REGULATION OF THE ANTIBODY RESPONSE TO TYPE III PNEUMOCOCCAL POLYSACCHARIDE BY CONTRASUPPRESSOR T CELLS

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Previous studies from this and other laboratories (1–5) have established that the antibody response of mice to the so-called thymus independent (TI)[1] antigen type III pneumococcal polysaccharide (S3) is strongly influenced by regulatory T cells such as suppressor T cells (Ts) and amplifier T cells (Ta). Like other polysaccharide antigens, S3 was shown to be a potent tolerogen in mice; in addition, tolerance induced either by low doses of S3 (low dose paralysis) (5) or by S3 coupled to syngeneic spleen cells (S3-SC) (6) was dependent on the presence of T lymphocytes. Although those observations suggested that tolerance induced by S3 or S3-SC might be mediated by Ts, T cells obtained from mice given S3 or S3-SC several days or weeks previously did not suppress the antibody response to S3 after transfer to normal mice (2, 7, 8). Subsequent studies, however, indicated that S3-specific Ts could be detected in mice given S3-SC (8–10) or low doses of S3 (7, 11) but only under very defined conditions. For example, T cells from mice given low doses of S3 were able to suppress the S3 response if the cells were transferred within 6–24 h after the donors were injected with S3 (7). Cells transferred at later times, 3–7 d after injection of S3, did not suppress the response (7) but rather were able to augment it (11). Cells from mice injected with S3-SC were able to suppress the S3 response after transfer if donors were given cyclophosphamide (Cy) before injection of S3-SC (10) or if cells from mice given S3-SC were cultured in vitro with S3 and a soluble membrane component of S3-SC (S3-SCSM) before transfer (8, 9).

The fact that S3 and S3-SC did indeed activate Ts but that the Ts could only be demonstrated under defined and rather unusual conditions suggested that S3 and S3-SC also activated a second type of cell which could interfere with the activity of Ts. T cells with the ability to interfere with Ts activity have been previously described (12) and have been termed contrasuppressor T cells (Tcs). The present studies were initiated in an attempt to determine if Tcs might be

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution; Cy, cyclophosphamide; FCS, fetal calf serum; PFC, plaque-forming cell; PVP-SCSM, soluble membrane component of polyvinylpyrrolidone-coupled spleen cells; S3, type III pneumococcal polysaccharide; S3-SC, S3 coupled to spleen cells; S3-SCSM, soluble membrane component of S3-SC; SE, sheep erythrocyte; Ta, amplifier T cell; Tcs, contrasuppressor T cell; Th, helper T cell; TI, thymus independent; Ts, suppressor T cell; *V.v.*, *Vicia villosa*.

detectable in mice injected with S3-SCSM. Tcs were found in such mice and some of the properties of these cells are described below.

Materials and Methods

Mice. CAF_1 female mice, 8–12 wk old, were used for all experiments. They were obtained from The Jackson Laboratories, Bar Harbor, ME. BALB.B mice were raised in our breeding colonies at the University of Missouri.

Preparation of S3-SC. S3 was coupled to normal CAF_1 or BALB.B spleen cells with chromic chloride as previously described (6). Mock SC were treated with chromic chloride alone. S3-SCSM, prepared by freeze-thawing S3-SC four times followed by centrifugation at 10,000 *g* to remove debris (8), was used for all experiments. Mock SCSM was prepared in the same way using mock SC.

Induction and Assay to Ts. Mice were injected intraperitoneally with 100 mg/kg Cy (Cytoxan; Mead Johnson & Co, Indianapolis, IN) and 2 d later were given mock SCSM or S3-SCSM (amount equivalent to 5×10^7 SC). 8 d later, 5×10^6 spleen cells from these donor mice were transferred intravenously to syngeneic recipients which were immunized 4–6 h later with 0.6 μg S3 (10). In one experiment, S3-specific Ts were induced by injecting mice intravenously with S3-SCSM. 5 d later spleen cells from these mice were cultured in vitro for 48 h with mock SCSM (controls) or S3 plus S3-SCSM as previously described in detail (8). 5×10^6 control or Ts cells were transferred intravenously to syngeneic recipients which were immunized the following day with 0.6 μg S3 (8, 9). S3-specific plaque-forming cells (PFC) were enumerated 5 d later in all experiments (8). Previous studies have shown that S3-specific Ts induced by the two methods described above belong to different T cell subsets (9).

Induction and Assay of Tcs. Mice were injected intravenously with S3-SCSM (amount equivalent to 5×10^7 SC). 8 d later spleen cells from these mice were separated on plates coated with the lectin *Vicia villosa* (*V.v.*) (E. Y. Laboratories, San Mateo, CA) as described in detail below. Cells which adhered to *V.v* were mixed with either of the Ts populations described above just before intravenous injection (5×10^6 *V.v.*-adherent cells and 5×10^6 Ts). In some experiments, *V.v.*-adherent cells were treated with various antisera and complement (C) before transfer (see below). Other cell populations that were tested for Tcs activity in some experiments included *V.v.*-nonadherent cells from mice given S3-SC 8 d previously or *V.v.*-adherent cells from mice given mock SCSM or Cy plus S3-SCSM 8 d previously.

