FEEDBACK SUPPRESSION OF THE IMMUNE RESPONSE IN VITRO

I. Activity of Antigen-stimulated B Cells*

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Powerful and often complex regulatory mechanisms that maintain homeostasis are a *sine qua non* of all carefully studied physiologic activities. Thus, it is not surprising that the immune system has evolved a sophisticated regulatory apparatus for preventing excessive responses to antigenic stimulation. The specific mechanisms so far described include (*a*) classical antibody feedback which is believed to operate by interference with cellular recognition of antigenic determinants (1, 2) and (*b*) development of suppressor T lymphocytes (T_s)[1] (3, 4), recognizing either antigenic (5) or idiotypic (6) determinants and functioning in part by the release of subcellular effector molecules (7–10). Until now, these two broad categories have been considered as largely independent pathways. However, it would seem likely that to most efficiently control humoral immunity, some interrelationship between these modalities would have evolved.

The initial purpose of these studies was to further characterize the triggering and mode of action of suppressor T cells and T-suppressor factors (T_sF). Previous data have shown that T_s play a major role in the regulation of the Ir gene-controlled response to L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT): GAT priming of nonresponder mice leads primarily to stimulation of GAT-specific T_s (11), which produce a soluble GAT T_sF factor (GAT-T_sF) (9) bearing I-region determinants and sharing a common idiotype with anti-GAT antibodies (12) that stimulates virgin T cells to develop T_s activity (GAT-T_sF-induced T_s [T_{s_2}]) (13). Furthermore, similar T_s and T_sF can be obtained from responder lymphocytes, provided that antigen-presenting adherent cells are removed before GAT exposure (14). These data, in agreement with those of others (15), indicated that predominant T_s stimulation occurred when antigen-presentation to helper T cells was compromised.

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[1] *Abbreviations used in this paper:* B6, C57BL/6; BDF, (C57BL/6 × DBA/2)F_1; FACS II, fluorescence-activated cell sorter; FCS, fetal calf serum; GAT, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-T_sF, soluble GAT T-suppressor factor; HRBC, horse erythrocyte(s); MDM, Mishell-Dutton medium; 2-ME, 2-mercaptoethanol; MEM, minimum essential medium; MIg, mouse myeloma Ig; NMS, normal mouse serum; NRIg, normal rabbit Ig; PBS, phosphate buffered saline; PBS-5% FCS, PBS containing 5% FCS; PEC, peritoneal exudate cells; PFC, plaque-forming cell(s); RAMIg, rabbit anti-mouse Ig antibody; sIg^+, surface Ig positive; sIg^-, surface Ig negative; SRBC, sheep erythrocyte(s); T_s, suppressor T lymphocytes; T_sF, T suppressor factor; T_{s_2}, GAT-T_sF-induced T_s.

More recently, Eardley and Gershon (16), Eardly et al. (17), and Cantor et al. (18, 19) have shown that T-cell suppression can also be generated as a result of activation of a unique T-inducer subset during the course of an active, ongoing immune response. Their studies indicate that antigen-stimulated Lyt 1^+:Qa 1^+ inducer cells (*a*) activate B lymphocytes to produce anti-sheep erythrocyte (SRBC) plaque-forming cells (PFC) in a second culture stimulated with SRBC and (*b*) activate a set of T_s (Lyt $1^+2^+3^+$: Qa 1:I-J^+) cells present in unselected spleen cell suspensions (17, 18). Similar data have also been obtained in vivo (19). These findings, i.e., antigen-primed T cells induce target T cells to become suppressive, are strikingly similar to the induction of T_{s_2} by GAT-T_sF. It was therefore of interest to ask if the T-cell subsets involved in GAT-specific suppression were the same as those identified in the SRBC model. The initial experiments attempted to define conditions leading to the in vitro production of SRBC-specific T_sF, to be studied in parallel with GAT-T_sF production and activity. However, during these studies it was found that T lymphocytes alone were inefficient under our experimental condition in generating suppressive activity upon antigen (SRBC) exposure. This report documents that T-dependent B lymphocytes are necessary for the production of potent in vitro suppression of SRBC responses, and that, in fact, it is the B, not T, cells recovered from SRBC-educated spleen cell populations that are crucial for the transfer of suppression in this model. The possibility that these suppressor B cells are involved in antibody feedback and/or T_s generation is considered.

Materials and Methods

Mice. (C57BL/6 × DBA/2)F_1 (BDF) mice, males or females, 2–6 mo old, were used in most experiments and purchased from The Jackson Laboratory, Bar Harbor, Maine, or from the Health Research Inc., West Seneca, N. Y. C57BL/6 (B6) males or females and DBA/2 female mice of the same age were obtained from The Jackson Laboratory. The mice were maintained in our animal facilities on standard laboratory chow and acidified, chlorinated water.

