THE SPECIFICITY OF CELLULAR IMMUNE RESPONSES IN GUINEA PIGS

I. T Cells Specific for 2,4-Dinitrophenyl-*O*-Tyrosyl Residues

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Responses of thymus-derived (T) lymphocytes to hapten-protein conjugates are very largely specific for carrier- and conjugate-specific determinants; such responses rarely show the hapten specificity which predominates in the responses of thymus-independent bone marrow-derived (B) lymphocytes to similar antigens (1). These findings, and similar observations in other antigen systems, have led to the suggestion that T and B cells have distinctive libraries of specific receptors, or perhaps very different requirements for activation (2–4). However, it has not in fact been possible to precisely analyze T-cell receptor specificity, except in a few cases, because simple, well defined chemical determinants can rarely be employed for this purpose. Nevertheless, T-cell responses apparently specific for hapten occur under certain circumstances, as described by Leskowitz et al. (5) in the *p*-azo-benzenearsonate system, and in recent extensions of these studies by Alkan et al. (6). In addition, Benacerraf and Gell (7) reported that immunization of guinea pigs with 2,4,6-trinitrophenylated *Mycobacteria* induced a state of delayed hypersensitivity (DH)[1] which could be elicited by 2,4,6-trinitrophenyl (TNP) coupled to several proteins, and these results have recently been confirmed by Trefts et al.[2]

In the present experiments, the specificity of T lymphocytes has been explored by immunizing inbred guinea pigs to 2,4-dinitrophenyl (DNP) *Mycobacteria*. We confirm that DH responses to a variety of DNP proteins can be obtained in such animals and in addition we show that contact reactivity to 1-fluoro-2,4-dinitrobenzene (DNFB) and in vitro DNA-synthetic and migration inhibition responses are obtained. However, several lines of evidence clearly demonstrate

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[1] *Abbreviations used in this paper:* DAG, DNP-β-alanyl-glycyl-glycyl-azide; D-GL, copolymer of D-glutamic acid and D-lysine; DH, delayed hypersensitivity; DNFB, 1-fluoro-2,4-dinitrobenzene; GPA, guinea pig serum albumin; H37, *Mycobacterium tuberculosis* of strain H37Ra; IFA, incomplete Freund's adjuvant; KLH, giant keyhole limpet hemocyanin; L-GL, copolymer of L-glutamic acid and L-lysine; oNP, orthonitrophenyl; OVA, ovalbumin (chicken egg albumin); PECs, peritoneal exudate cells; PELs, peritoneal exudate lymphocytes; pNP, paranitrophenyl; TGA, copolymer of tyrosine, glutamic acid, and alanine; TGL, copolymer of tyrosine, glutamic acid, and lysine.

[2] Trefts, P. E., and J. W. Goodman. Personal communications.

that even in this system, determinants contributed by regions of the carrier contiguous with the hapten are essential for T-cell activation. In particular, DNP coupled directly to the hydroxyl group of tyrosine appears to be one important determinant in T-cell recognition.

Materials and Methods

Animals. Guinea pigs of the inbred strains 2 and 13 were obtained from the Animal Production Section, NIH, Bethesda, Md., and were used between 3 and 6 mo of age.

Antigens. Ovalbumin (OVA) was obtained as five times crystallized hen's egg albumin from Pentex Biochemical, Kankakee, Ill. Guinea pig albumin (GPA) was prepared from normal guinea pig serum as described by Schwert (8). Giant keyhole limpet hemocyanin (KLH) was purchased in dry form from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. A variety of linear synthetic polypeptides were used; their composition, molecular weight, and the abbreviations used in the text are given in footnote 1 and Table I. The copolymer of L-glutamic acid and L-lysine (L-GL)

TABLE I

Composition of Synthetic Antigens Used in these Studies

Linear copolymer	Molar proportions of amino acid residues in the polypeptide	Molecular weight
L-GL	6:4	40,000
D-GL	6:4.1	40,600
L-TGL	1:10.4:6	61,000
L-TGA	1:6.4:5.7	23,000
D-TGA	1:5.8:5.1	33,800

