MECHANISMS OF SUPPRESSION IN THE TRANSFER OF CONTACT SENSITIVITY

Analysis of an I-J* Molecule Required for Ly2 Suppressor Cell Activity*

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The nature of cellular interactions involved in the expression of suppressor cell activity is a central question in immunoregulation. The effector cell of suppression in the in vitro plaque-forming cell (PFC) response of naive spleen cells to sheep erythrocytes (SRBC) has been identified as an Lyl*,2* I-J- cell that can directly suppress spleen cell cultures depleted of Ly2+ cells (1). It has been previously reported that suppressor effector cells require an induction signal from an Lyl*, 2+ I-J+ cell to express optimal suppressor activity (2). The biological activity of both these cells can be replaced by cell free products secreted by these cells. Both the Lyl+-derived suppressor inducer factor and Ly2+-derived suppressor-effector factor require two separate macromolecules: one that is antigen binding, and one that is I-J* for functional activity (3, 4). A number of other investigators also report similar antigen-specific suppressor factors composed of two separate molecules (5, 6).

We have extensively studied an antigen-specific suppressor factor (TNP-TsF) that can inhibit the adoptive transfer of contact sensitivity to the picryl chloride (PCI; 7–9). The biological activity of this factor also requires the interaction of two separate macromolecules. The first is an antigen-binding I-J* product secreted by an Lyl*,2* I-J- cell that was previously activated by painting the flank skin of donor mice with PCI (PCI-F). The second, an antigen nonbinding I-J- macromolecule secreted by a cell that expresses the same phenotype as suppressor effector cells (Lyl*2*, I-J-), is obtained from spleen and lymph node cells of

* Supported by grants CA29606, CA16359, and AI10497 from the U. S. Public Health Service.
† Supported by grants from the Maria Skodowski-Curie Fund (Polish-American Agreement) and the Polish Academy of Sciences (10.5).
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Abbreviations used in this paper: BSS, balanced salt solution; CS, contact sensitivity; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; LN, lymph node; Lyl TsF, an SRBC-specific T suppressor-inducer factor made by Lyl*,2* cells; MHC, major histocompatibility complex; OX, 4-ethoxymethylene-2-phenyloxazolone; PCI, picryl chloride; PCI-F, supernatant from antigen-primed T cells, TNP-labeled spleen cells, and PCI-F intermediate cultures; PFC, plaque-forming cell; SRBC, sheep erythrocytes; Tacc, T acceptor cell; TNBSA, trinitrobenzenesulfonic acid; TNP, 2,4,6-trinitrophenyl; TsF, T suppressor factor; Ttrans, T suppressor-transducer cell.
mice immunized with 2,4,6-trinitrobenzene sulfonic acid (TNBSA-F). The presence of both these molecules is prerequisite for suppressive activity to be manifest (9).

While these two molecules work in tandem to effect suppression, the exact mechanism by which they interact at a functional level is still unknown. In the present set of experiments, we investigated the nature of TNP-TsF activity while at the same time unifying elements from two distinct suppressor cell systems. In the results described here, we found that the suppressive activity of TNP-TsF required the presence of Ly-2 I-J\(^+\) cells in the assay population, and this requirement could be overcome by the addition of either an Ly1 I-J\(^+\) T cell product obtained from animals hyperimmunized to SRBC, or an Ly2 I-J\(^+\) T cell product induced in an intermediate culture system by PCI-F and TNP-labeled spleen cells. These I-J\(^+\) molecules interact only with TNBSA-F, the subfactor of TNP-TsF generated from Ly2 cells, to form an antigen nonspecific suppressor factor that inhibited the adoptive transfer of contact sensitivity and the anti-SRBC PFC response of primed T cells in vitro.

Materials and Methods

Mice

CBA/J mice (6–8-wk old males) were obtained from The Jackson Laboratory (Bar Harbor, ME) and rested for 1 wk after arrival at the Yale University School of Medicine before use.

Antigens

Sheep erythrocytes (SRBC) were obtained from Colorado Serum Co. Denver, CO. Trinitrobenzene sulphonic acid (TNBSA) was obtained from Eastman Organic Chemicals (Rochester, NY), Picryl chloride (PCI) from Chemotronix (Swannonoa, NC), and 4-ethoxyethylene-2-phenyloxazolone (oxazolone, OX) from British Drug House (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY).

Antisera

Monoclonal anti-Ly sera were generously supplied by F.-W. Shen, Memorial Sloan-Kettering Cancer Center, New York, NY. Monoclonal anti-Thy-1 reagents were generously provided by Dr. Jonathan Sprent, University of Pennsylvania, Philadelphia, PA. Anti-I-J\(^+\) serum was prepared by hyperimmunizing B10.A (3R) recipients with a mixture of B10.A (5R), spleen, and lymph node cells (antiserum ASM-18) (we thank D. Murphy for preparing this antiserum). IB3 is a monoclonal antibody that was produced in this laboratory by immunization of normal rats with TNP-TsF. This antibody was found to specifically react with the TNBSA-F portion of TNP-TsF (Ptak, manuscript in preparation). Depletion of cells bearing Lyt (or Thy-1) markers was achieved by incubating 1 × 10\(^7\) cells/ml of antibody appropriately diluted in balanced salt solution (BSS), washing, and incubating with complement for 45 min at 37°C. Complement used in these experiments was serum from rabbits (guinea pigs) selected for low natural cytotoxicity to mouse cells.

