Inhibition of Rat Mixed Lymphocyte Cultures by Suppressor Macrophages

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Normal rat spleens contain suppressor cells which can inhibit proliferative and cyto-toxic responses of lymphocytes to alloantigens in vitro. The suppressor cells are ad-herent, phagocytic, resistant to treatment with ATS and C, radioresistant, resistant to treatment with mitomycin C, apparently absent from the thymus, and found in very high concentrations in peritoneal exudates. These characteristics indicate that the sup-pressor cell is a macrophages and not a T cell. When suppressor cells were removed from spleen cell suspensions, strong in vitro proliferative and cytotoxic responses to alloantigens could consistently be observed.

INTRODUCTION

In vitro lymphoproliferative responses have been difficult to demonstrate in the spleens of rats (1). Consistent demonstration of in vitro generation of high cyto-toxic reactivity to alloantigens which is dependent on a proliferative response (2–4) required the use of lymph node cells as responders (5). In contrast, in mice, the spleen is routinely used as a source of effector cells for MLC-CML[5] reactions and thus the poor reactivity in the rat spleens has not been easily explained. Two main possibilities need to be considered: either the rat, unlike the mouse, does not have alloreactive cells in the spleen, suggesting a different functional T-cell distribution in closely related species, or the rat spleen contains suppressor cells which can inhibit proliferative responses. A recent study of CML in rats has shown that their spleens do have cells capable of a proliferative and cytotoxic response to allo-antigen, but these responses were demonstrable only after depletion of an adherent

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[5] Abbreviations used in this paper: MLC, mixed lymphocyte culture; CML, cell-mediated lympholysis (in vitro generation of a cytotoxic response to alloantigen); GIA, growth inhibi-tion assay; MLTI, mixed lymphocyte tumor interactions; ATS and C, anti-T cell serum and complement; BSS, Hanks' balanced salt solution; PEC, peritoneal exudate cells; W/Fu, Wistar Furth; BN, Brown Norway; 2-ME, 2-mercaptoethanol; FBS, fetal bovine serum; ^{3}H-TdR, tritiated thymidine; LNC, lymph node cell; PBL, peripheral blood leukocyte; MMC, mitomycin C.

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cell population from the responding cells prior to culture (6). The adherent cell population, however, was not characterized. Folch and Waksman (7) have presented data which they interpreted as demonstrating an adherent T-suppressor cell which can strongly inhibit MLC in rat spleens (7). From that work, it would appear that an adherent T-suppressor cell was responsible for the inability of unfractionated rat spleen cells to respond in MLC or to generate CML.

Our laboratory has done extensive testing to suppressor cell activity in both tumor and non-tumor systems (8–12) and has found adherent suppressor cells with the characteristics of macrophages and not T cells. Recently, normal rat spleens, unlike normal mouse spleens, were found to have activity in the growth inhibition assay, which has been shown to measure cytostatic macrophage activity (13, 14). In addition, we found that cells active in the growth inhibition assay were also active in suppression of the *in vitro* secondary proliferative response to tumor-associated antigens (MLTI) (13). This appeared to be in contrast to the findings of Folch and Waksman, but since our findings were in a tumor system involving a secondary response and they had studied a primary response to alloantigen, it was possible the findings were not comparable. In order to compare our findings directly, we have developed an MLC-CML system in rats and have characterized the adherent suppressor cells in the normal rat spleen. Our data indicate that the suppressor cells in normal rat spleens which inhibit both MLC and CML are macrophages and not T cells. Possible reasons for these disparate findings are discussed.

MATERIALS AND METHODS

Animals

Eight- to 10-week-old male W/Fu (Ag-B2) rats usually served as donors for responding cells. Ex-breeder male and female BN (Ag-B3) rats usually served as donors of stimulating cells. All rats were obtained from Microbiological Associates, Inc., Walkersville, Md.

Preparation of Lymphoid Cells

Spleen, lymph node and thymus cell suspensions were prepared as previously described (15). Peripheral blood lymphocytes (PBL) were obtained by cardiac puncture. Ten milliliters of heparinized blood were diluted with 10 ml of BSS and carefully layered over 10 ml of Ficoll Hypaque (LSM, Litton Bionetics, Rockville, Md.) at 22°C. The gradient was then centrifuged at 250g at 22° for 30 min after which the cells layered at the interface of the Ficoll and BSS were carefully collected, washed and resuspended in complete medium (see below). This procedure resulted in a lymphoid population free of granulocytes and consisting of small lymphocytes and monocytes. Peritoneal exudate cells (PEC) were collected from the peritoneal cavity 4 days after injection of 20 ml of light mineral oil i.p. Latex particle ingestion techniques consistently showed that 45–55 percent of the cells obtained were phagocytic; the remainder appeared under light microscopy to be small lymphocytes.

