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)[1] 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 (T_s) activity (7).

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

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[1] *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, *N*-ε-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; T_{DH}, delayed hypersensitivity T cells; T_s, suppressor T cells; TNP-LC, trinitrophenyl-modified lymphoid cells.

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(afferent limb) (8). 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 allogeneic, 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*-ε-DNP-L-lysine HCl (DNP-lysine) were obtained from Sigma Chemical 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_6-BSA, DNP_{26}-OVA, and DNP_{15}-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 trinitrophenylated by incubating equal volumes of spleen cells at 20×10^6/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°C in dry ice-acetone and thawing at 37°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 T_s. Mice were injected i.v. with 5×10^7 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 T_s consisted of lymph node cell suspensions from mice injected with sham-modified lymphoid cells (LC).

Induction of DNFB-immune T_{DH}. DNFB-immune T_{DH} 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-2^d (B.10 αB10.D2) or anti-H-2^k (B.10 αB10.BR) serum. 4×10^8 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 T_s. T_s to be tested were treated at a concentration of 10^8 cells/ml of DNP-lysine (100 μg/ml); DNP_6-BSA (500 μg/ml); DNP_{26}-OVA (500 μg/ml); DNP_{15}-MGG (500 μg/ml); or DNP-LC membranes (10^8 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.

T_s Assay: Efferent Blockade. T_s were assayed for suppressive ability by testing their effect on passive transfer of contact sensitivity by immune T_{DH}. 5×10^7 T_s (either normal or treated with DNP-congeners) were mixed with 5×10^7 T_{DH} and immediately transferred by i.v. injection to normal recipients syngeneic to the T_{DH} donor. Recipients were ear challenged with 20 μl of 0.2% DNFB 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 T_{DH} and T_s (experimental) with those receiving T_{DH} and sham T_s (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 T_s recognize by determining their ability to be blocked by various DNP-congeners (Fig. 1). To accomplish this, T_s were treated in suspension with the various DNP-congeners, washed, and 5×10^7 cells were cotransferred to normal, syngeneic recipients along with 5×10^7 T_{DH}. Recipients were ear challenged within 1 h of transfer and the degree of passive transfer determined 24 h later by measuring ear-swelling responses. Lack of ear swelling in the recipient mouse indicates suppression by active T_s. In contrast, a positive ear-swelling response in the recipient mouse indicates that T_s were blocked by the preincubation and are thus incapable of inhibiting the expression of CMI by the transferred T_{DH} cells.

The Ability of Various DNP-Congeners to Block the Suppressive Action of T_s. Initial experiments were designed to ask the question: what form of DNP is able to inhibit active suppression by T_s? To accomplish this, a pool of BALB/c T_s were treated with the indicated concentrations of DNP-congeners, washed extensively, and cotransferred to normal BALB/c recipients along with BALB/c T_{DH}. 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, T_s pre-incubated with sham-modified,

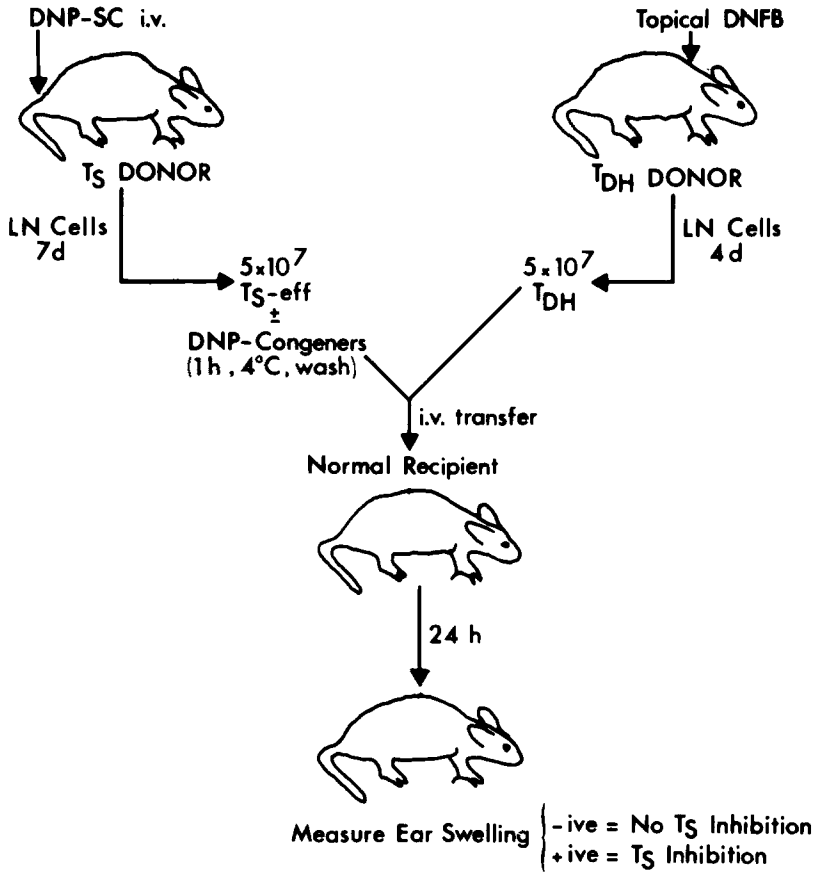

FIG. 1. Experimental protocol. DNP-SC, dinitrophenylated spleen cells; eff, efferent; LN, lymph node; +ive, positive; −ive, negative.

syngeneic BALB/c membrane preparations still suppressed passive transfer an average of 85.0% (group A). Pretreatment of T_s with 100 μg/ml of monomeric DNP-lysine (group B), or with 500 μg/ml of various DNP-protein preparations (DNP_6-BSA [group C], DNP_{26}-OVA [group D], and DNP_{15}-MGG [group E]) had no significant effect on the ability of T_s to suppress the expression of CMI by T_{DH}. However, pretreatment of the T_s 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 T_s activity. T_s apparently recognize DNP associated with membrane determinants. It should be pointed out that concentrations of DNP_{26}-OVA up to 2.0 mg/ml had no effect on T_s activity, and that concentrations of DNP-lysine >100 μg/ml were toxic.

