THE SPECIFICITY OF CELLULAR IMMUNE RESPONSES IN GUINEA PIGS

III. The Precision of Antigen Recognition by T Lymphocytes

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Thymus-derived (T) lymphocytes from guinea pigs immunized with a hapten coupled directly to mycobateria will respond to that hapten coupled to many, although not all, carriers (1–3). By manipulating the structure of the hapten, the carrier, or the bond joining them, chemically characterized antigenic determinants can be generated. The functional binding activity of the T-cell receptor for antigen can be inferred from the response or lack of response of immune T lymphocytes to such antigenic determinants. Previous studies have shown that guinea pigs immunized with 2,4-dinitrophenyl (DNP) coupled directly to mycobacteria have T lymphocytes that respond to a determinant made up of the hapten, its bond to the carrier, the amino acid residue to which the DNP is coupled, and adjacent amino acid residues, the number of which is not known. DNP groups linked to either the ϵ-amino group of L-lysyl residues (DNP-Lys) or to the hydroxyl group of tyrosyl residues (O-DNP-Tyr) are recognized by T lymphocytes, although the former are apparently the important residues in DNP proteins (2). These results suggest that the T-cell receptor for antigen recognizes a large, complex antigenic determinant.

Two further questions arise from such results. The first is, whether the responses of T cells from DNP-mycobacteria-primed animals to various DNP conjugates are mediated by T lymphocytes of a single specificity, or alternatively by several subpopulations of cells, each of a distinct specificity. If the former were true, it would strongly suggest that T lymphocytes with a high degree of specificity for DNP exist, since the only common feature of all the conjugates is the DNP group, whereas the latter would suggest that T lymphocytes recognize and discriminate between more complex antigenic determinants comprising hapten and carrier, as we would anticpate from previous analyses of T-lymphocyte specificity. The second question is whether the responses of such T cells to DNP conjugates can be inhibited by DNP in a nonstimulatory form. Successful inhibition would imply that the T-cell receptor is capable of binding hapten with high avidity, but that further steps, beyond simple recognition, are required for cell activation.

The object of the present experiments was to answer these two questions. Briefly, our results show that different subpopulations of immune T cells

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derived from guinea pigs immunized to DNP-mycobacteria respond to DNP on different carriers. No evidence was found for inhibition of T-lymphocyte activation by nonstimulatory DNP compounds. These results suggest that the affinity of T-cell receptors for DNP itself is relatively low and are consistent with the concept that these receptors are composed of several subsites, each binding with low affinity a different portion of a complex antigenic determinant.

Materials and Methods

Animals. Inbred strain 2 and strain 13 guinea pigs from the Animal Production Section, NIH, Bethesda, Md., 2- to 6-mo old, were used throughout. Where cells were pooled, animals of one sex, age, and strain were used.

Antigens and Immunizations. DNP conjugates of killed *Myobacterium tuberculosis* organisms of strain H37Ra (H37),[1] ovalbumin (OVA), guinea pig albumin (GPA), keyhole limpet hemocyanin (KLH), and of a series of synthetic amino acid copolymers were prepared as previously described (1, 2). Subscripts denote the moles of hapten per mole of carrier or, for KLH, per 10^5 daltons. The amino acid copolymers, their abbreviations, their molar compositions, and their mean molecular weights are indicated in Table I.

In addition, trinitrophenyl (TNP) derivatives of GPA and KLH were prepared using 2,4,6-trinitrochlorobenzene and a DNP derivative of guinea pig albumin was synthesized using 2,4-dinitrobenzene sulfonate (DNBS). This compound is referred to as DNBS-GPA. ε-DNP-L-lysine, mono-*O*-DNP-L-tyrosine, and di-*O*,*N*-DNP-L-tyrosine were purchased from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. DNP-H37 and H37 were emulsified to incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) at a concentration of 2.5 mg/ml. Guinea pigs received 0.1 ml of emulsion in each foot pad for a total dose of 1.0 mg of H37 or DNP-H37.

In Vitro Proliferation. Purified peritoneal exudate lymphocytes (PELs) (1, 4) were cultured with antigen as previously described (1). Results are expressed as counts per minute of tritiated thymidine incorporated by cells in wells containing antigen minus counts per minute incorporated by cells in control wells without added antigen (E-C); control counts per minutes are given in parentheses at the top of each column in the tables.

Treatment with 5-Bromodeoxyuridine (BUdR) and Light. 2×10^6 PELs in 1-ml portions were cultured in 12×75 mm capped plastic tubes (Falcon no. 2058; Falcon Plastics, Div. of BioQuest, Oxnard, Caif.) in medium with or without antigen. After 2 days, freshly prepared, sterile BUdR (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp.) in saline was added to yield a final concentration of 3×10^{-6} M (5, 6). 24 h later, the tubes were tightly capped, and the bottoms centerd over openings in the wire mesh base of a test tube rack to permit optimal light exposure of the cell pellets. The rack was then placed directly on three fluorescent light bulbs (Westinghouse Cool-Ray; Westinghouse Electric Corp., Lamp Parts Dept., Bloomfield, N. J.) and illuminated for 90 min. The cells were washed four times with Hanks' balanced salt solution (HBSS), resuspended in complete medium, and counted in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). The BUdR- and light-treated cells were recultured, in the presence or absence of antigen, in microtiter U trays at 2×10^6 cells/ml, either alone or mixed 1:1 with peritoneal exudate cells (PECs) that had been inactivated by culturing for 30 min at 37°C in 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.). The mitomycin C-treated PECs were washed four times with HBSS before mixing with the PELs. The mixed cells were cultured for 3 days and 1 μCi of tritiated thymidine was added for the last day. The results for each group of precultured cells were calculated individually as E-C for that group.

