CELL-MEDIATED LYMPHOLYSIS OF TRINITROPHENYL-MODIFIED AUTOLOGOUS LYMPHOCYTES

Confirmation of Genetic Control of Response to Trinitrophenyl-Modified H-2 Antigens by the Use of Anti-H-2 and Anti-Ia Antibodies

BY ANNE-MARIE SCHMITT-VERHULST, DAVID H. SACHS, AND GENE M. SHEARER

(From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

The in vitro induction of T-cell-mediated lympholysis (CML) to trinitrophenyl (TNP)-modified autologous spleen cells has been demonstrated in the mouse (1). Lysis of modified splenic target cells was obtained when there was K or D serological region homology between the target cells and the cells used in the sensitizing phase (2, 3). Experiments using F~ responding lymphocytes sensitized and assayed with TNP-modified parental cells indicated that homology between effector and target cells was not sufficient to obtain lysis, but that the homology required was either between modified stimulating and modified target cells, or among responding, modified stimulating and modified target cells (2). These findings are compatible with the hypothesis that TNP is modifying cell surface components controlled by the K and D serological regions of the major histocompatibility complex, and that the responding lymphocytes are reacting against these new antigenic specificities.

Earlier studies have shown that sensitization of B10.A responding lymphocytes with TNP-modified autologous spleen cells generated effector cells which lysed TNP-modified K-end-matched target cells, but not TNP-modified D-end-matched targets (2, 4). This finding contrasts with the results obtained with B10.D2 effector cells which lysed TNP-modified H-2K- and H-2D-region-matched targets equally well (2, 4). This observation was unexpected, since the same D-end products are involved in the new antigenic specificities formed in both strains. This difference in response to TNP-modified H-2D^d products of B10.A and B10.D2 splenic lymphocytes has been attributed to a dominant H-2-linked immune response (Ir) gene(s), since (B10.A × B10.D2)F_1 lymphocytes generated lytic activity toward TNP-modified H-2D^d products when sensitized with B10.A-TNP stimulating cells (4).

Nabholz et al. have demonstrated that the lytic phase of an allogeneic CML can be blocked by antisera directed against the serological region of the target cell to which the responding lymphocytes were sensitized (5). A similar approach has been used in this study to: (a) determine whether antisera directed against H-2 serological specificities expressed by the target cells would affect the lytic phase of the TNP autologous CML, and (b) independently verify by selective blocking with anti-H-2K or D-end sera that in the CML generated by B10.A splenic lymphocytes against TNP-modified autologous cells, only modified K-end products would be involved in the lytic phase, in contrast to B10.D2 in which both K- and D-end products are involved in the lysis.

THE JOURNAL OF EXPERIMENTAL MEDICINE • VOLUME 143, 1976 211
Material and Methods

**Mice.** All mice used in the experiments were males, 6–9 wk of age. The C57BL/10 congenic strains and AKR/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. The A.TL, A.TH, and A.AL mice used for preparation of antisera were raised in our animal colony from breeders kindly provided by Doctors D. Shreffler and C. David, Department of Human Genetics, University of Michigan, Ann Arbor, Mich.

**In Vitro Cell Culture and TNP Modification of Spleen Cells.** The methods employed for the primary 5-day in vitro CML sensitization and the TNP modification of stimulating and target cells have been reported previously (1, 2, 4).

**Blocking of Effector Phase with Antisera.** A vol of 10 µl containing 5 × 10^4 TNP-modified ⁵¹Cr-labeled target cells was preincubated for 30 min at 37°C with 20 µl of undiluted normal sera or alloantisera. Based on their known cytotoxic titers for the specificities being studied, these antisera were in antibody excess under the experimental conditions used. The sera were absorbed on TNP-modified spleen cells of the strain of origin in order to remove any natural anti-TNP antibody. 5 × 10⁶ effector cells in a vol of 20 µl were then added to the target cell-antisera mixture, and this was incubated for 3.5 h at 37°C in a CO₂ atmosphere for the ⁵¹Cr-release assay (1, 2).

