SUPPRESSOR T-CELL MECHANISMS IN CONTACT SENSITIVITY

III. Apparent Non-Major Histocompatibility Complex Restriction is a Result of Multiple Sets of Major Histocompatibility Complex-Specific Suppressor T Cells Induced by Syngeneic 2,4-Dinitrophenyl-modified Lymphoid Cells*

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Specific immune suppression is one of the mechanisms capable of regulating the immune response. Membrane-bound haptens and protein antigens have become powerful tools for the study of both positive and negative aspects of the immune response. Subcutaneous injection of hapten- or antigen-modified lymphoid cells leads to the induction of significant cell-mediated immunity (CMI) responses as assessed by either ear swelling or footpad swelling after challenge with free or cell-bound antigen (1-3). In contrast, as first noted by Battisto and Bloom (4) in a guinea pig system, and later expanded upon by our laboratory and others, i.v. injection of hapten- or antigen-modified syngeneic lymphoid cells leads to a profound, efficient state of unresponsiveness of both humoral immunity and CMI (1-6).

Our efforts have concerned the mechanisms of tolerance in mice with contact sensitivity to 1-fluoro-2,4-dinitrobenzene (DNFB), where tolerance was induced by 2,4-dinitrophenyl (DNP)-modified lymphoid cells (DNP-LC). Mice, thus treated, are specifically unresponsive to epicutaneously applied DNFB (5). Further, we have shown that this tolerant state can be due to either or both of two antigen-specific mechanisms: (a) a rapidly induced, long-lasting, cyclophosphamide (CY)-insensitive period of inhibition of reactive T-cell clones (clone inhibition), and (b) a transient, CY-sensitive, infectious period of suppressor T-cell (Tₜ) activity (7).

Ts induced by the injection of syngeneic DNP-LC (syninduced Ts) have been shown to inhibit the expression of DNFB-immune, lymph node delayed hypersensitivity T cells (TDH) (efferent limb of sensitivity) without affecting the development of the TDH

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Abbreviations used in this paper: BSA, bovine serum albumin; BSS, Mishell-Dutton balanced salt solution; CMI, cell-mediated immunity; Cy, cyclophosphamide; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; DNP-BSA, DNP-bovine serum albumin; DNP-LC, DNP-modified lymphoid cells; DNP-lysine, Nα-DNP-lysine HCl; DNP-MGG, DNP-mouse gamma globulin; DNP-OVA, DNP-ovalbumin; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; LC, lymphoid cells; MGG, mouse gamma globulin; MHC, major histocompatibility complex; OVA, ovalbumin; TDH, delayed hypersensitivity T cells; Tₜ, suppressor T cells; TₜDH, trinitrophenyl-modified lymphoid cells.
Efferent inhibition by syninduced $T_s$ was shown by the fact that
syninduced $T_s$ could suppress previously sensitized recipients. Furthermore, the
cotransfer of syninduced $T_s$ and DNFB-immune $T_{DH}$ prevented the passive transfer
of contact sensitivity into normal recipients. Inhibition of passive transfer by these $T_s$
was shown to be both dose-dependent and antigen-specific.

In addition, we have shown that syninduced $T_s$ were active in mediating hapten-
specific suppression regardless of the recipients' genetic background (9, 10). That is,
BALB/c DNP-LC can induce $T_s$ in BALB/c donor mice that are capable of
transferring suppression to BALB/c, CBA, and C57Bl/6 recipients. Similarly, these
syninduced $T_s$ of BALB/c origin were able to suppress the passive transfer of immunity
by DNFB-immune BALB/c $T_{DH}$ or CBA $T_{DH}$ when cotransferred to normal recipients
(8). The induction of syninduced $T_s$ has been shown to be restricted to combinations
where the DNP-LC tolerogen and the $T_s$ donor strain share the H-2D region of the
major histocompatibility complex (MHC) (11).

The present experiments were designed to ask what it is that $T_s$ in the system
recognize; i.e., what is their receptor(s) directed against? They were also designed to
examine the possible mechanisms by which syninduced $T_s$ exert suppression of
allogenic, DNFB-immune $T_{DH}$. The results indicate that their receptor is directed
against DNP-modified determinants encoded for by the MHC and that the wave of
apparently non-MHC-restricted suppression is, in fact, polyclonal in nature, i.e.,
composed of a collection of distinct MHC-restricted $T_s$. This is true, because the
ability of $T_s$ to suppress a particular allogeneic $T_{DH}$ can be specifically inhibited by
absorption with DNP-membranes, MHC-compatible with the target $T_{DH}$, leaving
intact the ability of the $T_s$ to suppress $T_{DH}$ derived from other strains.

Materials and Methods

**Mice.** 2- to 4-mo-old female BALB/c mice were obtained from Cumberland Farms, Clinton,
Tenn. Female CBA, A/J, and C57Bl/6 mice were obtained from The Jackson Laboratory, Bar
Harbor, Me. A.TH mice were obtained from Dr. J. W. Moorhead, University of Colorado
Medical Center, Denver, Colo.

**Cell Lines.** DBA/2-derived P-815 mastocytoma cells were obtained from Dr. D. W. Talmage
(University of Colorado Medical Center) and maintained by serial passage in RPMI-1640
(Grand Island Biological Co., Grand Island, N. Y.) medium containing 5% fetal calf serum.

