ANTIGEN RECEPTORS ON MURINE T LYMPHOCYTES IN CONTACT SENSITIVITY

I. Functional Inhibition of Effector T Cells by Monovalent 2,4-Dinitrophenol: Implication for a Two-Receptor Model*

By JOHN W. MOORHEAD‡

From the Departments of Microbiology and Immunology and of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Numerous studies have shown that activation of T cells requires recognition of antigen in association with certain gene products of the major histocompatibility complex (MHC).[1] This so-called MHC restriction or dual specificity has been shown for all functional T cell subsets (1–11). Two basic models have been proposed to explain these findings: a one-receptor or altered-self-model (12) and a two-receptor model (13, 14). The essentials of these models and their variations are well known (10, 11, 15–18). In the two-receptor model, it is important to note that the receptors may exist either as separate but linked entities or as two distinct subsites of a single complex receptor. A critical difference between the two theories is that the two-receptor model predicts that the T cell has specificity for both the antigen and self-MHC product, whereas the altered self-model predicts that the T cell has specificity for neither determinant alone. At the present time, neither proposal can be totally excluded.

Several studies exploring the interaction of T cells with antigen have attempted to block T cell functions in vitro by the addition of excess free antigen (19–26). In general, these studies have given negative results and have been used as indirect arguments to support the altered-self-hypothesis. Exactly what influence the in vitro conditions have on these negative findings is unknown. Ideally, one would like to treat T cells in vitro with antigen and then measure their functional capacity in vivo, under physiological conditions, away from any artifactual in vitro influence. Contact sensitivity (CS) to 2,4-dinitrofluorobenzene (DNFB) is a T cell-mediated response that lends itself to this type of experimentation. CS is easily transferred to naïve recipients with purified T cells from sensitized donors, and the response can be measured within 24 h of transfer (27).

Using this approach, I have investigated the effect of treating DNFB-immune T cells with various monovalent or multivalent 2,4-dinitrophenol (DNP) ligands. The

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C, rabbit complement; CS, contact sensitivity; DNBS, 2,4-dinitrobenzene sulfonate; DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenol; HBSS, Hanks' balanced salt solution; LN, lymph node; MGG, mouse gamma globulin; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; Ox, 4-ethoxy methylene-2-phenyl oxazolone; RGG, rabbit gamma globulin; SIS, suppressive immune serum; T_{DR}, effector T cells of the CS reaction; TNBS, 2,4,6-trinitrobenzene sulfonate; TNP, 2,4,6-trinitrophenol; Ts-aux, auxiliary suppressive T cells.

results show that treatment of the T cells with these ligands in vitro inhibits the transfer of CS to naïve recipients. The inhibition is hapten specific, requires divalent cations during the treatment period, and is not due to activation of suppressor cells. Furthermore, adsorption studies indicate that anti-idiotypic antibodies and the hapten DNP compete for the same site(s) on the immune T cells.

Materials and Methods

Mice. Female BALB/c mice were obtained from the AMC Cancer Research Center, Lakewood, Colo. Male CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Haptens, Hapten-Protein Conjugates, and Chemicals. ϵ-DNP-L-lysine and 2,4-6-trinitrobenzene sulfonate (TNBS) were purchsed from Sigma Chemical Co., St. Louis, Mo. DNFB and 2,4-dinitrobenzene sulfonate (DNBS) were obtained from Eastman Kodak, Rochester, N. Y. 4-Ethoxy methylene-2-phenyl oxazolone (Ox) was obtained from BDH Chemicals Ltd., Poole, Eng. The haptens DNBS and TNBS were coupled to bovine serum albumin (BSA, Mile' Laboratories, Elkhart, Ind.), rabbit gamma globulin (RGG, Miles Laboratories), or ammonium sulfate precipitated-, Sephadex G-200-purified mouse gamma globulin (MGG) according to the method of Little and Eisen (28) and yielded the following coupling ratios: DNP_{15}-BSA, trinitrophenol $(TNP)_{19}$-BSA, DNP_{29}-RGG, TNP_{24}-RGG, and DNP_{11}-MGG. The hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was coupled to MGG according to the method of Brownstone et al. (29) and yielded a coupling ratio of NIP_9-MGG. Hanks' balanced salt solution (HBSS) and Ca^{++}, Mg^{++}-free HBSS were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, N.Y.).

Sensitization with DNFB or Ox. To serve as donors of immune lymph node (LN) cells, mice were sensitized with DNFB by two daily paintings with 25 μl of 0.5% DNFB on the shaved abdomen and 5 μl on the footpads and ears. In some experiments mice were doubly sensitized with DNFB and Ox. This was done by painting the left abdomen, forepaw, and ear with DNFB (as above), and the right abdomen, forepaw, and ear with 50 μl and 5 μl, respectively, of 3% Ox.

Transfer of CS. 3 d after the last skin painting, single cell suspensions of draining LN were prepared and 4×10^7 to 5×10^7 cells injected intravenously into normal syngeneic recipients. All recipient groups contained at least four animals. The recipients and negative controls were challenged within 1 h after cell transfer by applying 20 μl of 0.2% DNFB or 20 μl of 1% Ox on the dorsal side of each ear. Increased ear swelling (δ) was measured 24 h later with an engineer's micrometer and expressed in units of 10^{-4} in.