Induction of Tolerance. Guinea pigs were given daily intraperitoneal injections of 2 mg DNP-D-GL or 1.5 mg DNP-D-GAT in saline for 5 days. 3 days later they were primed with DNP-H37. The

[1] *Abbreviations used in this paper:* ABC, antigen-binding capacity; BUdR, 5-bromodeoxyuridine; DAG, DNP-β-alanyl-glycylglycyl; DNBS, 2,4-dinitrobenzene sulfonate; GPA, guinea pig serum albumin; HBSS, Hanks' balanced salt solution; H37, *Mycobacterium tuberculosis* of strain H37Ra; KLH, giant keyhole limpet hemocyanin; OVA, hen's egg albumin; PEC, peritoneal exudate cell; PEL, peritoneal exudate lymphocyte; PPD, purified protein derivative of tuberculin; TNP, trinitrophenyl.

TABLE I
Composition of Synthetic Antigens Used in these Studies

Abbreviations	Composition	Molar proportions of amino acids	Mol wt
		%	
L-GL	L-glutamic acid,	60	90,000
	L-lysine	40	
D-GL	D-glutamic acid,	59	40,000
	D-lysine	41	
L-GLA^{30}	L-glutamic acid,	42	32,000
	L-lysine,	28	
	L-alanine	30	
L-GLA^{40}	L-glutamic acid,	36	45,000
	L-lysine,	24	
	L-alanine	40	
L-GT	L-glutamic acid,	54	22,600
	L-tyrosine	46	
L-GLT	L-glutamic acid,	60	61,000
	L-lysine,	34	
	L-tyrosine	6	
L-GAT	L-glutamic acid,	60	55,000
	L-lysine,	30	
	L-tyrosine	10	
L-GLAT	L-glutamic acid,	36	30,000
	L-lysine,	24	
	L-alanine,	35	
	L-tyrosine	5	
L-TGA	L-glutamic acid,	49	23,000
	L-alanine,	43	
	L-tyrosine	8	
D-TGA	L-glutamic acid,	49	33,800
	L-alanine,	43	
	L-tyrosine	8	

degree of tolerance achieved was assessed by titrating anti-DNP antibody using a modified Farr assay (7) with 0.5×10^{-8} M ^{3}H-ε-DNP-L-lysine as ligand. Antibody with principal specificity for *O*-DNP-Tyr groups was measured by the binding of 0.5 μg/ml ^{125}I-DNP-L-GAT [labeled by the chloramine-T method, (8)]; bound ligand was precipitated with rabbit anti-guinea pig immunoglobulin serum. Results are expressed as antigen-binding capacity (ABC) measured at 33% antigen bound. Values for individual animals are shown, including those of the animals tested for T-cell tolerance by in vitro proliferation with antigen.

Inhibition of In Vitro Proliferation. PELs were prepared, mixed with inhibitors, and 2×10^{5} placed in each well of microtiter U plates. Stimulating antigens were added in the form of antigen-pulsed, mitomycin C-inactivated PECs (9). PECs at 6×10^{6}/ml were pulsed with antigen at the concentrations shown in the tables for 30 min at 37°C in medium containing 50 μg/ml mitomycin C, washed four times, and counted; 2×10^{5} PECs were added to each well. The expected stimulation was calculated as the sum of the stimulation given by inhibitors in the presence of

unpulsed PECs plus the stimulation given by pulsed PECs to which no inhibitor had been added. Inhibition is expressed as the percent reduction in the response found compared to that expected.

Results

The Demonstration of Subpopulations of T Lymphocytes Responsive to Different DNP Conjugates in PELs from Guinea Pigs Immunized with DNP-H37. The technique employed to determine whether PELs from guinea pigs immunized with DNP-H37 includes a population of T lymphocytes of a single specificity, responsive to a wide variety of DNP conjugates, or consists of multiple subpopulations, each responsive to a different antigenic determinant, involved culturing the cells twice in succession. In the first culture, cells responding to the stimulating antigen were selectively destroyed using BUdR and light. The second culture assessed the degree of diminution in response to the stimulating antigen used in the first culture and the effect of this negative selection on responses to other antigens. The use of BUdR and light to delete populations of proliferating cells has been described in detail (5). It is based on the incorporation of BUdR, a thymidine analogue, into DNA and the subsequent cross-linking of DNA strands as a result of light activation of BUdR. This leads to a permanent block in cell replication. Thus, any cell which is stimulated to synthesize DNA in the original culture should be unable to respond in the second culture; cells not responding in the first culture should be unaffected by the BUdR and light treatment. Preliminary experiments showed that both BUdR and light were required for these effects; neither alone inhibited cellular responses.

Table II summarizes a series of experiments which demonstrate the specificity of the negative selection technique. Thus, if cells were initially cultured with purified protein derivative of tuberculin (PPD) in the presence of BUdR and light, their subsequent response to PPD is 6.5% of the response of a comparable population treated with BUdR and light in the absence of antigen. On the other hand, cells precultured with BUdR and light in the presence of PPD display a response to a wide series of DNP and TNP conjugates which is 74.9% of the response of cell populations precultured with BUdR and light without antigen. This selective ablation of antigen-responsive populations is also observed when cells are precultured with either DNP-KLH, DNP-GLA[30], DNP-L-GAT, DNP-GLAT, TNP-KLH, DNP-GPA, TNP-GPA, or DNP-OVA and their subsequent responses to the antigen initially present and to PPD are compared. Indeed a summary of a large series of experiments indicates that the response to the antigen initially present is 12.1% of the control response while the response to an unrelated antigen is 81.3% of the control response (Table II).