Separation of Spleen Cells on V.v. Plates. Spleen cells were separated on *V.v.* essentially as described by Iverson et al. (13). Briefly, *V.v.* diluted to 500 μg/ml in saline citrate buffer, pH 5.5, was added to 100-mm tissue culture grade petri plates (Falcon 3003; Falcon Labware, Oxnard, CA) for 45–60 min at room temperature. The *V.v.* solution was removed, plates were washed twice in pH 7.2 phosphate-buffered saline, and balanced salt solution (BSS) containing 2–3% fetal calf serum (FCS) was then added for 10–15 min. Spleen cells (10×10^6/ml in BSS/FCS were then added (6–7 ml/plate) and plates were incubated at 37°C for 45 min. Cells nonadherent to the plates were removed after gentle swirling of the plates and plates were rinsed one or twice in BSS. Cells adherent to the plates were eluted by adding 5 ml BSS containing 1 mg/ml *N*-acetyl-D-galactosamine (Sigma Chemical Co., St. Louis, MO) and incubating the plates 5–10 min at 37°C. Adherent cells were then removed by vigorous pipetting. Cells were washed once in BSS and counted. Generally, 50–60% of the spleen cells were recovered as nonadherent cells and 15–25% as adherent cells.

Antiserum Treatment. For some experiments, *V.v.*-adherent spleen cells were treated with anti-Thy-1.2, anti-Lyt-2.2, anti-Lyt-1.2, or anti-$I\text{-}J^b$ and C before use. The preparation, activity, and specificity of the anti-Lyt antisera were described previously (14). Anti-Thy-1.2 was ascites from mice injected with the anti-Thy-1.2 hydridoma HO 13.4, obtained from Dr. Michael Misfeldt, University of Missouri. The monoclonal anti-$I\text{-}J^b$ reagents WF9.40.5 and WF9.1.4 were kindly supplied by Dr. Carl Waltenbaugh, Northwestern University, Chicago, IL. WF9.1.4 is cytotoxic for Tcs cells in the sheep erythrocyte

(SE) system while WF9.40.5 is cytotoxic for Ts but not for Tcs (D. R. Green, personal communication). The culture supernatants containing these monoclonals were used at a final concentration of 1:50. Cells were treated with antiserum and low toxicity rabbit complement by a two-step cytotoxicity procedure (14).

PFC Assay. IgM PFC were enumerated as previously described (8) using S3 coupled to sheep erythrocytes (S3-SE) as indicator cells (15). Results are presented as PFC/spleen but the conclusions would not differ if results were expressed as PFC/10^6 spleen cells.

Results

Mice Injected with S3-SCSM Have Cells that Interfere with Ts Activity. Previous studies (10) from this laboratory have shown that S3-specific Ts could be induced by S3-SCSM in Cy-treated mice but not in normal mice (10). Similarly, the data shown in groups *A–C* of Table I illustrate that spleen cells from Cy-treated mice injected with S3-SCSM (group *B*) are able to suppress the IgM PFC response to S3 whereas cells from mice given S3-SCSM but no Cy (group *C*) have no detectable suppressor activity. The suppressor cells induced in Cy-treated mice with S3-SCSM have been previously described and are S3-specific Lyt-1^-2^+, $I\text{-}J^+$ T cells (9, 10). The necessity to treat mice with Cy to obtain detectable Ts suggested that a Cy-sensitive cell which could interfere with the activation or expression of Ts in this system might be present in normal mice given S3-SCSM. Cells with the ability to interfere with Ts function, i.e., Tcs, have been shown by others to be adherent to *V.v.* (13, 16). If spleen cells from normal mice injected with S3-SCSM do contain Tcs, *V.v.*-adherent cells from those mice should be able to abrogate the function of the Ts induced in Cy-treated mice. To investigate this possibility, spleen cells from normal mice given S3-SCSM 8 d previously were separated on *V.v.* plates and the *V.v.*-adherent and -nonadherent cells or unseparated cells from these mice were mixed with an equal number of Ts induced in Cy-treated mice. The ability of these Ts to suppress the response to S3 (group *B* in Table I) was not affected by spleen cells from donors given Cy and mock SCSM (group *D*) or by unfractionated spleen cells (group *E*) or *V.v.*-nonadherent cells (group *F*) from mice given S3-SCSM. By contrast, *V.v.*-adherent cells from mice given S3-SCSM almost completely abrogated Ts activity

TABLE I

V.v.-adherent Spleen Cells from Mice Given S3-SCSM Have Contrasuppressor Activity

Donor mice given:*	Cells transferred‡	S3 PFC/spleen§
A. Cy + mock SCSM	A	13,875 ± 1,451
B. Cy + S3-SCSM (Ts control)	B	7,680 ± 1,428 (45)
C. S3-SCSM (no Cy)	C	16,050 ± 2,415 (0)
D.	A + B	4,425 ± 542 (69)
E.	B + C	6,144 ± 759 (56)
F.	B + *V.v.* nonadherent of C	5,356 ± 597 (62)
G.	B + *V.v.* adherent of C	13,175 ± 1,789 (7)

* CAF_1 mice were given 100 mg/kg Cy 2 d before mock- or S3-SCSM (*A* and *B*). Group *C* received S3-SCSM and no Cy. 8 d later, spleen cells were removed and transferred as indicated to normal CAF_1 recipients which were immunized 4 h later with 0.6 μg S3.

‡ 5×10^6 cells of each type per recipient.

§ Mean PFC/spleen ± SEM of four to five mice per group 5 d after immunization. The percent suppression relative to group *A* (positive control) is given in parentheses.

(group *G* vs. group *B* in Table I). The results suggest that cells from normal mice injected with S3-SCSM have the ability to interfere with S3-specific Ts activity. The necessity to separate the spleen cells on *V.v.* to demonstrate the contrasuppressor activity (group *E* vs. group *G*) may be due to the fact that the contrasuppressor cells in the unfractionated spleen cells are 'neutralized' by Ts present in the same spleen cell population (see below).

