ONTOGENY OF CULTURE-GENERATED SUPPRESSOR CELLS*

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Murine spleen cells, after culture for 3–5 d, suppress the ability of normal cells to produce antibody (1–3), to proliferate both in response to allogeneic cells (4) and mitogens (5, 6), or to generate alloreactive cytotoxic cells (4, 5, 7–9). In this report we show that the maturation of such suppressor functions in ontogeny parallels that of the functions they regulate in vitro. It has been shown that cells which proliferate in response to allogeneic cells in the mixed lymphocyte reaction (MLR)[1] appear in ontogeny before precursors of cytotoxic cells (10, 11). Accordingly, spleen and hemopoietic liver cells from young animals, when precultured, were able to suppress only the proliferative response of adult spleen cells in the MLR. The ability to suppress the generation of alloreactive cytotoxic T cells (CTL) in adult spleen was obtained only from spleens and hematopoietic livers of animals one week of age or older.

Materials and Methods

Mice. C57BL/6J (B6) and DBA/2J (D2) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, maintained on standard laboratory chow and water ad lib., and used at ~2–6 mo of age. Breeding pairs were set up using one male plus three females per cage. Pregnant mice were removed twice a week, and placed in separate cages, where they were checked daily for litters.

Cell Cultures. Cell cultures were carried out in complete medium (CM) consisting of Eagle's complete minimum essential medium (MEM), supplemented with 15% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, 1% MEM nonessential amino acids mix, and 2 mM L-glutamine. All media and sera were obtained from Grand Island Biological Co., Grand Island, N. Y. Batches of sera were screened for both high plating efficiency, and low background mitogenicity for mouse lymphocytes. Tumor cell lines were passed three times weekly and were split 1:1 the day before use in the ^{51}Cr-release assay. Cell lines used in this assay were obtained from investigators at the Sloan-Kettering Institute, New York: EL-4, a B6 benzpyrene-induced T-cell leukemia, and P815, a methylcholanthrene-induced D2 mastocytoma, were supplied by Dr. Peter Ralph; SL-8, a spontaneous T-cell lymphoma of AKR origin, was provided by Dr. J. Tung.

Preparation of Precultured Cells. Precultured (P) cells were prepared according to a modification of the method of Nadler and Hodes (4). B6 spleen cells were pressed gently through a sterile 60-mesh screen into warm Hanks' balanced salt solution + 5% FCS (HBSS + FCS). The clumps were broken up by passage through a 26-gauge needle. The cells were centrifuged at 200 *g* for 10 min at room temperature, the pellet resuspended in 1 ml HBSS + FCS and

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[1] *Abbreviations used in this paper:* CM, complete medium; CTL, cytotoxic T lymphocyte(s); FCS, fetal calf serum; HBSS + FCS, Hanks' balanced salt solution + 5% fetal calf serum; LM, lysis medium; 2-ME, 2-mercaptoethanol; MEM, Eagle's complete minimum essential medium; MLC, mixed lymphocyte culture; MLR, mixed lymphocyte reaction; P, precultured; TM, test medium.

treated with 8 ml Tris-NH_4Cl lysis medium (LM) (one part 2.1% Tris + nine parts 0.8% NH_4Cl) for 15 seconds. The LM was diluted 10-fold with HBSS + FCS and the cells were centrifuged again, adjusted to 2×10^6/ml in test medium (TM) (composition same as CM except that 10% heat-inactivated FCS was substituted for 15% FCS), supplemented with 2-mercaptoethanol (2-ME) to a final concentration of 5×10^{-5} M and plated in 160-mm Petri dishes (35–40 ml/plate) (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). 4 d later, the nonadherent and loosely adherent cell populations were harvested by gentle pipetting, and are referred to as P cells.

MLR. The mixed lymphocyte culture (MLC) was carried out in Falcon 3040 microtiter plates (Falcon Labware, Div. of Becton, Dickinson & Co.) in a total vol of 0.2 ml, according to the method of Peck and Bach (12). A constant number (2×10^5) of irradiated D2 (allogeneic) or B6 (syngeneic) spleen cells was incubated with varying numbers of normal or precultured cells, or a mixture of the two, as responders. After 5 d incubation (37°C, 5% CO_2 in air), 0.2 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.) was added (sp act 2 Ci/mmol). 8 h later, the cells were harvested on glass filter-paper discs using an automatic cells harvester (Skatron, Flow Laboratories, Inc., Rockville, Md.). The discs were dried overnight, and counted in a Packard scintillation counter, using Permablend I (Packard Instrument Co., Inc., Downers Grove, Ill.) as scintillation fluid.