The modest reduction in responsiveness to an unrelated antigen which occurs as a result of preculturing with antigen in the presence of BUdR and light might reflect an unrecognized degree cross-reactivity between these antigens. However, it is more likely that this reduction is due to the deletion of a population of cells which proliferate nonspecifically in response to factors produced by specific cells. In the experiments that follow, in which the effect of preculturing with one DNP conjugate on the responsiveness to another DNP conjugate is tested, we will use the diminution in responsiveness to PPD as an index of the nonspecific

TABLE II

Specific Inhibition of T-Lymphocyte Proliferative Responses by Preculturing with Antigen in Presence of BUdR and Light

Antigen used in first culture	Second culture		
	Antigen	N*	Residual response (%)‡
			Mean ± *SE*
PPD	PPD	7	6.5 ± 2.1
	DNP + TNP conjugates§	32	74.3 ± 5.7
DNP-KLH	DNP-KLH	5	10.4 ± 2.6
	PPD	5	80.9 ± 12.5
DNP-GLA$_{30}$	DNP-GLA$_{30}$	5	13.6 ± 6.0
	PPD	5	74.0 ± 3.4
DNP-L-GAT	DNP-L-GAT	6	18.6 ± 4.1
	PPD	6	88.1 ± 12.5
DNP-GLAT	DNP-GLAT	2	15.2 ± 11.0
	PPD	2	88.7 ± 6.2
TNP-KLH	TNP-KLH	2	9.4 ± 2.1
	PPD	2	72.9 ± 4.5
DNP-GPA	DNP-GPA	2	15.9 ± 9.9
	PPD	2	137.9 ± 17.3
TNP-GPA	TNP-GPA	2	6.3 ± 6.3
	PPD	2	123.4 ± 31.3
DNP-OVA	DNP-OVA	1	7.3
	PPD	1	97.3
Summary	Initial antigen	32	12.1 ± 1.6
	Alternative antigen‖	57	81.3 ± 4.2

In the first culture, PELs were exposed to antigen for 2 days, BUdR was then added (final concentration, 3×10^{6} M) and culture was continued for one more day. The cells were exposed to light for 90 min and washed. In some experiments mitomycin C-treated PECs from syngeneic donors were added at a 1:1 ratio. The cells were then cultured (second culture) with the indicated antigen for 3 days and ^{3}H-TdR incorporation measured at the end of that time.

* N, number of experiments.

‡ Residual response (%) = 100 (Δcpm of cells precultured with indicated antigen/Δcpm of cells precultured without antigen).

§ DNP and TNP conjugates are DNP-KLH, DNP-GLA$_{30}$, DNP-L-GAT, DNP-GLAT, TNP-KLH, DNP-GPA, TNP-GPA, and DNP-OVA.

‖ Alternative antigen: in cultures initially exposed to DNP or TNP conjugates, the alternative antigen is PPD; in cultures initially exposed to PPD, the alternative antigens are DNP or TNP conjugates.

TABLE III

The Effect of Preculturing PELs from Strain 2 Guinea Pigs Primed with DNP-H37 with Various Antigens on their Subsequent in Vitro Proliferative Responses to Antigen

Stimulating antigen‡	BUdR- and light-treated PELs precultured with:*				
	0	PPD	DNP_8-KLH	DNP-GLA_{30}	DNP-L-GAT
None	(1,456)§	(5,188)	(5,084)	(4,697)	(10,810)
PPD	103,351‖	11,165¶ [10.8]**	61,105 [59.1]	79,749 [77.2]	46,790 [45.3]
DNP_8-KLH	29,293	22,972 [78.4]	3,102 [10.6]	17,741 [60.6]	23,408 [79.9]
DNP-GLA_{30}	30,979	24,416 [78.8]	8,534 [27.5]	2,345 [7.6]	9,255 [29.9]
DNP-L-GAT	20,268	11,873 [58.6]	15,973 [78.8]	26,088 [128.7]	1,763 [8.7]

* PELs cultured with the antigens shown across the top of the table for 2 days, then made to 3×10^{-6} M with BUdR for 24 h, followed by 90-min illumination. PELs washed four times, made to 2×10^6 cells/ml, and recultured with the antigens shown in the vertical column.

‡ Antigen concentrations for primary and secondary stimulation: PPD, 20 μg/ml; all others, 100 μg/ml.

§ Uptake of ^{3}H-TdR – (cpm).

‖ Net uptake of ^{3}H-TdR [Δ cpm]; cpm incorporated by cultures exposed to antigen minus cpm incorporated by nonstimulated cultures (value in brackets).

¶ Underlined values, lowest stimulation with each antigen.

** Residual response (%), 100 (Δcpm of cells precultured with antigen/Δcpm of cells precultured without antigen).

component and will consider inhibition significantly in excess of that as reflecting the cross-reactivity of the two antigens.

The experiment reported in Table III illustrates the effect of culturing PELs from strain 2 guinea pigs, immunized with DNP-H37, with a series of DNP conjugates and with PPD. The important point illustrated by the data in this table is that preculturing with DNP-KLH, DNP-GLA^{30}, or DNP-L-GAT reduces the subsequent response to the antigen initially used to a greater extent than it does the response to any of the other DNP conjugates, indicating that specific subpopulations of cells respond to each of these DNP conjugates. This is especially well illustrated by the responsiveness of cells precultured with DNP-GLA^{30} and exposed to BUdR and light. Their subsequent responses to DNP-GLA^{30} is 7.6% of control values but their response to DNP-KLH and DNP-L-GT are very well maintained. On the other hand, the responses of cells precultured with DNP_8-KLH and with DNP-L-GAT indicate a degree of cross-reaction between DNP-KLH and DNP-GLA^{30} and between DNP-L-GAT and DNP-GLA^{30}, respectively. However, cells precultured with these antigens in this experiment also show reduced responsiveness to the unrelated antigen PPD, so the data must be interpreted with caution.

