TRINITROPHENYL MODIFICATION OF H-2^k AND H-2^b SPLEEN CELLS RESULTS IN ENHANCED SEROLOGICAL DETECTION OF K^k-LIKE DETERMINANTS

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Haptens such as trinitrophenyl (TNP),[1] fluorescein isothiocyanate (FITC), and *N*-iodoacetyl-*N'*-(5-sulfonic-1-napthyl)ethylenediamine (AED) have been instrumental in elucidating the specificity and regulation of cytotoxic T lymphocyte (CTL) responses restricted by major histocompatibility complex (MHC) gene products (1–12). Most hapten- (as well as virus) self CTL responses exhibit preferential recognition, that is, the predominant lytic activity is restricted by a single class I product (3, 4, 9, 13–15). Although a number of genetic and regulatory mechanisms have been proposed to account for this effect (3, 5, 16–18), little biochemical evidence exists that can explain preferential recognition of certain class I molecules in conjunction with foreign antigens (19). H-2K^k, in particular, is the major restricting element for several haptens and viruses including TNP (3, 4), FITC (9), Sendai, and influenza (16, 20, 21). For example (17), in the H-2^a haplotype (K^k/D^{d}L^d), the response against TNP-self is predominantly directed against K^k-TNP antigens.

In the present study several anti-MHC monoclonal antibodies (mAb) have been examined for their ability to bind to TNP-modified and unmodified spleen cells. The results indicate that certain anti-K^k mAb exhibit significantly enhanced binding to TNP-modified, compared with unmodified, B10.A spleen cells. In addition, anti-K^k mAb were shown to specifically bind to TNP-modified, but not to untreated, allogeneic cells, and that cell surface expression of class I MHC antigens is required for enhanced binding. These observations suggest that TNP-derivatization of cell surface products can result in the generation of neodeterminants that resemble alloantigens as detected by serological means.

Materials and Methods

Monoclonal Antibodies. The following mAb were used in the present study: 36.7.5, anti-K^k (22); 11.4.1, anti-K^k and cross-reactive with K^{q,p,r} (23); 27.11.13, anti-D^b and cross-reactive with D^{d,q} (24); 28.14.8, anti-D^b and cross-reactive with L^{d,q} and D^q (24); 100.3,

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[1] *Abbreviations used in this paper:* AED, *N*-iodoacetyl-*N'*-(5-sulfonic-1-napthyl)-ethylenediamine; CTL, cytotoxic T lymphocyte; FcγR, receptors for the Fc portion of IgG; FITC, fluorescein isothiocynate; mAb, monoclonal antibodies; MHC, major histocompatibility complex; TNP, trinitrophenyl.

which represents mAb H100-30/3 (25), anti-$K^{k.s}$, and cross-reactive with r, b, and q, and 2.4G2, anti-mouse IgG2bFc receptor (FcγR) (26).

Cells. Spleen cells were isolated from B10 or B10.A mice and treated with either 10 mM trinitrobenzene sulfonate (TNBS) (Pierce Chemical Co., Rockford, IL) or 0.5 mM fluorescein isothiocyanate (FITC) (Research Organics, Cleveland, OH) as previously described (3). The R1 (TL^+) and R1 (TL^-) cell lines were obtained from Dr. Robert Hyman of The Salk Institute, San Diego, CA, and were grown in tissue culture. The R1 (TL^+) line was originally derived from a spontaneous thymoma in a C58 mouse, and expresses the H-2^k haplotype. The R1 (TL^-) variant was obtained by selecting mutagenized R1 (TL^+) cells against TL expression. It has concomitantly lost the ability to express MHC class I molecules, but not other cell surface antigens (27). Cells from both lines were modified with 10 mM TNBS, using the same procedure as for the spleen cells. Both spleen and lymphoma cells were centrifuged over Lympholyte M (Cedarlane Laboratories, Hornby, Ontario) to remove dead cells.

