SELECTIVE SUPPRESSION OF THE MAJOR IDIOTYPIC COMPONENT OF AN ANTIHAPTEN RESPONSE BY SOLUBLE T CELL-DERIVED FACTORS WITH IDIOTYPIC OR ANTI-IDIOTYPIC RECEPTORS*

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The existence of regulatory molecules derived from suppressor T cells has been reported in a number of experimental systems. Antigen-specific (1-11), allotype-specific (12), and subgroup-specific (13) soluble factors, regulating humoral and cell-mediated responses, have been identified and partially characterized. In some instances T cell-derived soluble suppressor factor (TsF) has been shown to bear antigenic determinants characteristic of major idiotypes associated with humoral antibodies that react with the same antigen for which the TsF has specificity (14, 15). It has been suggested that the idiotypic determinants are crucial to the regulatory role of such suppressor factors. However, it has not been practical to demonstrate selective suppression of the idiotypic component of an immune response. The data presented here demonstrate the existence of TsF which can selectively suppress a major idiotype without greatly diminishing the over-all antihapten response. The idiotype investigated is that associated with the anti-p-azophenylarsonate (Ar) antibodies of the A/J strain; studies of hybridoma products have indicated that these antibodies comprise a family of idiotypes that are closely related but are nonidentical serologically and with respect to amino acid sequences (16, 17).

Soluble suppressor factors were obtained from spleen cells of mice that had been immunologically suppressed with respect to the expression of the cross-reactive idiotype, hyperimmunized, and allowed to rest for 3 wk. Evidence is presented for the existence of two types of idiotype-suppressor factor, one bearing idiotypic, and the other anti-idiotypic receptors. Either suppressor factor selectively suppresses the biosynthesis of the cross-reactive idiotype without major suppression of the total antibody response to the Ar hapten group. Both suppressor factors bear determinants encoded by genes of the H-2 complex, and the active material in both cases is eluted from a Sephacryl column in a fraction corresponding to a mol wt of 50,000-100,000.

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1 Abbreviations used in this paper: anti-BAT, anti-mouse brain-associated T cell antigen; Ar, p-azophenylarsonate; BGG, bovine IgG; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CRI, cross-reactive idiotype common to anti-Ar antibodies of A/J mice; FCS, fetal calf serum; HIS, immunologically suppressed with respect to the expression of the CRI, then hyperimmunized with KLH-Ar; IBC, idiotypic-binding capacity; Id, idiotype(-ic); KLH, keyhole limpet hemocyanin; TsF, T cell-derived soluble suppressor factor.
Materials and Methods

Mice. Inbred mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. In experiments on suppression, A/J mice of one sex (usually male) were used in any given experiment. Recipients of TsF were A/J males, 13–15 wk old.

Preparation of Idiotypic and Anti-Idiotypic Antibodies: Assays. A/J mice were immunized with keyhole limpet hemocyanin (KLH)-Ar, and anti-Ar antibodies were purified from ascites by affinity chromatography as previously described (18, 19). Rabbit anti-Id (idiotype) was prepared by immunization with purified anti-Ar antibody, emulsified in complete Freund's adjuvant (CFA) (20). Rabbit antisera were adsorbed twice on columns of crude, nonimmune mouse globulin (18% sodium sulfate fraction) conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.). All precipitating activity against normal mouse globulin was lost after passage through the first column. Quantitative assays for cross-reactive idiotype common to anti-Ar antibodies of A/J mice (CRI) were carried out by inhibition in a radioimmune assay as previously described (20). 10 ng of 125I-labeled purified A/J anti-Ar was used as ligand, and complexes were precipitated with goat antirabbit Fc. Each assay mixture contained 5 μl of nonimmune A/J serum to ensure saturation of any residual antibody directed to nonidiotypic determinants. Anti-Ar titers in sera were determined by the method of Klinman et al. (21) using polyvinyl microtiter plates (Cooke-type, Fisher Scientific Co., Pittsburgh, Penn.) that were coated with bovine serum albumin (BSA)-Ar; 125I-labeled specifically purified rabbit antimouse Fab was used as the developing reagent after exposure to serum. The standard was an immune serum whose anti-Ar titer had been determined by quantitative precipitin analysis.

Hyperimmune, Idiotypically Suppressed (HIS) Mice: CRI(−) Anti-Ar. A/J mice, 8–10 wk old, were suppressed with respect to the CRI by two i.p. inoculations, with a 3 d interval, of rabbit anti-Id antiserum (25 μg idiotype-binding capacity [IBC]/inoculation) (22). Starting 2 wk later the mice were immunized i.p. three times at 2-wk intervals with 0.1 mg KLH-Ar in CFA. Only those mice were used as a source of suppressor factor which had produced high titers of anti-Ar with undetectable CRI (less than 50% inhibition in the radioimmune assay by 25,000 ng of anti-Ar).

Anti-Ar antibody without detectable CRI was specifically purified (18) on a bovine IgG (BGG)-Ar-Sepharose column from the sera and ascites (23) of HIS A/J mice. The antibody was passed over an anti-Id-Sepharose column before use.

Cell Fractionation. To enrich for T cells, single cell suspensions of spleen were exposed at 0°C for 5 min to a solution containing 0.155 M NH4Cl, 0.01 M KHCO3, and 0.1 mM EDTA, pH 7.4, to lyse erythrocytes (24). The remaining cells were enriched for T cells by the method of Mage et al. (25). In brief, 3 × 107 leukocytes in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 5% heat-inactivated FCS (Grand Island Biological Co.), 2 mM L-glutamine, 5 × 10−5 M 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml). A cell suspension containing 2 × 107 viable nucleated cells/ml was cultured in a 60-mm Falcon tissue culture dish (Becton Dickinson & Co., Orangeburg, N. Y.) for 24 h (except as specified) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Culture supernatants, recovered by centrifugation for 10 min at 1,500 g, were dialyzed against RPMI-1640 and stored at −60°C before testing as a source of suppressor factor.
Assay for Suppressor Factors. Cell-free culture supernatants to be tested for suppressor factor were dialyzed against fresh RPMI-1640 medium. A/J spleen cells from nonimmune mice were incubated at a cell density of $2 \times 10^7$/ml in undiluted culture supernatant from HIS or normal cells (control). (1 ml of cell-free culture supernatant corresponded to material from $2 \times 10^7$ spleen cells.) After incubation for 4 h at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air, the cells were washed twice with RPMI-1640 and transferred into irradiated syngeneic recipients (560 rad; $5 \times 10^7$ cells/mouse). The irradiation was carried out over a 3 min period in a $^{137}$Cs gamma irradiator (J. L. Sheperd & Associates, Glendale, Calif.). Cell viability (80–90%) was not significantly affected by the treatment with culture supernatant. Recipients of cells were inoculated i.p. with 100 µg of KLH-Ar in CFA on days 3 and 17 after the adoptive transfer. 25 and 35 d after the adoptive transfer of cells mice were bled, and serum concentrations of anti-Ar antibody and CRI were determined.