The experiment presented in Table III involved culturing PELs, a mixture of macrophages and lymphocytes, primarily T cells, with continuous antigen in both the first and second culture. Since microphages or other adherent PECs are critical to the activation of T cells it is formally possible that the negative selection observed acted on the adherent PECs, rather than on the T lymphocyte. Therefore, the experiments were repeated, using PELs from strain 2 guinea pigs primed with DNP-H37. These cells were cultured initially with antigen, treated with BUdR and light, and recultured with added, mitomycin C-inactivated PECs from either H37- or DNP-H37-primed syngeneic guinea pigs. The results are given in Table IV. It can be seen that the results are very similar to those in Table III. These data again indicate that the population of cells responding to one DNP conjugate is at least partially independent of those

TABLE IV

The Effect of Preculturing PELs from DNP-H37-Primed Strain 2 Guinea Pigs with Various Antigens and Treatment with BUdR and Light

Stimulating antigen*	Stimulation of PELs after preculture with:‡					
	0	PPD	DNP-KLH	TNP-KLH	DNP-GLA^{30}	DNP-L-GAT
In the presence of PECs from H37-primed guinea pigs						
None	(289)§	(2,1118)	(623)	(1,410)	(1,155)	(2,874)
PPD	[49,982]‖	<u>6.4</u>¶	52.1	68.4	69.1	68.2
DNP-KLH	[23,075]	56.2	<u>5.1</u>	44.7	35.3	55.0
TNP-KLH	[12,923]	47.4	15.0	<u>7.3</u>	44.3	95.4
DNP-GLA^{30}	[18,359]	48.1	13.6	51.6	<u>10.2</u>	57.5
DNP-L-GAT	[10,996]	64.9	44.4	59.8	50.4	<u>16.8</u>
In the presence of PECs from DNP-H37-primed guinea pigs						
None	(537)	(2,150)	(1,160)	(1,498)	(1,469)	(3,655)
PPD	[39,369]	<u>4.7</u>	72.3	77.4	86.2	101.1
DNP-KLH	[20,479]	64.1	<u>5.2</u>	33.3	31.1	63.4
TNP-KLH	[13,892]	47.0	16.3	<u>11.4</u>	32.2	85.6
DNP-GLA^{30}	[16,872]	70.2	19.8	49.1	<u>12.6</u>	57.1
DNP-L-GAT	[10,510]	92.3	58.6	76.0	54.9	<u>15.5</u>

* Antigen concentrations for primary and secondary stimulation: PPD, 20 μg/ml; TNP-KLH, 50 μg/ml; all others, 100 μg/ml.

‡ PELs cultured with antigen for 2 days, then made to 3×10^{-6} M with BUdR for 1 day, followed by 90-min illumination. PELs washed four times, mixed 1:1 with fresh, mitomycin C-inactivated PECs, and recultured.

§ Uptake of ^{3}H-TdR (cpm).

‖ Net uptake of ^{3}H-TdR [Δcpm].

¶ Residual response (%); lowest stimulation with each antigen is underlined.

responding to a second. In these experiments, it appears that the cells responsive to DNP-GLA^{30} and DNP-L-GAT are largely independent, while a degree of cross-reactivity between DNP-KLH and DNP-GLA^{30} clearly exists. In addition, these data strongly suggest that T cells from animals primed with DNP-H37 can discriminate between DNP and TNP on the same carrier. Thus, preculture with TNP-KLH diminishes the response to DNP-KLH to only a modest degree, although it eliminates responsiveness to TNP-KLH. Preculture with DNP-KLH lowers the response to DNP-KLH to a greater extent than it lowers the response to TNP-KLH, though the latter is quite strikingly reduced.

In order to explore the nature of T-lymphocyte cross-reactivity more fully, we studied responses to DNP derivatives of related polymers. DNP-L-GLAT resembles both DNP-L-GAT and DNP-L-GLA^{30} chemically. It would therefore be expected that preculturing immune T cells with DNP-L-GLAT would reduce responses to all three of these conjugates. The experiments shown in Table V confirm these predictions. Preculturing with DNP-GLAT diminished the subsequent response to DNP-GLAT, DNP-GAT, and DNP-GLA^{30} to essentially the same extent. Similarly, this type of analysis showed tht preculturing with DNP-GAT diminished responses to DNP-GT and to the closely related polymer, DNP-TGA to the same degree as the response to DNP-GAT was inhibited. On the other hand, these experiments again show that cells responding to DNP-GAT

TABLE V
T-Cell Cross-Reactivity between DNP-L-GLA[30], DNP-L-GAT, and DNP-L-GLAT in Strain 2 Guinea Pigs Immunized with DNP-H37

Stimulation antigen*	Stimulation of BUdR- and light-treated PELs precultured with:‡				
	0	PPD	DNP_{12}-L-GAT	DNP_{14}-L-GLA^{30}	DNP_{13}-L-GLAT
Exp. 1					
None	(265)§	(685)	(731)	(274)	(243)
PPD	[59,729]‖	1.5¶	95.4	68.1	82.5
DNP_{12}-L-GAT	[8,160]	60.1	9.4	45.6	5.7
DNP_{14}-L-GLA^{30}	[11,446]	46.2	50.4	1.3	3.7
DNP_{13}-L-GLAT	[12,883]	57.1	74.8	22.2	4.2
Exp. 2					
None	(506)	(1,130)	(1,512)	(315)	(624)
PPD	[20,626]	16.4	83.5	69.6	94.8
DNP_{12}-L-GAT	[5,653]	80.0	33.3	90.1	38.7
DNP_{14}-L-GLA^{30}	[2,070]	60.6	113.3	36.4	22.0
DNP_{13}-L-GLAT	[2,254]	95.2	108.7	105.8	26.1
DNP_{16}-L-GLA^{60}	[1,806]	82.9	164.1	43.9	23.4
DNP_{24}-L-GT	[1,766]	110.0	35.8	96.4	40.9
DNP_{7}-L-TGA	[2,642]	80.4	33.7	98.8	31.6

* Antigen concentration for primary and secondary cultures: PPD, 20 μg/ml; all others 100 μg/ml.

‡ PELs cultured for 2 days with the antigen shown across the top of the table, then made to 3×10^{-6} M BUdR, incubated a further 24 h, and lighted for 90 min. The cells were washed four times, mixed 1:1 with PECs from a normal strain 2 guinea pig, which had been inactivated with 50 μg/ml mitomycin C and washed four times; the mixture was recultured with the antigens shown in the vertical column.

