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ROLE OF SELF CARRIERS IN THE IMMUNE RESPONSE AND TOLERANCE

VII. Importance of H-2 Antigens in the *in Vitro* Induction of Tolerance and Suppression to Hapten-Modified Self[1]

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The ability of trinitrophenylated spleen cells to inhibit the anti-TNP plaque-forming cell response of normal splenocytes to a TNP-immunogen *in vitro* is well established. Using a 2-stage culture technique, we recently demonstrated that, as in the *in vivo* model system, suppressor T cells are generated in response to modified self *in vitro*. In this report, we compare the abilities of 2 cell lines, presumed identical except for the expression of H-2 antigen, to induce tolerance and suppression of the B cell response *in vitro* in order to analyze the role of H-2 in the response to modified self. The results of the comparison using a single-stage culture reveal that at high cell doses and under optimal coupling conditions (i.e., with 10 mM TNBS solution), TNP-R1$^-$ (H-2K$^-$, H-2D$^-$, and TL1,2,3$^-$) is as effective a blocking agent as TNP-R1$^+$ (H-2K$^+$, H-2D$^+$, and TL1,2,3$^+$). At limiting hapten doses, however, it is apparent that the H-2 and TL-positive cell line is significantly more inhibitory than its H-2-negative variant. Moreover, comparison of TNP-R1$^+$ and TNP-R1$^-$ in the 2-stage culture system reveals that heavily haptenated TNP-R1$^+$ induces tolerance and suppression as expected, whereas suboptimally haptenated (with 1 mM TNBS) TNP-R1$^+$ could induce tolerance but not suppression. Furthermore, although TNP-R1$^-$ induced tolerance in the preculture as effectively as similarly trinitrophenylated TNP-R1$^+$, such H-2-negative cells failed to generate suppressor cells detectable in our cell-mixing protocol. These results demonstrate that tolerance can be induced *in vitro* by modified self in the absence of detectable suppression, and suggest that suppressor cells may be generated primarily in response to modified major histocompatibility complex encoded determinants.

It is well established that the systemic administration of hapten-modified spleen cells renders the host specifically tolerant in terms of both the humoral and delayed-type hypersensitivity (DTH)[4] responses (1–7). Moreover, tolerance in both systems has been shown to involve suppressor T cells that are induced by major histocompatibility complex- (MHC) encoded determinants on haptenated cells (8–11).

There is evidence that suggests, however, that direct haptenation of MHC-encoded antigens is not an absolute requirement for the induction of suppression and tolerance (2, 10–13). Our earlier studies, for instance, demonstrated that trinitrophenylated (TNP) teratoma cells, which lack detectable surface H-2 antigens (14), were fully capable of inducing tolerance and suppression *in vivo* (2, 10). This effect, however, is potentially attributable to an H-2-like oncofetal antigen expressed by teratoma cells (15).

In this report, we have further investigated the role of modified H-2 in the induction of tolerance and suppression by utilizing a pair of cultured mouse thymoma lines, R1.1$^+$ (abbreviated R1$^+$) and its selected variant R1.E$^-$ (abbreviated R1$^-$). These 2 lines, derived from a spontaneous C58(H-2^k) tumor (16), are considered identical except for the expression of surface MHC antigens. The R1$^-$ line lacks detectable surface H-2 and TL antigens as measured serologically (16) or by the ability of the cells to sensitize (17, 18), inhibit (17, 19), and serve as targets (17–19) for cytotoxic T cells directed against H-2^k. Both cell lines, however, are haptenated to an equal extent by trinitrobenzenesulphonate (TNBS) (19). Pierres *et al.* (12) have recently demonstrated that recipient mice injected with splenocytes from donors treated with TNP-R1$^+$ or TNP-R1$^-$ show equally depressed contact sensitivity responses when compared with naive controls. This supports the notion that haptenated H-2 *per se* is not an absolute requirement for the induction of tolerance or suppression by modified self.