and copolymer of D-glutamic acid and D-lysine (D-GL) were obtained from Pilot Chemicals, Inc., Waltham, Mass. The other polypeptides were a kind gift of Professor Michael Sela, The Weizmann Institute for Science, Rehovot, Israel, and have been described fully elsewhere (9). Killed *Mycobacterium tuberculosis* of strain H37Ra (H37) was purchased from Difco Laboratories, Detroit, Mich. DNP proteins and polypeptides were prepared with DNFB (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) as previously described (3). OVA and GPA were also conjugated with DNP-β-alanyl-glycyl-glycyl-azide (DAG) by the method of Inman et al. (10). KLH and OVA were conjugated with 1-fluoro-2-nitrobenzene, 1-fluoro-4-nitrobenzene, picryl chloride (all from Eastman Organic Chemicals Div., Eastman Kodak Co.), and 1-chloro-2,6-dinitrobenzene (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) to yield orthonitrophenyl (*o*NP), paranitrophenyl (*p*NP), TNP, and 2,6-DNP proteins. Conjugation ratios (moles hapten per mole protein) were calculated from spectrophotometric and Kjeldahl nitrogen determinations and are denoted subsequently by a subscript (e.g., $oNP_{1.1}$-KLH). Dried H37 were ground in a mortar and pestle with 1% K_2CO_3 and conjugated with 8 μl of DNFB dissolved in 1,4-dioxane/100 mg H37 to produce DNP-H37; the mixture was dialysed repeatedly against a large volume of 0.01 M potassium phosphate buffer, pH 7.4. No attempt was made to measure conjugation ratio. ϵ-DNP-L-lysine, mono-*O*-DNP-L-tyrosine, and di-*O,N*-DNP-L-tyrosine were purchased from Nutritional Biochemical Corp., Cleveland, Ohio.

Thiolysis of DNP-Synthetic Polypeptides. Some DNP conjugates of synthetic antigens were treated with 2.5 M 2-mercaptoethanol at pH 8.0 in 0.25 M potassium phosphate buffer for 8 h at room temperature to remove DNP coupled to the hydroxyl group of tyrosine. *S*-DNP-2-mercaptoethanol was recovered by dialysis against a small volume of 0.01 M potassium phosphate buffer, and the amount produced determined by spectrophotometric measurement as described by Shaltiel (11). The

compounds were then thoroughly dialysed against large volumes of the same buffer, and aliquots either tested directly or reconjugated with DNFB, dialysed again, and tested.

Immunization: DNP-proteins and DNP-L-GL were emulsified in incomplete Freund's adjuvant (IFA, Difco Laboratories) at 250 μg/ml to which 2.5 mg/ml H37 had been added; 0.1 ml was injected into each foot pad. DNP-H37 or H37 were emulsified in IFA at 2.5 mg/ml and 0.1 ml injected into each foot pad, so that all animals received a total dose of 1 mg H37 (for DNP-proteins) or DNP-H37.

In Vitro Stimulation with Antigen. Peritoneal exudate lymphocytes (PELs) were purified from oil-induced peritoneal exudates, as previously described (12–14). 4×10^6 PELS were cultured in 0.2 ml of medium RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 5 U/ml each of penicillin and streptomycin, 2 mM L-glutamine, 0.015 M HEPES buffer and 10% fetal bovine serum in Mictotiter U plates as previously described (12), and 20 μl of antigen or medium added to triplicate culture wells. PELs were pulsed after 2 days in culture with 1 μCi[^{3}H]thymidine (New England Nuclear, Boston, Mass., 6.7 Ci/mol), harvested 1 day later with a Harrison (15) microharvester, and counted. Means and standard errors for incorporation of ^{3}H in triplicate culture wells were calculated. Standard errors rarely exceeded 20% and have been omitted from the tables. Data in all cases is expressed as average counts per minute (cpm) incorporated in experimental (i.e., antigen containing) wells minus cpm in control (i.e., no antigen) wells (E-C). The value of the control is given in parentheses in each table. Stimulation greater than twice control values has been arbitrarily considered significant and such values have been underlined in the tables. The data presented comes from representative experiments; all the experiments have been performed on several occasions with comparable results.

Nylon Wool Columns. In some experiments, PELs were suspended in complete medium with 5% fetal bovine serum and passed over washed nylon wool (Leukopak, Fenwal Laboratories, Inc., Morton Grove, Ill.) columns according to the technique of Julius et al. (16). These cells were either cultured directly or mixed 1:1 with peritoneal exudate cells (PECs) which had been treated with 50 μg/ml Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) for 30-min at 37°C and washed four times. They were also tested for immunoglobulin-positive cells by direct staining with a fluorescein conjugate of rabbit antiguinea pig immunoglobulin.

Inhibition of Macrophage Migration. Inhibition of macrophage migration was performed directly using PECs as previously described (17). Results are expressed as percent of control migration plus or minus one standard error.

Skin Testing. Immune and normal guinea pigs were shaved and 50 or 100 μl of antigen in phosphate-buffered saline were injected into several sites. Skin tests were measured at 4 and 24 h. Results of 24-h skin reactions are given as the mean diameter of induration and erythema in millimeters. 25 μl 0.5% DNFB in 1:1 acetone:olive oil was spread in a 2 x 2-cm square to test for contact sensitivity. Results were read from 0 to 4+ for degree of erythema and induration.