Antigens. SRBC were prepared from sheep blood in Alsever's solution (14) purchased from Gibco Diagnostics, Gibco Invenex Div., Chagrin Falls, Ohio, and from Colorado Serum Co., Denver, Colo. SRBC from three individual sheep were selected because they stimulated a high PFC response in vitro for B6 and BDF cells and these three types of SRBC were used interchangeably for the experiments. Horse erythrocytes (HRBC) were similarly obtained from horse blood purchased from Colorado Serum Co. as pools from several animals.

Cell Cultures

EDUCATION WITH SRBC. The protocol developed by Eardley and Gershon (16) was used. Spleen cell suspensions or selected cell populations were suspended in complete Mishell-Dutton medium (MDM) (20) buffered with 10 mM Hepes (14) but without 2-mercaptoethanol (2-ME) except where mentioned. The medium contained 10% fetal calf serum (FCS) obtained from Reheis Chemical Co., Phoenix, Ariz. (lot R61306). The cells were suspended at a concentration of 10^7 viable cells (as assessed by counting in trypan blue)/ml in MDM. 3–3.5 ml was added to 60- × 15-mm tissue culture dishes (Falcon 3002; Falcon Labware, Div. of Becton, Dickinson & Co. Oxnard, Calif.) or 1 ml was added to 35- × 10-mm dishes (Falcon 3001) with or without 2×10^6 SRBC/ml. Cells were cultured for 4 d (except where otherwise stated) on a rocking platform at 37°C in a humidified 10% CO_2, 7% O_2, and 83% N_2 atmosphere. Cultures were fed daily with a feeding mixture (70 μl/ml starting vol) composed of 50% nutritional cocktail (20) and 50% FCS. Viable cell recoveries on day 4 were 15–35% for unseparated spleen cells.

TEST CULTURES. These were set up as 7.5×10^6 viable spleen cells in 1 ml MDM per 16-mm well of flat-bottom tissue culture plates (FB-16-24 TC; Linbro Chemical Co., Hamden, Conn.), and stimulated with 2×10^6 SRBC or 3×10^6 HRBC/culture. Cells that had been educated in the presence or absence of SRBC were washed three times in Eagle's minimum essential

medium (MEM) (Microbiological Associates, Walkersville, Md.) buffered with 10 mM Hepes, before the indicated numbers of viable cells in 100 μl MEM were added into the test cultures. Test cultures lasted 4 d (except where otherwise stated) under the same incubation conditions as for education cultures.

Lymphocyte Separation Techniques

NYLON-WOOL PASSAGE. Nylon-wool passed, T cell-enriched populations were obtained according to the method of Julius et al. (21). Between 12 and 20% of the input cells were recovered as nonadherent cells; <5% of these cells were surface Ig positive (sIg^+) as assessed by immunofluorescence with fluoresceinated rabbit anti-mouse Ig antibody in the presence of 0.2% NaN_3. Viable cell recovery after in vitro education of such T-cell preparations was 3–5% in the absence and 16–23% in the presence of 5×10^{-5} M 2-ME. In some experiments, nylon-wool-passed cells were supplemented with irradiated peritoneal exudate cells (PEC) which were obtained as described (14). Viable cell recovery after education in the presence of 10^5 PEC/10^7 nylon-wool-passed cells was 25–30%.

SEPARATION ON ANTIBODY-COATED POLYSTYRENE DISHES. The basic protocol of this technique was that developed by Mage et al. (22) and Wysocky and Sato (23), for the separation of spleen cells into sIg^+ and surface Ig negative (sIg^-) fractions. Purified rabbit anti-mouse Ig antibody (RAMIg) was prepared by affinity chromatography of hyperimmune rabbit anti-mouse Ig antiserum on a mouse Ig-Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) immunoadsorbent. The purified antibody was filtered and stored at 4°C as a 1 mg/ml solution in phosphate-buffered saline (PBS). Normal rabbit immunoglobulin (NRIg), used in control experiments and for dilution of purified RAMIg, was obtained by two precipitations of pooled normal rabbit serum with Na_2SO_4, followed by dialysis against PBS, and also stored as 1 mg/ml sterile solution at 4°C.

For the coating of dishes with Ig, 5 ml of a 1 mg/ml solution of RAMIg, RAMIg diluted 1:10 in NRIg, or NRIg alone was added to 100- × 20-mm Petri dishes (Falcon 1005), and incubated for 1 h at room temperature. Unbound antibody was removed, the plates were washed extensively with PBS, and 5 ml of PBS containing 5% FCS (PBS-5% FCS) was added to the dishes and left for 30–60 min before adding the cells to be separated. In some experiments, to specifically block the RAMIg activity on the dishes, plates coated with RAMIg were washed with PBS and then treated for 2 h at 4°C either with 10% normal mouse serum (NMS) in PBS or with mouse myeloma Ig at a concentration of 140 μg/ml in PBS. Such dishes were then washed with PBS-5% FCS before adding the cells. The mouse myeloma Ig (MIg) used was a mixture of equal amounts of MOPC 104E (μ/λ) and MOPC 31C (γ/κ) proteins.