Hapten and MHC Specificity of T_s Blocking by Hapten-modified Membrane Preparations. Having established the efficiency of T_s blocking by syngeneic DNP-membrane preparations, it was next of interest to ask what the hapten and MHC specificities were. BALB/c syninduced T_s were thus treated with 10^8 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

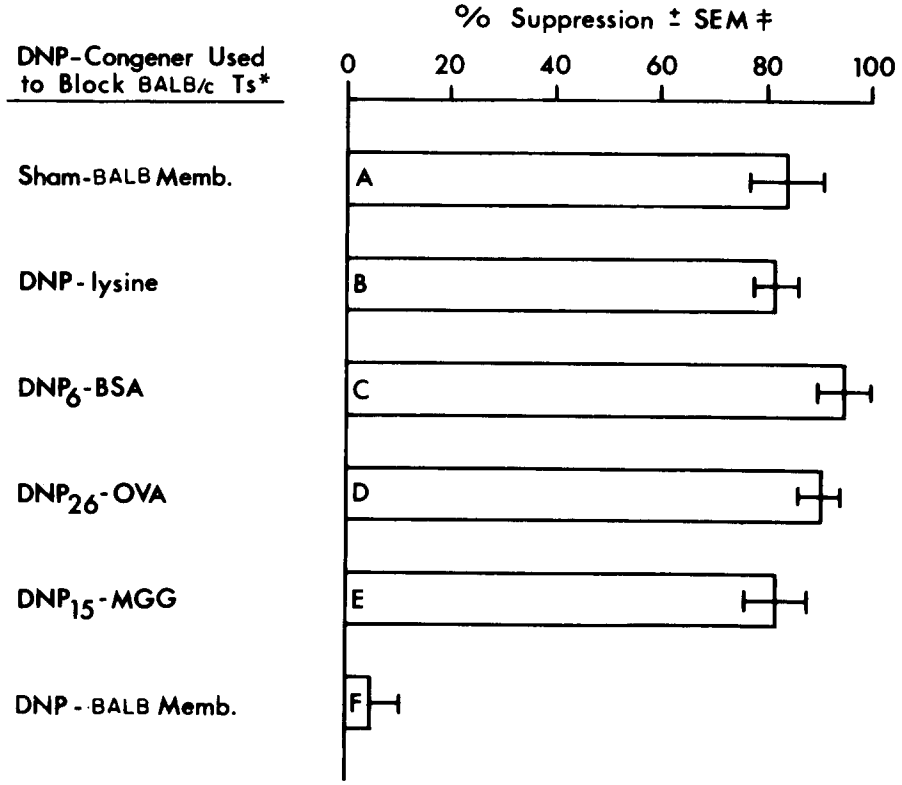

FIG. 2. The ability of various DNP-congeners to block the suppressive activity of syninduced T_s.

* BALB/c T_s were treated with the indicated DNP-congeners as described in Materials and Methods and 5×10^7 cells cotransferred with 5×10^7 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, 5×10^7 treated T_s were mixed with 5×10^7 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

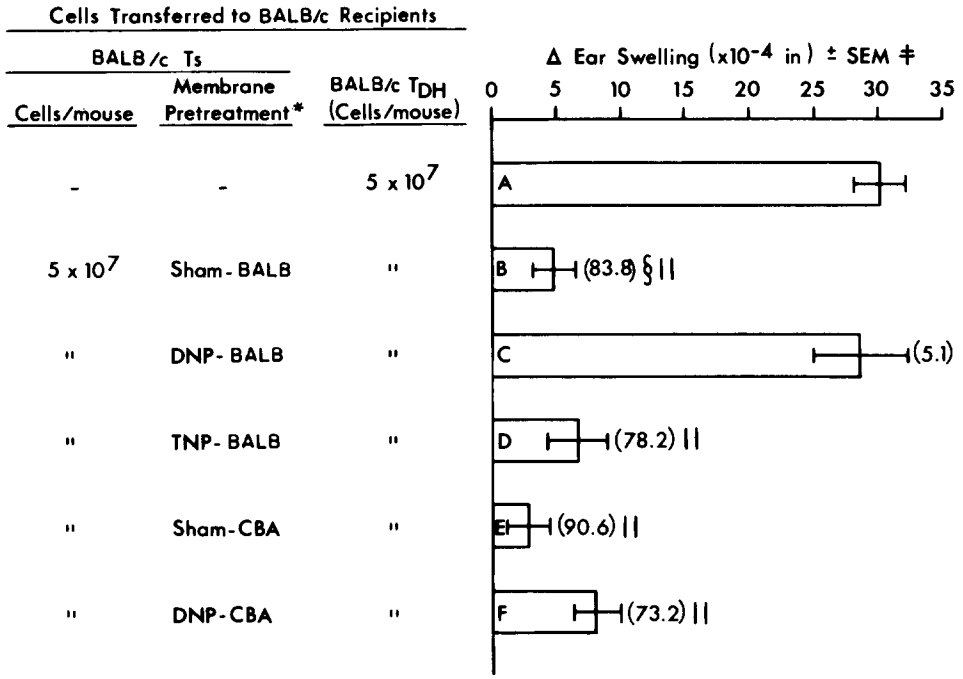

FIG. 3. Hapten and MHC specificity of T_s inhibition by hapten-modified LC membranes.

