

ONTOGENY OF CELL-MEDIATED IMMUNITY

I. Early Development of Alloantigen-Specific
Cytotoxic T-Cell Precursors in Postnatal Mice*

By LINDA M. PILARSKI

(From the Department of Immunology, Medical Sciences Building, University of Alberta,
Edmonton, Alberta)

Although the development of B-cell precursors in neonatal mice has been well charted (1-3), no similar studies exist with respect to the ontogenic development of cytotoxic T-cell precursors. Mosier has shown that both responsiveness to T-cell mitogens (phytohemagglutinin, Concanavalin A) and the ability of cells to proliferate in mixed lymphocyte reactions appear at any early stage in ontogeny, but no functional studies were done (4). The development in this laboratory of a very efficient method for generating cytotoxic T cells (5) has allowed culture of neonatal spleen cells with allogeneic, irradiated stimulator cells yielding a detectable cytotoxic T-cell response as early as 2-3 days after birth. Thus, this represents the first demonstration of cell-mediated immunity at such an early stage of development. Since other studies (6) indicated an absolute requirement for antigen-specific helper T cells in the generation of a cytotoxic T-cell response, neonatal spleen cells were cultured with and without extra helper T cells (6). Addition of helper cells did not facilitate cytotoxic T-cell generation in neonates.

Materials and Methods

Mice. CBA/CaJ neonates and BALB/cCr neonates were obtained through the cooperation of Dr. J. Chlumecky of the University of Alberta Animal Breeding Establishment. Adult BALB/cCr and CBA/CaJ were from the same source and were used at 12-14 wk of age. (BALB/cJ × C57BL/6)F₁ mice were from The Jackson Laboratory, Bar Harbor, Maine and were used at 7-10 wk of age. A total of 194 postnatal mice were used in these studies.

Newborn mice – a total of 9 litters (46 mice) were tested in four separate experiments.
2-3 days – 12 litters (60 mice) tested in four experiments.
4-5 days – 8 litters (40 mice) tested in four experiments.
6 days – 4 litters (20 mice) tested in two experiments.
8, 9, and 12 days – 2 litters each, tested in two experiments.

The majority of experiments included mice of several age groups and all experiments included adult positive controls.

Culture System. 1-10 × 10⁶ neonatal spleen cells were cultured in Marbrook acrylamide tissue culture rafts with 8-16 × 10⁶ irradiated allogeneic stimulator cells (6). Assay was as previously described (6). Number of lymphocytes needed to kill one target cell was calculated as follows:

$$\text{Number of lymphocytes per same} \div \frac{\text{sample cpm} - \text{spontaneous cpm}}{\text{cpm released/target cell}} = N$$

This is expressed in Fig. 3 as the reciprocal of N. This is necessary to allow comparison of experiments in which varying numbers of responder cells were cultured.

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TABLE I
Representative Experiment Showing Cytotoxicity by Neonatal Spleen Cells

Day after birth		⁵¹ Cr cpm released by lymphocytes × 10 ⁻⁵ (% specific lysis)		Viable cells per culture
		20	6.7	
Day 2	a	738 ± 15 (7.5%)	557 ± 21	7.5 × 10 ⁶
	b	451 ± 10 (-)	447 ± 23	20 × 10 ⁶
	c	468 ± 24 (-)	457 ± 11	12 × 10 ⁶
	d	455 ± 8 (-)	453 ± 11	7.5 × 10 ⁶
Day 3	a	563 ± 33 (4%)	530 ± 17	33 × 10 ⁶
	b	682 ± 2 (6.4%)	522 ± 64	23 × 10 ⁶
	c	647 ± 39 (5.7%)	596 ± 22	23 × 10 ⁶
	d	772 ± 39 (8.1%)	662 ± 13	21 × 10 ⁶
Day 4	a	2,332 ± 97 (38.6%)	1,368 ± 12	31 × 10 ⁶
	b	2,244 ± 19 (36.8%)	1,356 ± 24	18 × 10 ⁶
	c	2,128 ± 11 (34.6%)	1,187 ± 7	12 × 10 ⁶
	d	2,667 ± 64 (45.1%)	1,616 ± 31	20 × 10 ⁶
Irradiated cells		387 ± 22		
		2.0	0.7	0.3
Adult		3,850 ± 159 (68%)	2,967 ± 58 (50%)	2,096 ± 66 (34%)

1 × 10⁷ neonatal BALB/c spleen cells, or 2 × 10⁶ adult BALB/c spleen cells, were cultured with 16 × 10⁶ (BALB/c × C57BL/6)F₁ irradiated spleen cells as stimulators. The anti-C57BL/6 response was measured on 10⁶ ⁵¹Cr-labeled EL4 target cells at day 5 of culture. a-d represent individual cultures.

