THE SPECIFICITY OF CELLULAR IMMUNE RESPONSES
IN GUINEA PIGS

II. The Structure of Antigenic Determinants Leading to T-Lymphocyte
Stimulation*

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In a previous report (1), guinea pigs primed with the hapten 2,4-dinitrophenyl
(DNP) coupled directly to mycobacteria were shown to possess thymus-derived
(T) lymphocytes responsive to DNP on a wide variety of carriers. This system is
of interest for several reasons. First, it contrasts with most T-cell responses to
hapten-carrier conjugates, which are specific for carrier or for specific conjugate
determinants, but not for the same hapten on heterologous carriers (2). Second,
it allows the comparison of responses of T lymphocytes to those of thymus-
independent, bone-marrow-derived (B) lymphocytes to the same, chemically
defined determinant. Finally, it permits the precise analysis of the structure of
antigenic determinants recognized by such T cells; since it is necessary that the
determinant contain the hapten for T-cell responses to occur, the hapten be-
comes a marker for the determinant. If the specificity of such T-cell responses
could be shown to resemble closely the specificity of B-cell responses to DNP,
and of anti-DNP antibody, then a strong argument could be made for the
identity of the specific receptors for antigen on T and B lymphocytes; B-
lymphocyte receptors are generally believed to be immunoglobulin (Ig) in na-
ture (2-4). On the other hand a clear difference in the specificity of T-lymphocyte
responses compared to B-lymphocyte responses might imply that these two
types of lymphocytes use entirely different molecules to perform a similar
function, namely specific antigen recognition.

Previous studies (1) of guinea pigs immunized with DNP-mycobacteria have
shown that the in vitro proliferative response of a purified population of guinea
pig T lymphocytes to DNP conjugates is independent of the few B cells in the
system, and is indeed a T-cell response. The determinant recognized involves
both hapten and carrier, since neither alone can stimulate such T cells. The

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hapten itself, and especially its para-nitro group, was shown to be specifically recognized. Furthermore, when conjugates were prepared in which DNP was coupled to the carrier via a tripeptide spacer, no response was obtained; this suggests that the determinant involves the hapten-carrier bond and contiguous portions of carrier. One determinant recognized by such T cells was clearly shown to be DNP coupled to the hydroxyl group of tyrosine. DNP coupled to a copolymer of L-glutamic acid and L-lysine would not stimulate such T cells, nor would certain other DNP conjugates.

The object of the present experiments was to further define the specificity characteristics of these T-cell responses, in order to describe as precisely as possible the antigenic determinants which such cells recognize. It was found that DNP coupled either to the ε-amino group of lysyl residues (DNP-lysyl) or to the hydroxyl group of tyrosyl residues (O-DNP-tyrosyl) can be recognized by these T cells. Furthermore, amino acids to which DNP is not coupled are also recognized as part of the antigenic determinant. It was also shown that while the hapten used for immunization must be identical to that used for the later stimulation of T-cell responses, the hapten need not be DNP; thus, the hapten 3-nitro-4-hydroxy-5-iodophenacetyl (NIP) gave results identical to those obtained using DNP. Different inbred strains of guinea pigs immunized with DNP-mycobacteria gave rise to T cells of differing specificity, suggesting a genetic control of these responses. Finally, similar T-cell responses were obtained using immunogens other than haptenated mycobacteria. Taken together, these data suggest a receptor on T cells that recognizes a more extensive determinant than that recognized by B cells; B cells respond to DNP essentially regardless of carrier or of the hapten-carrier bond (5). They also suggest a mechanism by which DNP-mycobacteria primes T lymphocytes for such responses.

Materials and Methods

**Animals.** Adult male and female guinea pigs of the inbred strains 2 and 13 were used throughout these studies. They were obtained from the Animal Production Section, NIH, Bethesda, Md.

**Antigens.** Ovalbumin (OVA), guinea pig serum albumin (GPA), giant keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and Mycobacterium tuberculosis of strain H37Ra (H37) and their DNP conjugates were obtained and prepared as previously described (1). Conjugation ratios in DNP groups per mole or per 10^5 daltons (KLH) are given as subscripts in the tables. Some DNP conjugates in the present study were prepared using 2,4-dinitrobenzene sulfonate (DNBS) by the method of Little and Eisen (6), instead of 1-fluoro-2,4-dinitrobenzene (DNFB) as previously employed; to distinguish such conjugates the former are labeled DNBS-X. Thiolysis of DNP conjugates with 2.5 M 2-mercaptoethanol to remove DNP from tyrosyl residues was carried out as previously described (1, 7). Synthetic antigens used in these studies are listed in Table I along with the symbols used to denote them. Compounds L-TGA (253), d-TGA (236), and L-TGL (252) were a kind gift of Professor M. Sela, Weizmann Institute, Rehovoth, Israel, and have been described previously (8). The other compounds were synthesized by one of us (P. H. M.), and

1 Abbreviations used in this paper: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DAG, DNP-β-alanyl-glycylglycyl; DNBS, 2,4-dinitrobenzene sulfonate; DNFB, 1-fluoro-2,4-dinitrobenzene; GPA, guinea pig serum albumin; H37, Mycobacterium tuberculosis of strain H37Ra; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; L-BSA, lipid-conjugated BSA; NAG, 3-nitro-4-hydroxy-5-iodophenacetyl-β-alanyl-glycylglycyl; NIP, 3-nitro-4-hydroxy-5-iodophenacetyl; NP, 3-nitro-4-hydroxyphenacetyl; OVA, hen's egg albumin; PBS, phosphate-buffered saline; PELs; peritoneal exudate lymphocytes; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin; t-BuOH, tertiary butyl alcohol.
**Table I**

