ROLE OF SELF CARRIERS IN THE IMMUNE RESPONSE AND TOLERANCE

V. Reversal of Trinitrophenyl-modified Self Suppression of the B-Cell Response by Blocking of H-2 Antigens*

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Genes associated with the major histocompatibility complex (MHC)[1] have been shown to exert profound control of both humoral and cell-mediated immune responses (1, 2). One approach to clarifying the cellular and molecular bases of these systems involves the definition of immune function attributed to antigens coded for by these genes. For example, the importance of the products of the *K* and *D* regions of the *H-2* complex as target antigens for T-cell lympholysis induced by allo-immunization is well established (3). In addition, it has become increasingly clear that both the K and D antigens play a critical role in the generation of cytotoxic T cells in response to syngeneic cells that are chemically or virally modifed (4–6). Antigens coded for in the *I* region, on the other hand, are not only important in T-cell interactions with both B cells and macrophages in antibody formation, but also in the T-cell-mediated transfer of delayed hypersensitivity (7–9). Moreover, *H-2*-linked immune response genes can control preferential induction of suppressor or helper T-cell function to many antigens (9, 10). Ia determinants have been detected on both T-suppressor and T-helper factors (11, 12).

With these observations in mind, Miller et al. (13, 14) have recently shown that tolerance for contact hypersensitivity to dinitrofluorobenzene can be mediated by suppressor T cells, and, furthermore, that recognition of MHC products is critical for this process. Interestingly, they found that the funtion of T suppressors appears to require recognition of modified self *H-2D*-region determinants (14), whereas cytotoxic T cells are capable of recognizing both modified *H-2K* and *H-2D* products (4–6). Recently, Cantor and Boyse (15, 16) suggested that suppression of the plaque-forming

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[1] *Abbreviations used in this paper:* DNP-SC, dinitrophenyl lymphoid cells; HMS, hapten-modified self; Ir, immune response; MHC, major histocompatibility complex; PFC, plaque-forming cells; POL, polymerized flagellin; TNP, trinitrophenyl(ated); TNP-SC, TNP syngeneic spleen cells.

cell (PFC) response to hapten-modified self also reflects stimulation of certain suppressor T-cell subsets by cell surface structures associated with autologous H-2K and H-2D molecules.

We and others have previously shown that haptenated syngeneic spleen cells can induce B-cell tolerance, which is mediated largely by suppressor T cells (17–21). We recently reported that anti-Ly2+C-treated spleen cells produced a small but significant PFC response to trinitrophenyl (TNP)-modified syngeneic spleen cells in the absence of extrinsic immunogen, whereas normal spleen cells are unresponsive (17, 20). The nature of the TNP-modified determinants that stimulated this response had not yet been determined. Because regulation of the immune response may reflect the recognition of different cell surface structures (K/D vs. I), we have directly analyzed the antigens recognized in the T-cell control of B-cell responsiveness to hapten-modified self with antibodies to MHC determinants. Thus, TNP-modified and antibody-coated spleen cells were cultured with normal syngeneic spleen cells and the anti-TNP-PFC response was measured in the absence or presence of extrinsic antigen. The results indicate that modified *H-2K-* (and, in some cases, *H-2D-*) region products are recognized by T-suppressor cells, and that modified I-A antigens stimulate presumptive helper T cells. Haptenated syngeneic spleen cells can, therefore, activate different T-cell subsets simultaneously depending upon the MHC determinants recognized. Although both suppression and help are present, suppression of the B-cell response dominates.

Materials and Methods

Animals. CBA/J mice, 10–14 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine. A/J, CBA/St, and C3H/St mice of the same age were acquired from the colony of Dr. D. B. Amos, Duke University Medical Center, Durham, N. C. B10.A(4R) also of the same age were produced from the mouse colony of Dr. Frelinger at the University of Southern California Medical School, Los Angeles, Calif., or were generously supplied by Dr. Gene Shearer, National Cancer Institute, Bethesda, Md. All animals were age and sex matched for each experiment.