Production of Factors

TNP- and OX-specific T suppressor factors and subfactors were generated as previously described (9). A brief description of the procedure used to generate each factor is given as follows.

TNBSA-F. Mice were injected intravenously with 0.35 ml of a 1% solution of TNBSA in distilled water (adjusted to pH 7.2 with sodium hydroxide) on days 0 and 4. 3 d later single-cell suspensions were prepared from spleen and peripheral lymph nodes and
cultured in vitro at a cell density of $1.5 \times 10^7$ cells/ml for 48 h at 37°C in serum-free RPMI 1640 medium supplemented with glutamine and antibiotics (culture medium). Cells were removed by centrifugation and supernatants were stored at -70°C.

**PCI-F.** Mice were painted with 0.15 ml of a 5% solution of PCI in an ethanol/acetone mixture (3:1 vol/vol) in all four paws and the skin of the clipped abdomen. Spleen and LN cells of these mice were taken on day 4 and incubated as described above.

**OX-Th-F.** An oxazolone equivalent to TNBSA-F (OX-Th-F) was prepared by injecting mice intravenously with $7 \times 10^7$ mouse thymocytes to which oxazolone had been conjugated on days 0 and 4, after which spleen cells were harvested on day 7, and cultured in the same fashion as were mice that made TNBSA-F.

**TNP-TsF.** Was prepared by intravenous injection of TNBSA solution on days 0 and 4, followed by skin painting with PCI on day 6. Cultures of spleen and lymph node cells were set up on day 8.

**OX-TsF.** Was prepared by intravenous injection of OX-labeled thymocytes ($7 \times 10^7$/mouse) on day 0 skin painting with oxazolone on day 7, followed on day 9 by culturing spleen and lymph node cells for 48 h as described for TNBSA-F.

**LyTsF.** Preparation of LyTsF specific for SRBC has been previously described (7). Briefly, a suspension of spleen cells from mice hyperimmunized to SRBC were treated with anti-Ly2 antibody and rabbit complement, and subsequently cultured in vitro for 48 h in culture medium supplemented with 10% fetal calf serum (FCS) at a concentration of $10^7$ cells/ml. After culture, supernatant fluids were cleared and passed through a Millipore filter (Millipore/Continental Water Systems, Bedford, MA).

**In Vitro Induction of I-J* Subfactor by PCI-F**

CBA/J mice were skin sensitized with OX as previously described and 4 d later, single cell suspensions of spleen and LN cells were prepared. Lymphoid cells ($3 \times 10^8$) were incubated with 15 ml of undiluted PCI-F for 1 h at 37°C, washed, and then added to TNP-labeled normal spleen cells ($10^8$) and subsequently incubated for 3 h at 37°C in 5 ml of RPMI 1640 medium supplemented with glutamine, antibiotics, and 2% FCS. Depletion of cells bearing Ly or I-J markers was accomplished by treating the cells with antibody and complement before the addition of PCI-F. Supernatants were then cleared and either used directly, or concentrated approximately fivefold with polyethylene glycol (mol wt 20,000, Sigma Chemical Co., St. Louis, MO) and applied to immunosorbent columns.

**Absorption of Soluble Factors**

Absorption of LyTsF with erythrocytes was done by mixing 1 ml of culture supernatants with 0.1 ml of a 50% suspension of sheep erythrocytes for 1 h on ice. The erythrocytes were removed by centrifugation. TNBSA-F was purified by passage over a 1B3 column prepared by conjugation of the monoclonal antibody to cyanogen-bromide-activated Sepharose 4B. Purified TNBSA-F was then attached to CNBr-activated Sepharose 4B. For absorption of factors over anti-I-J or TNBSA-F immunosorbents, supernatant was passed over relevant Sepharose column, washed extensively, then eluted with 0.2 M sodium carbonate, pH 11.0. Filtrate and eluate were then concentrated to original volume and dialyzed against first PBS, and then RPMI 1640.

**Adoptive Transfer of Contact Sensitivity (CS)**

Mice were skin sensitized by application of 0.15 ml of a 5% picryl chloride solution or a 3% oxazolone solution to the skin of shaved abdomen and four paws. 4 d after sensitization single cell suspensions of spleen and LN cells were prepared and then treated with anti-Ly-2.1 antibody and complement where noted. Cells were then incubated in the appropriate factors for 1 h at 37°C before being injected into naive recipients ($5-6 \times 10^7$ cells/mouse). If mixtures of factors were used, factors were preincubated for 1 h at 37°C before being added to to cells. Recipients were then immediately challenged on the ears with the appropriate antigen (0.8% PCI or OX in olive oil). CS responses are expressed as the increment of ear thickness from duplicate measurements (using an engineer's
micrometer (Mitotope) prior to challenge and 24 h later. The increment in ear thickness is expressed as mean ± SD in units of 10⁻³ cm. Values given are the average and the SEM for five individual samples from each group. These results are given as typical results from three independent experiments. Percent response was calculated according to the formula:

(Experimental-negative control)/(Positive control-negative control) × 100 (reference 13)

Statistical analysis was performed on these numbers using a Student's t test comparing the suppressed values to the control nontreated sample.

