HETEROGENEITY OF MURINE REGULATORY T CELLS

I. Subpopulations of Amplifier and Suppressor T Cells*

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That thymus-derived lymphocytes play a critical role in either amplifying or suppressing both cell-mediated and humoral responses is clear (reviewed in 1-3). However, several crucial questions concerning mechanisms resulting in both the generation and the mediation of these regulatory forces remain. Thus, it appears that while such factors as dose and molecular configuration of the immunogen (4-6), as well as other influences under genetic control (7, 8), are responsible for determining whether amplifying or suppressive regulatory forces predominate, it is also possible that feedback signals occurring between regulatory cells and other effector cells may be important in this regard (1, 9). Additionally, while amplification and suppression may represent either synergistic or antergistic interaction between a single regulatory T population and effector cells (1), evidence has accumulated suggesting that these regulatory antagonists may represent the capabilities of distinct T-cell populations (10-14). As regards heterogeneity existing within regulatory T populations, it is not clear whether the reported T-dependent suppression of primary and secondary antibody responses as well as delayed hypersensitivity reactions represent the suppressive capability of a single or distinct T-cell populations.

In this report we have attempted to analyze some of these questions. Thus, the data to be presented suggest that T-dependent amplification and suppression of antibody responses reflect the capabilities of distinct T-cell populations separable by differences in their buoyant density. Additionally, it appears that the generation of T-dependent suppressive influences, at least in the system utilized here, may depend on "feedback signals" from other regulatory or effector cells. Finally, suppression of direct and indirect plaque-forming cells (PFC)[1] responses appears to reflect the regulatory capacity of distinctive T-cell populations.

Materials and Methods

Animals.   6-8-wk-old male C3H/HeJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and maintained for 2-3 wk, before use, in animal facilities at the Mayo Medical School.

Experimental Protocol.   As illustrated in Fig. 1, regulatory T-cell populations were generated by intraperitoneal (i.p.) immunization with either 4-5 × 10⁹ sheep erythrocytes (SRBC, Grand Island Biological Co., Grand Island, N. Y.) or pigeon erythrocytes (PRBC, Colorado Serum Co.,

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FCS, fetal calf serum; M199, medium 199; PFC, plaque-forming cells; PRBC, pigeon erythrocytes.
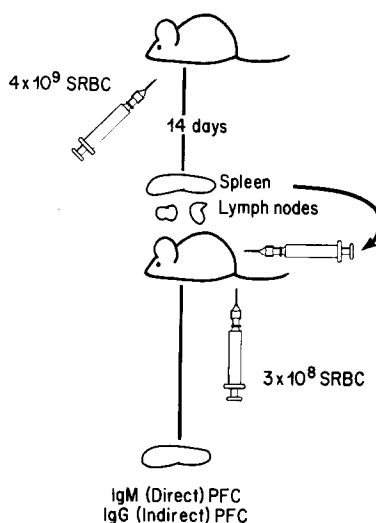
FIG. 1.   Experimental protocol for the generation of regulatory T-cell populations.

Denver, Colo.). 2 wk after immunization the spleens or inguinal and mesenteric lymph nodes were removed and single cell suspensions prepared. Various numbers of these cells were then infused intravenously into normal syngeneic recipients along with 3×10^8 SRBC or PRBC. The number of direct or indirect PFC were assayed 4 and 8–10 days later, respectively, utilizing a modification of the Jerne plaque technique (15). Indirect PFC were developed with rabbit anti-mouse immunoglobulin prepared by the immunization of rabbits with mouse Ig (Miles Laboratories Inc., Elkhart, Ind.). The antisera were heated at 56°C for 30 min and absorbed with SRBC before use. Indirect PFC were calculated by subtracting the direct PFC from the total PFC obtained with the rabbit anti-mouse Ig. Three to five recipients were utilized in each experimental group, and the results represent the mean PFC/10^6 spleen cells.

  Lymphocyte Subpopulations.   Spleen and lymph node cells were depleted of Ig-bearing and adherent cells by passage over nylon wool columns (LP-1; Leuko-Pak Leukocyte Filters, Fenwal Laboratory, Morton Grove, Ill.), equilibrated with 10% fetal calf serum (FCS) in medium 199 (M199) at 37°C as described by Julius et al. (16). The relative frequency of thy-1^b and Ig-bearing cells in the effluent populations was determined by an immunofluorescent technique as previously described (17). Anti-thy-1^b antisera were prepared as described previously (17) and absorbed with AKR thymocytes before use. The frequency of protein synthesizing cells was determined by methyl green pyronin staining.

  Density Gradient Separation of Lymphoid Cells.   Effluent spleen or lymph node populations obtained from the nylon wool columns were centrifuged on discontinuous bovine serum albumin (BSA) density gradients, utilizing the method of Raidt et al. (18). Briefly, 35% BSA (pathocyte 5, Miles Laboratories Inc.) was diluted to 33, 29, 26, 23, and 10% with M199. 1 ml of each fraction was layered into cellulose nitrate tubes. The cells, suspended in 10% BSA, were then layered on top and the gradient centrifuged at 12,000 g max (middle of the tube) in a swinging bucket rotor at 4°C for 30 min. Cells at each interface were removed with a Pasteur pipette, washed twice in M199, and counted under a hemocytometer before adoptive transfer. The viability of cells in each fraction was assessed by trypan blue exclusion and was always greater than 95%.