Not All V.v.-adherent Cells Have Contrasuppressor Activity. The results in Table I established that *V.v.*-adherent cells from mice injected with S3-SCSM could interfere with Ts activity but that unfractionated or *V.v.*-nonadherent cells did not interfere with Ts activity. Before concluding that S3-SCSM induced cells with contrasuppressor activity, it was important to establish that all *V.v.*-adherent spleen cell populations did not have this activity. Therefore spleen cells from normal CAF_1 mice or from mice given S3-SCSM or polyvinylpyrrolidone-coupled SCSM (PVP-SCSM) 8 d previously were separated on *V.v.* and the *V.v.*-adherent fractions were each mixed with an equal number of Ts as in the previous experiments (Table II). As before, the *V.v.*-adherent cells from mice injected with S3-SCSM almost completely abrogated the ability of the Ts to suppress the S3 response (group *D* vs. group *B*). By contrast, *V.v.*-adherent cells from normal mice (group *E*) or from mice injected with PVP-SCSM (group *F*) had no effect on Ts activity. Thus cells with the ability to interfere with S3-specific Ts are apparently induced by S3-SCSM.

Contrasupressor Cells Are Not Present in Cy-treated Mice Given S3-SCSM. We previously suggested (10) that the ability to induce S3-specific Ts with S3-SCSM in Cy-treated mice but not in normal mice was due to the fact that cells which interfered with Ts activity were eliminated by Cy, thus allowing Ts activity to be expressed. To directly test this possibility, spleen cells from both normal and Cy-treated mice given S3-SCSM 8 d previously were separated on *V.v.* and the *V.v.*-adherent cells were mixed with an equal number of unfractionated spleen cells

TABLE II

Not All V.v.-adherent Cells Have Contrasuppressor Activity

Donor mice given:*	Cells transferred‡	S3 PFC/spleen§	
		Exp. 1	Exp. 2
A. Cy + mock SCSM	A	14,667 ± 2,763	12,523 ± 2,486
B. Cy + S3-SCSM (Ts control)	B	6,200 ± 470 (58)	3,356 ± 443 (74)
C. S3-SCSM (no Cy)	C	9,163 ± 1,902 (38)	ND
D.	B + *V.v.* adherent of C	11,675 ± 1,183 (20)	9,350 ± 1,632 (26)
E.	B + *V.v.*-adherent normal	5,590 ± 988 (62)	3,300 ± 750 (75)
F.	B + *V.v.*-adherent PVP-SCSM	ND	4,433 ± 733 (65)

* See footnote * in Table I for experimental design.

‡ 5×10^6 cells of each type per recipient. In group *E*, the *V.v.*-adherent cells were from normal CAF_1 mice and in group *F*, they were from CAF_1 mice injected 8 d previously with PVP-SCSM.

§ Mean PFC/spleen + SEM of 5–12 mice per group determined 5 d after immunization. The percent suppression relative to group *A* is given in parentheses.

from Cy-treated mice given S3-SCSM (Ts control) (Table III). As in the previous experiments, *V.v.*-adherent cells from mice given S3-SCSM and no Cy (group *D*) abrogated the ability of Ts to suppress the S3 response. By contrast, *V.v.*-adherent cells from mice given the same S3-SCSM and Cy (group *E*) were unable to interfere with Ts activity, suggesting that cells with contrasuppressor activity are Cy sensitive.

Removal of Contrasuppressor Cells Reveals Ts Activity in Cells from Mice Injected with S3-SCSM. Having established that mice injected with S3-SCSM have cells which can interfere with the activity of Ts induced by S3-SCSM in Cy-treated mice, it was of interest to determine whether the contrasuppressor cells prevented the activation of Ts or merely prevented their expression. To begin to assess this question, we asked whether the removal of contrasuppressor cells from a spleen cell population would enable the remaining cells to suppress the S3 response. Therefore mice were injected with S3-SCSM or with mock SCSM as a control. 8 d later spleen cells from the mice given S3-SCSM were separated on *V.v.* and the various unseparated or separated cell populations were injected into mice that were then immunized with S3. The results of two experiments of this type are shown in Table IV. As shown previously (see above), unfractionated spleen

TABLE III

Contrasuppressor Cells Are Not Present in Cy-treated Mice

Donor mice given:*	Cells transferred‡	S3 PFC/spleen§
A. Cy + mock SCSM	A	13,250 ± 2,106
B. Cy + S3-SCSM (Ts Control)	B	6,111 ± 833 (54)
C. S3-SCSM (no Cy)	C	9,683 ± 659 (27)
D.	B + *V.v.* adherent of C	13,321 ± 907 (0)
E.	B + *V.v.* adherent of B	5,575 ± 999 (58)

* See footnote * in Table I for experimental design.

‡ 5×10^6 cells of each type per recipient.

§ Mean PFC/spleen ± SEM of 4–10 mice per group determined 5 d after immunization. The percent suppression relative to group *A* is given in parentheses.