Generation of CTL. Generation of CTL was carried out in a total vol of 6 ml TM + 2-ME, following a modification of the method of Burton et al. (13). 12×10^6 irradiated D2 spleen cells were incubated with an equal number of B6 spleen cells plus P cells at 1:10 or 1:2 in 17- × 100-mm test tubes (Falcon Labware, Div. of Becton, Dickinson & Co.). The tubes were incubated for 5 d as in the MLC, after which, the viable cells were adjusted to the desired concentration and used in the ^{51}Cr-release assay.

^{51}Cr-release Assay. The ^{51}Cr-release assay was performed according to a modification of the method of Brunner et al. (14). Nonadherent target cells were incubated with 100 μCi of ^{51}Cr (as Na[^{51}Cr]O_4) (New England Nuclear), for 30 min at 37°C. The cells were washed three times before use. Radioactively labeled targets were plated in 50 μl at 2×10^3/well in Cooke round-bottom microtiter plates (Cooke Engineering Co., Alexandria, Va.). Effector cells at the appropriate concentration were added in 100 μl TM. After a 4-h incubation at 37°C, the plates were centrifuged at 200 *g* for 10 min and 100 μl supernate was removed and counted in a gamma scintillation counter. Spontaneous release of isotope was measured by incubating the targets with 100 μl medium alone. Maximum release was determined by freezing and thawing the targets plus 100 μl medium three times. The percentage of cytotoxicity was determined according to the following formula:

$$\text{Percentage of cytotoxicity} = \frac{\text{counts per minute (experimental)} - \text{counts per minute (spontaneous)}}{\text{counts per minute (maximum)} - \text{counts per minute (spontaneous)}}.$$

Results

P cells appear to lose proliferative capacity after 4 d in vitro, and when mixed with normal cells at ratios of 1:10 or 1:2, suppressed the normal cell MLR in proportion to their numbers in culture (Fig. 1). This suppression is statistically significant at the 1% level, and is not a result of crowding, because controls also included total numbers of responders per well (2.2×10^5 and 3×10^5) in addition to the mixtures.

P cells alone generated almost no cytotoxicity, and if they were added to fresh responders at the sensitization step, suppressed the generation of alloreactive CTL. Suppression, in proportion to the numbers of P cells present, was seen over a wide range of effector to target ratios (0.5:1 through 10:1). Fig. 2 shows typical cytotoxicity curves generated by B6 responders cocultured with irradiated D2 spleen cells with or without B6 P cells, tested against the D2 tumor P815. It can be seen that P cells from adult animals are highly effective in suppressing the generation of alloreactive CTL.

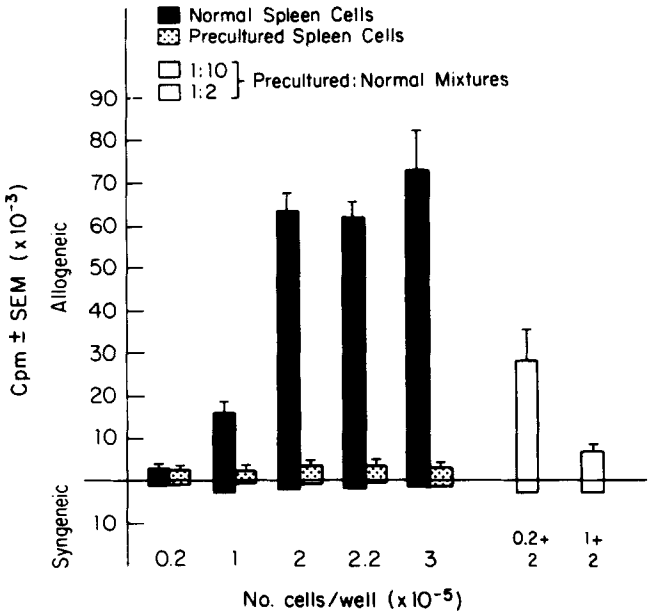

FIG. 1. Suppression of proliferation in MLR by P cells. [^{3}H]Thymidine uptake of normal, precultured, or mixtures cocultured with irradiated B6 (syngeneic) or D2 (allogeneic) cells. Cell concentration/well ranged from 0.2×10^5 to 3×10^5, including mixtures of P plus normal cells at ratios of 1:10 and 1:2. Results of a representative experiment ± SEM ($n = 6$).