**Alloantisera.** The antisera used in this study were prepared by immunization of appropriate groups of animals with skin grafts and lymphoid cells as previously described (6). The following sera were used in this study: B10.BR anti-C57BL/10 (kkkkkk abbbbbb); B10.BR anti-B10.D2 (kkkkkk adddddd); B10.D2 anti-B10.BR (dddddd addaaaa); A.TL anti-A.AL (akkkkk akkkkkk); A.TH anti-A.TL (sssssd askkkkd); (C57BL/10 x A/J)F₁ anti-B10.D2 (bbbbb x kkkddd adddddd); B10.BR anti-B10.A (kkkkkk akkkkkk); and B10.A anti-C57BL/10 (kkkddd abbbbbb). Anti-TNP, kindly provided by Dr. Pierre Henkart, Immunology Branch, National Cancer Institute, Bethesda, Md., was the immunoglobulin fraction of anti-TNP-keyhole limpet hemocyanin serum prepared in C57BL/10 mice.

Results and Discussion

The results shown in Table I verify that B10.A effector cells generated by sensitization with TNP-modified autologous cells lyse B10.A-TNP (homology throughout H-2) and B10.BR-TNP (H-2 homology at K, I-A, and I-B) targets equally well, but not B10.D2-TNP (H-2 homology at I-C, S, and D), nor C57BL/10-TNP (no H-2-region homology) targets. However, B10.D2 effectors, generated by sensitization with TNP-modified autologous spleen cells, lysed both B10.D2-TNP (homology throughout H-2) and B10.A-TNP (H-2 homology at I-C, S, and D) targets, but not B10.BR-TNP nor C57BL/10-TNP (no H-2-region homology).

The lympholysis of B10.A-TNP targets by effectors generated from sensitization of B10.A and B10.D2 lymphocytes with either TNP-modified autologous or allogeneic spleen cells and the effect of various antisera are shown in Table II. A complete inhibition of lysis by B10.D2 effectors was obtained when the B10.A-TNP targets were preincubated with sera directed against D-end serological specificities (section A). In contrast, this antisera had no effect on the lysis of targets by B10.A effector cells. Antisera directed against products of K, I-A, and I-B regions of B10.A target cells had no effect on the lysis by B10.D2 effectors, but significantly inhibited the lysis by B10.A effector cells. The data in the lower part of section A demonstrate that these sera have only minimal effects on the lytic capacity of effector cells of these strains directed against H-2<sup>b</sup> alloantigens. It is interesting to note that the only appreciable decrease in lysis seen in these

---

1 Lowercase letters indicate haplotype of origin of the six presently defined regions and subregions of the H-2 complex, i.e. K, I-A, I-B, I-C, S, and D (7).
allogeneic controls occurred when a possible D-region public specificity was blocked and when B10.D2 but not B10.A was the effector cell donor.

The differential effect of the anti-H-2\(^a\) serum on the inhibition of lysis by B10.A and B10.D2 effector cells can be attributed exclusively to differences in the lytic potential of the effector cells, since the same antiserum and target cells were used in both cases. These results demonstrate that inhibition was observed only in the presence of antisera directed against those modified H-2 specificities toward which the effectors were sensitized, and verify that B10.A responding lymphocytes are nonresponders to TNP-modified autologous D-end components.

The selectivity of the H-2 components involved in the blocking of the lytic phase of CML by antibodies was further analyzed for B10.A responding cells sensitized against TNP-modified autologous cells and assayed on B10.A-TNP target cells. The data presented in section B show that antiserum directed against the H-2K\(^a\) region (A.TL anti-A.AL) can completely inhibit the lysis of B10.A effectors on B10.A-TNP target cells, whereas sera directed against (I-A to S)\(^d\) regions (A.TH anti-A.TL) or against (I-C to D)\(^d\) (B10.BR anti-B10.D2) do not affect lympholysis. As shown in the controls in the lower part of section B, these sera directed against H-2 antigens expressed on effectors sensitized against H-2\(^b\) alloantigens did not reduce the lysis measured on C57BL/10-TNP target cells.