Radioiodination and Binding Assay. The purification and iodination of mAb, and the binding assay is described in detail elsewhere (28). Briefly, purified mAb were radiolabeled by a modified chloramine T method (28). Splenic or R1 lymphocytes were labeled with ^{51}Cr, then mixed with graded concentrations of ^{125}I-antibody. After incubation at 4°C in medium containing 0.2% sodium azide for a time predetermined to allow the system to reach equilibrium, the cells were sedimented through a mixture of phthalate oils in 400-μl microfuge tubes (28). The numbers of molecules of bound antibody/cell were calculated from the $^{125}I/^{51}Cr$ ratios in the tips of the microfuge tubes. Nonsaturable binding was determined from controls containing a large excess of unlabeled antibody in addition to the labeled antibody and cells, and was subtracted from the total binding. Since the phthalate oil technique does not require a wash step, it measures both high and low avidity interactions. In order to assure accurate cell counts of ^{51}Cr-labeled cells, three aliquots of the same cell sample were counted microscopically. The resulting values of ^{51}Cr cpm/cell were estimated to be accuate to ± 5%.

Generation of CTL and Assay for Cytotoxicity Against TNP-Self. CTL were generated by co-culturing mouse spleen cells with autologous spleen cells treated with 10 mM TNBS, as previously described (3). 5 d later, the resulting effector cells were assayed on ^{51}Cr-labeled TNBS-treated and untreated concanavalin A–stimulated splenic blasts (3). The percentage of lysis and standard error of the mean were calculated based on triplicate assay samples. Cells modified with FITC (Sigma Chemical Co., St. Louis, MO) were incubated at 37°C for 15 min at a concentration of 200 μg/ml, as previously described (4).

Results

Spleen cells from B10.A mice modified with 10 mM TNBS or with 200 μg/ml FITC were incubated with various concentrations of three different anti-K^k and one anti-D^d mAb. The average numbers of antibody molecules bound per cell were then determined as described in Materials and Methods. The results of Fig. 1 *A* show the binding of 36.7.5 (anti-K^k), to unmodified, TNP-modified, or FITC-modified cells. The concentration of haptenic reagents used were those typically used to induce cytotoxic T cell responses. While 4.4×10^4 molecules per cell of 36.7.5 were bound to unmodified spleen cells under saturating conditions, significantly enhanced binding (approximately twofold) was detected on cells modified with 10 mM TNBS (Fig. 1 *A*). A similar enhancement of 36.7.5 binding was not detected following hapten modification with FITC (Fig. 1 *A*). Two additional anti-K^k reagents, 11.4.1 (Fig. 1 *B*) and 100.3 (Fig. 1 *C*) showed similar increases in binding to TNP-modified spleen cells when compared with unmodified B10.A spleen cells. To assess whether any anti–class I mAb would exhibit increased binding after TNBS modification, a mAb directed against the