Cell Separation Procedures

The medium used for all experiments and cell separation procedures was as follows: RPMI medium 1640 (Grand Island Biological Co., Grand Island, N. Y.)

containing 25×10^{-3} M HEPES solution (Microbiological Associates, Inc., Bethesda, Md.), 5×10^{-5} M 2-mercaptoethanol (Schwarz-Mann Division, Becton, Dickinson & Co., Orangeburg, N. Y.), 100 units of penicillin per ml, 100 μg of streptomycin per ml and 60 μg/ml of gentamicin, 2 mM of glutamine and 10% FBS (all reagents from Grand Island Biological Co.). This will be referred to as complete medium.

Carbonyl iron and magnet. Spleen cells (1×10^8) in 5 ml of medium were combined with 200 mg of carbonyl iron powder (GAF Corp., New York, N.Y.) and incubated for 1 hr at 37°C in a shaking water bath. Following the incubation period, the cell suspension was diluted to 10 ml and serially poured from test tube to test tube using a strong magnet to retain cells having phagocytized the iron powder at each step. A series of 6–8 test tubes was used. This procedure resulted in a decrease from 8–10% to 1–2% in the proportion of normal spleen cells which could ingest latex particles.

Passage of spleen cells through a rayon column. Thirty-five milliliter syringes were disassembled, and the barrel was filled with three rayon cotton balls (Parke Davis Co., Detroit, Mich.). The assembly was then autoclaved and prepared for use as follows: A sterile stopcock was placed on the tip of the syringe and 50 ml of BSS were rapidly passed through the column. Then $5–8 \times 10^8$ spleen cells in media were placed on the column and incubated at 37°C in a 5% CO_2 humid atmosphere. After 20 min incubation, the nonadherent cells were eluted with 50 ml of warm (37°C) BSS, centrifuged at 250g for 10 min, and resuspended in complete medium. Twenty to 30% of the cells were recovered by this procedure. Latex particle ingesting cells decreased from 8–10% of the normal spleen population to 1–2%. This procedure had virtually no effect on the percentage of cells bearing surface immunoglobulin (40–50% of normal spleen population as measured by membrane surface immunofluorescence with fluorescein-conjugated goat anti-rat immunoglobulin [Lot #4020201, prepared for NCI by Huntington Research Center, Brooklandville, Md.]) The levels of cell-mediated cytotoxicity of immune W/Fu spleen cells against (C58NT)D, a syngeneic Gross virus-induced lymphoma, which is T-cell dependent (16, 17), were either unchanged or increased after column passage.

Removal of T Cells

A burro anti-rat thymocyte antiserum (ATS) was used to specifically remove T cells. The methods for producing and absorbing this antiserum have been described (16). One-tenth milliliter of this antiserum (diluted 1:5) and 0.1 ml of undiluted guinea pig serum as a source of complement were added to 0.1 ml of 1×10^7 spleen cells, and the mixture was incubated at 37°C for 1 hr. The cells were then washed twice with 50 ml of BSS and resuspended in complete medium as previously reported (16). This procedure consistently and completely abrogated the proliferative response of the spleen cells to PHA and the cytotoxicity of immune lymphocytes against (C58NT)D. The number of antibody-producing spleen cells from rats immunized against sheep red blood cells was not decreased. In addition, macrophages shown to be active in the GIA were not decreased (13).

Assays of Functional Activity

Mixed lymphocyte culture (MLC) was done as follows: 1×10^8 stimulating cells (usually BN spleen cells) were treated with 50 μg of mitomycin C in 1 ml of complete medium for 45 min and washed twice in BSS and resuspended in complete medium. Responding cells were placed in culture with mitomycin C-treated stimulating cells in 0.2 ml of medium in microtiter U plates (Cooke Engineering Co., Alexandria, Va.). Cell concentrations of both stimulating and responding cells were varied from 4×10^6 cells/ml to 0.5×10^6 cells/ml to obtain optimum conditions. Culture periods varied from 48 hr to 120 hr. One microcurie of ^{3}H-thymidine ([^{3}H-TdR] specific activity 6 Ci/mmol; Schwarz-Mann, Orangeburg, N.Y.) was added to each well for the final 16 hr of the incubation period, and the cultures were harvested and further processed as previously described (8). All cultures were carried out in triplicate. All standrad errors were less than 5% and are not included in the tables.