Composition of the Synthetic Polypeptides Used in These Studies

| Abbreviation | Molar proportions of amino acid residues in the polymers | Mol wt |
|--------------|--------------------------------------------------------|--------|
| L-GL         | 60% L-Glutamic acid, 40% L-lysine                      | 90,000 |
| D-GL         | 59% D-Glutamic acid, 41% D-lysine                      | 40,000 |
| L-GA         | 62% L-Glutamic acid, 38% L-alanine                     | 22,000 |
| L-GT         | 54% L-Glutamic acid, 46% L-tyrosine                    | 22,600 |
| L-GLT        | 57% L-Glutamic acid, 38% L-lysine, 5% L-tyrosine       | 47,000 |
| L-GAT        | 60% L-Glutamic acid, 30% L-alanine, 10% L-tyrosine     | 55,000 |
| L-TGL        | 6% L-Tyrosine, 60% L-glutamic acid, 34% L-lysine       | 61,000 |
| L-TGA        | 8% L-Tyrosine, 49% L-glutamic acid, 43% L-alanine      | 23,000 |
| L-TGA        | 8% L-Tyrosine, 49% D-glutamic acid, 43% D-alanine      | 33,800 |
| L-GLAT       | 57% L-Glutamic acid, 38% L-lysine, 5% L-alanine        | 40,000 |
| L-GLA*       | 54% L-Glutamic acid, 36% L-lysine, 10% L-alanine       | 70,000 |
| L-GLA*       | 48% L-Glutamic acid, 32% L-lysine, 20% L-alanine       | 90,000 |
| L-GLA*       | 42% L-Glutamic acid, 28% L-lysine, 30% L-alanine       | 32,000 |
| L-GLA*       | 36% L-Glutamic acid, 24% L-lysine, 40% L-alanine       | 45,000 |
| L-GLA*       | 24% L-Glutamic acid, 18% L-lysine, 60% L-alanine       | 30,000 |
| GLA3° (LLD)  | 42% L-Glutamic acid, 28% L-lysine, 30% D-alanine       | 12,000 |
| GLA3° (LDDL)| 42% L-Glutamic acid, 28% D-lysine, 30% L-alanine       | 7,000  |
| GLA3° (DLL)  | 42% D-Glutamic acid, 28% L-lysine, 30% L-alanine       | 8,000  |
| D-GLA*       | 42% D-Glutamic acid, 28% D-lysine, 30% D-alanine       | 30,000 |
| L-GLAT       | 36% L-Glutamic acid, 24% L-lysine, 35% L-alanine, 5% L-tyrosine | 30,000 |
| (GLAGly)3*   | 25% L-Glutamic acid, 25% L-lysine, 25% L-alanine, 25% glycine | 11,000 |

* This molecule was formed by polymerizing the tetrapeptide subunits L-Glu-L-Lys-L-Ala-Gly and therefore has a known, repeating sequence.

Their synthesis and characterization have been previously described (9-12). ε-DNP-L-lysine, and di-O,N-DNP-L-tyrosine were purchased from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. Phytomagglutinin (PHA) was obtained from Burroughs Welcome & Co., Greenville, N. C.

The hapten NIP was also conjugated to H37, to several proteins, and to L-GL and L-GLA3°. NIP-t-BOC hydrazide (Regis Chemical Co., Morton Grove, Ill.) was converted to NIP-azide by using the technique of Inman and co-workers (13-15), and coupled as described. 3-nitro-4-hydroxyphenacyl (NP)-t-BOC hydrazide (Regis Chemical Co.) was also converted to the azide and coupled to protein. NIP linked to a β-alanyl-glycylglycyl spacer (NAG) was prepared, converted to the azide, and coupled to protein as previously described (13-15). Conjugation ratios were determined by absorption at 430 nm.

BSA, doubly conjugated with DNP and 12-carbon fatty acid groups, was prepared by a method similar to that previously described (16). 1 gram of BSA (Sigma Chemical Co., St. Louis, Mo.) was dissolved at 25 mg/ml in 0.5 M carbonate buffer, pH 9.5. A 200-fold molar excess of dodecanedic anhydride (Pfaltz & Bauer Inc., Flushing, N. Y.) in 6 ml tertiary butyl alcohol (t-BuOH) was added dropwise and the solution was stirred overnight at room temperature. After extensive dialysis against 25% t-BuOH in phosphate-buffered saline (PBS) and then against H2O, the lipid-conjugated BSA (L-BSA) was lyophilized. This product was then dinitrophenylated by the addition of 50 µl of DNFB (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) to 250 mg L-BSA in 15 ml of 20% t-BuOH in 0.1 M carbonate buffer, pH 9.5, and stirring for 1 h, followed by dialysis against many changes of PBS. The extent of fatty acid substitution of lysine groups was determined by the reaction of L-BSA with trinitrobenzenesulfonic acid according to the method of Habeeb (17). The amount of DNP present in the lipid-conjugated antigen (DNP-BSA-lipid) was determined by absorption at 360 nm (6). These analyses indicated that 27 lipid and 26 DNP groups were bound per molecule of BSA.

**Immunization.** Guinea pigs were immunized with H37 alone, DNP-H37, or NIP-H37 by injecting 1 mg emulsified in incomplete Freund's adjuvant (IFA) divided equally among the four foot pads. Animals were immunized with 100 µg DNP-BSA-lipid mixed with 1 mg H37 emulsified...
in IFA also divided equally among the four feet. Animals were tested 2–10 wk after immunization; responses were optimal about 1 mo after priming.

In Vitro Stimulation with Antigen. In vitro stimulation was carried out by culturing peritoneal exudate lymphocytes (PELs) that had been purified over rayon wool columns as previously described (1, 18). 4 × 10^5 cells were cultured in 0.2 ml in wells of Microtiter U trays (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Arlington, Va.) in a medium consisting of RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), 5 U/ml each of penicillin and streptomycin, 2 mM L-glutamine, 15 mM HEPES buffer, and 10% fetal bovine serum. In experiments using animals primed with DNP-BSA-lipid, all cells were cultured in complete medium containing 5% heat-inactivated normal guinea pig serum instead of fetal bovine serum. 20 μl medium (control) or antigen was added to triplicate wells at the start of the culture period. PELs were cultured in a moist atmosphere of 5% CO₂ in air for 2 days, pulsed with 20 μl containing 1 μCi ³H-thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.), harvested 1 day later using a Multiple Automated Sample Harvester (MASH II; Microbiological Associates, Bethesda, Md.), and the incorporated ³H counted in a liquid scintillation counter. Counts were punched on paper tape and processed by a PDP-11 computer (Digital Equipment Corp., Maynard, Mass.) to give mean counts per minute and standard error for triplicate culture wells. Standard errors rarely exceeded ±10%, and have not been presented. Results are expressed as average counts per minute incorporated in experimental cultures (those containing antigen) minus counts per minute in control (medium alone) wells (E-C). The value of the control is given in parentheses for each preparation of cells. Stimulation greater than twice the control value is considered significant and such values have been underlined in the tables. Where several experiments were averaged, geometric means were calculated.

Test for Trans-dinitrophenylation. The ability of DNP groups to transfer to other proteins during culture was tested as follows: 20 μl medium containing 1 mg/ml GPA plus 20 μl antigen in 0.15 M saline buffered with 0.015 M PBS, pH 7.4, or 20 μl medium plus 20 μl antigen, were incubated for 24 h. PELs from strain 13 guinea pigs primed with DNP-L2-GPA were then added and cultured for 72 h as usual. The extent of trans-dinitrophenylation was then determined by subtracting counts per minute obtained when antigens were precultured in medium from counts per minute obtained with antigen preincubated with GPA. As a standard, DNP-GPA at various conjugation ratios was used to stimulate these cells at 100 μg DNP-GPA/ml final concentration. It should be noted that preincubations were carried out in the presence of 5% fetal bovine serum, which was found not to affect results of this assay.