**Antigens.** DNFB and N-e-DNP-L-lysine HCl (DNF-lysine) were obtained from Sigma Chem-
ical Co., St. Louis, Mo. 2,4-dinitrobenzene-1-sulfonic acid sodium salt was obtained from
Eastman Kodak Co., Rochester, N. Y. Picryl sulfonic acid was obtained from Matheson,
Coleman, & Bell, East Rutherford, N. J. Ovalbumin (OVA) and bovine serum albumin (BSA)
were obtained from Miles Laboratories, Inc., Kankakee, Ill. Mouse gamma globulin (MGG)
was prepared from pooled mouse serum by ammonium sulfate precipitation.

**Preparation of DNP-Proteins.** BSA, OVA, and MGG were dinitrophenylated by the method of
Little and Eisen (12). The approximate molar ratios were DNP$\text{-}$BSA, DNP<sub>26</sub>-OVA, and
DNP<sub>15</sub>-MGG.

**Preparation of Hapten-modified Lymphoid Cells.** Erythrocyte-free spleen cell suspensions were
prepared in Hanks' balanced salt solution (HBSS) and spleen cells were dinitrophenylated
exactly as previously described (5) and are termed DNP-LC. Spleen cells were trinitrophenyl-
lated by incubating equal volumes of spleen cells at $20 \times 10^7$/ml in HBSS and 10 mM picryl
sulfonic acid (in HBSS) for 30 min at room temperature; these are termed trinitrophenyl-
modified lymphoid cells (TNP-LC).

**Preparation of Hapten-modified Lymphoid Cell Membranes.** Membranes were prepared similar to
the method of Greene, et al. (2) by subjecting DNP-LC or TNP-LC to four alternate cycles of
snap freezing at $-78^\circ$C in dry ice-acetone and thawing at $37^\circ$C, followed by centrifugation at
10,000 g for 45 min. The soluble membrane fragments were then dialyzed overnight against
phosphate-buffered saline, pH 7.4, and adjusted to the desired number of membrane equivalents per milliliter. Membranes prepared from sham-modified cells were used as controls.

**Induction of Ts.** Mice were injected i.v. with $5 \times 10^7$ syngeneic DNP-LC on day -7. On day 0, peripheral and mesenteric lymph nodes were collected and single-cell suspensions were prepared in Mishell-Dutton balanced salt solution (BSS). Control Ts consisted of lymph node cell suspensions from mice injected with sham-modified lymphoid cells (LC).

**Induction of DNFB-immune TDH.** DNFB-immune TDH were obtained from donors contact sensitized with 0.5% DNFB in 4:1 acetone:olive oil. Donor mice were sensitized with 25 µl of DNFB on the shaved abdomen and 5 µl on each ear on days 0 and 1. They also received 5 µl on each front paw on day 1. Draining (inguinal, axillary, brachial, and cervical) lymph nodes were removed on day 4 and single-cell suspensions were prepared in BSS.

**Affinity Chromatography.** Cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) was coupled with hyperimmune anti-H-2d (B10 aB10.D2) or anti-H-2k (B10 aB10.BR) serum. $4 \times 10^8$ membrane equivalents of BALB/c DNP-LC membranes was applied to the columns and washed through with phosphate-buffered saline, pH 7.2. The eluate was collected and concentrated to the original volume (4.0 ml) by negative-pressure dialysis.

**Blocking of Ts.** Ts to be tested were treated at a concentration of $10^8$ cells/ml of DNP-lysine (100 µg/ml); DNP6-BSA (500 µg/ml); DNP26-OVA (500 µg/ml); DNP15-MGG (500 µg/ml); or DNP-LC membranes (10° membrane equivalents/ml) for 1 h at 4°C. All DNP-congeners were diluted in BSS. Following the initial incubation, the cells were washed three times in BSS and adjusted to the proper concentration for testing their suppressive ability.

**Ts Assay: Efferent Blockade.** Ts were assayed for suppressive ability by testing their effect on passive transfer of contact sensitivity by immune TDH. $5 \times 10^7$ Ts (either normal or treated with DNP-congeners) were mixed with $5 \times 10^7$ TDH and immediately transferred by i.v. injection to normal recipients syngeneic to the TDH donor. Recipients were ear challenged within 1 h of cell transfer and the degree of passive transfer was assessed 24 h later by measuring the increment in ear thickness (in units of 10⁻⁴ in) with an engineer's dial thickness gauge. The percentage of suppression was calculated by comparing the ear-swelling response of mice receiving both TDH and Ts (experimental) with those receiving TDH and sham Ts (positive controls), and negative (ear challenged only) control mice: percentage of suppression = [(Positive control - experimental)/(positive control - negative control)] × 100%.

**Results**

**Rationale.** The experiments were designed to ask what it is that mature Ts recognize by determining their ability to be blocked by various DNP-congeners (Fig. 1). To accomplish this, Ts were treated in suspension with the various DNP-congeners, washed, and $5 \times 10^7$ cells were cotransferred to normal, syngeneic recipients along with $5 \times 10^7$ TDH. Recipients were ear challenged within 1 h of transfer and the degree of passive transfer was assessed 24 h later by measuring ear swelling. Lack of ear swelling in the recipient mouse indicates suppression by active Ts. In contrast, a positive ear-swelling response in the recipient mouse indicates that Ts were blocked by the preincubation and are thus incapable of inhibiting the expression of CMI by the transferred TDH cells.