* BALB/c T_s were treated with the indicated membrane preparations as described in Materials and Methods and 5×10^7 cells cotransferred with 5×10^7 BALB/c T_{DH} 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-2^d or anti-H-2^k affinity columns. The materials not adherent to the columns were collected, concentrated, and tested for their ability to block BALB/c syninduced T_s (Fig. 4). 5×10^7 untreated BALB/c T_s suppressed the passive transfer by 5×10^7 BALB/c T_{DH} by 87.2% (group B). Treatment of T_s with the material recovered after passage of BALB/c DNP-LC membranes over an irrelevant anti-H-2^k 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-2^d column rendered the membranes unable to block the T_s (group D, 75.6%). Thus, T_s 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 T_s. Experiments were done to define the DNP-modified MHC-encoded membrane determinants responsible for blocking the suppressive action of syninduced T_s. Initial experiments examined the blocking effects of A/J DNP-LC membranes on BALB/c and CBA syninduced T_s (Fig. 5). Untreated, BALB/c syninduced T_s suppressed the response of syngeneic BALB/c T_{DH} 96.8% when transferred to BALB/c recipients (group B). Treatment of these T_s with A/J DNP-LC membranes (compatible at the IC → D regions) completely reversed their suppressive action (group C). Also, CBA syninduced T_s suppressed the response of syngeneic CBA T_{DH}

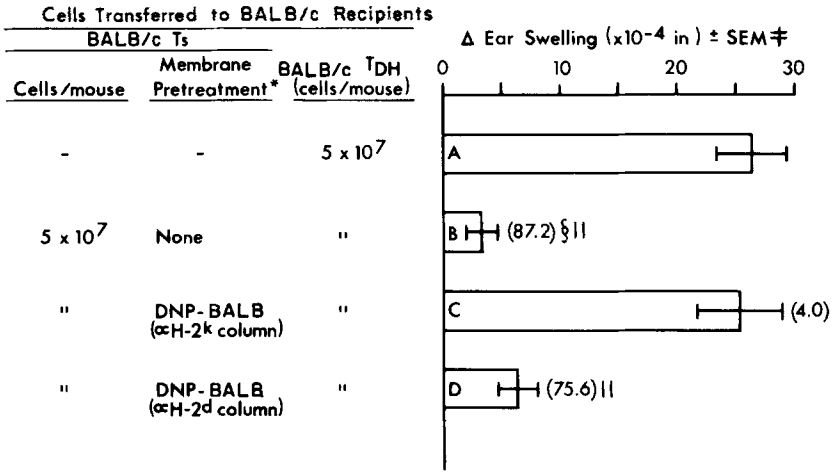

FIG. 4. Inhibition of T_s function requires DNP-modified MHC determinants.

* BALB/c T_s were treated with BALB/c DNP-LC membrane eluates from either anti-H-2^k or anti-H-2^d affinity columns and 5×10^7 cells cotransferred with 5×10^7 BALB/c T_{DH} 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) $P < 0.001$.

88.6% when transferred to CBA recipients (group E). However, pretreatment of CBA T_s 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 T_s 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 T_s blocking, both DBA/2 (H-2^d)-derived P-815 membranes (which carry H-2K^d and H-2D^d antigens, but no detectable I-region antigens [D. C. Shreffler, personal communication]) and A.TH DNP-LC membranes (which share only the H-2D^d region) were tested for their ability to block BALB/c (H-2^d) syninduced T_s (Fig. 6). As shown previously, untreated BALB/c T_s suppressed the T_{DH} 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 T_s (groups D and E). These data indicate that T_s recognize DNP-H-2D-region determinants, as H-2D-region compatibility between syninduced T_s and the DNP-LC membrane preparation is required for blocking of suppression.

Allosuppression by Syninduced T_s is Not Blocked by Pretreatment with DNP-LC Membranes Syngeneic to the T_s. The data to this point clearly show that pretreatment of syninduced T_s with DNP-LC membranes compatible at the H-2D region block the ability of the T_s to suppress syngeneic T_{DH}. As we have previously shown that syninduced T_s are not genetically restricted and will suppress allogeneic T_{DH} 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