Results

Recognition of DNP Lysyl Residues by T Cells. In a large number of experiments, T cells from guinea pigs primed with DNP-H37 did not respond to DNP-L-GL, but did respond to conjugates containing DNP-O-tyrosyl residues (1). In order to determine whether the responses of such T cells to DNP-protein conjugates were due to recognition of DNP-lysyl residues or of DNP coupled to other amino acid residues, DNP-protein conjugates were prepared using DNBS and compared with DNP conjugated to the same protein using either DNFB or DNP linked to a β-alanyl-glycylglycyl spacer (DAG). DNBS and DAG couple only to amino groups, primarily of lysyl residues, while DNFB also couples to tyrosyl, histidyl, and cysteinyl residues (7). The results of one such experiment are shown in Table II. It can be seen that unmodified carrier (OVA) does not cause these T cells to divide, in agreement with the failure of all unmodified carriers to stimulate throughout these experiments. However, proteins coupled with DNP using either DNFB or DNBS stimulate strong proliferative responses by PELs from animals primed with DNP-H37. Thus, DNP coupled to residues other than tyrosine, and most likely to the e-amino group of lysine, can be recognized by these T cells. Moreover, where conjugation ratios are equivalent, as for DNP₁₂-GPA and DNBS₁₂-GPA, (or for DNP₂-OVA and DNBS₂-OVA, not
In Vitro Proliferative Responses of Strain 13 PELs after DNP-H37 Priming: Stimulation with DNP-Proteins Prepared with 2,4-DNBS

| Stimulating antigen (100 μg/ml) | Proliferation (cpm, E-C) |
|---------------------------------|-------------------------|
| None                            | (2,456)*                |
| PPD†                            | 35,857§                 |
| OVA                             | 0                       |
| DNP-OVA                         | 23,212                  |
| DAG-OVA                         | 0                       |
| DNBS-OVA                        | 4,114                   |
| DNPSr-GPA                       | 11,844                  |
| DAGs-GPA                        | 828                     |
| DNBSr-GPA                       | 2,1096                  |
| DNP-L-GL                        | 0                       |

* 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.

Responses to DNBS conjugates were as great as those to DNP conjugates. Therefore, responses to DNP proteins are most likely directed primarily at the DNP-lysyl-containing determinants. It is important to note that these same T cells do not respond to DNP-L-GL which contains only DNP-lysyl residues. This implies that the determinant recognized in DNBS-GPA involves more than DNP-lysyl residues and must therefore include portions of the carrier as well. That the portion of the antigenic determinant contributed by the carrier is contiguous with the DNP-lysyl residue is strongly suggested by the lack of stimulation seen with DAG-protein conjugates. If only the hapten and distant portions of the carrier needed to be recognized for stimulation to occur, as is true for T-B collaboration, then the interposition of the tripeptide spacer molecule should not abolish their stimulatory activity. Indeed, DAG and DNP conjugates of the same carrier have been shown to induce equivalent T-B cell collaboration.

That T cells from guinea pigs do respond to determinants containing DNP-lysyl residues is further demonstrated by the results given in Table III. In these experiments, PELs from either strain 2 or strain 13 guinea pigs primed with DNP-H37 were tested for responsiveness to a wide variety of DNP-L-GL conjugates, and for their responses to a series of DNP conjugates of related compounds which preserve the ratio of L-glutamic acid to L-lysine (6:4) in L-GL but contain, in random sequence, increasing proportions of L-alanine (L-GLA-L-GLA\textsuperscript{6}). The final carrier tested (L-GLAT) is a further modification of L-GL containing 35% alanine and 5% L-tyrosine at random. The data are the average responses obtained in two to three experiments. It can be seen that none of the DNP-L-GL conjugates tested elicited significant proliferative responses with PELs of either strain. By contrast, there is a significant response to the compounds DNP-GLA\textsuperscript{10}-DNP-GLA\textsuperscript{40} by PELs from strain 2 guinea pigs primed with DNP-H37. This response increases with increasing alanine content in the carrier, a point...
TABLE III

Stimulation of PELs from Strain 2 or Strain 13 Guinea Pigs Immunized with DNP-H37 with Various DNP-L-GL and DNP-L-GLA Compounds

| Stimulating antigen (100 μg/ml) | DNP concentration | Proliferation (cpm, E-C) of PELs from:* |
|-------------------------------|-------------------|---------------------------------------|
|                               | µM                | Strain 2 | Strain 13 |
| None                          | 3,823             | (4,014) |
| DNP₁₄-L-GL                   | 36                | 646      | 0         |
| DNP₃₆-L-GL                   | 10                | 924      | 0         |
| DNP₅₄-L-GL                   | 44                | 750      | 993       |
| DNP₁₂₁-L-GL                  | 646               | 1,970    | 127       |
| DNP₂₃₀-L-GLA                 | 121               | 2,193    | 0         |
| DNP₂₇₀-L-GLA                 | 69                | 3,283    | 253       |
| DNP₂₈₇-L-GLA                 | 67                | 5,842    | 223       |
| DNP₃₄₅-L-GLA                 | 54                | 7,950    | 480       |
| DNP₄₃₁-L-GLA                 | 63                | 14,234   | 1,445     |
| DNP₅₃₅-L-GLA                 | 53                | 20,466   | 3,485     |
| DNP₆₃₉-L-GLAT                | 43                | 19,703   | 9,453     |

* Mean values, two to three experiments.

that is more clearly seen in a separate experiment shown in Fig. 1. Since DNP groups can not couple to alanyl residues of L-GLA, which are aliphatic, but couple only to the ε-amino groups of lysyl residues, and perhaps to the α-amino groups of the N-terminal of the polypeptide chain, the stimulatory activity of DNP-L-GLA in this system confirms that such T cells can recognize DNP-lysyl-containing determinants. That DNP coupled to the α-amino group is unimportant in this system is shown by the lack of stimulatory activity found with DNP coupled to a copolymer of l-glutamic acid and L-alanine (DNP-L-GA) (data not shown). The results in Table III and Fig. 1 also suggest, as was seen with DNBS conjugates of proteins, that amino acid residues adjacent to the DNP-lysyl residue are essential for recognition of these determinants by T cells. Thus, DNP-L-GL is not significantly stimulatory, whereas similar compounds with increasing amounts of alanine are increasingly stimulatory. Finally, Table III shows a clear difference in the responsiveness of PELs from strain 2 and strain 13 guinea pigs to the DNP-L-GLA compounds, a finding confirmed by subsequent experiments (see below, Table VI).

It could be argued that the reason DNP-L-GLA conjugates stimulate PELs from strain 2 guinea pigs while DNP-L-GL does not, is that the alanine in DNP-L-GLA, being a neutral amino acid, alters the overall net charge of the carrier and may also decrease intramolecular interactions between glutamyl and lysyl residues, thus affecting its interaction with cells in general. Alternatively, the alanyl residues in DNP-L-GLA may be a component of the antigenic determinant itself. A third possibility is that, since increasing alanine content leads to increasing helical content of the polymer, new conformational determinants are formed in DNP-L-GLA that are absent in DNP-L-GL. The results shown in the right-hand panel of Fig. 1 suggest that the alanyl residues actually comprise a part of the antigenic determinant, either as such, or by producing specific
conformations. Thus, strain 2 PELs primed with DNP-H37 respond significantly only to DNP-GLA<sup>3°</sup> conjugates in which all the amino acids are of the L configuration (DNP-L-GLA<sup>3°</sup>) or in which only glutamic acid residues are of D configuration. DNP-GLA<sup>3°</sup> in which either all the residues are of the L-configuration or in which the lysyl or alanyl residues are of the D-configuration are not significantly stimulatory. That DNP coupled to an ordered sequence linear polymer built of repeating units of L-glutamyl-L-lysyl-L-alanyl-L-glycyl tetrapeptides [DNP-L-(GLAgly)<sub>n</sub>] is stimulatory suggests that alanyl residues do not have to be on both sides of the DNP-lysyl residue in order for a stimulatory determinant or conformation to be generated. Thus, the experiments in this section demonstrate two major features of the recognition of antigen by T cells raised in guinea pigs immunized with DNP-H37: first, determinants in which DNP is coupled to the ε-amino group of lysyl residues can be recognized by such T cells, and second, such determinants also involve amino acid residues adjacent to the DNP-lysyl residues, perhaps only in the proper conformation.