Serological Studies. Antibody to DNP was measured using a modified Farr assay (3) with [^{3}H]ε-DNP-L-lysine at 0.5×10^{-8} M as ligand. Inhibition studies were carried out in the same system, using a dilution of antiserum that bound 50–70% of the ligand, and an equal volume of the inhibitor. Data from these experiments are expressed as I_{50}, the molar hapten concentration required for 50% inhibition of maximum binding of DNP-lysine.

Results

Carrier Specificity in the Proliferative Response of Guinea Pigs Primed with DNP-Protein Conjugates. Strain 2 guinea pigs were immunized with a variety of DNP-carrier conjugates. PELs from these animals were cultured with various proteins and DNP-protein conjugates, and incorporation of tritiated thymidine measured. The results given in Table II manifest the predominant carrier or conjugate specificity characteristic of T-cell responses. PELs from all the animals respond well to purified protein derivative of tuberculin as expected. PELs from guinea pigs immunized with DNP-OVA, DNP-KLH, and DNP-L-GL respond optimally to the conjugate used for immunization and almost equally

TABLE II

In Vitro Proliferative Responses of PELs of Strain 2 Guinea Pigs Primed with DNP-Proteins

Stimulating antigen	Stimulation (CPM, E-C) after priming with:			
	DNP_8-OVA	DNP_6-KLH	DNP_{7}-L-GL	DNP_{22}-GPA
100 μg/ml				
0	(5,328)*	(5,978)	(1,300)	(2,450)
PPD‡	104,099	120,283	74,474	113,174
OVA	139,552	856	64	0
DNP_8-OVA	149,203	1,265	1,661	15,425
KLH	4,204	82,151	53	0
DNP_6-KLH	4,666	114,324	1,181	6,799
DNP_{22}-GPA	5,232	830	2,354	42,141
DNP_{7}-L-GL	2,269	0	148,042	237

* The value of control is given in parentheses in each table.

‡ 1 μg/ml.

§ Underlined values in each table represent stimulation greater than twice control values.

well to the unconjugated homologous carrier. By contrast, DNP conjugates of unrelated carriers stimulate very meager responses, as has been previously observed (18). There is however a significant response to DNP-OVA and DNP-KLH by PELs from the DNP-GPA-immunized donor. This result was obtained with cells from some but not all guinea pigs primed with this conjugate and requires relatively high concentrations of antigen for its elicitation. It is presented here to demonstrate that this type of response can occasionally be generated by conventional DNP-protein immunization.

Hapten Specificity in the Responses of T Cells from Guinea Pigs Immunized with DNP Coupled Directly to Mycobacterium Tuberculosis. While T-cell responses to DNP conjugates of proteins unrelated to the immunizing DNP conjugate are generally weak or undetectable, substantial responses to DNP-proteins are regularly obtained with PELs from guinea pigs immunized with DNP-H37. Table III presents representative experiments using PELs from guinea pigs immunized with H37 and DNP-H37. Significant in vitro proliferative responses are elicited by DNP-KLH, DNP-OVA, and DNP-GPA. These responses are not obtained when unsubstituted carriers are used, nor are DNP-proteins stimulatory if the guinea pig has been immunized with *M. tuberculosis* (H37) alone. Thus the DNP group is clearly essential for these responses.

PELs are rich in highly active T cells and contain relatively few immunoglobulin-bearing lymphocytes (13, 14). To test the possibility that the response of PELs to DNP-proteins in vitro is a function of the few B cells present, PELs were further purified by passage over nylon wool columns, reducing the frequency of immunoglobulin-bearing lymphocytes in the experiment shown in Table III from 5% to 1%. It can be seen that this increased or left unchanged the response of PELs to DNP-proteins, providing strong evidence that this response is not due to

TABLE III

In Vitro Proliferation of PELs and Delayed Skin Reactions in Guinea Pigs Immunized with DNP-H37 or H37

Stimulating antigen*	Stimulation of PELs (CPM, E-C) after priming with:					24-h skin reaction (average diameter, mm) in strain 2 guinea pigs after priming with:	
	Strain 13			Strain 2			
	H37	DNP-H37		H37	DNP-H37	H37	DNP-H37
		Prenylon‡	Postnylon§				
0	(2,002)	(4,003)	(3,028)	(1,809)	(1,361)	—‖	—
PPD¶	25,691	56,036	53,137	127,797	95,051	17	14
OVA	415	86	—	0	0	0	0
DNP_7-OVA	252	13,936	27,324	297	6,177	0	9
KLH	243	37	—	99	0	0	3
DNP_9-KLH	0	30,439	30,767	1,662	21,055	2	10
DNP_{12}-GPA	157	12,831	32,857	603	6,861	0	13
DNP_7-L-GL	289	1,341	229	1,282	1,372	0	4