Treatment of Immune LN Cells In Vitro with Hapten Ligands. Immune LN cells were suspended in RPMI 1640 medium (Gibco Laboratories) at 10^8 cells/ml. In some experiments, 1 mg/ml BSA was added to the medium. Replicate experiments showed that addition of BSA did not affect the results and it was not always used. Various concentrations of the hapten or the carrier were then added to the cell suspensions, and the mixture incubated for 1 h on ice unless otherwise indicated (see Results). The cells were then washed three times, resuspended in RPMI medium, and 4×10^7 to 5×10^7 cells injected intravenously into normal syngeneic recipients. Ear challenge and measurement of ear swelling were one as described. The effect of the hapten treatment was determined by its ability to inhibit the expression of CS by the tpansferred LN cells. The results are expressed as the percent inhibition of transfer compared to untreated or carrier-treated immune cells.

Antiserum. Anti-Ia^k serum was prepared by repeated injections of A.TH mice with spleen and LN cells from normal A.TL donors. The cytotoxicity and specificity of this antiserum has been previously described (27). Monoclonal anti-Lyt-2.2 antibodies were generously provided by Dr. Kappler and Dr. Marrack from National Jewish Hospital. This reagent was obtained from a hybridoma cell line produced by Dr. Paul Gottlieb (30). The cytotoxicity and specificity of this monoclonal antibody has been described in detail (30). For treatment of immune LN cells, 10^8 cells/ml were suspended in 1:10 diluted anti-Ia^k or 1:400 anti-Lyt-2.2 and incubated for 1 h at 4°C. This dilution of anti-Lyt-2.2 eliminates suppressor cell activity in LN cells from mice injected intravenously with DNP-coupled syngeneic spleen cells (not shown). After

washing, anti-Ia-treated cells were resuspended in 1:20 rabbit complement (C) and anti-Lyt-treated cells were resuspended in 1:6 guinea pig C and incubated for 10 min at 4°C and then 30 min at 37°C. The washed cells were then resuspended in RPMI medium at 10^8 cells/ml and treated with hapten.

Purification of T Cells. Lymph node T cells were purified on nylon wool according to the method of Julius et al. (31). 4–5 × 10^8 LN cells in 1.0 ml were washed into 1.5–2.0-g nylon wool columns with warm medium, and the columns were incubated at 37°C. After 20 min incubation, the cells were slowly moved down the column by allowing 0.5–1 ml of medium to pass through. The column was replaced at 37°C and after an additional 20 min incubation, the above procedure was repeated. After a total incubation period of 60 min at 37°C, nonadherent cells were collected by washing the column slowly with 25–30 ml of warm medium. The eluted cells were washed and resuspended at 10^8 cells/ml in RPMI medium. The nonadherent T cells were monitored for B cell contamination by immunofluorescent staining with fluorescein-labeled polyvalent rabbit anti-mouse Ig and for macrophage contamination by esterase staining. In the experiments reported here (Table III and VIII), the T cell populations contained a maximum of 1% B cells and <1% macrophages.

Results

Dose-dependent Inhibition by DNP-Lysine of Transfer of CS. To determine whether soluble hapten would interfere with transfer of CS, DNFB-immune LN cells were incubated for 1 h at 4°C with different concentrations of monovalent DNP-lysine. The cells were then washed to remove free hapten and 5 × 10^7 cells injected intravenously into each of four syngeneic recipients. These animals were then ear challenged, and ear swelling was measured 24 h later. Results of two experiments are given in Table I and show a dose-dependent inhibition by DNP-lysine of transfer of CS to DNFB. Similar inhibition curves have been obtained using DNP-glycine, DNP_{11}-MGG, DNP_{15}-BSA, and DNP_{29}-RGG (not shown).

Specificity of Hapten-mediated Inhibition of Transfer of Immunity. To test the specificity of inhibition, we compared various DNP-, TNP-, and NIP-protein conjugates. The treatment protocol was the same. Immune LN cells (10^8/ml) in RPMI medium were

TABLE I

Dose-dependent Inhibition by DNP-Lysine of Transfer of CS to DNFB

Group	Pretreatment of immune LN cells with DNP-lysine	Experiment I		Experiment II	
		Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
	μg/10^8 cells/ml	*× 10^{-4} in. ± SEM*	*%*	*× 10^{-4} in. ± SEM*	*%*
A	—	25.0 ± 2.8		33.6 ± 3.1	
B	0.1	19.9 ± 3.4	20	21.0 ± 3.0‡	38
C	1.0	9.9 ± 1.1§	60	9.4 ± 2.1§	72
D	10.0	0.2 ± 1.2§	99	2.3 ± 1.1§	93
E	100.0	1.1 ± 0.7§	96	2.0 ± 0.9§	94

BALB/c mice were sensitized with DNFB on days 0 and 1, and immune LN cells harvested on day 4. The cells were resuspended in RPMI 1640 medium at 10^8 cells/ml and treated with different doses of DNP-lysine for 1 h at 4°C. The cells were then washed three times and transferred to normal syngeneic recipients that were ear challenged with DNFB. Increased ear swelling was measured 24 h later as an index of transfer of CS.

* Ear swelling in recipients of immune LN cells minus ear swelling in negative controls (ear challenge only).

‡ $P < 0.05$.

§ $P < 0.001$.

treated for 1 h at 4°C by the addition of 1 μg/ml of the particular hapten-protein conjugate. A group of cells treated with 1 μg/ml DNP-lysine was included in all experiments for comparison. After washing, 5×10^7 cells were injected intravenously into naïve recipients that were then ear challenged, and ear swelling was measured 24 h later. Representative results of several replicate experiments are given in Fig. 1. In experiment I, pretreatment of the immune LN cells with either DNP-lysine or DNP_{11}-MGG inhibited the transfer of immunity by >60% (compare groups B and C with A), whereas treatment of the LN cells with NIP_9-MGG had no effect (compare groups D and A). Similar results were obtained in experiment II where DNP- and TNP-protein conjugates were compared. Pretreatment with DNP-lysine, DNP_{15}-BSA, or DNP_{29}-RGG inhibited transfer by 75% or more (compare groups B, C, and E with A). In contrast, treatment with TNP_{19}-BSA or TNP_{24}-RGG caused no significant inhibition (compare groups D and F with A).