Affinity Chromatography of Suppressor Factors. Solid-phase immune adsorbents were prepared according to the procedure of Cuatrecasas (27), using cyanogen bromide and Sepharose 4B. After conjugation of the protein, residual active sites of the Sepharose were blocked by treatment with BSA, and the washed beads were packed into 12-ml disposable syringes. To minimize nonspecific adsorption, columns were further treated with RPMI-1640 medium containing 10% FCS before loading culture supernatants containing suppressor factor. Antigen-binding capacities were determined by using small portions of the Sepharose beads and $^{125}$I-labeled proteins. The protein contents of the various Sepharose 4B columns used were as follows. Normal mouse globulin, 6 mg/g of gel; BGG-Ar, 6 mg/g; rabbit antimouse Fab, 160 µg of IgG-binding capacity/g; rabbit anti-CRI, 54 µg IBC/g; CRI(+) anti-Ar, 500 µg KLH-Ar-binding capacity/g; CRI(−) anti-Ar, 500 µg KLH-Ar-binding capacity/g. The last two columns were adjusted to the same KLH-Ar-binding capacity by the addition of unconjugated beads.

Adsorptions were carried out by using suppressor factor from $1 \times 10^9$ to $2 \times 10^9$ spleen cells in the first step. After incubation in the column for 90 min at 4°C, the column was washed with 30 ml of PBS. Effluents were collected and the column washed again with at least 100 ml of phosphate-buffered saline. Elutions of bound protein at low pH were carried out by rapidly adding four column volumes of 0.1 M glycine-HCl buffer, pH 2.8. Eluates were neutralized immediately to pH 7.5 with 1 N NaOH. Filtrates and eluates were concentrated by negative pressure dialysis in the cold. FCS was added to a final concentration of 1%, except for the preparations used in tests of enzymatic digestion. Solutions were dialyzed against fresh culture medium immediately before use.

Preparation of Alloantiserum. B10-anti-B10.A antiserum was prepared in 20 C57BL/10 mice by the method of Greene et al. (9). Sera were obtained and pooled after eight biweekly i.p. injections of $4 \times 10^7$ B10.A spleen cells depleted of erythrocytes. The cytotoxic titer of antibody required for 50% lysis of $1 \times 10^6$ target cells, using 0.1 ml of alloantisemum, was 640. The antiserum was not cytotoxic for C57BL/10 cells.

Enzymes. Trypsin immobilized on Sepharose was obtained from Worthington Biochemical Co., Freehold, N. J. DNase and RNase were obtained from Sigma Chemical Co., St. Louis, Mo.

Results

Suppression of Id Synthesis by Spleen Cells Incubated with Cell-free Culture Supernatants. Nearly all experiments reported here were carried out with culture supernatants from spleens of A/J mice that had been immunologically suppressed with respect to the CRI, hyperimmunized, and allowed to rest for 3 wk before sacrifice. Cell-free culture supernatants were prepared from spleen cells and incubated with nonimmune spleen cells; the latter were then tested by adoptive transfer for their suppressive activity, as described under Methods.

The data in Table I show that nonimmune A/J spleen cells incubated with spleen cell culture supernatants from HIS mice adoptively transferred a state of suppression with respect to the production of CRI; this is indicated by the values for weight of anti-Ar antibody required for 50% inhibition in the radioimmune assay. The sup-
Table I

Suppression of Id Synthesis by Cell-free Culture Supernatants of Spleen Cells from Idiotypically Suppressed Hyperimmunized Mice

| Culture supernatants from (A/J spleen cells) | Recipients challenged with | Anti-Ar titer | Anti-Ar Ab required for 50% inhibition |
|---------------------------------------------|---------------------------|---------------|-----------------------------------|
| Nonimmune KLH-Ar                            |                           | 32, 44, 70, 75, 294 | 40, 4, 4, 4, 19 | 2,600, 600, 3,600, 1,02, 8, 18, 13, 24 |
| Nonimmune Edes.-Ar                           |                           | 48, 51, 92, 108, 210 | 1,200, 420, 76, 22, 13 | 470, 210, 600, 900, 105, 100, 21, 16 |
| KLH-Ar-immunized                            |                           | 16, 22, 38, 50, 50, 70, 112 | 400, 500, 32, 14, 14 | 270, 360, 1,190, 560, 460, 360, 64, 25 |
| KLH-Ar-immunized                            |                           | 30, 37, 40, 45, 70, 70, 90 | >750, >900, >1,000, | 310, 840, 560, 7,800, >21,000, >14,000, |
| Id-suppressed, KLH-Ar-immunized             |                           | 52, 72, 84, 100, 180 | >1,000, >2,200, >2,100, | 1,050, 1,700, >26,000, 8,400 |
| Id-suppressed, KLH-Ar-immunized             |                           | 52, 72, 84, 100, 180 | >2,500, >4,500, >4,500, | 530, 230, >13,000, >5,800 |

* Each recipient was given 5 × 10^7 A/J spleen cells that had been incubated with culture supernatant from 5 × 10^7 spleen cells specified in the first column. Idiotypically suppressed donor mice were allowed to rest for 3 wk after hyperimmunization before culturing their cells. The recipients were inoculated i.p. with 0.1 mg of KLH-Ar in CFA on days 3 and 17 after the adoptive transfer and were bled on days 25 and 35. Each value in the table represents an individual mouse.