All cell populations to be separated were incubated for 4 min at 37°C with Tris-NH_4Cl buffer to lyse erythrocytes. All separations were done at 4°C. In experiments where normal or educated spleen cells passed over dishes coated with RAMIg were compared to cells passed over various control dishes, the nonadherent cells were recovered after a single 1-h incubation of 6×10^7 cells in 5 ml PBS-5% FCS per dish. For all other separations of sIg^- and sIg^+ cells a double cycle protocol was used: 12×10^7 cells in 5 ml PBS-5% FCS were added per dish in the first step. After 1 h, the nonadherent sIg^- cells as well as the adherent sIg^+ cells were then recovered and each fraction was again added for 1 h to a dish coated with RAMIg. When normal spleen cells were separated, the first step and the purification of sIg^+ cells in the second step were done using dishes coated with RAMIg diluted 1:10 in NRIg. Otherwise, sIg^+ cells could not be recovered by flushing and aspiration with a Pasteur pipette. When educated cells were separated, it was, however, necessary to use undiluted RAMIg to retain sIg^+ cells and anti-SRBC PFC on the dishes. Recovery and purity of the cell populations (counts of sIg^+ cells and of anti-SRBC PFC) will be shown in the Results.

Treatment with Anti-Lyt Antisera. The preparation of the anti-Lyt antisera has been described (24). The sIg^+ cell fraction from BDF spleen cells used for the reconstitution of sIg^- cells was treated with a mixture of anti-Lyt antisera (anti-Ly 1.1, 1.2, 2.1, 2.2, and 3.2) for 35 min at room temperature, washed, and then exposed to selected rabbit complement for 30 min at 37°C. The whole treatment was then repeated. The SRBC-educated cells from B6 were also treated in two steps. In the first step, a mixture of anti-Ly 1.2 and 2.2 was used; in the second step, a mixture of anti-Ly 1.2 and 3.2 was used.

Treatments with Anti-H-2^d Antiserum. An antiserum raised in B6 mice by hyperimmunization with the DBA/2 (H-2^d) tumor P815 was used for the selective removal of BDF sIg^- cells from

an educated mixed population of such cells and B6 sIg^+ cells. 4×10^6 educated cells were treated with 0.3 ml of antiserum (diluted 1:3.5 in MEM) for 30 min at 4°C, washed once, and then treated with 1 ml of selected rabbit complement (diluted 1:8 in MEM).

Assay for PFC. The direct (IgM) anti-SRBC or anti-HRBC PFC and the facilitated (IgG) anti-SRBC PFC were assayed by a slide modification of the Jerne hemolytic plaque assay as described (25). For the assay of IgG PFC the formation of IgM plaques was inhibited by addition of goat anti-mouse μ-chain antiserum into the assay mixture as described (25). The IgM and IgG anti-SRBC PFC in educated cells or separated fractions from such cells were usually measured 24 h after the transfer of the cells into test cultures to aid in detecting IgG PFC which were just developing at day 4–5. The background level of anti-SRBC or HRBC PFC in control cultures without erythrocyte antigens was always <8%, and in most experiments <5% of the response in cultures stimulated with SRBC or HRBC and was subtracted from all results.

Results

Generation and Specificity of SRBC Suppressor Cells. To enable a comparison of the T-cell interactions involved in GAT- and SRBC-specific T-cell suppression, a SRBC-specific feedback suppression model was established according to the protocol developed by Eardley and Gershon (16). Spleen cells from B6F or B6 mice were educated for 4 d in vitro under Mishell-Dutton type conditions in the presence of SRBC (2×10^6/ml) and, in all experiments, also in the absence of SRBC. On day 4, the educated or control-educated cells were titrated into fresh SRBC-stimulated spleen cell test cultures (with 7.5×10^6 spleen cells and 2×10^6 SRBC/culture) at the time of culture initiation. The suppression of the anti-SRBC IgM PFC response in test cultures receiving SRBC-educated cells was then measured on day 4 and compared with the PFC response in the presence of control educated cells. A representative experiment is shown in Table I. $3–5 \times 10^5$ SRBC-educated cells suppressed the test response by >80%. An education period of 4 d was found to be optimal. Cells educated for only 3 d had no suppressive activity, whereas after 5 d of education, the control cells often exhibited considerable nonspecific suppressive activity. Day-4 educated cells could suppress the IgG as well as IgM PFC response of test cultures. Such day-4 SRBC-educated cells did not suppress an anti-HRBC IgM PFC response in cultures stimulated with HRBC alone or simultaneously with HRBC and SRBC (Table II), demonstrating the specificity of suppression mediated by educated cells.