Detergent release, 5,127 ± 192 cpm.

Spontaneous lysis, 355 ± 17 cpm.

Machine background of 97 ± 10 has not been subtracted.

Results

The CBA anti-BALB/c and BALB/c anti-C57Bl/6 cytotoxic responses were measured as a function of time after birth. Table I illustrates a representative experiment showing that the competence of spleen cells to generate cell-mediated cytotoxicity in vitro has begun to develop by 3 days after birth and reached substantial levels by 4 days after birth. Peak cytotoxic activity was measured after a culture period of 5 days. Table I also shows the high level of cytotoxicity generated in adult spleen cells which is detected at lymphocyte to target ratios of 0.3:1-2:1. To obtain comparable levels of cytotoxicity from 4-day-old spleen, a ratio of 6.7:1 to 20:1 was required. Cytotoxicity by spleen cells from postnatal mice was eliminated by treatment with anti-theta serum plus complement, and was found to be specific (data not shown).

Newborn mice yielded no splenic precursors of cytotoxic cells even when 10⁷ cells were cultured (equivalent to three newborn spleens per culture) (Fig. 1). At 2 days of age very few precursors were present in 10⁷ cells, but by day 3 significant and reproducible cytotoxicity could be detected in both strains (10⁷ cells was equivalent to two spleens per culture). When the two strains of mice were compared, it was apparent that spleen cells from 4-day-old BALB/c mice yielded a cytotoxic response that was not paralleled in CBA mice, which required 8-9 days of development before comparable cytotoxicity levels were achieved. In both strains, 12-day-old spleen cells contained many precursors, with cytotoxicity at approximately 23-32% of adult levels.

The apparent lack of cytotoxic T-cell precursors in the period from birth to 2 days of age could reflect a lack of cooperating cells rather than an absence of precursors. This was tested by culturing neonatal spleen cells with or without irradiated adult normal spleen cells which have been shown to provide helper

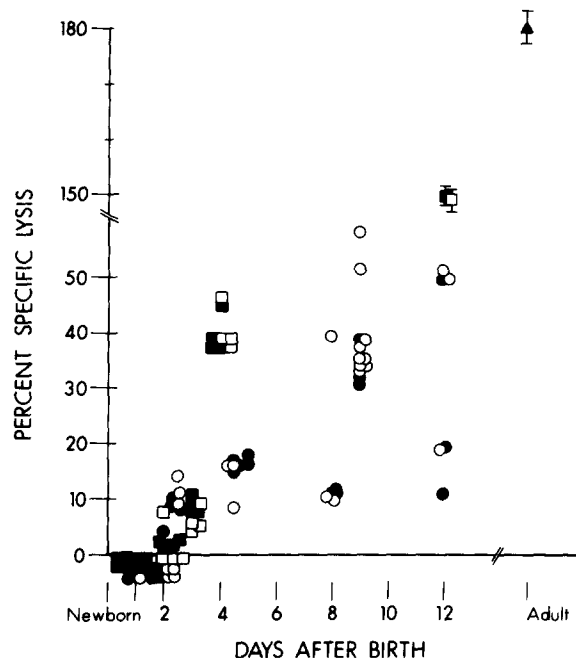


FIG. 1. The response of neonatal spleen cells to alloantigens when 10^7 cells are cultured. \square , BALB/cCr neonatal spleen cells responding to 16×10^6 irradiated (BALB/CJ \times C57BL/6) F_1 spleen cells. \blacksquare , BALB/cCr neonatal spleen cells responding to 8×10^6 irradiated (BALB/CJ \times C57BL/6) F_1 spleen cells plus 8×10^6 irradiated BALB/cCr spleen cells (helpers). \circ , CBA/CaJ neonatal spleen cells responding to 16×10^6 irradiated BALB/cCr spleen cells. \bullet , CBA/CaJ neonatal spleen cells responding to 8×10^6 irradiated BALB/cCr spleen cells plus 8×10^6 irradiated CBA/CaJ spleen cells (helpers). \blacktriangle , 1×10^6 adult BALB/cCr spleen cells responding irradiated (BALB/CJ \times C57BL/6) F_1 spleen cells plus or minus irradiated BALB/cCr spleen cells. Assay was at day 5. Each point represents the percent specific ^{51}Cr release by $1/5$ of a culture, except points with error bars which represent the mean \pm one standard deviation. Values above 100% were derived by extrapolation from the cytotoxicity obtained at various dilutions of the culture, to the amount of cytotoxicity which would be expected from $1/5$ of a culture.

