IMMUNOREGULATORY CIRCUITS AMONG T-CELL SETS

II. Physiologic Role of Feedback Inhibition In Vivo: Absence in NZB Mice*

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There is increasing evidence that the immune response is regulated by a series of interactions among distinct T-lymphocyte subclasses (1, 2). In the preceding paper we have analyzed one such regulatory interaction: antigen-activated Ly1 helper cells induce a second nonimmune set of T cells (cell surface phenotype $Ly1^+2^+3^+Qa1^+$) to exert potent "feedback" inhibitory effects which are directly proportional to the level of antigen-induced T-helper activity (3). This T-T interaction was defined by using an in vitro antibody response as a model system, since isolation of different lymphocyte sets is achieved most readily under such in vitro conditions. However, we do not know whether this in vitro T-T interaction (or, for that matter, any cellular interaction that may regulate in vitro antibody responses) plays a physiologic role in the regulation of in vivo immune responses.

We have therefore examined the influence of $Ly123^+$ T cells upon in vivo antibody responses. We find that mice lacking such cells do not exhibit feedback suppression, and that the provision of $Ly123^+$ T cells to such mice restores this activity. In addition, the relevance of feedback suppression to the physiology of the immune system is underlined by the finding that development of autoimmunity in NZB mice is associated with (*a*) absence of feedback suppression and (*b*) a defect in the differentiation and/or function of T cells expressing the $Ly123^+$ phenotype.

Materials and Methods

Animals. C57BL/6 (B6) mice and Ly congenic stock were obtained as described previously (3). NZB and BALB/c mice were bred by H. Cantor from breeders supplied by The Jackson Laboratory, Bar Harbor, Maine.

Production and Use of LY and Thy1.2 Antisera. Preparation and use of Ly-1.2, Ly-2.2, Ly-3.2 and Thy-1.2 antisera have been described (3, 4). The proportion of T cells expressing one or more Ly components was estimated from the lytic effects after sequential exposure to two different Ly antisera with selected rabbit sera as a source of complement (C) according to a protocol detailed

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previously (5). An additional specificity control was employed to rule out the possibility that a portion of lysis seen after treatment of NZB spleen cells with anti-Ly1.2 + C reflected anti-natural killer[1] (NK) reactivity, an additional antibody that has been shown to be present in this serum (6): Ly-1.2 antisera (1:10 dilution) was absorbed with equal volumes of spleen and lymph node cells from B6-Ly-1.1 (NK^+) congenic mice. This serum had no effect on NK activity mediated by NZB or B6 spleen cells and did not exert detectable lytic activity (above normal mouse serum [NMS] + C) against lymph node, spleen, or thymocyte populations from B6-Ly1.1 congenic donors.

Production of "B" Mice. B6 mice were thymectomized at 6 wk of age, lethally irradiated (750 rads; ^{137}cesium source) at 8 wk of age, followed immediately by intravenous infusion of 5×10^6 bone marrow cells that had been doubly treated with anti-Thy1 + C, according to reference (3). Adult thymectomy (ATx) was also performed at 6 wk of age according to a method described previously (7).

Cellular Repopulation of Irradiated or Nonirradiated Adoptive Hosts. Highly purified Ly1 cells from mice immunized with 10^8 sheep erythrocytes (SRBC) 10–30 days previously were obtained according to reference (3) and 5×10^5 were injected intravenously along with 10^6 SRBC into irradiated (750 rads) or unirradiated syngeneic recipients. In some cases, nonimmune syngeneic spleen cells or unselected T cells (3) were added to the inocula. Anti-SRBC plaque-forming cell (PFC) responses were measured 5 days later.