Controls for crowding were performed by adding normal fresh cells or irradiated normal cells in place of P cells to the tubes in the sensitization step, and no changes in the number of CTL generated was seen (data not shown). Specificity of the CTL was tested using EL-4 of B6 origin, or SL-8 of AKR origin. Neither of these two cell lines were killed by normal or precultured cells. This was tested under conditions that gave 100% cytotoxicity by the sensitized cells on the appropriate targets (data not shown).

Because the ontogeny of cell-mediated responses in the mouse has been well described (10, 11), we investigated the ontogeny of the culture-induced suppressor cell to gain insight into its possible function in vivo. Table I, line 4 shows that hematopoietic liver cells from 17-d-old embryos were suppressive of adult spleen MLC, when precultured, but not if added fresh (line 6). Although others (15, 16) have found fresh liver cells from fetal mice to be suppressive in the MLR, the ratios of liver cells to responders were much higher than those used in this report. It is possible that the relative numbers of suppressor cells present in fetal livers is so low that their effect can be demonstrated only in high numbers, or upon enrichment during the preculture period.

Although hematopoietic fetal liver P cells were suppressive in the MLR, no suppression of the generation of CTL was seen by fetal liver P cells (Table II, line 4). This is true also whether the added cells were precultured or fresh (line 6).

Later ages were tested in an attempt to determine when the suppressor of CTL appears in ontogeny, and as a control, suppression of the MLR was monitored for all ages tested for CTL suppression. It can be seen that P cells from mice of all ages were able to suppress the MLR (Table I). Fresh cells were also tested, as a control for

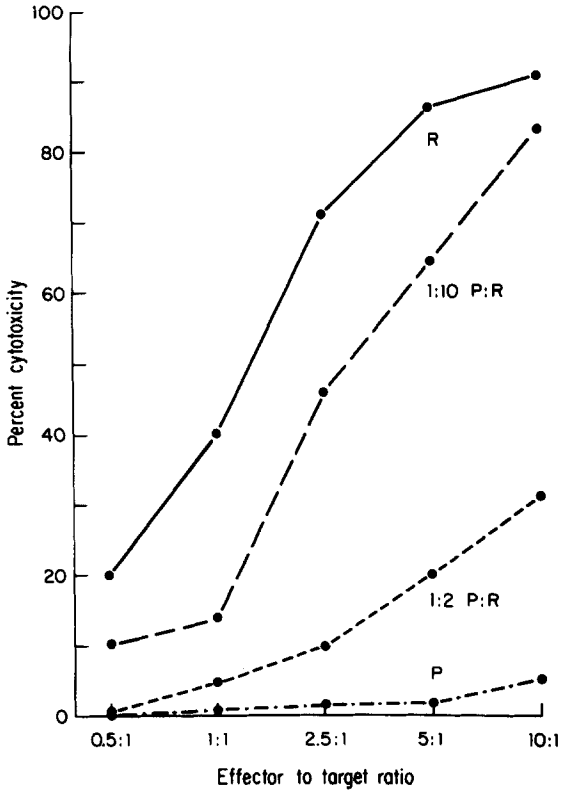

FIG. 2. Suppression of generation of alloreactive CTL by P cells. The percentage of cytotoxicity generated by: (R) 12 × 10^6 irradiated D2 spleen cells + 12 × 10^6 normal B6 spleen cells; (P:R) 12 × 10^6 irradiated D2 spleen cells + 12 × 10^6 normal B6 cells + 12 × 10^6 P (1:10) or 6 × 10^6 P (1:2) cells; (P) 12 × 10^6 irradiated D2 spleen cells + 12 × 10^6 P cells.

crowding in the wells, and to document the existence of the newborn suppressor cell reported in the literature (11). Suppression was seen when fresh 3-d-old spleen cells were added to normal adult responders (Table I, line 10). This effect disappeared by 6 d of age (Table I, line 14), as has been reported by others (17). Thymus cells, whether precultured or added fresh, were never suppressive (data not shown), and in later experiments were omitted from the protocol.