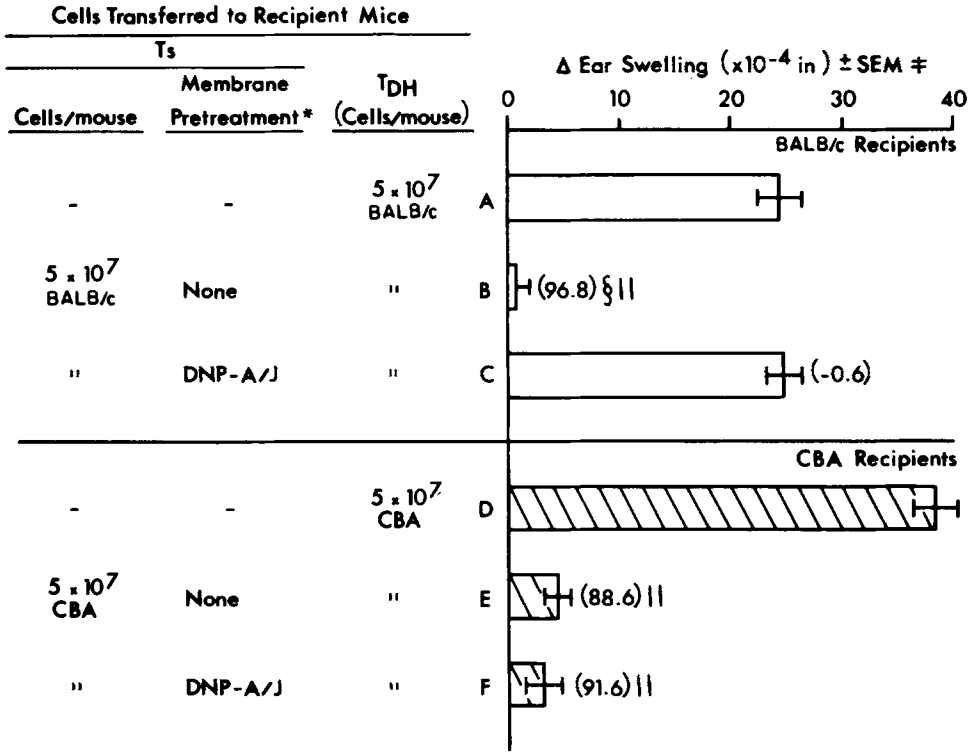

FIG. 5. Inhibition of syngeneic suppression requires right-end MHC identity.

* BALB/c and CBA T_s were treated with A/J DNP-LC membrane preparations as described in Materials and Methods. 5×10^7 BALB/c T_s were cotransferred with 5×10^7 BALB/c T_{DH} to normal BALB/c recipients (upper panel, open bars) and 5×10^7 CBA T_s were cotransferred with 5×10^7 CBA T_{DH} 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 T_s were pretreated with either DNP-BALB/c or DNP-CBA LC membranes and cotransferred with BALB/c or CBA T_{DH} into normal recipients syngeneic to the T_{DH} donor. Untreated BALB/c T_s suppressed the passive transfer of CMI by either BALB/c (group A) or CBA (group D) T_{DH}. As shown previously, BALB/c T_s treated with DNP-modified BALB/c LC membranes could no longer suppress BALB/c T_{DH} (group B), but they still retained their allosuppressive ability for CBA T_{DH} (group E). BALB/c T_s pretreated with allogeneic DNP-modified CBA LC membranes could still suppress syngeneic BALB/c T_{DH} (group C and Fig. 3), but were no longer able to suppress allogeneic CBA T_{DH} (group F). In experiment two, BALB/c T_s pretreated with CBA DNP-LC membranes were tested for their ability to suppress passive transfer of sensitivity by BALB/c, CBA, and C57Bl/6 T_{DH}. As expected, untreated BALB/c T_s suppressed passive transfer mediated by T_{DH} of each haplotype (groups G, I, and K). Those T_s pretreated with CBA DNP-LC membranes suppressed BALB/c (group H) and third-party C57Bl/6 (group L) T_{DH}, but could no longer suppress CBA T_{DH} (group J). These data indicate that the allosuppressive

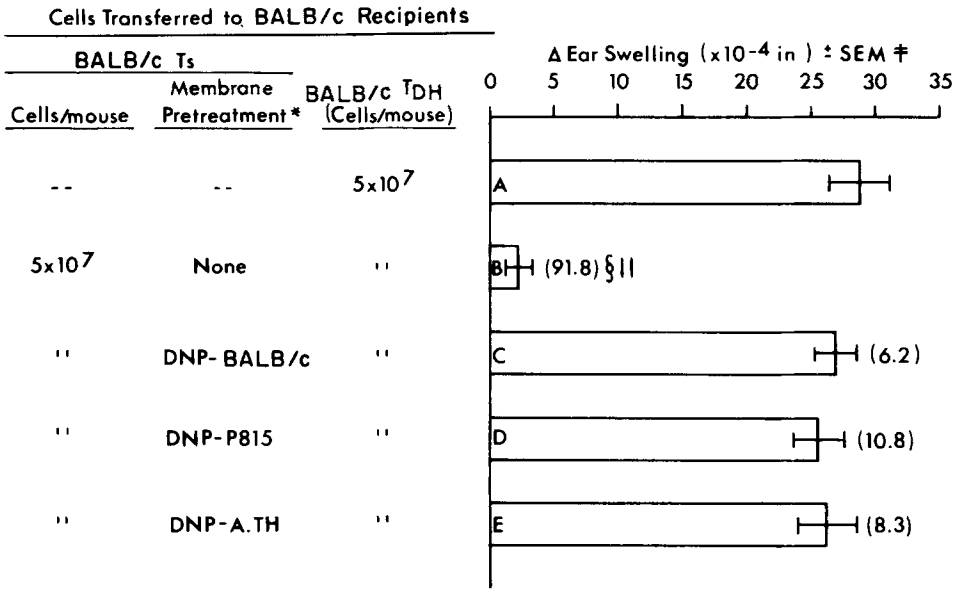

FIG. 6. Inhibition of syngeneic suppression maps to the H-2D Region of the MHC.

* BALB/c T_s were treated with the indicated DNP-LC membrane preparations as described in Materials and Methods and 5×10^7 cells cotransferred with 5×10^7 BALB/c T_{DH} 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 T_s is not blocked by treatment with DNP-LC membranes syngeneic to the T_s, but allosuppression is blocked by pretreatment of the T_s with DNP-LC membranes syngeneic to the T_{DH} donor. Thus, it appears that i.v. injection of DNP-modified syngeneic LC leads to a polyclonal wave of T_s, separate members of which can suppress the response of T_{DH} from various haplotypes. Allosuppression can be blocked by pretreatment of the T_s with DNP-LC membranes syngeneic to the immune T_{DH} in question, but this treatment has no effect on the ability of the T_s to suppress T_{DH} derived from the same strain as the T_s or third-party-derived T_{DH}.