Results

A Subset of $Ly1^+2^+3^+$ Cells Exerts Feedback Suppressive Effect during in Vivo Anti-SRBC Responses (Tables I, II). B6 mice depleted of T cells ("B-mice"—see Materials and Methods for details of preparation) did not produce significant anti-SRBC PFC responses after challenge with 10^6 SRBC (Table I, group A). Recipients repopulated with 0.3×10^6 Ly1 cells from syngeneic donors immunized to SRBC 1 wk previously produced substantial numbers of anti-SRBC PFC 5 days after immunization with 10^6 SRBC (group B). To determine whether a population of nonimmune T cells might exert feedback suppressive effects in vivo, an additional group of B mice was repopulated with nonimmune unselected T cells as well as SRBC-immune Ly1 cells. The anti-SRBC PFC response of these mice was reduced to almost that of unrepopulated B-mice (group C). Since B mice repopulated with nonimmune T cells were capable of producing substantial anti-SRBC responses (group D), these findings are consistent with the hypothesis drawn from similar experiments in vitro (3): highly purified, antigen-stimulated Ly1 cells induce nonimmune T cells to exert substantial feedback suppressive effects. Finally, elimination of $Ly123^+$ cells from the nonimmune T-cell population abolished suppression (group E), indicating that the surface phenotype of cells responsible for feedback suppression in vivo is the same as that ascertained in vitro (3). Identical conclusions are drawn from experiments in which lethally-irradiated hosts (rather than B mice) were repopulated with different T-cell sets together with purified B cells (Table II).

Biologic Properties of Feedback Suppressor Cells in Vivo

FEEDBACK SUPPRESSOR CELLS ARE SENSITIVE TO SMALL DOSES OF CYCLOPHOSPHAMIDE IN VIVO (Fig. 1). The PFC responses of mice given 10 mg/kg of cyclophosphamide 24 h before SRBC immunization did not differ significantly

[1] *Abbreviations used in this paper:* B6, C57BL/6; C, complement; NK, natural killer; Ly1 cells, $Ly1^+23^-$ cells; Ly23 cells, $Ly1^-23^+$ cells; ATx adult thymectomy; NMS, normal mouse serum; NTA, naturally occurring thymocyte autoantibody; SRBC, sheep erythrocytes.

TABLE I

Demonstration of Feedback Suppression by Nonimmune Lyl23 Cells in B Mice Repopulated with T-Cell Subclasses

	Cellular repopulation of B mice:			PFC/Spleen*	
	SRBC-Immune Lyl (0.3×10^6)	Nonimmune T-cells (8×10^6)	PFC/10^6 Cells	Mean	Range
A.	–	–	8	970	(150–2,100)
B.	+	–	145	12,100	(6,000–16,500)
C.	+	Unselected	22	1,760	(400– 2,600)
D.	–	Unselected	361	31,600	(19,050–40,100)
E.	+	(Lyl + Ly23)	155	14,650	(10,500–33,600)

* Three to four recipients/group. Mixtures of 8×10^6 nonimmune Lyl + Ly23 cells were obtained by combining 4×10^6 anti-Ly1.2 + C-treated T cells with 4×10^6 anti-Ly2.2 + C-treated T cells.

TABLE II

Demonstration of Feedback Suppression by Lyl23 Cells in Lethally Irradiated Hosts

Cellular repopulation of lethally irradiated hosts:			Anti-SRBC PFC Responses*	
SRBC-Immune T-cells + nonimmune B-cells		Nonimmune T-cells (10^7)	PFC/10^6 Cells (Mean)	PFC/Spleen (Mean and Range)
SRBC-Immune Lyl (10^6) + 5×10^6 B cells	+	None	310	1,155 (1,079–1,574)
		Unselected	24	276 (83–498)
		(Lyl + Ly23)	560	1,245 (950–1,555)

* Three to five recipients/group.