* Antigen concentration in vitro, 100 μg/ml; antigen amount in skin test, 50 μg/site.
‡ 5% Ig-positive lymphocytes.
§ 1% Ig-positive lymphocytes.
‖ Not tested.
¶ PPD used at 10 μg/ml in vitro, and at 2 μg/site for skin tests.

a small number of hapten-specific B cells. Moreover, skin tests performed on identically primed strain 2 guinea pigs at the time PELs were harvested showed an excellent concordance between stimulation in vitro and delayed hypersensitivity, further strengthening the contention that these responses represent the activity of specific T lymphocytes. The skin tests were repeated using strain 13 guinea pigs immunized with DNP-H37 with comparable results (not shown), and in addition, skin painting such animals with DNFB led to strong contact reactions at 24 h; animals primed with H37 did not respond to DNFB.

Although PELs from guinea pigs immunized with DNP-H37 respond to DNP conjugated to a variety of unrelated protein carriers, DNP-L-GL does not cause PELs from these animals to proliferate in vitro nor will it elicit delayed hypersensitivity reactions, although strain 2 guinea pigs are genetic responders to DNP-L-GL (Table II). These results have been confirmed with several preparations of DNP-L-GL at differing conjugation ratios and at a variety of antigen concentrations. Furthermore, neither DNP-D-GL nor several preparations of DNP-Ficoll elicit proliferative responses in PELs from DNP-H37-primed donors. Thus, while PELs from guinea pigs immunized with DNP-H37 show specificity for many DNP-proteins, they do not respond to DNP on all carriers.

The role of DNP-O-Tyrosyl Residues in the Response of DNP-H37 Immune T Cells. DNFB can react not only with amino groups, but also with the hydroxyl

group of tyrosine, the imidazole of histidine, and the sulfhydryl group of cysteine. In DNP-L-GL, DNP substitution is confined to the ϵ-amino groups of lysine and to the free α-amino group because of the absence of other reactive amino acids. The fact that this conjugate is not stimulatory suggests that DNP derivatized to tyrosine, histidine, or cysteine may be critical for activation of DNP-H37 immune PELs. The role of DNP-*O*-tyrosyl residues was explored using DNP conjugates of synthetic polypeptide carriers containing tyrosine. The results are shown in Table IV. The tyrosine-containing polymers (tyrosine-GL and TGA) prove to be excellent carriers for DNP in the stimulation of PELs from

TABLE IV

Role of DNP-O-Tyrosyl Residues in the In Vitro Stimulation of PELs from Strain 13 Guinea Pigs Immunized with DNP-H37

Stimulating antigen	Stimulation of PELs (cpm, E-C) from guinea pigs immunized with:				
	Exp. 1		Exp. 2		
			H37	DNP-H37*	
	H37	DNP-H37		Prenylon‡	Postnylon‡
100 μg/ml					
0	(636)	(957)	(1,013)	(630)	(833)
PPD§	24,321	24,532	29,399	27,308	27,083
OVA	220	107	0	0	0
DNP_7-OVA	82	13,435	31	5,056	5,346
DAG_7-OVA	60	474	223	449	288
DNP_{12}-GPA	−38	10,280	155	7,881	9,395
DAG_{14}-GPA	−38	602	0	440	134
DNP_7-L-GL	159	323	42	898	659
L-TGL	360	661	0	1,004	1,775
DNP_{44}-L-TGL‖	1,901	14,683	113	5,622	6,961
Thiolysed DNP_{42}-L-TGL	1,697	2,263	302	2,581	2,906
Re-DNP_{68}-L-TGL¶	1,659	6,263	1,455	5,441	5,917
L-TGA	78	155	167	57	0
DNP_1-L-TGA**	852	16,990	567	9,260	10,356
Thiolysed $DNP_{0.2}$-L-TGA	251	39	178	46	0
Re-DNP_1-L-TGA	731	11,090	303	6,950	9,124
D-TGA	189	299	—	—	—
DNP_2-D-TGA‡‡	738	5,619	—	—	—
Mono-O-DNP-L-tyrosine§§	95	100	—	—	—
Di-O,N-DNP-L-tyrosine§§	−68	459	—	—	—

* H37-primed mitomycin C-treated PECs mixed 1:1 with PELs.

‡ Percent Ig-positive lymphocytes: 3% prenylon wool column, 1% postnylon wool column.

§ 1 μg/ml.

‖ 9 DNP-*O*-tyrosyl residues per mole by thiolysis.

¶ Reconjugation of the compound with DNP after thiolysis.