Allosuppression by Syninduced T_s 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 T_s. A/J DNP-LC membranes were used to treat either CBA or BALB/c syninduced T_s. The allosuppressive ability of these T_s was then tested. The results (Fig. 8) show that CBA syninduced T_s suppress BALB/c T_{DH} by 99% (group B) and that pretreatment of those T_s with A/J DNP-LC membranes reverses their allosuppressive ability to only 5.7% (group C). Thus, pretreatment of T_s with DNP-LC membranes compatible at the IC → D regions of the MHC with the allogeneic target T_{DH} is sufficient for blockade of allosuppression. In the converse experiment, it can be seen that BALB/c syninduced T_s suppress passive transfer of CMI by CBA T_{DH} 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 T_s on syngeneic

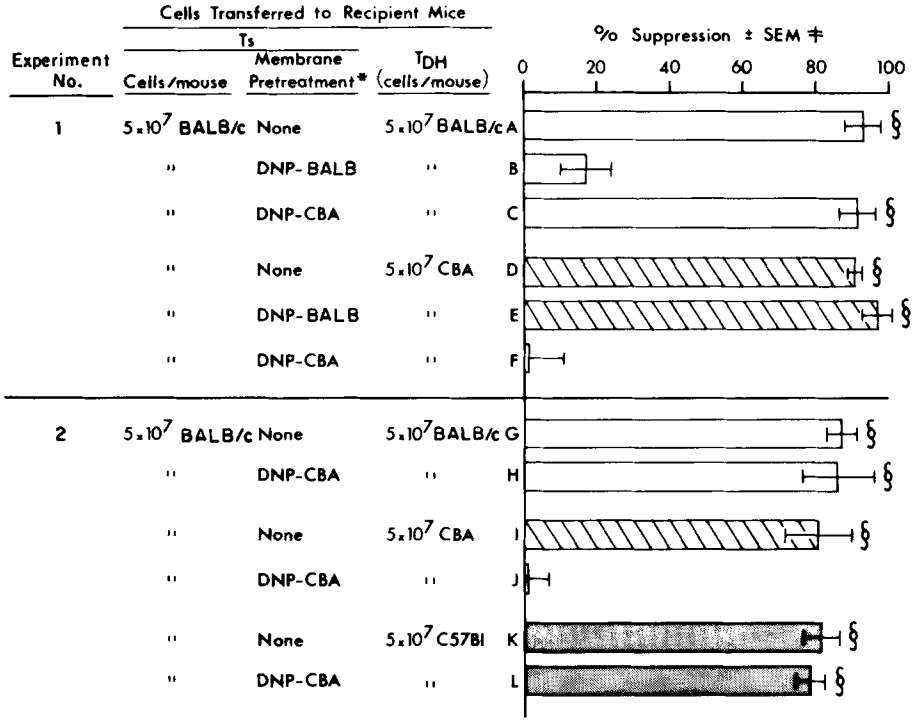

FIG. 7. Allosuppression is inhibited by treatment with DNP-LC membranes syngeneic to the target T_{DH}.

* In experiment 1 (upper panel), BALB/c T_s were treated with BALB/c or DBA DNP-LC membrane preparations as described in Materials and Methods, and 5×10^7 cells were cotransfered with either 5×10^7 BALB/c T_{DH} (open bars) or 5×10^7 CBA T_{DH} (hatched bars) to the appropriate normal recipients. In experiment 2 (lower panel), BALB/c T_s were treated with CBA DNP-LC membrane preparations, and 5×10^7 cells were cotransferred with either 5×10^7 BALB/c T_{DH} (open bars), 5×10^7 CBA T_{DH} (hatched bars), or 5×10^7 C57BL/6 T_{DH} (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 T_{DH} (Fig. 5, group C). Thus, pretreatment of syninduced T_s with DNP-LC membranes compatible at the K → IE loci of the MHC with the target T_{DH} 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 T_s for DNP-modified Syngeneic and Allogeneic Determinants. It was of interest to determine the relative affinities of blocking of T_s by syngeneic DNP-LC membranes on suppression of syngeneic T_{DH}, and blocking by allogeneic DNP-LC membranes on allosuppression by T_s. To accomplish this, BALB/c syninduced T_s 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 T_{DH} (Fig. 9). In terms of syngeneic suppression, BALB/c T_s treated with 10^8 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 10^6 DNP-LC membrane equivalents were much less effective in blocking suppression, yielding