Antisera. The following antisera were prepared and tested for specificity at the University of Southern California by cytotoxicity and absorption analyses: A.TH anti-A.TL, anti-Ia^k (entire *I* region); A.TL anti-A.TH, anti-Ia^s (entire *I* region); (A.AL × B10)F_1 anti-A.TL, anti-H-$2K^s$; A.TL anti-A.AL, anti-H-$2K^k$; (A.AL × B10)F_1 anti-B10.K, anti-H-$2D^k$; (A × B10.D2)F_1 anti-B10.A(2R), anti-H-$2D^b$. These sera were prepared by immunization of recipients with mixtures of spleen, thymus, and lymph node cells as previously described (22). All sera were also confirmed by immunofluorescence to bind to TNP-modified cells of the appropriate specificity. This was an important control to verify labeling of stimulator cells in later studies. Rabbit anti-TNP (affinity purified), goat anti-mouse or donkey anti-rabbit Ig were prepared as described earlier (17, 23). These sera also specifically labeled (TNP) mouse spleen cells sensitized with the appropriate antiserum.

Preparation of Antiserum-blocked Hapten-modified Self (HMS) Lymphoid Cells. Trinitrophenylated spleen cells were prepared as previously described using 10 mM trinitrobenzene sulfonic acid for 20 min at 20°C or 10 min at 37°C before extensive washing (4, 24). After washing these HMS cells, 0.5 ml antiserum at a final concentration of 1:10 for each 5×10^6 spleen cells was added to the cell pellet. These were mixed and then incubated for 60 min at 0–4°C. The cells were washed three times and 0.5 ml of goat anti-mouse Ig or donkey anti-rabbit antisera (as required) was added at a dilution of 1:10 to each 5×10^6 spleen cells. After a further incubation at 37°C for 90 min to facilitate capping, the tubes were washed three times and exposed to 2,000 R from a ^{137}Cs source (Gamma Cell 40, Atomic Energy of Canada, Ottawa, Canada). The cells were counted, washed, and resuspended at a final concentration indicated below.

In Vitro Culture

SINGLE STAGE MICROCULTURE. 1×10^6 spleen cells were cultured for 3–4 d in flat-bottomed microtiter plates (Flow Laboratories, Inc., Rockville, Md.) in the presence of antisera-blocked HMS, or control cells. Stimulator (HMS):responder ratios varied from 1:10 to 1:40. Each experiment also contained both normal spleen cells and antisera-blocked HMS (or unblocked HMS) with or without TNP-polymerized flagellin (POL) as extrinsic immunogen at 100 ng/ml. Microculture medium was as described earlier (17).

DOUBLE-STAGE PRECULTURE. 5×10^6 normal spleen cells were cultured in 24-well IS-FB-24 Linbro (Linbro Chemical Co., Hamden, Conn.) plates $\pm 1 \times 10^6$ antisera-blocked (or unblocked) HMS for 24–48 h. The cells were harvested and washed three times. 1×10^6 precultured, washed cells were then placed in each microtiter well (in 0.2 ml) and cultured with or without TNP-POL for 3 d. In some experiments, precultured cells were mixed at a 1:1 ratio (i.e., 5×10^5 of each) with fresh, normal spleen cells to test for suppression of the B-cell response to TNP-POL.

Assay of Responsiveness. All cultures were harvested and tested for anti-TNP PFC in Cunningham chambers with TNP-goat erythrocytes as targets (17, 25). Four replicate wells were analyzed for each group and the geometric mean (⨲ standard error factor) calculated. In general, the standard error was <15% of the mean PFC count and is not presented.