Is the Response of T Cells from DNP-Mycobacteria-Primed Guinea Pigs to O-DNP-Tyrosyl Residues Due to Trans-dinitrophenylation? In previous studies (1), it was shown that PELs from guinea pigs primed with DNP-H37 responded strongly to conjugates containing DNP coupled to the hydroxyl group of tyrosine. Since O-DNP-tyrosine is known to be unstable chemically (20), it is possible that such conjugates are stimulatory only because their DNP groups transfer to other proteins or cells in culture, becoming DNP-lysyl residues instead. In order to test this hypothesis, the following experiments were performed.
Strain 13 guinea pigs were immunized with DNP$_{4s}$-GPA. T cells from these animals respond very strongly to DNP-GPA, as shown in Table IV, right-hand column, even at very low conjugation ratios. Their responses to other DNP-conjugates are low or absent (1). 1 day before the PELs from the DNP-GPA-primed animals were cultured, a variety of DNP conjugates were incubated in a small volume of medium with or without added GPA (see Materials and Methods). If the DNP groups of these conjugates can transfer to GPA, then the responses of PELs from guinea pigs primed with DNP-GPA should be higher in cultures containing GPA than in cultures without added GPA, and the difference should be a function of the extent to which this trans-conjugation occurs. Table IV gives a typical result for such an experiment. All compounds containing O-DNP-tyrosyl residues do in fact show substantial degrees of trans-conjugation to DNP-GPA, including the hapten-coupled amino acid, O,N-DNP-L-tyrosine, whereas DNP proteins and conjugates containing only DNP-lysyl residues do not show trans-conjugation. The maximum trans-conjugation is observed using DNP$_{4s}$-L-GT, stimulation being that expected if DNP$_{4s}$-GPA were produced. Although compounds containing O-DNP-tyrosyl can therefore transfer their DNP groups to other carriers, comparison of the results in Table IV with those in Table V, in which the same compounds are used to stimulate PELs from guinea pigs primed with DNP-H37, suggests that trans-dinitrophenylation is not a significant factor in the response of these latter T cells to determinants containing O-DNP-tyrosyl residues. Thus, O,N-DNP-L-tyrosine

| Compound tested       | Δcpm | Control compound  | cpm, E-C |
|-----------------------|------|------------------|---------|
| PBS                   | -196 | PBS              | (2,373) |
| ε-DNP-L-lysine        | -413 | DNP$_{4s}$-GPA   | 46,197  |
| O,N-DNP-L-tyrosine    | 31,988 | DNP$_{4s}$-GPA | 55,459  |
| DNP$_{3s}$-GL         | -237 | DNP$_{4s}$-GPA   | 76,732  |
| DNP$_{3s}$-GLAT       | 2,916 | DNP$_{4s}$-GPA   | 87,880  |
| DNP$_{3s}$-GLT        | 15,600 | DNP$_{4s}$-GPA | 105,103 |
| DNP$_{3s}$-GLAT       | 19,564 | DNP$_{4s}$-GPA | 106,944 |
| DNP$_{3s}$-GT         | 46,130 | DNP$_{4s}$-GPA | 96,270  |
| DNP$_{3s}$-GAT        | 35,077 | DNP$_{4s}$-GPA | 106,944 |
| DNP$_{3s}$-OVA        | 3,390 | DNP$_{4s}$-GPA   | 96,270  |
| DNP$_{3s}$-KLH        | 388  | DNP$_{4s}$-GPA   | 96,270  |

* 20 µl of the various DNP conjugates at 100 µg/ml for DNP amino acids or 1 mg/ml for DNP proteins or polypeptides were incubated for 24 h with 20 µl of medium or of medium containing 1 mg/ml GPA. 4 x 10$^5$ PELs from strain 13 guinea pigs immunized with DNP$_{4s}$-GPA were then added, and the cultures carried out as usual for 3 days in a final vol of 200 µl. DNP-GPAs tested at final concentration of 100 µg/ml. The degree of trans-conjugation of DNP to GPA is assessed by subtracting the cpm given by an antigen in medium alone from that found when the antigen was preincubated with GPA. The number of DNP groups per molecule of GPA required to give a particular amount of proliferation can be assessed by reference to the proliferation given by the DNP-GPAs conjugated to varying degrees (control compounds).
TABLE V
Stimulation of PELs from Strain 13 Guinea Pigs Immunized with DNP-H37 with the Compounds Tested for Trans-Dinitrophenylation

| Stimulating antigen (100 µg/ml) | Stimulation (cpm, E-C) of PELs primed with: |
|-------------------------------|---------------------------------------------|
|                               | H37                                         | DNP-H37                                     |
| None                          | (1,843)                                     | (1,584)                                     |
| PPD*                          | 7,396                                       | 16,217                                      |
| ε-N-DNP-L-lysine†             | 408                                         | 323                                         |
| Di-O,N-DNP-L-tyrosine‡         | 179                                         | 1,252                                       |
| DNP₄₋L-GL                     | 408                                         | 1,045                                       |
| DNP₄₋L-GLA³⁶                 | 1,238                                       | 6,100                                       |
| DNP₄₋L-GLT                   | -14                                         | 4,387                                       |
| DNP₄₋L-GLAT                  | -107                                        | 10,311                                      |
| DNP₄₋L-GT                    | -112                                        | 5,889                                       |
| DNP₄₋L-GAT                   | 3                                           | 11,310                                      |
| DNP₄₋OVA                     | -604                                        | 7,319                                       |
| DNP₄₋KLH                     | -772                                        | 6,280                                       |
| DNP₄₋GPA                     | -746                                        | 2,631                                       |
| DNP₄₋ε-GPA                   | -674                                        | 3,582                                       |
| DNP₄₋ε-GPA                   | -692                                        | 4,266                                       |
| DNP₄₋δ-GPA                   | -787                                        | 3,791                                       |
| DNP₄₋ε-GPA                   | -616                                        | 6,143                                       |
| DNP₄₋GPA                     | -611                                        | 6,848                                       |

* 20 µg/ml.
‡ 10 µg/ml.

does not stimulate such T cells although it trans-conjugates to the same extent as DNP-L-GAT, which is highly stimulatory. It seems more likely that O-DNP-tyrosyl residues are recognized as such by PELs from guinea pigs primed with DNP-H37. This is strongly supported by experiments presented in the subsequent paper (21).