In the present study, we have compared the abilities of TNP-R1$^+$ and TNP-R1$^-$ to induce tolerance and suppression of the humoral response in 2 *in vitro* model systems: 1) a single-stage culture where responder splenocytes, TNP-immunogen, and putative tolerogen (TNP-R1$^+$ or TNP-R1$^-$) all reside in the same culture well, and 2) a 2-stage culture system wherein responder cells are first precultured with tolerogen, then harvested, washed, and recultured in the presence of TNP-immunogen, with or without freshly prepared responder cells (as a test for suppression and tolerance, respectively) (20). As recent studies have suggested that antigen dose and antigen density may be critical for differentiation of lymphocyte subsets within

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[4] Abbreviations used in this paper: Baθ, brain-associated θ antigen; DTH, delayed-type hypersensitivity; TNBS, trinitrobenzene sulphonic acid; TNP-POL, TNP-polymerized flagellin; TNP-SC, trinitrophenylated splenocytes; MHC, major histocompatibility complex.

a hapten-specific lymphocyte population (12), we have titrated both the cell number and TNBS concentration in order to increase the sensitivity of our model systems.

The results show significant differences in the ability of TNP-R1$^+$ and TNP-R1$^-$ to induce tolerance using the single-stage culture system over a range of cell numbers, but only minimal differences when the TNBS concentration used to modify the cells was varied. Moreover, in the 2-stage culture system, both cell lines haptenated with 10 mM or 1 mM TNBS were equally tolerogenic, but only 10 mM TNBS-modified R1$^+$ was able to generate suppression. Thus, in our *in vitro* systems, the presence of directly modified H-2 antigen is apparently not required for the induction of tolerance but may be required for the generation of suppressor cells. That is, tolerance can be achieved under conditions where suppressor cell activity is undetectable. The significance of these findings to the understanding of the role of H-2 in hapten-modified self-induced tolerance and suppression is discussed.

MATERIALS AND METHODS

Animals. CBA/J female mice, 10 to 14 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were age matched for all experiments.

Cell lines. R1$^+$ is a cell line derived from a spontaneous thymoma of a C58 mouse (21) and is known to express the MHC antigens H-2K^k, H-2D^k, and TL 1,2,3 (16). The cell line R1$^-$ is a variant that was selected from R1$^+$ by repeated treatment *in vitro* with anti-TL antiserum and complement (C) and is negative for H-2K, H-2D, and TL expression (16). Both cell lines were cultured in Dulbecco's modified essential medium with added glutamine, pyruvate, antibiotics, 10^{-5} M mercaptoethanol, and 15% horse serum. The phenotypes of the 2 cell lines were confirmed by immunofluorescence and the ability to serve as targets for anti-H-2^k cytotoxic T lymphocytes. Cells were exposed to 10,000 rads gamma radiation (^{137}Cs unit) before use in experimental culture. The cell lines were a gift from Dr. Mark I. Greene, Harvard Medical School.

Trinitrophenylation of cells. Cell were haptenated using a modification of published haptenation methods (2). Briefly, 10^8 nucleated cells (freed of red blood cells in the case of splenocytes) in 0.5 ml phosphate-buffered saline (PBS) were incubated at 37°C for 10 min with 1 ml of 10 mM, 1 mM, or 0.1 mM recrystallized TNBS in PBS, pH 7.3. Haptenated cells were washed a minimum of 3 times with medium containing fetal calf serum before use.

Antigens. TNP-LPS was prepared from *Escherichia coli* 1(O55:B5) and TNP-POL from polymerized flagellin of *Salmonella adelaide* SW 1338 according to published methods and used in culture at a concentration of 50 to 100 ng/ml, respectively (22, 23).

In vitro culture: Single-stage culture. Normal spleen cells were cultured in 96-well Linbro trays (IS-FB-96) at 10^6 cells/well with or without haptenated cells as indicated, in the presence or absence of TNP-LPS. Microculture medium was prepared as previously described (24). The anti-TNP plaque-forming cell (PFC) response was assayed after 3 days.

Two-stage culture. Normal spleen cells were precultured in 12-well Linbro plates (IS-FB-12) at 16×10^6 cells/well with 1.6×10^6 irradiated (10,000 rads for R1$^+$ and R1$^-$, 1000 rads for trinitrophenylated splenocytes [TNP-SC]), haptenated cells (or unhaptenated cells as controls) for 48 hr. Precultured cells were then harvested, washed extensively, mixed at a 1:1 ratio with either irradiated spleen cells (1000 rads, as a test for tolerance) or freshly prepared normal spleen cells (as a test for suppression) and recultured in microtiter plates (10^6 total cells/well) with or without TNP-LPS. Cultures were assayed for anti-TNP PFC after 3 additional days.