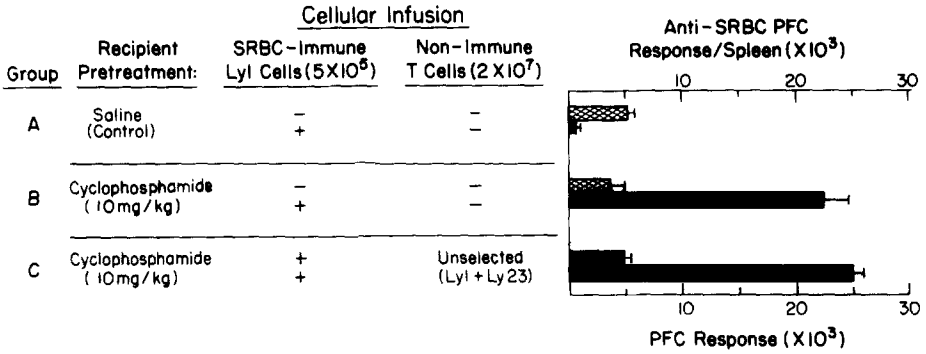

FIG. 1. Lyl23 cells mediating feedback inhibition are sensitive to small doses of cyclophosphamide in vivo (see Materials and Methods). The indicated cell populations were injected intravenously along with 2×10^6 SRBC into 8–10 wk old syngeneic B6 mice that had been given saline or cyclophosphamide (10 mg/kg i.p.) 24–36 h previously. Purified "SRBC-immune" Lyl cells (see Materials and Methods for preparation) were obtained from B6 donors immunized with 2×10^8 SRBC i.v. 10–30 days previously. The mean and SE of the α-SRBC PFC response of each group (three to five mice/group) was determined 5 days after injection of SRBC. Similar results were obtained in three additional experiments.

from untreated controls. Adoptive transfer of SRBC-immune Ly1 cells to untreated control mice resulted in over a 90% reduction of the anti-SRBC PFC response (Fig. 1, group A). By contrast, adoptive transfer of identical populations of SRBC-immune Ly1 cells to recipients that had been pretreated with 10 mg/kg cyclophosphamide i.p. 1 day previously resulted in a substantial increase (four to sixfold) in the anti-SRBC PFC response (group B). Thus, the adoptive transfer of purified SRBC-immune Ly1 T-helper cells into normal, untreated hosts failed to increase the host anti-SRBC response; in fact, it resulted in a profound reduction of the response which was associated with the activation of a cyclophosphamide-sensitive population of host cells.

Since the phenotype of nonimmune T cells responsible for feedback suppression in vitro and in vivo (Tables I and II) was $Ly1^+2^+3^+$, we asked whether the same cell population was sensitive to in vivo administration of small doses of cyclophosphamide (10 mg/kg i.p.). SRBC-immune Ly1 cells were inoculated into recipients that had been pretreated with cyclophosphamide on day -2 and repopulated with unselected spleen cells on day -1. Such repopulation reversed the effects of cyclophosphamide: i.e., SRBC-immune Ly1 cells induced potent feedback suppressive effects in spleen cell-repopulated hosts. If $Ly123^+$ cells had been removed from the nonimmune lymphoid population, feedback suppression was abolished (Fig. 1, group C).

Finally, we tested whether the set of cyclophosphamide-sensitive $Ly123^+$ cells responsible for feedback suppression in vivo was also responsible for in vitro feedback effects described previously (3) (Fig. 2): SRBC-immune Ly1 cells were added to culture containing purified B-cells alone (group A), B cells combined with nonimmune T cells from untreated donors (group B), or donors given 10 mg/kg cyclophosphamide i.p. 1 day previously (group C). Feedback suppression was noted only in group B cultures.

IN VIVO FEEDBACK SUPPRESSIVE ACTIVITY IS REDUCED SHORTLY AFTER ADULT THYMECTOMY (Fig. 3). Since at least a portion of cells belonging to the Ly123 subclass are sensitive to the short-term effects of adult thymectomy (5), we examined the effects of adult thymectomy upon in vivo feedback suppressive activity (Fig. 3). Infusion of SRBC-immune Ly1 cells into animals that had been sham-thymectomized 6 wk earlier induced strong feedback suppressive effects (group A). By contrast, infusion of SRBC-immune Ly1 cells into mice thymectomized 6 wk previously resulted in slightly enhanced responses (group B): and this loss of feedback suppression was regained if adult thymectomized animals were reconstituted with syngeneic spleen cell populations containing cells of the Ly123 subclass (group C).

