

DEVELOPMENT OF CYTOLYTIC T LYMPHOCYTE
PRECURSORS IN ORGAN-CULTURED MOUSE
EMBRYONIC THYMUS RUDIMENTS*

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Various lines of evidence have established the importance of the thymus in the development of the T cell compartment (1-3). The developmental pathways by which the different T cell subpopulations arise, and the relative importance of intra- and extra-thymic differentiation are, however, less clear. A number of the functional activities of thymic lymphoid cells including mitogen and mixed leukocyte culture (MLC) reactivity, as well as graft vs. host and helper activity, have been reported to arise in the developing mouse thymus around the time of birth (4, 5). Similarly, the immediate precursors of alloreactive cytolytic T lymphocytes (CTL-P), which can be demonstrated in the adult thymus (6), first become detectable in the perinatal period when they increase in frequency and number as measured in limiting dilution assays (7-9). CTL-P appear later in the spleen than in the thymus, thus suggesting that they arise in the thymus and migrate to the periphery (7, 8). Conclusive evidence that the thymus provides a sufficient environment for the differentiation of these cells is, however, still lacking.

Studies using organ culture of isolated embryonic thymus rudiments have already been used to show that maturation of mitogen and MLC reactivity can proceed entirely within the thymic environment (10, 11). In this report, we show that a sensitive limiting dilution assay (12) can be used to demonstrate the generation of CTL-P in organ cultures of isolated 14-d mouse embryo thymus. Moreover, in this closed system, CTL-P accumulate so as to reach frequencies that are as high, if not higher, than those found in adult thymus. These observations open the way to studies on the functional maturation of T cells and the acquisition of tolerance to MHC antigens in chimeric thymuses constructed entirely in vitro.

Materials and Methods

Mice. Adult female mice of the inbred strains C57BL/10 (B10) and BALB/c were obtained from the animal colony maintained at the University of Birmingham Medical School. Embryonic material was obtained from timed matings. The day of detection of a vaginal plug was designated day 0.

Cell Suspensions. Normal thymus and spleen cell suspensions were prepared in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 10 mM Hepes buffer. Normal thymuses were removed from a minimum of two 4-6-wk-old C57BL/10 mice, taking care to avoid removal of parathymic lymph nodes. Normal spleens were removed from 10-12-wk-old BALB/c mice. Fetal, neonatal, and organ-cultured fetal thymuses were teased

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into phosphate-buffered saline containing 5% FBS, cell clumps removed, and the cell suspension washed and resuspended in DMEM supplemented as above.

Organ Cultures. Thymic lobes were removed from 14-d-old B10 fetuses and cultured on the surface of 0.8 μm nucleopore filters supported on gelatin sponge rafts as described previously (13). At various intervals after the initiation of organ culture, thymuses were used as responder cells in microcultures as described below (the age of the organ-cultured thymuses described is in addition to its original gestational age).

Mixed Leukocyte Microcultures (Micro-MLC). Micro-MLC were prepared as detailed elsewhere (12) in DMEM supplemented with additional amino acids, 10 mM Hepes, 5×10^{-5} 2-mercaptoethanol, 10% (vol/vol) FBS, and 10% (vol/vol) supernatant from secondary MLC (2° MLC SN) (14). Limiting numbers of responder cells were cultured (24–32 microcultures per cell dose) with 10^6 irradiated (1,000 rad from an Andrex portable x-ray machine) spleen cells from BALB/c mice in a final volume of 200 μl in round-bottomed microtiter plates (Greiner, Nürtingen, Federal Republic of Germany). Responder cells were thymus cells from organ-cultured fetal thymuses or from fetal or neonatal thymuses removed directly from the animal. In all experiments normal B10 thymocytes were included as a positive control. Microplates were maintained for 7 d at 37°C in plastic boxes containing a water-saturated 5% CO_2 in air atmosphere.