function (6). Figs. 1 and 2 illustrate the effect of additional helper cells on the degree of cytotoxicity generated from young spleen cells. Even in the presence of helper cells, no cytotoxic T-cell precursors were detected in newborn or 2-day-old mice, while spleen cells from 3 or 4-day-old mice yielded the same degree of cytotoxicity with or without extra helper cells. At 12 days, however, increased levels of cytotoxicity were detected in cultures containing irradiated syngeneic helper spleen cells. Since irradiated spleen cells are a rich source of adherent/accessory cell activity (A cell) (7), the addition of such cells also indicates a lack of A-cell function is not a limiting factor in these cultures.

An estimate of precursor frequency was made by culturing spleen cells at several different cell concentrations. Fig. 2 illustrates the cytotoxicity generated by the progeny of a low concentration of spleen cells ($1-2 \times 10^6$ cells/culture which is equivalent to $1/3-1/37$ of a spleen depending on the age of the donor). There were virtually no precursors present in 10^6 cells at day 2, 3, and 4 after birth for CBA mice. BALB/c mice yielded significant cytotoxicity from 10^6 cells

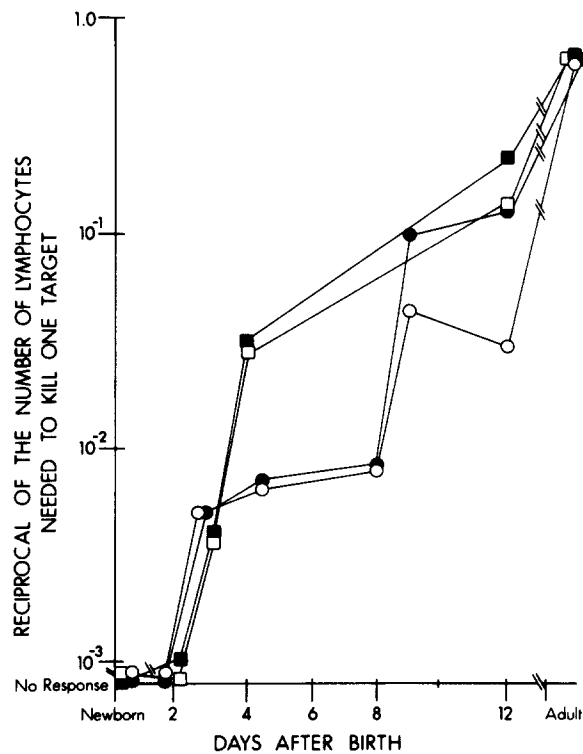


FIG. 3. Normalized cytotoxic responses of neonatal spleen cells to alloantigens as a function of the time after birth. Symbols are as described in Fig. 1. The number of lymphocytes needed to kill one target was calculated for each dose of lymphocytes cultured and the lowest value obtained is indicated on the graph. A series three to four cell doses of from 0.5 to 10×10^6 cells per culture were tested depending on the age of spleen cells being analyzed. All doses were cultured with and without extra helper cells.

pressive; i.e. there are precursors present but they are inhibited from responding. Experiments are in progress to determine which of these possibilities is correct.

These experiments conclusively demonstrate firstly, that cell-mediated immune responses exhibit the same pattern of ontogenic development as do humoral responses. Secondly, the lack of a cell-mediated response in cells from mice at birth to 2 days of age is not the result of limiting helper or accessory cell function. And, lastly, there is an apparent difference in the rate at which cytotoxic T cells develop in different strains of mice.

Summary

Cytotoxic T-cell precursors have been shown to occur in spleens of 2-3-day-old mice. By 12 days after birth, the cytotoxic T-cell response of spleen cells to alloantigens has reached 23-32% of adult levels. Addition of extra T-helper cells did not permit cytotoxic T-cell development in spleen cells from newborn to 2-day-old mice suggesting either a lack of precursors or suppression of precursors. The ontogeny of cell-mediated immune functions has thus been shown to corre-

late well with other work on the development of humoral immunity, accessory cells, and graft versus host reactivity.

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