Precocious Decline of Ly123 Cells and Feedback Suppressive Activity in NZB Mice. NZB mice spontaneously develop an autoimmune disorder characterized by the production of a variety of autoantibodies and a clinical syndrome resembling human systemic lupus erythematosus (8, 9). The suggestion has been put forward that this disorder is due, in part, to abnormal thymus-dependent differentiation, resulting in an imbalance between T-helper cells and T-suppressor cells (8, 9). To examine this question, we first determined proportions of Ly^+ T-cell subclasses in the spleens of NZB and BALB/c mice during the first 12 mo of life. These studies clearly show that during this time

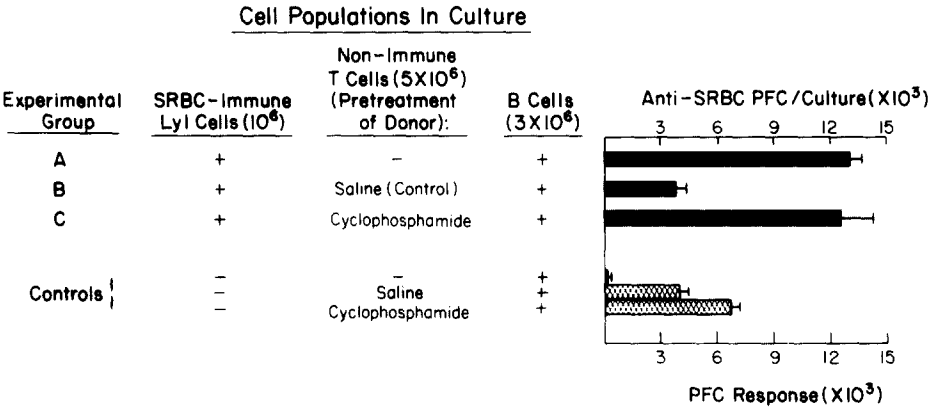

FIG. 2. Nonimmune T cells exerting feedback inhibition in vitro are sensitive to cyclophosphamide. Cultures containing the indicated cell populations were stimulated with 3×10^6 SRBC (3) $\times 5$ days. Cyclophosphamide (10 mg/kg) was administered i.p. 24–36 h before sacrifice and peparation of nonimmune spleen cells; purified Lyl cells and purified B cells were obtained as described previously (3). The mean α-SRBC PFC and standard errors for each group are based on the PFC responses of triplicate cultures. Similar results were obtained in four additional experiments.

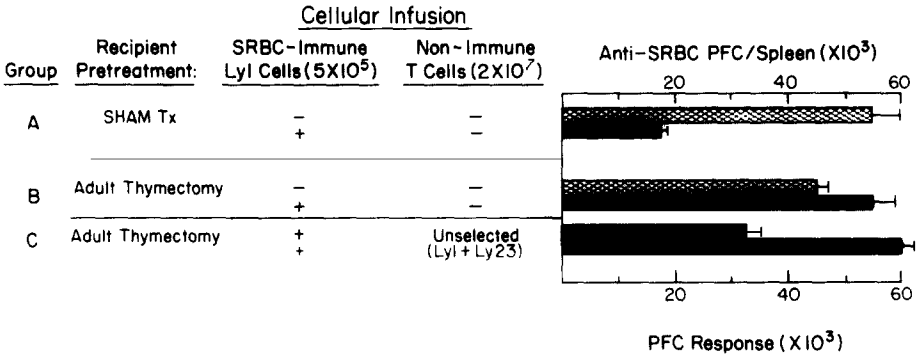

FIG. 3. Ly123 cells mediating feedback inhibition in vivo are sensitive to the short-term effects of adult thymectomy. B6 mice were thymectomized or sham thymectomized at 6 wk of life, and injected with the indicated cell populations at 10 wk of age. The protocol for adoptive transfer of SRBC-immune Lyl cells and nonimmune T-cells, immunization of adoptive hosts with SRBC and measurement of PFC were performed as outlined in the legend to Fig. 1; three to four mice per group. Similar results were obtained in two additional experiments.

period, NZB mice develop inordinately *high* proportions of Lyl and Ly23 cells, and substantially *reduced* concentrations of $Ly123^+$ cells compared with age and sex-matched BALB/c mice (Fig. 4).

In view of the substantial levels of $Ly23^+$ cells, one explanation of the immunoregulatory defect noted in NZB mice is that the suppressive $Ly23^+$ T-cell set is functionally defective and not capable of generating suppressive activity. This is unlikely since in vitro stimulation of NZB T-cells by high concentrations of SRBC induces potent $Ly23^+$ SRBC-specific suppressive activity, as tested in fresh cultures of NZB spleen cells (10).

