SEPARATION OF HELPER T CELLS FROM SUPPRESSOR T CELLS EXPRESSING DIFFERENT Ly COMPONENTS

I. Polyclonal Activation: Suppressor and Helper Activities are Inherent Properties of Distinct T-Cell Subclasses*

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Peripheral T lymphocytes can be subclassified on the basis of differential expression of Ly components on their surfaces (1-3). Approximately 50% of peripheral T cells manifest all three Ly components analyzed so far (phenotype Ly123), about 33% only Ly1 (phenotype Ly1), and about 5-10% Ly2 and Ly3 (phenotype Ly23). Functional studies indicate that Ly1 T cells can generate helper function during an adoptive primary antibody response, but do not generate appreciable killer activity to alloantigens, whereas the reverse is true of Ly23 T cells. In this report we examine the participation of T-cell subclasses of different Ly phenotypes from the standpoint of their regulatory or suppressive functions (4).

Our approach to this question involves the use of a polyclonal activator, concanavalin A (Con A).[1] Con A stimulates T cells to undergo proliferative and differentiative events similar to those which T cells undergo when they encounter specific antigen (5, 6), and Con A-activated T cells can under appropriate circumstances perform helper, suppressor, and killer functions (5, 6). We analyze here whether each of these functions is confined to a distinct subclass of T cells.

Materials and Methods

Animals. C57BL/6 (B6) mice 10-14 wk of age were obtained from The Jackson Laboratory, Bar Harbor, Maine. The congenic lines B6-*Ly-1*^{a} and B6-*Ly-2*^{a} (see Klein, reference 7), phenotypes Ly-1.1, 2.2,3.2 and Ly-1.2,2.1,3.2, respectively, were produced and supplied by E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York.

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[1] *Abbreviations used in this paper:* α, anti; Con A, concanavalin A; B6, C57BL/6; NMS, normal mouse serum; PBS-FBS, phosphate-buffered saline plus 10% fetal bovine serum; PFC, plaque-forming cells; SIRS, soluble immune response suppressor; [^{3}H]TdR, tritiated thymidine.

Antisera. For details of the preparation and use of antisera to Ly-1.2, Ly-2.2, and Thy-1.2 see Shen et al., (8) which also provides a bibliography of the Ly systems.

Isolation of Ly Subclasses. 20–50 × 10^6 cells/ml were incubated with Ly antiserum at a final dilution of 1:40 in phosphate-buffered saline plus 10% fetal bovine serum (PBS-FBS) for 1/2 h at 20°C. After washing once, the cells were brought up in 1 ml of freshly thawed selected rabbit serum (diluted 1:12 in PBS) as the source of complement (C), and were incubated for another 1/2 h at 37°C. Ly specificity was confirmed for each group of experiments by substituting cells from congenic B6-*Ly-1*^{a} and *2*^{a} donors (negative controls) as described previously (1, 3). In each case, anti(α)-Ly activity was absorbed from Ly antiserum only by B6 cells and not by cells of the respective B6-Ly congenic lines.

Polyclonal Activation of T Cells with Con A. B6 spleen cells that had been treated with the various Ly sera were incubated at 10^7 cells/ml in RPMI 1640 or Eagle's minimal essential medium (MEM) containing 10% FBS (lot no. M26302; Reheis Chemical Co., Kankakee, Ill.) alone (control) or with 1 μg/ml Con A (ICN Nutritional Biochemical Div., International Chemical & Nuclear Corp., Cleveland, Ohio) for 48 h. The cells were harvested, washed four times, and added in graded numbers of viable cells to test cultures to assess their influence on the generation of cytotoxic lymphocytes or plaque-forming cells (PFC). In other experiments, the cells were activated with Con A first, and then treated with the various Ly sera plus C, before addition to test cultures.

Con A-activated T cells produce a factor(s), soluble immune response suppressor (SIRS), which nonspecifically suppresses antibody responses in vitro (9). To determine which T-cell subclass produces SIRS, the different Ly subclasses were prepared from spleen and incubated (in numbers corresponding to 10^7 of the starting spleen cell population) in MEM containing 2% FBS with or without 1 μg/ml Con A for 48 h. Supernatant fluids were harvested, absorbed with Sephadex G50 to remove residual Con A, sterilized by membrane filtration, and added (at a final dilution of 1:40) to test cultures to assay activity on PFC responses (9).