The suppressive potential of precultured or fresh cells from mice of various ages was tested on the generation of CTL. Table II, line 8 shows that precultured spleen cells from 3-d-old animals were not effective in suppressing CTL generation. As later ages were tested for suppression of the generation of CTL, it was found that at 6 d of age, spleen cells were slightly suppressive (line 12), when precultured, but not if added fresh (line 14), on the generation of CTL. Suppression was also seen by precultured, not fresh, hematopoietic liver cells from animals of the same age (data not shown). Table II shows the maturation of the cell which suppresses CTL generation when precultured, showing that precultured spleen cells were suppressive only when taken from mice 6 d of age or older.

The maturation data as shown is consistent with the interpretation that two subpopulations of suppressors may be generated under the same in vitro culture

TABLE I

Effect of P Spleen or Hemopoietic Liver Cells from Mice of Different Ages on the MLR Response of Adult Spleen Cells

	Cells tested*	[^{3}H]Thymidine uptake‡		Percentage of suppression
		Syngeneic§	Allogeneic§	
				%
1	Normal responders	1,759	63,326	—
2	Normal responders + adult P cells	1,804	7,275	89
3	Normal responders	1,523	31,121	—
4	Normal responders + 17-d fetal liver (P)	1,479	6,957	78
5	Normal responders	577	24,214	—
6	Normal responders + 17-d fetal liver (F)	1,010	24,941	+3
7	Normal responders	1,374	39,367	—
8	Normal responders + 3-d spleen (P)	4,430	6,183	85
9	Normal responders	1,374	39,367	—
10	Normal responders + 3-d spleen (F)	5,541	9,943	75
11	Normal responders	408	65,364	—
12	Normal responders + 6-d spleen (P)	2,229	5,778	92
13	Normal responders	366	16,125	—
14	Normal responders + 6-d spleen (F)	1,588	15,718	3
15	Normal responders	2,611	83,367	—
16	Normal responders + 20-d spleen (P)	3,745	4,480	95
17	Normal responders	366	16,125	—
18	Normal responders + 20-d spleen (F)	974	24,539	+52

* P or fresh (F) cells incubated at a ratio of 1:2 with 2×10^5 normal adult spleen cells + 2×10^5 irradiated B6 (syngeneic) or D2 (allogeneic) spleen cells as stimulators.

‡ 8-h pulse of [^{3}H]thymidine. SEM ($n = 6$): 10% for all groups.

§ Day-to-day variability in MLR results made it necessary to perform all controls and experimental groups for each determinations as in Fig. 1.

conditions. One, the suppressor of proliferation, is present in 17-d-old fetal liver, whereas the other, the suppressor of CTL generation matures in the spleen at ~6 d after birth.

Discussion

There is some disagreement concerning the onset of adult-type lymphoid functions in young mice. Thus, some investigators have stated that murine spleen cells attain both MLR stimulatory and reactive capacity at about 1–2 wk after birth (18), although others (10) report significant in vitro reactivity to allogeneic lymphocytes in newborn mice. Whatever the controversy concerning proliferative responses in vitro, it is generally agreed upon that cytotoxic reactivity develops at ~1 wk of age (10, 11).