An alternative explanation of the immunoregulatory disorder in NZB mice is

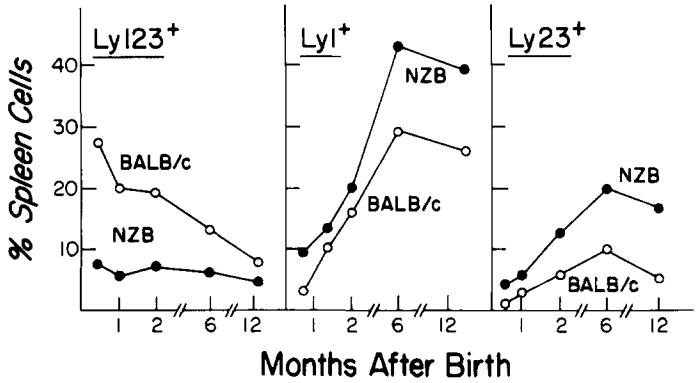

FIG. 4. The ontogeny of Ly^+ T-cell sets in NZB and BALB/c mice. The proportions of $Ly123^+$, $Ly1^+$ ($Ly1^+23^-$), and $Ly23^+$ ($Ly1^-23^+$) T-cell subclasses in the spleen cell populations of (♀) NZB and BALB/c mice of increasing age were measured as described previously (5); specificity controls were performed as indicated in Materials and Methods. Ly phenotypes of spleen cell populations from each age group were determined using pooled spleen cell populations from four to six donors. It should be noted that at 6 mo of age, almost 70% of NZB spleen cells were Ly^+ (7% $Ly123^+$; 41% $Ly1^+$; 20% $Ly23^+$). In two additional experiments, spleen cell populations obtained from 6 mo old NZB mice contained 54–70% Ly^+ lymphocytes.

that the formation of autoantibodies is, in part, due to high levels of Ly1 T_H activity and the absence of a set of Ly123 cells that normally mediates feedback suppressive activity. To test this hypothesis, spleen cells from NZB mice of different ages were tested for the presence of feedback-suppressive activity in vitro. As early as 1.5 mo after birth, little or no feedback-suppressive activity is detectable in nonimmune NZB spleen cells, in sharp contrast to the potent feedback suppressive activity expressed by spleen cells for age and sex-matched BALB/c controls (Fig. 5). These findings suggest that, both by the criterion of cell surface phenotype and that of in vitro function, NZB mice progressively lose a set of T cells responsible for feedback-suppressive activity.

Discussion

These findings show that T-T interactions responsible for feedback regulatory effects of in vitro antibody responses, also play a central role in regulating in vivo antibody responses. Repopulation of "B" mice with SRBC-immune Ly1 cells resulted in the acquisition of immunocompetence and the formation of large numbers of anti-sheep erythrocyte PFC after immunization. Infusion of unselected, nonimmune T-cells to these mice resulted in a dramatic reduction of the anti-SRBC response. Removal of $Ly123^+$ cells from the nonimmune T-cell population before adoptive transfer eliminated suppression. The set of Ly123 cells responsible for feedback suppression in vivo was shown to be sensitive to small doses of cyclophosphamide and ATx.[2]

[2] These findings indicate that failure of lymphoid cells from immune donors to transfer secondary antibody responses to adoptive, nonirradiated recipients probably reflects induction of feedback suppressive effects by radiosensitive or cyclophosphamide-sensitive host T cells, rather than a requirement for "space" in host lymphoid tissues. This feedback mechanism should be taken into account in designing clinical protocols aimed at either specific augmentation of immunity by adoptive cellular immunotherapy, or nonspecific augmentation by adjuvant-like materials. Relevant, too, is that the combination -ATx + cyclophosphamide- is synergistic.