Assay for anti-TNP PFC. All cultures were assayed for anti-TNP PFC in Cunningham chambers using TNP-goat red blood cells as targets (25). All groups were cultured in quadruplicate, and the geometric mean with standard error factor of PFC counts was calculated. Mean background PFC counts from unstimulated cultures were subtracted from PFC values of identical groups cultured in the presence of TNP-LPS. In general, the standard error factor was less than 1.15, and in some figures is not presented for simplicity.

RESULTS

Specific inhibitory effect of TNP-R1$^+$. The ability of TNP-SC to block the anti-TNP PFC response of normal spleen cells to TNP-immunogen is well established (26). Our initial efforts were directed toward the determination of the inhibitory activity of haptenated R1$^+$ (which expresses H-2K^k and H-2D^k) relative to that of TNP-SC. The results of a direct comparison between the blocking activity of irradiated syngeneic TNP-SC and irradiated R1$^+$ tumor cells haptenated with 10 mM TNBS (Fig. 1) show that TNP-SC and TNP-R1$^+$ are equally efficient in suppressing the anti-TNP PFC response. Better than 50% inhibition was achieved by as few as 2×10^4 haptenated cells (a 50:1 responder:"stimulator" ratio), and approximately 70% inhibition at a 10:1 responder:haptenated cell ratio. It should be noted that except at the highest cell dose, irradiated, unmodified R1$^+$ exhibited no inhibitory effect, demonstrating the hapten specificity of the inhibition. Furthermore, neither TNP-R1$^+$ nor TNP-SC significantly affected the response of normal spleen cells to fluoresceinated LPS, confirming the specificity of the suppression (data not shown).

Comparison of TNP-R1$^+$ and TNP-R1$^-$ in a single-stage culture. Having established that TNP-R1$^+$ was equally as effective as TNP-SC in specifically blocking the anti-TNP PFC response to TNP-LPS, we sought to directly compare the inhibitory activities of TNP-R1$^+$ and TNP-R1$^-$. Inbar and colleagues (19) have previously reported that R1$^+$ and R1$^-$ are

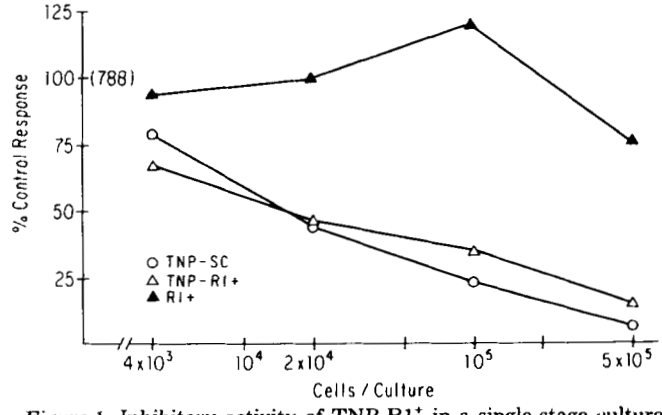

Figure 1. Inhibitory activity of TNP-R1$^+$ in a single-stage culture. Normal CBA splenocytes were cultured in microtiter plates (10^6 cells/well) in the presence of varying numbers of a) 1000-R irradiated TNP-SC (○); b) 10,000-R irradiated TNP-R1$^+$ (△); or c) irradiated unmodified R1$^+$ (▲). Haptenation of cells was performed by using a 10 mM TNBS solution. Cultures were stimulated with TNP-POL (100 ng/ml) and the anti-TNP PFC response was assayed after 3 days of culture. Number in parentheses indicates the mean number of PFC per culture of control cultures (normal splenocytes cultured without additional cells). While unhaptenated R1$^+$ shows essentially no inhibitory activity, TNP-R1$^+$ inhibits as effectively as TNP-SC.

haptenated to an equal extent with TNBS. Thus, any observed differences in the ability to inhibit could presumably be attributed to the presence or absence of haptenated H-2 or TL antigens.