Role of Amino Acid Residues to which DNP is Not Coupled in the Antigenic Determinant Recognized by T Cells from Guinea Pigs Primed with DNP-Mycobacteria, and the Effect of Strain on the Response. As discussed above (Table III), DNP-L-GL does not stimulate PELs from guinea pigs primed with DNP-H37, while DNP-L-GLA compounds do. The experiments shown in Table VI show that the addition of alanine to other synthetic polypeptides distinct from L-GL also enhances the activity of their DNP conjugates in stimulating PELs from DNP-H37 immune strain 2 or strain 13 guinea pigs. The examples shown here are DNP-L-GLT vs. DNP-L-GLAT and DNP-L-GL vs. DNP-L-GAT as well as DNP-L-GL vs. DNP-L-GLA. In each instance, the DNP conjugate of the polymer-containing alanine is far more stimulatory than that lacking alanine. This is further evidence for a role of amino acids other than those to which DNP is coupled in the antigenic determinant to which such T cells respond.

Table VI also compares the responses of PELs from strain 2 guinea pigs primed with DNP-H37 with those of PELs from identically immunized strain 13 guinea pigs. Geometric mean responses of four to six experiments are given; in each experiment, both types of PELs were cultured simultaneously. Since strain
### TABLE VI

**The In Vitro Proliferative Response of PELs from Strain 2 and Strain 13 Guinea Pigs Primed with DNP-H37 to DNP on Various Carriers: the Effect on the Response of Strain and of the Alanine Content of the Carrier**

| Stimulating antigen | Stimulation (cpm, E-C) of PELs from:* | Strain 2 | Strain 13 | Normalized strain 13§ |
|---------------------|--------------------------------------|---------|----------|-----------------------|
| None                | (4,754)                             | (4,206) |
| PPD                 | 66,300                               | 48,552  | 66,522   |
| DNP, KLH            | 30,886                               | 22,949  | 31,507   |
| DNP, GPA            | 7,438                                | 12,166  | 15,673   |
| DNP, GLT            | 1,089                                | 110     | 156      |
| DNP, GLA °          | 27,239                               | 5,318   | 7,291    |
| DNP, GT             | 4,844                                | 1,169   | 1,607    |
| DNP, GAT            | 29,266                               | 18,475  | 25,317   |
| Thiolysed DNP, GLAT|| 21,630                               | 9,977   | 13,674   |
| Re-DNP, GLAT        | 27,346                               | 16,550  | 22,679   |
| DNP, GT             | 8,197                                | 8,666   | 11,878   |
| DNP, GAT            | 17,898                               | 15,039  | 20,611   |

* Geometric mean of four to six experiments; relative standard error (RSE), 1.3 or less except the response of strain 2 to DNP-L-GT (RSE, 1.58) and of strain 13 to Re-DNP-GLAT (RSE, 1.39).
† Antigen concentration, 100 μg/ml except PPD at 10 μg/ml.
§ Normalized on the basis of response to PPD and to DNP-KLH.
|| 4 DNP-O-tyrosyl residues cleaved by treatment of DNP, L-GLAT with 2.5 M 2-mercaptoethanol.

13 animals consistently responded less well to purified protein derivative of tuberculin (PPD) and to DNP-KLH, a third column in which these two responses have been used to normalize the responses to other compounds is also shown. It can be seen that the responses of PELs from strain 2 guinea pigs to certain conjugates is significantly greater than the response of strain 13 PELs to that conjugate; in other instances, the opposite is true. Thus, the responses of immune strain 2 PELs to DNP-L-GLA ° and to thiolysed DNP-L-GLAT (which is closely similar to DNP-L-GLA ° chemically since thiolysis selectively removes DNP from tyrosyl residues) are greater than those of strain 13 PELs to the same compounds, while strain 13 PELs respond more strongly to DNP-GPA than do strain 2 PELs. These findings are interesting for two reasons. They suggest that the T cells primed by immunization with DNP-H37 in one strain of guinea pigs differ significantly from those induced by the same immunogen in a different strain. This in turn suggests that T cells responding to one DNP conjugate are different from those responding to a second DNP conjugate. This has been confirmed by further experiments reported in the subsequent paper (21). Second, the differences between strains suggest that such responses may be under the control of histocompatibility-linked immune response (Ir) genes, since strain 13 guinea pigs have previously been shown to be high responders to immunization with low doses of DNP-GPA while strain 2 animals are low responders (22); and strain 2 guinea pigs are responders to immunization with L-GLA ° whereas strain 13 animals are nonresponders (reference 23; P. H. Maurer, unpublished observations).

It can also be seen from Table VI that PELs from strain 2 guinea pigs primed
with DNP-H37 recognize DNP-L-GLAT primarily as it resembles DNP-L-GLA\textsuperscript{40}, since thiolysis of DNP-L-GLAT lowers the response by only 25%. By contrast, PELs from similarly primed strain 13 guinea pigs respond to DNP-L-GLAT more as it resembles DNP-L-GAT, since thiolysis of DNP-L-GAT lowers the response by about 50%.

The Effect of the Degree of Conjugation on the Response. It has been proposed by some authors (24–26) that the general finding of a failure to observe T-cell responses to haptens results from the multivalent nature of haptenic determinants in conventional (i.e. multiply substituted) hapten-protein conjugates; according to this view, multivalent antigens would inactivate T cells bearing receptors of that specificity. It was therefore of interest to determine the effect of the conjugation density of DNP-carrier conjugates in this system. Fig. 2 shows the response of PELs from strain 13 guinea pigs primed with DNP-H37 to DNP-OVA and to DNP-KLH, conjugated to varying degrees. The data are plotted as counts per minute vs. concentration of DNP, and it can be seen that the response is essentially a direct function of hapten concentration in the cultures, up to plateau levels, for both sets of conjugates. DNP\textsubscript{150}-KLH (Fig. 2 B) is clearly toxic. Thus, these experiments fail to give evidence for significant numbers of hapten-specific T cells responding to very lightly conjugated DNP proteins.