Results

  Specific Suppression of Direct and Indirect SRBC PFC Responses.   As previously reported (6), immunization of mice with a large number of SRBC (4×10^9) yields a spleen cell population which can, on adoptive transfer, suppress recipients' PFC responses to SRBC (Table I). To determine (*a*) the antigen specificity

TABLE I
Specificity of Suppression

RBC used to induce sup-pressor cells	Recipient PFC response/10^6 spleen cells			
	Direct		Indirect	
	SRBC	PRBC	SRBC	PRBC
0	446 ± 26	169 ± 15	206 ± 46	ND
SRBC	131 ± 42	146 ± 57	80 ± 34	ND
PRBC	790 ± 66	74 ± 8	309 ± 22	ND

C3H/HeJ mice were immunized with no RBC (0), 4 × 10^9 SRBC or 4 × 10^9 PRBC. 2 wk later 60 × 10^6 (for direct PFC) or 15 × 10^6 (for indirect PFC) spleen cells from each group were transferred to syngeneic hosts along with 3 × 10^8 SRBC or PRBC. Direct and indirect PFC were measured at 4 and 8–10 days, respectively. Results represent geometric mean ± SE for two experiments with three to five recipients/experiment. ND, not done.

of the suppression, (*b*) if the low recipient PFC responses merely represented a shift in the subsequent dose of SRBC required to elicit maximal PFC, perhaps related to "antigen carry-over," or (*c*) if the decreased response reflected a temporal change in the occurrence of maximal direct or indirect PFC in the recipients, the following experiments were performed.

Firstly, two antigens (SRBC and PRBC) which have previously been shown not to cross-react at the T- or B-cell level (19) were used to induce suppressive spleen cell populations. Groups of animals were immunized i.p. with 4 × 10^9 SRBC, 4 × 10^9 PRBC, or no RBC. 2 wk later, 60 × 10^6 spleen cells from each group were transferred intravenously with 3 × 10^8 SRBC or PRBC to normal syngeneic animals and the subsequent direct and indirect PFC responses measured. Note (Table I) that while spleen cells from animals previously immunized with 4 × 10^9 SRBC effect a 3.4- and 2.6-fold decrease in recipients' direct and indirect PFC response to SRBC, no significant suppression of the direct PFC responses to PRBC occurred. Similarly, spleen cells from mice previously immunized with 4 × 10^9 PRBC suppressed recipients' responses to PRBC and not to SRBC. Thus, the noted suppression is specific, at least as regards the antigenic challenge required to elicit suppression. Indirect PFC responses to PRBC were not performed because of technical difficulties. Recipients receiving either spleen cells from nonimmunized donors or no spleen cells manifested comparable PFC responses. Thus, such recipients are used interchangeably as controls for future experiments.

Secondly, to exclude the possibility that the observed suppression of recipient PFC responses might reflect a shift in the subsequent dose of SRBC required to elicit maximal recipient PFC responses, two groups of animals were prepared. The first group received 60 × 10^6 spleen cells from animals immunized 2 wk previously with 4 × 10^9 SRBC, while the second group received spleen cells from nonimmunized animals. The two groups were then immunized intravenously with no SRBC, 3 × 10^7, or 3 × 10^8 SRBC, and the direct PFC response was determined 4 days later. For animals receiving nonimmune spleen cells, the responses to the three immunization schedules were 4, 56 ± 7, and 153 ± 4 direct PFC/10^6, respectively. This compares to the response of 5, 23 ± 6, and 25 ± 4

direct PFC/10^6 spleen cells respectively noted in animals who received spleen cells from donors immunized with 4×10^9 SRBC. Thus, administration of spleen cells from animals immunized with 4×10^9 SRBC resulted in a subsequent "flattening" rather than significant shifting of recipients' dose response curve. Moreover, the noted low direct PFC response of recipients who received the immune spleens but who were not subsequently immunized with SRBC (five PFC) would argue against carry over of significant amounts of SRBC antigen.

Finally, any temporal change in the occurrence of maximal recipients' direct or indirect PFC response induced by transferred spleen cells was analyzed as follows. 30 million spleen cells from animals immunized 2 wk previously with 4×10^9 SRBC were transferred to syngeneic recipients along with 3×10^8 SRBC. Direct and indirect PFC responses were assayed at various intervals after transfer and compared to the responses noted for animals who received normal spleen cells (Fig. 2). Note that no appreciable shift in the time-dependent appearance of maximal direct and indirect PFC could be detected in animals receiving the immune spleen cells. Moreover, although suppression of both direct and indirect PFC occurred throughout the response, this was most marked during relatively late direct and indirect PFC responses, a finding previously noted by others (20).

Suppression Can be Mediated by Nonadherent, Non-Ig-Bearing Cells. To determine if splenic T cells were required for suppression of recipients' PFC responses, spleen cells from animals immunized with 4×10^9 SRBC 2 wk previously were passed over nylon wool columns equilibrated with 10% FCS at 37°C. The ability of effluent cells to suppress the SRBC response of syngeneic recipients was then compared to spleen cells simply incubated for comparable intervals in 10% FCS at 37°C or 4°C. The relative frequency of thy-1^b and Ig positive lymphocytes as well as the frequency of plasma cells, as indicated by staining with methyl green pyronin, was determined for each population.

It is evident from the data in Table II that when compared to spleen cells incubated at 4°C, simple incubation of cells at 37°C resulted in a 1.6-fold decrease in their ability to suppress recipients' direct SRBC PFC responses. Whether this represents death of a portion of suppressor cells or elution from cell surfaces of soluble substances capable of mediating suppression is not known. Nonetheless, when compared to spleen cells which were incubated at 37°C for 45 min, equivalent numbers of nylon column effluent populations were enriched in their ability to suppress recipient direct PFC responses. This coincided with a 1.8-fold increase in the relative frequency of cells bearing thy-1^b and a 22-fold decrease in the frequency of Ig-bearing lymphocytes.