† Edes.-Ar: Edestin-p-azophenylarsonate

Expression of Id was evident when either KLH-Ar or edestin-Ar was used as the immunogen after adoptive transfer. In each case the CRI was virtually undetectable in the immunized animals. The fact the CRI levels were very low irrespective of the carrier indicates that suppression of Id is not mediated through the protein carrier. The suppression of Id is evident in bleedings taken on days 25 or 35. The prolonged suppression is suggestive of an active mechanism rather than passive blockade of B cells; further evidence for this is discussed below.

There is an indication of a decrease in the total anti-Ar titer on day 35 in the idiotypically suppressed animals that were immunized with KLH-Ar; more data would be required to validate this conclusion. Also, there is some evidence for Id suppression when culture supernatants were obtained from spleen cells of KLH-Ar-immunized, but nonsuppressed mice; two of the mice in this group showed definite evidence for suppression of Id, although suppression was much less profound than that observed with HIS spleen cells. The possibility of obtaining Id-specific suppressor factor from immunized, nonsuppressed mice was not pursued in the present investigation.

Mice that were immunized with edestin-Ar rather than KLH-Ar produced somewhat lower titers of anti-Ar antibody, and there was also evidence for a reduced titer of CRI, particularly on day 25. (It should be noted that the data on nanograms required for 50% inhibition reflect the concentration of Id per unit weight of antibody.)

Identification of the Suppressor Factor as a T Cell Product. The data relevant to this conclusion are shown in Table II. Again, the culture supernatants were obtained from the spleens of HIS A/J mice that were allowed to rest for 3 wk after hyperimmunization, before sacrifice. Culture supernatants of unfractionated HIS spleen cells, when incubated with normal spleen cells which were subsequently transferred into irradiated recipients, strongly suppressed biosynthesis of the CRI while having relatively little effect on the total amount of anti-Ar produced. Cells that were nonadherent to anti-Fab, linked to polystyrene Petri dishes by the method of Mage et al. (25), yielded
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Identification of the Suppressor Factor as a T Cell Product*

| Culture supernatants from HIS A/J spleen | Day 25 | | Day 35 | | |
| Anti-Ar titer | Anti-Ar Ab required for 50% inhibition | Anti-Ar titer | Anti-Ar Ab required for 50% inhibition |
|---|---|---|---|
| Unfractionated cells | | | |
| 1217 | 1,100, 1,600, 2,400 | | |
| 96, 98 | 2,400, 3,000 | | |
| Cells nonadherent to anti-Fab§ | 38, 44, 86, 500 | | |
| 32, 114 | 3,000, 3,000, 3,000 | | |
| Treated with anti-BAT + C§ | 28, 30, 38, 280, 100, 30, 10, 12 | | |
| 40, 92 | 420, 510, 960, 810 | | |
| Control (culture medium) | 34, 86, 105, 4, 18, 30, 5, 6 | | |
| 210, 490 | 280, 660, 850, 5, 14, 42, 5, 12 | | |

* See first footnote, Table I. Each value represents an individual mouse.
† The anti-Fab was bound to polystyrene Petri dishes.
§ Culture conditions: TsF from 1 × 10^7 anti-BAT-treated, viable cells incubated with 2 × 10^7 normal cells/ml; 5 × 10^7 cells transferred to each recipient.

Time-Course of Appearance of TsF in Culture Supernatants *

| Duration of culture | Serum titers, day 30 |
|---|---|
| Anti-Ar | Anti-Ar Ab required for 50% inhibition |
| h | µg/ml | ng |
| 0 | 510, 660, 780 | 14, 66, 30, 64, 92 |
| 6 | 340, 650, 810, 1,050, 1,400 | 8,600, 16,000, 20, 15, 82 |
| 12 | 600, 780, 960, 1,350, 1,590 | 6,000, 66, 4,800, >33,000, 8,000 |
| 24 | 840, 840, 1,200, 1,300, 1,400 | >21,000, >21,000, >30,000, >34,000, >35,000 |
| 48 | 90, 470, 480, 750, 1,200 | >2,300, >11,000, >12,000, >18,000, >30,000 |

* The spleen cells cultured were from mice that had been idiotypically suppressed, hyperimmunized, and allowed to rest for 3 wk. See first footnote, Table I.

Culture supernatants that were strongly suppressive of CRI. Experiments on killing of cells with anti-BAT antiserum plus complement indicated that the nonadherent cells were mainly T cells. Moreover, culture supernatants from spleen cells that had been pretreated with anti-BAT plus complement had little CRI-specific suppressive activity (Table II). The same conclusions are derived from the data obtained from bleedings taken on days 25 or 35 after the adoptive transfer. The results indicate that most if not all the suppressor activity is derived from T cells.

Time-Course of Appearance of TsF in Culture Supernatants and Dosage Effects. The data on appearance of TsF as a function of time again were obtained with cultures of spleen cells of HIS A/J mice. The data in Table III indicate that TsF was present as early as 6 h, since two of five recipient mice were suppressed; however, maximal suppression occurred with supernatants taken from 24-h cultures. The data are consistent with continued biosynthesis and accumulation of TsF. On the basis of these data, subsequent experiments were carried out with supernatants from 24-h cultures.

The data in Table IV indicate that 2 × 10^6 HIS cells provided an amount of TsF that caused suppression of CRI in only two of nine recipients. About half of the recipients were suppressed when TsF from 1 × 10^5 HIS cells was used for each
recipient, and eight of ten were idiotypically suppressed when TsF from $5 \times 10^7$ cells was used. In the eight suppressed mice the CRI was virtually undetectable. TsF from $5 \times 10^7$ HIS cells was used for each recipient in the following experiments, except as indicated.

Failure of Id or Anti-Id Antibody to Replace TsF. The possibility was considered that cells from HIS mice might have elaborated small amounts of CRI, or of rabbit anti-Id, when cultured and that one of these substances was the active factor. Both possibilities were considered remote since the mice were idiotypically suppressed and were not synthesizing CRI, and because about 9 wk had elapsed between the initial injection of rabbit anti-Id and the sacrifice of the mice. The half-life of rabbit IgG in mice is approximately 6 d (28). Nevertheless, normal A/J spleen cells were cultured with rabbit Anti-Id (10 µg IBC/ml) or CRI-positive A/J anti-Ar antibodies (2 µg/ml), both of which had been heated at 56°C for 30 min to inactivate complement. After culturing for 4 h, the cells were washed and transferred into irradiated recipients. None of the mice receiving cells that had been incubated with CRI-positive anti-Ar was suppressed with respect to Id synthesis (Table V). One of nine mice that received cells which had been preincubated with rabbit anti-Id was idiotypically suppressed, presumably through the adherence of rabbit anti-Id to the normal cells. However, the concentration of rabbit anti-Id used was probably far in excess of its concentration in vivo. These results, together with the fact that T cells are the major or sole source of suppressor factor, indicate that the factor is not anti-Ar antibody or rabbit anti-Id. Supporting this are the data on molecular weights and on the presence of H-2 determinants on suppressor factors, to be discussed below.