§ Uptake of ^{3}H-TdR (cpm).

‖ Net uptake of ^{3}H-TdR [Δ cpm].

¶ Residual response (%); Underlined values, highly significant inhibition of response to secondary stimulation.

are distinct from those which respond to DNP-GLA[30]. These results indicate that T cells discriminate between DNP-Lys and DNP-*O*-Tyr residues even when the rest of the carrier is quite similar, as is true of DNP-GAT and DNP-GLA[30] which share L-glutamic acid and L-alanine.

Similar experiments have been carried out using PELs from strain 13 guinea pigs primed with DNP-H37. The results are given in Table VI. It can again be seen that the lowest response to each antigen is always given by cells precultured with that antigen. In these experiments, we have included a series of nitrophenyl-protein conjugates. It appears that PELs from DNP-H37-primed donors do display a partial cross-reactivity between these conjuates. For example, preculturing with DNP-OVA decreases responses to DNP-GPA and DNP-KLH. Furthermore, as was found prevously, preculturing with DNP proteins lowers the response to TNP protein to a much more considerable degree than is true of the response to DNP proteins of cells precultured with TNP proteins.

From the data presented in this section, it seems safe to conclude that the capacity of cells from DNP-H37-primed donors to respond to different DNP conjugates is based, at least in part, on the capacity of each conjugate to stimulate independent clones of cells. Since the hapten is the same in all

TABLE VI

T-Cell Cross-Reactivity between Various DNP Proteins in Strain 13 Guinea Pigs Immunized with DNP-H37

Stimulating antigen*	Stimulation of BUdR- and light-treated PELs precultured with:‡						
	0	PPD	DNP$_8$-OVA	DNP$_{12}$-GPA	TNP$_{33}$-GPA	DNP$_8$-KLH	DNP$_{12}$-L-GAT
Exp. 1							
None	(309)§	(737)	(343)	(367)	(450)	(522)	–
PPD	[39,616]‖	3.5¶	97.3	120.6	92.1	108.2	–
DNP$_8$-OVA	[8,147]	44.2	7.3	25.1	62.9	19.6	–
DNP$_{12}$-GPA	[5,169]	41.7	17.4	6.0	51.2	30.6	–
TNP$_{33}$-GPA	[4,423]	81.8	36.4	42.1	12.5	56.2	–
DNP$_8$-KLH	[6,423]	44.9	24.9	30.9	62.9	12.0	–
Exp. 2							
None	(1,014)	(4,480)	–	(2,971)	(3,799)	(2,680)	(5,920)
PPD	[60,218]	11.9	–	155.2	154.6	112.7	134.9
DNP$_8$-OVA	[14,584]	61.8	–	62.4	83.5	54.6	85.6
DNP$_{12}$-GPA	[8,479]	67.2	–	25.7	76.1	101.8	133.1
TNP$_{33}$-GPA	[8,199]	79.0	–	28.8	0	18.5	49.4
DNBS$_{12}$-GPA	[7,727]	75.9	–	27.6	58.1	67.4	84.4
DNP$_8$-KLH	[13,649]	32.8	–	139.7	47.1	19.3	43.2
DNP$_{12}$-L-GAT	[17,989]	164.3	–	126.6	106.0	76.6	28.1
DNP$_{14}$-L-GLAT	[15,585]	132.3	–	115.8	109.7	92.4	44.1
DNP$_{24}$-L-GT	[5,796]	172.2	–	74.7	103.1	30.0	0

* Antigen concentration in primary and secondary cultures: PPD, 20 μg/ml; all others, 100 μg/ml.

‡ PELs precultured with the antigens shown across the top of the table for 2 days, then made to 3×10^{-6} M with BUdR and cultured for one more day. Cells then treated 90 min with light, washed four times, mixed 1:1 with normal strain 13 PECs treated with 50 μg/ml mitomycin C for 30 min and washed four times. The mixture was recultured with the antigens shown in the vertical column.

§ Uptake of ^{3}H-TdR (cpm).

‖ Net uptake of ^{3}H-TdR [Δ cpm].

¶ Residual response (%). Underlined values, lowest response to secondary antigen.

instances, this distinction must be based on the recognition of determinants involving both hapten- and carrier-, or conjugate-specific determinants.

The effect of DNP-Specific Tolerance on T-Cell Priming with DNP-H37. The characteristics of the specificity of responses of DNP conjugates of T lymphocytes from DNP-H37-primed donors indicates that the binding site of the T-cell receptor recognizes an extensive antigenic determinant of which the hapten is an essential part. However, since populations of T cells responsive to individual DNP conjugates are partially independent, the degree of contribution of the hapten is uncertain. An alternative approach to assess the contribution of the haptenic group is to attempt to inhibit responses in a hapten-specific manner either by induction of tolerance with DNP derivatives of tolerogenic carriers or by blocking activation with DNP conjugates which themselves are not stimulatory. Tolerance induction was attempted with the DNP derivatives of the copolymer of D-glutamic acid and D-lysine (DNP-D-GL) and with the DNP derivative of the copolymer of D-tyrosine, D-glutamic acid and D-alanine (DNP-D-TGA). DNP-D-GL has previously been shown to induce a profound state of B-cell tolerance in guinea pigs and mice (10–12) and D-TGA is highly tolerogenic in mice (13). DNP-D-GL (10 mg) or DNP-D-TGA (7.5 mg) was administered over a 5 day period to strain 13 guinea pigs and 3 days later the animals and controls were immunized with DNP-H37. Antibody responses measured 3–5 wk later indicated a profound inhibition in the anti-DNP response of the tolerized animals, as determined by binding of ^{3}H-ϵ-DNP-L-lysine and, in the case of DNP-D-

TABLE VII

Effect of Pretreatment of Strain 13 Guinea Pigs with DNP-D-GL on the Cellular and Humoral Response to DNP-H37 Immunization