TABLE IV

Removal of V.v.-adherent Cells Reveals Suppressor Activity in Spleen Cells of Mice Injected with S3-SCSM and No Cy

Donor mice given:*	Cells transferred‡	S3 PFC/spleen§	
		Exp. 1	Exp. 2
A. Mock SCSM	A	13,375 ± 1,938	15,250 ± 1,950
B. S3-SCSM	B	9,163 ± 2,097 (32)	14,250 ± 2,593 (7)
C.	*V.v.* nonadherent of B	5,850 ± 1,660 (66)	3,500 ± 959 (77)
D.	*V.v.* adherent of B	10,838 ± 1,990 (29)	12,063 ± 1,595 (21)
E. Cy + S3-SCSM	E	2,780 ± 121 (79)	4,410 ± 327 (71)

* CAF_1 mice were given mock SCSM or S3-SCSM (no Cy) (groups *A* and *B*) or 100 mg/kg Cy followed 2 d later by S3-SCSM (group *E*). 8 d later, cells were transferred as indicated to normal CAF_1 mice which were then immunized with 0.6 μg S3.

‡ 5×10^6 cells transferred per recipient.

§ Mean PFC/spleen ± SEM of four to five mice per group determined 5 d after immunization. The percent suppression relative to group *A* is given in parentheses.

cells from normal mice given S3-SCSM (group *B*) had little effect on the S3 response. The *V.v.*-adherent cells obtained from these spleen cells (group *D*) behaved similarly to the unfractionated cells whereas the *V.v.*-nonadherent cells (group *C*) suppressed the S3 response. In some experiments (e.g., Exp. 2 in Table IV), these *V.v.*-nonadherent cells suppressed the response as effectively as did spleen cells from Cy-treated mice injected with S3-SCSM (group *E*), although in the majority of experiments the Ts induced in Cy-treated mice were more effective (e.g., Exp. 1 in Table IV). These results suggest that contrasuppressor cells in mice injected with S3-SCSM act by preventing the expression of Ts activity rather than by preventing the activation of Ts. The fact that both suppressor and contrasuppressor cells are present in spleen cells from mice given S3-SCSM also explains the inability of unfractionated spleen cells from these mice to abrogate the activity of Ts induced in Cy-treated mice (Table I), since there may be insufficient contrasuppressor cells to neutralize both populations of suppressor cells. It is also important to note that *V.v.*-adherent cells (group *D* in Table IV) injected alone do not augment the response to S3, indicating that these cells are not helper or amplifier cells.

Contrasuppressor Cells Are T Cells. Having demonstrated that *V.v.*-adherent cells from mice injected with S3-SCSM could abrogate the activity of Ts (Tables I and II), it was important to determine if these cells might be similar to the Tcs described by others (16). Therefore spleen cells from mice injected 8 d earlier with S3-SCSM were separated on *V.v.* plates. The *V.v.*-adherent cells were then treated with anti-Thy-1.2, anti-Lyt-1.2, anti-Lyt-2.2 and C, or with C alone before transfer with Ts to recipient mice (Table V). As shown above with untreated *V.v.*-adherent cells, the *V.v.*-adherent cells treated with C alone (group *D*) almost completely abrogated the ability of Ts to suppress the S3 response (group *B*). Treatment of these cells with either anti-Thy-1.2 (group *E*) or anti-Lyt-1.2 (group *F*) and C completely eliminated the ability of the *V.v.*-adherent

TABLE V

Contrasuppressor Cells Are Lyt-1$^+$ T Cells

Donor mice given:*	Cells transferred‡	S3 PFC/spleen§	
		Exp. 1	Exp. 2
A. Cy + mock SCSM	A	15,800 ± 2,312	9,263 ± 628
B. Cy + S3-SCSM	B	5,550 ± 910 (65)	2,750 ± 380 (70)
C. S3-SCSM (no Cy)	C	ND	9,850 ± 660 (0)
D.	B + *V.v.* adherent of C (C treated)	11,120 ± 534 (30)	7,755 ± 695 (16)
E.	B + *V.v.* adherent of C (α-Thy-1.2 + C)	2,650 ± 539 (83)	2,700 ± 398 (71)
F.	B + *V.v.* adherent of C (α-Lyt-1.2 + C)	4,588 ± 1,295 (71)	3,080 ± 661 (67)
G.	B + *V.v.* adherent of C (α-Lyt-2.2 + C)	6,562 ± 1,327 (58)	4,887 ± 1,140 (47)

* See footnote *, Table I for experimental design.

‡ In groups *D–G*, the *V.v.*-adherent cells were treated with antiserum and/or complement (C) as indicated before mixing with group *B* cells. 5×10^6 cells of each type were transferred per recipient.

§ See footnote §, Table IV.

cells to abrogate Ts activity, indicating that the contrasuppressor cells are Lyt-1^+ T cells. Treatment with anti-Lyt-2.2 and C (group *G*) had a variable effect in these as well as several other experiments. In general, contrasuppressor activity was only partially eliminated by anti-Lyt-2.2 so that it was not possible to draw any definite conclusion concerning the presence or absence of Lyt-2 on these cells. It should be noted that the same anti-Lyt-2.2 serum used here does abrogate the activity of two different subsets of S3-specific Ts (9, 10) so that the results in Table V cannot be due to the inactivity of the antiserum used. It has been shown that the Tcs described by Green et al. (16, 21) have I-J determinants that are recognized by some monoclonal anti-$I\text{-}J^b$ reagents (e.g., WF9.1.4) but not by others (e.g., WF9.40.5) (D. R. Green, personal communication). To determine if the Tcs described here also had similar I-J determinants, BALB.B mice were injected with S3-SCSM. 8 d later, spleen cells from these mice were separated on *V.v.* The *V.v.*-adherent cells were treated with C alone or with the monoclonal anti-$I\text{-}J^b$ reagents WF9.1.4 or WF9.40.5 and C before transfer with Ts induced in BALB.B mice with Cy and S3-SCSM (Table VI). As noted above with CAF_1 mice, spleen cells from BALB.B mice given Cy and S3-SCSM suppressed the S3 response in recipient mice (group *B* vs. group *A*) while cells from mice given S3-SCSM and no Cy had no effect on the S3 response (group *C*). *V.v.*-adherent cells from these mice abrogated the activity of the Ts (group *D*). Treatment of the *V.v.*-adherent Tcs cells with the monoclonal WF9.40.5 had little effect on Tcs activity (group *E*) while the other monoclonal (WF9.1.4) almost completely eliminated the ability of these cells to abrogate Ts activity (group *E* vs. group *B*). Thus, Tcs induced by S3-SCSM apparently bear I-J determinants that are defined by the same monoclonal anti-$I\text{-}J^b$ reagent that eliminates Tcs in the SE system.