In Vitro Anti-SRBC Response

Anti-SRBC responses from immune mice were generated in vitro using a modification (13) of a cell culture technique described initially by Mishell and Dutton (14). Briefly, naive CBA/J mice were immunized with 0.2 ml of a 1% (vol/vol) solution of SRBC-BSS intraperitoneally. 10 d later, spleens were removed and T cells were prepared by adding spleen cells to plastic petri dishes coated with goat anti-mouse immunoglobulin and harvesting the nonadherent fraction (14). This procedure removed 95% of the Ig⁺ cells as determined by fluorescence. Thy-1-depleted spleen cells (B cells) were prepared from unprimed spleen cells by treating the cells with monoclonal anti-Thy-1 and complement. All cells were suspended in culture medium consisting of RPMI 1640 supplemented with antibiotics, 10% FCS, 100 mM glutamine, 25 mM Hepes, and 5 × 10⁻³ M 2-mercaptoethanol, at a final concentration of 2 × 10⁶ immune T cells, and 2 × 10⁶ B cells in 1 ml. To each culture 0.05 ml of a 1% SRBC suspension was added and cultures were maintained in Falcon 3008 plates (Falcon Labware, Div. Becton, Dickinson & Co, Oxnard, CA) for 5 d in a 5% CO₂, 95% air incubator at 37°C. The number of PFC was determined by using the Cunningham modification of the Jerne-Nordin plaque assay (16).

Results

T Suppressor Factors that Suppress in Adoptive Transfer Hapten-specific CS Are Antigen-specific and Composed to Two Distinct Subfactors. CS to the haptens PCI and OX can be passively transferred by immune spleen cells into naive recipients. The results in Table I show that TNP-TsF can significantly inhibit the passive transfer of CS to PCI, while the same factor has little effect on the CS reaction of immune cells to OX. The converse is also true. OX-TsF could only inhibit the CS of OX-immunized cells while not affecting the ability to transfer CS to PCI. Group 3 shows that complete suppression of CS to PCI can be seen by the mixture of two subfactors obtained from cultures of PCI-immune and TNBSA-immune cells. Supernatants from cell cultures in which only PCI-immune (PCI-F) or TNBSA-immune (TNBSA-F) cells were present contained no biological activity (groups 4 and 5).

Inability of TsF to Suppress the Passive Transfer of CS in Cells Depleted of Ly2 Cells Can Be Overcome by Addition of I-J⁺ Chain from SRBC-specific T Cell Factor. We investigated the cellular requirements for TsF suppressive activity (Table II). While removal of Ly2⁺ cells from the immune population did not abrogate their ability to transfer CS, there was a marked difference in the ability of TsF to suppress this transfer. Under these conditions, neither TNP-TsF or OX-TsF could alter the transfer of CS to their respective antigens. Likewise, incubation of Ly2 cell-depleted PCI-immune cells with a mixture of subfactors that yield a complete TNP-TsF (TNBSA- and PCI-F) had little affect on their ability to transfer CS, as did an I-J⁺ material from a SRBC-specific Ly1 Tₐ,F. However, whole TsF, or the TsF made up of subfactors, supplemented with Ly1 Tₐ,F I-J⁺
TABLE I

| Group | Cells immunized with | Source of TsF incubated with immune cells* | CS response in adoptive recipients  \( \pm SD \) | % Control response | Suppression |
|-------|----------------------|--------------------------------------------|---------------------------------|-------------------|-------------|
| 1 PCI | --                   | 8.2 ± 1.23                                 | Standard                        | Standard          |            |
| 2 PCI | PCI-TNP-TsF          | 3.0 ± 0.54                                 | 36                              | + (p < 0.01)      |            |
| 3 PCI | PCI-F² + TNBSA-F*    | 3.7 ± 0.81                                 | 45                              | + (p < 0.01)      |            |
| 4 PCI | PCI-PCI-F*           | 8.1 ± 0.76                                 | 98                              |                   |            |
| 5 PCI | PCI-TNBSA-F          | 8.4 ± 0.90                                 | >100                            |                   |            |
| 6 PCI | PCI-OX-TsF           | 8.0 ± 0.74                                 | 97                              |                   |            |
| 7 OX  | --                   | 9.4 ± 1.08                                 | Standard                        | Standard          |            |
| 8 OX  | OX-TNP-TsF           | 8.9 ± 1.17                                 | 94                              |                   |            |
| 9 OX  | OX-OX-TsF            | 3.8 ± 0.71                                 | 40                              | + (p < 0.01)      |            |

* Lymphoid cells from mice immunized with either PCI or OX 4 d earlier were mixed with supernatant from mice immunized to produce TNP- of OX-TsF. Immune cells were incubated with TsF for 1 h, then \( 6 \times 10^7 \) cells were injected into normal recipients.

† The 48-h supernatant from cultures of cells from mice whose skins were painted with PCI 24 h before culture.

‡ The 48-h supernatants from cultures of cells from mice injected intravenously with TNBSA on days 0 and 4 before harvesting for culture on day 8. Immune cells were incubated with a 50:50 mix of PCI-F and TNBSA-F.