Suppressor cell activity was demonstrated as described previously (13). Briefly, various numbers of spleen cells from the populations being tested for suppression were added to a fixed concentration (2×10^6 cells/ml) of responding cells. Prior to the assay, the cells being tested for suppressor activity were either treated with mitomycin C or x-irradiated with 2500 R. MLC was then done as above.

In Vitro Generation of Cytotoxic Lymphocytes

MLC was carried out as described above except that instead of adding ^{3}H-TdR and harvesting 16 hr later, the cells were gently resuspended in each well with a 0.1 ml semiautomatic pipette (Schwarz-Mann, Orangeburg, N.Y.) and 2×10^3 BN lymphoma [a Kirsten virus-induced lymphoma (18) most generously provided by Dr. Frank Fitch, Department of Pathology, University of Chicago, Chicago, Ill.] target cells, labeled with ^{51}Cr were added to each well. The plates were then incubated on a rocking platform for 4 hr at 37°C in a 5% CO_2 humidified atmosphere and then centrifuged at 500g for 10 min. One-tenth milliliter of the supernatant was then carefully withdrawn from each microwell and counted for 10 min in a Searle gamma counter. Microwells with 0.2 ml of distilled water to which labeled target cells were added served as controls for total releasable ^{51}Cr. Supernatants drawn from wells containing distilled water after the 4-hr incubation period contained 80–90% of the total ^{51}Cr incorporated into the target cells. Baseline release was measured from microwells containing either labeled target cells alone or mitomycin C-treated stimulating cells and target cells. Values obtained with either method were always within 1.0% of each other and were consistently between 5 and 10% of the distilled water controls.

Percent cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{cpm in sup of test group} - \text{cpm in sup of baseline control}}{\text{cpm in sup of distilled water control} - \text{cpm of background}} \times 100.$$

RESULTS

Comparison of MLC Reactivity of Various Lymphoid Populations

To ensure optimal conditions when comparing various lymphoid populations for MLC reactivity, various concentrations of both stimulating and responding cells,

from 5×10^5 to 4×10^6 cells per ml, were mixed. BN stimulating cells were blocked with mitomycin C and never incorporated ^{3}H-TdR greater than background levels when cultured alone. W/Fu responding cells incorporated similar amounts of ^{3}H-TdR whether cultured alone or with various concentrations of syngeneic cells treated with mitomycin C. In most experiments the responding cells were cultured alone as controls. Figure 1 shows the results of an experiment comparing MLC reactivity of lymph node cells (LNC), peripheral blood lymphocytes (PBL), and spleen cells to BN alloantigens. A responding cell concentration of 2×10^6 per ml was optimal to demonstrate a proliferative response although a broad dose response relationship was seen when the responding cell concentrations were varied. Stimulating cells also produced strong proliferative responses over a broad range of concentrations. For subsequent experiments, 5×10^5 cells per ml was used as the stimulating cell concentration because that concentration consistently elicited the best proliferative response. Figure 1 also shows the kinetics of the reactivity of the various populations. Peak reactivity of the PBL was seen at about Day 4. This was variable, however, with similar levels of reactivity frequently being demonstrated both on Days 4 and 5. LNC reactivity was frequently demonstrable by Day 3 but usually peaked on Day 4 and dropped off sharply by Day 5. The responses of the spleen cells, on the other hand, usually peaked on Day 3, if any significant reactivity could be seen at all (Fig. 1c and d). The responses shown in Figure 1a, 1b, and 1c are from one experiment using the same pool of BN stimulating cells. Figure 1d is included as a separate kinetic study of the MLC reactivity of rat spleens, again showing low MLC reactivity of the spleen cells. These data indicate that although W/Fu rats responded well to BN alloantigen, as demonstrated by the MLC responses of LNC and PBL, spleen cells responded poorly or not at all.