To assess the proliferative responses of T-cell subclasses to Con A, spleen cells or nylon wool-enriched T cells (1, 2), after treatment with the various Ly sera or NMS, plus C, were incubated at 5 × 10^6 or 10^7 viable cells/ml in RPMI 1640 containing 10% FBS with 0, 1, and 2 μg/ml Con A. At 60 h, 1 μCi tritiated thymidine ([^{3}H]TdR) was added, at 72 h the cultures were terminated and [^{3}H]TdR incorporation was assessed.

Test Cultures for Assay of Activity of Con A-Activated T Cells and Their Products. In most experiments, the activities of Con A-activated cells were assayed simultaneously in two laboratories. A modified Mishell-Dutton culture system was used to generate αSRBC PFC (10, 11): 4–8 × 10^6 lymphocytes were incubated in 16-mm wells (dispose-trays; Linbro Chemical Co., New Haven, Conn.) with 3 × 10^6 SRBC in 1 ml of RPMI-1640 containing 10% FBS and 2 × 10^{-5} 2-mercaptoethanol. Cultures were rocked (8–12 times/min) at 37°C in an atmosphere of 8% CO_2, and fed daily with a nutrient mixture (11) for 5 days, harvested, and assayed for αSRBC PFC activity. PFC responses were measured using either the Cunningham assay or the slide modification of the Jerne localized-hemolysis-in-gel assay (10) and are expressed as PFC per culture. Cytotoxic responses generated during mixed lymphocyte reactions were measured in ^{51}Cr-release assays as described previously (1). Cytotoxic activity was calculated as follows:

$$\%\ \text{Cytotoxicity} = \frac{\text{cpm (sensitized cells)} - \text{cpm (unsensitized cells)}}{\text{cpm (freeze-thaw control)}} \times 100.$$

Ly Subclass Notation. Because there is so far no evidence that Ly2 and Ly3 can be expressed independently of one another, i.e. no cells of Ly-2^-3^+ or Ly-2^+3^- phenotypes have yet been identified (1), there are as yet only three well-defined Ly subclasses: Ly1, Ly23 and Ly123. Therefore in the following account we have used the following notations: (*a*) Ly1 signifies the selected T-cell population obtained by treating spleen cells or T cells with αLy-2 or αLy-3, plus C; (*b*) Ly23 signifies the equivalent T-cell population remaining after treatment with αLy-1 plus C.

Results

I. Proliferative Responses of Separate Ly Subclass Populations to Stimulation by Con A (Table I). Ly1 and Ly23 populations were prepared from (*a*) nylon-enriched, and (*b*) unfractionated, spleen cells of B6 mice, and then stimulated in

TABLE I

Thymidine Incorporation by T-Cell Ly Subclass Populations Exposed to Con A

Starting populations from B6 mice	Treated with C and:	T-cell population derived	Thymidine incorporation after 48 h		
			No Con A	Con A, 1 μg/ml	Con A, 2 μg/ml
				cpm	
Nylon-enriched splenic T cells	NMS	All	4,059	25,372	28,646
	αLy-1.2	Ly23	3,426	21,414	26,631
	αLy-2.2	Ly1	3,081	23,702	29,594
	αLy-3.2	Ly1	3,300	23,573	25,959
Unfractionated spleen cells	NMS	All	2,473	25,573	28,658
	αLy-1.2	Ly23	2,376	18,975	24,438
	αLy-2.2	Ly1	2,384	21,167	21,504

vitro with 1 or with 2 μg Con A/ml. According to [^{3}H]TdR incorporation by equal numbers of cells all these populations responded to Con A to roughly the same extent, and to about the same extent as control spleen cells treated only with normal mouse serum (NMS). Thus the responses of the Ly1 and Ly23 populations to the indicated concentrations of Con A are not markedly different from one another or from the T-cell population as a whole.

II. Suppressor Activities of Separate Ly Subclass Populations Stimulated by Con A (Fig. 1). Having established that the Ly1 and Ly23 populations are equally stimulated by Con A (I above), we asked whether these discrete nonspecifically activated populations would exhibit differences in regard to helper-suppressor capabilities.

For this purpose the Ly1 and Ly23 populations from B6 spleen were exposed to Con A (10^7 viable cells/ml culture containing 1 μg/ml Con A), harvested at 48 h, washed four times, and introduced in graded numbers (viable cell count) into cultures of 5×10^6 sheep erythrocyte (SRBC)-stimulated B6 spleen cells. As few as 10^5 Con A-activated spleen cells that had not been fractionated (treated only with NMS plus C before Con A activation) markedly suppressed the PFC response; 10^5 Con A-activated Ly23 cells gave somewhat more suppression; but Con A-activated Ly1 cells gave no demonstrable suppression, in fact their addition in large numbers to normal spleen cell cultures slightly increased the PFC response. Similar results (not shown) were obtained by substituting αLy-3.2 for αLy-2.2 (i.e. once again the Ly2 and Ly3 phenotypes are concordant). Thus polyclonally activated Ly23 cells, but not Ly1 cells, exhibit suppressor activity in the αSRBC system.