Preliminary experiments demonstrated that using standard trinitrophylation conditions (i.e., with 10 mM TNBS) at a 10:1 responder:TNP-cell ratio, TNP-R1$^+$ and TNP-R1$^-$ are both equally suppressive (Fig. 2). Thus, in this single-stage culture system, the anti-TNP PFC response of normal splenocytes to TNP-LPS can be effectively inhibited by the presence of a haptenated cell that expresses no H-2 antigens. At lower TNP-cell doses, however, it became evident that R1$^+$ was a significantly more efficient "carrier" for TNP than R1$^-$ with respect to inhibitory activity (Fig. 2). For instance, in this experiment at a 100:1 responder:modified cell ratio, TNP-R1$^+$ reduced the control response by >75%, whereas at the same ratio, TNP-R1$^-$ exhibited virtually no blocking activity. Thus, at limiting antigen doses, it is apparent that the presence of modified H-2 antigen significantly contributes to the blocking efficiency of haptenated cells.

Recent studies have suggested that the level of haptenation may be critical in determining how lymphocyte populations are affected by modified self (12, 27, 28). We therefore next assayed the blocking activity of TNP-R1$^+$ and TNP-R1$^-$ modified over a range of TNBS concentrations and cultured at a standard 10:1 responder:modified cell ratio. The results shown in Figure 3 demonstrate that TNP-R1$^+$ and TNP-R1$^-$ modified with 10 mM TNBS are equally inhibitory, as expected, and that TNP-R1$^+$ modified with 1 mM TNBS is significantly more inhibitory than TNP-R1$^-$ similarly modified. It is interesting to note that both cell lines are equally poor in blocking the anti-TNP response when modified with only 0.1 mM TNBS, a concentration at which H-2 antigens are reported not to be effectively modified (29). The inhibitory effectiveness of TNP-SC modified over a range of TNBS concentrations essentially paralleled that for TNP-R1$^+$ (data not shown).

Comparison of TNP-R1$^+$ and TNP-R1$^-$ in a 2-stage culture. Analysis of tolerance of the humoral and DTH responses induced *in vivo* by modified spleen cells has established that suppressor T cells are generated in response to modified MHC determinants on the haptenated cell surface (8–11). In contrast,

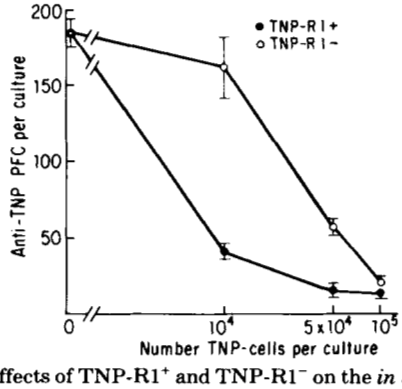

Figure 2. Effects of TNP-R1$^+$ and TNP-R1$^-$ on the *in vitro* response of normal spleen cells to TNP-LPS. Normal CBA spleen cells were microcultured (10^6/culture) with and without TNP-LPS (50 ng/ml). Various numbers of TNP-R1$^+$ or TNP-R1$^-$ (haptenated under standard conditions by using 10 mM TNBS and irradiated with 10,000 R) were included in culture. Comparisons of the anti-TNP PFC response 3 days later shows that, although at the highest concentration both TNP-R1$^+$ and TNP-R1$^-$ dramatically reduce the anti-TNP-PFC response, at lower concentrations TNP-R1$^+$ exhibits more effective inhibitory activity than TNP-R1$^-$.

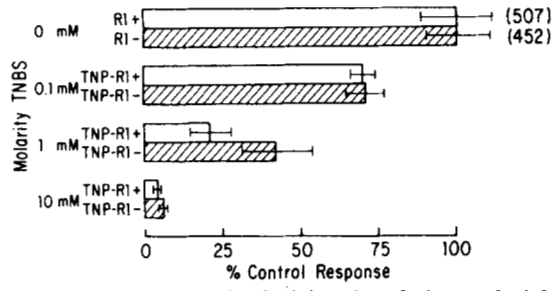

Figure 3. Effects of suboptimal trinitrophenylation on the inhibitory activities of TNP-R1$^+$ and TNP-R1$^-$ *in vitro*. Normal CBA splenocytes were microcultured (10^6/culture) with or without TNP-LPS (50 ng/ml) and the anti-TNP PFC response assayed after 3 days. Irradiated R1$^+$ or R1$^-$ cells haptenated with 10 mM, 1 mM or 0.1 mM TNBS, were included in cultures as indicated. Numbers in parentheses indicate mean PFC counts of control cultures to which unmodified R1$^+$ or R1$^-$ were added. TNP-R1$^+$ and TNP-R1$^-$ modified with 1 mM TNBS is significantly more inhibitory than similarly haptenated TNP-R1$^-$. Neither cell line exhibited blocking activity when modified with 0.1 mM TNBS.