MLC Reactivity of Normal Spleen Cells after Depletion of Various Populations

Using the conditions found to be optimal for MLC reactivity, responding spleen cells were depleted of adherent or phagocytic cells and then tested for their ability
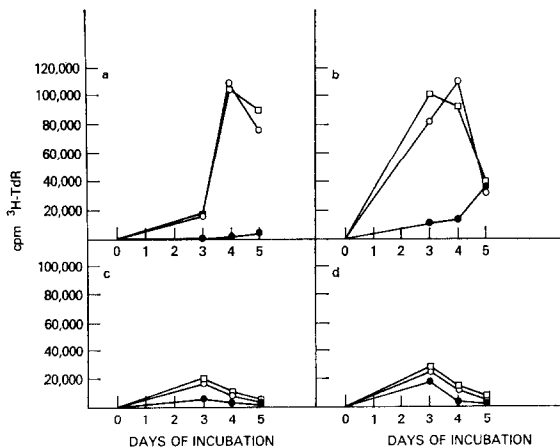

Fig. 1. Kinetic study of MLC reactivity in various lymphoid populations. (a) PBL, (b) LNC, (c) spleen cells, (d) spleen cells. ● = cpm of responding cells alone (2×10^6 cells/ml). ○ = cpm in MLC with 0.5×10^6 cells/ml as BN stimulating cell concentration. □ = cpm in MLC with 1×10^6 cells/ml as BN stimulating cell concentration.
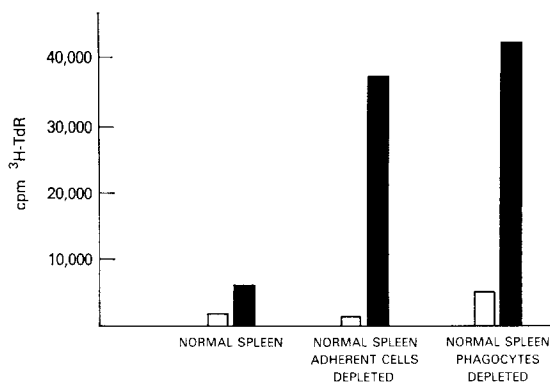
FIG. 2. Comparison of MLC reactivity before and after depletion of adherent (column passage) or phagocytic (treated with iron and magnet) cells. Open bars represent cpm of effector cells incubated alone. Closed bars represent cpm of effector cells stimulated with BN spleen cells treated with mitomycin C.

to respond in the MLC. The results in Figure 2 show the MLC response was greatly improved after depletion of adherent or phagocytic cells. The results of this experiment indicate that rat spleens contain cells which can respond vigorously to BN alloantigens, thus ruling out the possibility that the decreased MLC reactivity observed with spleen cells was due to the absence of a highly reactive subpopulation of T cells. However, although surface immunofluorescence studies have shown the T-cell/B-cell ratio to be relatively undisturbed after column passage or iron treatment, the possibility remained that the enhanced reactivity seen after

TABLE 1

Effect of Addition of Third Party Cells to MLC

Stimulating cells	Responding cells		Third party cells added[b]	
	Normal spleen unfractionated	Normal spleen column passage	+ Normal spleen	+ Normal spleen column passage
Experiment 1				
Media	9,385[a]	2,277	6,034	2,073
BN spleen	24,126	39,837	18,679	36,625
	Normal spleen unfractionated	Normal spleen column passage	+ Normal spleen	+ Normal spleen iron and magnet
Experiment 2				
Media	1,675	1,210	3,025	6,010
BN spleen	5,845	34,460	10,395	31,420
	Normal spleen unfractionated	Normal spleen column passage	+ Normal spleen	+ Normal spleen 2500 R
Experiment 3				
Media	5,511	722	1,878	1,953
BN spleen	11,706	37,550	14,914	11,031
			+ Thymus	+ Normal spleen ATS and C
Media			4,835	1,626
BN spleen			33,570	3,754

[a] Cpm ^{3}H-TdR.

[b] Third party cells were treated with mitomycin C unless otherwise noted and added in concentrations equal to that of the column-passaged responding cells.

these purification procedures was the result of increasing the percentage of a reactive subpopulation of T cells rather than depleting suppressor cells.

