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Immunology

Definition of two pathways for generation of suppressor T-cell activity

(feedback inhibitory circuit/inducer cell/Ig-H-linked gene)

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ABSTRACT **Antigen-stimulated Ly1 cells induce T cells from nonimmune donors to develop potent feedback suppressive activity. Suppression is mediated by Ly23 suppressor T (Ts) cells, which are generated from either Ly23 or Ly123 precursors. Ts activity generated from Ly23 precursors requires a strong inducer signal and is rapidly expressed but short lived. In contrast, Ts activity from Ly123 precursors is relatively long lived and is efficiently generated by relatively low levels of inducer signals. Induction of both Ly123 and Ly23 precursors to become Ts cells requires that both cells share genes linked to the *Ig-H* locus.**

There is good evidence that the immune response is regulated by a series of interactions among inducer, suppressor, and effector T cells (1). Each of these sets expresses a unique genetic program that combines information for a particular pattern of cell surface glycoproteins and a specialized function (2, 3). Inducer cells, bearing the surface phenotype $Thy1^+Ly1^+Ly2^-$, stimulate various target cells to divide or differentiate (2–7). A portion of these cells specifically activate $Ly12^+$ cells to suppress Ly1 inducer activity: this interaction has been termed "feedback" inhibition because the level of suppression is directly proportional to the number of antigen-activated Ly1 cells and because one target of suppression is the inducer cell itself (5–7). Although Ly123 cells play a critical role in the development of $Ly23^+$ suppressor T (Ts) cells, the mechanism by which they influence the suppression circuit has not been directly defined.

One approach to this question involves the isolation of each T-cell set that participates in this complex interaction and definition of the contribution of each to the generation of Ts activity. This general approach has been used to define other complex biologic interactions, such as the cascade of events resulting in complement (C) activation or blood clotting. We show that antigen-activated Ly1 inducers are essential for conversion of both Ly23 and Ly123 precursors into Ly23 Ts cells and that the Ly123 → Ly23 Ts transition is a substantially more effective pathway for Ts generation than activation of resting Ly23 cells to become Ly23 Ts. Finally both T-cell inducer–acceptor interactions are controlled by the genes regulating expression of the Ig heavy chain (i.e., the *Ig-H*-linked genes).

MATERIALS AND METHODS

Mice. C57BL/6 (B6) and BALB/c mice, 8–12 wk old, were obtained from The Jackson Laboratory. CB.20 mice were from the breeding colony at Sidney Farber Cancer Institute, Boston, MA.

Antisera. Ly1.2 and Ly2.2 antisera prepared as described (8) were provided by F. W. Shen. Monoclonal anti-Thy1.2 was the gift of A. Marshak-Rothstein (Boston University Medical School) and noncytolytic monoclonal anti-Ly1 and anti-Ly2 were purchased from Becton Dickinson. Low-toxicity rabbit C was obtained from Cedarlane Laboratory Limited, Ont., Canada.

Antigen and Immunization. Sheep erythrocytes (SRBC) were purchased from Colorado Serum, Denver, CO. Mice were immunized intraperitoneally with 2×10^7 SRBC in 0.2 ml of balanced salt solution four days before use.

Preparation of Lymphoid Cell Populations. *B cells.* B cells were positively selected (9, 10) as follows. Goat anti-mouse Ig was incubated in Fisher Petri dishes (100 μg per dish) with 10 ml of 0.05 M Tris buffer (pH 9.5) for 40 min at room temperature. Then, the dishes were extensively washed with balanced salt solution/2% fetal calf serum, 3 ml of spleen cell suspension in balanced salt solution/5% fetal calf serum (10^7 cells per ml) was added, and suspensions were incubated for 70 min at 4°C with occasional swirling. The nonadherent cells were removed, the plates were washed gently (three times), 5 ml of balanced salt solution/5% fetal calf serum was added, and the plates were then slowly rocked for 30 min at 37°C. Adherent cells were washed from the plates by vigorous pipetting. Eluted cells were incubated with monoclonal anti-Thy1.2 (final dilution, 1:1,000), anti-Ly1.2 (final dilution, 1:60), and anti-Ly2.2 (final dilution, 1:20) for 35 min on ice (3×10^7 cells per ml); washed; and then incubated for an additional 35 min with rabbit C. The recovered cells were washed twice before use.

$Ly1^+23^-$ cells. The nonadherent cells recovered from the B-cell preparation dishes were applied to monoclonal anti-Ly1-coated plates (11). Adherent cells were eluted as described above and incubated with anti-Ly2.2 (3×10^7 cells per ml; final dilution, 1:20) and then with rabbit C. This procedure resulted in a cell population >98% $Thy1^+Ly1^+23^-Ig^-$, as determined by immunofluorescence.