Contrasuppressor Cells Do Not Abrogate the Activity of S3-specific Ts Induced In Vitro. The results presented above demonstrate that Tcs induced by S3-SCSM

TABLE VI

Tcs Bear I-J Determinants Defined by a Particular Monoclonal Anti-I-J Reagent

Donor mice given:*	Cells transferred‡	S3 PFC/spleen§	
		Exp. 1	Exp. 2
A. Cy + mock SCSM	A	17,513 ± 1,562	19,650 ± 3,465
B. Cy + S3-SCSM	B	7,065 ± 718 (60)	8,270 ± 3,825 (68)
C. S3-SCSM (no Cy)	*V.v.* adherent of C	13,610 ± 1,792 (22)	28,075 ± 1,401 (0)
D.	B + *V.v.* adherent of C	13,195 ± 1,320 (25)	28,860 ± 4,683 (0)
E.	B + *V.v.* adherent of C (WF9.40.5 + C)	11,339 ± 1,453 (35)	26,550 ± 6,819 (0)
F.	B + *V.v.* adherent of C (WF9.1.4 + C)	5,690 ± 1,044 (68)	10,110 ± 2,875 (49)

* BALB.B mice were given 100 mg/kg Cy 2 d before mock- or S3-SCSM (prepared using BALB.B SC) (*A* and *B*). Group *C* received the same S3-SCSM and no Cy. 8 d later, spleen cells were transferred as indicated to normal BALB.B recipients which were immunized 4 h later with 0.6 μg S3.

‡ In groups *E* and *F*, *V.v.*-adherent cells were treated with the indicated monoclonal anti-$I\text{-}J^b$ and C before transfer. 5×10^6 cells of each type were transferred per recipient.

§ Mean PFC/spleen ± SEM of four (Exp. 2) or six to nine (Exp. 1) mice per group determined 5 d after immunization. The percent suppression relative to group *A* is given in parentheses.

can abrogate the activity of S3-specific Ts induced in Cy-treated mice. We have also described and characterized (8, 9) a subset of S3-specific Ts that are induced by culturing spleen cells from mice injected with S3-SCSM in vitro with S3 and S3-SCSM. These in vitro induced Ts belong to a separate T cell subset from the Ts induced in Cy-treated mice (9) and previous studies had indirectly suggested that the activity of these Ts could be neutralized by Lyt-1^+ T cells present in the same spleen cell populations (8). It was therefore of interest to determine directly if *V.v.*-adherent spleen cells from mice injected with S3-SCSM could also abrogate the suppression mediated by the in vitro induced Ts. Thus, spleen cells from mice injected with S3-SC were cultured with either mock SCSM (controls) or with S3 and S3-SCSM (Ts) as described in Materials and Methods. At the time of cell transfer, some of the Ts were mixed with an equal number of *V.v.*-adherent cells from mice given S3-SCSM 8 d previously as a source of Tcs (see above). Cells were then transferred to normal CAF_1 mice that were immunized on the following day with S3; PFC were determined 5 d later (Table VII). Clearly, *V.v.*-adherent cells had no effect on the ability of the in vitro induced Ts to suppress the S3 response (group *B* vs. group *C*). Because a 24-h interval between cell transfer and immunization of recipients is required to demonstrate optimal suppression with the in vitro induced Ts, it was important to determine whether the lack of effect of Tcs was due to a real difference in the ability of Tcs to abrogate the activity of different subsets of Ts or whether Tcs activity could not be demonstrated at all when the interval between cell transfer and immunization was 24 h rather than the 4–6 h used in all of the previous experiments. Thus, mice were also injected with Cy and mock SCSM (control) or with Cy and S3-SCSM (Ts). Ts induced either by this latter method or Ts induced in vitro were then mixed with the same population of *V.v.*-adherent

TABLE VII

V.v.-adherent Cells from Mice Given S3-SCSM Have No Effect on S3-specific Ts Induced In Vitro

Donors given (in vitro activation):	Cells transferred[‡]	S3 PFC/spleen[§]		
		Exp. 1	Exp. 2	
A. S3-SCSM (cultured with mock SCSM) (control)[‖]	A	10,320 ± 1,772	9,733 ± 2,799	
B. S3-SCSM (cultured with S3 + S3-SCSM) (Ts)[‖]	B	5,462 ± 877 (47)	1,375 ± 275	(86)
C. S3-SCSM (no Cy)	B + *V.v.* adherent of C	2,280 ± 448 (78)	1,725 ± 319	(82)
D. Cy + mock SCSM*	D	ND	7,600 ± 717	
E. Cy + S3-SCSM*	E	ND	1,550 ± 202	(80)
F.	E + *V.v.* adherent of C	ND	5,413 ± 110	(29)
G. S3-SCSM (no Cy)	G	ND	6,467 ± 1,098	(15)

* See footnote *, Table I.