Addition of Various Populations of Cells as Third Parties to the MLC

If normal spleen cells contained suppressor cells, addition of unfractionated cells as third parties to MLC with column-purified responder spleen cells should suppress the reactivity. To test this possibility, unfractionated spleen cells were treated with mitomycin C or with 2500 R x-irradiation to prevent a proliferative response and added as third parties into the MLC in concentrations equal to the responding cells. Table 1 shows the results of three representative experiments. In each experiment, the MLC reactivity of the unfractionated population was compared to that of the column-passaged population. In addition, some of the third party cells were pretreated to deplete various subpopulations. In experiment 1, addition of unfractionated spleen cells greatly reduced the MLC of the column-passaged cells. In contrast, addition of the same number of column-passaged cells did not significantly decrease the MLC of the column-passaged responders. This indicates that adherent suppressor cells are present in the spleen.

Experiment 2 shows that pretreatment of the third party spleen cells with iron and magnet to deplete phagocytes was also highly effective in removing suppressor cells. In experiment 3, the suppressor cells were shown to be resistant to 2500 R, absent from the thymus, and resistant to treatment with ATS plus complement. These experiments indicate that the rat spleen has cells capable of inhibiting a proliferative response to alloantigen and that the cells are resistant to mitomycin C, x-irradiation, and treatment with a specific heterologous T-cell serum plus complement. In addition, they are adherent and phagocytic and not found in the thymus.

TABLE 2

Kinetics of *In Vitro* Generation of Cytotoxicity with Unfractionated Spleen Cells

Stimulating cells	Days in culture		
	Day 3	Day 4	Day 5
Experiment 1			
Media	2.3[a]	1.6	−0.3
BN spleen (0.5)[b]	8.9	14.4	8.4
Experiment 2			
Media	0.7	1.2	0.4
BN spleen (0.5)	10.4	24.8	7.7
Experiment 3			
Media	3.4	1.5	
BN spleen (0.5)	12.7	38.7	
Experiment 4			
Media	0.1	0.5	0.7
BN spleen (0.5)	16.5	34.4	15.5
BN spleen (1)	10.9	23.9	23.3

[a] Percent cytotoxicity.
[b] Cells/ml $\times$ 10^{-6}.

TABLE 3

Comparison of CML Generated with Various Populations of Responding
Cells After 4 Days of Culture

Stimulating cell concentration	Responding populations		
	Unfractionated spleen	Spleen after column passage	Spleen after iron and magnet
Experiment 1			
Media	−1.4[a]	2.0	
BN spleen (0.5)[b]	11.1	64.0	
Experiment 2			
Media	2.4	2.3	
BN spleen (0.5)	3.6	34.1	
BN spleen (1)	3.3	46.6	
Experiment 3			
Media	0.5		1.5
BN spleen (0.5)	34.4		54.9
BN spleen (1)	23.0		55.7
	Unfractionated lymph node cells		Lymph node cells after iron and magnet
Experiment 4			
Media	−0.4		0.6
BN spleen (0.5)	63.3		66.8

[a] Percent cytotoxicity.
[b] Cells/ml $\times$ 10^{-6}.

In Vitro Generation of Cytotoxic Effector Cells to Alloantigen Using Rat Spleen Cells as Responders

The ability of spleen cells to become cytotoxic after MLC was also examined. Pilot kinetic experiments with unfractionated spleen cells showed the highest cytotoxicity was on the fourth day of culture. Various stimulator/responder cell ratios were tested and the concentrations which were best for the proliferative response were most consistently optimal for the development of cytotoxic effector cells. After 4 days of culture, 2×10^5 viable cells remained in each microwell (about 50% of the original number). Addition of 2×10^3 labeled target cells, for an attacker/target cell ratio of 100 to 1, consistently resulted in the highest percentage of target cell lysis. Under these conditions, several experiments were performed to more carefully determine the kinetics of the CML of unfractionated spleen cells (Table 2). As in the pilot experiments, peak cytotoxicity consistently occurred on Day 4.

Depletion of the Adherent and Phagocytic Cells from the Responding Cells in the CML

In experiments similar to those described for measuring proliferation, the responding spleen cells were placed through a rayon column to remove adherent cells

or treated with iron and magnet to remove the phagocytes. Table 3 summarizes the results of several experiments. The levels of cytotoxicity generated with unfractionated spleen cells varied considerably among experiments, from undetectable responses to moderately high levels. In all cases, however, significant increases in the cytotoxic responses generated were observed after depletion of adherent or phagocytic cells. LNC became highly cytotoxic even without treatment and the cytotoxicity was not significantly improved by depletion of phagocytes.