Evidence for the Existence of Distinct Suppressor Factors with Id or Anti-Id Receptors. In preliminary experiments it was found that TsF could not be removed completely by adsorbent columns to which either Id (specifically purified A/J anti-Ar antibody) or rabbit anti-Id (globulin fraction) was conjugated, but that successive passage through the two columns removed all activity. The possibility was considered that two types
Failure of Id or Anti-Id Antibody to Replace TsF*

| Material cultured with normal spleen cells | Bleeding |
|-------------------------------------------|----------|
|                                            | Anti-Ar titer | Anti-Ab required for 50% inhibition | Anti-Ar titer | Anti-Ab required for 50% inhibition |
|                                            | µg/ml | ng | µg/ml | ng |
| None (culture medium)                      | 140, 170, 240, 620, 630 | 27, 52, 117, 150, 19 | 770, 1,500, 1,400, 2,100, 2,630 | 25, 57, 130, 180, 15 |
| Rabbit anti-id (10 µg IBC/ml)              | 64, 74, 88, 140, 160, 210, 300, 310, 320 | 60, 150, 150, 160, 20, 100, 150, 63, 22 | 730, 840, 720, 110, 99, 100, 43, 24, 200, 12 | 2,300, 890, 1,200, 1,300, 23, 75, 10, 11 |
| A/J Anti-Ar (2 µg/ml)                      | 56, 75, 86, 100, 120, 240, 250, 300 | 70, 80, 430, 86, 15, 30 | 520, 430, 1,200, 680, 230, 370, 620 | 16, 23, 150, 14, 62, 90, D, 30 |

* Normal A/J spleen cells were cultured for 4 h alone, or in the presence of rabbit anti-id or specifically purified A/J anti-Ar antibodies. After washing, the cells were adoptively transferred to A/J recipients which were immunized as described in Table I.

† D., deceased.

FIG. 1. Fractionation procedure used for TsF from 2 × 10⁹ spleen cells of HIS mice. The IgG-binding capacity of the anti-Fab-Sepharose column was 160 µg/g, and an 8-g column was used. Glycine (0.1 M)-HCl, pH 2.8, was used for elution.

of TsF molecules are elaborated by HIS cells; one with Id and another with anti-Id receptors. To investigate this possibility in detail the experiments outlined in Fig. 1 were carried out. The data are presented in Table VI.

TsF was first passed over anti-Fab-Sepharose. The amount of TsF used was 2 × 10⁹ cell equivalents, and the IgG-binding capacity of the column was 800 µg. Each recipient was given 5 × 10⁷ normal spleen cells incubated with a volume of TsF solution that would have contained 5 × 10⁷ cell equivalents if all the TsF applied had been recovered in that fraction (filtrate or eluate). This protocol was also used in subsequent fractionation experiments. The filtrate (fraction A) was found to possess suppressor activity (Table VI). Upon passage over a column in which Id (specifically purified A/J anti-Ar antibody) was conjugated to Sepharose, both the filtrate (B) and acid eluate (C), when incubated with normal spleen cells, rendered them suppressive for CRI. When fraction B, which had passed through the Id-Sepharose column without being bound, was in turn passed over an Anti-Id column, the filtrate (D) had little, if any, suppressor activity whereas the acid eluate from this column possessed such activity. The fact that TsF in fraction B was not bound by an Id-containing
Evidence for TsF with Id or Anti-Id Receptors

| Fraction (Fig. 1) | Anti-Ar titer | Anti-Ar Ab required for 50% inhibition |
|------------------|---------------|--------------------------------------|
|                  | µg/ml         | ng                                   |
| A                | 100, 160, 220, 330 | 2,500, >4,200, >5,200, 4,100          |
| B                | 42, 150, 180, 250, 430, 450 | >1,000, 3,700, 30, >6,400, >10,800, 45 |
| C                | 230, 270, 360, 430, 560 | >5,620, >6,700, 9, 11,000, >14,000   |
| D                | 60, 150, 150, 330, 360, 660 | 8, 19, 39, 41, 18, 16                |
| E                | 32, 104, 150, 180, 240, 300 | >800, 210, 750, 18, 23, >7,500       |
| F                | 58, 120, 120, 160, 200, 430 | >1,500, 3,000, 1,500, >4,100, 24, 11,000 |
| G                | 44, 56, 90, 270, 330 | 11, 1,400, 45, 13, 11                |
| H                | 180, 220, 240, 430, 840, 990 | 4,500, 450, 960, 2,100, 4,300, 5,000  |
| I                | 150, 180, 210, 210, 210, 360 | 750, 180, 2,700, >5,300, >5,300, 720  |
| Control (culture medium) | 150, 180, 230, 630, 640 | 27, 52, 110, 130, 19 |

* TsF was generated and recipient mice were immunized as described in first footnote, Table I. The fractionation procedure is shown in Fig. 1. Each recipient was given 5 × 10^7 A/J spleen cells that had been incubated with an amount of TsF corresponding to 5 × 10^7 A/J spleen cells (assuming that all the TsF was in that fraction). Recipient mice were bled on day 25 after the adoptive transfer.

The presence of TsF in the acid eluate from an Id-Sepharose column (fraction C) indicates that there is a TsF which possesses anti-Id receptors. This is supported by the failure of the active substance in fraction C to be bound by an anti-Id column. Five of six mice receiving cells exposed to fraction F (filtrate from anti-Id column) were suppressed, whereas only one of five mice was suppressed when the acid eluate (fraction G) was used. The suppression of the single mouse might be explained on the basis of a small amount of nonspecific adsorption of Id TsF to Id receptors on the Id-Sepharose column or of anti-Id TsF to the anti-Id-Sepharose column. In any event it is clear that a substantial amount of TsF is bound by an Id-Sepharose column which, after elution at low pH, is not bound by an anti-idiototype-Sepharose column. These results are consistent with the presence of TsF possessing anti-Id receptors.