§ 24-h increase in ear swelling (measured in units of \( 10^{-3} \) cm) after painting with the specific contactant was measured. The nonspecific swelling (that of mice that did not receive immune cells) is subtracted, so the results are presented as net increase in swelling, ± SD.

\( p \) value determined using a Student's t test.

TABLE II

The Requirement of Ly2 Cells in the Passive Transfer of TsF Activity Can Be Overcome by the Addition of an I-J K+ Molecule Isolated From an SRBC-specific Ly1 T Suppressor Factor

| Group | Cells immunized with | Source of factor incubated with Ly1 immune cells* | CS response in adoptive recipients  \( \pm SD \) | % Control response | Suppression |
|-------|----------------------|--------------------------------------------|---------------------------------|-------------------|-------------|
| 1 PCI | --                   | 7.8 ± 1.46                                 | Standard                        | Standard          |            |
| 2 PCI | PCI-TNP-TsF          | 7.7 ± 1.10                                 | 99                              |                   |            |
| 3 PCI | PCI-F² + TNBSA-F*    | 8.1 ± 1.24                                 | >100                            |                   |            |
| 4 PCI | PCI-I-J K            | 7.6 ± 1.33                                 | 98                              |                   |            |
| 5 PCI | PCI-TNP-TsF + I-J K  | 2.1 ± 0.67                                 | 27                              | + (p < 0.01)      |            |
| 6 PCI | PCI-F² + TNBSA-F* + I-J K | 2.4 ± 1.16                  | 31                              | + (p < 0.01)      |            |
| 7 OX  | --                   | 7.2 ± 0.61                                 | Standard                        |                   |            |
| 8 OX  | OX-OX-TsF            | 8.2 ± 0.64                                 | >100                            |                   |            |
| 9 OX  | OX-OX-TsF            | 3.1 ± 0.87                                 | 36                              | + (p < 0.01)      |            |

* Immune cells depleted of Ly2 cells were treated with the appropriate TsF as described in footnote *. Table I. The I-J K+ molecule was obtained from a 48-h culture supernatant of SRBC-immune spleen cells depleted of Ly2 cells by absorption of the supernatant with an anti-I-J K+ immunosorbent as detailed in materials and methods. I-J K+ material was added at a final concentration of 50%.

† See footnote †, Table I.

‡ See footnote ‡, Table I.

§ See footnote §, Table I.
material significantly reduced the ability of immune Ly1 cells to transfer the CS reaction.

Two important points should be noted here: (a) both TNP-TsF and the mixture of TNBSA-F and PCI-F require the presence of an Ly2 T transducer (acceptor) cell for biological activity. The need for this cell can be overcome by the addition of an I-J+ material from Ly1+ cells; and (b) the I-J+ material, secreted by cells immunized to SRBC, shows no antigen specificity in that it can work with either TNP- or OX-specific TsF to overcome the need for Ly2+ cells in the immune population.

The Combination of TNBSA-F and I-J+ Material Forms a Suppressive Complex That Is Antigen Nonspecific. The fact that two subfactors (TNBSA- and PCI-F) can be added together to form a complete suppressor factor allowed us to investigate whether either subfactor alone could interact with the I-J+ material to give suppressive activity (Table III). While none of the subfactors alone exhibited any suppressive activity, mixture of TNBSA-F with I-J+ material significantly suppressed the ability of PCI immune cells to passively transfer the CS reaction. However, the suppressive activity of the TNBSA-F I-J+ suppressor complex was no longer antigen specific in that it could equally suppress the passive transfer of CS to OX (group 6). Likewise, the OX equivalent of TNBSA-F (OX-Th-F) formed a suppressor complex with the I-J+ material that could inhibit the transfer of CS to either PCI or OX (group 8). In contrast, mixtures of the I-J+ material with the PCI-F subfactor were completely ineffective in suppressing PCI-immune Ly1+ cells (group 4).

The Inability to Suppress the Passive Transfer of CS in Cells Depleted of Ly2 Cells Can Be Overcome by Addition of an I-J+ Molecule Induced from Antigen-primed Ly2 I-J+ Cells by PCI-F. We postulated that since the combination of TNBSA-F and I-J+ material forms a nonspecific suppressor complex that suppresses Ly2 cell-

| Group | Source of TsF incubated with Ly1 immune cells | CS response in adoptive recipients |
|-------|---------------------------------------------|-----------------------------------|
|       |                                             | PCI                               | OX      |
| 1     | None                                        | 6.3 ± 1.23                       | 6.5 ± 1.02 |
| 2     | I-J+                                        | 6.4 ± 0.59                       | 7.2 ± 1.56 |
| 3     | PCI-F                                       | 6.4 ± 0.75                       | ND†      |
| 4     | PCI-F + I-J+                                 | 5.7 ± 1.75 (93)                  | ND       |
| 5     | TNBSA-F†                                     | 5.9 ± 0.75                       | 5.9 ± 125 |
| 6     | TNBSA-F + I-J+                               | 2.9 ± 1.45 (46, p < 0.01)        | 2.6 ± 0.77 (40, p < 0.01) |
| 7     | OX-Th-F†                                     | 6.1 ± 0.87                       | 6.5 ± 0.94 |
| 8     | OX-Th-F + I-J+†                              | 2.2 ± 0.68 (40, p < 0.01)        | 1.7 ± 0.80 (26, p < 0.01) |