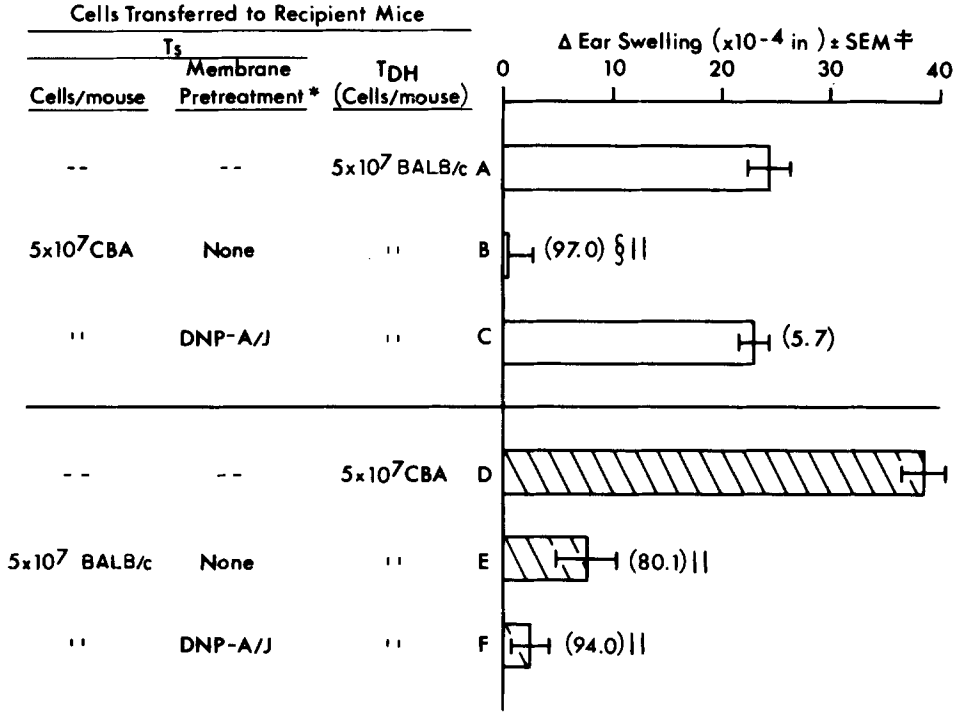

FIG. 8. Allosuppression involves recognition of allogeneic DNP-modified H-2D-end determinants.
* CBA T_s (upper panel) and BALB/c T_s (lower panel) were treated with A/J DNP-LC membrane preparations as described in Materials and Methods. 5×10^7 CBA T_s were cotransferred with 5×10^7 BALB/c T_{DH} into normal BALB/c recipients (open bars) and 5×10^7 BALB/c T_s were cotransferred with 5×10^7 CBA T_{DH} into normal CBA recipients (hatched bars).
‡ Values represent mean 24-h ear swelling in recipient mice (four per group) ± SEM.
§ Numbers in parentheses represent the percentages of suppression of control passive transfer.
‖ Significant suppression as compared to positive controls (groups A or D) $P < 0.001$.

46.9% (group D) and 84.7% (group E) suppression, respectively. Thus, at least 10^8 DNP-membrane equivalents are required to reverse suppression of syngeneic T_{DH}. In contrast, treatment of these same T_s with from 10^8 to 10^6 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 T_s able to recognize DNP-modified allogeneic determinants is of much higher affinity than is receptor recognition of DNP-modified syngeneic determinants (toward which the T_s population was generated).

Discussion

This report has examined the nature of the suppressive interaction of DNP-specific syninduced T_s (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 T_s is effectively blocked only when the T_s 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

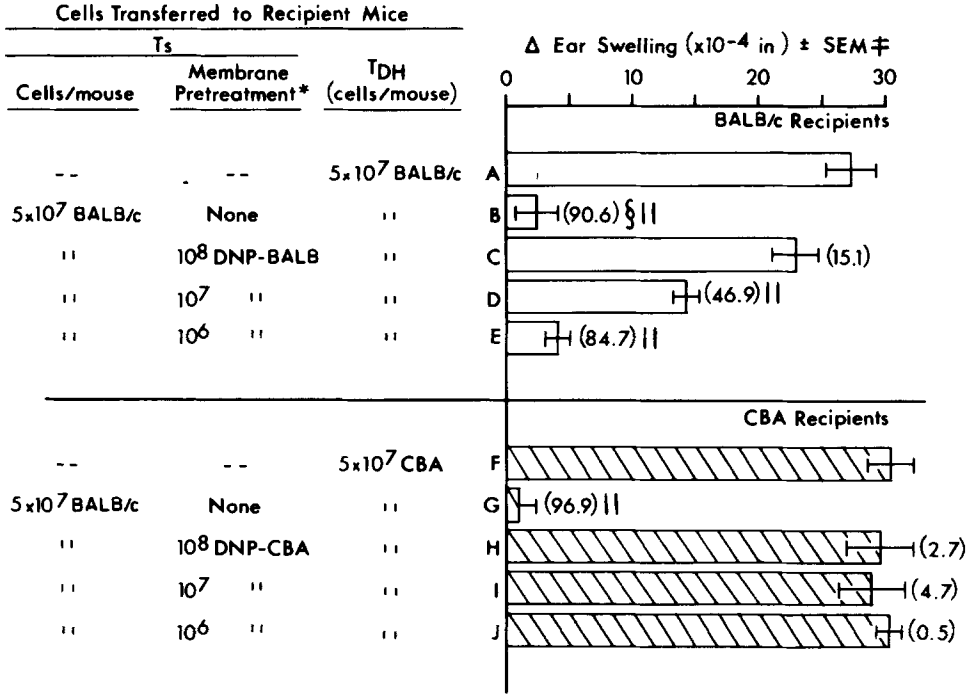

FIG. 9. Relative affinity of syninduced T_s for DNP-modified syngeneic and allogeneic determinants.

* BALB/c T_s were treated with varying concentrations of BALB/c DNP-LC membrane preparations and 5 × 10^7 cells were cotransferred with 5 × 10^7 syngeneic BALB/c T_{DH} into normal BALB/c recipients (upper panel, open bars). In the lower panel, BALB/c T_s were treated with varying concentrations of CBA DNP-LC membrane preparations and 5 × 10^7 cells cotransferred with allogeneic CBA T_{DH} 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.

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

membranes do not block DNP-specific T_s. In terms of MHC requirements for blocking the suppressive action of T_s on syngeneic T_{DH}, it was found that DNP-LC membranes which shared only the H-2D region with the T_s were sufficient and necessary for inhibiting T_s function, a restriction we had earlier reported for the induction of syninduced T_s by DNP-LC (11).