Attempts were next made to generate suppressor cells from purified T cells. Nylon-wool passed spleen cells were used as a T cell-enriched population for education with SRBC. Using such cells, however, no suppressive activity was generated. Modifying the culture conditions did not permit generation of suppressor T cells in this system; neither reconstitution of nylon-wool-passed cells with PEC nor addition of 2-ME to the cultures permitted the development of an active suppressor population using nylon-wool-passed T cells.

Education of sIg^- Cells. Because it has been reported that nylon wool removes some T-cell subsets that may be crucial in suppressor cell activity (26), a second technique was used to obtain T cell-enriched cells. Spleen cells were depleted of sIg^+ cells by separation on plastic dishes coated with RAMIg, and the sIg^- cells were then educated with SRBC. The sIg^- cells obtained after a single passage over these dishes developed only 1/10 the suppressor cell activity of unseparated SRBC-educated cells after 4 d of culture with antigen (Fig. 1). It was also found that this small amount of suppressive activity obtained with sIg^- cells correlated with the number of anti-SRBC PFC

TABLE I

Suppressive Activity of SRBC-educated Cells

Cells added/culture	Anti-SRBC IgM PFC/culture		Suppression
	Control-educated cells	SRBC-educated cells	
			%
5×10^5	9,910	980	90
1.5×10^5	10,030	1,050	90
5×10^4	11,470	2,700	76
1.5×10^4	11,950	6,850	43
5×10^3	11,150	8,670	23
Control response (no cells added)	11,030		

Spleen cells from BDF mice were educated for 4 d in the presence of 2×10^6 SRBC/ml and then tested for suppressive activity on a test culture of 7.5×10^6 cells. The values for the percent suppression represent specific suppression compared to the effect of cells educated in absence of SRBC.

TABLE II

Antigen Specificity of SRBC Suppressor Cells

	IgM PFC/test culture			
	Experiment 1: SRBC and HRBC in separate cultures		Experiment 2: SRBC and HRBC in the same cultures	
	Anti-SRBC	Anti-HRBC	Anti-SRBC	Anti-HRBC
Control response	6,740	1,080	9,830	505
5×10^5 control-educated cells	5,020	900	10,005	555
5×10^5 SRBC-educated cells	990	890	1,160	545
Suppression	80%	1%	88%	2%

Educated spleen cells from BDF mice were added to cultures stimulated separately or simultaneously with SRBC and HRBC, in different experiments.

generated during the education. Moreover, double cycle purified sIg^- cells that generated almost no PFC had no detectable suppressive activity after either 4 or 5 d of education. As is further shown in Fig. 1, various control experiments were performed to test whether the adherence of Fc-receptor-bearing sIg^- cells was responsible for the depletion of suppressor cells from the dish-nonadherent fraction. It can be seen that the nonadherent cells recovered from dishes coated with NRIg or from dishes coated with RAMIg on which the anti-mouse Ig activity was blocked with NMS or MIg were not depleted of the cells needed to generate suppression. Thus, depletion of such cells was only observed when there was specific removal of sIg^+ cells even under conditions in which immune complexes were intentionally generated on the dish to bind Fc receptor-positive cells.

Generation of Feedback Suppression by Reconstituted sIg^- and sIg^+ Cells. Effective generation of suppressor activity was observed when double cycle purified sIg^- and sIg^+ were educated together but not when either fraction was educated separately with SRBC (Table III). The anti-SRBC PFC response was also reconstituted in these experiments. One should mention that for separate education the sIg^+ cells had to be

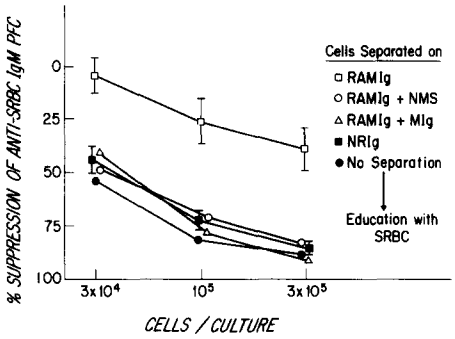

FIG. 1. Suppressive activity of BDF spleen cells passed over plastic dishes coated with RAMIg or control dishes and then educated with SRBC. The nonadherent, predominantly sIg^- cells (20–35% recovery, <3% sIg^+) were recovered after a single passage on RAMIg and generated 235 ± 33 SRBC IgM PFC/10^6 educated cells after 4 d of education. Unseparated cells or cells passed over control dishes (70–95% recovery, 45–60% sIg^+) generated 2,460 ± 205 PFC/10^6 educated cells. Control dishes were coated with NRIg, or were coated with RAMIg and then blocked by preincubation with NMS or by preincubation with MIg. The results show the means (± SE) of the percent suppression of the test culture response. This is specific suppression which is compared to the effects of the respective cell populations when educated in the absence of SRBC. The mean control response in test cultures was 7,270 ± 1,400 anti-SRBC IgM PFC.

supplemented with splenic adherent cells to obtain viable cells after education because most macrophages are contained in the sIg^- fraction under these separation conditions. In contrast, sIg^- cells alone had an excellent viability. Each fraction alone generated almost no PFC. The same result was obtained when sIg^- cells as well as the sIg^+ cells were supplemented with adherent cells.