studies of B cell tolerance induced *in vitro* by modified self in a single-stage culture system as described above suggested that the inhibition observed was T cell independent (2). It was recognized, however, that soluble TNP-moieties shed into the culture medium might directly block the potential B cell responses to TNP-immunogen, masking any presumptive T cell involvement (2). We recently used a 2-stage culture system to demonstrate that when soluble TNP-moieties are washed out, suppressor activity of regulator T cells can be detected (20). Responder cells are precultured with modified self in the absence of TNP-immunogen for 48 hr, then harvested, washed, mixed with freshly prepared spleen cells or irradiated spleen cells (as a test for suppression and tolerance, respectively), and recultured in microtiter trays with TNP-immunogen. Since suppressor cells generated by modified self are thought to recognize modified H-2 (8–11), we believed that the inferior effectiveness of TNP-R1$^-$ (compared with that of TNP-R1$^+$) in blocking the B cell response to TNP-LPS (Figs. 2 and 3 above) might be due to reduced or nonexistent ability to generate T suppressor cells. We therefore compared the abilities of TNP-R1$^+$ and TNP-R1$^-$ to induce tolerance and suppression of the anti-TNP PFC response in a 2-stage culture.

Examination of the data shown in Figure 4 reveals that when precultured cells are mixed with irradiated spleen cells (as "filler" cells) and challenged with TNP-LPS in the secondary culture (as a test for B cell tolerance), TNP-R1$^+$ and TNP-R1$^-$, whether modified with 10 mM or 1 mM TNBS, were equally capable of inducing tolerance. Modification with 10 mM TNBS, however, resulted in slightly more complete tolerance than modification with 1 mM TNBS (>90% tolerance *vs* about 70%).

When precultured cells are mixed with freshly prepared normal spleen cells at a 1:1 ratio before reculture with TNP-LPS (as a direct test for suppression), those spleen cells precultured with TNP-R1$^+$ modified with 10 mM TNBS suppressed the anti-TNP PFC response of normal spleen cells to TNP-LPS to 25% of control values. Interestingly, spleen cells precultured with TNP-R1$^-$ modified with 10 mM TNBS exhibited no suppression. When 1 mM TNBS was used for coupling, neither TNP-R1$^+$ nor TNP-R1$^-$ generated detectable suppression. In fact, splenocytes precultured with 1 mM TNBS-modified TNP-R1$^-$ show a reproducible enhancing activity when mixed with normal spleen cells (although generally more modest that in the experiment shown in Fig. 4). This effect is presently under investigation.