** 6 DNP-*O*-tyrosyl residues per mole by thiolysis.

‡‡ 9 DNP-*O*-tyrosyl residues per mole by thiolysis.

§§ 10 μg/ml.

DNP-H37-primed animals. Thus, DNP-L-TGA, DNP-D-TGA and DNP-L-TGL are all stimulatory, while DNP-L-GL elicits no response. Furthermore, DNP conjugated to protein using a tripeptide spacer of β-alanyl-glycyl-glycyl (DAG) uniformly fails to stimulate PELs from DNP-H37-primed animals. In these conjugates DNP is coupled only to β-alanyl residues, and no DNP-*O*-tyrosyl residues are present.

It should be noted that the conjugation ratios given in all the tables are based on measurements of absorption at 360 nm which detects DNP-lysyl residues but not DNP-*O*-tyrosyl residues. The number of DNP-*O*-tyrosyl residues can be determined by thiolysing the compound with excess 2-mercaptoethanol which selectively removes DNP from tyrosine while leaving DNP-lysyl residues intact. By this technique, DNP-L-TGA has approximately 6 DNP-*O*-tyrosyl residues per mole, DNP-L-TGL has approximately 9, and DNP-D-TGA has approximately 9. The fact that DNP-L-GL was not stimulatory while a DNP derivative of a polymer differing only in possessing 7% tyrosine (DNP-L-TGL) was stimulatory strongly implicates DNP-*O*-tyrosyl residues in this specific response. That it was the DNP-*O*-tyrosyl residues, and not simply the increased complexity of the carrier, that made DNP-L-TGL and the two other tyrosine-containing polypeptides stimulatory is shown by the lack of stimulation given by the thiolysed compounds, in which the DNP groups have been cleaved from tyrosine. Furthermore, thiolysis did not simply destroy or irreversibly alter the carrier molecules as shown by the ability of thiolysed compounds which have been reconjugated with DNFB to stimulate DNP-H37 immune PELs. That these are T-cell responses has again been confirmed (exp. 2) using PELs passed over nylon wool columns.

Frey et al. have pointed out that DNP-*O*-tyrosyl residues are relatively unstable chemically. They have shown that mono-*O*-DNP-tyrosine may induce immune responses under some conditions but that this ability is due to transconjugation of the DNP groups to other carrier molecules (19). That such a transconjugation might explain the stimulatory activity of DNP-*O*-tyrosyl-containing antigens is therefore a very important consideration. The fact that mono-*O*-DNP-L-tyrosine and di-*O*, N-DNP-L-tyrosine, at concentrations somewhat greater than the concentration of DNP-*O*-tyrosyl residues in DNP-L-TGL, DNP-L-TGA, and DNP-D-TGA used in these experiments, cause no stimulation of DNP-H37 immune PELs (Table IV) is strong evidence that a transconjugation mechanism is not principally responsible for the stimulatory capacity of the latter DNP conjugates.

Inhibition of Macrophage Migration in DNP-H37-Immunized Guinea Pigs. PECs from strain 13 guinea pigs immune to DNP-H37 or H37 were cultured in capillary tubes in the presence or absence of antigen and the antigen-dependent inhibition of macrophage migration determined. The results (Table V) essentially confirm those obtained previously by skin testing and by in vitro proliferation. DNP-protein conjugates and DNP-L-TGA, but neither carrier protein alone, DNP-L-GL, nor DAG-protein conjugates cause inhibition of migration. The excellent correlation between three different assays of T-cell function is further evidence that T-cell specificity is being measured in these experiments.

TABLE V

Inhibition of Migration of PECs from Strain 13 Guinea Pigs Immunized with DNP-H37 or H37

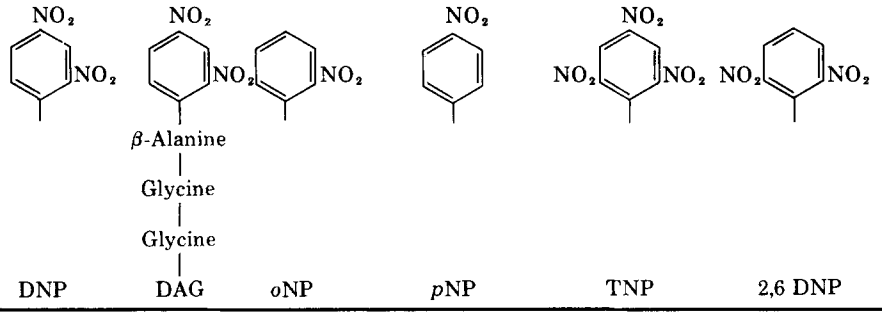
Antigen	Migration (% of control) By PECs primed to:	
	H37	DNP-H37
100 μg/ml		
0	100 ± 8	100 ± 10
PPD	7 ± 1	9 ± 1
OVA	84 ± 9	117 ± 5
DNP_8-OVA	100 ± 9	31 ± 3
DNP_{12}-GPA	124 ± 3	35 ± 4
DAG_{14}-GPA	108 ± 7	91 ± 6
DNP_7-L-GL	113 ± 5	113 ± 4
DNP-L-TGA	80 ± 5	20 ± 3