To examine the specificity of inhibition in a more critical way, the following experiment was done. BALB/c mice were doubly sensitized with DNFB and Ox (see Materials and Methods). A single cell suspension of pooled LN was prepared, diluted to 10^8 cells/ml, and divided into two tubes. One tube received 1 μg/ml DNP-lysine and the other received an equal amount of lysine. After 1 h at 4°C, 5×10^7 washed cells were injected intravenously into normal syngeneic recipients. Half of the recipients that received control lysine-treated LN cells and half that received DNP-lysine-treated cells were ear challenged with DNFB. The remaining recipients were ear challenged with Ox. Increased ear swelling was measured 24 h later. Results of two such experiments are given in Table II. Transfer of contact sensitivity to DNFB by LN cells from the doubly sensitized mice was inhibited by treating the cells with DNP-lysine (compare group B with A). In contrast, the ability of these same LN cells to transfer contact sensitivity to Ox was unaffected by the DNP-lysine treatment (compare groups C and D). Collectively, these results indicate that the inhibition of transfer of CS by treating immune LN cells with DNP-lysine or DNP-protein conjugates is hapten specific. Furthermore, the results in Table II show that the 1-h

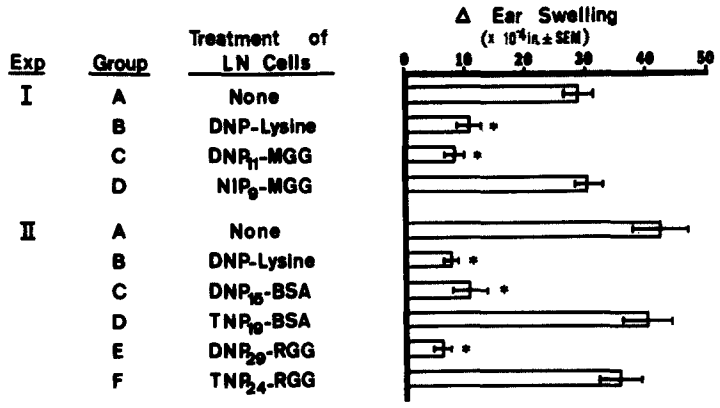

FIG. 1. Hapten specificity of inhibition of transfer of immunity. BALB/c mice were sensitized with DNFB on days 0 and 1, and immune LN cells harvested on day 4. The cells were suspended in RPMI medium at 10^8 cells/ml and treated for 1 h at 4°C with either DNP-lysine or different DNP-, NIP-, or TNP-protein conjugates. The cells were then washed three times and 5×10^7 cells transferred intravenously into normal recipients (four per group). The recipients were ear challenged with DNFB, and ear swelling was measured 24 h later. * $P < 0.01$.

TABLE II

Specificity of DNP-Lysine Inhibition of Transfer of CS to DNFB

Group	LN cells transferred	Treatment of LN cells from DNFB- plus Ox-sensitized mice	Challenge antigen	Experiment I		Experiment II	
				Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
	$\times 10^7$			$\times 10^{-4}$ in. ± SEM	%	$\times 10^{-4}$ in. ± SEM	%
A	5.0	Lysine	DNFB	23.7 ± 1.8		43.7 ± 4.1	
B	5.0	DNP-lysine	DNFB	9.9 ± 1.6‡	60	9.6 ± 2.3‡	79
C	5.0	Lysine	Ox	38.9 ± 4.0		47.0 ± 4.6	
D	5.0	DNP-lysine	Ox	43.6 ± 3.1	0	53.2 ± 3.9	0

BALB/c mice were doubly sensitized with DNFB and Ox on days 0 and 1, and immune LN cells were harvested and pooled on day 4. The cells were suspended in RPMI 1640 medium at 10^8 cells/ml, and half of the total population were treated with 1 μg/ml DNP-lysine for 1 h at 4°C. Control cells were treated with 1 μg/ml lysine. The cells were then washed and transferred to normal syngeneic recipients. Half of the recipients that received control LN cells and half that received DNP-lysine treated cells were ear challenged with DNFB. The remaining recipients were challenged with Ox. Increased ear swelling was measured 24 h later as an index of transfer of immunity.

* See footnote in Table I.

‡ $P < 0.001$.

treatment with the monovalent hapten is not toxic for the LN cells since only the DNP-specific response is inhibited.

DNP-Lysine Inhibits T Cells That Are Ia⁻. CS to DNFB can be transferred to naïve recipients by Ia⁻ LN T cells (27). Thus, experiments were done to determine whether purified Ia⁻ T cells were hapten inhibitable. Results of these experiments are given in Table III. In experiment I, unseparated and nylon wool-purified DNFB-immune BALB/c LN T cells were compared. Both cell populations transferred similar levels of immunity and both were sensitive to inhibition by the hapten. In experiment II, purified LN T cells from DNFB-immune CBA mice were treated with normal mouse serum or anti-Ia^k serum plus C and then tested for their sensitivity to inhibition by hapten. Antiserum treatment did not alter either the ability of the cells to transfer immunity or their sensitivity to inhibition by DNP-lysine. These results indicate that inhibition of transfer of immunity is mediated by interaction of the hapten DNP with the effector T cells of the CS reaction.

