MECHANISMS OF REGULATION OF CELL-MEDIATED IMMUNITY

VII. Suppressor T Cells Induced by Suboptimal Doses of Antigen Plus an I-J-specific Allogeneic Effect*

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Thymus-derived suppressor cells (Ts)[1] regulate humoral (1, 2), cell-mediated (3, 4), or proliferative (5) responses, and the characterization of these suppressor systems reveals that there are probably several distinct types of Ts. This has been further complicated by the demonstration in certain systems that one Ts or another immune cell may help induction of other phenotypically distinct Ts (6–10). These latter studies have raised the possibility that various Ts may in fact be part of a larger interrelated network of suppressor cells (11).

Studies from this laboratory (7, 8, 10) designed to analyze the suppressor T cell network revealed that in several systems, antigen presented on syngeneic cells induces an antigen-specific I-J$^+$ suppressor T cell, Ts_1, from which an antigen-specific, idiotype$^+$, I-J$^+$ suppressor factor, TsF_1, can be obtained. TsF_1, in turn, stimulates the development of a second set of suppressor T cells, Ts_2, which are anti-idiotypic, I-J$^+$, and which suppress antigen-specific immune cells.

To better understand suppressor T cell networks we must determine what signals stimulate suppressor T cells. Compared with our detailed knowledge of the I region-controlled requirement for the stimulation of helper T cells by antigen, very little is known of the conditions for suppressor T cell stimulation. These experiments were designed to address this issue. We explored whether a major histocompatibility complex (MHC) requirement for the stimulation of suppressor T cells could be revealed by administering syngeneic spleen cells derivatized with a low density of hapten, a stimulus which is incapable of triggering suppression unless allogeneic cells are added, thus demonstrating the requirement of an allogeneic effect in the development of suppressor T cell responses. We then were able to verify that allogeneic T cells responding to I-J-coded differences on suppressor T cells provided the necessary stimulus together with antigen, at the low dose used, for the development of specific suppression. The significance of this finding is discussed in relation to MHC restrictions in the development of suppressor T cell responses.

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[1] *Abbreviations used in this paper:* AEF, allogeneic effect factor; CS, contact sensitivity; DNFB, 2,4-dinitrofluorobenzene; DNP, dinitrophenyl; HBSS, Hanks' buffered saline solution; MHC, major histocompatibility complex; PCl, 2,4,6-trinitrochlorobenzene (picryl chloride); TNBS, 2,4,6-trinitrobenzene sulfonic acid; Ts, thymus-derived suppressor cell; Ts_1, first order suppressor cell; Ts_2, second order suppressor cell; TsF, soluble suppressor factor.

Materials and Methods

Animals. All mouse strains were purchased from The Jackson Laboratory, Bar Harbor, Maine and housed in our animal facilities.

Antigens. 2,4,6-trinitrobenzene sulfonic acid (TNBS), 2,4-dinitrofluorobenzene (DNFB), and 2,4,6-trinitrochlorobenzene (picryl chloride, PCl) were purchased from Eastman Kodak Company, Rochester, N. Y.

Antiserum. A monoclonal anti-Thy 1.2 ascitic fluid used at 1:1,000 was kindly provided by Dr. P. Lake, University College, London.

TNP-derivatized Cell Preparation. Spleen cells were suspended in chilled Hanks' buffered saline solution (HBSS). Erythrocytes were lysed by treatment with 0.83% ammonium chloride in Tris buffer, pH 7.6. After washing, cells were resuspended to 5×10^7 cells/ml in HBSS containing TNBS, pH 7.4 (adjusted with NaOH), at the appropriate molarity. The cells were incubated for 30 min at room temperature with gentle stirring. The cell suspensions were then washed three times with HBSS and resuspended to appropriate final concentrations. For 0.01 mM TNBS solutions, the concentration used throughout these experiments, cell viability was always 50–70%.

Generation of Suppressor Cells. Animals were injected intravenously with 5×10^7 live, syngeneic, haptenated cells and were either immunized on the same day (nontransfer protocol) or served as donors of Ts 7 d later (transfer protocol). In the transfer protocol, 5×10^7 live spleen cells were transferred from these Ts donors to appropriate recipients that had been immunized 1–2 h earlier.

Immunization and Challenge. Animals were immunized by skin painting with one 100-μl dose of a 7% PCl solution or two daily doses of 25 μl of a 0.5% DNFB solution in acetone:olive oil (4:1) on the shaved abdomen. 5 d later, 20 μl of a 0.5% PCl solution or a 0.2% DNFB solution in acetone:olive oil was applied to both ears of the appropriately immunized animals. The extent of the contact sensitivity (CS) reaction is expressed as the difference in thickness of each ear measured with an engineer's micrometer immediately before and 24 h after challenge. All groups contained three to five mice and the mean and standard error of the mean (SEM) are depicted. *P* values reflect differences obtained using the Student's two-tailed *t* test.