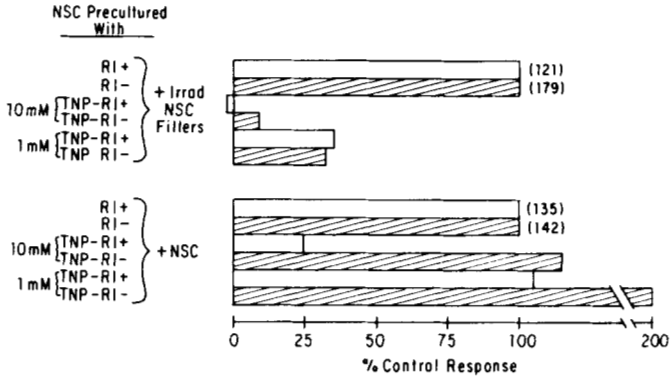

Figure 4. Induction of tolerance and suppression by TNP-R1$^+$ and TNP-R1$^-$ in a 2-stage culture. Normal CBA spleen cells precultured with 10,000 R-irradiated TNP-R1$^+$ or TNP-R1$^-$ in 12-well Linbro plates (16×10^6 NSC and 1.6×10^6 stimulators per culture). Haptenation was performed under standard conditions (10 min at 37°C) by using 10 mM or 1.0 mM TNBS (1 ml for 10^8 cells). Control groups (responses indicated in parentheses) were co-cultured with unhaptenated R1$^+$ or R1$^-$. After 48 hr cells were harvested, washed 3 times, mixed at a 1:1 ratio with fresh NSC or irradiated NSC and recultured in microculture with or without TNP-LPS (50 ng/ml) at 10^6 total cells per culture. The anti-TNP PFC response was assayed 3 days later. When haptenated with 10 mM TNBS, TNP-R1$^+$ was able to induce both tolerance (*upper bars*) and suppression (*lower bars*), while TNP-R1$^-$ similarly haptenated could induce tolerance, but not suppression. More lightly haptenated (1 mM) TNP-R1$^+$ or TNP-R1$^-$ could both induce tolerance (although less effectively than heavily haptenated cells) but neither could generate suppression. Control groups (cells co-cultured with unhaptenated R1$^+$ or R1$^-$) generated PFC response not significantly different from normal spleen cells precultured alone (data not shown).

Thus, even in a 2-stage culture, tolerance to challenge with a TNP-immunogen can be achieved *in vitro* by modified self in the absence of hapten-modified H-2. Furthermore, tolerance can be induced *in vitro* by modified cells that express H-2 antigen in the absence of detectable suppressor activity. This dissociation of tolerance and suppression has previously been reported, albeit in somewhat different model systems, by Claman and Miller (7), and more recently by Sherr and co-workers (30). In any case, only preculture with cells carrying heavily haptenated (with 10 mM TNBS) H-2 on the cell surface (i.e., TNP-R1$^+$) resulted in the induction of both tolerance and suppression. In the presence of less than optimally haptenated H-2 (TNP-R1$^+$ coupled with 1 mM TNBS) or in the absence of H-2 (i.e., TNP-R1$^-$ coupled with 10 mM or 1 mM TNBS), no suppression could be generated. Both TNP-R1$^+$ and TNP-R1$^-$, when haptenated with 0.1 mM TNBS, failed to induced either tolerance of suppression (data not shown).

DISCUSSION

Previous studies have indicated that suppressor T cells are generated during the induction of tolerance by hapten-modified spleen cells (7–12). Furthermore, a number of laboratories have demonstrated that the suppressor cells are generated in response to modified MHC determinants both *in vivo* and *in vitro* (8–11). However, some evidence has appeared that suggests that modified H-2 is not an absolute requirement for the induction of tolerance or suppression (2, 10–13). For instance, TNP-modified teratoma cells (which are serologically H-2 negative) could induce both tolerance and suppression in inbred mice (2, 10). Similarly, Pierres and co-workers (12) showed that TNP-R1$^-$ (another H-2-negative cell line) could generate hapten-specific suppressor cells for DTH.

In this report, we have compared the abilities of modified R1$^+$ and R1$^-$ to induce tolerance and suppression of a B cell response *in vitro* in order to analyze the role of H-2 in the response to modified self. As described above, R1$^-$ was selected from R1$^+$ by repeated anti-TL and C treatment and has been shown by several assays not to express MHC determinants (16–19). To the best of our knowledge, then, the only difference between the cell lines R1$^+$ and R1$^-$ is that the former expresses H-2K^k, H-D^k, and TL1,2,3 on its surface and the latter does not. Therefore, since both cell lines are trinitrophenylated to an equivalent extent by TNBS (19), any difference in the abilities of TNP-R1$^+$ and TNP-R1$^-$ to induce tolerance and/or suppression is presumably a reflection of the participation of modified H-2 or modified TL antigens. For simplicity of discussion, we will consider R1$^-$ as an H-2K/D-negative cell line, although the lack of TL is clearly recognized.

Our preliminary studies revealed that TNP-R1$^+$ was indistinguishable from TNP-SC with respect to inhibitory activity in a single-stage culture (Fig. 1) or in a 2-stage culture (data not shown). Inasmuch as R1$^+$ (and R1$^-$) does not carry lymphocyte-activating determinants and is thought not to express Ia antigens (18), this result suggests that modified Ia is not required for the *in vitro* induction of tolerance and suppression.