Hapten-mediated Inhibition of Transfer of Immunity Is Not due to Activation of Suppressor Cells. Skin painting of mice with DNFB induces at least two distinct T cell subpopulations: effector T cells of the CS reaction (T_{DH}) and auxiliary suppressor T cells (Ts-aux) (32). This latter population is required for suppression of the efferent limb of CS by suppressor T cells (32). Because the immune LN cells contain both T cell subsets, it was possible that inhibition of transfer of immunity was due to activation of Ts-aux cells by the hapten. To test this possibility, cotransfer experiments were done. Immune LN cells were prepared and a portion treated with DNP-lysine. After washing, half of the treated cells were injected intravenously into naïve recipients (5×10^7/recipient). The remaining cells were mixed with untreated immune LN cells (1:1) and 10^8 cells injected into naïve recipients. A third group of recipients received 5×10^7 untreated immune LN cells. All mice were then ear challenged, and ear swelling was measured 24 h later. As shown in Table IV, cotransfer of DNP-lysine-

TABLE III

Transfer of CS by Purified DNFB-immune T Cells Is Inhibited by DNP-Lysine

Exp.	DNFB-immune LN cells transferred	Serum treatment	DNP-lysine	Experiment I		Experiment II	
				Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
			1 μg/10⁸ cells/ml	*× 10⁻⁴ in. ± SEM*	%	*× 10⁻⁴ in. ± SEM*	%
I	Unseparated	None	−‡	29.6 ± 2.0		36.0 ± 3.0	
	Unseparated	None	+	8.4 ± 0.9§	72	11.7 ± 2.0§	68
	Purified T cells	None	−	25.4 ± 1.9		34.8 ± 1.3	
	Purified T cells	None	+	3.1 ± 1.1§	88	8.7 ± 0.7§	75
II	Purified T cells	NMS‖ + C	−	37.8 ± 4.2		27.4 ± 3.0	
	Purified T cells	NMS + C	+	12.1 ± 2.0§	68	4.1 ± 1.0§	85
	Purified T cells	Anti-Ia¶ + C	−	39.3 ± 2.7		27.2 ± 2.5	
	Purified T cells	Anti-Ia + C	+	9.2 ± 1.7§	77	4.9 ± 2.2§	82

BALB/c (experiment I) or CBA (experiment II) mice were sensitized with DNFB on days 0 and 1, and immune LN cells harvested on day 4. In experiment I, a portion of the cells were purified on nylon wool columns. Unseparated and purified T cells were suspended in RPMI 1640 medium at 10^8 cells/ml, and half of each population were treated with 1 μg/ml DNP-lysine for 1 h at 4°C. The cells were then washed three times and transferred to normal syngeneic recipients.

* See footnote in Table I.

‡ 1 μg/ml lysine.

§ $P < 0.001$.

‖ Normal mouse serum.

¶ Anti-Ia^k serum.

TABLE IV

DNP-Lysine Inhibition of Transfer of CS to DNFB Is Not Due to Suppressor Cells

Group	Untreated immune LN	DNP-lysine-treated immune LN	Experiment I		Experiment II	
			Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
	× 10⁷	*× 10⁷*	*× 10⁻⁴ in. ± SEM*	%	*× 10⁻⁴ in. ± SEM*	%
A	5.0	—	41.8 ± 3.6		54.2 ± 3.3	
B	—	5.0	9.1 ± 1.7‡	78	14.6 ± 3.1‡	73
C	5.0	5.0	39.1 ± 2.7	7	64.6 ± 5.6	0

BALB/c mice were sensitized with DNFB on days 0 and 1, and immune LN cells were harvested on day 4. The cells were suspended in RPMI 1640 medium at 10^8 cells/ml and a portion treated with 1 μg/ml DNP-lysine for 1 h at 4°C. These cells were then washed three times, and half were injected into normal syngeneic recipients. The remaining cells were mixed with an equal number of untreated immune LN cells, and the mixture injected into normal syngeneic recipients. An additional group of recipients received only untreated immune cells. All animals were ear challenged, and increased ear swelling was measured 24 h later as an index of transfer of immunity.

* See footnote in Table I.

‡ $P < 0.001$.

treated cells with untreated immune cells had no effect on the ability of the latter population to transfer immunity.

To further test for the possible role of suppressor T cells, the following experiment

was done. Immune LN cells were prepared, and four replicate tubes were treated with monoclonal anti-Lyt-2.2 antibody plus C. The washed cells were resuspended at 10^8 cells/ml in RPMI medium, treated for 1 h at 4°C with lysine or DNP-lysine, and then tested for their ability to transfer CS. Results of two experiments are given in Table V and show that anti-Lyt-2.2 plus C treatment did not alter the sensitivity of the LN cells to inhibition by DNP-lysine. These results indicate that DNP-lysine inhibition of transfer of CS is not due to activation of suppressor cells.

Effect of Temperature, Time of Treatment, and Divalent Cations on DNP-Lysine Inhibition. Certain in vitro conditions have been found to be critically important for studying T cell interaction with antigen. Thus, experiments were done to determine the minimal in vitro conditions necessary for DNP-lysine-mediated inhibition of transfer. Three parameters were examined and representative results are given in Table VI. First, the temperature during the incubation period was tested. Immune LN cells (10^8 cells/ml) were incubated with 1 μg/ml DNP-lysine for 1 h at 4°, 22°, and 37°C. Lysine-treated cells were incubated at 37°C. After washing, the cells were tested for their ability to transfer CS. As shown in experiment I, incubation temperature had no effect on the ability of DNP-lysine to inhibit transfer of immunity. Next, we determined the minimum amount of time necessary to treat the cells. Replicate tubes of immune LN cells (10^8 cells/ml) were treated with 1 μg/ml DNP-lysine at 4°C for 15, 30, or 60 min, washed, and 5×10^7 cells transferred to naïve recipients. As shown in experiment II, significant inhibition of transfer was obtained by treating the cells for only 15 min (compare group B to A). Finally, we examined the requirement for divalent cations. Immune LN cells were suspended in HBSS or in Ca^{++}, Mg^{++}-free HBSS at 10^8 cells/ml and treated for 1 h at 4°C with 1 μg/ml DNP-lysine. Control cells were treated with lysine and incubated in Ca^{++}, Mg^{++}-free HBSS. After washing, 5×10^7 cells per recipient were transferred to naïve recipients, and ear swelling was measured 24 h later. As shown in experiment III, transfer to CS by immune LN cells treated