Since B10.D2 cells sensitized against autologous TNP-modified spleen cells appeared to react against both \((K, I-A, I-B)\)^d and H-2D\(^d\)-modified components, the influence of sera directed against products of the whole H-2\(^d\) complex, \((K, I-A, I-B)\)^d regions, \((I-C, S, D)\)^d regions, and against the shared I-A subregion specificity \(\text{Ia.8} \) (6) were examined in the lytic phase assayed on B10.D2-TNP spleen targets. The results, shown in section C of Table II, indicate that a complete inhibition of the lysis measured by B10.D2 cells sensitized against autologous TNP-modified cells was obtained only in the presence of serum directed against products of the whole H-2 complex. Sera directed against either \((K, I-A, I-B)\)^d or \((I-C, S, D)\)^d regions of the unmodified targets partially inhibited lympholysis, whereas serum against the \(\text{Ia.8} \) specificity had no significant effect on CML. Controls indicating that none of these sera affect lympholysis by B10.D2 effectors sensitized against H-2\(^a\) alloantigens when assayed on B10.BR-
**TABLE II**

Selective Effect of Specifically Directed Anti-H-2 Sera and Anti-TNP-KLH Serum on the Lysis of TNP-Modified Target Cells by Effector Cells Generated by Sensitization with TNP-Modified Autologous Stimulating Cells

| Responding cells | Stimulating cells | Target cells | % Specific lysis ± SE of target cells preincubated in the presence of: |
|------------------|-------------------|--------------|---------------------------------------------------------------|
|                  |                   |              | Normal serum | B10.A TNP | ddAAA | B10.BR | B10.D2-TNP | B10.D2-TNP |
| A                | B10.A             | C57BL/10     | Normal serum | 36.0 ± 3.0 | 38.0 ± 3.8 | 45.7 ± 1.9 | 45.7 ± 1.9 | 45.7 ± 1.9 |
|                  | B10.A-TNP        | C57BL/10-TNP | ddAAA | 48.4 ± 4.8 | 56.7 ± 3.8 |
|                  | B10.A-TNP        | B10.BR       | ddAAA | 46.0 ± 4.8 | 46.0 ± 4.8 |
|                  | B10.BR           | B10.D2-TNP   | ddAAA | 6.6 ± 2.7 | 6.6 ± 2.7 |
|                  | B10.BR           | AKR/J        | ddAAA | 51.5 ± 2.6 | 51.5 ± 2.6 |
|                  | B10.BR           | C57BL/10     | ddAAA | 49.2 ± 3.7 | 49.2 ± 3.7 |
|                  | B10.BR           | C57BL/10-TNP | ddAAA | 54.9 ± 2.7 | 54.9 ± 2.7 |
|                  | B10.BR           | B10.A       | ddAAA | 48.7 ± 4.9 | 48.7 ± 4.9 |
|                  | B10.BR           | B10.D2       | ddAAA | 6.6 ± 2.7 | 6.6 ± 2.7 |
|                  | B10.BR           | B10.BR       | ddAAA | 51.5 ± 2.6 | 51.5 ± 2.6 |
|                  | B10.BR           | B10.BR-TNP   | ddAAA | 46.0 ± 4.8 | 46.0 ± 4.8 |
|                  | B10.BR           | B10.BR-TNP   | ddAAA | 54.9 ± 2.7 | 54.9 ± 2.7 |
|                  | B10.BR           | B10.A       | ddAAA | 48.7 ± 4.9 | 48.7 ± 4.9 |
|                  | B10.BR           | B10.D2       | ddAAA | 6.6 ± 2.7 | 6.6 ± 2.7 |

* See footnote (*) of Table I. Products of the regions in bold italics are potentially blocked by the serum used on a genetic basis. Public H-2 specificities have not been indicated in the table for simplicity, since they do not appear to have a significant effect on target cell lysis in these studies.