The results obtained when half of the initial filtrate (A) was passed over an anti-Id-Sepharose column are consistent with the above conclusion. Both the filtrate (H) and acid eluate (I) possessed suppressor activity. These data indicate that some but not all molecules of TsF possess Id determinants. The fact that all activity was removed by successive passage over Id-Sepharose and anti-Id-Sepharose (fraction D) is consistent with the presence of Id or anti-Id receptors on all of the TsF.

The possibility was considered that the adherence of TsF to Id-Sepharose columns might be mediated through bound antigen rather than through the presence of anti-Id receptors; i.e., that all TsF bears Id receptors but that some TsF also contains bound multivalent antigen which permits binding to an Id-Sepharose column. If the latter premise were correct, then the TsF that we had identified as anti-Id should bind equally well to anti-Ar Sepharose columns in which the anti-Ar possesses or lacks the CRI; i.e., if binding were mediated by antigen it should be independent of the presence of Id on the anti-Ar antibodies bound to Sepharose.

The protocol used to investigate this possibility is outlined in Fig. 2, and the data
TABLE VII

Evidence That Adherence of TsF to CRI-positive Anti-Ar Is Mediated by Id Determinants on Anti-Ar*

| Fraction (Fig. 2) | Day 25 |
|------------------|--------|
|                  | Anti-Ar titer | Anti-Ar Ab required for 50% inhibition |
|                  | µg/ml | ng |
| A                | 270, 360, 390, 540, 600, 690 | 1,100, 720, 10, >14,000, 1,200, 1,400 |
| B                | 100, 270, 270, 420, 540, 810 | >2,500, 1,300, 1,300, 500, 27, 16,000 |
| C                | 210, 240, 270, 390, 420, 690 | 26, 600, 52, 390, 120, 57 |
| D                | 190, 360, 540, 800, 1,080, 1,200 | 200, 180, 33, 50, 27, 14 |
| E                | 360, 290, 330, 510, 570, 710 | 5,200, >7,200, 41, 2,600, 600, 350 |
| Control (culture medium) | 360, 390, 480, 720, 870, 990 | 120, 50, 30, 65, 27, 24 |

* See footnote, Table VI. The fractionation procedure used for TsF is shown in Fig. 2. Other procedures are as described in the first footnote, Table I.

are shown in Table VII. TsF from HIS mice was first passed over a column of Id-Sepharose containing bound, specifically purified anti-Ar antibodies from immunized A/J mice. The acid eluate (A) possessed suppressor activity. Portions of this eluate were passed over columns of anti-Ar Sepharose in which the anti-Ar antibodies were either CRI negative (from HIS mice) or CRI positive; the antigen-binding capacities of the two columns were the same (Materials and Methods). It is evident that nearly all of the suppressor activity passed through the CRI-negative column without being retained (fraction B) whereas essentially all of the activity was retained by the CRI-positive column. The filtrate from the latter column (D) lacked activity whereas the acid eluate (E) was strongly suppressive. These results are consistent with the conclusion that the TsF initially eluted from a CRI-positive column at low pH had anti-idiotypic receptors.

Presence of an Antigen-binding Site on TsF with Id Receptors; Further Evidence for Two Types of TsF. The protocol of these experiments is shown in Fig. 3, and the data are in Table VIII. TsF from 1 x 10^9 spleen cells was first passed over a column of BGG-Ar-Sepharose, then incubated with normal A/J spleen cells which were assayed for suppressor activity. In all cases the amount of TsF tested was that associated with 5 x 10^7 cells, obtained from HIS mice which were rested for 3 wk before sacrifice. Id-specific suppressor activity was present in both the filtrate and the acid eluate (fractions B and C, Table VIII); this result is again consistent with the existence of two distinct suppressor factors. The data obtained with fractions D and E indicate
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Fig. 3. Fractionation procedure used for TsF from $1 \times 10^8$ spleen cells of HIS mice. The filtrate and acid eluate from the BGG-Ar-Sepharose column were each divided in half for the subsequent step.

TABLE VIII
Further Evidence for TsF with Id or Anti-Id Receptors: Binding of Id but Not Anti-Id TsF to BGG-Ar*

| Fraction (Fig. 3) | Anti-Ar titer | Anti-Ar Ab required for 50% inhibition |
|------------------|---------------|--------------------------------------|
|                  | ng/ml         |                                      |
| A                | 320, 340, 480, 750, 1,050 | >8,200, >8,500, 960, >18,000, >27,000 |
| B                | 240, 390, 480, 630, 1,100 | >6,000, >9,800, >12,000, 2,500, 70 |
| C                | 330, 600, 1,000, 1,050, 1,320 | 600, >15,000, 5,900, >26,000, >30,000 |
| D                | 210, 240, 570, 870, 1,350 | 1,050, 6,000, 1,800, 11, 17 |
| E                | 870, 960, 990, 1,050, 1,230 | 72, 12, 25, 13, 30 |
| F                | 330, 340, 570, 630, 870 | 14, 21, 13, 78, 14 |
| G                | 180, 450, 630, 990, 1,050 | 4,500, 1,100, 39, 980, 26 |
| Control (culture medium) | 440, 930, 1,170, 1,240 | 15, 55, 9, 150 |

* See footnote, Table VI. The fractionation procedure is shown in Fig. 3. Recipient mice were immunized as described in the first footnote of Table I and bled 25 d after the adoptive transfer.

that the suppressor factor which passed through the BGG-Ar column had anti-Id receptors, since its activity was completely lost on further passage through an Id-Sepharose column (E), but not through an anti-Id-Sepharose column (D).

In contrast, suppressor factor eluted from BGG-Ar-Sepharose appears to have Id receptors since all of its activity was lost upon passage over an anti-Id-Sepharose column (F) but its activity was retained after passage through Id-Sepharose (G). In addition to supporting the existence of two factors the results indicate that TsF(Id) is bound by BGG-Ar-Sepharose. Experiments are in progress to analyze the antigen-binding specificity of the factor.

