CYTOTOXIC T CELLS DISTINGUISH BETWEEN TRINITROPHENYL- AND DINITROPHENYL-MODIFIED SYNGENEIC CELLS

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Studies analyzing the specificity of receptors on thymus-derived (T) cells and their ability to distinguish between structurally related antigens in comparison to that of humoral antibody has provided insight into the nature of the T-cell receptor (1, 2). One approach to directly evaluate the specificity of a particular subpopulation of T cells is to assay for cytotoxicity in the cell-mediated lympholysis (CML) assay. Evidence that the cytotoxic T cell recognizes specific antigen includes the findings that such cells can be absorbed on antigen-specific monolayers, T-cell-mediated cytotoxicity can be blocked by adding an excess of unlabeled antigen-bearing target cells, and antisera against structures recognized by these T cells blocks killing (reviewed in 3).

Cytotoxic T-cell activity has been examined in detail against trinitrophenyl(TNP)-modified syngeneic cells (3, 4). The specificity of this response has been shown to be controlled by the H-2 gene complex in that cytotoxic effector cells generated against syngeneic TNP-modified stimulator cells express cytotoxicity against only H-2-related TNP-modified target cells (4, 5). In addition, cross-reactivity of the cytotoxic T-cell response towards cells derivatized with different haptens does not occur to a significant extent (6).

In the present experiments I have tested the specificity of the cytotoxic effect of splenic T cells sensitized against either TNP- or dinitrophenyl (DNP)-modified spleen cells in the CML assay. Since these haptens are structurally very similar and couple to proteins by attaching primarily to the same amino acid residues (7), the fine specificity of the receptor on the cytotoxic T cell and its ability to discriminate between closely related antigenic molecules can be analyzed.

Materials and Methods

Generation of Cytotoxic Effector Cells, Preparation of Target Cells, and Assay for CML Activity. The method used for generating and assaying cytotoxic T-cell activity against TNP-modified spleen cells has been described in detail in previous communications (5, 8). The method used to generate anti-DNP-cytotoxic cells was identical to that used to generate anti-TNP effector cells with the following exceptions: stimulator or target cells were suspended at a concentration of 3 × 10^7 cells/ml in a 10-mM solution of dinitrobenzenesulfonic acid (DNBS) (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in phosphate-buffered saline at pH 8.5 for 30 min at 37°C. After this treatment, the cells were washed two times in a balanced salt solution containing 5% fetal calf serum before use.

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TABLE I

Specificity of Splenic Effector Cells Sensitized to TNP- or DNP-Modified Stimulator Cells

| Responder | Stimulator | Target | Inhibitor | Net release | Experiment number |
|-----------|------------|--------|-----------|-------------|-------------------|
| BALB/c    | BALB/c-TNP | BALB/c-TNP | 15.3 | 48.3 | 31.4 | 1 |
| A/J       | BALB/c-DNP | BALB/c-DNP | 0.1 | 5.7 | 13.3^§ | 2 |
| BALB/c-TNP | BALB/c-DNP | BALB/c-DNP | 8.5 | 14.6 | 10.4 | 3 |
| BALB/c-TNP | BALB/c-DNP | BALB/c-DNP | -0.6 | 6.6 | -6.1 | 4 |
| BALB/c-TNP | BALB/c-TNP | BALB/c-TNP | 22.2 | 17.1 (25)** | 18.6 (16) | 1 |
| (0.5 × 10^6) | | | | | |
| (1 × 10^6) | | | | | |
| BALB/c-DNP | BALB/c-DNP | BALB/c-DNP | 9.3 (58) | 8.4 (63) | | |
| (0.5 × 10^6) | | | | | |
| (1 × 10^6) | | | | | |
| BALB/c-DNP | BALB/c-DNP | BALB/c-DNP | 27.9 (26) | 20.1 (9) | | |
| (0.5 × 10^6) | | | | | |
| (1 × 10^6) | | | | | |

* Effector to target cell ratio, 100:1, target cells are 10^4 3H-Cr-labeled Con A lymphoblasts.
† Net release = isotope release in the presence of immune cells - nonimmune cells.
§ A/J rather than BALB/c cells were used.
¶ The cross-reactivity observed in experiment 3 was not noted in three additional experiments (data not shown).
** Number in parentheses indicates percent inhibition of net release (See reference 8).

The spontaneous isotope release from target cells ranged from 25 to 35% for TNP-modified targets and from 25 to 45% for DNP-modified targets.

Cold Target Competition Assay. The method used is identical to that described in a previous communication (5). In Vivo Immunization of Animals with Haptenated Cells. Spleen cells were modified with trinitrobenzenesulfonic acid (TNBS) or DNBS, adjusted to a concentration of 40 × 10^6 cells/ml, and each mouse received 0.25 ml of the suspension intraperitoneally. The concentration of TNBS and DNBS used for derivatization of the spleen cells was 10 mM. 7-10 days after priming, the animals were sacrificed, their spleens removed, and the cells used for in vitro culturing in the CML assay.