TABLE V

Lyt-2$^+$ T Cells Are Not Required for DNP-Lysine Inhibition of Transfer of CS

Group	Serum treatment of immune LN	DNP-lysine	Experiment I		Experiment II	
			Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
		μg/10^8 cells/ml	*× 10^{-4} in. ± SEM*	%	*× 10^{-4} in. ± SEM*	%
A	Anti-Lyt-2.2 + C	−‡	27.0 ± 2.0		21.0 ± 1.4	
B	Anti-Lyt-2.2 + C	1	6.2 ± 1.3§	77	ND‖	
C	Anti-Lyt-2.2 + C	10	10.2 ± 1.0§	62	9.7 ± 1.5¶	54
D	Anti-Lyt-2.2 + C	100	5.2 ± 0.5§	81	4.3 ± 0.8§	80

BALB/c mice were sensitized with DNFB on days 0 and 1. Immune LN cells were harvested on day 4 and treated with monoclonal anti-Lyt-2.2 (1:400) plus C. The cells were then treated with different concentrations of DNP-lysine for 1 h at 4°C, washed and transferred to naïve recipients. The recipients were ear challenged with DNFB, and ear swelling was measured 24 h later.

* See footnote in Table I.

‡ 10 μg/ml lysine.

§ $P < 0.001$.

‖ Not done.

¶ $P < 0.01$.

TABLE VI

Effect of Different In Vitro Conditions on DNP-Lysine Inhibition of Transfer of CS to DNFB

Experiment	Group	Treatment of immune LN cells before transfer	Δ Ear swelling*	Inhibition
			$\times 10^{-4}$ *in.* ± *SEM*	%
I	A	Lysine—37°C	42.2 ± 3.8	
	B	DNP-lysine—4°C	13.6 ± 2.6‡	68
	C	DNP-lysine—22°C	12.8 ± 2.8‡	70
	D	DNP-lysine—37°C	11.2 ± 3.0‡	74
II	A	Lysine—4°C, 60 min	24.0 ± 1.3	
	B	DNP-lysine—4°C, 15 min	14.9 ± 2.4§	38
	C	DNP-lysine—4°C, 30 min	1.8 ± 1.8‡	93
	D	DNP-lysine—4°C, 60 min	3.1 ± 2.2‡	88
III	A	Lysine in Ca^{++}, Mg^{++}-free HBSS	30.4 ± 3.6	
	B	DNP-lysine in HBSS	6.3 ± 2.1‡	80
	C	DNP-lysine in Ca^{++}, Mg^{++}-free HBSS	28.5 ± 3.2	6

BALB/c mice were sensitized were DNFB on days 0 and 1 and immune LN cells were harvested on day 4. In experiments I and II the cells were suspended in RPMI 1640 medium at 10^8 cells/ml and treated with 1 μg/ml DNP-lysine or lysine as indicated. In experiment III cells were suspended in HBSS or Ca^{++}, Mg^{++}-free HBSS at 10^8 cells/ml and treated with 1 μg/ml DNP-lysine or lysine for 1 h at 4°C. In all three experiments, after treatment the cells were washed three times and transferred to naïve recipients that were then ear challenged. Increased ear swelling was measured 24 h later as an index of transfer of immunity.

* See footnote in Table I.

‡ $P < 0.001$.

§ $P < 0.05$.

with DNP-lysine in Ca^{++}, Mg^{++}-free HBSS was not inhibited (compare group C to A), whereas the transfer by cells treated in HBSS containing divalent cations (group B) was inhibited by 80%. Collectively, these results indicate that the interaction of DNP-lysine with immune LN T cells in vitro is rapid, temperature independent, and requires the presence of divalent cations in the treatment medium.

DNP-Lysine Inhibition of DNFB-immune T Cells Is Reversible. To begin to explore the mechanism of the hapten inhibition, two possibilities were considered. First, the hapten may bind to receptors for DNP and block the T cell from interacting with immunogenic forms of DNP in association of MHC gene products, i.e., receptor blockade. Alternatively, interaction with hapten alone may provide some negative signal to the T cell, making it refractory to stimulation. If so, the cells should not become functional after a short incubation period in vitro in hapten-free medium. However, if inhibition is due to hapten blocking the receptor, then incubation in vitro may reverse the effect by allowing the hapten to dissociate from the cell surface. To attempt to distinguish between these two possibilities, the following experiment was done. Four replicate tubes of immune LN cells were treated with DNP-lysine in vitro according to the standard protocol. After washing, one group of cells was injected intravenously into syngeneic recipients. The remaining cells were resuspended in RPMI medium and incubated at 37°C for 1 h. These cells were then washed and one group was injected intravenously into syngeneic recipients. The remaining two tubes of cells were resuspended in RPMI medium, placed at 4°C, and one tube was again

treated with 1 μg/ml of DNP-lysine. After 1 h incubation, these cells were washed and transferred to naïve recipients. Control, lysine-treated immune cells were carried through all three incubation periods and then injected into recipient mice. All recipients were then ear challenged, and ear swelling was measured 24 h later. The results are given in Table VII. Immune LN cells treated for 1 h with DNP-lysine and then transferred were inhibited (compare group B to A). This inhibition was reversed by incubating the treated cells for 1 h at 37°C in hapten-free medium (compare group C with A and B). However, if similarly treated cells were reincubated at 4°C for 1 h and retreated with DNP-lysine, their ability to transfer immunity was once again inhibited (compare group E to A and D). These results favor the interpretation that the inhibition is mediated by hapten binding to the T cell receptors thus blocking the cell from interacting with antigen-presenting cells at the skin test site.