Presence of H-2 Determinants on TsF. The data in Table IX indicate that TsF with Id or anti-Id receptors adheres to a column containing bound anti-H-2 antibodies directed against determinants controlled by the H-2d locus. The antiserum used was B10-anti-B10.A with cytotoxic activity. A crude globulin fraction, precipitated by 18% sodium sulfate, was conjugated to Sepharose 4B. The column contained the equivalent of 3.5 ml of antiserum with a cytotoxic titer of 640. The TsF that was passed over the column was obtained by culturing $5 \times 10^8$ spleen cells from HIS mice. The TsF was separated into Id and anti-Id fractions by the protocol shown in Fig. 2. Neutralized acid eluates from each column were passed over the anti-H-2 columns in the presence of 5% FCS. The data in Table IX indicate that both factors
Evidence That Id and Anti-Id Suppressor Factors Bear H-2 Region-encoded Determinants

| Source of TsF | Treatment |
|---------------|-----------|
| Id TsF‡       | Untreated  |
|               | 60, 90, 100, 100, 290 | 60, 90, 100, 100, 290 | 36 |
|               | 120, 190, 200, 360, 660 | 600, >4,800, 1,000, 560, 1,200 |
|               | 100, 150, 180, 330, 1,000 | 100, 150, 200, 10, 27 |
|               | 150, 190, 270, 380, 490 | 150, 200, 9, 10, 12 |
|               | 90, 110, 110, 120, 240 | >2,300, 2,700, >2,700, 360, 62 |
| Anti-Id TsF¶  | Untreated  |
|               | 90, 90, 110, 160, 170 | 450, >2,200, 200, 3,800, 4,200 |
|               | 90, 210, 270, 360, 570 | >2,200, 1,100, 33, 720, >14,000 |
|               | 60, 90, 120, 160, 230 | 30, 11, 3,000, 150, 6 |
|               | 300, 430, 930, 1,000, 1,000 | 10, 220, 23, 34, 5,000 |
|               | 60, 120, 240, 270, 450 | 1,500, >3,000, 6,000, >6,700, >11,000 |
| Culture medium, no TsF | 240, 270, 360, 840 | 15, 33, 45, 21 |

* See first footnote, Table I. Mice were bled 25 d after the adoptive transfer.
‡ Obtained by eluting TsF from an anti-Id Sepharose column at pH 2.8.
§ Crude globulin fraction obtained by precipitating serum with 18% sodium sulfate.
¶ Obtained by eluting TsF from an Id-Sepharose column at pH 2.8.

Molecular Size of TsF. This question was approached by gel filtration using Sephacryl S-200. The column was standardized by measuring the volumes required for elution (peak values) of human IgG (mol wt, 150,000); BSA (67,000); ovalbumin (43,000); and horse cytochrome c (12,000). A good straight line was obtained for a plot of log molecular weight vs. elution volume. The data in Table X show that nearly all the activity of TsF with either Id or anti-Id receptors was eluted from the column in fraction III, which has a molecular weight range of 50,000–100,000. In the case of the factor with Id receptors, a small amount of TsF was eluted in fractions II and IV, of higher and lower molecular weight; the Id in one of five mice in each group was suppressed by normal spleen cells incubated with fractions II or IV.

Evidence That the Suppressor Factors Contain Polypeptides. The data in Table XI show that TsF with either Id or anti-Id receptors lost its suppressor activity upon treatment with trypsin but not with DNase or RNase. The results indicate that both suppressor factors are, at least in part, polypeptides.
TABLE X

Estimation of Molecular Weight of TsF by Gel Filtration *

| Fraction from Sephacryl | TsF (anti-ld) | TsF (id) |
|-------------------------|--------------|----------|
|                         | Anti-Ar Ab required for 50% inhibition | Anti-Ar Ab required for 50% inhibition |
|                         | µg/ml ng     | µg/ml ng |
| Unfractionated         | 93 150, 140 1,900, >1,600, >3,600, 250, 190 1,200 1,900 | 540 390, 370, 510 >13,000, 2,300, 1,900 |
| Fraction I (mol wt >200,000) | 420, 700, 200, 26, 43, 12, 11, 70 | 260, 590, 450, 880, 260 |
| Fraction II (mol wt 200,000-100,000) | 90, 140 9 11 9 17 19 | 290, 450, 840, 230, 930 7,400 18 280, 30, 1, 24 |
| Fraction III (mol wt 100,000-50,000) | 160, 120, 46, 1,500 3,100 >1,200 42 >9,700, 620 | 250, 510, 810, 390, 490 5,900 >13,000 >20,000 |
| Fraction IV (mol wt 50,000-20,000) | 190, 160, 320, 11, 15, 330, 15, 19 | 640, 680, 1,300, 830, 620 31, 6,000, 11, 21, 52 |
| Fraction V (mol wt <20,000) | 310, 250, 470, 19, 27, 21, 130, 17 | 840, 310, 400, 810 35, 10, 25, 16 |

* TsF(Id) and TsF(anti-ld) were obtained by the procedure indicated in Fig. 2. The fractions of varying molecular weight were obtained by gel filtration on a 1.5 × 90-cm column of Sephacryl S-200. The TsF used was obtained from 5 × 10⁸ spleen cells of HIS mice. Fractions were concentrated before incubation with normal spleen cells. Each value in the table represents an individual mouse that received 5 × 10⁷ spleen cells incubated with 5 × 10⁷ cell equivalents of TsF (assuming all TsF was eluted in that fraction). Recipients were immunized as indicated in Table I and bled 27 d after the adoptive transfer.

TABLE XI

Effect of Trypsin, DNase, and RNase on TsF Activity *

| Source of TsF | Treatment of TsF | Day 35 |
|---------------|------------------|--------|
|               | Anti-Ar titer | Anti-Ar Ab required for 50% inhibition |
|               | µg/ml ng | µg/ml ng |
| Acid eluate from CRIP-Sepharose§ | No enzyme treatment || 270, 390, 1,400, 1,600, 2,700, 1,900, 45, 8,100 |
|               | Immunobilized trypsin (10 U/ml) | 570, 930, 990, 1,000, 14, 15, 62, 33 |
|               | DNase (150 Kunitz U/ml) | 360, 390, 960, >9,000, >10,000, >24,000, 1,300, 12,000 |
|               | RNase (10 Kunitz U/ml) | 240, 660, 780, >6,000, >17,000, >20,000, 1,000, 26 |
| Acid eluate from anti-Id-Sepharose§ | No enzyme treatment || 270, 600, 780, >6,000, >17,000, >20,000, 1,500, 16 |
|               | Immunobilized trypsin (10 U/ml) | 510, 960, 1,200, 22, 15, 18, 40 |
|               | DNase (150 Kunitz U/ml) | 240, 330, 570, >6,000, >8,000, 2,900, 1,400, 12 |
|               | RNase (10 Kunitz U/ml) | 420, 600, 1,200, >11,000, 15,000, 1,100, 13 |
| Control (culture medium) | None | 640, 750, 840, 34, 36, 18 |

* See first footnote, Table I.

