Brief Definitive Report

CELL-MEDIATED IMMUNITY: DIFFERENTIAL MATURATION OF MIXED LEUKOCYTE REACTION AND CELL-MEDIATED LYMPHOLYSIS*

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Two in vitro assays of cell-mediated immunity, mixed leukocyte culture (MLC) and cell-mediated lympholysis (CML) are frequently used as models of the afferent recognition and efferent destruction phases of allograft rejection, respectively (1, 2). Together these two tests serve as powerful tools in delineating the cellular and genetic elements involved in histocompatibility and graft rejection (3, 4).

Several studies have shown that cell-mediated immunity in the mouse may arise quite early in development. For example, neonatal thymi of various mouse strains can induce a graft-vs.-host reaction (GvHR) in vivo (5) or in vitro (6), while MLC and mitogen responses can be demonstrated for thymus cells of embryonic mice (7). Embryonic liver and yolk sac cells, moreover, appear competent to initiate a GvHR even at the earliest stages of development (day 12 and day 9 embryos, respectively) (8, 9). For the spleen, significant variation exists among different reports on the timing of onset of cell-mediated immunocompetence (reference 10; and R. Auerbach and L. Kubai, unpublished observations; cf.5). At one extreme, Adler et al. (10) reported that spleen cells from C57BL/6 mice less than 2–3 wk of age fail to respond to allogeneic stimulation in one-way MLC, while at the same time Auerbach and Kubai (unpublished observation) found that 1-day old spleen cells, also from C57BL/6 animals, could initiate a GvHR in vitro.

The present report deals with the ontogeny of the spleen as measured by the ability of spleen cells to respond in one-way MLC and their ability to lyse both specific and third-party (partially cross-reactive) target cells in CML.

Materials and Methods

For ontogeny studies male and female mice from newborn to 1 mo of age were used; in experiments involving older animals only male mice were employed. C57BL/10J (B10), B10.D2, and B10.BR were obtained primarily from Jackson Laboratories, Bar Harbor, Maine although some animals from our own colony were used.

MLC tests were carried out using the micromethod of Alter et al. (4). The cultures contained 1×10^6 responding and 1×10^6 X-irradiated (190 R/min for 10 min) stimulating cells in a total of 0.2 ml RPMI 1640 medium containing 5% heat-inactivated human plasma. Cultures were labeled with tritiated thymidine (2 μCi/well) for 12 h after 90–92 h of incubation, with assays carried out on five replicate cultures.

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To generate cytotoxic effector cells, 25–70 × 10^6 responding cells were mixed with a similar number of X-irradiated cells for 4–5 days. Phytohemagglutinin (PHA)-stimulated lymph node cells were labeled with radioactive sodium chromate (^{51}Cr) and used as target cells in CML with 100 effector cells per target cell. The percent CML was calculated as previously reported (4). Assays were carried out on triplicate cultures. Experiments for different aged responding mice were done at different times, but each experiment was repeated two to three times with similar results. For mice beyond 16 days of age, effector cells were harvested after 96 h of MLC incubation and then mixed with target cells for 3 h; for animals of 16 days and younger, initial incubation time was 115–120 h and the CML assay time was 4.5 h to obtain the strongest possible response.

The stimulation ratio (SR) was calculated as the ratio of counts per minute in the allogeneic mixture to counts per minute in the syngeneic control mixture. *t* tests were performed on the log converted counts per minute of the allogeneic mixture compared with the log counts per minute in the isogeneic control mixture, with the significance of stimulation then expressed as a *P* value.

Results and Discussion

The experiments (Table I) show that spleen cells from neonatal B10 mice are capable of responding to allogeneic stimulation in MLC. While there is a general trend toward higher stimulation ratios in MLC with increasing age it is important to note that significant MLC reactivity is present as soon as the mouse is born (day 0). In contrast, CML activity as measured by killing of specific target cells (B10.D2) was not seen until day 7. Moreover, the cross-killing which is seen on third-party cells from B10.BR mice after sensitization with B10.D2 is not significant until day 27. Thus in the ontogeny of these reactions, spleen cells respond by proliferation in MLC before they are able to mediate CML, and even after CML potential is developed against specific target cells there is an interval in development where such specific killing activity is distinct from the cross-killing capacity towards third-party target.

The dichotomy of MLC and CML ontogeny in spleen cells is not likely to be due simply to the fact that the ratio between responding and stimulating cells is not optimal since varying the responder-to-stimulator ratios from 1:2 to 8:1 still does not result in significant CML by spleen cells from 4- and 5-day old B10 mice (data not shown). One possible interpretation of the early MLC response is that a "back reaction" is taking place, i.e., that adult X-irradiated B10.D2 stimulator cells respond to the antigen(s) on the B10 responding cells and release a factor which then stimulates the B10 cells to divide. That this is probably not the case is demonstrated by results of experiments not part of the present series in which 2-day old B10 spleen cells were stimulated with X-irradiated BDF_1 spleen cells derived from adult thymectomized, lethally irradiated, bone marrow-reconstituted animals; positive MLC responses were still obtained.