Assay for Cytolytic Activity. Two assay procedures were used. For normal thymocytes, the assay was done directly by removing 100 μl of culture supernatant from 7-d cultures and adding 100 μl of DMEM 5% FBS containing 5×10^3 ^{51}Cr -labeled P815 target cells. For all other thymus-responder cells, two aliquots of 80 μl of each microculture were transferred to separate V-bottomed microtiter plates (Greiner), and ^{51}Cr -labeled target cells added to give a final volume of 200 μl . One half microculture was assayed directly, the other in the presence of phytohemagglutinin. Because all cultures showing direct cytotoxicity also showed PHA-dependent killing, only the direct cytotoxicity results will be presented in this report.

After a 4-h incubation at 37°C in a 5% CO_2 atmosphere, 100 μl of supernatant from each well was removed and counted in a γ -counter. Spontaneous release from 5×10^3 ^{51}Cr -labeled target cells was determined by incubating labeled cells in wells containing irradiated BALB/c cells alone. Maximum release was determined by lysing labeled target cells in 0.1 N HCl. Cytolytically positive microcultures were defined as those with ^{51}Cr release values exceeding the mean spontaneous release by >3 SD (the minimum positive ^{51}Cr release value). Minimal estimates of CTL-P frequencies were calculated from the Poisson distribution by the statistical method of log likelihood maximization as described in detail by Taswell (15).

Results and Discussion

Previous studies have established that thymic CTL-P do not develop until shortly after birth in CBA (8) or C57BL/6 (9) mice. To test the possibility that functional CTL-P could develop in organ-cultured fetal thymuses, three separate experiments were carried out using such responder cells in micro-MLC. In each experiment, responder cells from one or several groups of organ-cultured C57BL/10 thymuses (established at 14 d of gestation) were stimulated in micro-MLC with irradiated H-2 allogeneic (BALB/c) spleen cells in the presence of an optimal concentration of 2° MLC SN as a source of interleukin 2. For comparison, fetal and/or neonatal thymocytes, as well as adult (4–6-wk-old) thymocytes were also used as a source of responding cells. Fig. 1 shows the cytotoxicity data from one experiment in which groups of 24 microcultures were established with various doses of normal adult, 19-d fetal, or 10-d organ-cultured thymuses. It can be seen that at a dose of 10,000 responder cells per well, 24 of 24 cultures containing organ-cultured thymus, 20 of 24 adult, and 1 of 24 fetal thymuses show positive cytotoxicity. The cytotoxicity data shown in Fig. 1 are replotted in Fig. 2 as the proportion of negative cultures on a logarithmic scale vs. the dose of responder cells on a linear scale. When plotted in this way, the anti-H-2^d CTL-P frequency in each population was determined to be