‡ 5×10^6 cells of each type were transferred per recipient. Recipients were immunized 1 d after cell transfer.

§ Mean PFC/spleen ± SEM of four to five mice per group 5 d after immunization. The percent suppression relative to the positive controls (group *A* or *D*) is given in parentheses.

‖ See Materials and Methods for details of the induction of Ts.

spleen cells from mice given S3-SCSM 8 d previously and all recipients were immunized 24 h after cell transfer (Table VII, Exp. 2). The *V.v.*-adherent spleen cells had no effect on the ability of the in vitro induced Ts to suppress the S3 response (group *C* vs. group *B*), while the suppressive activity of the Ts induced in Cy-treated mice was largely abrogated by the same cell population. Thus Tcs appear to have different effects on different subsets of Ts.

Discussion

The studies described here have established that normal mice injected with S3-SCSM have in their spleens two types of regulatory T cells, Ts (Table IV) and a second T cell which is adherent to *V.v.* and which acts to abrogate or contrasuppress the activity of S3-specific Ts (Table I). Because these two T cell populations are both induced by S3-SCSM, unfractionated spleen cells from mice injected with S3-SCSM have little, if any, ability to suppress the antibody response to S3 (Table I, group *C*) or to contrasuppress the activity of Ts induced in Cy-treated mice (Table I, group *E*). By contrast, separation of the spleen cells on *V.v.* reveals suppressor activity in the *V.v.*-nonadherent cells (Table IV) and contrasuppressor activity in the *V.v.*-adherent cells (Table I, group *G*). The finding that S3-SCSM activates Tcs in addition to Ts in normal mice provides an explanation for our inability to detect Ts in such spleens in these as well as previous studies (8–10). As shown previously, S3-specific Ts are readily detected when donor mice are given Cy before the injection of S3-SCSM (10 and Table I, group *B*). We previously hypothesized (10) that Cy acted by eliminating cells which interfered with Ts activity. Direct evidence for that hypothesis was obtained in the present studies since *V.v.*-adherent cells from Cy-treated mice given S3-SCSM were unable to abrogate Ts activity, whereas the same cell population obtained from mice not given Cy did abrogate Ts activity (Table III). The finding that cells with contrasuppressor activity are Cy sensitive is consistent with the results of a recent study which showed that a Cy-sensitive T cell can abolish the activity of a Cy-resistant subset of Ts. Both of these cells were induced by injection of trinitrophenyl (TNP)-SC into the anterior chamber of the eye (17, 18). Moreover, a serum factor with antisuppressor activity has been recently described and this factor was not produced by Cy-treated mice (19).

Gershon et al. (12) first showed that T cells with contrasuppressor activity can regulate the in vitro antibody response to SE. They defined a Tcs circuit in which Lyt-2^+ T cells (contrasuppressor inducers) acted on Lyt-1^+2^+ transducer cells which in turn activated Lyt-1^+2^- contrasuppressor effector cells (12, 16, 20). In their system (16), contrasuppressor effector cells were shown to act on helper T cells (Th) to render them resistant to Ts signals. The contrasuppressor cell we describe here has also been shown to be a T cell and to be Lyt-1^+ (Table V). Whether the Tcs described here is a Lyt-1^+2^- contrasuppressor effector cell or a Lyt-1^+2^+ T cell perhaps similar to the contrasuppressor transducer cell (12, 16, 20) is not known, since treatment with anti-Lyt-2 and C partially but never completely eliminated contrasuppressor activity (Table V). The Tcs described here also were found to bear I-J determinants (Table VI) similar or identical to the I-J determinants found on Tcs that regulate responses to SE (D. R. Green, personal communication), which suggests their similarity to Tcs described in

other systems (12, 20, 21). However, while the Tcs described here may be similar to those described by Green et al. (16, 21), it is unlikely that the Tcs in our system act by making Th resistant to Ts signals (16), since the antibody response to S3 presumably does not involve Th interaction with B cells (22). The mechanism by which Tcs regulate the S3 response is not known at this time. Two likely possibilities are that Tcs interact directly with Ts and neutralize their activity or that Tcs somehow make S3-specific B cells resistant to Ts signals. Studies are currently in progress to determine which, if either, of those mechanisms are operative in this system.