Will Other Haptens Conjugated to Mycobacteria Elicit Similar Responses? In order to determine if the responses of T cells from guinea pigs after immunization with conjugates of H37 were unique to the hapten DNP or would work with other, noncross-reacting haptens, parallel experiments were carried out using the hapten NIP. To test the contribution of the 5-iodo substituent in NIP, NP proteins were prepared, and to test the role of the hapten-carrier bond, NIP was coupled to protein via the NAG. The result of a typical experiment, along with the structures of the relevant haptens, are shown in Table VII; they illustrate several points. There is essentially complete discrimination between NIP conjugates and DNP conjugates by these T cells, which respond only to the hapten used for priming, with two exceptions: PELs from NIP-H37 primed strain 2 animals respond to DNP-L-GL (as do DNP-H37-primed strain 2 guinea pigs), a finding shown subsequently to be due to contamination of this preparation of DNP-L-GL (C. A. Janeway, unpublished observation); and strain 2 guinea pigs primed with DNP-H37 respond, consistently in several experiments, to NP-KLH, probably a true cross-reaction with DNP in this case. NP proteins don't stimulate T cells from NIP-H37-primed animals, demonstrating an important function for the 5-iodo group on NIP, similar to the finding that the para-nitro group of DNP was required to stimulate T cells from animals primed with DNP-H37. NAG proteins, like DAG conjugates in previous experiments, will not stimulate T cells from guinea pigs primed with NIP-H37. NIP-L-GL is not stimulatory while NIP-L-GLA\textsuperscript{30} is strongly stimulating, a finding again analogous to the DNP system. Unfortunately, NIP-L-GAT in which NIP was coupled to tyrosyl residues could not be prepared, so stimulation by determinants containing NIP-tyrosyl has not been demonstrated. The response of T cells from guinea pigs primed with NIP-H37 to NIP conjugates is in every other respect analogous to the response of T cells from DNP-H37-primed guinea pigs to DNP proteins; furthermore, these T cells discriminate between the two haptens.
Fig. 2. The effect of dose and conjugation ratio of (A) DNP-OVA and (B) DNP-KLH on the response of PELs from strain 13 guinea pigs immunized with DNP-H37. Response expressed as total counts per minute incorporated. Control responses to OVA or to KLH at 100 μg/ml shown by dashed horizontal line. (A) Responses to DNP0.4-OVA (Δ), DNP1.3-OVA (□), DNP2.9-OVA (○), DNP4.3-OVA (◇), and DNP8.5-OVA (●). (B) Responses to DNP1.3-KLH (Δ), DNP3.0-KLH (○), DNP4.6-KLH (◇), DNP5.0-KLH (□), DNP6.3-KLH (◇), and DNP10.0-KLH (●). Conjugation ratios expressed as DNP groups per 10⁶ daltons for DNP-KLH.

absolutely. Similar results have been reported by Alkan and El-Khateeb (27).

The Induction of Hapten-Reactive T Cells by Various Immunogens. One of the most interesting and most difficult questions arising from these experiments is why immunization with hapten coupled directly to mycobacteria should elicit T-cell responses to that hapten on a wide variety of carriers. The results of two types of experiments that are relevant to this question will be presented here and are discussed fully later in this paper. In the first (Table VIII), guinea pigs were primed with DNP-H37 prepared in three different ways: DNP-H37, the usual preparation of H37 coupled with DNFB; DNP-H37 (gly), DNP-H37 dialyzed for a week against five changes of 1 M glycine at neutral pH; and DNBS-H37, H37 coupled with DNBS and dialyzed against phosphate buffer. It can be seen from the results presented in Table VIII that there is little difference in the responses of T cells from strain 13 guinea pigs primed with the various immunogens to a variety of DNP conjugates. Of particular interest is the response of PELs from guinea pigs primed with DNBS-H37 to DNP-L-GT and DNP-L-GAT,
**Table VII**

*The In Vitro Proliferative Response of PELs from Strain 2 and Strain 13 Guinea Pigs Primed with DNP or NIP Coupled to Mycobacteria*

| Stimulating antigen (100 µg/ml) | Strain 2 primed with | Strain 13 primed with |
|-------------------------------|----------------------|----------------------|
|                               | NIP-H37 | DNP-H37 | NIP-H37 | DNP-H37 |
| None                          | (1,846) | (2,413) | (801)   | (1,040) |
| PPD*                          | 80,249  | 67,054  | 23,515  | 21,120  |
| PHA†                          | 26,252  | 26,244  | 12,706  | 10,936  |
| NIP₆-OVA                      | 12,279  | 413     | 743     | 0       |
| NIP₆-OVA                      | 0       | 282     | 0       | 0       |
| NAG₆-OVA                      | 0       | 20,056  | 93      | 9,981   |
| NIP₆-GPA                      | 18,312  | 569     | 2,809   | 0       |
| NIP₆-GPA                      | 1,000   | 422     | 0       | 0       |
| NAG₆-GPA                      | 0       | 305     | 59      | 0       |
| DNP₆-GPA                      | 88      | 13,507  | 304     | 7,337   |
| NIP₁₂-KLH                     | 13,152  | 632     | 1,954   | 0       |
| NIP₁₂-KLH                     | 1,106   | 9,941   | 37      | 467     |
| DNP₁₂-KLH                     | 213     | 26,952  | 0       | 5,961   |
| NIP₆,L-GL                     | 1,247   | 1,566   | 0       | 0       |
| DNP₆,L-GL                     | 3,035   | 7,577   | 441     | 205     |
| NIP₆,L-GAT                    | 495     | 326     | 89      | 10      |
| DNP₆,L-GAT                    | 340     | 27,062  | 305     | 7,545   |
| NIP₆,L-GLA₉⁰                   | 27,789  | 1,869   | 3,420   | 0       |
| DNP₆,L-GLA₉⁰                   | 1,959   | 48,098  | 254     | 1,234   |

![Chemical structures]

* 20 µg/ml.
† 1 µg/ml.

since DNBS will not conjugate to tyrosyl residues, while the latter conjugates have DNP coupled almost exclusively to tyrosyl residues.

The second approach involved the use of lipid-substituted DNP-BSA as immunogen, since guinea pigs immunized with such conjugates have previously been shown to exhibit delayed hypersensitivity to a variety of DNP proteins (16). The results of an experiment in which PELs from guinea pigs primed with DNP-BSA-lipid in complete Freund's adjuvant (CFA) containing H37 were cultured with DNP conjugates are shown in Table IX. These data illustrate several...
Table VIII
Immunization of Strain 13 Guinea Pigs with Different Preparations of
DNP-H37

| Stimulating antigen (100 µg/ml) | Stimulation (cpm, E-C) of PELs from animals primed with: |
|-------------------------------|-------------------------------------------------|
|                               | DNP-H37 | DNP-H37 (gly)* | DNBS-H37‡ |
| None                          | (2,166) | (5,020)        | (4,480)   |
| PPD§                          | 53,319  | 74,589         | 67,713    |
| DNP₆-KLH                      | 7,071   | 12,151         | 7,386     |
| DNP₇-GPA                      | 9,864   | 27,726         | 23,485    |
| TNP₉-GPA                      | 16,995  | 21,253         | 16,550    |
| DNP₈-OVA                      | 11,730  | 27,822         | 15,097    |
| DNP₉-L-GL                     | 0       | 0              | 0         |
| DNP₉-L-GLA<sup>ao</sup>       | 5,925   | 9,158          | 6,351     |
| DNP₉-L-GT                     | 16,797  | 17,054         | 16,588    |
| DNP₉-L-GAT                    | 20,185  | 35,707         | 15,280    |
| DNP₉-L-GLT                    | 2,234   | 5,256          | 240       |
| DNP₉-L-GLAT                   | 19,833  | 26,127         | 6,361     |