For indirect PFC, no loss of suppression could be detected among spleen cells incubated at 37°C for 45 min. However, despite an increase in the relative frequency of T cells, 15×10^6 column effluent cells were not enriched in their ability to suppress recipients' indirect PFC responses. This cannot be explained by the possibility that maximal suppression of indirect PFC was already achieved with 15×10^6 of the control cell populations, as transfer of 30×10^6 spleen cells, which were simply incubated at 37°C, resulted in 90% suppression of recipients' indirect PFC. The explanation for the failure of T-enriched populations to demonstrate increased suppression is not known, but has been noted by others (19).
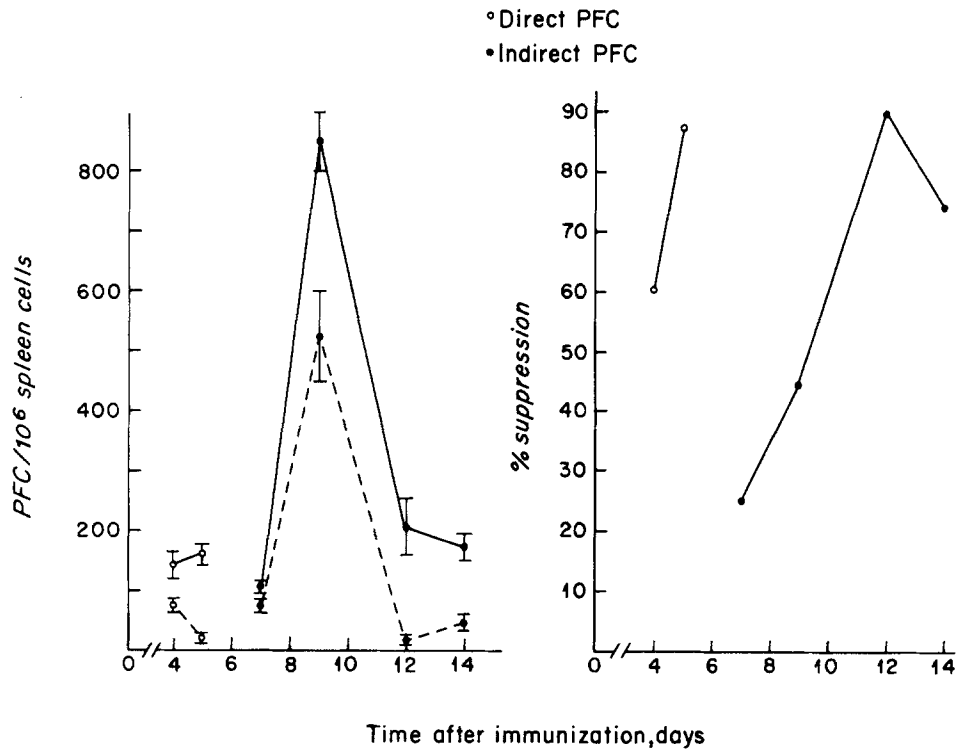
FIG. 2.   Suppression of early and late PFC responses. 30 × 10⁶ spleen cells from normal C3H/HeJ mice (—) or mice which had received, 2 wk previously, 4 × 10⁹ SRBC (- - -) were transferred intravenously with 3 × 10⁸ SRBC into syngeneic recipients. Direct (O) and indirect PFC (●) were measured at the indicated times postimmunization and are represented as PFC/10⁶ spleen cells (left) or as percent suppression (right). Each point represents the geometric mean ± SE for four to five recipients.

Thus, while these experiments do not delineate the characteristics of all spleen cells capable of suppressing subsequent PFC responses, they do indicate that populations markedly depleted of adherent cells, B cells, and plasma cells can exert suppressive regulatory influences. Thus, all subsequent experiments utilized, unless otherwise noted, column-passed lymph node or spleen cells.

Peripheral Localization of Suppressor Cells.   Previous work (21, 22) has indicated that T cells capable of abrogating immunologic reactivities are included within a relatively short-lived population of cells preferentially localizing to the spleen. To determine the relative frequency of T cells capable of suppressing direct and indirect SRBC PFC responses in the mesenteric and inguinal lymph nodes, as well as the spleens of animals immunized with 4 × 10⁹ SRBC, graded numbers of column-passed mesenteric and inguinal lymph node cells, as well as spleen cells obtained from these animals, were infused with 3 × 10⁸ SRBC into syngeneic recipients. The subsequent direct and indirect PFC responses were then determined. For spleen cells, note that as few as 3 × 10⁶ T cells resulted in significant suppression of direct PFC responses with maximal suppression noted after infusion of 30 × 10⁶ splenic T cells (Table III). For

TABLE II

Suppression by Nonadherent, Non-Ig-Bearing Spleen Cells

Cells transferred	Percent positive cells			Recipient PFC response	
	Thy 1	Ig	MGP	Direct	Indirect
				% suppression	
Control (4°C)	49	46	30	90	61
Control (37°C)	50	44	—	55	75
Column passed	90	2	3	84	60

Spleen cells from animals immunized 2 wk previously with 4×10^9 SRBC were incubated at 4°C or 37°C for 45 min or passed over nylon wool columns at 37°C. 30×10^6 (for direct PFC) or 15×10^6 (indirect PFC) cells from each population were transferred into syngeneic hosts and their subsequent response to immunization with 3×10^8 SRBC tested. The frequency of Thy-1-bearing and Ig-bearing lymphocytes as well as the frequency of cells staining positively with methyl green pyronin (MGP) were determined as described in Materials and Methods. Results represent the mean of two experiments.

TABLE III

Relative Ability of Spleen and Lymph Node Cells to Suppress PFC Responses

No. Cells Transferred (10^6)		Direct	Indirect
Spleen	0	423 ± 50	206 ± 46
	3	280 ± 24	229 ± 39
	30	32 ± 7	14 ± 7
	90	25 ± 5	11 ± 7
Lymph node	0	185 ± 29	81 ± 13
	3	168 ± 10	165 ± 16
	30	121 ± 21	31 ± 4
	60	174 ± 13	27 ± 7

The indicated number of column passed spleen or lymph node cells from animals previously immunized with 4×10^9 SRBC were transferred into normal syngeneic recipients. The subsequent direct and indirect PFC response of the recipients to immunization with 3×10^8 SRBC is presented as the geometric mean ± SE for 4–5 recipients.

indirect PFC, significant suppression of recipients' responses required the transfer of 30×10^6 splenic T cells.