‡ 3 × 10⁷ cell equivalents of TsF were treated with each enzyme for 1 h at 37°C. Each recipient was given 5 × 10⁷ spleen cells incubated with 5 × 10⁷ cell equivalents of TsF from HIS mice.

§ Glycine-HCl, pH 2.8.

‖ Control, incubated for 1 h at 37°C with Sepharose conjugated with BSA.
Discussion

The data presented establish the existence of TsF which can selectively suppress the major Id component of an immune response. Selective suppression of an Id has previously been demonstrated in the humoral response to the phenylarsonate hapten group (22, 29), group A streptococci (30, 31), and phosphorylcholine (32). Anti-Id antibodies or suppressor T cells rather than soluble suppressor factors were used in those experiments.

Our results also demonstrate that there are suppressor factors with anti-Id receptors. It was found that TsF with Id or anti-Id receptors, either of which can selectively suppress the Id component of the anti-Ar response, coexist in an idiotypically suppressed, hyperimmunized mouse. In the method used to assay TsF, which is based on that described by Watanabe et al. (33), cell-free culture supernatants from A/J HIS spleens are incubated with normal spleen cells, which are then transferred into irradiated A/J recipients. In all recipients high titers of anti-Ar antibodies were obtained upon subsequent immunization, but in many recipients the CRI was undetectable or present at an extremely low concentration. TsF was taken from 24-h cultures of HIS cells; 6-h cultures possessed much less activity (Table III). Suppression of Id was independent of the carrier used for immunization of recipients against the Ar hapten.

Evidence that the suppressor factors are T cell products was the failure of cells forming the factors to be bound by anti-Fab-coated Petri dishes and the destruction of the active cells by a T cell-specific anti-BAT antiserum plus complement. That the factor is not anti-Ar antibody with the CRI or residual rabbit anti-Id (used in the initial suppression of donor mice) was indicated by the failure of each of these two substances to exhibit substantial suppressor activity when incubated with normal spleen cells. The great superiority of T cells over B cells in producing the factors, the nonadherence of suppressor factors to an antimouse Fab adsorbent, data on molecular weight, and the presence of H-2 antigens provide further evidence that the factors are not immunoglobulins.

Strong evidence was obtained for the existence of distinct Id-suppressor factors with Id and anti-Id receptors. Neither an Id-Sepharose nor an anti-Id-Sepharose column was capable of removing all of the suppressor factor, but successive passage through the two columns removed all activity. Evidence for the existence of Id TsF was the failure of an Id-Sepharose column to remove all TsF activity. The unbound TsF was, however, removed by anti-Id-Sepharose, and material with suppressor activity could be eluted from the latter column at low pH (Table VI). In addition, TsF which was bound to BGG-Ar-Sepharose and then eluted at low pH was in turn bound by anti-Id-Sepharose (from which it could be eluted at low pH). It was not bound by an Id-Sepharose column (Table VIII). The results indicate the existence of TsF with Id receptors, which can be bound either by anti-Id or by BGG-Ar. Additional experiments are needed to establish the hapten-binding specificity of the factor. However, an Id-bearing TsF that suppresses DTH to the Ar hapten has been shown to possess hapten-binding specificity and idiotypic determinants (15).

Evidence for TsF with anti-Id receptors was, first, the failure of anti-Id-Sepharose to remove all suppressor activity. Second, material active in suppression was bound by, and could be eluted from an Id-Sepharose column (purified A/J anti-Ar antibodies conjugated to Sepharose). The eluted material was not adherent to anti-Id Sepharose.
The possibility was considered that the binding to Id-conjugated Sepharose was an artifact; i.e., that all TsF is idiotypic and that binding to Id-Sepharose is mediated by multivalent antigen present in culture supernatants. This was ruled out by the failure of the presumptively anti-Id TsF to be bound by anti-Ar-Sepharose in which the A/J anti-Ar antibodies lack the major CRI. Such TsF was, however, bound by CRI-positive A/J anti-Ar conjugated to Sepharose, and could be eluted from the column at low pH (Table VII).

The approximate molecular weight of TsF with Id or anti-Id receptors was estimated by gel filtration on Sephacryl S-200. In both cases nearly all suppressor activity was eluted in a fraction corresponding to a mol wt range of 50,000-100,000. In the case of the Id TsF a small amount of activity was found in the adjoining fractions of higher and lower molecular weight. The data on molecular weights correspond quite well with the values obtained by Greene et al. for TsF that suppresses DTH against the Ar hapten (9); by Thèze et al. for TsF that suppresses humoral responses to a synthetic copolymer of glutamic acid, alanine and tyrosine (5); and by Taniguchi et al. for TsF that suppresses humoral responses to protein antigens (7, 34). The TsF of Taniguchi was obtained from a T cell hybridoma as well as splenic T cells. In contrast, Taussig and Holliman report a mol wt of approximately 200,000 for a TsF with specificity for sheep red blood cells, produced by a T cell hybridoma (35).

We have not proven that our TsF is a single molecular species. If it is an aggregate the actual molecular weight would be lower than the estimated value. Also, since precautions were not taken against proteolysis, it is possible that the actual molecular weight is higher than 100,000 and that breakdown products retain suppressor activity.

That the suppressor factors are, at least in part, polypeptides was indicated by the loss of activity after treatment with trypsin, and the resistance of each factor to RNase and DNase (Table XI).

The data on molecular weights and the fact that the idiotypic and anti-idiotypic factors are fractionated independently suggest that they do not form complexes with one another to any substantial degree. This may be attributable to a low concentration, relative to their binding affinities.