Stimulating antigen	Stimulation (cpm, E-C) at various times after DNP-H37 immunization			
	22 Days after priming		35 Days after priming	
	Control	DNP-D-GL treated*	Control	DNP-D-GL treated
(100 μg/ml)				
None	(525)	(1,846)	(4,033)	(2,831)
PPD‡	14,520	26,900	56,036	43,436
OVA	37	184	86	0
DNP_7-OVA	6,218	8,912	13,936	12,735
DAG_7-OVA	67	41	591	437
DNP_{12}-GPA	3,431	10,612	12,831	15,221
DAG_{14}-GPA	87	0	325	116
KLH	–§	0	31	427
DNP_6-KLH	10,484	13,530	30,439	30,760
DNP_7L-GL	193	393	1,341	620
L-GLT	–	–	1,272	1,001
DNP_{44}-L-GLT	4,690	13,400	29,354	26,143
DNP-L-TGA	–	–	33,019	38,063
Serum anti-DNP	690‖	0.25	2,500	
Antibody titer	900	0.25	3,700	1.0
(ABC33 × 10^{-8} M)	1300		7,500	

* 2 mg DNP-D-GL injected intraperitoneally daily for 5 days; priming with DNP-H37 3 days later.
‡ 1 μg/ml.
§ –, not done.
‖ Values for individual animals whose cells were used in the in vitro testing.

TGA-tolerized animals, of the binding of ^{125}I-DNP-L-TGA. The proliferative response to a variety of DNP conjugates of cells harvested from DNP-D-GL-tolerized and control immune animals as essentially the same (Table VII). This experiment has been repeated several times and no evidence of DNP-specific T-cell tolerance has been obtained using DNP-D-GL.

The results obtained using PELs from animals pretreated with DNP-D-TGA are somewhat less clear. Proliferative responses to PPD are similar in the tolerant and control cells; PELs from the tolerized donors display a diminished response to all the DNP derivatives tested although the degree of diminution is variable and in some instances is very modest (Table VIII).

Failure to Inhibit In Vitro Proliferative Responses with Nonstimulatory DNP Compounds. The other "inhibition" technique which might allow the demonstration of a high affinity receptor for DNP on T cells from animals primed with DNP-H37 would be to culture the cells in the presence of nonstimulatory DNP-containing compounds. As has been pointed out previously (9), this analysis is complicated because such inhibitors can block the binding of antigen to cytophilic antibody on macrophages. To circumvent this problem, antigen in these experiments is presented in the form of antigen-pulsed, mitomycin C-treated

TABLE VIII

Induction of Tolerance by Injection of DNP-D-TGA before Immunization of Strain 13 Guinea Pigs with DNP-H37

Stimulating antigen	Stimulation (CPM, E-C) of PELs from:			
	21 Days after priming		24 Days after priming	
	Control	DNP-D-TGA treated*	Control	DNP-D-TGA treated
100 μg/ml				
None	(3,189)	(2,245)	(2,456)	(4,665)
PPD‡	28,549	21,287	35,857	30,940
DNP_7-OVA	18,937	5,808	23,212	10,960
DAG_7-OVA	18	173	0	479
DNP_{12}-GPA	12,821	3,962	11,844	4,947
$DNBS_{12}$-GPA	19,178	4,249	21,096	4,709
DAG_{14}-GPA	941	0	828	0
DNP_9-KLH	20,258	8,023	33,313	15,026
DNP_{14}-L-GL	0	0	0	0
DNP_7-L-TGA	20,258	7,923	31,946	23,570
DNP_{10}-D-TGA	7,053	1,249	8,717	2,331
DNP_{44}-L-GLT	17,746	6,294	19,301	14,569
DNP_{17}-L-GT	6,319	4,311	8,365	1,592
DNP_{42}-L-GLA_{40}	–	–	5,488	4,432
DNP_{21}-L-GLAT	–	–	28,006	22,908
Anti-DNP antibody§ titer (ABC × 10^{-8} M)	28, 22	1.8	21, 30	0.3
Anti-DNP-L-TGA antibody‖ titer (μg bound/ml)	44, 45	0.7	77, 47	0.2

* 1.5 mg DNP_6-D-TGA intraperitoneally in saline daily for 5 days; priming with DNP-H37 5 days later.

‡ 1 μg/ml.

§ Titered with 0.5 × 10^{-8} M ^{3}H-DNP-L-Lysine as ligand.

‖ Titered with 0.5 μg/ml ^{125}I-DNP-L-TGA as ligand.

PECs. Thus, any inhibition seen should reflect a blocking of the T-cell receptor. A further problem in such experiments is the nonspecific toxicity of the inhibitors. To control for this, the effect of the inhibitors on the response of PELs from DNP-H37 primed guinea pigs to PECs pulsed with PPD has also been measured. Data from two representative experiments are presented in Tables IX and X. The experiments in Table IX principally involves nonstimulatory DNP derivatives of proteins and polymers. These compounds are DNP-β-alanyl-glycyglycyl (DAG) derivatives of OVA and GPA and DNP conjugated to the copolymer of L-glutamic acid and L-lysine (DNP-L-GL). These compounds neither stimulate response nor do they block responses to other DNP derivatives. In Table X, the results obtained with ε-DNP-L-lysine, mono-*O*-DNP-L-tyrosine and di-*O*,*N*-DNP-L-tyrosine are shown. The highest concentrations of both tyrosine derivatives are inhibitory but they inhibit responses to PPD as well as to DNP-L-TGA and DNP-KLH. At lower concentrations, none of the derivatives is markedly inhibitory. Thus, inhibition experiments offer no evidence in favor of a T-cell receptor with a binding site with high affinity for the DNP group itself.