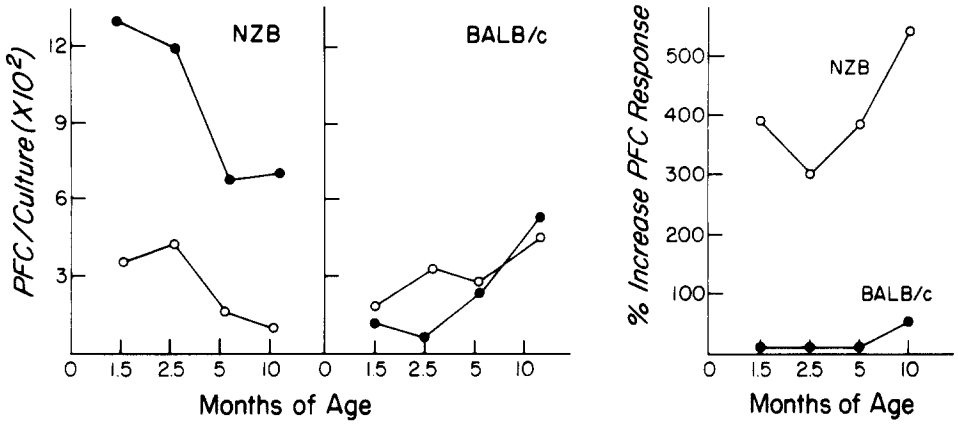

FIG. 5. Failure of spleen cell populations from NZB mice to express feedback inhibition. Purified Ly1 cells were obtained from 10 wk old NZB or BALB/c mice that had been immunized with 2×10^8 SRBC i.v. 10 days previously. 2×10^5 SRBC-immune Ly1 cells were added to SRBC-stimulated cultures containing 5×10^6 syngeneic spleen cells from non-immune NZB or BALB/c mice of the indicated age. The mean α-SRBC-PFC responses of SRBC-stimulated spleen cell cultures from nonimmune NZB or BALB/c mice of increasing age in the absence of added SRBC-immune Ly1 cells (○) are compared to the PFC responses of cultures containing spleen cells combined with 2×10^5 syngeneic SRBC-immune Ly1 cells (●). The percent increase in α-SRBC PFC noted after addition of syngeneic SRBC-immune Ly1 cells to age and sex-matched NZB or BALB/c spleen cells is shown on the right.

A second important indication of the physiologic importance of Ly123-mediated feedback suppressive activity comes from studies of NZB mice. These mice (*a*) spontaneously develop a variety of autoantibodies including anti-erythrocyte and anti-DNA antibodies (11), (*b*) acquire cellular and immunologic competence earlier than most other inbred strains (12), perhaps due to accelerated processing of pro-thymocytes by the thymus (13), and (*c*) are difficult to render tolerant after exposure to high concentrations of foreign proteins (14). The present studies clearly establish that throughout the 1st yr of life, spleen cells of NZB mice contain high concentrations (and absolute numbers) of cells of the Ly1 (T_H) set, high levels of Ly23 (T_S) cells, but harbor substantially reduced numbers of Ly123 T-cells. Consonant with this latter finding is the inability of spleen cells from either young or old NZB mice to mediate significant feedback suppressive activity in vitro. These findings, taken together, suggest an association between a "natural" defect in feedback suppression and a loss of immunoregulation resulting in the formation of autoantibodies. These experiments do not establish the cause of this subset-specific T-cell defect. Two possibilities must be considered: (*a*) NZB mice (as well as senescent mice) contain relatively high levels of "naturally-occurring" thymocyte autoantibody (NTA) (15); administration of NTA to young NZB mice can accelerate the disease (15). Possibly this antibody may selectively eliminate the $Ly123^+Qa1^+$ T-cell set responsible for feedback suppression. A second possibility is that NZB mice may undergo chronic, continuous antigen stimulation by virus-associated antigens. Such chronic stimulation might result in accelerated T-cell differentiation and continuous induction of Ly1 and Ly23 T-cell sets, at the expense of a

regulatory Ly123 precursor pool. Each of these explanations is clearly testable.[3]

We have not yet established the developmental relationship between the set $Ly123^+Qa1^+$ T-cells that mediate feedback suppression and other T-cell sets; nor do we have direct evidence bearing on whether suppression is directly mediated by Ly123 cells, or whether, after stimulation by Ly1 T_H cells, these cells differentiate to "mature" suppressor cells, expressing the Ly23 surface phenotype (17). Tada (18) has indicated that after immunization, products of Ly23 cells can induce immune but not resting Ly123 cells to generate increased antigen-specific $Ly23^+$ T_S activity. A similar recruitment or augmentation circuit has been implied for generation of mature T_H cells: products of Ly1 T_H cells induce $Ly123^+$ T-cells from immune donors to differentiate to mature antigen-specific T_H (Ly1 cells). Both T-T interactions represent *amplification* circuits, in which mature antigen-specific T cells recruit additional T_H or T_S cells from a partially differentiated T-cell population. Both "positive-feedback" circuits may be useful for potentiation of either the helper or suppressive mode after stimulation by antigen. By contrast, the T-T interaction described here represents a "negative-feedback" circuit that is useful for homeostatic regulation of the duration and intensity of the immune response.