$Ly1^-23^+$ cells. Nonadherent cells from the anti-Ig dishes were applied to anti-Ly2-coated plates. These plates were prepared by the coating method used for anti-Ly1 plates. Adherent cells were eluted as described above and incubated with anti-Ly1.2 (3×10^7 cells per ml; final dilution, 1:60) and then with rabbit C. Approximately 95–98% of the recovered cells were $Thy1^+Ly1^-23^+Ig^-$.

$Ly1^+23^+$ cells. After positive selection of Ig^- cells on anti-Ly2-coated plates, the eluted cells were incubated in dishes coated with anti-Ly1. The recovered adherent cell population was >98% $Thy1^+Ly1^+23^+Ig^-$.

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Abbreviations: Ts cells, suppressor T cells; *Ig-H* locus, genes regulating expression of the Ig heavy chain; SRBC, sheep erythrocytes; pfc, plaque-forming cell(s); C, complement.

***In Vitro* Stimulation of Ly1 Cells with SRBC.** SRBC-stimulated Ly1 cells were prepared as follows. Highly purified Ly1 cells (1×10^7) were incubated with 2×10^6 SRBC for 4 days in Falcon 3008 tissue culture plates containing 1 ml of Mishell–Dutton culture medium (Hepes-buffered RPMI/10% fetal calf serum/20 mM L-glutamine/50 μM 2-mercaptoethanol) at 37°C in humidified 5% CO_2/95% air. On day 4, cells were harvested and viability was determined by trypan blue exclusion.

***In Vitro* Induction of Ts Cells.** Groups of *in vitro* SRBC-stimulated Ly1 cells were incubated with highly purified Ly23 and Ly123 cells and 2×10^6 SRBC in 1 ml of Mishell–Dutton culture medium for 18 or 40 hr. After harvesting, $Ly1^+$ and $Ly12^+$ cells were removed by incubation with anti-Ly1.2 + C.

Assay for Ts Cells. The ability of Ly23 cells retrieved from induction cultures to suppress a primary anti-SRBC plaque-forming cell (pfc) response was determined by adding 1×10^5 Ly23 cells to mixtures of 1×10^5 Ly1 cells and 5×10^5 B cells from SRBC-immunized animals in a final volume of 0.2 ml of culture medium containing 1×10^6 SRBC (12). Anti-SRBC pfc were measured 5 days later by the Cunningham and Szenberg (13) modification of the technique originally described by Jerne. In all cases, anti-SRBC pfc were determined from triplicate cultures.

RESULTS

Both Ly123 and Ly23 Cells Require Induction by Ly1 Cells to Develop Ts Activity. To induce Ly23 Ts cells, 10^6 Ly1 cells were incubated with either Ly23 cells, Ly123 cells, or a mixture of the two with SRBC for 2 days. After removing $Ly1^+$ cells (Ly1 and Ly12 cells) by treatment with anti-Ly1 + C, we tested the ability of retrieved cells to inhibit the anti-SRBC responses of mixtures of Ly1 and B cells (Table 1). Neither inducer cells nor $Ly2^+$ cells incubated separately with SRBC significantly suppressed anti-SRBC pfc responses. Ly23 Ts were generated only after incubation of mixtures of Ly1 inducer cells with either Ly23 or Ly123 T cells. Mixtures containing Ly1 and Ly123 cells together generated at least 10-fold more Ly23 Ts activity than $Ly2^+$ cells alone. Thus, (*i*) antigen-activated Ly1 cells do not directly suppress anti-SRBC pfc responses and (*ii*) they are necessary to generate Ly23 Ts from either Ly23 or Ly123 precursors.

Table 1. Requirement of Ly1 induction for generation of Ts activity

Cell population in induction culture				Suppression of pfc response: % suppression		Ly23 cells required for suppression*
Group	1×10^6	5×10^5	5×10^5	Exp. 1	Exp. 2	(no. $\times 10^4$)
A	Ly1			0	0	—
B		Ly23		21	16	>10
C			Ly123	38	25	>10
D	Ly1			0	0	—
E	Ly1	Ly23		**93**	**86**	3
F	Ly1		Ly123	**95**	**88**	1
G	Ly1	Ly23	Ly123	**91**	**85**	1
H		Ly23	Ly123	17	6	>10

Cells recovered from groups A–C were added to mixtures of Ly1 cells, B-cells, and SRBC (assay cultures). Cells recovered from groups D–H were treated with anti-Ly1.2 + C to abolish $Ly1^+$ activity before addition to assay cultures. Cells retrieved from the induction culture medium were tested for ability to suppress the anti-SRBC response of mixtures of Ly1 and B cells. In group D, cells remaining after anti-Ly1.2 + C treatment ($\approx 1 \times 10^3$) were tested. Values in boldface type indicate significant suppression (>50%).

