to anti-H-2 serum; the implication of this finding in thymus cell maturation has been discussed.

The author is grateful to Professor Robert Auerbach for his encouragement and criticisms during the course of this work. This study was supported in part by Research Grant CA 13548 from the National Institute of Health.

1. Nossal, G. J. V., and Pike, B. L., Advan. Exp. Med. Biol. **29,** 11 (1972).
2. Stobo, J. D., and Paul, W. E., Cell. Immunol. **4,** 367 (1972).
3. Decker, J. M., Clarke, J., Bradley, L. M., Miller, A., and Sercarz, E. E., J. Immunol. **113,** 1823 (1974).
4. Gelfand, M. C., Elfenbein, G. J., Frank, M. M., and Paul, W. E., J. Exp. Med. **139,** 1125 (1974).
5. Spear, P. G., and Edelman, G. M., J. Exp. Med. **139,** 249 (1974).
6. Stites, D. P., Carr, M. C., and Fudenberg, H. H., Cell. Immunol. **11,** 257 (1974).
7. Chakravarty, A., Kubai, L., Sidky, Y., and Auerbach, R., Ann. N. Y. Acad. Sci. **249,** 34 (1975).
8. Howe, M. L., and Manziello, B., J. Immunol. **109,** 534 (1972).
9. Byrd, W. J., von Boehmer, H., and Rouse, B. T., Cell. Immunol. **6,** 12 (1973).
10. Mosier, D. E., J. Immunol. **112,** 305 (1974).
11. Owen, J. J. T., and Raff, M. C., J. Exp. Med. **132,** 1216 (1970).
12. Cohen, M. W., Thorbecke, G. J., Hochwald, G. M., and Jacobson, E. B., Proc. Soc. Exp. Biol. Med. **114,** 242 (1963).
13. Ritter, M. A., Transplantation **12,** 279 (1971).
14. Chiscon, M. O., and Golub, E. S., J. Immunol. **108,** 1379 (1972).
15. Claman, H. N., Chaperon, E. A., and Triplett, R. F., J. Immunol. **97,** 828 (1966).
16. Hartmann, K.-U., J. Exp. Med. **132,** 1267 (1970).
17. Sjöberg, O., Möller, G., and Andersson, J. Clin. Exp. Immunol. **13,** 213 (1973).
18. Click, R. E., Benck, L., and Alter, B. J., Cell. Immunol. **3,** 264 (1972).
19. Cunningham, J. A., and Szenberg, A., Immunology **14,** 599 (1968).
20. Ball, W. D., Exp. Cell Res. **31,** 82 (1963).
21. Clark, W. R., Cell. Immunol. **17,** 505 (1975).
22. Raff, M. C., Nature New Biol. **229,** 182 (1971).
23. Tyan, M. L., and Herzenberg, L. A., J. Immunol. **101,** 446 (1963).
24. Hofman, F., and Globerson, A., Eur. J. Immunol. **3,** 179 (1973).

Received May 19, 1976. P.S.E.B.M. 1977, Vol. 154.