III. Ly Phenotypes of Effector-Suppressor Cells Generated by Con A (Fig. 2). The data above indicate that the resting T-cell population, before stimulation by Con A, already contains Ly23 cells that are programmed for suppression (nature unspecified) of the αSRBC response, as well as Ly1 cells that are not so programmed. To decide whether the Ly23 phenotype remains unchanged during the process of activation by Con A, we conducted tests to ascertain the Ly phenotype of the effector-suppressor cell generated by Con A. This entailed activating the whole spleen cell population with Con A, and then testing the

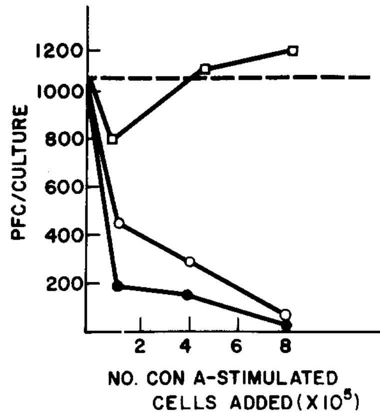

FIG. 1. Suppressor activity of Ly23 cells activated by Con A. Lyl and Ly23 populations were isolated from B6 spleen and activated by Con A (see II in Results) and added in graded numbers (abscissa) to fresh spleen cells (5×10^6 per culture; plus 3×10^6 SRBC). The PFC responses were assayed on day 5 (ordinate). (●), Ly23 cells; (□), Lyl cells; (○), intact spleen population (control; NMS plus C); and (---), no cells added.

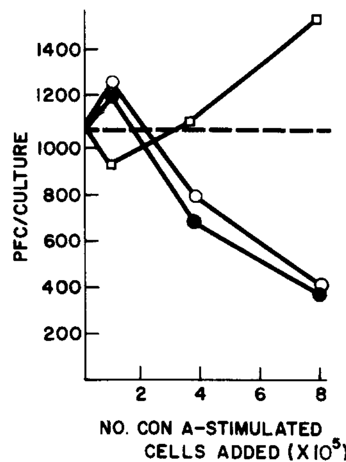

FIG. 2. Ly23 phenotype of effector-suppressor cells. The test system is the same as that shown in Fig. 1, except that the Ly subclass populations were separated after the intact spleen cell population had been activated by Con A. (●), Ly23 cells; (□), Lyl cells; (○), intact spleen population (control; NMS plus C); and (---), no cells added.

suppressor capabilities of Ly subclass populations separated from the already activated population.

Again suppression was effected by Ly23 cells but not by Lyl cells. Thus in the process whereby effector-suppressor cells are generated from antecedent cells already committed to suppressor function, there is no demonstrable change in Ly phenotype.

IV. Elaboration of SIRS by Ly⁺ T-Cell Subclasses (Table II). Supernatant fluids from Con A-activated spleen cells contain a factor (or factors), SIRS, made by T cells, that inhibits primary αSRBC PFC responses in vitro (9). We there-

TABLE II
Suppressive Effects of Supernates Generated from Treated Spleen Cells

Supernate of cultured spleen population:	PFC/culture	
	IgM	IgG
A. Intact	3,700	770
B. Intact (NMS)	3,780	810
Intact (NMS) + Con A	390	170
C. Ly23	1,490	540
Ly23 + Con A	500	140
D. Lyl	4,090	1,040
Lyl + Con A	3,340	1,230
E. C + D	3,180	880
C + D + Con A	630	170

Supernates were obtained after 24 h from cultures containing 10^7 viable spleen cells, either intact or after the usual elimination of Lyl cells (group C) or Ly23 cells (group D). The data show the effects of these supernates at a final dilution of 1:40, upon a primary in vitro αSRBC response.

fore asked whether SIRS was produced by the same subclass of T cells (Ly23) that suppressed the SRBC response in the previous experiments (II and III above). For this purpose Lyl and Ly23 populations were prepared from B6 spleen cells, at a starting concentration of 10^7/ml, and incubated, without readjustment of viable cell concentration, with or without 1 μg/ml of Con A for 48 h. Supernatant fluids were harvested, absorbed with Sephadex, and tested for SIRS activity in SRBC-stimulated B6 spleen cell cultures. These data indicate that SIRS is produced by cells of the Ly23 T-cell subclass, the same subclass of T cells responsible for suppressor T-cell activity (II and III above).