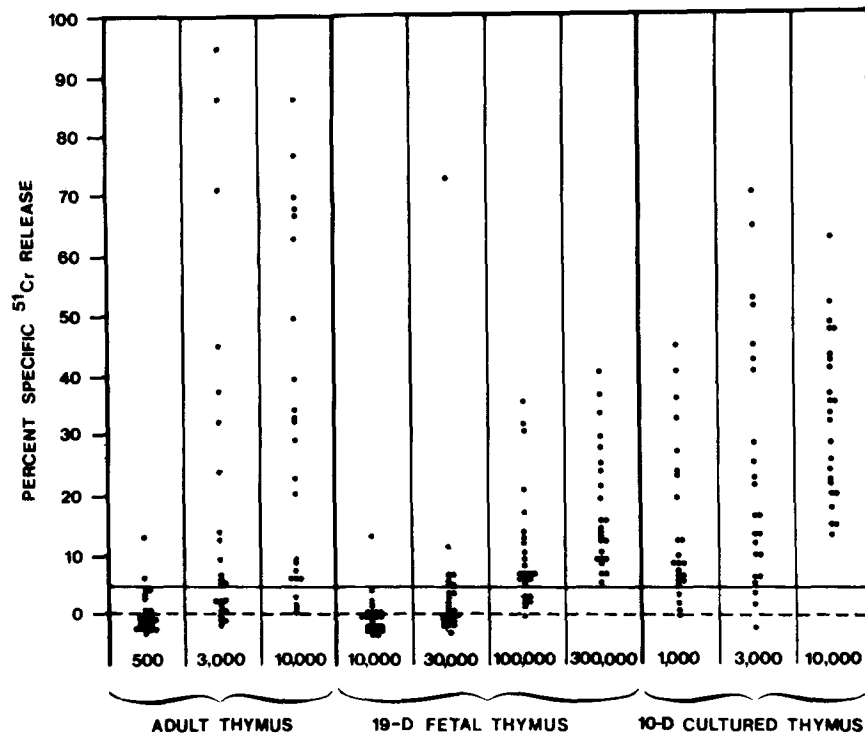


FIG. 1. Cytolytic activity of individual micro-MC using either normal adult, 19-d fetal, or 10-d organ-cultured fetal C57BL/10 thymuses. The indicated number of responder cells were cultured with 10^6 irradiated BALB/c spleen cells and 2° MLC SN. After 7 d the microcultures were assayed for cytolytic activity to ^{51}Cr -labeled P815 target cells. Each point represents an individual microculture. (—) Represents the mean ^{51}Cr release in spontaneous cultures (---) plus 3SD and defines the minimum positive ^{51}Cr release value.

1/1,057 for 10-d organ-cultured fetal thymus, 1/4,889 for normal adult thymus, and 1/80,100 for 19-d fetal thymus.

The results of all experiments using organ-cultured, fetal, and neonatal thymuses are summarized in Table I. Fetal thymuses from B10 mice, removed at day 14 of gestation and organ-cultured for 7, 10, 14, or 21 d contained anti-H-2^d CTL-P at a frequency similar to, or higher than, that found in adult thymuses. In contrast, no CTL-P were detectable in the fetal thymus until at least 19 d of gestation, as observed previously for two other mouse strains (8, 9).

At day 14 of gestation the thymic rudiment contains a population of blast-like lymphoid cells that are relatively undifferentiated in terms of morphology. These cells lack demonstrable functional characteristics and are only beginning to acquire the antigenic markers associated with adult thymocyte populations (4, 5). The absence of CTL-P in such rudiments when examined at day 14 (data not shown), and their subsequent appearance in increasing frequency after a period in a closed organ culture system, provides direct evidence that alloreactive CTL-P can develop from the blast cells present at the time of explantation. The possibility that the appearance of CTL-P in these organ cultures simply reflects the selective survival of rare cells present at the outset of the culture is rendered unlikely by the fact that there is considerable proliferation (~10-fold) of the lymphoid elements during the first 7 d of culture (10).