Addition of Various Lymphoid Populations to the CML as Third Parties to Characterize the Suppressor Cells

Lymphoid cells from various sources were either x-irradiated or treated with mitomycin C to prevent a proliferative response and were added in concentrations equal to that of the responding cells as third parties (Table 4). Experiment 1 shows again the enhanced cytotoxic response of cells depleted of adherent cells. This

TABLE 4

Effect of Addition of Third Party Cells on *In Vitro* Generation of Cytotoxicity

Stimulating cells	Responding cells		Third party cells added[c]	
	Unfractionated spleen	Column passage spleen	Spleen MMC	Column passage spleen
Experiment 1				
Media	0.2[a]	0.2	−1.3	−1.3
BN spleen (0.5)[b]	36.2	59.9	27.4	46.2
	Unfractionated spleen	Column passage spleen	Spleen 2500 R	Iron and magnet spleen
Experiment 2				
Media	1.5	0.0	0.0	1.1
BN spleen (0.5)	38.6	65.5	23.5	55.8
	Unfractionated spleen	Column passage spleen	Spleen 2500 R	ATS and C spleen
Experiment 3				
Media	2.4	2.3	1.8	0.4
BN spleen (0.5)	3.3	46.6	10.8	3.3
			Thymus	PEC
Media			2.1	0.8
BN spleen (0.5)			56.6	1.6

[a] Percent cytotoxicity.

[b] Cells/ml $\times 10^{-6}$.

[c] Third party cells were treated with mitomycin C unless otherwise noted and added in concentrations equal to that of the column-passaged responding cells.

TABLE 5

Addition of Various Concentrations of Third Party Cells into CML Cultures
With Column-Passaged Spleen Cells as Responders

Stimulating cells	Source of third party cells	Concentration of third party cells $\times 10^{-6}$/ml added						
		0	0.12	0.25	0.5	1.0	2.0	4.0
Experiment 1								
Media	Unfractionated spleen	2.3[a]				1.4	1.8	1.6
BN spleen (0.5)[b]		46.6				28.5	10.8	3.3
Media	Thymus					3.4	2.1	3.6
BN spleen						46.3	56.6	48.2
Media	PEC		1.0	0.9	0.6	0.1	0.8	
BN spleen (0.5)			9.6	4.9	2.9	1.2	1.6	
Experiment 2								
Media	Unfractionated spleen	−0.2			0.3	−0.2	−0.4	0.2
BN spleen (0.5)		46.7			34.9	33.7	17.2	0.7
Media	Thymus					−0.8	−0.1	0.7
BN spleen (0.5)						38.6	37.5	40.7
Media	PEC		−0.7	0.1	−1.5	−0.2	1.0	0.9
BN spleen (0.5)			18.7	3.5	8.6	0.9	0.6	1.5

[a] Percent cytotoxicity.
[b] Cells/ml $\times 10^{-6}$.

increased response was largely abrogated by addition of mitomycin C-treated unfractionated spleen cells to the cultures. In contrast, pretreatment of the third party cells with rayon column passage largely abrogated the suppressive effect. In experiment 2, addition of irradiated cells instead of cells treated with mitomycin C demonstrated that the suppressive effect is radioresistant. In addition, pretreatment of the spleen cells with iron and magnet to deplete the phagocytic cells largely abrogated the suppressive effect of the unfractionated population. Experiment 3 shows that cells treated with ATS plus complement were even more suppressive than the unfractionated controls and this has been a consistent finding. Addition of thymocytes into the CML cultures frequently stimulated the generation of cytotoxicity as shown in this experiment. In contrast, addition of light mineral oil-induced PECs always completely abrogated the cytotoxic response. Table 5 illustrates the relative suppressor activities of unfractionated spleen cells compared to thymus and PEC. In these experiments, column-passaged responding spleen cells were combined with various concentrations of the third party cells and the cytotoxicity generated measured on the fourth day of culture. In both experiments a concentration of spleen cells 16 times the concentration of PECs was required to give similar amounts of suppression. At no concentration tested did the addition of thymocytes suppress the cytotoxic response as much as the smallest dose of PECs.