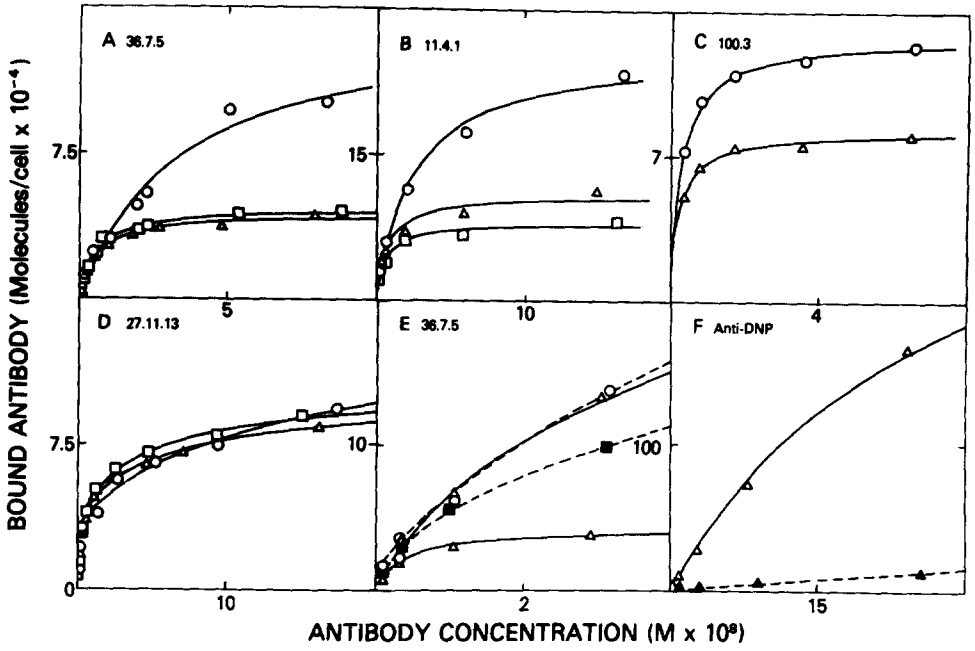

FIGURE 1. The binding of radiolabeled antibodies to modified or unmodified B10.A spleen cells plotted on a linear scale. In panels *A–D* open triangles indicate binding to unmodified cells, open circles to TNP-modified cells, and open squares to FITC-modified cells. *A–C* show the binding of three different anti-K^k monoclonal antibodies, *D* shows the binding of 27.11.13, an anti-D^d monoclonal antibody, *E* shows the binding of 36.7.5 (anti-K^k) to (—△—) unmodified cells, (—○—) to TNP cells, (– –△– –) to TNP cells in the presence of hapten (0.25 mM DNP-ϵ-aminocaproate), and (– –■– –) to TNP cells in the presence of 0.8 mg/ml 2.4G2 anti-FcγR monoclonal antibody; *F* shows the binding of rabbit anti-DNP antibodies to (—△—) TNP cells or (– –▲– –) TNP cells in the presence of 0.25 mM DNP-ϵ-aminocaproate.

D^d molecule (27.11.13) was tested in this system. In contrast to the anti-K^k monoclonals, this antibody bound to similar extents to unmodified, TNP-modified, or FITC-modified spleen cells (Fig. 1*D*).

To exclude the possibility that the anti-K^k antibodies cross-reacted with TNP, 36.7.5 was incubated with B10.A-TNP cells in the presence or absence of 0.25 mM DNP-ϵ-aminocaproate (a high concentration of inhibitor). No inhibition of the anti-K^k antibody binding was observed in the presence of the hapten (Fig. 1*E*). Free hapten, however, did inhibit the binding of anti-DNP antibody to B10.A-TNP cells (Fig. 1*F*). Together, these data indicate that the increase in 36.7.5 binding was not the result of a heteroclitic reaction with TNP. In another experiment, spleen cells were treated with a saturating concentration of 2.4G2, anti-murine FcγR mAb (26), to prevent the binding of anti-class I mAb to cells by their Fc portions. The data of Fig. 1*E* demonstrate that in this experiment ~⅓ of the increased binding could have occurred through FcγR.

Our findings that TNP modification results in enhanced binding of anti-H-$2K^k$ but not of anti-H-$2D^d$ mAb correlate with the results of studies of CTL specificity demonstrating preferential recognition of K^k over D^d in responses to B10.A TNP-modified spleen cells (3, 4). When the same B10.A spleen cells used for the binding experiments in Fig. 1 were used to sensitize B10.A spleen cells

against TNP-self (Fig. 2), a predominant K^k-restricted CTL response was induced. Thus, if TNP-modification of B10.A cell surface products results in the presence of additional K^k-like determinants, this could contribute to the predominant K^k restriction of anti-TNP self CTL responses.

Since extensive cross-reactivity has been reported between H-2 alloantigens and TNP-self by CTL from H-2^b mice (29–35), we investigated whether anti-K^k mAb would recognize TNP-modified B10 cells. The results of Fig. 3*A* indicate that 36.7.5, which does not bind to unmodified B10 spleen cells, does in fact

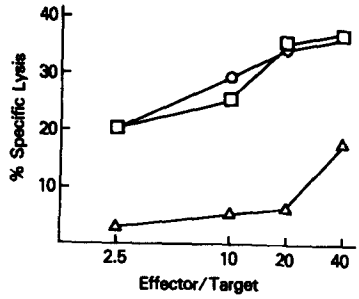

FIGURE 2. B10.A spleen cells were sensitized against autologous B10.A-TNP stimulating cells and assayed against concanavalin A–stimulated, ^{51}Cr-labeled spleen cells: B10.A-TNP, (□); B10.BR-TNP (○); and B10.D2-TNP (△). The lysis against unmodified target cells was B10.A, 2.7%; B10.BR, 6.6%; and B10.D2, 1.3%.