Additional experiments have shown that this reversal of inhibition is temperature independent, i.e., reversal occurs if the treated cells are incubated for 1 h in hapten-free medium at 4°, 25°, or 37°C. Also, we have found that addition of fetal calf serum (FCS) to the hapten-free medium retards the reversal in a concentration-dependent way. In other words, hapten-treated immune LN cells incubated for 1 h at 37°C in hapten-free medium containing 20% FCS are still inhibited by 50% or more, whereas cells incubated in medium containing 10 or 5% FCS are progressively less inhibited (data not shown).

Competition of DNP-Lysine and Anti-Idiotype Antibodies for Binding Sites on DNFB-immune T Cells. Previous studies from our laboratory have shown that the duration of the CS response to DNFB is regulated by anti-idiotype antibodies produced by the host

TABLE VII

Inhibition by DNP-Lysine of Transfer of CS to DNFB Is Reversible by Incubating the Treated Cells In Vitro

Group	1st treatment of immune LN cells (4°C)	2nd treatment of immune LN cells (37°C)	3rd treatment of immune LN cells (4°C)	Experiment I		Experiment II	
				Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
				× 10^{-4} in. ± SEM	%	× 10^{-4} in. ± SEM	%
A	Lysine	None	Lysine	50.7 ± 3.9		34.4 ± 3.6	
B	DNP-lysine (cells injected after 1st treatment)			19.2 ± 1.4‡	62	7.5 ± 2.8‡	78
C	DNP-lysine	None (cells injected after 2nd treatment)		47.2 ± 3.6	7	31.6 ± 3.0	8
D	DNP-lysine	None	None	44.8 ± 3.1	11	31.1 ± 3.3	10
E	DNP-lysine	None	DNP-lysine	15.3 ± 3.1‡	70	11.2 ± 1.9‡	67

BALB/c mice were sensitized with DNFB on days 0 and 1, and immune LN cells harvested on day 4. The cells were suspended in RPMI 1640 medium at 10^8 cells/ml and aliquoted into five replicate tubes. Cells in tube A were carried through all three 1-h incubations and treated with 1 μg/ml lysine as indicated. Tubes B–E were initially treated with 1 μg/ml DNP-lysine at 4°C and washed. Cells in tube B were then injected intravenously into syngeneic recipients. Cells in tubes C–E were resuspended in RMPI 1640 medium, incubated for 1 h at 37°C, and washed. Cells in tube C were then injected into syngeneic recipients. Cells in tubes D and E were then resuspended in RPMI 1640 medium, 1 μg/ml DNP-lysine added to tube E, and the two tubes incubated for 1 h at 4°C. After washing, these cells plus the cells from tube A were injected intravenously into three groups of syngeneic recipients. All recipients were then ear challenged with DNFB, and increased ear swelling was measured 24 h later.

* See footnote in Table I.

‡ $P < 0.001$.

(33). Suppressive immune serum (SIS) which contains these antibodies is obtained by bleeding DNFB-immune mice 9–15 d after sensitization. Treatment of immune LN cells with SIS plus C eliminates transfer of CS to DNFB but has no effect on transfer of immunity to TNCB or Ox. Similarly, the anti-idiotype activity of SIS is removed by adsorption with DNFB-immune LN cells but not by TNCB- or Ox-immune cells (33). We used this adsorbing ability of DNFB-immune LN cells to determine whether DNP-lysine and anti-idiotypic antibodies were binding to the same sites on the immune T cells. The following protocol was used. Nylon wool-purified or unseparated immune LN cells were treated in vitro for 1 h at 4°C with hapten. After washing, 2×10^8 treated or control cells were used to adsorb 2 ml of 1:2 diluted SIS for 30 min at 4°C. To test for remaining anti-idiotype activity, freshly prepared DNFB-immune LN cells were then treated with adsorbed or unadsorbed SIS plus C and transferred to naïve recipients. These animals were ear challenged, and ear swelling was measured 24 h later. Results of these experiments are shown in Table VIII. Two different pools of SIS were used in the two experiments shown. In experiment I, untreated and DNP-lysine-treated nylon wool-purified DNFB-immune LN T cells were compared for their ability to adsorb SIS. Unadsorbed SIS plus C inhibited transfer of immunity by 71% (compare group B to A), whereas serum adsorbed with lysine-treated LN T cells inhibited transfer by only 12% (compare group C to A). In contrast, serum adsorbed with DNP-lysine-treated T cells inhibited transfer by 68% (group D vs. A). Thus, DNP-lysine treatment of the T cells blocked their ability to adsorb the activity of the SIS. In experiment II, the specificity of the inhibition of adsorption was tested by comparing immune LN cells treated with DNP-lysine, DNP_{11}-MGG, or NIP_9-MGG. This SIS was somewhat less active as the unadsorbed serum plus C inhibited transfer