Results

Recognition of H-2K-end Antigens on Modified H-2^k Cells. Spleen cells from mice rendered unresponsive via the injection of TNP-modified syngeneic lymphoid cells contain hapten-specific suppressor cells capable of modulating the B-cell response to TNP (20, 21). This has been shown by limiting dilution precursor analysis, reversal of suppression by anti-Thy-1+C treatment (which allows a normal T-independent response to TNP-POL) and direct mixing experiments (21). The latter approach also suggested that these suppressor cells were Ly-1$^+$,2$^+$,3$^+$ T cells (21). Because spleens from which Ly-2$^+$,3$^+$ (and Ly-1$^+$,2$^+$,3$^+$) cells have been removed by anti-Ly2+C treatment make a small response to TNP-spleen cells in vitro (17, 20), it seemed that some haptenated cell surface moiety was being recognized by the remaining cells which led to an anti-TNP response. Presumably, in the intact spleen, Ly-1$^+$,2$^+$,3$^+$ suppressor cells recognized this or another TNP moiety and inhibited the PFC response. To ascertain which cell surface products were involved in this recognition, we performed a series of antibody modulation or blocking experiments on TNP-modified spleen cells and used these cells as stimulators of an anti-TNP response by normal syngeneic cells. We also tested the ability of these blocked cells to induce suppression of the PFC response to a challenge immunogen, TNP-POL. The data in Table I indicate that blocking of H-2K antigens on TNP-modified C3H stimulator cells leads to a small but significant response by normal C3H spleen cells. Anti-TNP, anti-mouse Ig, anti-Ia, and irrelevant (anti-H-2^s) antiserum treatment of stimulator cells have no apparent effect. It should be noted that brief treatment of responder cells with these antisera does not affect the response to TNP-POL (D. W. Scott. Unpublished observations.). These data implied that recognition of modified H-2K by normal spleen cells was important in the suppression of the PFC response by HMS. Subsequently, we asked which other MHC products were involved in this recognition. Spleen cells from CBA (*H-2^k*) mice were TNP-modified and blocked with specific antisera at the *K*- or *D*-ends or over the entire *I*-region. Some groups also received modified cells treated with mixtures of these antisera. The data in Fig. 1 establish that, in CBA mice (and as shown below in *H-2^k* mice or *H-2^a* mice, which are *K^k*, *I-A^k* ... *I-E^k*), it is TNP-modified H-2K which must be recognized by presumptive

TABLE I

*Effect of Antiserum Treatment of TNP-modified Splenocytes on PFC Responses**

Group	TNP-SC	TNP-POL	Antiserum (α) pre-treatment of TNP-SC	Anti-TNP response	
				Experiment 1	Experiment 2
				PFC/well	
1	−	−	—	56 (1.07)‡	65 (1.08)‡
2	+	−	—	55 (1.05)	24 (1.08)
3	+	−	α-TNP	45 (1.06)	48 (1.07)
4	+	−	α-Mouse Ig	55 (1.06)	47 (1.07)
5	+	−	α-Ia^k	56 (1.07)	52 (1.11)
6	+	−	α-Ia^s	55 (1.09)	53 (1.10)
7	+	−	α-H-$2K^k$	116 (1.04)	125 (1.03)
8	+	−	α-H-$2K^s$	42 (1.08)	31 (1.13)
9	−	+	—	486 (1.03)§	382 (1.03)§
10	+	+	—	111 (1.03)‖	133 (1.04)‖

* 1×10^6 C3H/St spleen cells were cultured ± antiserum-treated TNP-C3H/St spleen cells or untreated TNP-C3H/St spleen cells for 4 d in microculture. Underlined values (group 7, α-H-$2K^k$) are significantly greater than background (group 1).

‡ Normal background PFC with no immunogen. Standard error factors of the geometric means are indicated in parentheses.

§ Primary PFC response to TNP-POL.

‖ Typical suppression of primary response by TNP-SC equivalent to 87 and 79% unresponsiveness.

suppressor cells; H-2D molecules are not involved. Moreover, blocking Ia antigens on the modified cells reverses the stimulating capacity of anti-H-2^k-blocked TNP-spleen cells, an observation which will be consistently repeated herein. It is noteworthy that the specificity of hapten-specific cytotoxic T cells in such *H-2*^{k} mice is also for modified *H-2K*^{k} not *H-2D*^{k} (4, 6, 26).

Effect of Blocking H-2 and Ia Antigens in a Two-Stage Culture System. Although the responsiveness observed by normal spleen cells to H-2-blocked cells was significant, it never approached the response to TNP-POL, nor fully reversed unresponsiveness (Table I; Fig. 1; and J. J. Jandinski and D. W. Scott. Unpublished observations.). Presumably this is a result of a receptor blockade-type effect by excess TNP moieties shed during the culture period as suggested earlier (17). Therefore, we developed a two-stage culture system to remove excess antigen.[2] Hence, any shed cell surface moieties could be removed in a two-step system. The data in Table II indicate that spleen cells cultured with TNP-modified syngeneic spleen cells (at 5:1 responder: stimulator ratio) become unresponsive to challenge with TNP-POL within 24–48 h. These unresponsive cells, moreover, are able to suppress the responsiveness of fresh normal splenocytes. This effect is specific and is not a result of antigen carryover because the PFC response to fluorescein-POL is generally unaffected and no suppression of the anti-TNP response is observed when unresponsive precultured cells are irradiated before mixing with normal splenocytes.[2]