Our results show that in addition to a different rate of maturation of proliferative and cytotoxic responses, there is also a maturation of suppressor function. The results also suggest that regulation of MLR and CTL functions could be mediated by different suppressor cell subpopulations generated in the same way. Because proliferation in the MLR (largely in response to I-region determinants) can be suppressed by lymphoid cells from fetal mice, and because I-region determinants are found on very young mice (19), it would seem that regulation of this response develops concordantly with the function itself. Cytotoxicity directed against alloantigens, however, does not mature until ~1 wk of age (10, 11), just when regulation of the

TABLE II

Effect of P Spleen or Hemopoietic Liver Cells on the Generation of Alloreactive CTL

	Cells tested*	Percentage of cytotoxicity (E:T)		
		10:1	5:1	1:1
			%	
1	Normal spleen	90	85	40
2	Normal spleen + adult spleen (P)	30	10	2
3	Normal spleen	100	95	37
4	Normal spleen + 17-d fetal liver (P)	98	82	34
5	Normal spleen	88	77	31
6	Normal spleen + 17-d fetal liver (F)	72	63	27
7	Normal spleen	100	59	15
8	Normal spleen + 3-d spleen (P)	84	62	15
9	Normal spleen	99	83	23
10	Normal spleen + 3-d spleen (F)	101	89	34
11	Normal spleen	100	77	23
12	Normal spleen + 6-d spleen (P)	56	39	12
13	Normal spleen	99	75	46
14	Normal spleen + 6-d spleen (F)	96	89	76
15	Normal spleen	100	59	15
16	Normal spleen + 20-d spleen (P)	70	41	8
17	Normal spleen	99	97	46
18	Normal spleen + 20-d spleen (F)	97	89	44

* 12×10^6 irradiated D2 stimulators + 12×10^6 normal B6 spleen cells + 6×10^6 P or fresh (F) cells as indicated (ratio of 1:2), incubated for 5 d to generate cytotoxic cells.

‡ 4-h ^{51}Cr-release assay against P815. SEM ($n = 4$): 5% for all groups. Effector-to-target (E:T) ratios of 10:1, 5:1, and 1:1 are shown.

response is seen in the form of culture-generated suppressors.

In addition to the maturation data, we have also found that serum requirements differ for the two kinds of suppressors (20). Thus, spleen cells from adult mice, when precultured in medium containing heat-inactivated horse serum substituted for FCS were capable of suppressing the proliferative response to alloantigens only if they were recultured in FCS. Suppression of CTL generation, however, showed no such serum restrictions. Cells which suppressed this response could be generated in medium containing calf or horse serum, and exerted their effects in horse or calf serum.

Furthermore, when precultured cells from adult animals were subjected to 1,000 rad in vitro before being added to MLR or sensitization cultures for generation of CTL, it was found that suppression of the MLR occurred, but the ability to suppress the generation of alloreactive CTL was lost (F. M. Rollwagen. Unpublished data.). However, both the young and adult suppressor activity acting either on MLR or alloreactive CTL generation are mediated by Thy-1-negative, macrophage-like cells (F. M. Rollwagen and O. Stutman. Characterization of the culture-induced suppressor cells. Manuscript in preparation.).

Recently, it has been postulated that feedback suppressors exist for every clone of antibody-forming cells (21) or CTL (22) activated. In addition, new surface antigens may be displayed on cells that have been activated by mitogens (23), or histocompatibility antigens (24). It follows, therefore, that antigens in the medium or on the cells may act as activators of polyclonal suppressors effective in suppressing cells bearing these neoantigens.

These results permit the conclusion that P cells from adult mice consist of at least two subpopulations—one, which is activated by serum components, is relatively radioresistant, is present in mice <1 wk of age, and suppresses the proliferative response to alloantigen; the second subpopulation is not dependent on the culture serum used, is radiosensitive, and suppressses the generation of alloreactive CTL. The ontogeny data presented in this report, therefore, reflect the maturation of this second subpopulation. The fact that the maturation patterns of the in vitro-induced suppressor cell for MLR and CTL generation parallels the maturation of the functions they regulate, suggests that such mechanisms may have significance in vivo.

Summary

Culture of murine lymphoid cells without added antigen results in the generation of cells which suppress a variety of in vitro immune responses, such as the mixed lymphocyte response (MLR) and the generation of alloreactive cytotoxic T cells (CTL). The ontogeny of this phenomenon was studied. Cells which suppressed the MLR after preculture were isolated from spleens and hematopoietic livers of fetal and young (<1 wk old) mice. On the other hand, the generation of alloreactive CTL could be suppressed only by precultured spleen cells taken from 1-w-old or older mice. The parallel between the development of the suppressor functions and the maturation of the responses they regulate, suggests a possible biological significance of the effect.

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