Effect of Hapten Structure on the Response of DNP-37 Immune T Cells to Hapten-Protein Conjugates. To determine the effect of hapten structure on the in vitro proliferative response of PELs from guinea pigs immunized with DNP-H37, a variety of hapten congeners of DNP were conjugated to KLH and used for stimulation. The results are given in Table VI along with the structures of the various haptens. Serum from the same guinea pigs was used to test the ability of these conjugates to inhibit binding of DNP-lysine by anti-DNP antibody. It can be seen that DNP, TNP, and *p*NP conjugates of KLH all stimulate DNP-H37 immune PELs and inhibit anti-DNP antibody binding of DNP-lysine, as shown by low I_{50} values. These compounds are similar in that each possess a paranitro group while they differ in having one, two, or no orthonitro groups, respectively. On the other hand, *o*NP-KLH gives no stimulation and 2,6-DNP-KLH yields only a modest response. *o*NP-KLH does not significantly inhibit binding of DNP-lysine by serum antibody and 2,6-DNP-KLH does so only at high concentrations. These haptens have one or two orthonitro groups, respectively, but both lack a paranitro group. While the conjugation ratio of the nonstimulatory *o*NP-KLH is low, possibly affecting its ability to stimulate PELs, it should be noted that *p*NP-KLH and DNP-KLH at similar degrees of conjugation are both excellent stimulants of such cells (Table VI and unpublished observations). Comparable results have been obtained using the same haptens conjugated to OVA. Thus it appears that both the stimulation of DNP-H37 immune PELs and inhibition of DNP-lysine binding by anti-DNP antibody requires the presence of the paranitro group of DNP, with the orthonitro group contributing relatively little to either activity. Table VI also shows the effect of immunizing strain 13 guinea pigs with DAG-H37. Anti-DNP antibody is produced in somewhat higher titer than with DNP-H37 immunization, but the specificity of the two types of antiserum is essentially identical. Nevertheless, PELs from DAG-H37-primed guinea pigs respond neither to DNP-proteins nor to DAG-proteins in vitro (Table VI and unpublished observations). Although DAG-proteins fail to stimulate PELs from guinea pigs primed with DNP-H37,

TABLE VI

Effect of Hapten Structure on In Vitro Proliferation of PELs and on Binding by Anti-DNP Antibody from the Same Animal

Antigen	Hapten concentration for stimulation*	Stimulation (CPM, E-C) after priming with:			Inhibition (I_{50}, hapten $\times 10^{-8}$ M) of binding of DNP-lysine by antibody from animals primed with:‡		
		Strain 13 DAG-H37	Strain 13 DNP-H37	Strain 2 DNP-H37	Strain 13 DAG-H37	Strain 13 DNP-H37	Strain 2 DNP-H37
	$M \times 10^{-8}$						
0	—	(1,802)	(4,574)	(6,867)	—	—	—
PPD§	—	41,842	65,320	46,692	—	—	—
KLH‖	—	100	3,075	72	—	—	—
DNP_8-KLH	172	1,704	43,573	46,161	1.6	2.2	2.0
$oNP_{1.1}$-KLH	172	1,901	3,979	1,238	>4,000	>4,000	>4,000
$pNP_{1.2}$-KLH	20	1,212	16,556	35,669	0.67	0.28	0.8
TNP_{14}-KLH	26	1,519	11,086	27,125	2.0	2.3	5.0
2,6-$DNP_{2.6}$-KLH	620	1,136	6,694	7,380	520	41	64
DNP_7-L-GL	2,640	0	0	2,803	15	18	25
ε-DNP-L-lysine	—	—	—	—	7.9	3.7	9.2



* The concentration of hapten giving the greatest stimulation in all groups.

‡ The concentration of hapten required to cause 50% inhibition of binding of 0.33×10^{-8} M ^{3}H-labeled ε-DNA-L-lysine by anti-DNP antibody = I_{50} ($M \times 10^{-8}$).

§ 1 μg/ml.