In contrast to the noted ability of splenic T cells to suppress direct PFC responses, T cells from the lymph nodes of animals immunized with high dose SRBC did not decrease recipients' direct PFC responses, irrespective of the number of cells transferred. Transfer of 30×10^6 lymph node T cells did, however, suppress recipients' indirect PFC responses. Thus, while T cells capable of suppressing the indirect PFC response are present in both the lymph nodes and spleens of animals immunized with 4×10^9 SRBC, T cells capable of suppressing recipients' direct PFC appear to be absent from the lymph node. That the noted inability of lymph node T cells to suppress recipients' direct PFC

response did not simply represent a relative preponderance of amplifier over suppressor cells will be demonstrated subsequently.

Density Gradient Separation of Amplifier and Suppressor T Cells.   To this point, we have demonstrated that T cells capable of specifically suppressing recipients' direct and indirect PFC do exist among the peripheral lymphoid organs of animals immunized with 4×10^9 SRBC. To determine if this reflected either a complete absence of T-cell populations potentially capable of amplifying recipient responses or a relative preponderance of suppressor over amplifier T cells, the following experiments were performed.

Nylon column purified T cells from the spleens of animals immunized with 4 $\times 10^9$ SRBC were sedimented over a discontinuous gradient consisting of 10, 23, 26, 29, and 33% BSA (See Materials and Methods). Cells sedimenting in the top two interfaces (10/23, 23/26) were pooled and compared to cells sedimenting to the bottom of the gradient (pellet) in their ability to effect recipient PFC responses to SRBC. Both fractions contained >95% viable cells, as determined by the exclusion of trypan blue. Whereas 2.5×10^6 splenic T cells from layers 1 and 2 amplified recipients' PFC responses, comparable numbers of T cells sedimenting in layer 5 suppressed recipients' direct and indirect PFC responses (Table IV).

Similar density gradient fractionation of spleen cells from animals immunized 2 wk previously with 4×10^9 PRBC demonstrated no suppression of SRBC PFC responses by 2.5×10^6 high density (layer 5) T cells. However, comparable numbers of low density cells (layer 1 + 2) from these animals did augment the indirect SRBC PFC response 1.2-fold, while no significant augmentation of direct PFC was noted.

That the amplifying and suppressive activity of low and high density cells did represent distinct regulatory functions is supported by the data in Fig. 3. Thus, increasing the number of splenic T cells transferred from layers 1 and 2 over a 10-fold range augmented recipient's direct and indirect PFC response to 3×10^8 SRBC. Similarly, transfer of increasing numbers of cells sedimenting in layer 5 consistently resulted in suppression of direct and indirect PFC responses. Finally, transfer of admixtures containing equal numbers (2.5×10^6) of cells from layers 1 + 2 and 5 into normal animals resulted in a direct PFC response which could be predicted on the basis of the relative regulatory effect known to reside within each population.

An analogous situation also existed for lymph node T cells. Thus, while column passed, whole lymph node T cells suppressed recipient indirect PFC responses (Table III), density gradient separation of these cells revealed the presence of low density amplifier and high density suppressor cells (Table IV). However, T cells capable of suppressing direct PFC responses could not be detected in any gradient layer, even when the number of cells from each layer transferred was increased to 10×10^6. This then would suggest that the inability of whole lymph node T cells to suppress direct PFC responses resulted from an absence of high density suppressor cells rather than from a relative preponderance of amplifier cells.

Similar density gradient fractionation of lymph node T cells from animals immunized with 4×10^9 PRBC failed to demonstrate the presence of cells capable of suppressing recipient direct or indirect PFC responses to SRBC.

TABLE IV

Density Gradient Enrichment of Suppressor and Amplifier Cells

| Percent BSA | Layer no. | Percent recovered cells in each layer | | PFC of recipient/10^6 spleen cells | | | |
| | | | | Spleen | | Lymph node | |
		Spleen	Lymph node	Direct	Indirect	Direct	Indirect
10	1						
23		4	19	424 ± 55	396 ± 15	170 ± 8	253 ± 31
	2						
26							
	3	33	60	289 ± 19	404 ± 34	126 ± 33	270 ± 33
29							
	4	49	11	116 ± 8	300 ± 40	151 ± 54	146 ± 10
33							
Pellet	5	14	10	95 ± 27	98 ± 9	138 ± 32	160 ± 50
No. cells transferred				216 ± 19	200 ± 19	98 ± 10	197 ± 39

Lymph node or spleen cells from animals previously immunized with 4 × 10^9 SRBC were passed over nylon wool columns. Effluent populations were centrifuged over discontinuous BSA gradients as described in Materials and Methods. 2.5 × 10^6 cells from each layer were transferred into syngeneic recipients and the subsequent PFC response of the recipients to 3 × 10^8 SRBC determined. Results represent the geometric mean ± SE for two or three experiments with three recipients per layer per experiment.

Moreover, when compared to the effect of low density T cells (layers 1 + 2 + 3) from animals immunized with 4 × 10^9 PRBC, low density T cells from mice immunized with 4 × 10^9 SRBC augmented recipient responses 1.7-fold.

Effects of Antigen Dose and Presence of Amplifier T Cells on Generation of Suppressor Cells.   In all experiments to this point, only spleen or lymph node T cells from animals immunized with 4 × 10^9 SRBC have been the source of suppressor or amplifier cells. To determine if the generation of these antagonistic regulatory forces was related to immunogen dose, mice were immunized with either 3 × 10^7, 3 × 10^8, or 3 × 10^9 SRBC. 2 wk later the spleens from each group were removed, passed over nylon wool columns, and effluent T cells centrifuged on discontinuous BSA density gradients. 2.5 million cells from gradient layers 1 + 2 or 5 were infused into syngeneic recipients along with 3 × 10^8 SRBC to test for the regulatory effect on recipients' direct and indirect PFC responses. Control animals received 3 × 10^8 SRBC along with 2.5 × 10^6 T cells from layers 1 + 2 or 5 prepared from the spleens of nonimmunized animals. Note (Fig. 4) that transfer of 2.5 × 10^6 cells from layers 1 + 2 obtained from spleens of animals immunized with 3 × 10^7 SRBC effected little change (suppression or amplification) in the recipients' direct and indirect PFC responses. In contrast, immunization with 3 × 10^8 and 3 × 10^9 SRBC resulted in relative increases in amplifying activity among these cells. Similarly, increases in immunogen dose resulted in increasing suppressive reactivity when the effect of comparable numbers of cells sedimenting in layer 5 were compared. No significant differences in the regulatory effect of cells from gradient layers 3 or 4 isolated from the spleens of animals immunized with the three doses of SRBC were noted.