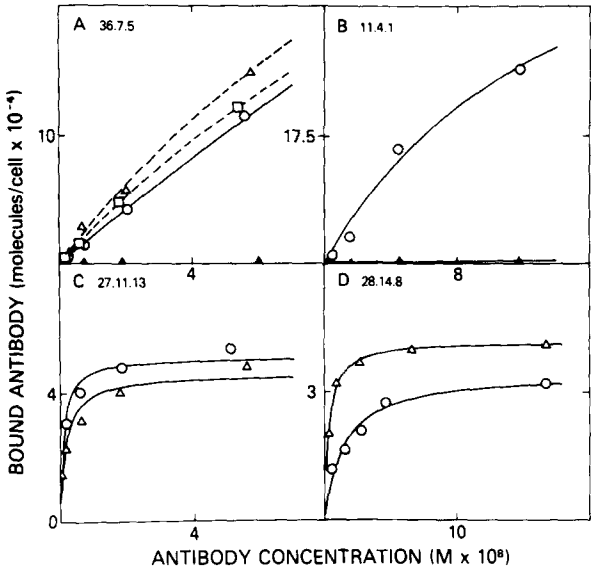

FIGURE 3. The binding of radiolabeled monoclonal antibodies to unmodified or modified B10 spleen cells. (*A*). The binding of 36.7.5 (anti-K^k) to (—▲—) unmodified B10 spleen cells, (—○—) to TNP-modified B10 spleen cells, (--□--) to TNP-spleen cells in the presence of 0.25 mM DNP hapten, and (--△--) to TNP-spleen cells in the presence of 0.8 mg/ml 2.4G2 anti-FcγR antibody. (*B*) The binding of 11.4.1 (anti-K^k) to (—▲—) unmodified and (—○—) TNP-spleen cells. (*C*) The binding of 27.11.13 (anti-$D^{b,d}$) to (—△—) unmodified and (—○—) TNP-spleen cells. (*D*) The binding of 28.14.8 (anti-D^bL^d) to (—△—) unmodified and (—○—) TNP spleen cells.

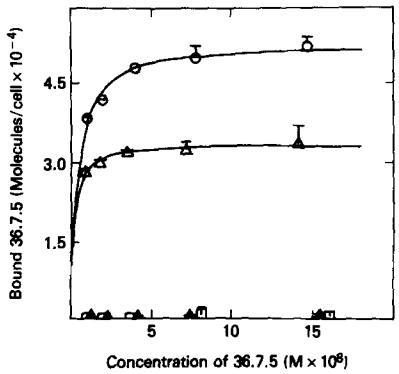

FIGURE 4. The binding of ^{125}I-36.7.5 to unmodified or TNP-treated R1 (TL$^+$) and R1 (TL$^-$) cells. (△) unmodified R1 (TL$^+$); (○) TNP-modified R1 (TL$^+$); (□) unmodified R1 (TL$^-$); and (▲) TNP-modified R1 (TL$^-$). The TL$^+$ and TL$^-$ cells were treated with the same solution of TNBS and were both about equally yellow. The numbers of TNP molecules/cell were estimated by lysing cells in 0.5% NP_{40} and reading the optical density at 365 nM. By this analysis the TNP-R1 (TL$^+$) had 1.4×10^8 TNP/cell, while the TNP-R1 (TL$^-$) cells had 1.0×10^8 TNP/cell. Error bars indicate standard errors from triplicate determinations.

bind to TNP-modified B10 cells. Furthermore, this binding activity was not inhibited by free hapten, nor by the anti-FcγR monoclonal antibody 2.4G2 (26), demonstrating that 36.7.5 does not bind to TNP or FcγR on the modified B10 cells. A second anti-K^k mAb, 11.4.1, which failed to react with B10 spleen cells, also bound significantly to TNP-modified B10 cells (Fig. 3*B*). However, two mAb directed against D^b antigens, 27.11.13 and 28.14.8, failed to show increased binding to B10 cells after TNP modification (Fig. 3, *C* and *D*). Although we have not made an extensive survey of the binding of 36.7.5 to TNP-modified spleen cells of various haplotypes, we have observed that TNP-modified ATL spleen cells and TNP-B10 cells (data not shown) bind 36.7.5 to a similar extent. This is of interest because the 36.7.5 antibody was derived from ATL spleen cells (i.e., ATL anti-AAL).

To determine whether the enhanced binding of anti-K^k mAb by TNP-modified cells was dependent on expression of class I MHC molecules, the R1 (TL$^+$) and R1 (TL$^-$) cell lines were modified with TNP and tested for 36.7.5 binding. As shown in Fig. 4, 36.7.5 bound saturably to the TL$^+$ cell line and exhibited enhanced binding to TNP-modified R1 (TL$^+$). In contrast, the TL$^-$ line, a variant that does not express class I molecules (27), did not bind 36.7.5, either unmodified or when modified with TNP. These data indicate that class I MHC molecules are required to obtain enhanced binding of anti-K^k mAb to TNP-modified H-2^k cells.