Discussion

This paper has two principal findings. The first is the high precision with which T lymphocytes discriminate similar antigenic determinants; the second is

TABLE IX

Attempt to Inhibit the Response of PELs from Strain 13 Guinea Pigs Primed with DNP-H37 to Antigen-Pulsed Macrophages

Inhibitor	Inhibitor concentration	Response (cpm, E-C) to PECs pulsed with:*				
		0	PPD	DNP_8-OVA	DNP_{12}-GPA	DNP_9-KLH
	μg/ml					
None	–	(975)	8,662	6,122	7,171	6,670
PPD	1	18,876	24,374 (11)‡	26,096 (−4)	29,526 (−13)	30,362 (−19)
DNP_7-OVA	100	3,485	9,013 (26)	8,175 (15)	9,712 (9)	9,645 (5)
DAG_7-OVA	1,000	−19	6,261 (28)	4,466 (27)	5,840 (18)	3,831 (42)
DNP_{12}-GPA	100	4,794	12,749 (5)	9,546 (13)	11,805 (1)	9,483 (17)
DAG_{14}-GPA	1,000	−17	5,887 (32)	4,645 (24)	5,704 (20)	5,617 (16)
DNP_6-KLH	100	14,423	20,176 (13)	21,513 (−5)	28,341 (−31)	16,109 (24)
DNP_{14}-L-GL	1,000	272	7,338 (18)	5,977 (7)	8,212 (−10)	6,239 (10)
ϵ-DNP-L-Lysine	§	87	8,430 (4)	6,404 (−3)	7,111 (2)	4,238 (37)

* DNP-H37 immune PECs cultured with 50 μg/ml mitomycin C and either no antigen, 4 μg/ml PPD, 4 mg/ml DNP_8-OVA, 4 mg/ml DNP_{12}-GPA, or 4 mg/ml DNP_9-KLH for 30 min at 37°C, then washed four times and added 1:1 to PELs in the presence of the inhibitor.

‡ Percent inhibition calculated from the expected response given by the sum of the response to the inhibitor in cultures with unpulsed macrophages plus that given by PELs and pulsed macrophages in the absence of inhibitors, divided into the response to pulsed macrophages in the presence of the inhibitor. Negative values indicate that in the presence of inhibitor the response was greater than expected.

§ 10^{-5} M

TABLE X

Attempt to Inhibit the In Vitro Proliferative Response of Strain 13, DNP-H37 Immune Peritoneal Exudate Lymphocytes to DNP Conjugates with DNP-Amino Acids

Inhibitor	Inhibitor concentration	Response (cpm, E-C) and inhibition of response (% in parentheses) to PECs pulsed with various antigens*			
		0	PPD	DNP_1-L-TGA	DNP_9-KLH
	μM				
None	–	(489)	2,160	3,045	3,659
ϵ-DNP-L-lysine	160	−100	1,901 (12)	2,414 (21)	3,095 (15)
ϵ-DNP-L-lysine	16	23	2,658 (−23)	3,895 (−28)	3,756(−3)
ϵ-DNP-L-lysine	1.6	24	2,059 (5)	3,657 (−20)	4,470 (−22)
Mono-*O*-DNP-L-tyrosine	140	394	942 (56)	2,047 (33)	1,977 (46)
Mono-*O*-DNP-L-tyrosine	14	305	2,198 (−2)	3,822 (−26)	4,546 (−24)
Mono-*O*-DNP-L-tyrosine	1.4	−8	2,452 (−14)	3,642 (−20)	4,952 (−35)
Di-*O*, *N*-DNP-L-tyrosine	100	248	594 (73)	698 (77)	10 (99)
Di-*O*, *N*-DNP-L-tyrosine	10	409	2,707 (−25)	2,489 (19)	4,571 (−25)
Di-*O*, *N*-DNP-L-tyrosine	1	104	2,636 (−22)	2,308 (24)	4,927 (−35)

* DNP-H37 immune PECs cultured with 50 μg/ml mitomycin C and either no antigen, 2.5 μg/ml PPD, 1.7 mg/ml DNP_1-L-TGA, or 2.5 mg/ml DNP_9-KLH for 30 min at 37°C, then washed four times, and mixed 1:1 with PELs to which inhibitor had already been added. Negative figures in parentheses indicate percent stimulation.

the failure of partial antigenic determinants to block activation of T lymphocytes by fully complementary (i.e., stimulating) determinants.

T cells from guinea pigs primed with DNP coupled covalently to mycobacteria respond to a wide variety of DNP conjugates. However, since not all DNP conjugates will trigger such cells it was postulated that their receptors are not strictly DNP specific (1, 2). Stimulation of such T cells with DNP conjugates accompanied by BUdR and light treatment has demonstrated that their re-

sponses to a wide variety of DNP conjugates rests upon the presence of subpopulations or clones of cells, each responsive to a particular conjugate. Since DNP is required for this activation (1, 2) and is present on all conjugates, this discrimination by T cells must reflect recognition of antigenic determinants composed not only of DNP but also portions of the carrier molecule. In the case of DNP-L-GLA^{30} and DNP-L-GAT, in which DNP, L-glutamic acid, and L-alanine are common to both and which differ primarily in the amino acid to which DNP is linked (lysine in the former and tyrosine in the latter) this discrimination is complete within the limits of the technique (Tables III–V). The experiments in Table VI suggest that diferent DNP proteins also stimulate unique subpopulations of cells, although in this case there is some overlap. Since DNP-Lys residues seem to be part of the active determinants in DNP proteins (2), this cross-reaction may be due either to a subpopulation of cells responding to any DNP-Lys residue or to cross-reacting new antigenic determinants shared amongst the proteins, and perhaps DNP-L-GLA^{30} as well (Tables III, IV, and VI). The former hypothesis seems unlikely since a wide variety of DNP-L-GL conjugates, containing ample DNP-Lys residues, are not recognized by these T cells, nor can DNP-lysine, DNP-L-GL, or DNP-D-GL block their activity (Tables VII, IX, and X). Thus it seems more likely that similar new antigenic determinants are generated by conjugation with DNP on what would seem to be unrelated proteins, namely an avian egg protein (OVA), a mammalian serum protein (GPA), and an invertebrate heme protein (KLH). If this is so, then the description by several authors (14–17) of hapten-specific helper effects, based on comparisons of hapten conjugates of such proteins, may result instead from T cells recognizing such cross-reactive new antigenic determinants. Indeed, in one such instance in which DNP coupled to poly-L-Lys was tested, little or no helper effect was found (17), a result strikingly similar to the failure of DNP-L-GL to activate T cells in the present experiments.