It is to be remembered (Table IV) that the number of cells sedimenting in
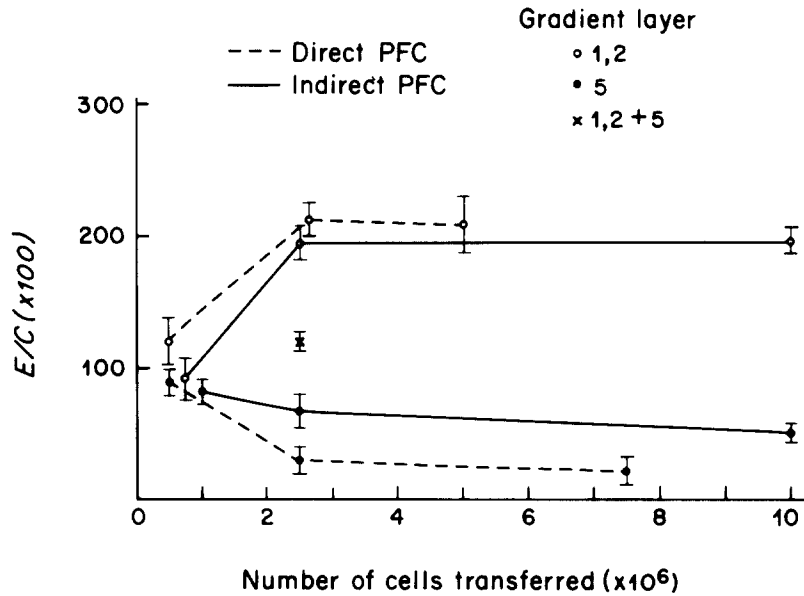
FIG. 3. Regulatory effect of increasing numbers of low density amplifier or high density suppressor cells. Column-passed spleen cells from animals previously immunized with 4 × 10^9 SRBC were fractioned over discontinuous BSA gradients. The indicated numbers of cells from layers 1, 2 (O), or 5 (●) were transferred with 3 × 10^8 SRBC into syngeneic hosts. Direct (- - -) and indirect (—) PFC were measured at 4 and 10 days later and are expressed as PFC in animals receiving fractionated cells/PFC in animals not receiving spleen cells (E/C). The effect of transferring mixtures containing equal numbers of cells from layers 1, 2, and 5 on recipients' direct PFC is indicated by X. Each point represented the geometric mean ± SE for three to four recipients.

layers 1 + 2 and 5 of the gradient represent 4 and 14%, respectively, of the total recoverable cells. As admixtures containing equal numbers of cells from layer 1 + 2 and 5 resulted in an intermediate response representative of a balance between amplification and suppression, it would be predicted that nonseparated column T cells from animals immunized with 3 × 10^9 SRBC would manifest more suppression than those obtained from animals immunized with 3 × 10^8 SRBC. Indeed, this was confirmed experimentally (data not shown).

Thus, increases in the dose of antigen resulted in increases in both amplifying and suppressive regulatory effects of fractionated spleen cells, with suppressive effects predominating. It is possible that the generation of suppressor T cells then resulted directly from interactions between high antigen concentrations and certain T-cell subpopulations. Alternatively, the generation of suppressor cells might occur indirectly in response to increases in amplifying regulatory reactivity. To distinguish between these two possibilities, advantage was taken of the fact that immunization of mice with 3 × 10^7 SRBC resulted in no detectable suppressive activity among gradient separated spleen cells. Simply, the experiment as outlined in Fig. 5 consisted of assessing the effect of amplifier cells on the appearance of suppressor activity among cells sedimenting in layer 5 of separated spleen cells from animals immunized with 3 × 10^7 SRBC. Note that when compared to animals immunized with only 3 × 10^7 SRBC, significant

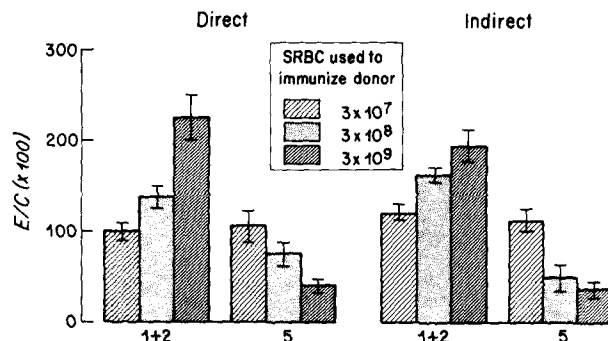FIG. 4. Generation of amplifier and suppressor T cells in response to various doses of immunogen. Groups of animals were immunized with either 3×10^7, 3×10^8, or 3×10^9 SRBC. 2 wk later, nylon wool column effluent spleen cells from each group were fractionated over discontinuous BSA gradients. 2.5 million cells from layer 1 + 2 or 2.5 cells from layer 5 were transferred with 3×10^8 SRBC into syngeneic hosts. Direct and indirect PFC were measured 4 and 10 days later. Results are expressed as $PFC/10^6$ in animals receiving fractionated regulator cells divided by $PFC/10^6$ in animals receiving fractionated cells from donors immunized with PRBC (E/C), and represent the geometric mean ± SE for two experiments.

suppressor activity was apparent among layer 5 cells from animals who received, in addition, amplifier cells (layers 1 + 2 from mice immunized with 4×10^9 SRBC). Thus, it would appear that the generation of suppressor cells by low doses of immunogen can, at least for direct PFC, be facilitated by the presence of amplifier cells.