It was now possible to ascertain whether suppression of the extrinsic response to TNP-POL could be reversed by blocking modified H-2 antigens on the stimulator cells. Antibody-blocked HMS were cultured with normal spleen cells for 24 h, at which time these cultures were washed and aliquoted in microculture with or without

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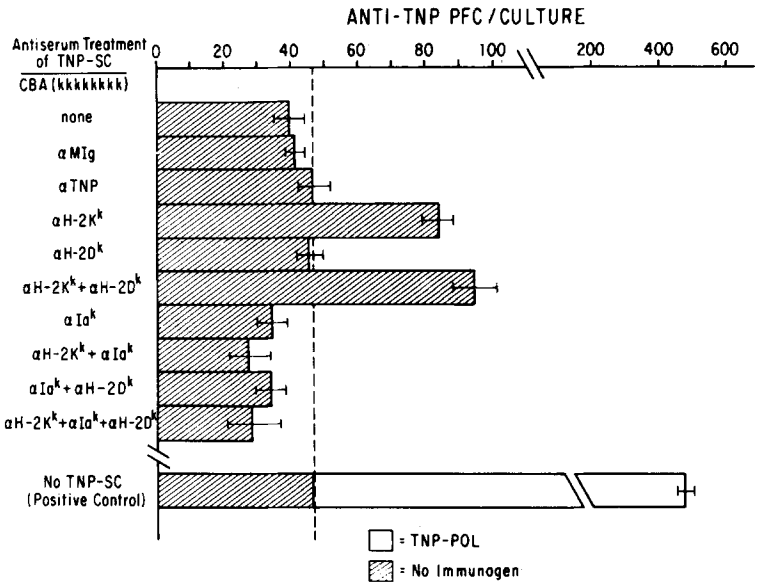

FIG. 1. Stimulation of an anti-TNP PFC response by culturing CBA spleen cells with anti-H-2-blocked TNP-CBA spleen-cell stimulators. Protocol as in Table I. Note that anti-H-2K^k but not anti-H-2D^k blocking of the stimulator cells allows immunogenic recognition, and anti-Ia^k treatment reverses this process. The dotted line represents the background PFC obtained with 10^6 CBA spleen cells cultured alone for 4 d, whereas the open bar is the positive control response of those cells to 100 ng/ml TNP-POL.

TABLE II

Induction of Tolerance (Suppression) to TNP by Preculture with TNP-SC

Group	Time	Anti-TNP*
	h	*PFC/well*
Experiment 1		
1 NSC‡	24	333 (1.08)
2 NSC + TNP-SC	24	19 (1.15)
Experiment 2		
3 NSC	48	192 (1.12)
4 NSC + TNP-SC	48	41 (1.23)
Experiment 3		
5 NSC	48	115 (1.28)
6 NSC + TNP-SC	48	5 (1.47)
5 + fresh NSC§	48	155 (1.13)
6 + fresh NSC§	48	47 (1.29)

* TNP-POL induced PFC on day 3 after preculture and wash. Background subtracted from all groups. Standard error factors of the geometric means are indicated in parentheses.

‡ NSC, normal spleen cell(s).

§ 1:1 mixture of precultured cells with fresh NSC.

TNP-POL challenge antigen. The data in Figs. 2 and 3 confirm that blocking of H-2K^k on TNP-modified CBA (Fig. 2) or TNP-A/J (Fig. 3) splenocytes permits an anti-TNP response to be elicited in the absence of TNP-POL. In this two-stage culture, this response approached that elicited by TNP-POL. Finally, unresponsiveness is now almost completely reversed (Fig. 2). As indicated above, the addition of anti-Ia (A.TH