An unexpected observation in these studies was the finding that the Tcs which consistently abrogated the suppression mediated by S3-specific Ts induced in Cy-treated mice injected with S3-SCSM had no effect on S3-specific Ts induced in vitro (Table VII). Previous studies have established that both of these Ts are S3 specific and are Lyt-1^-2^+, I-J^+ T cells, but differ in several other properties, indicating that they are separate subsets of Ts (9). The Ts induced by S3-SCSM in Cy-treated mice bind to S3-SCSM but not to anti-S3–coated plates (10), appear to directly suppress S3-specific B cells (9), and are apparently not V_H restricted (Braley-Mullen, unpublished results). By contrast, the in vitro induced Ts bind to anti-S3–coated plates (9), require a Cy-sensitive T cell in the recipient to mediate suppression (9), and are V_H restricted (unpublished results). These later Ts are thus similar to the second order Ts (Ts_2) described by others (reviewed in 23). Although it has been suggested that all types of Ts may not be regulated by cells in the contrasuppressor circuit (21), considerable indirect evidence suggests that the in vitro induced Ts are regulated by contrasuppressor cells. For example, our initial studies indicated that suppression mediated by these cells was lost if supraoptimal numbers of T cells were transferred to recipient mice (8), suggesting that cells which could interfere with Ts were also present in the cultured spleen cell population. In addition, the degree of suppression obtained with low numbers of transferred cells was often, although not always, increased when cells were treated with anti-Lyt-1.2 and C before transfer (9). Moreover, the need to culture the cells to obtain detectable Ts suggests that at least the precursor of the Ts is influenced by contrasuppressor cells, the activity of which might be diminished or lost during culture. The finding that Tcs obtained from mice injected 8 d earlier with S3-SCSM do not affect the activity of the in vitro induced Ts suggests the possibility that, in spite of the indirect evidence discussed above, this Ts subset is not affected by Tcs. Alternatively, it is possible that more than one subset of Tcs exists and that the Tcs detected in mice injected 8 d previously with S3-SCSM do not affect all subsets of Ts. Recent evidence in another system (24) indicates that distinct subsets of Tcs regulate suppression mediated by distinct subsets of Ts, so that this is not an unlikely possibility. It is also posible that only the precursors of the in vitro induced Ts are susceptible to Tcs so that an effect of Tcs might have been observed if the Tcs had been added at the initiation of culture. Studies currently in progress should help to resolve these questions.

The specificity of Tcs in the SE system has been reported (12, 21) to be less stringent than that of Ts or Th in that the activity of a Tcs factor induced by SE could be adsorbed by other types of erythrocytes (12, 21). Similarly, Rohrer and

Kemp (24) have shown that at least one Tcs subset is highly cross-reactive although another is SE specific. Our studies indicate that the Tcs described here are induced by S3-SCSM but not by another TI antigen, PVP-SCSM, and that the Tcs are not present in normal mice (Table II). The fact that PVP-SCSM does not activate the Tcs which regulate the S3 response suggests that Tcs are not entirely antigen nonspecific, but our data do not exclude the possibility that they could be activated by more closely related antigens such as other pneumococcal polysaccharides.

Finally, it is important to emphasize that the Tcs described here do not by themselves have any discernible effect on the S3 response (Table IV, group *D*). This strongly suggests that Tcs are not identical to Ta, which have been shown to interact antagonistically with Ts to regulate the S3 response (3, 11). Athough Tcs and Ta are both Ly-1$^+$ cells (Table V and reference 11), Ta markedly augments the antibody response to S3 when injected into normal mice (11) whereas Tcs clearly do not have this effect. Thus the antibody response of mice to a so-called TI antigen such as S3 can be influenced by multiple subsets of regulatory T cells that can now be separated both functionally and physically so that their properties and roles in the immune response can be studied in detail. It will be of particular interest to determine if Tcs such as those described here can be activated by free S3. If so, Tcs may prove to be important in enabling polysaccharide antigens such as S3 to elicit a positive immune response, since these types of antigens tend to induce tolerance very effectively. (2, 5, 22). It has previously (25) been suggested that the activation of T cells which can block Ts activity may be essential if an immune response is to take place. Tcs have also been shown (26) to be capable of interfering with tolerogenic signals in vivo and are important in allowing neonatal cells to exhibit positive immune function (16, 21). Clearly further studies will be required to fully define the role of Tcs in the regulation of the immune response to S3 as well as other types of antigens.

Summary

A soluble membrane component of type III pneumococcal polysaccharide–coupled spleen cells (S3-SCSM) induces S3-specific suppressor T cells (Ts) in mice. These Ts can be detected only if mice are pretreated with cyclophosphamide (Cy) or if cells adherent to the lectin *Vicia villosa* are removed from the spleen cell population prior to transfer. The *V. villosa*–adherent spleen cells from mice injected with S3-SCSM could abrogate suppression mediated by Ts induced by S3-SCSM in Cy-treated mice. The *V. villosa*–adherent contrasuppressor cells were shown to be T cells that were I-J$^+$ and of the Lyt-1 phenotype. Contrasuppressor T cells (Tcs) were not present in *V. villosa*–adherent spleen cell fractions obtained from normal mice, from mice injected with polyvinylpyrrolidone-coupled spleen cells, or from Cy-treated mice injected with S3-SCSM, i.e., mice in which Ts activity is dominant. The *V. villosa*–adherent cells that abrogated the activity of Ts induced by S3-SCSM in Cy-treated mice did not abrogate suppression mediated by a different subset of S3-specific Ts, suggesting that the Tcs described here do not have activity against all Ts subsets. The ability of S3-SCSM to activate Tcs in normal mice provides an explanation for the inability to detect S3-specific Ts in several previous studies.