* DNP-H37 (gly), DNP-H37 dialyzed vs. five changes of 1 M glycine for 1 wk.
‡ DNBS-H37, DNP-H37 prepared using DNBS instead of DNFB.
§ 20 µg/ml.

points. First, T cells from guinea pigs primed with DNP-BSA-lipid give responses similar to those of T cells from DNP-H37-primed animals, although as expected the former respond to BSA and very strongly to DNP-BSA. T cells from guinea pigs primed with DNP-BSA-lipid do not respond to DNP-L-GL but do respond to DNP-L-GLA<sup>ao</sup>, and the response to DNP-L-GLA<sup>ao</sup> is greater when such cells are from strain 2 guinea pigs than when they are from strain 13. Unlike PELs from guinea pigs primed with DNP-H37, T cells from animals primed with DNP-BSA-lipid in some but not all experiments respond to NIP conjugates as seen here, especially with strain 2 PELs. That these responses are not directed at the carrier is shown by the lack of stimulation by DAG-GPA (Table IX) and by the unconjugated carrier proteins (data not shown). Taken together, these findings suggest that DNP-H37 and DNP-BSA-lipid elicit T-cell responses of similar specificity, that is, they prime similar but not identical subpopulations of T cells.

Discussion

The principal aim of the present experiments was to define more fully and precisely those antigenic determinants that stimulate T cells primed by in vivo immunization of guinea pigs with DNP coupled directly to mycobacteria. This information is critical for the next stage of this study of T-cell specificity (21), which involves an analysis of the diversity of specificities expressed by individual responding T cells. A general discussion of T-cell receptor specificity in this system will be presented elsewhere (21, 28).

Several experiments point to the fact that T cells from animals primed with DNP-H37 can respond to DNP coupled covalently either to lysyl or tyrosyl
Table IX

| Stimulating antigen (100 μg/ml) | Strain 2 immunized with: | Strain 13 immunized with: |
|-------------------------------|--------------------------|--------------------------|
|                               | DNP-BSA-lipid            | DNP-H37                   |
|                               | DNP-BSA-lipid            | DNP-H37                   |
| None                          | (377)                    | (1,236)                  |
| PPD*                          | 132,638                   | 56,724                   |
| PPD†                          | 85,484                    | 20,959                   |
| PPD                           | 12,677                    | 143                      |
| DNP-L-BSA                     | 32,868                    | 896                      |
| NIP-L-OVA                     | 1,857                     | 395                      |
| DNP-L-OVA                     | 6,377                     | 9,146                    |
| NIP-L-GPA                     | 4,120                     | 173                      |
| DNP-L-GPA                     | 10,517                    | 3,826                    |
| DAG-L-GPA                     | 335                       | 0                        |
| NIP-L-KLH                     | 1,949                     | 7                        |
| DNP-L-KLH                     | 9,177                     | 11,973                   |
| DNP-L-L-GL                    | 151                       | 76                       |
| NIP-L-L-GLA<sup>30</sup>      | 2,286                     | 12                       |
| DNP-L-L-GLA<sup>30</sup>      | 7,185                     | 11,014                   |
| DNP-L-GAT                     | 5,361                     | 11,126                   |

* 20 μg/ml.
† 1 μg/ml.

residues. Thus, conjugates of proteins prepared using DNBS, which do not couple to tyrosyl residues, stimulate responses of these T cells comparable in magnitude to those elicited by DNFB conjugates of the same protein. Similarly, DNFB or DNBS (C. A. Janeway, unpublished observations) conjugates of L-GLA synthetic polypeptides, in which only the ε-amino groups of lysyl residues are available for hapten modification, stimulate strong in vitro proliferative responses of such T lymphocytes. In the previous paper (1) it was shown that these T cells also respond to antigenic determinants containing O-DNP-tyrosyl residues. This finding has been confirmed in the present experiments by the response to DNP-L-GT, and by the reduction of response after thiolysis of DNP-L-GLAT, which selectively removes DNP from tyrosyl residues. Since it has also been shown that O-DNP-tyrosyl residues can transfer their DNP groups to other proteins during culture, it is possible that the O-DNP-tyrosyl residues are not recognized as such by these T cells. However, data from trans-conjugation experiments strongly suggest that O-DNP-tyrosyl residues are directly recognized, in that the extent of trans-conjugation is not correlated with the ability to stimulate T cells from DNP-H37-primed guinea pigs, using comparable assays. Direct recognition of O-DNP-tyrosyl residues is strongly supported by studies of the specificity of subpopulations of the responding cells (21).

A second finding of these experiments is that the response of T cells from DNP-H37-primed guinea pigs is strongly influenced by amino acid residues to
which DNP is not coupled. This is clearly seen in the failure of DNP-L-GL to stimulate a response in T cells that respond strongly to DNP-L-GLA\textsuperscript{30}. The addition of alanine to other synthetic carriers also increases the responses to the DNP conjugates. The ability of DNBS proteins to stimulate such responses, supports the idea that amino acids in the carrier other than that to which the hapten is bound are recognized, since in this case DNP is also coupled to lysyl residues as in the nonstimulatory DNP-L-GL; the other amino acids in the carrier must therefore contribute to the stimulating activity of the conjugate. It could be argued that these findings do not reflect the specificity of the T-cell receptor, but are instead nonspecific in nature, resulting from an alteration in the net charge of the carrier, influencing its association with T cells or macrophages in general. In this regard, it should be pointed out that these same preparations of DNP-L-GL have been shown to associate functionally with T cells and macrophages in strain 2 guinea pigs immunized with DNP-L-GL (1). Furthermore, highly coupled DNP-L-GL, in which many of the (charged) lysyl residues are blocked by DNP does not stimulate T cells from guinea pigs primed with DNP-H37, nor does DNP-L-GL in which the total charge has been reduced by changing the ratio of glutamic acid to lysine from 6:4 to 4:6, coupling with DNP, and further blocking the lysyl residues by acetylation (C. A. Janeway and J. K. Inman, unpublished results). That the alanyl residues of DNP-L-GLA are recognized specifically by T cells from guinea pigs primed with DNP-H37 is also suggested by the findings presented in Fig. 1. DNP-L-GLA\textsuperscript{30} is highly stimulatory, whereas DNP-D-GLA\textsuperscript{30}, in which all the amino acids are of the D optical configuration, is not stimulatory. Conjugates of mixed polymers, in which only the alanyl or lysyl residues are of the D configuration, are also not stimulatory, whereas a mixed polymer in which glutamic acid is of the D configuration is stimulatory, albeit weakly. It must be pointed out that the mixed copolymers are of much smaller size than either L-GLA\textsuperscript{30} or D-GLA\textsuperscript{30}, which may reduce their stimulatory activity. Thus, responses to the conjugates of the optically mixed polymers probably cannot be compared quantitatively to responses to the larger polymers of L or D configuration. Nonetheless, comparison of responses between these three mixed polymers may be possible. Such a comparison then suggests that the optical configuration of lysyl and alanyl residues is important to the antigenic determinant recognized by T cells from guinea pigs primed with DNP-H37. Since D-alanyl residues should affect the charge of DNP-GLA\textsuperscript{30} identically to L-alanyl residues, it seems unlikely that the effect of the alanyl residues in DNP-GLA\textsuperscript{30} is simply alteration in charge. Rather, it seems more likely that they are actually recognized as part of the antigenic determinant. A second possibility is that the major effect of alanyl residues in these polymers is conformational, since increasing alanine content increases \(\alpha\)-helical conformation of the L-GLA polymers. Obviously, D-alanyl residues would not give rise to similar conformational effects. It seems most probable that alanyl residues contribute to the antigenic determinant both by being recognized as such and by altering the conformation of the polymer. The stimulatory activity of DNP-L-GLA\textsuperscript{30} in which every lysyl group has a glutamic acid residue on one side and an alanyl residue on the other, may suggest a role for an alanyl residue directly adjacent to a DNP-lysyl residue being part of an effective antigenic determinant.
Conjugation density (i.e., the number of hapten groups per molecule of carrier) per se, apparently does not greatly affect this response. Rather, the response seems to be a function of the total concentration of hapten groups in culture. Thus a significant number of truly hapten-specific T cells capable of responding only to monovalent DNP conjugates is not present in PELs from animals primed with DNP-H37. However, one can not say from such data that hapten-specific T cells responsive to such conjugates do not exist, only that they are not detected here.