V. Immunologic Activity of Lyl Cells Activated by Con A (Fig. 3). The experiments described above show that Ly23 cells, but not Lyl cells, mediate suppressor activity after polyclonal activation with Con A. We next tested whether Lyl cells might develop helper activity after polyclonal activation. Ly subclasses were prepared from Con A-activated B6 spleen cells in the usual way. These were added to cultures of B lymphocytes (spleen population treated with αThy-1 plus C) and stimulated with SRBC. Although 1.5×10^6 of the intact spleen cell population incubated for 48 h without Con A expressed significant helper activity (group B), the same number of Con A-activated spleen cells did not (group C). Thus either helper activity was lacking in the intact Con A-activated spleen population, or it was masked by Ly23 suppressor cells. Groups D and E give the answer: Removal of Ly23 cells (group E) reveals helper activity of the intact spleen population. This helper activity was substantially greater than the activity of unstimulated spleen cells (group B). Thus the helper activity of Con A-activated Lyl cells is masked in the intact population by suppressor Ly23 cells.

VI. The Effect of Con A-Activated Ly Subclasses on the Generation of Killer

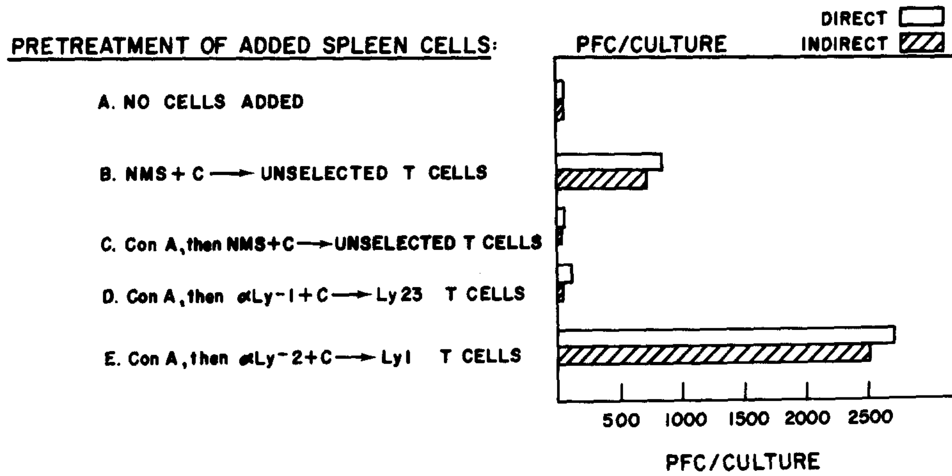

FIG. 3. The Con A-activated intact spleen population contains αSRBC helper cells of Ly1 phenotype. 1.5×10^6 remaining viable cells, after 2 days of incubation with or without 1 μg Con A, were added to cultures of 5×10^6 B cells (spleen cells treated with αThy-1 plus C) and 3×10^6 SRBC; assay for αSRBC on day 5.

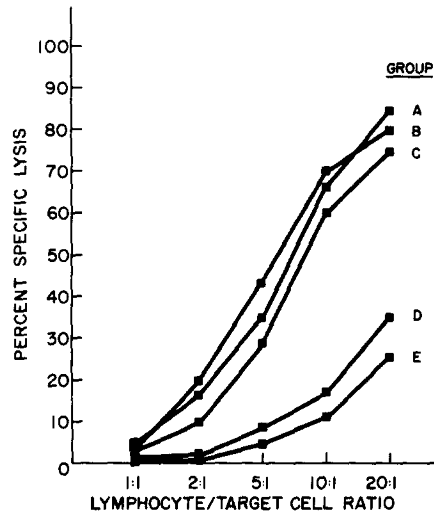

FIG. 4. Suppression of the generation of cytotoxic lymphocytes by Con A-activated Ly23 cells. Spleen cells were incubated for 48 h with 1 μg/ml Con A, and then treated with αLy-2 or αLy-1 as usual to yield the Ly1 and Ly23 subclass populations. 1.5×10^6 viable cells of each population were then added to fresh cultures containing 25×10^6 B6 (H-2^b) spleen cells plus 5×10^6 mitomycin C-treated BALB/c (H-2^d) cells. The figure shows cytotoxicity against ^{51}Cr-labeled P815 (H-2^d) target cells on day 5. Key: (A), no cells added; (B), 0.1 ml medium added; (C), Ly1 cells added; (D) Ly23 added; and (E), NMS-treated cells added.