Both Id and anti-Id TsF adhered to a Sepharose column containing bound antibodies prepared in C57BL/10 mice against B10.A splenic leukocytes. The antibodies were shown to have cytotoxic activity against B10.A cells and by this criterion possessed anti-H-2^a specificity. The results provide evidence for the presence of H-2 determinants on TsF. Other investigators have demonstrated the presence on TsF of determinants controlled by the I-J or I-C subregions of the H-2 MHC (1, 3, 5, 6, 36, 37).

The presence of Id determinants on TsF has previously been demonstrated by Bach et al. (15), who investigated delayed hypersensitivity to the Ar hapten, and by Germain et al. (14), who studied humoral responses to a copolymer of glutamic acid, alanine, and tyrosine. In those studies it was not practical to ascertain whether the major Id component of the response was selectively suppressed. The presence of Id determinants on suppressor T cells has been demonstrated or inferred in several systems (38-43).

Owen et al. (29) and Bona and Paul (44) have described suppressor T cells with anti-Id receptors, which specifically suppress the Id component of responses to the Ar hapten group and to the M460 Id of anti-DNP antibodies, respectively. In the experiments of Owen et al., HIS mice were allowed to rest for 8 wk before examination...
of their Id-suppressor cells, as compared to 3 wk in the present experiments. Our preliminary data indicate that in mice rested for 8 wk most but not all TsF activity is associated with molecules having anti-Id receptors.

The first demonstration of Id-suppressor T cells was that of Eichmann, who induced such suppressor cells with anti-Id antibodies (30). The induction by anti-Id antibody of T cells that suppress delayed-type hypersensitivity has been reported by Sy et al. (40) and by Yamamoto et al. (43). The fact that anti-Id can stimulate suppressor T cells suggests that the first cell involved in the induction of suppressor cells by anti-Id has Id receptors. Dohi and Nisonoff (18) and Sy et al. (45) have demonstrated suppression of a humoral idiotypic response or of delayed-type hypersensitivity, respectively, by administration of Id-coupled syngeneic leukocytes. In each case suppressor T cells were formed. In the suppression of delayed-type hypersensitivity, evidence has been obtained that the inoculation of Id-conjugated cells results in the formation of suppressor cells with anti-idiotypic receptors (M. I. Greene. Personal communication.). All of the data seems consistent with the hypothesis that anti-Id antibody initially induces the formation of suppressor cells with Id receptors, whereas the injection of Id-conjugated cells induces the formation of suppressor cells with anti-Id receptors.

It seems quite possible that the cell types may be mutually stimulatory. As already mentioned, Owen et al. (29) found that HIS mice possess a high percentage of Id-suppressor cells with anti-Id receptors; however, a prolonged waiting period (8–12 wk) was required after hyperimmunization of idiotypically suppressed mice. Since the reagent initially used was anti-Id, it seems probable that the initial targets were T cells with Id receptors and that this population was expanded upon immunization with KLH-Ar. Over a period of time, these cells may have stimulated the formation of large numbers of T cells with complementary, anti-Id receptors. This hypothesis is supported by the requirement for antigenic stimulation in the generation of large numbers of anti-Id cells (46). A requirement for antigen in the generation of large numbers of cells that suppress idiotype has also been demonstrated by Kim (47). The possibility that the two types of suppressor cell may be mutually stimulatory has been discussed by Dohi and Nisonoff (18) and by Sy et al. (45).

An interesting result was the presence of small amounts of suppressor activity in cell-free supernatants from mice that had been immunized with KLH-Ar but not immunologically suppressed (Table I). These results suggest the possibility that antigen alone may cause expansion of clones of Id-suppressor T cells.

The exact nature and function of suppressor factors is still unknown. It is possible, although unproven, that such factors represent receptors of T cells, which are released from the surface in culture. The potent activity of TsF suggest a physiological role; however this remains to be established. It is also possible, but uncertain, that all suppressor activity is mediated by released factors. It has been shown that suppressor factors can induce the formation of suppressor T cells in vivo or in vitro (48–51). In agreement with these findings we have observed that our unfractionated suppressor factors stimulated the development of suppressor T cells (Y. Hirai and A. Nisonoff. Unpublished observations.). It is not yet known whether such T cells bear Id or anti-Id receptors. The results of Moorhead (37) suggest that certain TsF may act directly on target cells, because time constraints in his experiments argue against the induction of suppressor cells.

Another area of uncertainty is the target of Id-suppressor T cells. Studies with the
anti-Ar Id have shown that secondary B cells are resistant to Id suppression (52, 18). This suggests the possibility that one target of suppression is the primary B cell, which presumably could interact with anti-Id suppressor T cells (or anti-Id TsF). An alternative possibility is that secondary B cells require much less help than primary cells and that helper T cells are the actual targets of suppression. Since there is evidence for the existence of helper T cells with Id (31, 53, 54) and anti-Id (54–57) receptors, such T cells are potential targets of suppressors with anti-Id or Id receptors, respectively. Alternatively, only one type of suppressor T cell might be the effector, but this cell type might be stimulated by the complementary (Id or anti-Id) T cell. Eardley et al. have shown that genes linked to the Igh locus govern the interaction between T inducers and T acceptors in the induction of feedback suppression; this suggests a possible role for Id interactions (58).

Jerne (59) has proposed that the immune system may be regarded as comprising a series of Id-anti-Id interactions. These interactions were postulated both to stimulate and regulate lymphocyte responses. The results from the many experimental systems mentioned above strongly support Jerne's original network hypothesis, particularly at the level of the T cell. Recognition that suppressor factors can selectively suppress the Id component of an immune response, and that there are two types of suppressor factor involved in Id-specific suppression, may provide new possibilities for exploring details of regulation in the immune system.

Summary
Evidence is presented for the selective suppression of the major idiotypic component of the humoral response to the phenylarsenate hapten by soluble factors derived from T cells (TsF). The existence of TsF with anti-idiotypic receptors was also demonstrated. It was found that TsF with idiotypic and anti-idiotypic receptors coexist in cultures of spleen cells prepared from idiotypically suppressed, hyperimmunized mice. By gel filtration the molecular weight of each factor was found to be 50,000–100,000. Each is sensitive to trypsin and is bound to a column containing anti-H-2^a antibodies. Evidence is discussed which suggests the possibility of mutual stimulation of suppressor T cells with idiotypic and anti-idiotypic receptors.

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