§ NT, not tested.
SCHMITT-VERHULST ET AL. BRIEF DEFINITIVE REPORT

TNP spleen targets are also shown. It is noteworthy that anti-Ia sera (sections B and C, Table II) had no appreciable effect on lysis of these splenic targets, despite the fact that at least 50% of spleen cells express Ia antigens (6).

The F(ab')2 fragment of antibodies directed against TNP protect TNP-modified targets from complement-dependent lysis by antisera directed against the unmodified H-2 specificities (A.-M. Schmitt-Verhulst, unpublished observation). This observation suggests that at least some of the TNP groups are closely associated with unaltered H-2 alloantigens. Therefore, we tested whether anti-TNP antibodies would inhibit the lysis of TNP-modified targets by effector cells generated by sensitization with either TNP-modified autologous or unmodified allogeneic sensitizing cells. The data, summarized in Table II, section D, indicate that the lytic phase of the CML of B10.BR effectors sensitized by TNP-modified autologous cells is partially inhibited by sera directed against H-2k alloantigens and is completely inhibited by serum directed against TNP. The lysis measured on C57BL/10-TNP targets by B10.BR effectors sensitized against unmodified H-2b alloantigens was not inhibited by the serum directed against the alloantigens of the effector cells (H-2k), but was completely blocked by antisera directed against the TNP present on the target cells. It is also shown in this section, in which B10.BR effector cells are sensitized against unmodified H-2b alloantigens, that the inhibition of the allogeneic effector phase by anti-TNP serum required the presence of TNP on the target cells. A partial inhibition was also obtained by serum directed against the H-2b alloantigens. These latter results using anti-TNP sera are compatible with the interpretation that antibodies attached to the haptenic moiety protect the alloantigens from attack by the effector cells.

The H-2K and H-2D antigens are known to be located on separate molecules (8) and to migrate independently on the cell surface (9). The blocking of the effector phase of the TNP-autologous CML with anti-H-2K, anti-H-2D, or anti-TNP sera suggests that some TNP groups are physically closer to the serologically defined antigens than K- and D-region antigens are to each other.

It has recently been found that H-2K or H-2D antigens are functionally associated with the lysis by cells sensitized against non-H-2 alloantigens (10) or sex-associated differences (11). In the latter case, K- and/or D-region homology was required between stimulator and target cells, but not between effector and target cells for lysis to occur (12). If a unitarian model is to be proposed for H-2K- or H-2D-restricted lysis observed for stimulations involving TNP modification (1-4), viral infection (13-15), and the non-H-2 differences (10, 11), at least two possibilities can be considered: (a) all of the above examples involve activation of T-cell clones recognizing modified H-2 components; or (b) TNP and viral modifications, as well as the other examples (10, 11), do not involve recognition of altered H-2 products, but lysis is effective only on targets sharing H-2K or H-2D antigens with the stimulating cells by some as yet undefined mechanism. The second possibility seems unlikely, since the recognition unit of the effector cell is blocked only by TNP- or viral-modified (2, 13), H-2-matched cells, and not by modified congenic cells that do not share serological region products with the stimulating cells. Furthermore, the examples presented in this report indicate that antibodies to the same D-region antigen do or do not block lysis depending
exclusively on the source of the effector cell. This observation would not be expected if any determinant not physically associated with H-2 were the target of lysis. Since a close association between TNP and the H-2 serological region products has been demonstrated in this report, it is difficult to distinguish between a model involving sensitization against modified H-2 products and one encompassing "hapten" in close association with or in the immediate environment of cell surface H-2 products.