Discussion

The data presented in this study demonstrate that modification of cell surface products with TNP can result in the formation of neo-antigenic determinants that mimic H-2K^k epitopes in their ability to bind anti-K^k mAb. The enhanced binding of anti-K^k mAb to TNP-modified cells is not due to a heteroclitic reaction with the TNP groups, since binding is not blocked by free hapten (Figs. 1 and

3). Furthermore, it is highly unlikely that three different anti-K^k mAb would each cross-react with some neo-determinant structurally distinct from the K^k epitope to which it was raised. Rather, the simplest and most likely interpretation of the data is that the neo-determinants that are produced by TNP modification of B10 and B10.A spleen cells are structurally similar to K^k epitopes.

The cross-reactivity of the anti-K^k mAb with TNP-modified B10 cells (Fig. 3) parallels the well-established cross-reaction that CTL raised against allogeneic stimulators exhibit when tested against TNP-self, especially in the B10 strain (29). It has also been demonstrated, both in bulk cultures (34) and at the clonal level (33), that B10 spleen cells stimulated with B10-TNP can lyse unmodified H-2^k targets. The similarities in the cross-reactions exhibited by both CTL and antibodies suggest that the CTL may recognize the same neo-antigens that are detected by the mAb.

The nature of the neo-determinants is unknown, although the data of Fig. 4 suggest that they require the expression of MHC class I molecules, since these neo-determinants were not detected on the TNP-modified class I negative variant line. One possibility is that the neo-antigens arise from TNP modification of class I molecules themselves. TNP derivatization of these products has been shown to occur (36), and since considerable homology between class I allelic products has been demonstrated (37), subtle modification of H-2^b MHC products might result in the formation of K^k-like determinants. Alternatively, TNP-modified non–class I molecules might interact on the cell surface with class I molecules and induce a change in epitope expression. This latter hypothesis is perhaps more attractive, since it provides a molecular basis for the function of MHC class I antigens, namely to serve as signal proteins that CTL could utilize to detect changes in cell surfaces resulting from, for example, viral infection.

We do not yet know whether the appearance of neo-determinants occurs only with TNP modification, or is a more general phenomenon that could involve other haptens or viruses. Some studies have reported that virally infected murine (38) and human (39) cells can bind alloantibodies, while other studies (32) have shown that CTL raised against virally infected self can cross-react with uninfected allogeneic cells. Recently it has been reported that neo-antigens that resemble HLA allodeterminants can be detected with mAb when human leukocytes are infected with human T cell leukemia virus (40). Finally, it has also been found (41) that a majority of mAb raised against virally infected syngeneic cells would not bind to either the virus alone or to uninfected syngeneic cells, but would react with infected cells. One explanation of these results is that the cell surface modifications resulting from viral infection included the production of class I–like neo-antigenic determinants, and that the mAb were directed against these determinants. It would be interesting to know whether such antibodies would bind to uninfected allogeneic cells.

The approach used in the present study may be valuable in elucidating the structural changes that can occur when class I molecules interact with foreign antigens on cell surfaces. Our data are consistent with the idea that antigen-induced altered regions on class I molecules serve as recognition sites for CTL and determine whether or not lysis will occur.

Summary

Several anti–H-2K^k but not anti–H-2D^d monoclonal antibodies (mAb) exhibited enhanced binding to B10.A murine spleen cells after modification of the cells with trinitrobenzene sulfonate (TNBS). The number of antibody molecules bound to TNP-modified B10.A spleen cells increased by a factor of two or more. The same anti-2K^k mAb that exhibited enhanced binding to modified B10.A cells did not bind to unmodified C57BL/10 spleen cells, as expected, but did bind to TNP-modified C57BL/10 spleen cells. This TNP-dependent binding was not a result of cross-reactions with cell surface TNP groups nor with Fc receptors. TNP modification of a variant cell line that does not express class I H-2 products did not result in enhanced binding by these mAb. These findings can account for preferential recognition of TNP-K^k by B10.A and B10.BR CTL, and also for cross-reactive lysis by C57BL/10 CTL stimulated by C57BL/10-TNP against unmodified H-2K^k targets.

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