**The Ability of Various DNP-Congeners to Block the Suppressive Action of Ts.** Initial experiments were designed to ask the question: what form of DNP is able to inhibit active suppression by Ts? To accomplish this, a pool of BALB/c Ts were treated with the indicated concentrations of DNP-congeners, washed extensively, and cotransferred to normal BALB/c recipients along with BALB/c TDH. The results (Fig. 2) show the average values for two-four experiments expressed as the percentage of suppression of control passive transfers. As can be seen, Ts pre-incubated with sham-modified,
syngeneic BALB/c membrane preparations still suppressed passive transfer an average of 85.0% (group A). Pretreatment of Ts with 100 μg/ml of monomeric DNP-lysine (group B), or with 500 μg/ml of various DNP-protein preparations (DNP₆-BSA [group C], DNP₉₆-OVA [group D], and DNP₁₅-MGG [group E]) had no significant effect on the ability of Ts to suppress the expression of CMI by T_DH. However, pretreatment of the Ts with soluble BALB/c DNP-LC membranes reversed the suppressive ability to an insignificant level of 4.7% (group F). Thus, monomeric DNP-lysine and polymeric DNP-protein conjugates are not capable of blocking Ts activity. Ts apparently recognize DNP associated with membrane determinants. It should be pointed out that concentrations of DNP₉₆-OVA up to 2.0 mg/ml had no effect on Ts activity, and that concentrations of DNP-lysine >100 μg/ml were toxic.

Hapten and MHC Specificity of Ts Blocking by Hapten-modified Membrane Preparations. Having established the efficiency of Ts blocking by syngeneic DNP-membrane preparations, it was next of interest to ask what the hapten and MHC specificities were. BALB/c syninduced Ts were thus treated with 10⁶ membrane equivalents/ml of sham-, DNP-, or TNP-modified BALB/c syngeneic LC, and with equivalent concentrations of allogeneic sham-modified CBA or DNP-modified CBA.
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**Fig. 2.** The ability of various DNP-congeners to block the suppressive activity of syninduced T$_s$.

* BALB/c T$_s$ were treated with the indicated DNP-congeners as described in Materials and Methods and $5 \times 10^7$ cells cotransferred with $5 \times 10^7$ BALB/c T$_{DH}$ into normal BALB/c recipients. Memb., membrane.

† Average values of percentage of suppression of control passive transfer for two-four experiments.

LC-membrane preparations. After washing, $5 \times 10^7$ treated T$_s$ were mixed with $5 \times 10^7$ T$_{DH}$ and transferred to BALB/c recipients. The results (Fig. 3) show that T$_s$ treated with sham-modified BALB/c LC membranes suppress passive transfer 83.8% (group B), and pretreatment with DNP-modified BALB/c LC membranes reversed this to 5.1% suppression (group C). Treatment of the DNP-specific T$_s$ with TNP-modified BALB/c LC membranes had no significant effect on their suppressive ability as they reduced passive transfer by 78.2% (group D). Thus, the T$_s$ receptor is exquisitely hapten-specific. Neither sham- (group E) nor DNP-modified (group F) CBA LC membrane preparations significantly reduced the suppressive ability of BALB/c T$_s$ on BALB/c T$_{DH}$. Thus, blocking of syngeneic suppression requires that DNP be present on syngeneic membrane determinants. We have also tested the ability of syngeneic and allogeneic DNP-membrane preparations to directly block DNFB-immune T$_{DH}$ and have found that both are effective blockers (Stephen D. Miller, data not shown). This observation may indicate a basic difference in the antigen recognition system used by T$_s$ as opposed to T$_{DH}$.

**T$_s$ Recognition Involves Hapten-modified MHC-encoded Determinants.** To directly determine if suppression of syngeneic T$_{DH}$ by syninduced T$_s$ required recognition of DNP-modified MHC-encoded determinants, BALB/c DNP-LC-membrane preparations
Cells Transferred to BALB/c Recipients

| BALB/c Ts | Membrane Pretreatment* | BALB/c TDH (Cells/mouse) | Δ Ear Swelling (x10^-4 in) | SEM ‡ |
|-----------|-------------------------|---------------------------|---------------------------|-------|
| -         | -                       | 5 x 10⁷                    | A                         |       |
| 5 x 10⁷   | Sham - BALB             |                           | B (83.8) §                 |       |
|           | DNP - BALB              |                           | C (5.1)                    |       |
|           | TNP - BALB              |                           | D (78.2)                   |       |
|           | Sham - CBA              |                           | E (90.6)                   |       |
|           | DNP - CBA               |                           | F (73.2)                   |       |

Fig. 3. Hapten and MHC specificity of Ts inhibition by hapten-modified LC membranes.

* BALB/c Ts were treated with the indicated membrane preparations as described in Materials and Methods and 5 x 10⁷ cells cotransferred with 5 x 10⁷ BALB/c TDH to normal BALB/c recipients.
‡ Values represent mean 24-h ear swelling in recipient mice (four per group) ± SEM. Δ, relative change in.
§ Numbers in parentheses represent the percentages of suppression of control passive transfer.
|| Significant suppression as compared to positive controls (group A) P < 0.001.

were prepared and applied to anti-H-2d or anti-H-2k affinity columns. The materials not adherent to the columns were collected, concentrated, and tested for their ability to block BALB/c syninduced Ts (Fig. 4). 5 x 10⁷ untreated BALB/c Ts suppressed the passive transfer by 5 x 10⁷ BALB/c TDH by 87.2% (group B). Treatment of Ts with the material recovered after passage of BALB/c DNP-LC membranes over an irrelevant anti-H-2k column reversed this suppression to an insignificant 4.0% (group C). However, removal of DNP-modified BALB/c MHC-encoded determinants on a specific anti-H-2d column rendered the membranes unable to block the Ts (group D, 75.6%). Thus, Ts recognize DNP-modified, syngeneic MHC-encoded determinants in a syngeneic suppression system.