* Minimum number of retrieved cells required for significant suppression of the pfc response.

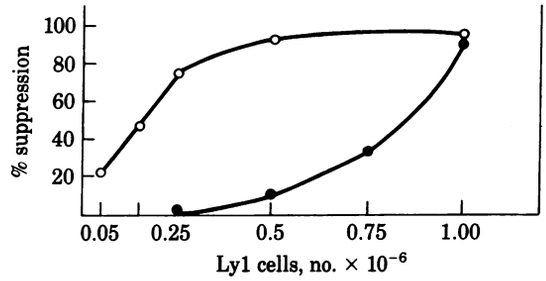

FIG. 1. Efficiency of Ts induction from Ls23 and Ly123 precursors. Purified Ly1 cells were stimulated with 2×10^6 SRBC for 4 days. Graded numbers of SRBC-activated Ly1 cells were mixed with 1×10^6 non-immune Ly23 (●) or Ly123 (○) cells for 40 hr. After treatment with anti-Ly1.2 + C, 1×10^5 cells from each culture were added to SRBC-stimulated cultures containing 1×10^5 Ly1 cells and 5×10^5 B cells from donors immunized intraperitoneally with 2×10^7 SRBC 4 days earlier. Anti-SRBC pfc responses were measured 5 days later.

Efficiency of Induction. We quantitated the number of inducer cells required for generation of Ly23 Ts activity from the two Ts precursors (Fig. 1). Although Ly1 inducer cells stimulated Ly23 Ts from either Ly23 or Ly123 precursors, Ts activity from Ly23 cells required 5- to 10-fold more Ly1 cells than did Ly123 precursors as judged by the number of cells required for 50% suppression.

Tempo of Ly23 Ts Generation from Ly23 or Ly123 Precursors. Graded numbers of inducer cells were mixed with two types of Ts precursors for either 18 or 40 hr. After 18 hr, Ly23 cells produced strong Ts activity in the presence of very large numbers of inducer cells (Fig. 2*A*). No further generation of Ts activity occurred over the next 22 hr (Fig. 2*B*). In some cases, longer periods of induction resulted in loss of Ts activity: from 88% suppression at 18 hr to 72% suppression at 40 hr. By contrast, cultures containing Ly123 cells did not generate substantial suppressor activity until 40 hr. Possibly, Ly23 cells were induced to terminally differentiate after 18 hr. Alternatively, rapid acquisition of Ts activity by Ly23 cells resulted in elimination of inducer cells and prevention of further Ts generation. In the latter case, but not the former, provision of high numbers of Ly1 inducer cells would favor Ts generation. In fact, generation of Ts activity from Ly23 cells required large numbers of Ly1 cells [5- to 10-fold more than required for Ly123 Ts precursors (Fig. 1)]. We therefore favor the latter view.

Genetic Requirement for Induction of Ts. We have previously shown that genes linked to the Ig heavy chain locus control the generation of Ts activity associated with feedback suppression (14). We directly tested the role of this locus in regulating Ts generation from purified Ly1 and $Ly2^+$ cells.

BALB/c and CB.20 mice are a congeneic pair differing only at *Ig-H*-linked genes (15). Mixtures of Ly1 inducers and Ly23

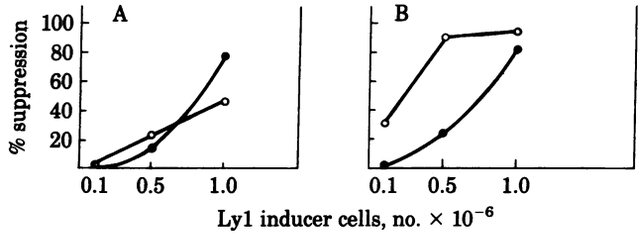

FIG. 2. Tempo of Ly23 Ts generation from Ly23 or Ly123 precursors. SRBC-stimulated Ly1 inducer cells were mixed with Ly23 (●) or Ly123 (○) cells at different ratios for 18 (*A*) or 40 (*B*) hr. Ly23 cells were retrieved from cultures after anti-Ly1.2 + C treatment and added to cultures containing Ly1 and B cells and SRBC.