DISCUSSION

The results of the present study provide an explanation for difficulties previously observed in generating a proliferative or cytotoxic response to alloantigens *in vitro*

using rat spleens as responding cells (1, 6). We have found that the poor pro-liferative and cytotoxic responses observed are due to the activity of suppressor cells present normally in rat spleens and not to the lack of cells capable of respond-ing. Experiments in which adherent or phagocytic cells were depleted from the responding cells showed greatly augmented MLC and CML activity. This sug-gested that the suppressor cells were phagocytic and adherent. This was supported by the finding that, although the addition of unfractionated cells into MLCs as third parties strongly suppressed both the proliferative and cytotoxic responses, spleen cells depleted of adherent or phagocytic cells had no such effect. Pretreat-ment of the cells with ATS and C never decreased the suppressor effect and usually enriched for it. Thymocytes contained very little if any suppressor activity. In con-trast, PECs consistently were very highly suppressive. In addition, the suppressor cell did not require proliferation to be effective as demonstrated by all the experi-ments in which the third party cells were either treated with mitomycin C or x-irradiation. These data indicate the suppressor cells are probably macrophages. This is consistent with previous reports from our laboratory. Tumor-bearing mice (8) and rats (9) and mice injected with *Corynebacterium parvum* (19) have been shown to have a population of splenic macrophages which can inhibit proliferative responses to mitogens and tumor-associated antigens and also the *in vitro* gener-ation of a secondary cytotoxic response to tumor-associated antigens. In addition, we have recently shown that normal rat spleens have suppressor macrophages which can inhibit secondary proliferative responses to tumor-associated antigens (13). Fernbach *et al.* (12) have shown that macrophages in PECs can inhibit MLC and CML in the mouse. The present study extends these observations to include the rat.

Spleen cells are routinely used in *in vitro* assays of cellular immunity in mice. From this study, it is apparent that unfractionated rat spleen cells may give sub-optimal responses in *in vitro* assays. Whether suppressor macrophages are only present in normal rat spleens and not in normal mouse spleens, or whether the suppressor activity in normal mouse spleens is just too low to be detected in the usual *in vitro* assays is unclear. However, results of the growth inhibition assay [which reflects antiproliferative effects of macrophages in mice (14) and rats (13)] suggest that suppressor macrophage activity may exist in normal mouse spleens also but to a far lesser degree (14). Thus, it is likely that either rat splenic macro-phages are more suppressive than mouse splenic macrophages or that rat lympho-cytes are more sensitive to the suppressor effects. It is possible that rats are naturally exposed to more antigenic stimuli with resulting increased baseline levels of macrophage activation, but against this is the finding that germ-free rat spleens were just as inhibitory in the GIA as spleen cells of conventionally bred rats (Oehler and Herberman, unpublished observations).

Folch and Waksman (7) have previously described suppressor cell activity in the spleens of normal rats, which could inhibit MLC responses. They characterized the suppressor cell as adherent and sensitive to pretreatment with cycloheximide and resistant to pretreatment with mitomycin C. However, since they have found suppressor cell activity to be largely abrogated by adult thymectomy, irradiation, and bone marrow reconstitution, they have concluded that it is a T cell.

Our data would support the characteristics they described for the suppressor cell, i.e., that it is adherent and does not require proliferation to be effective. The

main difference in our conclusions stems from the interpretation of the *in vivo* aspect of their studies, which indicated that B rats have little or no suppressor activity. A reasonable explanation for the disparate findings would be that rat splenic macrophages may require a certain level of T-cell activity *in vivo* in order to acquire or maintain their suppressor activity. In rats without *in vivo* T-cell influence, splenic macrophages may become less activated or may migrate from the spleen. There is abundant evidence that, for some functions, T cells are required for activation of macrophages (20-22). We are currently performing experiments to directly determine whether the activation of splenic suppressor macrophages is T cell dependent.

Of central importance is the *in vivo* significance of these findings. In the past few years, much attention has been directed toward regulation of the immune response and numerous reports have implicated T cells as the primary regulatory cell. Depending on the conditions present, T cells have been shown to either help or suppress B-cell responses to antigen (23). Similarly, T cells have been reported to suppress or enhance the responses of other T cells (4, 24–29). The present study shows that macrophage type non-T cells found in normal rat spleens can suppress T-cell responses and therefore may also be involved in the regulation of the immune response. In addition, it is possible that in some studies in which suppressor T cells have been described, as in the suppressor phenomenon reported by Folch and Waksman (7), the suppressor cells may in fact be T cell-dependent suppressor macrophages.

ACKNOWLEDGMENTS

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