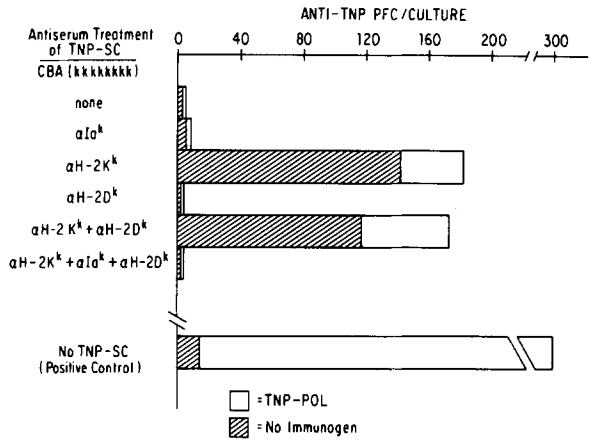

FIG. 2. Induction of an anti-TNP response by anti-H-2 blocking of TNP-CBA stimulator cells in a two-stage culture system ± challenge with TNP-POL. CBA spleen cells were cultured for 24 h with antisera-blocked or untreated TNP-CBA spleen cells, washed, and recultured alone (shaded bars) or challenged with TNP-POL. As in Fig. 1, blocking H-2K^k (but not H-2D^k) allowed a significant response, which was augmented by TNP-POL to near-positive control levels (bottom open bar).

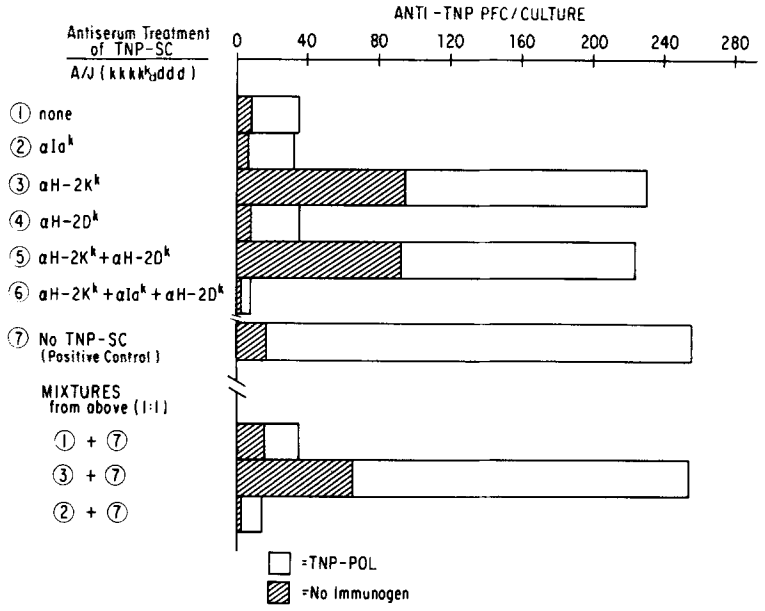

FIG. 3. Reversal of tolerance induction and suppression by antiserum blocking of TNP-A/J spleen cells. Protocol as in Fig. 2. Selected groups of precultured cells were also mixed to test for suppression and its reversal in this system. Anti-K^k treatment (group 3) reversed tolerance and suppression (mixture 3 + 7) to achieve control levels of PFC responsiveness (group 7).

anti-A.TL) to the modified stimulators prevents B-cell activation. These results map the stimulating moieties centromeric to the *I-C* region. Furthermore, gene products that map distal to *D* cannot be involved because both A and A.TH (the antiserum source) share the same *D-T1a* regions. The data in Fig. 3 also demonstrate that the induction of TNP-specific suppression of the normal B-cell response is reversed by

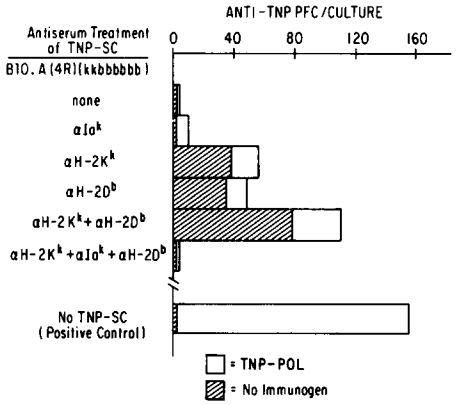

FIG. 4. Induction of an anti-TNP response in B10.A(4R) spleen cells requires blocking of both K and D ends. Protocol as in Fig. 2. Note that anti-K^k and anti-D^b treatment of TNP-B10.A(4R) each allow a small response, but that blocking both ends is required for more efficient reversal of tolerance.

blocking H-2 on the TNP-stimulator cells. That is, cells from group 3 (cultured with anti-H-2K^k-blocked HMS) fail to suppress the response of normal spleen cells (group 7), although other mixtures show suppression.