The failure of DNP-lysine, DNP-protein, and DNP-allogeneic membrane to block suppression of syngeneic T_{DH} by T_s is a strong indication that the MHC-unrestricted nature of the T_s cannot be explained by the fact that the T_s recognize only hapten. T_s apparently recognize DNP in association with the correct membrane determinant, i.e., DNP-H-2D. The fact that the T_s 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 T_s 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), $Lyl^-2^+3^+$, $I\text{-}J^+$ (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

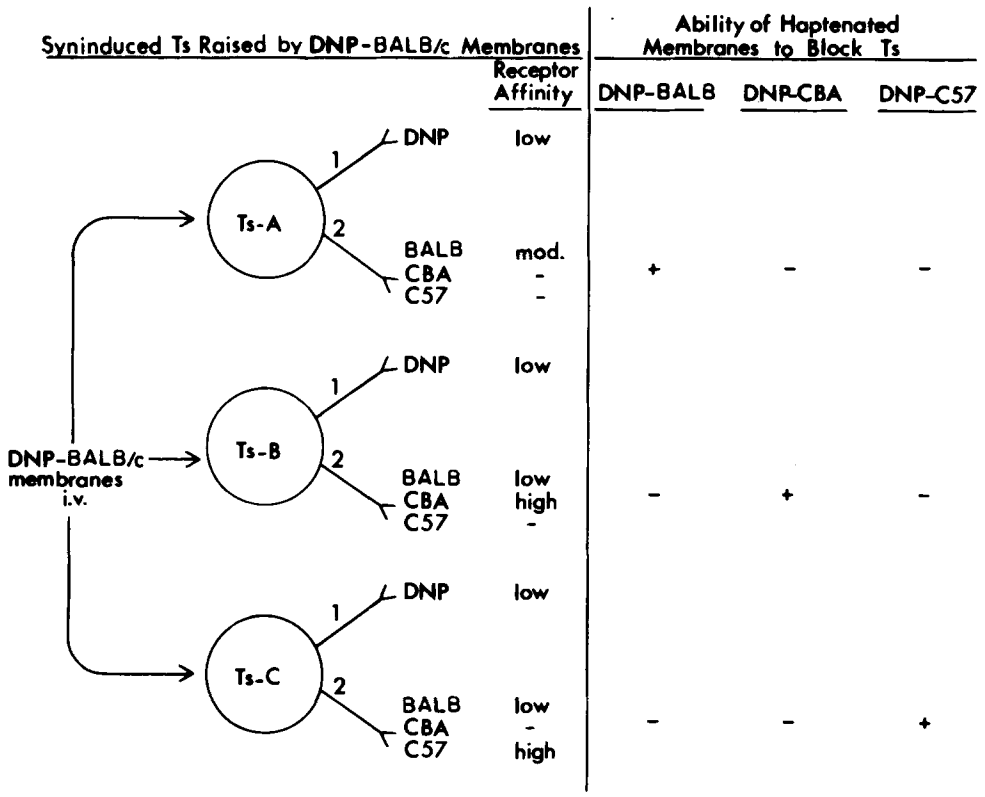

FIG. 10. Proposed model to explain the non-MHC restriction of syninduced T_s. mod., moderate.

vivo by DNP-modified syngeneic cells would be able to recognize both DNP-modified syngeneic and DNP-modified allogeneic determinants.

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-T_s 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 T_s-A, T_s-B, and T_s-C, for example (Fig. 10). Each has receptor No. 1 directed to DNP and V_H products in receptor No. 2 that recognize self (BALB/c) either with moderate affinity (T_s-A) or with low affinity (T_s-B, T_s-C, etc.). The aggregate of all these T_s 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 T_{DH}. However, one of these clones has a receptor No. 2 that fortuitously cross-reacts with the CBA MHC with high affinity (T_s-B). The activation of this clone by i.v. injected BALB/c membrane will thus generate what might be called a heteroclitic T_s which is efficient in suppressing CBA-immune T_{DH}.

In the blocking experiments, it is postulated that only T_s with an affinity for an MHC of moderate strength will be blocked by DNP-membranes, at least under the

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 T_{DH}. 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 T_{DH} (T_s-B) and C57Bl/6 T_{DH} (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 T_{DH}. 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^k) or T_s-C (high affinity for DNP-H-2^b); thus, T_s could still suppress CBA and C57Bl/6 T_{DH}. 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 T_{DH} (e.g., clones [T_s-C] that would cross-react with DNP-C57Bl/6 (H-2^b) 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 T_{DH} requires greater concentrations of DNP-LC membranes than blocking of suppression of allogeneic T_{DH}, 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 T_{DH} 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 T_{DH} in this system bear surface DNP associated with H-2K and H-2D determinants. One could envision the recognition of this DNP-H-2D, T_{DH} surface complex by T_s that either directly inactivate the T_{DH} 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 (T_{DH}). In terms of suppression of syngeneic T_{DH}, 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_{DH}. However, pretreatment of T_s with DNP-allogeneic membranes which were H-2D-end compatible to the allogeneic target T_{DH} eliminated their ability to suppress the specific allogeneic T_{DH}, leaving intact suppression of syngeneic or third party T_{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_s activity. Some members of this set of T_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_{DH} cells. These allosuppressive clones can thus be specifically inhibited only by pretreatment with DNP-LC membranes, MHC-compatible with the target T_{DH}. The data are discussed in terms of current models of T-cell cross-reactivity and T-cell-receptor recognition.