On the other hand, Vogt and Simpson (31), and Jandinski and co-workers (11) showed that modified Ia on haptenated cells could elicit helper activity. However, helper activity stimulated by modified I-A on TNP-SC *in vitro* was demonstrable only when H-2K/D was concomitantly modulated by specific antibody (11, 13). That is, H-2K/D antigens were shown to be suppressor epitopes and *dominant* over help (11, 13). Since TNP-R1$^+$ (H-2K$^+$, H-2K$^+$, Ia$^-$) and TNP-SC (H-2K$^+$, H-2D$^+$, Ia$^+$) induce tolerance and suppression *in vitro* to an equal extent, our conclusion that modified Ia antigens are not required for B cell tolerance in this system is consistent with this notion of immunodominance. Our findings should be compared with those of Conlon and colleagues (32), which indicated that modified Ia antigens on haptenated cells were required for the induction of immediate tolerance of the DTH response (which does not involve suppressor cells). Intravenous injection of TNP-SC does not result in immediate tolerance of the humoral response (2), however, which suggests that there might be significant differences in the mechanisms of tolerance induction of the humoral and the DTH responses.

The results of the comparison between TNP-labeled R1$^+$ and R1$^-$ using the single-stage culture system show that a) under optimal conditions TNP-R1$^-$ is as inhibitory as TNP-R1$^+$, but that b) at higher responder:stimulator ratios or using suboptimal coupling conditions, TNP-R1$^+$ is a more effective blocking agent than TNP-R1$^-$ (Figs. 2 and 3). When 1 mM TNBS is used as the coupling agent (the minimum concentration for effective H-2 haptenation) (29), a small but reproducible difference in the inhibitory properties of TNP-R1$^+$ and TNP-R1$^-$ is observed. This difference is not detected when 1 mM TNBS-haptenated cells are assayed for the ability to induce tolerance in a 2-stage culture, suggesting that soluble modified H-2 may possess singular suppressive properties. When 0.1 mM TNBS is used to couple the tumor cells (conditions that do not result in the modification of H-2 antigen) (29), TNP-R1$^+$ and TNP-R1$^-$ are equally poor in blocking the anti-TNP PFC response. Collectively, these results suggest that large amounts of hapten present in culture are sufficient to effect an inhibition of the response of spleen cells to TNP-LPS in the absence of modified H-2. At limiting hapten doses, the participation of modified H-2 in the inhibitory events becomes evident. Mechanistic interpretations of these findings will follow.

We recently developed a 2-stage culture system to separate the tolerizing and challenge events *in vitro* as is commonly performed in the *in vivo* situation (20). In this system, the induction of tolerance and suppression is achieved in a preculture of responder cells with tolerogen in the absence of immunogen. Precultured cells are harvested, washed, and only then are assayed for unresponsiveness or suppression by challenge with immunogen. Thus, potentially inhibitory soluble TNP-moieties shed by TNP-SC into the preculture medium are washed out before antigenic challenge. We have confirmed the hapten specificity of the tolerance and suppression induced by modified cells in this system and have demonstrated that little tolerogen is carried over from the first to the second culture (20). Comparison of the abilities of TNP-R1$^+$ and TNP-R1$^-$, haptenated with 10 mM or 1 mM TNBS, to induce tolerance and generate suppression in a 2-stage culture (Fig. 4) reveals that a) heavily haptenated (10 mM) TNP-R1$^+$ induces both tolerance and suppression in the preculture, b) suboptimally (1 mM) haptenated TNP-R1$^+$ induces tolerance but not suppression, and c) TNP-R1$^-$, whether coupled with 10 mM or 1 mM TNBS, induces tolerance as effectively as similarly haptenated TNP-R1$^+$, but d) such H-2$^-$ cells fail to generate suppression.

These results suggest that suppressor cells are indeed generated in response to modified H-2 as presented on TNP-R1$^+$ but not TNP-R1$^-$, as reported by a number of investigators (8–11). However, suboptimally haptenated H-2 (1 mM TNBS-modified TNP-R1$^+$) fails to generate detectable suppression while exhibiting significant tolerogenic activity. Dissociation of tolerance and suppression by modified self with respect to time has previously been reported by Claman and Miller (7), who postulated immediate clonal deletion and the transient presence of suppressor cells as 2 independent pathways to tolerance. More recently, Sherr and co-workers (30) demonstrated that the systemic administration of optimal amounts of modified self led to the induction of both tolerance and suppressor cells, but at limiting doses tolerance was achieved in the absence of suppression. Our results represent another example of such tolerance/suppression dissociation.