Discussion

Recently, there has been much interest in mechanisms involved in regulating immunologic reactivity. Thus, thymocytes, T cells, macrophages, and B cells have all been demonstrated to function in the regulation of other effector T and B cells (1-3, 23, 24). Additionally, a growing list of soluble substances have been demonstrated to affect both cell-mediated and humoral reactivity (25-28). Clearly, such studies are important for aberrancies in extrinsic regulatory forces, rather than intrinsic abnormalities in effector cells, and may play an important role in determining the immune response to exogenous stimuli as well as endogenous self-antigens.

The data demonstrated here indicate that immunization of C3H/HeJ mice with a high dose (4×10^9) of SRBC results in the generation of a heterogenous population of regulatory T cells. Thus, nonadherent T cells capable of augmenting or suppressing direct and indirect PFC responses appear to represent the capabilities of T-cell populations distinguishable by differences in their buoyant density. It is to be emphasized that differences in density do not necessarily reflect stable cell markers and certainly do not rule out the possibility that amplification and suppression represent different maturational stages within a single cell line. Whether or not the progenitors of amplifier and suppressor cells also differ in their buoyant density is currently under investigation. Nonetheless, data presented here support growing evidence indicating that, for a given immune response, T cells capable of augmenting or suppressing that response
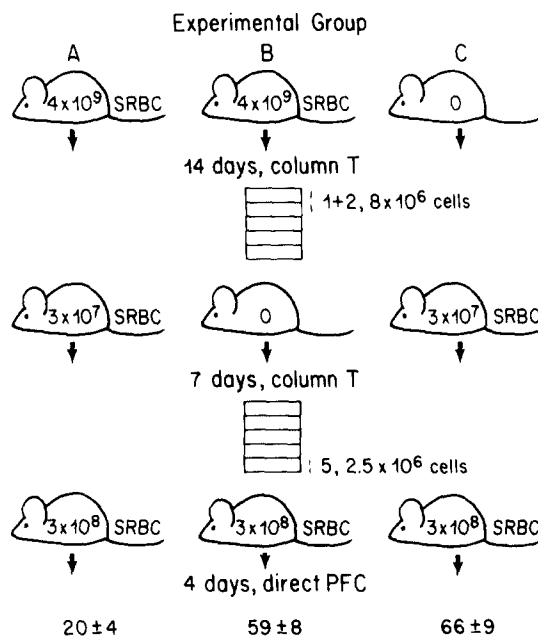
FIG. 5. Effect of amplifier T cells on the subsequent generation of suppressor T cells. Groups of mice were immunized with either 4 × 10⁹ SRBC (groups A, B) or no SRBC (group C). 2 wk later, nylon wool column passed spleen cells from each group were fractionated on discontinuous BSA density gradients and 8 million T cells localized to gradient layers 1 and 2 transferred into normal syngeneic recipients. Mice receiving low density cells from group A were simultaneously immunized with 3 × 10⁷ RBC, those receiving low density cells from group B were immunized with no (O) SRBC, and those receiving low density cells from group C were immunized with 3 × 10⁷ SRBC. 7 days later, column passed splenic T cells from these recipients were centrifuged on discontinuous BSA density gradients, and high density cells sedimenting in layer 5 were tested for their ability to suppress direct PFC responses. Results represent the geometric mean PFC ± SE/10⁶ spleen cells for four to five recipients.

can be delineated by differences in cell surface antigens (13), location in peripheral lymphoid tissue (21, 22), as well as by differences in their sedimentation at unit gravity (14). However, this does not negate the concept that the regulatory function of T cells is determined by "feedback" signals from responding cells (1, 9). For example, in this report the regulatory effect of distinctive T-cell populations was tested against a maximal direct or indirect PFC response (i.e., that PFC response achieved with immunization of 3 × 10⁸ SRBC). However, we have preliminary evidence to indicate that high density T cells which suppress this response may actually amplify suboptimal PFC responses generated by immunization with 3 × 10⁷ SRBC. Additionally, comparable numbers of low density cells which amplify optimal PFC responses may suppress suboptimal PFC responses. Whether this does indeed indicate that a given population of regulatory cells can suppress brisk and augment relatively weak responses is under investigation.

In addition to heterogeneity existing among regulatory T cells with respect to amplification and suppression, several observations presented here suggest that

subpopulations of suppressor T cells might exist as regards suppression of direct and indirect PFC. Firstly, while T cells capable of suppressing indirect PFC could be detected among both the lymph nodes and spleens of animals immunized with 4×10^9 SRBC, T cells capable of suppressing direct PFC could only be detected in the spleen. Moreover, this does not appear to merely reflect quantitative differences in the relative ability of direct and indirect PFC responses to be suppressed by a given number of suppressor T cells, nor does it reflect the masking of suppressive influence among lymph node T cells by an excess of amplifying influences. Thus, even when T cells capable of augmenting direct PFC were separated from the rest of the lymph node population by density gradient separation, T cells capable of suppressing direct PFC could not be detected. As the assay used to detect the regulatory function of T cells involved transfer of cells into normal syngeneic recipients, it is unlikely that the absence of suppressors for direct PFC in the lymph nodes represents the absence of another population of cells, such as macrophages, which are required to interact with suppressor T cells to inhibit direct but not indirect PFC. Thus, infusion of 30×10^6 lymph node T cells into syngeneic hosts should result in the localization of some cells to the spleen where they would be able to interact with another cell type, possibly a macrophage, and thus suppress direct PFC responses. However, this possibility is being investigated directly using admixtures of lymph node T cells from animals immunized with 4×10^9 SRBC and normal spleen cells to suppress the in vitro primary response to SRBC.