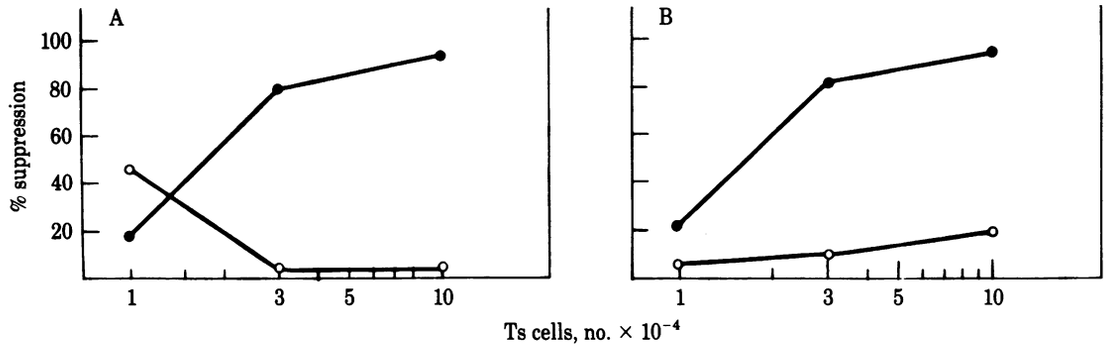

FIG. 3. Ig-restricted induction of Ts. Purified Ly1 cells from BALB/c mice were stimulated with SRBC for 4 days. Recovered Ly1 cells were mixed with Ly23 cells (*A*) or Ly123 cells (*B*) from either BALB/c (●) or CB.20 (○) mice for 40 hr. Ly23 cells were retrieved by anti-Ly1.2 + C treatment and then added to mixtures of Ly1 and B cells isolated from the $Ly2^+$ donor strain. Suppression of the anti-SRBC pfc response was tested 5 days later.

precursors identical at the *Ig-H* locus produced strong Ts effector activity. In contrast, mixtures of inducers and $Ly2^+$ Ts precursors that differed at the *Ig-H* locus did not produce significant suppression (Fig. 3).

DISCUSSION

These studies directly show that activated Ly1 cells induce Ly23 Ts cells from either resting Ly23 or Ly123 cell precursors. The cellular events leading to generation of Ly23 Ts activity from these two precursor pools differ considerably. Optimal Ts activity from Ly23 cells requires large numbers of Ly1 inducer cells and develops rapidly. Generation of Ts activity from Ly123 cells requires relatively low levels of inducer activity and a longer period of induction. Generation of Ts from either precursor is controlled by genes linked to the *Ig-H* locus.

Thus, resting $Ly2^+$ cells appear to constitute a store of receptor-positive intermediary cells that regulate the supply of Ly23 Ts activity. If stimulation of inducer cells by foreign material is relatively weak, the major source of Ts activity comes from Ly123 precursors. Production of Ts by this pathway is slow but progressive, allowing Ly1 induction of B cells before Ts cells dampen or abrogate this signal. In contrast to this homeostatic reaction, invasion of the immune system by large amounts of a strong antigen results in activation of large numbers of inducer cells that rapidly activate Ly23 cells. However, this may cause precocious inactivation of inducer cells and a rapid but short-lived suppressive response. After rapid induction of Ts activity from Ly23 precursors is reached, no further suppressive activity is generated. It is intriguing that NZB mice, which spontaneously generate autoantibodies, have high levels of inducer and Ly23 cells but substantially decreased concentrations of Ly123 cells (6). Possibly, reduced numbers of Ly123 cells in NZB mice might contribute to the loss of immunoregulation and result in the formation of large amounts of autoantibodies. A similar situation is seen in aged mice (6).

The net result of Ts generation from Ly123 cells is feedback suppression: Ly1 inducer cells that activate suppression and antibody formation are themselves inhibited by the Ly23 Ts cells they have induced, thus preempting further generation of suppression and antibody production. This feedback interaction between inducer cells and suppressor cells is in many ways analogous to enzyme–substrate interactions: Ly1 inducer cells act as a substrate for precursors of suppressive cells (enzyme) that in turn eliminate the substrate. This self-regulating inducer–acceptor circuit preempts the necessity for an endless network of dampening interactions among "suppressors of suppressors" carrying appropriate Ig-H markers. Instead, control of excessive $Ly2^+$ Ts activity reflects the loss of inducer cells required for generation and activation of Ts rather than continuous active generation of new regulatory cells.

A final point concerns the genetic regulation of inducer–acceptor interaction. Yamauchi *et al.* (16) have shown that Ly1 cells release molecules that bind antigen and also recognize Ig-H-linked products expressed by $Ly2^+$ cells. Control of the inducer–acceptor interaction by *Ig-H*-linked genes (Fig. 3) may reflect inducer cell recognition of antigen and Ig-H structures on Ly2 Ts precursors, analogous to corecognition of antigen and major histocompatibility structures on a subset of macrophages and B cells.

In summary, these data show that the intensity and duration of suppression are determined by two distinct pathways of Ts formation and that both are controlled by genes linked to the *Ig-H* locus.

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