Recognition of Modified D- and K-End Antigens. The data presented above suggest that spleen cells in *H-2*^{k} (or *H-2*^{a}) mice recognize modified H-2K antigens and suppress the PFC response induced by modified Ia antigens (to the left of *I-C*). This *K*-end recognition is typical in TNP-self cytotoxicity described by Shearer and Schmitt-Verhulst (6, 26) for *H-2*^{k} or *H-2*^{a} mice. The specificity of modified self cytotoxicity differs in *H-2*^{b} mice, which can recognize modified H-2K or D-end molecules. To map the recognition using the specific antisera as in the previous figures, we repeated the two-stage culture system with B10.A(4R) responders because they are *K*^{k}, *I-A*^{k} but *I-B*^{b} to *H-2D*^{b}. The results in Fig. 4 establish that in this strain both H-2K and D-end molecules are recognized by putative suppressor cells. For the best reversal of suppression, the HMS must be pretreated with both antisera together. This implies that some T suppressors see modified H-2K, whereas others see modified H-2D. Furthermore these data implicate that help in this system is localized within the *I-A* region per se because B10.A(4R) mice are *I-A*^{k}, *I-B*^{b}, and our anti-Ia is against the entire *I*^{k} region, although some k/b cross-reactivity cannot be completely ruled out.

Discussion

In 1966, Battisto and Bloom (18) first demonstrated that hapten or protein-modified autologous lymphoid cells were potent tolerogens for both cellular (contact sensitivity) and humoral (anaphylactic antibody) immunity. Since that time, numerous studies have confirmed the exquisite tolerogenicity of such conjugates (13, 14, 17–20) and explored the basis of this phenomenon (21, 27). Our earlier effort (28) demonstrated that trinitrophenylated syngeneic spleen cells (TNP-SC) specifically inhibited the PFC and delayed hypersensitivity responses to hapten in vivo. Moreover, such conjugates blocked the PFC response to T-dependent and T-independent TNP immunogens in vitro while simultaneously inducing a hapten-modified self specific

cytotoxic response (17, 24). The elegant studies of Shearer et al. (4, 6) on the induction of *H-2K/D*-restricted cytotoxic T-cell responses to TNP-self in vitro, and the observation by Forman et al. (29, 30) that modification of H-2 is necessary in this process, suggested (*a*) that T cells were involved in this form of B-cell unresponsiveness and (*b*) that recognition of H-2 antigens was critical for this process. Indeed, we were able to show that Ly-2^+,3^+ (and/or Ly-1^+,2^+,3^+) T cells were required for both the induction (17) and maintenance (21) of tolerance of TNP-SC, although a direct blockade of responsiveness could also occur (17). Identical observations have been made by Claman et al.(13, 14, 27) in their detailed studies on modified-self-induced tolerance for contact sensitivity. Our studies (21) and those of Cantor and Boyse (15, 16) also implied that, in the absence of Ly-2^+,3^+ (or Ly-1^+,2^+,3^+) T cells, a modest B-cell response to TNP-SC could be manifest.

Our experiments were aimed at analyzing which MHC-encoded antigens on haptenated lymphoid cells were being recognized during the induction of tolerance by modified self. By specific antiserum blocking of *K*-, *I*-, or *D*-region antigens on TNP-SC, we were able to determine that modified K or D antigens were being recognized by T-suppressor cells, and in the absence of such recognition, modified Ia antigens stimulated putative T-helper cells to generate an anti-TNP PFC response. In *H-2*^{k} or *H-2*^{a} mice, antibody blocking of H-2K^k but not H-2D^k on the TNP-syngeneic cells reversed suppression and led to a significant anti-TNP PFC response in the absence of extrinsic immunogen. This *K*-end restriction is reminiscent of that seen by Shearer *et al.* (4) in the modified-self-induced cytotoxic T-cell response. This responsiveness is under immune response (Ir) gene control, which maps telomeric to the *I-A* region (6). Thus B10.A(4R) spleen cells (which are *I-A*^{k} but *I-B*^{b} to *I-E*^{b}) responded to modified K and modified D antigens, whereas B10.A(2R) (which are *I-A*^{k}-*I-E*^{k}) are *K*-end restricted (6). Indeed, we find that suppression in our system maps similarly, because B10.A(4R) suppressor cells are blocked by antibody treatment of stimulator cells at either the *K* or *D* end (Fig. 3). This conclusion is tentative because definitive mapping studies with recombinant inbred mice have not yet been done.