‖ 100 μg/ml.

they inhibit the binding of DNP-lysine by serum from such animals 5- to 10-fold more efficiently than the same protein coupled with DNP directly. Other experiments using DAG-protein conjugates have shown that PELs from guinea pigs immunized with DAG-OVA and DAG-GPA respond well to the immunizing conjugates. Taken together, these results strongly indicate that all nitrophenyl proteins which stimulate cells from animals primed with DNP-H37 bind anti-DNP antibody efficiently, but not all compounds which bind antibody efficiently stimulate PELs. The data of this and the previous section suggest that determinants involving both the dinitrophenyl groups and contiguous portions of the carrier are required for T-cell recognition in this system.

Discussion

The object of the experiments reported here was to define the precise specificity of the receptors for antigen on T lymphocytes. To accomplish this, cell-mediated

immune responses of guinea pigs immunized with DNP coupled directly to *Mycobacteria* were examined. As previously reported by Benacerraf and Gell (7) in similar experiments, animals immunized in this way show delayed hypersensitivity to DNP coupled to a variety of carrier proteins, and are sensitive to skin painting with DNFB. Also, migration of PECs from guinea pigs immunized with DNP-H37 is inhibited by those DNP-protein conjugates which elicit DH. Purified PELs proliferate in vitro in the presence of these same DNP-containing compounds. Each of these responses has been associated with T cells. In order to confirm that the response being studied was indeed a T-cell response, PELs were further purified by passage over nylon wool columns. While this treatment removed the bulk of the cells with surface immunoglobulin (B cells), the in vitro proliferative response was either unchanged or increased. Thus, this is almost certainly a T-cell response. It should be noted that unconjugated carrier proteins failed to stimulate PELs from guinea pigs immunized with either DNP-H37 or H37, indicating that the responses observed with DNP-proteins were not due to the presence of determinants cross-reactive with these protein carriers within the *Mycobacteria*.

It thus seems quite clear that the responses observed are a function of T lymphocytes and are hapten specific in a general sense. However, the following considerations clearly demonstrate an essential role for some carrier components. While several DNP-proteins (including DNP-bovine serum albumin and DNP-bovine gamma globulin in addition to those shown here)[3] have elicited responses in these cells, certain DNP conjugates failed to elicit such responses. Among them was DNP-L-GL, which was a powerful stimulant of PELs from strain 2 guinea pigs which had been immunized with DNP-L-GL. In addition, DNP-D-GL and DNP-Ficoll failed to elicit responses by PELs from DNP-H37 immune animals. The carrier could be important in reacting with a helper T cell to stimulate a DNP-specific T cell, by analogy with T-B collaboration, as has been suggested by Mitchison (20). However, this seems very unlikely as carriers alone elicit no response, and DNP coupled to protein via a flexible tripeptide spacer is nonstimulatory. Other possibilities are either that some degree of complexity or heterogeneity of the carrier is essential for this response, and/or that DNP coupled to particular amino acid residues other than lysine make up important determinants recognized by these T cells. This latter hypothesis is strongly supported by the finding that DNP-L-TGL, a linear copolymer similar to DNP-L-GL but containing 7% tyrosine residues, was highly stimulatory, and stimulatory activity was greatly reduced by specifically removing DNP from tyrosyl residues by thiolysis. Thus it appears that the responses observed may be elicited by conjugates of proteins and polypeptides containing DNP-*O*-tyrosyl residues. Whether other residues conjugated with DNP or residues spatially near DNP groups are important is not clear from these data.

It has been demonstrated by Frey et al. (19) that DNP-*O*-tyrosyl groups may donate their DNP radicals to other amino acids. Thus, it is conceivable that in the course of immunization with DNP-H37, some DNP is transconjugated from DNP-*O*-tyrosyl residues or other labile DNP-amino acids to autologous carriers.

[3] Janeway, C. A., Jr. Unpublished observations.

A similar transconjugation could take place during the various assay procedures, from DNP-proteins or polypeptides, to create precisely the same DNP autologous carrier conjugate, the latter then actually giving rise to the observed stimulation. Although this mechanism can not be entirely ruled out as the explanation of the effect being studied, several findings mitigate against it. Di-*O*,*N*-DNP-tyrosine and mono-*O*-DNP-tyrosine, which might be expected to be excellent donors of DNP groups, are not stimulatory in this system. Secondly, in unpublished work, we have developed a functional assay for transconjugation based on the ability of DNP conjugates to stimulate lymphocytes from DNP-GPA-primed guinea pigs when the conjugates are preincubated with GPA. While there is indeed transconjugation from some DNP conjugates to GPA as measured in this way, there is no obvious correlation between the capacity to transconjugate and the relative stimulatory activity of a DNP conjugate for PELs from a DNP-H37-primed animal.