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References

1. Braley-Mullen, H. 1974. Regulatory role of T cells in IgG antibody formation and immune memory to type III penumococcal polysacchride. *J. Immunol.* 113:1909.
2. Braley-Mullen, H. 1978. Selective suppression of primary IgM responses by induction of low dose paralysis to type III pneumococcal polysaccharide. *Cell. Immunol.* 37:77.
3. Baker, P. J., P. W. Stashak, D. F. Amsbaugh, B. Prescott, and R. F. Barth. 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response of mice to type III penumococcal polysaccharide. *J. Immunol.* 105:1581.
4. Baker, P. J., N. D. Reed, P. W. Stashak, D. F. Amsbaugh, and B. Prescott. 1973. Regulation of the antibody response of mice to type III pneumococcal polysaccharide. I. Nature of regulatory cells. *J. Exp. Med.* 137:1431.
5. Baker, P. J., P. W. Stashak, D. F. Amsbaugh, and B. Prescott. 1974. Regulation of the antibody response to type III pneumococcal polysaccharide. IV. Role of suppressor T cells in the development of low dose paralysis. *J. Immunol.* 112:2020.
6. Braley-Mullen, H. 1980. Suppression of the antibody response to type III pneumococcal polysaccharide with antigen coupled to syngeneic lymphoid cells. *Cell. Immunol.*, 52:132.
7. Baker, P. J., D. F. Amsbaugh, P. W. Stashak, G. Caldes, and B. Prescott. 1982. Direct evidence for the involvement of T suppressor cells in the expression of low dose paralysis to type III pneumococcal polysaccharide. *J. Immunol.* 128:1059.
8. Braley-Mullen, H. 1980. Direct demonstration of specific suppressor T cells in mice tolerant to type III pneumococcal polysaccharide: two step requirement for development of detectable suppressor cells. *J. Immunol.* 125:1849.
9. Braley-Mullen, H. 1982. Activation of distinct subsets of T suppressor cells with type III pneumococcal polysaccharide coupled to syngeneic spleen cells. *Ann. NY Acad. Sci.* 92:156.
10. Braley-Mullen, H. 1983. Activation of type III pneumococcal polysaccharide-specific suppressor T cells in cyclophosphamide-treated mice: requirement for recognition of antigen and I-J determinants on antigen coupled to syngeneic spleen cells. *J. Immunol.* 131:2190.
11. Taylor, C. E., D. F. Amsbaugh, P. W. Stashak, G. Caldes, B. Prescott, and P. J. Baker. 1983. Cell surface antigens and other characteristics of T cells regulating the antibody response to type III pneumococcal polysaccharide. *J. Immunol.* 130:19.
12. Gershon, R. K., D. D. Eardley, S. K. Durum, D. R. Green, F. W. Shen, K. Yamauchi, H. Cantor, and D. B. Murphy. 1981. Contrasuppression: a novel immunoregulatory activity. *J. Exp. Med.* 153:1533.
13. Iverson, M., W. Ptak, D. R. Green, and R. K. Gershon. 1983. Role of contrasuppression in adoptive transfer of immunity. *J. Exp. Med.* 158:982.
14. Fraser, V., and H. Braley-Mullen. 1981. Characterization of suppressor T cells induced with the thymus independent antigen polyvinyl pyrrolidone coupled to syngneic cells. *Cell. Immunol.* 63:177.
15. Baker, P. J., P. W. Stashak, and B. Prescott. 1969. Use of erythrocytes sensitized with purified pneumococcal polysaccharides for the assay of antibody and antibody-producing cells. *Appl. Microbiol.* 7:422.

16. Green, D. R., D. D. Eardley, A. Kimura, D. B. Murphy, K. Yamauchi and R. K. Gershon. 1981. Immunoregulatory circuits which modulate responsiveness to suppressor cell signals: characterization of an effector cell in the contrasuppressor circuit. *Eur. J. Immunol.* 11:973.
17. Waldrep, J. C., and H. J. Kaplan. 1983. Anterior chamber-associated immune deviation induced by TNP-splenocytes (TNP-ACAID). II. Suppressor T cell networks. *Invest. Opthamol. Visual Sci.* 24:1339.
18. Waldrep, J. C., and H. J. Kaplan. 1983. Cyclophosphamide-sensitive contrasuppression in TNP-anterior chamber associated immune deviation (TNP-ACAID). *J. Immunol.* 131:2746.
19. Al-Meghazachi, A., S. T. Lee, and F. Paraskevas. 1983. Detection in vivo 6 hours after immunization of a mediator with possible antisuppressor activity. *Eur. J. Immunol.* 13:799.
20. Yamauchi, K., D. R. Green, D. D. Eardley, D. B. Murphy, and R. K. Gershon. 1981. Immunoregulatory circuits that modulate responsiveness to suppressor cell signals: failure of B10 mice to respond to suppressor factors can be overcome by quenching the contrasuppressor circuit. *J. Exp. Med.* 153:1547.
21. Green, D. R., P. M. Flood, and R. K. Gershon. 1983. Immunoregulatory T cell pathways. *Annu. Rev. Immunol.* 1:439.
22. Howard, J. G., G. H. Christie, B. M. Courtenay, E. Leuchers, and A. J. S. Davies. 1971. Studies on immunological paralysis. VI. Thymic independence of tolerance and immunity to type III pneumococcal polysaccharide. *Cell. Immunol.* 2:614.
23. Germain, R. N., and B. Benacerraf. 1981. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. *Scand. J. Immunol.* 13:1.
24. Rohrer, J. W., and J. D. Kemp. 1983. Heterogeneity of contrasuppressor T cell function and specificity. *Fed. Proc.* 42:690 (Abstr.).
25. DeKruyff, R. H., B. G. Simonson, and G. W. Siskind. 1981. Cellular interactions in immune regulation. Hapten-specific suppression by non-T cells and T cell-mediated reversal of suppression. *J. Exp. Med.* 154:1188.
26. Ptak, W., D. R. Green, S. K. Durum, A. Kimura, D. B. Murphy, and R. K. Gershon. 1981. Immunoregulatory circuits which modulate responsiveness to suppressor cell signals: contrasuppressor cells can convert an in vivo tolerogenic signal into an immunogenic one. *Eur. J. Immunol.* 11:980.