Requirement for H-2D-Region Compatibility for Blocking of Syngeneic Suppression by Syninduced Ts.

Experiments were done to define the DNP-modified MHC-encoded membrane determinants responsible for blocking the suppressive action of syninduced Ts. Initial experiments examined the blocking effects of A/J DNP-LC membranes on BALB/c and CBA syninduced Ts (Fig. 5). Untreated, BALB/c syninduced Ts suppressed the response of syngeneic BALB/c TDH 96.8% when transferred to BALB/c recipients (group B). Treatment of these Ts with A/J DNP-LC membranes (compatible at the IC → D regions) completely reversed their suppressive action (group C). Also, CBA syninduced Ts suppressed the response of syngeneic CBA TDH...
### Cells Transferred to BALB/c Recipients

| Cells Transferred to BALB/c Recipients | BALB/c Ts Cells Transferred to BALB/c Recipients |
|---------------------------------------|-----------------------------------------------|
| *BALB/c Ts*                         | Membrane Pretreatment* BEN                         |
| *Cells/mouse*                       | BALB/c TDH (cells/mouse)* 5 x 10⁷                  |
| -                                    | -                                             |
| 5 x 10⁷                              | None                                          |
| "                                   | DNP-BALB (K-H-2k column) "                     |
| "                                   | DNP-BALB (K-H-2d column) "                     |

| \(\Delta\) Ear Swelling (x10⁻⁴ in 1) \(\pm\) SEM | \(\Delta\) Ear Swelling (x10⁻⁴ in 1) \(\pm\) SEM |
|-----------------------------------------------|-----------------------------------------------|
| 0                                            | 0                                            |
| 10                                           | 10                                           |
| 20                                           | 20                                           |
| 30                                           | 30                                           |

| Fig. 4. Inhibition of Tₙ function requires DNP-modified MHC determinants. |

* BALB/c Ts were treated with BALB/c DNP-LC membrane eluates from either anti-H-2k or anti-H-2d affinity columns and 5 x 10⁷ cells cotransferred with 5 x 10⁷ BALB/c TDH to normal BALB/c recipients. |
† Values represent mean 24-h ear swelling in recipient mice (four per group) \(\pm\) SEM. Δ, relative change in. |
§ Numbers in parentheses represent the percentages of suppression of control passive transfer. |
‖ Significant suppression as compared to positive controls (group A) \(P < 0.001\). |

88.6% when transferred to CBA recipients (group E). However, pretreatment of CBA Ts with A/J DNP-LC membranes (compatible at the K → IE regions) failed to reverse their suppressive action (group F). These results indicate that the Ts are responding to DNP-modified determinants encoded for by the right end (IC → D regions) of the MHC.

To further define which DNP-modified membrane determinants were responsible for Ts blocking, both DBA/2 (H-2d)-derived P-815 membranes (which carry H-2Kᵈ and H-2Dᵈ antigens, but no detectable I-region antigens [D. C. Shreffler, personal communication]) and A.TH DNP-LC membranes (which share only the H-2Dᵈ region) were tested for their ability to block BALB/c (H-2ᵃ) syninduced Ts (Fig. 6). As shown previously, untreated BALB/c Ts suppressed the TDH response by 91.8% (group B), and pretreatment with syngeneic BALB/c DNP-LC membranes reversed suppression to a level of 6.2% (group C). Pretreatment with DNP-modified P-815 membranes (H-2K- and H-2D-region compatible) or with A.TH DNP-LC membranes (H-2D-region-only compatible) also reversed the suppressive ability of BALB/c Ts (groups D and E). These data indicate that Ts recognize DNP-H-2D-region determinants, as H-2D-region compatibility between syninduced Ts and the DNP-LC membrane preparation is required for blocking of suppression.

Allosuppression by Syninduced Ts is Not Blocked by Pretreatment with DNP-LC Membranes Syngeneic to the Ts. The data to this point clearly show that pretreatment of syninduced Ts with DNP-LC membranes compatible at the H-2D region block the ability of the Ts to suppress syngeneic TDH. As we have previously shown that syninduced Ts are not genetically restricted and will suppress allogeneic TDH upon cotransfer (8), it was of interest to examine the membrane requirements for blocking of their allosuppressive ability. Two experiments in this regard are shown in Fig. 7. In experiment
Fig. 5. Inhibition of syngeneic suppression requires right-end MHC identity.

* BALB/c and CBA Ts were treated with A/J DNP-LC membrane preparations as described in Materials and Methods. 5 x 10^7 BALB/c Ts were cotransferred with 5 x 10^7 BALB/c TDH to normal BALB/c recipients (upper panel, open bars) and 5 x 10^7 CBA Ts were cotransferred with 5 x 10^7 CBA TDH to normal CBA recipients (lower panel, hatched bars).

† Values represent mean 24-h ear swelling in recipient mice (four per group) ± SEM. Δ, relative change in.

§ Numbers in parentheses represent the percentages of suppression of control passive transfer.