The activation of PELs from guinea pigs immune to DNP-H37 by DNP-protein offers an important opportunity to evaluate the precise specificity of T-cell receptors for nitrophenyl ligands. To do this, a series of nitrophenyl conjugates of OVA and KLH were prepared. In both cases, it was clear that the presence of a paranitro group was very important in cell activation. Thus, *p*NP and TNP-proteins were similar to DNP-proteins in their stimulatory capacity, while *o*NP-proteins were nonstimulatory, and 2,6-DNP-proteins were weakly stimulatory or nonstimulatory. In this respect, the specificity of T-cell activation was remarkably similar to that of the relative avidity of anti-DNP antibodies for these conjugates.

Although DNP-*O*-tyrosyl residues are a principal determinant important for stimulation of T cells in this system, preliminary studies indicate that other, still uncharacterized determinants may also contribute to this response. Thus, PELs from strain 2 guinea pigs primed with DNP-H37 fail to respond to DNP-L-GL and to the DNP conjugate of the copolymer of L-glutamic acid and L-alanine, but are clearly stimulated by DNP coupled to a terpolymer of L-glutamic acid, L-lysine, and L-alanine. Furthermore, DNP protein conjugates prepared using dinitrobenzenesulfonate are essentially as stimulatory as those prepared using DNFB, although lacking detectable DNP-*O*-tyrosyl residues.

One final point which remains unresolved is the reason for substantial responses to all DNP-proteins being regularly obtained in guinea pigs primed with DNP-H37 but not in guinea pigs primed with DNP-proteins. Other examples of apparently similar types of responses are found in the extensive studies of Leskowitz et al. (5) and Alkan et al. (6) on *p*-azobenzenearsonate conjugates, especially *p*-azobenzenearsonate-*N*-acetyl-tyrosine. In addition, Dailey and Hunter (21) have reported that immunization of guinea pigs with DNP-bovine serum albumin which has been modified by conjugation with fatty acids yields delayed hypersensitivity reactions to several DNP-proteins. It is unlikely that simple association of hapten with adjuvant-like substances leads necessarily to such responses, as guinea pigs immunized with DAG-H37 or with DNP *Bordetella pertussis* conjugates[4] do not show responses to either DNP or DAG conjugates.

[4] Janeway, C. A., Jr. Unpublished results.

We regard the current experiments as providing important insights into T-cell specificity and as reinforcing the concept that T cells and B cells tend to have different libraries of receptors. Although this is consistent with a notion of basically different receptor molecules, it can not be used to definitively establish whether or not the portion of the T-cell receptor that is specific for antigen is derived from immunoglobulin variable region genes. Such studies may however provide insight into the type of ligand necessary to affinity label the T-cell receptor and thus allow its isolation and characterization.

Summary

Guinea pigs immunized with the hapten 2,4-dinitrophenyl (DNP) coupled directly to *Mycobacterium tuberculosis* of strain H37Ra (DNP-H37) show a variety of cell-mediated immune responses to DNP coupled to protein carriers. The cells responsible for this specific response are thought to be T lymphocytes for the following reasons: Guinea pigs immunized with DNP-H37 displayed delayed hypersensitivity reactions to several DNP-proteins and contact sensitivity to dinitrofluorobenzene. Peritoneal exudate lymphocytes (PELs) obtained from DNP-H37 immune animals respond to DNP-proteins with DNA synthesis and cause inhibition of macrophage migration. PELs are highly enriched in T lymphocytes and contain few immunoglobulin-bearing cells. Further depletion of immunoglobulin-bearing cells from this population does not diminish the in vitro proliferative response to antigen.

Nitrophenyl conjugates of proteins lacking a paranitro group stimulated DNA synthesis poorly or not at all, indicating the importance of the paranitro group of DNP in antigen recognition by T cells in this system. In this respect, the specificity of T cells resembles that of DNP-specific antibody from the same animals. On the other hand, DNP conjugates of copolymers of glutamic acid and lysine and DNP conjugated to proteins via an interposed β-alanyl-glycyl-glycyl spacer failed to stimulate DNA synthesis, although such compounds bind very efficiently to anti-DNP antibody. By contrast, DNP conjugates of synthetic polypeptide carriers containing as little as 7% tyrosine strongly stimulated DNA synthesis in DNP-H37 immune PELs. That the determinant responsible for this stimulation was DNP coupled to the hydroxyl group of tyrosine was shown by selective removal of DNP from tyrosine by thiolysis with 2-mercaptoethanol, which abolished their ability to stimulate T cells. This stimulatory capacity could be restored by redinitrophenylation, demonstrating that thiolysis had not simply inactivated the carrier. These data suggest that a major antigenic determinant recognized by such T cells is DNP coupled to the hydroxyl group of tyrosine. Nevertheless, either multivalent presentation or additional carrier structures are critical as mono-*O*-DNP-L-tyrosine and di-*O*,*N*-DNP-L-tyrosine failed to stimulate a proliferative response.

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