Results

Specificity of the Primary CML Response against TNP- and DNP-Modified Syngeneic Cells. BALB/c or A/J spleen cells sensitized against TNP-modified stimulator cells displayed a cytotoxic effect against TNP-modified concanavalin A (Con A) lymphoblast targets (Table I). If the spleen cells were sensitized to DNP-modified stimulators, however, little or no cytotoxicity was seen against TNP-modified target cells. The same result occurred in the reciprocal direction; that is, spleen cells sensitized against DNP-modified stimulators exerted a cytotoxic effect against DNP-modified targets, but not when they were sensitized with TNP-modified stimulators. In all experiments, a lower net isotope release was observed with anti-DNP effector cells compared to anti-TNP effectors. In addition, the spontaneous release from target cells modified with DNBS was generally higher due to the conditions used for modification (see Materials and Methods).

Specificity of the Secondary CML Response against TNP- and DNP-Modified Target Cells. While the specificity of the primary immune response in the CML test was specific for either TNP- or DNP-modified target cells, it is possible that during in vivo priming, cross-reactive anti-hapten T-cell clones are sensi-
Specificity of the Cytotoxic Effect of Spleen Cells from Primed Mice Rechallenged In Vitro and Tested against TNP- or DNP-Modified Targets

| Line | Responders | Stimulators | Targets | Net isotope release |
|------|------------|-------------|---------|--------------------|
| 1    | BALB/c     | BALB/c-TNP  | BALB/c-TNP | 37.6               |
|      | Imm-TNP    | BALB/c-DNP  | BALB/c-DNP | 6.1                |
| 2    | BALB/c     | BALB/c-DNP  | BALB/c-DNP | 33.4               |
|      | Imm-DNP    | BALB/c-TNP  | BALB/c-DNP | 8.2                |
| 3    | BALB/c     | BALB/c-DNP  | BALB/c    | -3.4               |
|      | Imm-TNP    | BALB/c-DNP  | BALB/c    | -2.1               |
| 4    | BALB/c     | BALB/c-TNP  | BALB/c-DNP | 2.1                |
|      | Imm-TNP    | BALB/c-DNP  | BALB/c-DNP | 15.5               |
| 5    | BALB/c     | BALB/c-DNP  | BALB/c-TNP | 12.0               |
|      | Imm-DNP    | BALB/c-TNP  | BALB/c-TNP | 38.0               |

* See footnote * in Table I.

1 Spleen cells from mice primed with TNP- or DNP-modified spleen cells 7-10 days previously were rechallenged in vitro with hapten-modified cells. tized, or that cells with receptors of higher avidities are activated which may give rise to cross-reactivity in a secondary cytotoxic response. Therefore, spleen cells from animals primed with TNP-modified spleen cells were cultured in vitro with TNP- or DNP-modified stimulator cells and then tested for their cytotoxic potential against TNP-modified target cells. The results indicate (Table II, line 1) that strong cytotoxicity was observed only when the in vitro stimulator and target cells were derivatized with the same hapten. Thus, mice primed with TNP-modified cells in vivo and rechallenged in vitro with DNP-modified stimulator cells did not cross kill TNP-modified targets to a significant extent. The same negative result was seen with spleen cells from animals primed to DNP-modified cells, rechallenged with TNP-modified stimulators, and tested against DNP-modified targets (Table II, line 2). Evidence that the cytotoxic response is specific is shown by the finding that the in vivo-primed and in vitro challenged effector cells show no cytotoxic activity against target cells that are not haptenated (Table II, line 3).

To further assess the specificity of cells from mice primed in vitro with hapten-modified cells, I rechallenged splenocytes from animals primed with TNP-modified cells in vivo with TNP-modified stimulators and tested for cytotoxic activity against DNP-modified targets. The results (Table II, line 4) show that no cytotoxic activity resulted. However, if the TNP immune spleen cells were cultured in vitro with DNP-modified stimulators cells, cytotoxicity was noted against DNP-modified target cells as expected, reflecting a primary anti-DNP-CML response. When animals were immunized in vivo with DNP-modified cells, rechallenged in vitro with DNP-modified stimulators, and tested against TNP-modified targets, some cytotoxic activity resulted (Table II, line 5). However, it was considerably less than seen against the specific targets (compare line 5 with line 2). A strong cytotoxic effect resulted, however, when the immune
cells were rechallenged with TNP-modified stimulators and tested against TNP-
modified targets (again reflecting a primary in vitro CML response).

Additional data indicates that the cytotoxicity can be completely abrogated by
treating the effector cells with an anti-T-cell serum and complement, and serum
from primed mice has no effect on inducing antibody-dependent cell-mediated
cytotoxicity in vitro (data not shown).

**Specificity of TNP Sensitized Effector Cells as Assessed by a Cold Target
Competition Assay.** To more directly assess the specificity of the cytotoxic
effector cells for these two haptens, I sensitized BALB/c spleen cells in vitro with
TNP-modified stimulators and determined the ability of unlabeled hapten-
modified Con A lymphoblasts to block the specific cytotoxic effect of the effector
cells generated. The results (Table I) show that spleen cells sensitized with TNP-
modified stimulator cells are cytotoxic for TNP-modified target cells. The addi-
tion of a 50- to 100-fold excess of unlabeled TNP-modified inhibitor cells in
relation to labeled targets blocked a large proportion of the cytotoxic effect.
However, target cells unmodified or modified with DNBS show little blocking
and in some cases enhancement of activity in the CML assay.