* The I-J+ material was obtained from CBA/J SRBC-specific Ly1 TsF by absorption of Ly1 TsF with SRBC.
† See footnote 1, Table I.
‡ The 48-h supernatants from cultures of cells from mice immunized intravenously with either TNBSA or mouse thymocytes (Th) conjugated with OX on days 0 and 4 before harvesting for culture on day 8.
§ See footnote 1, Table I. Numbers in parentheses are the percent of control response.
ND, not done.
depleted T cells, the function of PCI-F was to induce this I-J+ material in the assay population. We tested this in an intermediate culture system by incubating PCI-F, TNP-labeled spleen cells, and T cells from animals skin painted with oxazolone for 1 h, washing, incubating an additional 3 h, and asking whether this supernatant, when added to TNBSA-F, could form an antigen nonspecific suppressor complex that could suppress the ability of immune Ly1 cells to transfer CS. The results in Table IV show that TNBSA-F plus PCI-F induced supernatant (PCI-Fs) were able to suppress the adoptive transfer of CS to PCI in cells depleted of Ly2+ cells (line 4). This suppressive complex was antigen-nonspecific in that it could suppress the transfer of CS to both PCI and OX (groups 4 and 12). We also found this factor to bear I-Jh determinants by passage of the supernatant over anti-I-J immunosorbent columns (groups 5–8).

We then investigated the nature of the cell producing this factor by pretreatment of immune cells with various antisera before culture. The results in Table V show that while supernatant from PCI-F–induced cells worked quite well with TNBSA-F to suppress the ability of Ly1 cells to transfer CS to OX, removal of Ly2+ or I-J+ cells from these intermediate culture cells removes this activity. In contrast, treatment of immune cells with anti-Ly1 sera did not affect the ability of these cells to generate this I-J+ material. These results indicate that Ly2+ and I-J+ cells are required for the generation of this relevant I-J+ material.

The Combination of TNBSA-F and I-J+ Material Can Suppress the PFC Response of Immune Ly1 + Normal B Cells to SRBC. We then tested the ability of the TNBSA-

### Table IV

| Group | Lyl Cells immunized with | Source of factor incubated with Lyl immune cells | CS response in adoptive recipients | % Control response | Suppression |
|-------|--------------------------|-----------------------------------------------|----------------------------------|-------------------|------------|
| 1     | PCI                      | None                                          | 7.3 ± 0.6                        | Standard          | Standard   |
| 2     | PCI                      | TNBSA-F*                                      | 7.2 ± 0.4                        | 99                | —          |
| 3     | PCI                      | PCI-Fs                                        | 7.0 ± 1.1                        | 96                | —          |
| 4     | PCI                      | TNBSA-F + PCI-Fs                              | 1.1 ± 0.9                        | 15                | + (p < 0.001) |
| 5     | PCI                      | TNBSA-F + PCI-F, I-Jh-                      | 5.5 ± 0.6                        | 55                | − (p > 0.1) |
| 6     | PCI                      | TNBSA-F + PCI-F, I-Jh+                      | 0.9 ± 1.2                        | 12                | + (p < 0.001) |
| 7     | PCI                      | TNBSA-F + PCI-F, I-Jh-†                      | 1.4 ± 0.6                        | 19                | + (p < 0.001) |
| 8     | PCI                      | TNBSA-F + PCI-F, I-Jh+†                      | 8.1 ± 1.2                        | >100              | —          |
| 9     | OX                       | None                                          | 8.1 ± 0.7                        | Standard          | Standard   |
| 10    | OX                       | TNBSA-F                                      | 7.5 ± 0.8                        | 90                | —          |
| 11    | OX                       | PCI-Fs                                       | 7.8 ± 0.7                        | 96                | —          |
| 12    | OX                       | TNBSA-F + PCI-Fs                              | 1.3 ± 0.8                        | 16                | + (p < 0.001) |

* See footnote 6, Table I.
‡ PCI-Fs was supernatant prepared from cultures of antigen-primed T cells, TNP-labeled spleen cells and PCI-F as described in Materials and Methods. PCI-F was added at a final dilution of 50%.
† I-Jh− supernatant was filtrate from an anti-I-Jh (ASM 19) immunosorbent column while I-Jh+ supernatant was eluted material from this column.
†† I-Jh− is filtrate of supernatant passed over an anti-I-Jh (ASM 20) immunosorbent while I-Jh+ is eluate from this column. PCI-Fs was added to culture at a final dilution of 50% as described.
Both Ly2<sup>+</sup> and I-J<sup>+</sup> Cells Are Required for the Induction of the I-J<sup>+</sup> Subfactor Required for TNBSA-F Suppressive Activity

| Group | Ly1 cells immunized with | Source of factor incubated with Ly1 immune cells | Treatment of cells before induction by PCI-F | CS response in adoptive recipients | % Control response | Suppression |
|-------|--------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------|-------------------|------------|
| 1     | OX                       | —                                             | 0*                                            | 6.7 ± 1.8                         | Standard          | Standard   |
| 2     | OX                       | TNBSA-F                                      | 0                                             | 6.7 ± 1.8                         | 100               | —          |
| 3     | OX                       | TNBSA-F + PCI-F                              | 0                                             | 6.8 ± 2.0                         | >100              | —          |
| 4     | OX                       | TNBSA-F                                      | None                                          | 0.9 ± 1.3                         | 13                | + (p < 0.001) |
| 5     | OX                       | TNBSA-F                                      | Anti-Ly1<sup>†</sup>                          | 1.2 ± 1.7                         | 18                | + (p < 0.01) |
| 6     | OX                       | TNBSA-F                                      | Anti-Ly2                                      | 6.9 ± 1.6                         | >100              | —          |
| 7     | OX                       | TNBSA-F                                      | Anti-I-J<sup>K</sup>                          | 5.4 ± 0.6                         | 81                | —          |