Cells in vitro (Fig. 4). Con A-activated T cells have been shown to suppress the generation of cytotoxic lymphocytes in vitro (6). To determine the Ly phenotype of T cells that might suppress this cell-mediated immune response, Ly1 and Ly23 populations were prepared as usual from Con A-activated B6 spleen cells;

1.5×10^6 viable cells of each type were added to cultures of syngeneic responder cells and allogeneic (BALB/c; *H-2^d*) mitomycin C-treated stimulator cells, as detailed in Fig. 4. Cytotoxic responses assayed on ^{51}Cr-labeled P815 (*H-2^d*) target cells 5 days later show that the intact Con A-activated population (NMS plus C; group E) and the Ly23 subclass (group D) suppressed the generation of cytotoxic effector cells. Thus polyclonal activation of T cells gives rise to Ly23 cells that suppress not only humoral but also cellular immune responses.

Discussion

We have asked whether suppressor and helper functions are mediated by the same or different Ly subclasses separated from T cells that have been activated by Con A. The advantage of this approach is that any functional differences between such subclasses cannot be ascribed to preferential activation of particular Ly-distinctive T-cell subclasses by specific antigen.

The possibility that the suppression we observed reflects carry-over of Con A is extremely unlikely because: (*a*) elimination of Ly23 cells, but not of Ly1 cells, before Con A activation abolishes the development of suppressor T-cell activity; (*b*) inclusion of alpha-methyl-mannoside, a competitive inhibitor of Con A activation, does not influence suppressor activity (5), and (*c*) the amount of Con A actually carried over is two orders of magnitude below that required to suppress the generation of PFC to SRBC in vitro (9).

The findings are that: (*a*) Ly1 and Ly23 cells are equally activated by Con A to incorporate [^{3}H]TdR, and (*b*) Ly1 cells help, and Ly23 cells suppress. This rules out the possibility that suppression by Con A-activated T cells is due to some hypothetical mechanism involving an excess of helper activity, because activated Ly1 cells only help, they do not suppress, even in relatively high concentrations. Thus Ly1 and Ly23 cells are programmed for their respective helper and suppressor functions independently of their ability to discriminate and react to specific antigen. Moreover the same results can be obtained with Ly1 and Ly23 cells separated from the resting T-cell population before exposure to Con A. This confirms previous conclusions that programming for discrete T-cell functions, which is linked with the expression of particular Ly profiles, has already taken place before specific or nonspecific cellular activation (1, 2). Expression of those functions may of course depend upon activation.

Our interpretation of these data is that after polyclonal activation, immunosuppression is confined to a subclass of T cells (T_S) distinct from helper T cells (T_H). The Ly23 phenotype of T_S cells is the same as that expressed on cytotoxic T cells (T_C), although whether these two functions are mediated by the same population of Ly23 cells or by different populations of Ly23 cells that may be distinguishable by future immunogenetic analysis, and whether suppressor T cells act by killing the responding T or B cells, are questions yet to be decided.

The central implication is that even before activation, suppression is an obligatory response of a specialized T-cell subclass. We are now faced with the questions (*a*) whether suppression is also generated from this subclass as a normal consequence of immunization with antigen, and (*b*) whether the suppression generated is specific for the inducing antigens. These points are dealt with in the accompanying report (12).

Summary

Concanavalin A, a nonspecific polyclonal activator of T lymphocytes, activates Ly1 and Ly23 subclasses to the same degree. After activation, the Ly23 subclass, but not the Ly1 subclass, has the following properties: (*a*) Suppression of the antibody response to sheep erythrocytes (SRBC) in vitro. (*b*) Production of a soluble factor that suppresses the anti-SRBC response in vitro. (*c*) Suppression of the generation of cell-mediated cytotoxicity to H-2 target cells in vitro. Con A-activated cells of the Ly1 subclass, but not the Ly23 subclass, express helper function in the anti-SRBC response in vitro.

Because the intact Con A-stimulated T-cell population contains both cell types, these cells do not exert detectable helper effects in an anti-SRBC system in vitro, because the helper effect of Ly1 cells is masked by the suppressor effect of the Ly23 cells. Each function is revealed by eliminating one or the other population with the relevant Ly antiserum.

The resting T-cell population, before activation by Con A, also contains already programmed Ly1 and Ly23 cells with similar helper and suppressor potentials, respectively. This is revealed by experiments with Ly subclasses which have been separated from the resting T-cell population and then stimulated by Con A.

Thus helper and suppressor functions, as expressed in these systems, are manifestations of separate T-cell-differentiative pathways and do not depend upon stimulation of the cells by antigen.

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