Secondly, while incubation of spleen cells, at 37°C, from mice immunized with 4×10^9 did result in the dissipation of suppressive influences affecting direct PFC, suppressive forces capable of inhibiting indirect PFC were not lost. Indeed, preliminary data utilizing high density suppressor T cells demonstrate a similar preferential loss, during culture, of suppressive influences for direct but not indirect PFC. Whether this reflects a differential survival of T cells affecting direct and indirect PFC or whether this represents elution from T-cell surfaces of soluble materials involved in either the induction or mediation of suppressive influences for direct but not indirect PFC is under investigation. As regards this latter point, the ability to delineate subpopulations of suppressor T cells may be important in elucidating mechanisms by which such cells are generated, specifically as they may relate to possible interactions occurring between antibodies or antigen-antibody complexes and Fc receptor-bearing T cells (29-31).

Finally, while nylon wool column effluent cells were enriched, when compared to nonfractionated spleen cells incubated at 37°C, in their ability to suppress direct PFC, no enrichment for suppression of indirect PFC was noted despite a 1.8-fold increase in the relative frequency of thy-1^b-bearing cells. Although this may indicate that either adherent T cells, B cells, or macrophages may be involved in the suppression of indirect but not direct PFC, it does suggest that suppressive influences affecting these two PFC responses are not mutually inclusive.

As indicated previously, factors related to the generation of suppressive influences include dose of immunogen, physical characteristics of the immunogen, as well as other factors controlled at the genetic level. However, it is also possible that the generation of suppressive influences is part of a normal

regulatory response, and as pointed out by Eardley and Gershon (9), suppressive influences may exist among spleen cells which themselves manifest brisk immunologic reactivity. The data demonstrated in Fig. 4 would indicate that the relative appearance of high density suppressor cells is a direct function of the dose of antigen used to generate regulatory T cells. However, as the relative frequency of amplifier T cells also increases with the dose of immunogen, it is possible that suppressor cells are generated indirectly from "feedback" signals received from any increase in the frequency or activity of amplifier cells rather than directly via interactions with antigen. That this is so was suggested by data not presented which consisted of the following. Density gradient separation of column passed spleen cells obtained 2, 4, and 7 days after immunization with 4 × 10^9 SRBC demonstrated the appearance of low density amplifier cells before the detection of any high density suppressor cells, a temporal relationship noted previously (32). This then might suggest that suppressor cells are generated as an antagonist to, and thus reflect feedback signals from, amplifier cells. A more direct demonstration of this is the data presented in Fig. 6. Immunization of mice with 3 × 10^7 SRBC did not result in the appearance of substantial numbers of high density suppressive influences. However, in the presence of low density amplifier cells, this same dose of immunogen did yield significant numbers of high density suppressor T cells. It would appear then that increases in suppressive influences associated with increasing doses of antigen may indirectly reflect "feedback" signals from amplifier T cells rather than direct activation of suppressor cells by high doses of immunogen. Whether these "signals" are represented by proliferation of amplifier cells, production of antibody that activates suppressor cells by a process of "feedback inhibition," or reflect direct conversion of amplifier into suppressor cells is presently under investigation.

Two points are to be emphasized. Firstly, we have demonstrated the described relationship between amplifier and suppressive influences only for T cells which inhibit direct PFC responses. Whether a similar phenomena is also associated with T cells capable of suppressing indirect PFC is not known. Secondly, while the generation of suppressor T cells in the experimental system used here may reflect feedback signals from amplifier cells, this may not be true for all situations characterized by the presence of T-associated suppressive forces. Thus, in situations where unresponsiveness is under genetic control, T-dependent suppressive influences may arise via other mechanisms. If amplifier cells do in some way facilitate the generation of suppressors, it might be thought that infusion of amplifier cells would result in suppression rather than augmentation of recipient responses. However, it is clear that the regulatory effect of amplifier and suppressor T cells is dependent on their presence during early events in the generation of immune responses. Thus, unless given within 24 h of immunization, neither amplifier nor suppressor T cells exert a significant effect on humoral responses (20; Whisler and Stobo, unpublished observations). Suppressor cells generated then subsequent to the infusion of amplifier cells and antigen might not significantly inhibit an ongoing PFC response.

Many questions remain as to events leading to the induction and mediation of regulatory influences. The ability to delineate distinct populations of amplifier and suppressor T cells not only facilitates approaches to answer these questions,

but also allows studies of possible interactions between amplifier and suppressor T cells which result in net regulatory influences.

Summary

Immunization of C3H/HeJ mice with 4×10^9 SRBC yields a whole splenic T-cell population which can, upon transfer, specifically suppress recipient direct and indirect plaque-forming cells (PFC) responses to sheep erythrocytes (SRBC). Discontinuous bovine serum albumin density gradient fractionation of these T cells demonstrated a population of low density T cells which augmented and a population of high density T cells which suppressed recipient responses irrespective of the number of T cells transferred. Moreover, infusion of admixtures of low and high density cells resulted in intermediate regulatory functions which could be predicted by knowing the regulatory capacity of each population alone.

In addition to heterogeneity existing among regulatory T cells as regards amplification and suppression, it appeared that heterogeneity existed within the suppressor T population. Thus, T cells capable of inhibiting direct PFC could be distinguished from those suppressing indirect PFC by their differential localization in peripheral lymphoid tissue, differences in the dissipation of suppressive influences during incubation at 37°C, and by differences in the possible requirement for adherent cell populations.

While the relative frequency of both low density amplifier and high density suppressor cells increased with the dose of SRBC used for their induction, it appeared that suppressor cells might be generated in response to feedback signals from amplifier cells.

These studies indicate that further delineation of heterogeneity existing within suppressor populations may be helpful in defining mechanisms required for the induction and manifestation of suppressive regulatory forces.