The results shown in Figure 4 also indicate that TNP-R1$^-$, whether haptenated with 10 mM or 1 mM TNBS, induced tolerance in the absence of demonstrable suppression. This finding, when viewed in conjunction with the other data, is interpretable in at least 3 mutually nonexclusive ways:

a) Splenic B cells in the primary culture are tolerized upon exposure to modified self regardless of the presence or absence of TNP-H-2. Therefore, suppressor cells, which are generated only in response to heavily (10 mM) haptenated H-2, need not be essential for the induction or maintenance of the tolerance state induced *in vitro*. We have recently found, however, that anti-brain associated θ antigen (anti-Baθ) and C treatment of spleen cells before preculture with TNP-SC partially prevented the *in vitro* induction of tolerance and totally abrogated the generation of suppression (20). This suggests that the presence of T cells may in fact contribute to B cell tolerance in this system. Whether T cells are required for B cell tolerance included by TNP-R1$^-$ tumor cells is not known at present.

b) It is also possible that suppressor activity beyond the limits of detection of our system is indeed responsible (and essential) for *in vitro* induced tolerance. For instance, suboptimally haptenated H-2 or haptenated non-H-2 antigens may be reprocessed *in vitro* and presented in a form (e.g., in association with unmodified H-2 on an accessory cell) that can generate suppressor cells mediating tolerance but that are not detectable in our cell-mixing protocol. Supporting this contention are the recent findings of Heber-Katz and Shevach (33) that TNP-coupled membranes could stimulate T cells *in vitro* only when presented in association with Ia determinants on unmodified macrophages. Similarly, Korngold and Sprent (34) have demonstrated that antigen reprocessing plays an important role in activating H-2-restricted cytotoxic T cells specific for minor histocompatibility antigens *in vivo*. On the other hand, in an adoptive transfer system, the demonstration that allogeneic hapten-coupled cells induced suppressor T cells that could suppress the DTH response only of those recipients syngeneic with the tolerogen (8, 12) argues against antigen reprocessing. Clearly, further studies are necessary to resolve this issue.

c) A third interpretation contends that suppressor cells may be directly stimulated by modified non-MHC determinants. We have previously shown in a 2-stage culture that TNP-SC modulated with an anti-H-2K^k antiserum lose their ability to generate suppression and, in fact, can stimulate an anti-TNP PFC response. This stimulation was abrogated by further modulation with anti-Ia antiserum (11, 13). These anti-H-2K and anti-Ia-modulated TNP-SC, however, were able to generate hapten-specific suppressor cells, suggesting that in the absence of modified H-2, haptenated non-MHC determinants may trigger suppressor cells (11, 13).

The results of the comparison of TNP-R1$^+$ and TNP-R1$^-$ in the single-stage culture (Figs. 2 and 3) as well in the 2-stage system (Fig. 4) may thus be explicable if we assume there exists a spectrum of epitopes capable of eliciting suppression; that is, heavily haptenated MHC determinants (detectable in our 2-stage system) > suboptimally haptenated MHC determinants > modified non-MHC determinants.

In fact, Ciavarra and Forman (27) have recently suggested that TNP covalently coupled to H-2 antigens and TNP-protein noncovalently associated with H-2 stimulated two populations of cytotoxic T cells. Thus, our findings can be interpretated to suggest that separate suppressor subpopulations may be activated in response to different suppressor epitopes.

That is, although suboptimally haptenated H-2 antigens or modified non-MHC determinants may in fact activate a subpopulation of suppressor cells, the presence of heavily haptenated H-2 seems necessary for the elicitation of detectable suppression. It is believed that the suppressor cells are generated in response to modified H-2K/D antigens, although it is noted that the cell lines R1$^+$ and R1$^-$ differ in H-2K, H-2D, and TL1,2,3 expression.

Furthermore, as has been demonstrated for cytotoxic T cells (35), we have recently shown that suppressor cells from H-2^k mice are triggered by modified H-2K^k but not H-2D^k (11). Thus, investigation of the tolerogenic and suppressive properties of purified H-2K^k antigen is now in progress.

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