‖ Significant suppression as compared to positive controls (groups A or D) P < 0.001.

one, BALB/c syninduced Ts were pretreated with either DNP-BALB/c or DNP-CBA LC membranes and cotransferred with BALB/c or CBA TDH into normal recipients syngeneic to the TDH donor. Untreated BALB/c Ts suppressed the passive transfer of CMI by either BALB/c (group A) or CBA (group D) TDH. As shown previously, BALB/c Ts treated with DNP-modified BALB/c LC membranes could no longer suppress BALB/c TDH (group B), but they still retained their allosuppressive ability for CBA TDH (group E). BALB/c Ts pretreated with allogeneic DNP-modified CBA LC membranes could still suppress syngeneic BALB/c TDH (group C and Fig. 3), but were no longer able to suppress allogeneic CBA TDH (group F). In experiment two, BALB/c Ts pretreated with CBA DNP-LC membranes were tested for their ability to suppress passive transfer of sensitivity by BALB/c, CBA, and C57Bl/6 TDH. As expected, untreated BALB/c Ts suppressed passive transfer mediated by TDH of each haplotype (groups G, I, and K). Those Ts pretreated with CBA DNP-LC membranes suppressed BALB/c (group H) and third-party C57Bl/6 (group L) TDH, but could no longer suppress CBA TDH (group J). These data indicate that the allosuppressive
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**BALB/c Ts**

| Cells/mouse | Membrane Pretreatment* | BALB/c TDH (Cells/mouse) | Δ Ear Swelling (x10⁻⁴ in) | SEM † |
|-------------|------------------------|--------------------------|---------------------------|-------|
| 5x10⁷       | None                   | 5x10⁷                    | A                         |       |
| 5x10⁷       | DNP-BALB/c             | C                        | B (91.8) §                  |
| 5x10⁷       | DNP-P815               | D                        | C (6.2)                    |
| 5x10⁷       | DNP-A.TH               | E                        | D (10.8)                   |

Fig. 6. Inhibition of syngeneic suppression maps to the H-2D Region of the MHC.

* BALB/c Ts were treated with the indicated DNP-LC membrane preparations as described in Materials and Methods and 5 x 10⁷ cells cotransferred with 5 x 10⁷ BALB/c TDH to normal BALB/c recipients.

‡ Values represent mean 24-h ear swelling in recipient mice (four-five per group) ± SEM. Δ, relative change in.

§ Numbers in parentheses represent the percentages of suppression of control passive transfer.

∥ Significant suppression as compared to positive controls (group A) P < 0.001.

ability of syninduced Ts is not blocked by treatment with DNP-LC membranes syngeneic to the Ts, but allosuppression is blocked by pretreatment of the Ts with DNP-LC membranes syngeneic to the TDH donor. Thus, it appears that i.v. injection of DNP-modified syngeneic LC leads to a polyclonal wave of Ts, separate members of which can suppress the response of TDH from various haplotypes. Allosuppression can be blocked by pretreatment of the Ts with DNP-LC membranes syngeneic to the immune TDH in question, but this treatment has no effect on the ability of the Ts to suppress TDH derived from the same strain as the Ts or third-party-derived TDH.

Allosuppression by Syninduced Ts Involves Recognition of Allogeneic DNP-modified H-2D-End Determinants. Experiments were done next to determine the nature of the determinants involved in blocking the allosuppressive function of syninduced Ts. A/J DNP-LC membranes were used to treat either CBA or BALB/c syninduced Ts. The allosuppressive ability of these Ts was then tested. The results (Fig. 8) show that CBA syninduced Ts suppress BALB/c TDH by 99% (group B) and that pretreatment of those Ts with A/J DNP-LC membranes reverses their allosuppressive ability to only 5.7% (group C). Thus, pretreatment of Ts with DNP-LC membranes compatible at the IC → D regions of the MHC with the allogeneic target TDH is sufficient for blockade of allosuppression. In the converse experiment, it can be seen that BALB/c syninduced Ts suppress passive transfer of CMI by CBA TDH by 80.1% (group E); however, pretreatment with A/J DNP-LC membranes has no effect on their allosuppressive action (group F, 94.0%). It should be restated that A/J DNP-LC membranes are sufficient for blocking the suppressive ability of BALB/c Ts on syngeneic
### Cells Transferred to Recipient Mice

| Experiment No. | Ts Cells/mouse | Membrane Pretreatment | TDH Cells/mouse | % Suppression ± SEM |
|----------------|----------------|-----------------------|-----------------|--------------------|
| 1              | 5 × 10^7 BALB/c None | BALB/c CBA | 5 × 10^7 | § |
|                |                  | DNP-BALB               | B               | § |
|                |                  | DNP-CBA               | C               | § |
|                |                  | None                  | D               | § |
|                |                  | DNP-BALB               | E               | § |
|                |                  | DNP-CBA               | F               | § |
| 2              | 5 × 10^7 BALB/c None | BALB/c CBA | 5 × 10^7 | § |
|                |                  | DNP-CBA               | G               | § |
|                |                  | None                  | H               | § |
|                |                  | DNP-CBA               | I               | § |
|                |                  | None                  | J               | § |
|                |                  | DNP-CBA               | K               | § |
|                |                  | DNP-CBA               | L               | § |

**Fig. 7.** Allosuppression is inhibited by treatment with DNP-LC membranes syngeneic to the target TDH.

* In experiment 1 (upper panel), BALB/c Ts were treated with BALB/c or DBA DNP-LC membrane preparations as described in Materials and Methods, and 5 × 10^7 cells were cotransferred with either 5 × 10^7 BALB/c TDH (open bar) or 5 × 10^7 CBA TDH (hatched bar) to the appropriate normal recipients. In experiment 2 (lower panel), BALB/c Ts were treated with CBA DNP-LC membrane preparations, and 5 × 10^7 cells were cotransferred with either 5 × 10^7 BALB/c TDH (open bar), 5 × 10^7 CBA TDH (hatched bar), or 5 × 10^7 C57BL/6 TDH (shaded bars) to the appropriate normal recipients.