TABLE VIII

Adsorption of Anti-Idiotype Antibody by DNFB-immune T Cells Is Blocked by Pretreating the Cells with DNP-Lysine or DNP_{11}-MGG

Group	Serum treatment of immune LN cells	Serum preadsorbed with	Experiment I		Experiment II	
			Δ Ear swelling*	Inhibition	Δ Ear swelling	Inhibition
			$\times 10^{-4}$ in. ± SEM	%	$\times 10^{-4}$ in. ± SEM	%
A	NMS‡ + C	None	44.8 ± 4.1		29.2 ± 2.6	
B	SIS + C	None	13.0 ± 1.9§	71	16.3 ± 2.6‖	44
C	SIS + C	Lysine-treated T_{DH}	39.3 ± 3.1	12	31.3 ± 3.1	0
D	SIS + C	DNP-lysine-treated T_{DH}	14.4 ± 2.2§	68	16.0 ± 2.6‖	45
E	SIS + C	DNP_{11}-MGG-treated T_{DH}	ND¶		17.8 ± 2.3‖	40
F	SIS + C	NIP_9-MGG-treated T_{DH}	ND		29.9 ± 3.0	0

BALB/c mice were sensitized with DNFB on days 0 and 1, and immune LN cells harvested on day 4. The cells were resuspended in RPMI 1640 medium at 10^8 cells/ml and treated with 1 μg/ml lysine, DNP-lysine, DNP_{11}-MGG, or NIP_9-MGG. After washing, these cells were used to adsorb 2 ml of 1:2 SIS for 30 min at 4°C. Freshly prepared DNFB-immune LN cells were then treated with the adsorbed or unadsorbed SIS plus C and transferred to normal recipients. The recipients were ear challenged with DNFB, and increased ear swelling was measured 24 h later.

* See footnote in Table I.

‡ Normal mouse serum.

§ $P < 0.001$.

‖ $P < 0.01$.

¶ Not done.

by only 41% (group B vs. A). Nevertheless, adsorption of this serum with lysine-treated immune LN cells completely removed the inhibitory activity (group C vs. A), and this adsorption was blocked by treating the immune LN cells with either DNP-lysine or DNP_{11}-MGG (groups D and E). However, treating the immune cells with NIP_9-MGG had no effect as these cells completely adsorbed the inhibitory activity of the SIS (group F). These results indicate that DNP-lysine and the anti-idiotypic antibodies compete for the same binding sites on the DNFB-immune T cells.

Discussion

Exactly how T cells "see" antigen still is poorly understood. It seems clear that for activation of T cells, the cells must interact with antigen in the context of certain MHC determinants expressed on the surface of antigen-presenting cells or target cells (7). The molecular nature of this interaction, i.e., the type(s) of receptor(s) used by the T cells, is unknown. In the present study, I have investigated the first phase of T cell-antigen interaction by treating DNFB-immune T cells in vitro with free hapten or hapten-protein conjugates. The effect(s) of this treatment was measured by testing the ability of the treated cells to transfer CS to DNFB into naïve recipients. The experimental results establish the following points: (*a*) incubation of DNFB-immune LN cells or purified T cells in vitro with ϵ-DNP-L-lysine or DNP-protein conjugates inhibits, in a dose-dependent way, the transfer of CS; (*b*) the inhibition is hapten specific and is not due to toxic effects of the hapten; (*c*) the inhibition is not mediated by activation of suppressor cells; (*d*) inhibition of the T cells by hapten in vitro, presumably reflecting their interaction, is rapid and temperature independent but requires divalent cations; (*e*) inhibition is reversed by incubating the treated cells for 1 h in vitro in hapten-free medium. If these cells are retreated with hapten, transfer of immunity is again blocked; and (*f*) hapten-treated T cells are unable to adsorb specific anti-idiotype antibody, and this inhibition of adsorption is hapten specific.

CS to DNFB is mediated by Ia^- T cells (T_{DH}) (27). The finding here that transfer of CS by purified, Ia^- T cells, carefully monitored for macrophage and B cell contamination, is blocked by monovalent hapten indicates that the T_{DH} cells interact with the hapten. This is supported by the experiments which showed that activation of suppressor cells was not responsible for the inhibition. Thus, it appears that the inhibition of transfer of immunity is due to direct interaction of the effector T_{DH} population with the hapten and does not require or involve other cell types.

These findings differ from most other studies that have attempted to block T cell function with free antigen. For example, lysis of chromium-labeled target cells by virus- or hapten-specific cytotoxic T cells is not inhibited by unmodified cold target cells, virus-infected, or hapten-labeled allogeneic cells or by addition of free hapten (18, 20, 22, 26). Similarly, free antigen does not inhibit T cell proliferation in vitro or the binding of immune T cells to antigen-pulsed macrophages (19, 21, 23–25). These negative findings are somewhat surprising in light of studies that have shown T cell binding of soluble radiolabeled antigen (34–37). It is difficult to reconcile these apparently contradictory results, although it is possible that the T cells that bind soluble radiolabeled antigen do not belong to the same functional subset being stimulated or analyzed. Alternatively, the closed, in vitro conditions used to measure most T cell functions and that are almost always optimized, may so favor T cell activation that functional inhibition of the cells by free antigen may be extremely

difficult. In other words, if only a limited number of contacts (one or two perhaps) between the T cell and antigen-presenting cell or target cell are required for stable T cell binding and activation, these interactions may occur with time in vitro even in the presence of a large excess of antigen. Thus, our ability to inhibit CS by treating T cells with monovalent hapten may reflect, in part, the fact that we test the function of the cells in vivo rather than in vitro. Indeed, previous studies by Kantor (38) and Leskowitz and Jones (39) have shown that delayed hypersensitivity reactions are inhibited either by treating cells in vitro with highly substituted hapten-protein conjugates and then transferring the cells, or by injecting immune animals with hapten-amino acid or hapten-protein conjugates.