* No PCI-F induced supernatant was added.
† Cells were treated with anti-Ly1, anti-Ly2, or anti-I-J<sup>K</sup> serum as described in methods before being added to intermediate cultures.

Mixtures of I-J<sup>+</sup> Material and TNBSA-F Suppresses the Anti-SRBC PFC Response of Immune Ly1<sup>+</sup> Cells In Vitro

| Group no. | Cells added to cultures* | TsF added<sup>†</sup> | I-J material added | PFC/culture | % Control response<sup>‡</sup> | Suppression |
|-----------|--------------------------|-----------------------|-------------------|-------------|-----------------------------|------------|
| EX I      |                          |                       |                   |             |                             |            |
| 1         | Immune Ly1 T + B         | —                     | —                 | 3,500       | Standard                    | Standard   |
| 2         |                         | PCI-F                 | —                 | 3,000       | 91                          | —          |
| 3         |                         | TNBSA-F               | —                 | 3,700       | >100                        | —          |
| 4         |                         | Ly1<sup>‡</sup>       | —                 | 3,600       | >100                        | —          |
| 5         |                         | PCI-F                 | Ly1               | 5,100       | 93                          | —          |
| 6         |                         | TNBSA-F               | Ly1               | 1,200       | 26                          | + (p < 0.01) |
| EX II     |                          |                       |                   |             |                             |            |
| 7         | Immune Ly1 T + B         | —                     | —                 | 2,600       | Standard                    | Standard   |
| 8         |                         | PCI-F                 | —                 | 2,200       | 85                          | —          |
| 9         |                         | TNBSA-F               | —                 | 2,200       | 85                          | —          |
| 10        |                         | Ly2<sup>‡</sup>       | —                 | 2,200       | 85                          | —          |
| 11        |                         | PCI-F                 | Ly2               | 1,900       | 73                          | —          |
| 12        |                         | TNBSA-F               | Ly2               | 950         | 36                          | + (p < 0.01) |

* T cells from CBA/J mice immunized with 0.2 ml of a 1% (vol/vol) solution of SRBC 1 wk earlier were treated with anti-Ly2 and complement and 2 × 10<sup>6</sup> cells were added to 2 × 10<sup>6</sup> Thy-1 depleted (B) cells and SRBC. PFC responses were measured on day 5.
† See footnote *, Table I. Factors were added at a final concentration of 25%.
‡ See footnote *, Table II. I-J<sup>+</sup> material was added at a final concentration of 25%.
§ Ly2 I-J<sup>+</sup> material was added in culture by PCI-F as described in methods. I-J<sup>+</sup> material was added at a final concentration of 25%.
† Given by the formula: (experimental)/(positive control) × 100.

F-I-J<sup>+</sup> chain complex to suppress the in vitro response of immune Ly1<sup>+</sup> cells to SRBC (Table VI). Ly1<sup>+</sup> T cells from SRBC-primed animals were added to naive Thy-1-depleted spleen cells and T cell factors on day 0 and PFC response was measured on day 5. While none of the subfactors tested by itself were capable of suppressing the activity of Ly1 immune cells, the mixture of TNBSA-F and I-J<sup>+</sup>
MECHANISMS OF SUPPRESSION

TABLE VII
I-J* Material Binds to TNBSA-F

| Group | Source of TsF incubated with Ly1 immune cells* | CS response in adoptive recipients | % Control response | Suppression |
|-------|-----------------------------------------------|-----------------------------------|--------------------|------------|
| EX I  |                                               |                                   |                    |            |
| 1     | None                                          | 7.5 ± 2.0                         | Standard           | Standard   |
| 2     | TNBSA-F                                       | 7.1 ± 1.7                         | 95                 | —          |
| 3     | Ly1-I-Jk                                      | 7.6 ± 0.6                         | >100               | —          |
| 4     | TNBSA-F + Ly1-I-Jk                            | 2.0 ± 0.4                         | 27                 | + (p < 0.05) |
| 5     | TNBSA-F + Ly1-I-Jk filtrate†                  | 7.1 ± 0.9                         | 95                 | —          |
| 6     | TNBSA-F + Ly1-I-Jk eluate†                    | 2.2 ± 0.4                         | 29                 | + (p < 0.05) |
| EX II |                                               |                                   |                    |            |
| 7     | None                                          | 5.8 ± 1.6                         | Standard           | Standard   |
| 8     | TNBSA-F                                       | 5.3 ± 0.8                         | 91                 | —          |
| 9     | Ly2-I-Jk                                      | 5.5 ± 0.8                         | 95                 | —          |
| 10    | TNBSA-F + Ly2-I-Jk                            | 0.7 ± 1.1                         | 12                 | + (p < 0.05) |
| 11    | TNBSA-F + Ly2-I-Jk filtrate†                  | 4.0 ± 0.5                         | 69                 | —          |
| 12    | TNBSA-F + Ly2-I-Jk eluate†                    | 0.6 ± 0.9                         | 10                 | + (p < 0.05) |

* Immune cells of mice sensitized to oxazolone were incubated with TNBSA-F, I-J* material, or mixtures of both, then injected into normal recipients.
† I-J* material was passed over a TNBSA-F-Sepharose 4B column and filtrate or eluate was tested for its ability to give a suppressive activity to TNBSA-F.