The partial and largely unidirectional discrimination of these T cells between DNP and TNP is best explained by postulating that most TNP-reactive cells also react, to DNP, the immunizing hapten, while a substantial fraction of DNP-reactive cells fail to be stimulated by TNP derivatives. This in turn might result from some but not all DNP-specific sites in the receptor being able to accommodate the bulkier but closely related TNP group. There may also be some T cells that are heterolytic (18) for TNP, in that preincubation with TNP proteins more effectively reduces subsequent responses to TNP proteins than does preincubation with DNP protein (Tables IV and VI).

In any case, these experiments serve to illustrate yet again the great precision with which T cells discriminate between closely related antigenic determinants, all in this instance including the same or closely related haptens. Although this strongly suggests that the T cells being studied here are not strictly speaking hapten specific, they must have at least a portion of their specificity directed at the hapten, since changes in the haptenic group markedly affect the responsiveness of the cells. In order to demonstrate and measure this hapten recognition by T cells, attempts were made to block their responses with DNP-containing nonstimulatory compounds either in vivo (Table VII) or in vitro (Tables IX and X). Little or no inhibition was seen in either situation. However, an apparently specific reduction in response to most of the DNP conjugates was

obtained using in vivo treatment with the weakly stimulatory, slowly catabolizable (19) DNP-D-TGA. Surprisingly, since virtually all the DNP of DNP-D-TGA is coupled to the hydroxyl of D-Tyr residues, this reduction was more consistent for responses to DNP-Lys-containing determinants (i.e., DNBS-GPA) than for DNP-*O*-Tyr-containing determinants (i.e., DNP-L-TGA). DNP-D-TGA, however, did not act as an inhibitor when used in in vitro studies like that in Table X, showing instead stimulatory activity (data not shown) as had been found previously (1). The failure of DNP-D-GL to inhibit the immunization of T cells significantly, while virtually abolishing anti-DNP antibody responses, can be interpreted in a variety of ways. At the least, the results in Table VII would seem to rule out passively absorbed anti-DNP antibody as the T-cell receptor in this system. Also, taken together with the results in Table IX and X, they suggest that either T and B cells recognizing DNP have very different specificities or that T- and B-cell tolerance takes place by different mechanisms. In any case, it seems unlikely that the DNP-reactive T cells we are studying have receptors with high affinity for DNP, as such receptors should be blocked by hapten in nonstimulatory form. However, the data in these inhibition experiments, while suggestive, are essentially negative; unless effective inhibition of some T-cell receptors by such techniques can be demonstrated, they must be interpreted with caution.

On the basis of the data presented in this and the preceding papers (1, 2), a model for the antigen-binding site on the T cell has been proposed and is discussed in detail elsewhere (20). According to this model, the T-cell receptor for antigen consists of a single site, since no evidence for cooperation between sites or cells has been obtained in these studies. The site is composed of multiple subsites, each binding contiguous portions of the hapten-containing antigenic determinant. Quite precise complementation at all subsites would be required for cell activation. Since a subsite specific for hapten appears to exist, but can not be demonstrated by hapten-specific inhibition of the response, a relatively low affinity has been postulated for each subsite, the stability and the precision of the receptor-antigen complex thus deriving from multiple, low affinity interactions with contiguous portions of a single determinant. According to this model, the capacity of lipid-containing haptenated immunogens (such as DNP-H37 and DNP-lipidated-bovine serum albumin) to activate a wide range of T-cell clones with low affinity for the hapten itself is due to stabilizing nonpolar interactions between the cell membrane lipid and immunogen lipid. Ordinary hapten-protein conjugates prime only those cells which bind them in a highly precise manner, and thus prime for responses which can be elicited by the hapten conjugated to the immunizing carrier but not by hapten conjugates of an alternative carrier.

A number of problems remain to be resolved. In particular, the role of macrophages in this system requires further detailed study, as does the role of histocompatibility-linked immune response genes (2), and the possible contributions of cell membrane histocompatibility antigens to the specificities determined (21). Furthermore, the specificity of the functional receptor for antigen on B cells has not been measured in sufficient detail to determine if it can discriminate between antigens with the same precision as the T-cell receptor, although it appears not to do so (22). A particular problem for which there is no satisfac-

tory answer at present is the failure of DNP-H37 to prime cells responding to DNP-L-GL, although priming with DNP-L-GL itself in complete Freund's adjuvant will do so (1). A possible explanation is that the affinity for DNP itself of the T-cell receptor on this latter type of cell is too low for selective activation by DNP-H37 to occur. These and other questions are currently being investigated.

Summary

T lymphocytes from guinea pigs immunized with 2,4-dinitrophenyl (DNP) derivatives of mycobacteria respond to a variety of DNP conjugates. Preincubation of such cells with a given DNP conjugate under conditions which lead to the inactivation of responding cells causes a loss of the response to that conjugate, but has little effect on the response to DNP coupled to unrelated carriers. Thus, the responses of such cells to a variety of DNP conjugates can best be explained by the presence of a mixture of highly specific cells each responding to a different antigenic determinant rather than by the presence of T cells with specificity limited to the hapten itself. Furthermore, the activity of T cells from DNP-mycobacteria-primed donors could not be blocked by a variety of nonstimulatory DNP conjugates. This suggests that while such T cells clearly recognize DNP with great precision, the receptor does not contain a very high affinity site for the hapten. A possible model for such a T-cell receptor is discussed.

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