The interaction of T cells with hapten in vitro, as measured by inhibition of transfer of immunty, is rapid, temperature independent, and requires divalent cations in the incubation medium. Furthermore, the inhibition is reversed by incubating the treated cells for 1 h in hapten-free medium (see below). Our finding that 15–30 min incubation time is required to achieve inhibition of transfer is in agreement with other reports that have investigated T cell binding of radiolabeled antigen (34, 36, 37). However, these studies have found that significant binding occurs only at 37°C, whereas our results indicate that the temperature of incubation has no effect on the level of inhibition caused by the hapten. At present we have no explanation for this difference. The requirement for divalent cations is of particular interest since this aspect has been studied in a variety of other systems. For example, divalent cations have been shown to be required for adherence of cytotoxic T cells to target cells (40), for antigen-independent and antigen-dependent binding of lymphocytes to macrophages (41, 42), and for maintenance of antigen-dependent binding of T cells to macrophages (42). It has been suggested that divalent cations serve to form ionic bridges between cells, thus providing stable interaction at the membrane interface. The results presented here, however, indicate that divalent cations are required for the inactivation of T_{DH} cells by monovalent DNP-lysine without the participation of other cell types. The precise role the cations play in this process is unknown; it seems unlikely to involve ionic bridges between cells. One possible alternative explanation is that they serve to stabilize the antigen receptor in the T cell membrane, perhaps by maintaining the proper stereoconfiguration of subunits. This effect would be similar to Ca^{++}-dependent antigen-antibody reactions that are due to antibodies directed against determinants formed and/or maintained by Ca^{++}-induced alterations of the antigen (43). We are unaware of any studies of the divalent cation requirements for binding of radiolabeled antigen to T cells. We are currently studying this and other aspects of the hapten-T cell interaction.

The reversible nature of the hapten-inhibition in vitro at first presented a paradox since the inhibition is still present 24 h after the washed cells are transferred intravenously to naïve recipients. We believe, however, that the situation is clarified by our recent experiments which show that addition of serum to the incubation medium maintains the inhibited state. It appears that high protein concentration, i.e., 20% or more FCS, favors the continued association of the hapten with the T cells, whereas low or no protein favors dissociation. Thus intravenously injected cells would be suspended in the plasma equivalent of 100% serum that would maintain the hapten binding and would account for the inhibited response.

The experiments that showed hapten-specific blocking of T cell adsorption of anti-

idiotype antibody provide critical information with respect to the mechanism of hapten-inhibition of transfer of immunity. These results indicate that the hapten and the anti-idiotype antibody compete for the same site on the T_{DH} cell. Since both of these reagents are hapten specific in their effect, this site is most likely the antigen-specific receptor for DNP. Krammer (44) has reported similar findings for alloantigen-binding T cells. It follows then that the inhibition of transfer of CS is due to DNP-binding to the hapten-specific receptor on the T cell. It is likely that this binding prevents the T cells from interacting with immunogenic forms of the hapten, i.e., DNP plus MHC determinants, at the skin test site. This binding may be of relatively low affinity since the inhibition is reversed by incubating the treated cells in vitro for 1 h in hapten-free medium. We believe this reversal is due to dissociation of the hapten rather than shedding of receptors, because it occurs at 4°C and the cells are inhibited again by reincubation with hapten. It is unlikely that any significant receptor shedding or re-expression would occur at 4°C.

Finally, in a recent article discussing T cell recognition of antigen, it was suggested that five criteria be met before T cells could be characterized as hapten specific (17). Although these criteria were written with respect to activation of T cells, they are equally applicable to inhibition of T cells and are restated in this context as follows: T cells may be characterized as hapten specific if (*a*) cells are inhibited by the same hapten on different carriers; (*b*) carrier alone does not inhibit the cells; (*c*) a different hapten on the same carrier does not inhibit the cells; (*d*) free hapten can inhibit the response; and (*e*) cells are inhibited by the hapten coupled via spacer molecules to the carrier. The results presented here satisfy the first four of these five criteria and so the T cells that mediate CS to DNFB can be termed hapten specific. Furthermore, these results provide strong evidence that in this hapten-specific T_{DH} population, and regardless of other receptors, antigen recognition occurs via a receptor specific for the hapten DNP. These findings make the altered self model less likely, unless the one receptor that this model proposes and that is specific for a neoantigenic determinant still can recognize hapten alone.

Summary

Experiments were done to investigate the nature of the antigen receptor on lymph node (LN) T cells from mice sensitized to 2,4-dinitrofluorobenzene (DNFB). LN cells or purified T cells were treated in vitro with monovalent or different multivalent 2,4-dinitrophenol (DNP) ligands. The effect of this treatment was measured by testing the ability of the cells to transfer contact sensitivity (CS) to DNFB into naïve recipients. We found that treatment of the T cells in vitro with either ε-DNP-L-lysine or DNP-protein conjugates inhibits the transfer of CS in a dose-dependent way. The inhibition is hapten specific and is not mediated by activation of suppressor cells. Inhibition of the T cells by hapten in vitro is rapid (15–30 min) and temperature independent but requires divalent cations in the treatment medium. In addition, it was found that hapten-treated T cells are unable to adsorb specific anti-idiotype antibody, and this inhibition of adsorption is hapten specific. Collectively, these data indicate that DNFB-immune T cells express a receptor specific for the hapten DNP and provide evidence that supports a two-receptor model for T cell recognition of antigen.

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