The possibility that a T cell essential for T cell-mediated suppression separated with the sIg^+ cells and was reintroduced into the system with the sIg^+ cell fraction was further tested by treating the sIg^+ cells with anti-Ly antisera and complement. Equivalent suppressive activity was generated whether or not the sIg^+ cell fraction has been subjected to a combined treatment with anti-Ly 1, 2, and 3 antisera and complement before its use for the reconstitution of sIg^- cells. Therefore, both T and B cells were essential for the generation of suppression under these conditions (Table III).

sIg^+-educated Cells are Responsible for Suppression. In the following experiments, it was studied whether educated T cells or B cells caused the observed feedback suppression. Educated cells were separated into sIg^- and sIg^+ fractions using RAMIg-coated plastic dishes. It was found that the sIg^+ cells were enriched and the sIg^- cells relatively depleted of suppressor cells compared to unseparated educated cells (Fig. 2). Control separation experiments were done as before for noneducated cells (Fig.1) to test whether the separation was specific for sIg^+ cells rather than for Fc-receptor positive cells. SRBC-suppressor cells were not adherent to dishes coated with NRIg or to dishes coated with RAMIg which had been blocked by preincubation with NMS before adding the cells (data not shown). Thus, suppressor cells were only lost by passage over unblocked RAMIg. One should note that the sIg^- fractions had still significant suppressive activity (Fig. 2). But such cells also contained 1/7–1/10 as many anti-SRBC PFC as the unseparated cells, showing that the separation was not complete. In contrast to separations of uneducated cells, we were not able to get better

TABLE III

Generation of SRBC Suppressor Cells by Recombined sIg^- and sIg^+ Cells

Educated cells (3×10^6/culture)	Anti-SRBC PFC/10^6 educated cells		Anti-SRBC IgM PFC/culture		Mean suppression‡
	IgM*	IgG (Experiment 2)*	Experiment 1	Experiment 2	
					%
Control response			3,040	6,540	
Unseparated cells	3,630	—	440	2,780	72
sIg^- cells§	50	—	3,260	6,830	−6
sIg^+ cells§	130	—	3,140	5,500	7
sIg^- + sIg^+ cells	2,190	335	740	720	83
sIg^- + (sIg^+ treated with complement)‖	1,180	220	—	2,760	58
sIg^- + (sIg^+ treated with anti-Ly 1, 2, 3 + complement)‖	1,150	260	—	2,350	64

sIg^- (<2% sIg^+) and sIg^+ (>95% pure) cells were obtained from BDF spleen cells by double cycle separation on plastic dishes coated with RAMIg. These populations were educated for 4 d with SRBC, either separately or after reconstitution (sIg^-:sIg^+ = 4:6).

* The anti-SRBC IgM and IgG PFC in educated cells were measured 24 h after the transfer into test cultures.

‡ The values for the percent suppression represent mean suppression compared to the control response.

§ The sIg^+ cells in both experiments and the sIg^- cells used in experiment 1 but not in experiment 2 were supplemented with splenic adherent cells. Therefore, normal spleen cells (10^7/ml) were incubated for 3 h at 37°C in the culture dishes. The dishes were then washed and irradiated with 1,500 rad and the same number of sIg^+ cells was added. Viable cell recovery after education was 10–18% for sIg^+ cells supplemented with adherent cells compared to 0% for sIg^+ cells alone. Recovery was 21% for sIg^- cells in experiment 1 (supplemented with adherent cells) and 30% in experiment 2 (sIg^- cells alone).

‖ sIg^+ cells were treated twice with a mixture of anti-Ly 1.1, 1.2, 2.1, 2.2, and 3.2 antisera and complement or with complement alone.

separation of already educated cells as far as the levels of anti-SRBC PFC are concerned. Similar results to those observed with the dish separation technique were also obtained when educated cells were separated into sIg^- and sIg^+ cells using the fluorescence-activated cell sorter (FACS) after staining with a fluoresceinated rabbit $F(ab)'_2$ anti-mouse Ig. The sIg^+ fraction was enriched and the sIg^- fraction was >10-fold depleted of suppressor cells (data not shown).