These considerations also suggest that the response to a given antigenic determinant may reflect, in part, the amount of feedback inhibition generated after exposure to that antigen. B. Benacerraf et al., personal communication, have recently shown that pretreatment of genetically controlled nonresponder "suppressor" mouse strains with a small dose of cytoxan allows significant PFC responses to two Ir-regulated antigens, GT and GAT. R. K. Gershon has also found that ATx allows nonresponder SJL mice to produce antibody to GAT. These findings indicate that, at least for some Ir gene-controlled antigen responses, exaggerated induction of cytoxan and ATx-sensitive feedback inhibitory cells may be sufficient to mask delivery of the T_H signal to the B cell.

In sum, the data described in this report suggest that the following cellular events may ensue after stimulation of the immune system by foreign materials: activated antigen-specific T_H (Ly1) cells induce B cells to form antibody and induce resting $Ly123^+Qa1^+$ cells to inhibit T_H activity (2, 3). Reduction in T_H activity is accompanied by decreased induction of B cells as well as progressively decreasing induction of resting Ly123 cells: the net result is a progressive decrease in both antibody formation and supressor-cell induction.

According to this notion, the following predictions can be made: (*a*) after immunization, T-helper activity should always be accompanied by T-suppressor activity; (*b*) optimal generation of T_S activity requires the presence of both Ly1 cells and Ly123 cells during antigen stimulation; and (*c*) T-helper (Ly1) cells may produce cell-free factors which can induce resting Ly123 cells to express T_S activity. Evidence in support of predictions *a* and *b* have been published (19–21). We have obtained preliminary evidence in support of the third proposition

[3] It is worth noting that the proportions of Ly123 cells and associated feedback-suppressive activity are substantially reduced in aged mice of all inbred mouse strains so far examined (B6, BALB/c, CBA); possibly the NZB syndrome may, in part, represent a disorder best characterized as accelerated immunologic senescence. Indeed, autoantibodies similar or identical to those found in NZB mice can be found in senescent mice of various other inbred strains (16).

and these latter findings also indicate that T_H (Ly1) cell products that induce B cells to secrete antibody are not biochemically identical to those responsible for the induction of feedback suppression by Ly123 cells.

In general, these considerations suggest that the immune system responds in large part to signals continuously generated from *within* the system itself, and that detectable immune responses reflect alterations of these signals after the Ly1 system is perturbed by "antigen". Our data also indicates that a major role of cells of the Ly123 subclass is to (*a*) detect these messages and (*b*) pass them on to mature regulatory T cells of the Ly1 or Ly23 sets (perhaps via macrophages) and/or differentiate to mature regulatory T-cell sets. The net effect of these interactions after perturbation by antigen is to restore the homeostatic balance of the system, usually at a new level reflecting differentiation of antigen-specific clones belonging to the lymphocyte sets described in these studies.

Summary

We have shown that (*a*) purified T-helper cells induce cells of another T-cell set, expressing the $Ly123^+Qa1^+$ surface phenotype, to exert potent suppressive activity, (*b*) this T-T interaction plays an important role in regulating in vivo immune responses, and (*c*) this interaction represents an important barrier to protocols intended to augment the immune status of individuals by adoptive (or active) immunotherapy.

Our results also indicate that the $Ly123^+$ T-cell set mediating feedback suppression in vivo is sensitive to both low doses of cyclophosphamide and removal of the thymus in adult life. The importance of this T-T interaction to normal, physiologic regulation of the immune system is emphasized by the finding that the major T-cell deficit of NZB mice (an inbred strain of mice that spontaneously develops an autoimmune disorder) is the absence or malfunction of an $Ly123^+$ T-cell set responsible for feedback inhibition.

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