The critical role of the hapten (1, 27) in the antigenic determinants recognized by T cells from guinea pigs primed with hapten-coupled mycobacteria is confirmed here by the findings with the polar hapten NIP. T cells from guinea pigs primed with NIP or DNP mycobacteria appear to fully discriminate between NIP and DNP. Furthermore, the 5-iodo substituent of NIP is essential for T-cell recognition, as is the para-nitro group of DNP, since NP proteins are not stimulatory. Whether the 5-iodo group per se, or its effect on the pK of the 4-hydroxy substituent, determines this difference is not known.

There is a very interesting effect of the strain of guinea pig immunized with DNP-H37 on the response to certain DNP conjugates. Since, for instance, immunization with DNP-L-GLA shows strain 2 guinea pigs to be responders and strain 13 guinea pigs to be nonresponders, and the same pattern of responsiveness to DNP-L-GLA emerges from immunization with DNP-H37 it may be possible to assess the response of a single animal to a wide variety of compounds by immunization with haptenated mycobacteria and testing the response of its T cells to hapten conjugates. Direct immunization experiments could then be used to test only promising materials, thus saving many animals. These findings also suggest very strongly that immunization with DNP-H37 activates a population of T cells whose specificity characteristics differ significantly between strain 2 and strain 13 guinea pigs. Since DNP is common to all the antigens tested, the distinction must be based on recognition of determinants contributed, at least in part, by the carrier. This supposition has now been confirmed (21). Thus T cells recognize determinants comprising hapten and carrier, and the bond between them (c.f. Table VII).

The mechanism by which hapten coupled to mycobacteria activates those T cells which recognize determinants that we may call "conjugate specific," since they are a complex of hapten plus carrier, is not known. That DNP covalently linked to H37 will elicit such a response is particularly striking in view of the fact that the same hapten, coupled covalently to protein and mixed with H37 in CFA, will not (1, 29, 30). Several possible mechanisms for the action of DNP-H37 can be envisaged. First, it is possible that the DNFB is sequestered in reactive form in the waxyl coat of the mycobacteria, exiting and conjugating to host protein upon injection. According to this hypothesis, subsequent in vitro responses to conjugates containing O-DNP-tyrosyl residues then proceed via trans-dinitrophenylation of the same host proteins. That similar responses occur after immunization with DNBS-H37, including responses to conjugates containing DNP only as O-DNP-tyrosyl residues, renders this explanation unlikely, since DNBS is highly water soluble.

Second, the mycobacteria may be so complex and heterogeneous chemically as to resemble all of the carriers used in these studies; hence, immunization with
DNP-H37 would be like immunization with a mixture of all of these DNP conjugates. However, this hypothesis fails to account for the absence of stimulation by unconjugated carriers of cells obtained from animals immunized with DNP-H37. This explanation is also made unlikely by the finding of a response similar to that given by DNP-H37 when the DNP is presented as DNP-BSA-lipid. In this instance, it is hard to see how adding lipid to DNP-BSA would alter DNP-BSA so that it resembles all the DNP conjugates to which it elicits responses. Third, the mycobacteria may be such potent stimulators of cell-mediated immunity that the minor cross-reactivity, observed when immunization with DNP proteins is carried out, is greatly exaggerated if the hapten is covalently bound to the mycobacteria. This possibility can not be ruled out, but requires an explanation for the adjuvanticity of mycobacteria, a subject not dealt with here. Finally, it seems necessary to postulate that the T-cell receptor being studied has at least a low affinity for the hapten itself, an argument made in detail elsewhere (1, 28). If this is so, then one might explain the unique ability of DNP-H37 and DNP-BSA-lipid to prime T cells of this type by a dual interaction with the T-cell surface, the lipid in both immunogens bonding by nonspecific hydrophobic forces to T-cell membrane lipid, thus stabilizing the weak interaction between the DNP group and any receptor even partially specific for it. The nonlipid containing DNP-carrier conjugates used for in vitro testing, however, need to be bound with better fit and higher affinity to the receptor itself, in order that a sufficiently stable antigen-receptor bond be formed to cause cell activation. This hypothesis would explain how it would be possible for the antigenic determinants of the immunogen to differ significantly from those required for subsequent activation of the cell, as the present experiments clearly show they do, without having to postulate alterations in the specificity or behavior of the receptor after immunization. We favor this hypothesis, and current experiments strongly support it.

Summary

T cells from guinea pigs immunized with the hapten 2,4-dinitrophenyl (DNP)-coupled directly to mycobacteria are of interest since they recognize and respond to DNP conjugated to many but not all carriers. The experiments reported here further analyze the structure of the complex, chemically defined antigenic determinants recognized by such T cells. These antigenic determinants can have DNP coupled either to the ε-amino group of lysyl residues or to the hydroxyl group of tyrosyl residues. Furthermore, essential contributions to the determinant recognized by such T cells are made by amino acid residues to which the hapten is not attached. Such residues are thought to be close to the hapten group itself, since introducing a small spacer between hapten and carrier prevents recognition. The hapten itself is also recognized and discriminated from other haptens with great precision by these T lymphocytes. The strain of guinea pig immunized affects the precise specificity characteristics of the responding T cells, in a way that may reflect the activity of histocompatibility-linked immune response genes. Finally, the characteristics of the immunogen have been studied. It is thought that the lipid content of the mycobacteria may be critical in inducing the hapten-reactive T cells, and this is supported by finding similar responses in T cells from guinea pigs immunized with DNP protein to which
lipid has been covalently attached. Thus, the T-cell population being studied,
while recognizing haptens with great precision, appears to require a larger
determinant for activation than do hapten-specific B lymphocytes.

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