‡ Average values of percentage of suppression of control passive transfer (four mice per group) ± SEM.

§ Significant suppression as compared to positive controls P < 0.001.

BALB/c TDH (Fig. 5, group C). Thus, pretreatment of syninduced Ts with DNP-LC membranes compatible at the K → IE loci of the MHC with the target TDH is not sufficient for blockage of allosuppression. Therefore, allosuppression, like syngeneic suppression, is directed against DNP-modified H-2D-end determinants.

**Relative Affinity of Syninduced Ts for DNP-modified Syngeneic and Allogeneic Determinants.** It was of interest to determine the relative affinities of blocking of Ts by syngeneic DNP-LC membranes on suppression of syngeneic TDH, and blocking by allogeneic DNP-LC membranes on allosuppression by Ts. To accomplish this, BALB/c syninduced Ts were treated with varying concentrations of either BALB/c or CBA DNP-LC membranes, washed, and then tested for their suppressive action on both BALB/c and CBA TDH (Fig. 9). In terms of syngeneic suppression, BALB/c Ts treated with 10^6 BALB/c DNP-LC membrane equivalents reversed their suppressive ability from 90.6% (group B) to only 15.1% (group C). Dilutions of 10^7 and 10^8 DNP-LC membrane equivalents were much less effective in blocking suppression, yielding
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**Fig. 8.** Allosuppression involves recognition of allogeneic DNP-modified H-2D-end determinants.

* CBA Tₜ (upper panel) and BALB/c Tₜ (lower panel) were treated with A/J DNP-LC membrane preparations as described in Materials and Methods. 5 × 10⁷ CBA Tₜ were cotransferred with 5 × 10⁷ BALB/c T_DH into normal BALB/c recipients (open bars) and 5 × 10⁷ BALB/c Tₜ were cotransferred with 5 × 10⁷ CBA T_DH into normal CBA recipients (hatched bars).

**Table:**

| Cells Transferred to Recipient Mice | Membrane Pretreatment | T_DH | Δ Ear Swelling (x10⁻⁴ in) ± SEM  |
|------------------------------------|-----------------------|------|---------------------------------|
| 5x10⁷ CBA                         | None                  | B    | (97.0) § II                      |
|                                   | DNPA/J                | C    | (5.7)                           |
| --                                | 5x10⁷ CBA             | D    |                                 |
| 5x10⁷ BALB/c                      | None                  | E    | (80.1) § II                     |
|                                   | DNPA/J                | F    | (94.0) § II                     |

46.9% (group D) and 84.7% (group E) suppression, respectively. Thus, at least 10⁸ DNP-membrane equivalents are required to reverse suppression of syngeneic T_DH. In contrast, treatment of these same Tₜ with from 10⁶ to 10⁸ CBA DNP-LC membrane equivalents was sufficient to reverse their allosuppressive action on CBA T_DH from 96.9% (group G) to insignificant levels (groups H–J). These data indicate that the receptor on the clone(s) of Tₜ able to recognize DNP-modified allogeneic determinants is of much higher affinity than is receptor recognition of DNP-modified syngeneic determinants (toward which the Tₜ population was generated).

**Discussion**

This report has examined the nature of the suppressive interaction of DNP-specific syninduced Tₜ (induced by the i.v. injection of syngeneic DNP-LC) on the passive transfer of DNFB contact sensitivity by syngeneic and allogeneic immune T_DH. The results show that the syngeneic suppressive function of Tₜ is effectively blocked only when the Tₜ are pretreated with syngeneic, soluble DNP-LC membrane preparations, not by monomeric DNP-lysine or polyvalent DNP-protein conjugates. The blocking by hapten-modified membranes is also hapten-specific as TNP-modified syngeneic
Cells Transferred to Recipient Mice

| Cells/mouse | Membrane Pretreatment* | TDH (cells/mouse) | Δ Ear Swelling (x10^-4 in) ± SEM
|--------------|------------------------|-------------------|---------------------|
|             | 5x10⁷ BALB/c           |                   |                     |
| 5x10⁷ BALB/c None | 5x10⁷ BALB/c           |                   |                     |
| 5x10⁷ DNP-BALB | 5x10⁷ BALB/c           |                   |                     |
| 5x10⁷ DNP-CBA | 5x10⁷ BALB/c           |                   |                     |
| 5x10⁷ CBA    | 5x10⁷ CBA              |                   |                     |

Fig. 9. Relative affinity of syninduced Ts for DNP-modified syngeneic and allogeic determinants.

* BALB/c Ts were treated with varying concentrations of BALB/c DNP-LC membrane preparations and 5 x 10⁷ cells were cotransferred with 5 x 10⁷ syngeneic BALB/c TDH into normal BALB/c recipients (upper panel, open bars). In the lower panel, BALB/c Ts were treated with varying concentrations of CBA DNP-LC membrane preparations and 5 x 10⁷ cells cotransferred with allogeic CBA TDH into normal CBA recipients (hatched bars).

+ Values represent mean 24-h ear swelling in recipient mice (four per group) ± SEM. Δ, relative change in.
§ Numbers in parentheses represent the percentages of suppression of control passive transfer.
|$P < 0.001.
membranes do not block DNP-specific Ts. In terms of MHC requirements for blocking the suppressive action of Ts on syngeneic TDH, it was found that DNP-LC membranes which shared only the H-2D region with the Ts were sufficient and necessary for inhibiting Ts function, a restriction we had earlier reported for the induction of syninduced Ts by DNP-LC (11).