The possible role of small numbers of sIg^- cells (T cells) in mediating the observed suppression was further tested by treating educated cell populations with various antisera plus complement. First, it was found in two experiments that a treatment with anti-Thy-1 antiserum and complement did not decrease the suppressive activity of educated cells (data not shown). However, it has been reported previously that the T cells mediating suppression in this system are largely resistant to anti-theta antiserum and complement treatment (16). Therefore, educated spleen cells were treated with anti-Ly 1, 2, and 3 antisera and complement. The remaining cells were slightly more efficient than an equal number of complement-treated or untreated cells in suppressing the anti-SRBC test culture response (Table IV). The treated cells were also enriched for IgM and IgG anti-SRBC PFC.

Finally, experimental conditions were then chosen to use a treatment with anti-H-2 antiserum and complement to selectively eliminate the sIg^- cells in educated

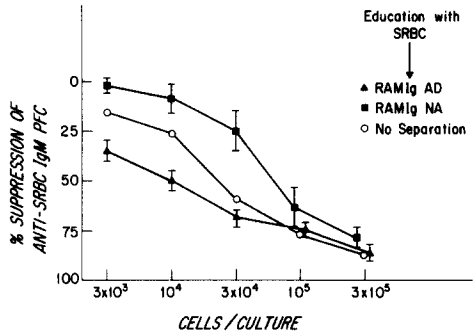

FIG. 2. Comparison of the suppressive activity of sIg^+ adherent (AD) and sIg^- nonadherent (NA) cell fractions obtained by double cycle separation of educated BDF spleen cells on dishes coated with RAMIg. The recovery for AD cells was 11–13%, for NA cells 25–27%. The anti-SRBC IgM and IgG PFC/10^6 recovered cells were measured 24 h after the transfer into test cultures and were: 1,350 ± 410 IgM, 347 ± 81 IgG for unseparated cells; 4,060 ± 1,540 IgM, 1,420 ± 650 IgG for AD cells; 170 ± 70 IgM, 55 ± 22 IgG for NA cells. The results are expressed as for Fig. 1. The mean control response in the test cultures was 9,080 ± 150 anti-SRBC IgM PFC.

TABLE IV

Effect of a Combined Treatment of SRBC-educated Cells with Anti-Ly 1, 2, and 3 Antisera and Complement

	Anti-SRBC PFC per 10^6 educated cells after treatment*		Cells added/culture	Anti-SRBC IgM PFC/test culture	Suppression
	IgM	IgG			
					%
Control response				5,595	
Control-educated cells	NT	NT	3×10^5	6,480	
			10^5	6,270	
			3×10^4	6,030	
SRBC suppressor cells	1,240	500	3×10^5	1,065	84
			10^5	2,340	63
			3×10^4	4,470	26
SRBC-suppressor cells + complement	1,570	490	3×10^5	765	88
			10^5	2,070	67
			3×10^4	4,560	24
SRBC-suppressor cells + anti-Ly 1, 2, 3 + complement	2,100	850	3×10^5	675	90
			10^5	1,245	80
			3×10^4	2,445	60

Educated spleen cells from B6 mice were treated with anti-Ly 1.2, 2.2, and 3.2 antisera and complement or with complement alone. NT, not tested.

* PFC in educated cells were measured 24 h after the transfer into the test cultures.

populations. For this purpose sIg^- cells from BDF ($H\text{-}2^{b/d}$) mice were reconstituted with sIg^+ cells from B6 ($H\text{-}2^b$) parental mice and educated with SRBC. After 4 d of education, the cell population was treated with a B6 anti-P815 (anti-$H\text{-}2^d$) antiserum and complement and tested for suppressive activity in test cultures with B6 cells. The results are shown on Table V. The treated cells were still as effective as cells treated

TABLE V

Effect of Selective Elimination of T Cells with Anti-H-2^d Antiserum and Complement on SRBC-suppressor Cell Activity Generated by Education of BDF T Cells Reconstituted with B6 B Cells

Composition of educated cells		Treatment of educated cells	Cells added per test culture			Anti-SRBC IgM PFC per test culture		Suppression
			Total viable cells	Anti-SRBC IgM PFC	Anti-SRBC IgG PFC	B6 culture	DBA/2 culture	
			× 10^{-5}					%
Control response						5,610	3,020	
(A) Selective killing of T cells								
T_{BDF}* + B_{B6}‡	CO‖	—	3	NT	NT	4,635		
	SC¶	Complement (C)	4.4	1,350	360	465		90
	SC	Anti-H-2^d + C	1.1	750	270	810		83
(B) Specificity controls for anti-H-2^d antiserum								
T_{BDF} + $B_{DBA/2}$§	CO	—	3	NT	NT		3,030	
	SC	C	2.8	480	190		410	86
	SC	Anti-H-2^d + C	0	0	0		3,340	−10
B6 spleen	CO	—	3	NT	NT	5,025		
	SC	C	3.2	1,340	360	960		81
	SC	Anti-H-2^d + C	2.7	1,370	350	870		83
DBA/2 spleen	CO	—	3	NT	NT		3,700	
	SC	C	3.2	530	171		960	74
	SC	Anti-H-2^d + C	0	8	0		3,240	12
BDF spleen	CO	—	3	NT	NT	5,220	2,920	82/75
	SC	C	3.4	1,220	363	950	720	−14/−22
	SC	Anti-H-2^d + C	0	27	0	5,930	3,560	

Equal amounts of educated cells were treated with B6 anti-P815 (anti-H-2^d) antiserum and C and then suspended in equal volumes of culture medium and tested for suppressive activity (Materials and Methods). It was also found that separate education of BDF T cells, B6 B cells, or DBA/2 B cells did not generate SRBC-suppressor cell activity (results within ± 6% range of control response). NT, not tested.