Interestingly, the reversal of unresponsiveness by anti-H-2 blocking was only partial in our single-stage culture system. When a two-stage system in which pretreated cells were washed extensively was employed, an excellent PFC response ensued (approximating that induced by TNP-POL). We assume this reflects the removal of shed TNP moieties that might reduce subsequent B-cell responsiveness. Presumably, any new, synthesized MHC antigens would not have TNP associated with them. Though some reexpression of TNP-Ia on guinea pig macrophages has been reported (31), we doubt if this plays a significant role in our system, especially in the presence of antibody.

Another striking finding was that anti-Ia^k treatment prevented the PFC response induced by anti-H-2^k-blocked TNP-stimulator cells in *H-2*^{k} mice. We interpret this finding to indicate that presumptive helper T cells recognize TNP-modified *I*-region antigens, but that this response is normally suppressed by modified *K*-end specific regulatory cells (which are Ly-2^+,3^+ or Ly-1^+,2^+,3^+) (21). That is, suppression here is dominant over help. In B10.A(4R) mice, which respond to both modified K- and D-end antigens (*vide infra* and [6]), it is modified *I-A*-subregion antigens that provide T-cell help for the anti-TNP response. We hope to confirm this in *H-2*^{k} mice by the use of monoclonal antibodies to the *I-A*^{k} region. It should be noted that we have not directly shown that I-A antigens are haptenated on our stimulator cells (29–31), only

that anti-Ia can block stimulation, which suggests that this is the case. Interestingly, anti-H-2K^k and -Ia^k-modulated TNP-SC were as effective as unblocked TNP-SC in inhibiting the response to extrinsic TNP-POL. This suggests that other modified self (non-MHC?) determinants can be suppressive in the absence of *I*-region induced help. Alternatively, anti-Ia-immune complexes may persist in our cultures to nonspecifically suppress the PFC response to TNP-POL. This needs to be explored.

Our results are in apparent contradistinction to those of Miller et al. (14) who found that suppression of contact sensitivity by dinitrophenyl lymphoid cells (DNP-SC) was dependent upon *H-2D* region identity between the DNP-SC donor and the recipient of suppressor cells. Aside from measuring a different response (contact sensitivity vs. PFC formation), it should be pointed out that our system reflects *H-2K*-end recognition during the tolerance induction phase rather than in the effector phase. We have not measured the H-2 restriction of the transfer of suppression. This is currently in progress.

Summary

Trinitrophenylated syngeneic spleen cells (TNP-SC) are potent tolerogens of the anti-TNP plaque-forming cell (PFC) response in vivo and in vitro. This unresponsive state requires T cells for both its induction and maintenance. Because *H-2K/D*-restricted cytotoxic T cells are also induced by exposure to TNP-SC, we determined the role(s) of histocompatibility antigens (K, I, and D) in the suppression of the PFC response by TNP-SC. We treated syngeneic TNP-modified stimulator cells with antiserum directed at *K*-, *I*-, or *D*-region determinants and found that blocking of H-2K or D antigens on TNP-SC transformed these tolerogens into immunogens capable of eliciting an anti-TNP PFC response in the absence of extrinsic immunogens like TNP-polymerized flagellin. In *H-2^k* or *H-2$^{a\,(k/d)}$* mice, only H-2K^k needs to be blocked on the stimulator cells, whereas H-2K or D recognition was apparent in B10.A(4R) mice. These observations indicate that suppression of the PFC response by TNP-SC shows the same restriction in recognition as does the cytotoxic T-cell response. Furthermore, our results suggest that TNP-I-A is recognized by the helper cells in this system as the intrinsic antigen. When both TNP-K and TNP-I-A are present and available on the same stimulator cell, suppression (via modified K recognition) is dominant over help.

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