We have extensively studied (7-9) a previously described (17) antigen-specific T cell factor that can suppress the transfer of delayed type hypersensitivity to PCI. What we found was a factor made up of two molecules (subfactors) secreted by cells that express distinct cell surface phenotypes and that are activated to secrete these factors by two different immunization regimens (9). The first subfactor, PCI-F, is the product of an Ly1*Ly2+I-J* cell that is activated in mice.

Conclusion

We have extensively studied (7-9) a previously described (17) antigen-specific T cell factor that can suppress the transfer of delayed type hypersensitivity to PCI. What we found was a factor made up of two molecules (subfactors) secreted by cells that express distinct cell surface phenotypes and that are activated to secrete these factors by two different immunization regimens (9). The first subfactor, PCI-F, is the product of an Ly1*Ly2+I-J* cell that is activated in mice.
by skin painting with PCI, an standard way of generating CS to PCI (13). The second, TNBSA-F, is the product of an Ly1-2+ I-J- T cell that is activated by intravenous injection of trinitrobenzene sulfonic acid (TNBSA), which is a standard way of generating tolerance to PCI (18). Both of these molecules are required for standard suppressive activity and, while only the PCI-F has demonstrable antigen-binding capacity, both appear to be antigen-specific (9).

The mechanism by which these two molecules effect suppression is by no means clear. Previous investigators (10, 11, 19) have shown the need for additional T cells in the assay culture that are not in the final targets of the suppressive activity. Sy et al. (19) report an antigen-primed T auxiliary cell (Ts- aux) required for the activity of efferent T suppressor cells. This cell is I-J+ and cyclophosphamide sensitive. Zembala et al. (11) also report the need for an auxiliary cell, which they call T acceptor cell (Tac), for the action of TsF. This cell is also I-J+, cyclophosphamide sensitive, and exhibits an Ly1-2+ phenotype. Our results are consistent with these given above, since we also find a need for an Ly2+ cell in the immune populations to effect suppression. In all likelihood, the three cells described here are identical. Zembala also reports that interaction of TsF with Tac results in the generation of an I-J+ antigen-nonspecific inhibitor of the transfer of contact sensitivity (Zembala, personal communication). This interaction required the presentation of antigen on haptenated spleen cells that were I-J matched with the cells that produced the TsF (10). It appears, then, that TsF-mediated suppression involves both an antigen-specific and an antigen-nonspecific T cell factor, the latter being the final effector of suppression.

We initially investigated this antigen-nonspecific phase of suppression using a novel approach. Our earlier results with Ly2 TsF, a T suppressor factor made by I-J-Ly2+ T cells, indicated that Ly2 TsF requires an I-J+ transducer T cell to effect suppressor function. the need for this transducer cell could be overcome by addition of an antigen-nonspecific I-J+ molecule secreted by immune T cells (4). We therefore asked the question: ¿Could the need for Ttrans cell in the suppression of CS to PCI be overcome by the addition of this antigen-nonspecific I-J+ molecule from SRBC immune T cells¿ Our finding was that the requirement for Ly2+ Ttrans cells in the immune population could be alleviated by the addition of this I-J+ molecule, and the resultant suppressive activity showed no antigen specificity. Our ability to look at the subfactors that make up a functional TsF led us to discover that the I-J+ molecule works only with one of the subfactors, the TNBSA-F. Earlier reports by Zembala (10) have shown that the antigen-binding molecule of TsF was necessary for the induction of this antigen nonspecific I-J+ inhibitor of CS, but found no evidence for its participation in subsequent suppressor activity. We have now shown that PCI-F alone can induce an I-J+ molecule, which can then act in tandem with the TNBSA-F to effect suppression. This molecular complex of TNBSA-F and I-J+ chain suppresses not only the passive transfer of DTH, but also the ability of immune Ly1 cells to stimulate B cells into becoming PFC. It appears likely that the antigen-nonspecific factors reported earlier may be identical to our suppressor complex of I-J+ material and TNBSA-F.