* T_{BDF}, BDF T cells.

‡ B_{B6}, B6 B cells.

§ $B_{DBA/2}$, DBA/2 B cells.

‖ CO, control-educated cells.

¶ SC, SRBC-educated cells.

with complement alone. The following controls were done in the same experiment. First, the sIg^- and sIg^+ cell fractions were also educated separately with SRBC and were found to have no suppressive activity. Second, educated spleen cells from B6, BDF, and DBA/2 (H-2^d) mice, and a population of educated sIg^- cells from BDF mice reconstituted with sIg^+ cells from DBA/2 mice were treated with B6 anti-P815 antiserum and complement. Treatment of B6 cells did not eliminate the suppression or the anti-SRBC PFC, but no viable cells and no suppressor activity were recovered in the other (H-2^d bearing) control groups, thus showing that the antiserum had specific activity.

Discussion

Under the conditions of experiments initially designed to duplicate the protocol of Eardley and Gershon for generating SRBC-T_s in vitro (16), we found that SRBC-stimulated B cells were best equipped to suppress an in vitro primary anti-SRBC PFC response. This conclusion is based on several observations: (*a*) stimulation of nylon-wool-passed cells or sIg^- cells for 4 or 5 d in the presence or absence of macrophages never led, in our hands, to generation of suppressor cells; (*b*) both sIg^- and sIg^+ cells were required to generate suppressive activity; the latter cell population could be obtained after treatment of spleen cells with anti-Ly 1, 2, and 3 antisera and complement; (*c*) sIg^+ cells obtained from educated spleen cells (using plastic dishes coated with RAMIg) were consistently enriched in suppressive activity, whereas the sIg^- fraction contained reduced activity. This cell separation depended upon anti-MIg activity on the dishes, and thus did not reflect selection of sIg^- Fc-receptor-positive cells in the sIg^+ fraction; (*d*) the suppressive activity of educated cells was maintained or enriched after selection of sIg^+ cells on the FACS II or after combined treatment with anti-Ly 1, 2, and 3 antisera and complement; (*e*) educated cells containing a mixture of BDF ($H\text{-}2^{b/d}$) sIg^- cells and B6 ($H\text{-}2^b$) sIg^+ cells retained suppressive activity after treatment with anti-$H\text{-}2^d$ and complement to remove all T cells. Because (*a*) the sIg^+ cell population (after fractionation on anti-Ig-coated plates or FACS II separation) was relatively depleted of macrophages and (*b*) suppression was antigen-specific, macrophages are unlikely to contribute to suppression in these experiments.

The initial purpose of these experiments was to obtain a soluble factor from T cells that might induce T_s in analogy with the GAT system (13). Therefore, the in vitro SRBC-education protocol developed by Eardley and Gershon (16) was followed in an attempt to generate an SRBC-specific T_s source from which to prepare T_sF. Using this model, Eardley and Gershon (16) and Eardley et al. (17), in a careful series of experiments, have found that T cells obtained by nylon-wool passage (16) or by passage through anti-MIg columns (17) could be educated with SRBC in the absence of B cells and then could mediate feedback inhibition. Feedback suppressor cells were also enriched in the nylon-wool-nonadherent fraction obtained from educated spleen cells (16). In subsequent studies it was shown that a subset of Ly 1 inducer cells ($Lyt\ 1^+$:$Qa\ 1^+$) activates suppression by stimulating nonimmune acceptor cells ($Lyt\ 1^+2^+3^+$: $Qa\ 1^+$) to either differentiate into $Lyt\ 2^+3^+$ T_s, or to augment suppressive activity of the latter cell set, both in vitro (17, 18) and in vivo (19). This same $Lyt\ 1^+$ cell has recently been shown to also modulate the in vitro primary response to streptococcal antigen (27). Further analysis of this T-T interaction in vitro showed that although $Lyt\ 1^+$-induced suppression of test cultures was often preceded by a precocious anti-SRBC response, this precocious activation of B cells did not necessarily correlate with the suppression observed (28).