References

1. Gershon, R. K. 1974. T cell control of antibody production. In Contemporary Topics in Immunobiology. M. D. Cooper and N. L. Warner, editors, Plenum Publishing Corporation, New York. 3:1.
2. Gershon, R. K. 1975. A disquisition on suppressor T cells. *Transplant. Rev.* 26:170.
3. Katz, D. H., and B. Benacerraf. 1972. The regulatory influence of activated T cells on B cell response to antigen. *Adv. Immunol.* 15:2.
4. Benjamin, D. C. 1975. Evidence for specific suppression in the maintenance of immunological tolerance. *J. Exp. Med.* 141:635.
5. Basten, A., J. F. A. P. Miller, J. Sprent, and C. Cheers. 1974. Cell-to-cell interaction in the immune response. X. T-cell-dependent suppression in tolerant mice. *J. Exp. Med.* 140:199.
6. Gershon, R. K., and K. Kondo. 1971. Infectious immunological tolerance. *Immunology.* 21:903.
7. Gershon, R. K., P. M. Maurer, and C. F. Merryman. 1973. A cellular basis for

genetically controlled immunological unresponsiveness in mice: tolerance induction in T cells. *Proc. Natl. Acad. Sci. U. S. A.* 70:250.

8. Benacerraf, B., J. A. Kapp, P. Debré, C. W. Pierce, and F. De La Croix. 1975. The stimulation of specific suppressor T cells in genetic nonresponder mice by linear random copolymers of L-amino acids. *Transplant. Rev.* 26:21.

9. Eardley, D., and R. K. Gershon. 1975. Feedback induction of suppressor T-cell activity. *J. Exp. Med.* 142:524.

10. Baker, P. J. 1975. Homeostatic control of antibody responses: a model based on the recognition of cell-associated antibody by regulatory T cells. *Transplant. Rev.* 26:3.

11. Scavalli, J., and R. W. Dutton. 1975. Competition between concanavalin A-induced stimulatory and inhibitory effects in the in vitro immune response to antigen. *J. Exp. Med.* 141:524.

12. Herzenberg, L. A., C. M. Metzler, and L. A. Herzenberg. 1974. Mechanism of allotype suppression in mice. *In* Immunological Tolerance. D. H. Katz and B. Benacerraf, editors. Academic Press, Inc., New York. 519.

13. Feldman, M., P. C. L. Beverley, M. Dunkley, and S. Kantiairen. 1975. Different Ly antigen phenotypes of in vitro induced helper and suppressor cells. *Nature (Lond.)*. 258:614.

14. Gerber, N. L., and A. D. Steinberg. 1975. Physical separation of "suppressor" from "helper" thymocytes. *J. Immunol.* 115:1744.

15. Cunningham, A. J., and A. Svenberg. 1968. Further improvements in plaque technique for detecting single antibody-forming cells. *Immunology.* 14:599.

16. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus derived lymphocytes. *Eur. J. Immunol.* 3:645.

17. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface θ or immunoglobulin determinants. *J. Exp. Med.* 138:71.

18. Raidt, D. J., R. I. Mishell, and R. W. Dutton. 1968. Cellular events in the immune response. Analysis and in vitro response of mouse spleen cell populations separated by differential flotation in albumin gradients. *J. Exp. Med.* 128:681.

19. Kettman, J., and M. C. Mathews. 1975. Radio resistance of cells responsible for delayed hypersensitivity reactions in the mouse. *J. Immunol.* 115:606.

20. Tada, T., and T. Takemori. 1974. Selective roles of thymus-derived lymphocytes in the antibody response. I. Differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. *J. Exp. Med.* 140:239.

21. Wu, C-Y., and E. M. Lance. 1974. Immunoregulation by spleen-seeking thymocytes. II. Role in response to sheep erythrocytes. *Cell. Immunol.* 13:1.

22. Gershon, R. K., E. M. Lance, and K. Kondo. 1974. Immunoregulatory role of spleen localizing thymocytes. *J. Immunol.* 112:546.

23. Gershon, R. K., M. B. Mokyr, and M. S. Mitchell. 1974. Activation of suppressor T cells by tumour cells and specific antibody. *Nature (Lond.)*. 250:594.

24. Gershon, R. K. 1974. T cell control of antibody production. Role of antibody. *In* Contemporary Topics in Immunobiology. M. D. Cooper and N. L. Warner, editors. Plenum Publishing Corporation, New York. 3:23.

25. Murgita, R. A., and T. B. Tomasi, Jr. 1975. Suppression of the immune response by alpha-fetoprotein. I. The effect of mouse alpha-fetoprotein on the primary and secondary antibody response. *J. Exp. Med.* 141:269.

26. Mortensen, R. F., A. P. Osmond, and H. Gewurz. 1975. Effects of C-reactive protein on the lymphoid system. I. Binding to thymus-dependent lymphocytes and alterations of their functions. *J. Exp. Med.* 141:821.

27. Menzoian, J. O., A. H. Glasgow, R. D. Nimberg, S. R. Cooperband, K. Schmid, I.

Saporoschetz, and J. A. Mannick. 1974. Regulation of T lymphocyte function by immunoregulatory alpha-globulin (IRA). *J. Immunol.* 113:266.

28. Benson, M. D., M. A. Aldo-Benson, T. Shirahoma, Y. Borel, and A. S. Cohen. 1975. Suppression of in vitro antibody response by a serum factor (SAA) in experimentally induced amyloidosis. *J. Exp. Med.* 142:236.

29. Sinclair, N. R., R. K. Lees, S. Abrahams, P. L. Chon, G. Fagon, and C. R. Stiller. 1974. Regulation of the immune response. X. Antigen-antibody complex inactivation of cells involved in adoptive transfer. *J. Immunol.* 113:1493.

30. Stout, R. D., and L. A. Herzenberg. 1975. The Fc receptor on thymus derived lymphocytes. II. Mitogen responsiveness of T lymphocytes bearing the Fc receptor. *J. Exp. Med.* 142:1041.

31. Basten, A., J. F. A. P. Miller, N. L. Warner, R. Abraham, E. Chia, and J. Gamble. 1975. A subpopulation of T cells bearing Fc receptors. *J. Immunol.* 115:1159.

32. Janeway, C. A. 1975. Cellular cooperation during in vivo anti-hapten antibody responses. I. The effect of cell numbers on the response. *J. Immunol.* 114:1394.