The failure of DNP-lysine, DNP-protein, and DNP-allogeic membrane to block suppression of syngeneic TDH by Ts is a strong indication that the MHC-unrestricted nature of the Ts cannot be explained by the fact that the Ts recognize only hapten. Ts apparently recognize DNP in association with the correct membrane determinant, i.e., DNP-H-2D. The fact that the Ts receptor is directed against hapten-modified MHC-encoded determinants is somewhat at odds with earlier studies concerning carrier-specific suppression in antibody systems. Okumura et al. (13) have reported that keyhole limpet hemocyanin (KLH)-specific Ts could be bound to an antigen immunoabsorbant column and therefore were enriched by this procedure. Interestingly, they also showed that KLH-specific helper T cells, run under identical conditions, were not retained on these columns. More recently, Taniguchi and Miller (14)
reported that human gamma globulin-specific $T_s$ could be enriched by adherence to antigen-coated Petri dishes. The current observations indicate that DNP-specific $T_s$ see antigen in the context of MHC gene products. A trivial explanation of these observations could be that the affinity of binding of $T_s$ to multideterminant antigen-coated beads or Petri dishes may be sufficient for their retention, whereas, our attempts to inhibit $T_s$ function in cell suspensions by treatment with DNP-lysine or DNP-proteins did not provide a sufficiently stable binding for functional inhibition. A more likely possibility is that the real antigen formed in contact sensitivity to DNFB is a DNP-conjugated, self-membrane component. As $T_s$ are also raised by immunization with DNP-syngeneic membranes, it is likely that they recognize, and are blocked by, DNP-membrane components and not by DNP on lysine, serum protein, or allogeneic membrane. Indeed, the fact that syngeneic DNP-membrane preparations were avid blockers of $T_s$ function (in suspension) reflects this type of receptor specificity of the $T_s$. It should also be noted that the phenotype of DNP-specific syninduced $T_s$ is Thy 1$^+$ (7), Lyt$^{-}2^+3^+$, I-J$^+$ (S. D. Miller, unpublished data) similar to the $T_s$ in the above-mentioned, carrier-specific $T_s$ systems. Thus, differences due to a phenotypically different effector $T_s$ in the systems does not appear likely.

We also investigated the mechanisms of allosuppression by syninduced $T_s$, because we have previously reported that syninduced $T_s$ are not genetically restricted and will suppress DNP-specific, allogeneic $T_DH$ upon cotransfer into the appropriate allogeneic recipient (8). The data reported here show that although pretreatment of syninduced $T_s$ with syngeneic DNP-LC membranes will block their suppressive action on syngeneic $T_DH$, these $T_s$ are still fully capable of suppressing allogeneic $T_DH$. This result again indicates that it is unlikely that allosuppression is directed toward hapten alone or directed against DNP-modified, public MHC determinants. That the allosuppressive action of syninduced $T_s$ is specific was shown by the fact that suppression directed against a specific allogeneic $T_DH$ could be inhibited only by pretreatment of the $T_s$ with DNP-LC membranes that were H-2D-end compatible with the target allogeneic $T_DH$ cells. Therefore, allosuppression appears to be specifically directed against the DNP-modified allogeneic H-2D-end determinants.

It appears that, after perturbation of the immune system by i.v. injected, DNP-modified, syngeneic LC, a polyclonal wave of $T_s$ is invoked. Some members of this set recognize DNP-modified syngeneic determinants with high affinity and syngeneic suppression can thus be inhibited by pretreatment of the $T_s$ with those DNP determinants. Other members of this set, once induced, display receptors directed against DNP-modified allogeneic determinants and thus suppress the passive transfer of CMI by allogeneic $T_DH$. These allosuppressive clones can be specifically inhibited by pretreatment with the correct allogeneic DNP-modified MHC-encoded determinants, without inhibiting the ability of the $T_s$ population to suppress syngeneic or third-party $T_DH$. T cells with receptors for autologous MHC products associated with antigen (hapten or virus) have been shown to cross-react extensively. The elegant experiments of Lemonnier et al. (15) and Burakoff et al. (16), using cytotoxic T cells, illustrate this point. They showed that cytotoxic T cells generated against allogeneic determinants can specifically lyse autologous cells coupled with TNP molecules (15). More recent work has shown that cytotoxic T cells stimulated by Sendai virus-modified syngeneic cells can lyse both syngeneic virus-coated targets as well as noninfected allogeneic cells (16). Thus, it is not unlikely that a set of $T_s$ stimulated in
The ability of haptenated membranes to block Ts is raised by DNP-BALB/c membranes. The table below summarizes the receptors for different determinants:

| Receptor Affinity | DNP-BALB | DNP-CBA | DNP-C57 |
|-------------------|----------|---------|---------|
| low               | -        | -       | -       |
| mod.              | +        | -       | -       |
| high              | -        | +       | -       |

The data can also be explained according to a dual receptor hypothesis of T-cell-antigen recognition similar to that advanced by Janeway et al. (17) and to a similar hypothesis outlined by Doherty et al. (18). We have previously invoked this model several years ago to interpret our results (10). According to this model, the i.v. injection of DNP-BALB/c LC into BALB/c mice activates a library of pre-Ts clones, perhaps independent of macrophage presentation (19, 20), each with low-to-moderate affinity receptors for DNP-BALB/c self-determinants. These clones are designated Ts-A, Ts-B, and Ts-C, for example (Fig. 10). Each has receptor No. 1 directed to DNP and VH products in receptor No. 2 that recognize self (BALB/c) either with moderate affinity (Ts-A) or with low affinity (Ts-B, Ts-C, etc.). The aggregate of all these Ts with affinity for BALB and DNP, or perhaps only those with moderate affinity for BALB and a receptor for DNP, is sufficient to suppress syngeneic BALB/c TDH. However, one of these clones has a receptor No. 2 that fortuitously cross-reacts with the CBA MHC with high affinity (Ts-B). The activation of this clone by i.v. injected BALB/c membrane will thus generate what might be called a heteroclitic Ts, which is efficient in suppressing CBA-immune TDH.