It was therefore surprising that under our experimental conditions, there was an absolute requirement for B cells (sIg^+, $Lyt\ 1^-2^-3^-$) for generating suppressor cells. This requirement distinguishes our test system from both GAT and previously studied SRBC models. It should be emphasized, however, that although the results do not duplicate the T cell-mediated feedback suppression obtained by Eardley and Gershon (16) and Eardley et al. (17) using similar methods, they by no means imply that such a process, i.e., the stimulation of suppressor T cells by activated $Lyt\ 1^+$ cells, does not

normally occur. They simply indicate that under certain in vitro conditions, antigen-stimulated B cells are required to obtain suppression, even for T-dependent antigens. Other investigators have described suppressor B cells, usually generated in vivo (29–33). The degree to which physiological (in vivo) immune responses to thymus-dependent antigens are regulated by antigen-activated T cells alone, or by suppressor pathways involving activated B cells, clearly remains to be determined. Related to this issue is the question of why we failed to observe the generation of either Lyt 1^+ inducer cells or SRBC T_s in our experiments, and it would seem important to delineate those (culture) conditions which favor the triggering of one or the other suppressor pathway.[2]

The mechanism of suppression by educated B cells has not been addressed in our report. The simplest possibility is that B cells suppress via the production of soluble antibody. Studies of classical antibody feedback showed that such antibodies, usually IgG from hyperimmune mice, might act by blocking antigenic determinants (1, 2, 34). If direct antibody feedback is involved here, it is astonishingly effective. Very few (two–three detectable) IgG-secreting cells are able to suppress a test culture which is in agreement with the report that the IgG fraction of the in vivo anti-SRBC response was suppressed after transfer of as few as 10^4 in vivo primed spleen cells containing only 30 IgG PFC (33).

Alternatively, feedback suppression of the anti-SRBC PFC response in vitro by B cells from in vivo primed mice has been shown to be dependent upon the presence of unprimed T cells (35, 36). More recent work has demonstrated that Ig-associated structures on activated B cells trigger Lyt 1^+2^- inducer cells to stimulate Lyt 2^+ T-suppressive activity in vivo (36) and in vitro.[3] The precise molecular basis of B-cell stimulation of T suppression was not defined in these analyses, but the report by Eardley et al. (37) that the inducer:acceptor T-T interaction that generates SRBC-specific T_s is controlled by V_H-linked genes may indicate the involvement of idiotypic recognition by the relevant B and T cell sets. Therefore, suppression by activated B cells as shown here might also result directly from B-cell secretion of anti-idiotypic antibody, by antibody interference with idiotype-related help, or by B-cell triggering of Lyt 1^+ inducer cells in the test culture, which in turn activate T-suppressive activity. Experiments designed to test these possibilities are in progress. In this regard we document in the accompanying paper (38), that suppression induced by activated B cells is also governed by V_H-linked genes. Further, a soluble material in supernates of these B cells, capable of being bound by anti-MIg immunoadsorbents, can suppress in vitro primary anti-SRBC responses only if the B cells in the assay culture share V_H-linked genes with the suppressor B cells. Taken together, these data indicate that V_H-restricted suppression can be initiated by either T inducer cells (37) or by B cells and their products.

Summary

Feedback regulation of the primary humoral immune response to sheep erythrocytes (SRBC) was studied in vitro. Whole spleen cells or spleen cell subpopulations were

[2] Recent experiments carried out in collaboration with R. K. Gershon have demonstrated that the batch of FCS used in the cultures is critical for the detection of T_s activity.

[3] Horowitz, M., F. W. Shen, H. Cantor, and R. K. Gershon. Activated B cells stimulate antigen-specific Ly 1^-2^+ T suppression in vitro. Manuscript submitted for publication.

incubated with antigen for 4 d under Mishell-Dutton conditions (education) and the surviving cells tested for regulatory activity in fresh anti-SRBC spleen cell cultures assayed by measuring plaque-forming cells on day 4. The data indicate that (*a*) whole spleen cells educated with SRBC exert potent antigen-specific suppression in the assay culture, (*b*) surface Ig^- (sIg^-) cells (T cells) prepared by either nylon-wool separation or fractionation on rabbit anti-mouse-Ig-coated polystyrene Petri dishes failed to generate suppressive activity when educated alone, in 2-mercaptoethanol, or in the presence of additional macrophages, (*c*) surface Ig (sIg^+) (B) cells educated alone also failed to generate suppressor cells, and (*d*) mixing sIg^- (T) and sIg^+, Lyt 123^- (B) cells reconstituted the ability to induce suppressor cells under these conditions. The antigen-primed cell actually required to transfer suppression was also characterized by separating cells using anti-Ig coated dishes, by fluorescence-activated cell sorting and by anti-Lyt treatment. All these methods clearly identified sIg^+ (B) and not sIg^- (T) cells as the important educated cells. It is concluded that under our conditions, T cell-dependent B cells triggered by antigen during primary in vitro cultures cause potent specific feedback suppression of humoral responses. Possible mechanisms for this suppression, including antigen blockade or anti-idiotypic responses, are discussed.

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