From these data, a general working model can be put forth to explain how these biologically active T cell-derived factors may work (Fig. 1). Skin painting
an animal with a contact sensitizer such as PCI activates a population of Ly1 T cells that secrete an antigen-specific T cell factor, PCI-F. PCI-F then interacts with a population of TNP-labeled spleen cells, which in turn activates an antigen-primed Ly2+ I-J+ cell to secrete an antigen-nonspecific I-J+ subfactor. The exact mechanisms by which these interactions take place are currently under investigation, but the fact that the antigen used to prime the cellular source of this I-J+ material is not important suggests that antigen priming serves only to clonally expand the population of Ly2+ T cells that serve as a source of these antigen-nonspecific I-J+ subfactors. This I-J+ chain can then complement the TNBSA-F either directly (group 1) or by activating an Ly1 I-J+ cell similar to the one activated by hyperimmunization with SRBC (group 2). Finally, these I-J+ subfactors then combine with TNBSA-F to form a functional suppressor complex that can suppress immune Ly1 T cells. Although these factors do not bind antigen with a measurable avidity, TNBSA-F does bind to the I-J+ molecule with which it cooperates in effecting suppression. This suggests that a physical association is required between the two molecular entities in order to form a suppressive molecular complex. It is not presently known what the molecular basis for interchain bonding is or whether there is any genetic restrictions in the ability of these factors to interact with one another. Preliminary results suggest, however, that there is a genetic restriction in the ability of TNBSA-F and I-J+ chain to suppress. The nature of these genetic restrictions are currently being investigated.

At present, we have no direct evidence linking the Ly2+ cell induced by PCI-F to secrete the I-J+ molecule needed for TsF activity with the SRBC-induced
Ly1+ T cell source of the I-J+ material that can also interact with TNBSA-F to form a nonspecific suppressor molecule. However, there are a number of striking similarities in the activation phase of both cell types: (a) both require the presence of the antigen-specific portion of TsF to be activated; (b) both require the presence of antigen and antigen presenting cells during activation; and (c) both combine with an Ly2+ T cell product to effect suppression. Therefore, it seems likely that the Ly1+ I-J+ cell in the SRBC system is playing a physiologic role very similar to the one played by the Ly2+ Ttrans cell in the suppression of adoptive transfer of DTH. This postulate is strengthened by the fact that secreted products from one cell can replace the need for the other cell to yield a functionally identical product.

It therefore appears that the biological activity of soluble products in the suppression of DTH depends upon the interaction of a number of T cell sets. The exact nature of these interactions is as yet unknown, but the evidence suggests the participation of at least three independent macromolecules. The fact that subfactors raised against SRBC can aid TNBSA-F in suppressing DTH responses, and vice versa, suggests a universality in the mechanism by which suppressor cells find and suppress their target cells. A second important point is that, in these experiments, the molecule that suppresses has no antigen-specific receptor on it, and at the same time shows no antigen-specific suppressor activity. However, in factors that exhibit an antigen-binding receptor, the suppressor molecule will only work on cells with which it can form an antigen bridge. In considering any antigen-nonspecific factor, it must be asked whether it is, in fact, a component of an antigen-specific complex. The loss of that antigen-specific portion of the molecule may result in a molecular complex that is then antigen-nonspecific but functions by utilizing an antigen-specific molecule supplied by the assay population. Immune Ly1+ T cells spontaneously produce antigen-specific regulatory molecules (3, 9). The use of T cell subfactors to dissect the molecular interactions necessary to deliver the suppressive signal will help us in analyzing these complexities of immunoregulation.

Summary

The passive transfer of contact sensitivity (CS) by immune cells can be inhibited with an antigen-specific T suppressor factor. This factor is composed of two subfactors: an antigen-specific subfactor made by an Ly1+ cell (PCI-F) and a antigen nonspecific subfactor made by an Ly2+ T cell (TNBSA-F). The suppressive activity of the complete factor can be eliminated by depleting the assay population of Ly2+ cells, even though it is the Ly1+ cell in the population that transfers the adoptive immunity. This suggests that the Ly2+ cell in the assay population is needed to transduce the suppressive signal to the Ly1+ effector cell of DTH.

We found that an Ly2+ cell from immune animals could be induced to produce a cell free subfactor that overcame the requirement for this Ttrans cell in the suppression of CS by TsF. The induction required only PCI-F, TNP-coupled

\(^2\) Flood, P. M., and R. K. Gershon. Mechanisms of Ly2 suppressor cell activity: analysis of the induction of an Ly1 I-J+ cell needed to transduce the suppressive signal. Manuscript submitted for publication.
spleen cells, and resulted in the production of an antigen-nonspecific I-J+ subfactor by immune Ly2+, I-J+ cells. The need for the Ly2+ transducer cell could also be overcome by addition of an I-J+ molecule secreted by Ly1 T cells hyperimmunized to SRBC. A suppressor complex made from mixing the I-J+ molecule with TNBSA-F could directly suppress the functional activity of immune T cells not only to transfer CS, but also to deliver help to B cells in an in vitro PFC response. This suppressive complex is antigen-nonspecific and does not require Ly2+ T cells in the assay population for suppressive activity.

These results indicate that effector factors of the suppressor circuit require two molecules; one that contains the functional suppressor material and one that serves as a "schlepper," a molecule needed to deliver the suppression to the appropriate target cell. The ability to construct a functional suppressor complex from two subfactors raised against different antigens, using different immunization procedures, which were isolated from factors exhibiting different functional activities suggests that certain cells of the immune system may play a universal role in "transducing" the suppressive signal.

We thank Dr. Robert Rosenstein for his help in preparing the 1B3 and TNBSA-F immunosorbent columns. We also acknowledge F. Bockleman for her expert technical assistance, and V. Fowler for her help in preparing this manuscript.

Received for publication 13 June 1983 and in revised form 16 August 1983.

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