In the blocking experiments, it is postulated that only Ts with an affinity for an MHC of moderate strength will be blocked by DNP-membranes, at least under the
SUPPRESSOR T-CELL MECHANISMS IN CONTACT SENSITIVITY

conditions used in these experiments. Thus, DNP modified-BALB/c LC membranes will block T_{s-A} and inhibit the ability of the syninduced T_s to suppress BALB/c TDH. However, as T_{s-B} and T_{s-C} have only low affinity for BALB/c, the ability of these T_s within the aggregate of syninduced T_s to block CBA TDH (T_s-B) and C57Bl/6 TDH (T_s-C) is unimpaired. Treatment of the T_s population with CBA DNP-LC membranes would inhibit only clone T_{s-B} (with a high-affinity receptor for DNP-CBA) and leave intact the ability of the T_{s-A} clone to suppress BALB/c TDH. This would explain the genetic restrictions of blocking of syngeneic suppression. In terms of allosuppression, pretreatment of BALB/c syninduced T_s with syngeneic BALB/c DNP-LC membranes would efficiently block only those clones with moderate affinity for self (T_{s-A}), but may not efficiently block clone T_{s-B} (with low affinity for self, but high affinity for DNP-H-2^D) or T_{s-C} (high affinity for DNP-H-2^K); thus, T_s could still suppress CBA and C57Bl/6 TDH. As explained above, pretreatment of BALB/c syninduced T_s with CBA DNP-LC membranes would only block those clones with high-affinity receptors for DNP-H-2^K (T_{s-B}), leaving the suppressive activity for self (by the aggregate of moderate-affinity T_{s-A}) and for third party TDH in this system bear surface DNP associated with H-2K and H-2D determinants with high affinity. A prediction of this model would be that allosuppressive clones would be fewer in number and/or have higher-affinity receptors for allogeneic DNP determinants. The results presented in Fig. 9, that show that blocking of suppression of syngeneic TDH requires greater concentrations of DNP-LC membranes than blocking of suppression of allogeneic TDH, would seem to support this hypothesis.

The mechanism of efferent limb blockade by syninduced T_s is not clear at this time. T_s may act by competing for the immunogenic form of DNP-self presented by stimulator macrophages at the each challenge site. Alternatively, T_s may act on the immune TDH cell by direct contact or via a soluble suppressor factor mechanism (21, 22). In terms of the latter possibility, it has been shown (J. W. Moorhead, unpublished data) that TDH in this system bear surface DNP associated with H-2K and H-2D determinants. One could envision the recognition of this DNP-H-2D, TDH surface complex by T_s that either directly inactivate the TDH or liberate an antigen-specific suppressor factor. Experiments to define the exact locus of suppression by syninduced T_s are currently in progress.

Summary

This report has examined the mechanisms by which major histocompatibility complex (MHC) non-restricted suppressor T cells (T_s), induced by the i.v. injection of 2,4-dinitrophenyl (DNP)-modified, syngeneic lymphoid cells (DNP-LC), suppress the passive transfer of contact sensitivity mediated by syngeneic and allogeneic immune delayed hypersensitivity T cells (TDH). In terms of suppression of syngeneic TDH, it was found that the suppressive action of the T_s was only blocked by pretreatment with soluble syngeneic DNP-LC membrane preparations. Monomeric DNP-lysine, polymeric DNP-protein conjugates, and syngeneic TNP-LC membranes did not inhibit T_s function. Further experiments showed that inhibition of syngeneic suppression could be achieved by DNP-modified-membrane preparations that were only H-2D-region compatible with the T_s donor. Thus, T_s antigen receptors in this system specifically recognize DNP-modified H-2D-region determinants.

In contrast, it was found that pretreatment of syninduced T_s with syngeneic DNP-
LC membranes did not inhibit the ability to suppress allogeneic T\textsubscript{DH}. However, pretreatment of T\textsubscript{s} with DNP-allogeneic membranes which were H-2D-end compatible to the allogeneic target T\textsubscript{DH} eliminated their ability to suppress the specific allogeneic T\textsubscript{DH}, leaving intact suppression of syngeneic or third party T\textsubscript{DH}. It is proposed that perturbation of the immune system by i.v. injection of syngeneic DNP-LC leads to the induction of a polyclonal wave of DNP-specific T\textsubscript{s} activity. Some members of this set of T\textsubscript{s} recognize DNP-self MHC determinants with moderate affinity and are thus specifically inhibited after pretreatment with those DNP-self determinants. Other members of this set display receptors which cross-react with high affinity with DNP-allogeneic determinants and thus suppress allogeneic T\textsubscript{DH} cells. These allosuppressive clones can thus be specifically inhibited only by pretreatment with DNP-LC membranes, MHC-compatible with the target T\textsubscript{DH}. The data are discussed in terms of current models of T-cell cross-reactivity and T-cell-receptor recognition.

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