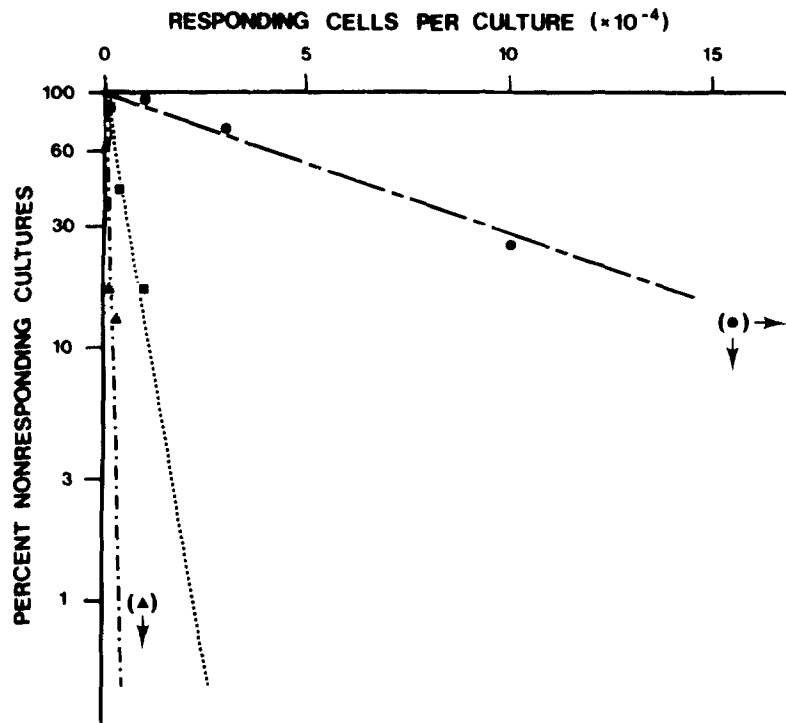


FIG. 2. CTL-P frequencies (f) in organ-culture fetal thymus. Cytotoxicity data from Fig. 1 are plotted as the proportion of nonresponding cultures, on a logarithmic scale, versus the dose of responder cells on a linear scale. ■, Normal adult thymus ($f = 1/4,889$); ●, 19-d fetal thymus ($f = 1/80,100$); ▲, 10-d organ-cultured fetal thymus ($f = 1/1,057$). CTL-P frequencies were calculated by the method of maximum log likelihood (15).

TABLE I
Frequency of Anti-H-2^d CTL-P in C57BL/10 Thymus*

Responders	CTL-P frequency	CTL-P/ 10^6 cells	Cells per thymus ($\times 10^{-6}$)	CTL-P per thymus
Adult 1	1/4,889	205	210	43,050
Adult 2	1/2,033	492	200	98,400
Adult 3	1/9,000	111	287	31,857
7-d culture‡	1/4,136	242	0.45	109
10-d culture	1/1,057	946	0.11	104
14-d culture	1/2,967	337	0.15	51
21-d culture	1/2,272	440	0.19	84
18-d fetal	1/677,170	1.5	3.6	5
19-d fetal	1/80,100	13	3.1	40
1-d neonatal	1/10,479	95	8.0	760

* The percent noncytolytic cultures at each of a minimum of three cell doses was used to compute the CTL-P frequency, as described in Materials and Methods.

‡ Thymuses removed at 14 d gestational age.

Thereafter the cell yield decreases (10, and Table I) and there also appears to be a slight decline in the absolute number of CTL-P. This may reflect a limited proliferative capacity by the stem cells in the 14 day rudiment that cannot be replenished by further inflow, although the involvement of a decline in culture conditions cannot be ruled out.

The emergence of alloreactive CTL-P is in line with other studies showing development of functional responses by thymocytes in organ-cultured rudiments, and with the ability of these cultures to support T cell differentiation as indicated by the appearance of various antigenic markers (4, 5). In this context we have recently found that cells of different Lyt phenotypes accumulate in embryonic thymus organ cultures (Van Ewijk et al. Manuscript in preparation). The system, therefore, may prove useful in correlating surface phenotype and function in the developing thymus.

Further studies are now required to determine whether CTL-P reactive to other (e.g., viral) antigens also appear in the organ culture system and, if so, whether they show MHC restriction. Our recent findings have suggested that it is possible to produce chimeric thymuses that will continue to develop in vitro by recombining embryonic thymus stroma and embryonic lymphoid blast cells of different haplotypes. The feasibility of using sensitive microassays to investigate the functional properties of cells developing in thymus organ cultures, as demonstrated in this study, suggests that this may provide an accessible system in which to investigate interactions between developing cells and the thymic environment, with particular reference to MHC-related phenomena.

Summary

The appearance of immunologically competent cells in the organ-cultured mouse fetal thymic rudiment has been investigated. Fetal thymuses removed at 14 d of gestation and cultured for 7–21 d were assayed for their content of cytolytic T lymphocyte precursors (CTL-P) directed against H-2^d alloantigens. Whereas CTL-P were undetectable within fetal thymus until 18–19 d of gestation, their frequency in the organ-cultured fetal thymus was similar to, or greater than, that found in the normal adult thymus. This direct demonstration of the appearance of alloreactive CTL-P in a closed in vitro system should provide an accessible model for the investigation of interactions between developing T cells and the thymic microenvironment.

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