Summary

Splenic lymphocytes from B10.A and B10.D2 mice were sensitized in vitro to trinitrophenyl (TNP)-modified autologous spleen cells. The effector cells generated were assayed in a $^{51}$Cr-release assay on TNP-modified syngeneic or congenic spleen target cells. Effector cells from B10.A donors lysed TNP-modified H-2$^{K\kappa}$ but not H-2D$^d$-region products, whereas B10.D2 effectors reacted with modified products of both the H-2$^{K\delta}$ and H-2D$^d$ regions. As an independent confirmation that this selective K-end lysis by B10.A effector cells is due to an H-2-linked responder cell defect (4), anti-H-2K$^\kappa$ but not anti-H-2D$^d$ sera were shown to inhibit the lysis of B10.A-TNP targets by B10.A effectors. In contrast, anti-H-2D$^d$ sera inhibited the lysis of B10.A-TNP targets by B10.D2 effectors. Anti-Ia antibodies had no detectable effect on lysis. Anti-TNP-keyhole limpet hemocyanin sera blocked the lysis of TNP-modified targets, irrespective of whether the effector cells were directed against TNP-modified autologous H-2 products or H-2 alloantigens. These results independently verify that B10.A responding lymphocytes do not generate effector cells to TNP-modified H-2D$^d$ products, whereas B10.D2 lymphocytes do (4), and suggest that some TNP groups are sterically close to (or part of) the serologically defined H-2K- and H-2D-region antigens.

The authors are grateful to Dr. Pierre Henkart for providing the anti-TNP-keyhole limpet hemocyanin sera and to Judith Jaworek for preparation of the manuscript.

Received for publication 22 September 1975.

References

1. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. Eur. J. Immunol. 4:527.
2. Shearer, G. M., T. G. Rehn, and C. A. Garbarino. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. J. Exp. Med. 141:1348.
3. Forman, J. 1975. On the role of the H-2 histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. J. Exp. Med. 142:403.
4. Schmitt-Verhulst, A.-M., and G. M. Shearer. 1975. Bifunctional major histocompatibility-linked genetic regulation of cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes. J. Exp. Med. 142:914.
5. Nabholz, M., J. Vives, H. M. Young, T. Meo, V. Miggino, A. Rijnbeck, and D. C.
Shreffler. 1974. Cell-mediated cell lysis in vitro: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* 4:378.

6. Sachs, D. H., and J. L. Cone. 1973. A mouse B-cell alloantigen determined by gene(s) linked to the major histocompatibility complex. *J. Exp. Med.* 138:1289.

7. Sachs, D. H., C. S. David, D. C. Shreffler, S. G. Nathenson, and H. O. McDevitt. 1975. Ir associated antigens. *Immunogenetics.* 2:301.

8. Cullen, S. E., B. D. Schwartz, S. G. Nathenson, and M. Cherry. 1972. The molecular basis of codominant expression of the histocompatibility-2 genetic region. *Proc. Natl. Acad. Sci. U. S. A.* 69:1394.

9. Neauport-Sautes, G., F. Lilly, D. Silvestre, and F. Kourilsky. 1973. Independence of *H-2K* and *H-2D* antigenic determinants on the surface of mouse lymphocytes. *J. Exp. Med.* 137:511.

10. Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (Lond).* 256:419.

11. Gordon, R. D., E. Simpson, and L. E. Samelson. 1975. In vitro cell-mediated immune responses to the male specific (H-Y) antigen in mice. *J. Exp. Med.* 142:1108.

12. Gordon, R. D., and E. Simpson. 1976. *H-2* restricted T-cell cytotoxicity responses to H-Y antigens. *In: Proceedings of the 10th Leukocyte Culture Conference.* V. P. Eijsvoogel, D. Roos, and W. P. Zeylemaker, editors. Academic Press, Inc., New York. In press.

13. Zinkernagel, R. M., and P. C. Doherty. 1975. *H-2* compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded for in *H-2K* and *H-2D*. *J. Exp. Med.* 141:1427.

14. Gardner, I. D., N. A. Bowern, and R. V. Blanden. 1975. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. III. Role of the *H-2* gene complex. *Eur. J. Immunol.* 5:122.

15. Koszinowski, U., and R. Thomssen. 1975. Target cell-dependent T cell-mediated lysis of vaccinia virus-infected cells. *Eur. J. Immunol.* 5:245.