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References

1. Miller, S. D., R. P. Wetzig, and H. N. Claman. 1979. The induction of cell-mediated immunity and tolerance with protein antigens coupled to syngeneic lymphoid cells. *J. Exp. Med.* **149:**758.
2. Greene, M. I., M. Sugimoto, and B. Benacerraf. 1978. Mechanisms of regulation of cell-mediated immune responses. I. Effect of route of immunization with TNP coupled syngeneic cells on the induction and suppression of contact sensitivity to picryl chloride. *J. Immunol.* **120:**1604.
3. Bach, B.-A., L. Sherman, B. Benacerraf, and M. I. Greene. 1978. Mechanisms of regulation of cell-mediated immune responses. II. Induction and suppression of delayed type hypersensitivity to azobenzene-arsonate-coupled syngeneic cells. *J. Immunol.* **121:**1460.
4. Battisto, J. R., and B. R. Bloom. 1966. Dual immunological unresponsiveness induced by cell membrane coupled hapten or antigen. *Nature (Lond.).* **212:**156.
5. Miller, S. D., and H. N. Claman. 1976. The induction of hapten-specific T cell tolerance using hapten-modified lymphoid cells. I. Characteristics of tolerance induction. *J. Immunol.* **117:**1519.
6. Cheung, N. K., D. H. Scherr, K. M. Heghinian, B. Benacerraf, and M. E. Dorf. 1978. Immune suppression in vivo with antigen-modified syngeneic cells. I. T-cell-mediated suppression to the terpolymer poly-(glu,lys,phe)$_n$. *J. Exp. Med.* **148:**1539.
7. Miller, S. D., M. S. Sy, and H. N. Claman. 1977. The induction of hapten-specific T cell tolerance using hapten-modified lymphoid cells. II. Relative roles of suppressor T cells and clone inhibition in the tolerant state. *Eur. J. Immunol.* **7:**165.
8. Miller, S. D., M. S. Sy, and H. N. Claman. 1978. Suppressor cell mechanisms in contact sensitivity. I. Efferent blockade by syninduced suppressor T cells. *J. Immunol.* **121:**265.
9. Miller, S. D., M. S. Sy, and H. N. Claman. 1977. H-2 restriction of suppressor T-cell induction by hapten-modified lymphoid cells in tolerance to 1-fluoro-2,4-dinitrobenzene contact sensitization. *J. Exp. Med.* **145:**1071.
10. Claman, H. N., S. D. Miller, and M. S. Sy. 1977. Suppressor cells in tolerance to contact

sensitivity active against hapten-syngeneic and hapten-allogeneic determinants. *J. Exp. Med.* **146:**49.

11. Miller, S. D., M. S. Sy, and H. N. Claman. 1978. Genetic restrictions for the induction of suppressor T cells by hapten-modified lymphoid cells in tolerance to 1,fluoro-2,4-dinitrobenzene contact sensitivity: role of the *H-2D* region of the major histocompatibility complex. *J. Exp. Med.* **147:**788.
12. Little, J. R., and H. N. Eisen. 1967. Preparation of immunogenic 2,4-dinitrophenyl and 2,4,6-trinitrophenyl proteins. *Meth. Immunol. Immunochem.* **1:**128.
13. Okumura, K., T. Takemori, T. Tokuhisa, and T. Tada. 1977. Specific enrichment of the suppressor T cell bearing I–J determinants. Parallel functional and serological characterizations. *J. Exp. Med.* **146:**1234.
14. Taniguchi, M., and J. F. A. P. Miller. 1977. Enrichment of specific suppressor T cells and characterization of their surface markers. *J. Exp. Med.* **146:**1450.
15. Lemonnier, F., S. J. Burakoff, R. N. Germain, and B. Benacerraf. 1977. Cytotoxic T lymphocytes specific for allogeneic stimulator cells cross-react with chemically modified syngeneic cells. *Proc. Natl. Acad. Sci. U. S. A.* **74:**1229.
16. Burakoff, S. J., R. Finberg, L. Glimcher, F. Lemonnier, B. Benacerraf, and H. Cantor. 1978. The biologic significance of alloreactivity. The ontogeny of T-cell sets specific for alloantigens or modified self antigens. *J. Exp. Med.* **148:**1414.
17. Janeway, C. A., H. Wigzell, and H. Binz. 1976. Two different V_H gene products make up the T cell receptor. *Scand. J. Immunol.* **5:**993.
18. Doherty, P. C., D. Götze, G. Trinchieri, and R. M. Zinkernagel. 1976. Models for recognition of virally modified cells by immune thymus-derived lymphocytes. *Immunogenetics.* **3:**517.
19. Feldmann, M., and S. Kontiainen. 1976. Suppressor cell induction *in vitro*. II. Cellular requirements of suppressor cell induction. *Eur. J. Immunol.* **6:**302.
20. Pierres, M., and R. N. Germain. 1978. Antigen-specific T cell-mediated suppression. IV. Role of macrophages in generation of L-glutemic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)-specific suppressor T cells in responder mouse strains. *J. Immunol.* **121:**1306.
21. Moorhead, J. W. 1977. Soluble factors in tolerance and contact sensitivity to 2,4-dinitrofluorobenzene in mice. I. Suppression of contact sensitivity by soluble suppressor factor released *in vitro* by lymph node cell populations containing specific suppressor cells. *J. Immunol.* **119:**315.
22. Moorhead, J. W. 1977. Soluble factors in tolerance and contact sensitivity to 2,4-dinitrofluorobenzene in mice. II. Genetic requirements for suppression of contact sensitivity by soluble suppressor factor. *J. Immunol.* **119:**1773.