**Discussion**

The fact that antigen-specific receptors on cytotoxic T cells can distinguish
between DNP- and TNP-modified cells indicates that the T-cell receptor can
discriminate between two closely related haptenic molecules. Although the
hapten plays an important role in antigen recognition by T cells, the neighbor-
ing amino acids to which small haptenic molecules are attached play a critical
role in determining T-cell specificity (9). Thus, it is possible that T cells may be
able to discriminate between DNP- and TNP-modified cells not on the basis of
the meta nitro group, but rather by the fact that the nitrophenyl haptens
coupled to different amino acid residues. However, under the conditions used for
modification in this study, the predominant residue derivatized by both of these
haptens is the e-amino group of lysine (7). Although these haptens may also
couple to nonprotein cell membrane components, we have found that greater
than 70% of labeled (3H) TNBS attaches to plasma membrane proteins. Therefore,
the ability of the T cell to distinguish between TNP- and DNP-modified
cells is most likely the result of the receptor discriminating between differences
in the two haptens themselves, rather than adjacent amino acids.

The specificity of T cells for these two haptens has been examined in other
assays including delayed type hypersensitivity responses (10), and T-cell prolif-
eration assays in vitro (2). While such responses are characterized by hapten
specificity, some cross-reactivity is observed between DNP and TNP derivatized
proteins (2, 10). Since none of these assays measure T-cell binding, but rather an
activity that follows as a consequence of antigen recognition, this cross-reactiv-
ity may in part be due to biological amplification and recruitment (11).

The specificity of cytotoxic T cells sensitized to hapten-modified cells and the
H-2 genetic restriction can be explained by two different hypotheses (3, 12).

1 Forman, J., E. S. Vitetta, and D. A. Hart. Relationship between trinitrophenyl and H-2
antigens on trinitrophenyl-modified spleen cells. III. Quantitation of trinitrophenol molecules and
trinitrophenyl-derivatized H-2 antigens on cells that specifically block cytotoxic effector cells.
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According to the altered self interpretation of T-cell-mediated cytotoxicity, T cells bear one clonally distributed receptor that recognizes H-2 controlled cell surface antigens which have been altered by extrinsic antigens (haptens and viruses), extrinsic antigens that have been altered by H-2 gene products, or some type of interaction antigen. In contrast, the dual receptor hypothesis states that T cells have two receptors, one clonally distributed and specific for antigen, the second receptor directed towards a self H-2 gene product. In this case, T-cell recognition would occur as a result of these two receptors interacting with antigen and H-2 markers on the stimulator and target cell.

Data obtained in other studies indicate that a certain degree of cross-reactivity occurs between TNP-modified H-2 haplotypes as well as between TNP-modified and H-2 allogeneic haplotypes (13, 14). This would be in agreement with the findings of Lindahl et al. (15) who noted some cross-reactivity between haplotypes in H-2 allogeneic CML. The results obtained in this study, as well as in those reported by using other haptens for cell modification (6) indicate that T cells do not cross kill target cells modified with different but related haptens. Therefore, these data are more consistent with the dual or two receptor hypothesis, one of which has the capability of distinguishing between closely related haptens. A likely possibility for this antigen receptor is an immunoglobulin V region similar to that described in the studies identifying idiotypes on T cells (16, 17). Consistent with this postulate, Klinman et al. (18) have shown that virgin B cells bear receptors with nonoverlapping specificities for DNP and TNP.

If T cells have two receptors, one for antigen (viz. TNP) and the other for self H-2 gene products, these may be of low affinity which would explain the lack of cross-reactivity between TNP and DNP. Interaction with both receptors would be required for high avidity binding between the T cell and target. Previous results (15, 16) indicating cross-reactivity occurs between haplotypes in TNP-CML would be consistent with the receptor for self H-2 products being more cross reactive, perhaps as a result of relatively few V region genes. This would be in agreement with recent estimates indicating that there is a high frequency of cytotoxic T cells responding to foreign H-2 haplotypes in CML responses and the fact that approximately 10% of clones activated in CML can react with H-2 unrelated haplotypes (19).

Summary

Spleen cells sensitized against trinitrophenyl (TNP)-modified stimulator cells displayed a cytotoxic effect against syngeneic TNP-modified but not dinitrophenyl (DNP)-modified target cells. The same finding was observed in the opposite direction; that is, effector cells sensitized against DNP-modified stimulator cells did not cross kill TNP-modified targets. The specificity of the anti-TNP effector cells was confirmed in a cold target competition assay.

Presensitization in vivo with hapten-modified cells followed by rechallenge and testing in vitro did not alter the specificity of the response between the haptons. These data indicate that the receptor(s) on the cytotoxic T cell can distinguish between two closely related haptenic molecules.

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