Since results from a number of studies (11–13) suggest that an MLC-proliferative response is a prerequisite for or at least highly helpful to the development of CML it is important to rule out as best as possible that the ontogenetic sequence described in our study is not simply a reflection of a gradual increase in the magnitude of the MLC-proliferative response. The data shown in Table I seem to argue against such an interpretation. First, with respect to the apparently delayed onset of a specific CML response compared with MLC proliferation, cells of mice either 13 or 16 days old show no greater MLC response as revealed by stimulation ratios than do cells of mice which are 4 or 5 days old. Yet in the older mice excellent CML occurs (13.6 and 40.9%), while in the younger animals no

TABLE I

*Ontogenesis of MLC and Lympholytic Responses (CML) of B10 Spleen Cells to B10.D2 Spleen Cells**

Age of responder cells	MLC (mean cpm ± SD)				% CML (mean ± SD)		
	Syngeneic MLC (B10 + $B10_x$)	Allogeneic MLC (B10 + $B10.D2_x$)	SR	*P*	B10 target	B10.BR target	B10.D2 target
days							
0	10,698 ± 997	18,988 ± 1,604	1.77	<0.0005	—	—	—
1	10,744 ± 1,947	24,722 ± 2,175	2.30	<0.0005	—	—	—
4	5,626 ± 615	12,077 ± 786	2.14	<0.0005	−19.8 ± 3.1	−13.8 ± 4.5	−13.3 ± 4.8
5	10,210 ± 787	13,263 ± 1,035	1.29	<0.005	−19.4 ± 3.4	−11.0 ± 4.7	−9.6 ± 4.5
7	43,116 ± 5,950	69,726 ± 5,579	1.61	<0.0005	−7.8 ± 4.3	−7.4 ± 2.5	14.6 ± 8.0
13	6,940 ± 623	11,862 ± 1,457	1.61	<0.0005	−25.7 ± 10.2	−2.3 ± 1.1	13.6 ± 2.8
16	8,684 ± 1,095	15,066 ± 1,531	1.73	<0.0005	−4.5 ± 4.6	−7.2 ± 2.4	40.9 ± 13.4
22	18,994 ± 3,982	54,750 ± 6,658	2.88	<0.0005	−3.9 ± 2.3	3.0 ± 0.8	24.4 ± 0.8
27	25,341 ± 6,227	89,522 ± 13,329	3.53	<0.0005	−3.5 ± 3.4	11.2 ± 1.7	40.4 ± 3.2
46	17,913 ± 1,973	76,957 ± 9,666	4.29	<0.0005	−24.0 ± 6.8	7.4 ± 11.0	60.5 ± 2.8
59	73,123 ± 8,120	179,496 ± 10,000	2.45	<0.0005	−0.2 ± 0.8	7.8 ± 1.4	36.1 ± 2.6
88	19,027 ± 2,618	81,257 ± 7,987	4.28	<0.0005	−4.0 ± 2.4	7.7 ± 3.0	39.8 ± 5.3
320	17,962 ± 3,736	84,097 ± 10,456	4.68	<0.0005	−3.0 ± 2.2	12.6 ± 2.0	76.2 ± 10.4

* See Materials and Methods for legends.

CML is seen (−13.3 and −9.6%). With respect to cross-reactivity of killing, while 16- and 27-day old mice show essentially the same degree of specific killing of B10.D2 cells (about 40%) the 16-day group gives no cross-killing at all while the 27-day group shows significant destruction of B10.BR cells.

We should emphasize that whereas the above findings are true for splenic lymphocytes, we have not yet described the ontogenic pattern for other organs such as the thymus, liver, or yolk sac. The pattern of development of immunocompetence is complex (cf. 5) and both differential maturation and selective migration of lymphoid cells may influence the appearance of functional cells in specific organs during development.

Several reports (2, 14–19) suggest heterogeneity of thymus-derived lymphocytes (T cells). For example, different subpopulations of T cells have been demonstrated by synergism studies in GvHR, one as effector cells and the other as amplifier cells (14). Later this was confirmed in MLC and CML reactions (15), also by demonstration of synergism between populations from different lymphatic organs. Different subpopulations of T cells have also been implicated for GvHR and helper function in antibody responses (16), for inhibition of macrophage migration and CML reaction (17), for responses to PHA and concanavalin A mitogens (18), and for GvHR and CML reactions (19). The cell adsorption method (2) to remove cytotoxic T cells provided perhaps the most direct indication for, although it did not prove, subpopulations reactive in MLC proliferation and CML cytotoxicity, correlating with genetic studies (3) which have shown a dichotomy for the genes controlling MLC and CML reactions.

The results presented in this paper demonstrate that in the spleen three phenomena associated with the development of CML arise at different times: proliferation induced by allogeneic stimulation, specific killing of target cells used for sensitization, and cross-killing of third-party target cells. These findings may be of particular significance in terms of the establishment of specific immunological unresponsiveness (tolerance) during embryogenesis (20), since

differential maturation of T-cell subfunctions could serve to alter the equilibrium between suppressor cells, cells serving as stimulators of antireceptor or blocking antibodies, cells essential for the generation of specific memory, etc. Similarly, the differential ontogeny of T-cell subfunctions[1] may provide the biological basis for the transient successful survival, then immunological destruction of potentially autoimmune or graft-vs.-host-reactive maternal lymphoid cells known to be present during fetal and neonatal periods of mouse development (21). Finally, the possibility that in the ontogeney of immune responses there is a gradual acquisition of the ability to cross-kill is an intriguing finding which may well relate to somatic development of specificity of recognition by T cells.

Summary

While spleen cells from neonatal B10 ($H\text{-}2^b$) are reactive (proliferate) in one-way mixed leukocyte culture, cell-mediated lympholysis reactivity does not arise until 7 days of age. When B10 cells are sensitized to B10.D2 ($H\text{-}2^d$), cross-killing of third-party B10.BR ($H\text{-}2^k$) target is always lower than the specific killing of B10.D2 targets and is not demonstrable until 27 days after birth.

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