Results

Epitope Density on Cell Surfaces Influences the Generation of Ts. After having shown that high hapten density (10 mM TNBS derivatization of tolerogen) on allogeneic cells could supersede and abrogate the allogeneic restrictions of Ts for CS (12), we next asked whether a very low density of hapten (0.01 mM TNBS derivatization of tolerogen) on syngeneic cells could possibly generate a syngeneically restricted Ts, since 1 mM derivatized allogeneic cells could generate allogeneically restricted Ts. Fig. 1 shows an experiment in which BALB/c mice received, intravenously, syngeneic cells haptenated at various concentrations of TNBS (0.01–10 mM). 1 wk later, these animals served as donors of putative Ts to either syngeneic BALB/c recipients or completely allogeneic B6 recipients, which were all immunized on the day of cell transfer. Contrary to our expectations, syngeneic Ts were not induced. In fact, Ts induced by progressively less haptenated tolerogen functioned less well in syngeneic recipients but continued to manifest a large degree of suppression in allogeneic recipients.

These results led us to conclude that a "pre-Ts" was being generated by low (0.01 mM) hapten densities and that it required a second, activating or differentiating signal which was being provided by an allogeneic effect. This idea was tested more directly as shown in Fig. 2. Syngeneic 0.01 mM haptenated spleen cells were injected intravenously into BALB/c recipients. 1 wk later their spleens were transferred to syngeneic BALB/c recipients or allogeneic CBA/J recipients. As before, the transferred

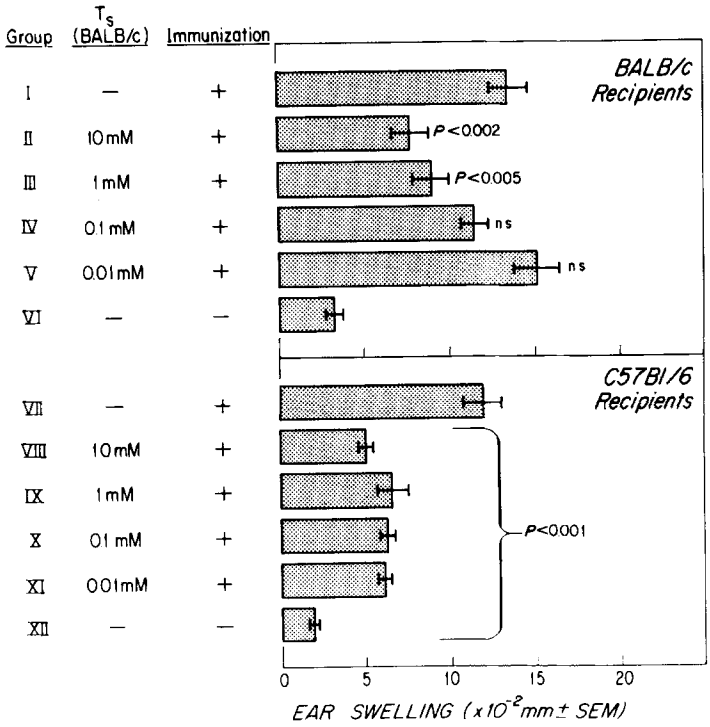


FIG. 1. Hapten density and allogeneic effects on the generation of suppressor cells. 5×10^7 10 mM, 1 mM, 0.1 mM, or 0.01 mM TNBS-derivatized BALB/c cells were injected intravenously into syngeneic recipients. 7 d later, 5×10^7 spleen cells from these groups of mice were transferred into syngeneic or allogeneic (C57BL/6) recipients, which were then immunized epicutaneously with PCl. 5 d thereafter, the separate groups were challenged on the ear with PCl, and T cell-dependent CS reactivity was determined 24 h later.

cells functioned only as Ts in the allogeneic strain. However, if an allogeneic signal is given concomitantly with the Ts to the syngeneic recipient, then suppression is manifested. Thus, if a BALB/c recipient receives both BALB/c Ts and allogeneic cells (3×10^7 CBA/J spleen cells), the CS reaction is suppressed. Neither cell population on its own is able to accomplish this.

Kinetics of Allogeneic Effects Necessary for Ts Induction. Additional experiments reveal that in a nontransfer protocol (i.e, one in which the recipient of the haptenated cells is tested directly for CS), haptenated syngeneic cells plus an allogeneic signal given on the day of abdominal immunization will suppress the CS reaction. Fig. 3 shows that if an animal is both immunized and given 0.01 mM haptenated syngeneic cells on day 0, then an allogeneic signal can be given from day −5 to 2 to significantly suppress a challenge on day 5. Thus, the kinetics of induction of these Ts is short. Notably, neither haptenated cells nor allogeneic cells alone suppress the CS response. Although this nontransfer protocol is less cumbersome than the transfer protocol, we have decided to pursue the latter because it permits one to deliver two unlinked signals (antigen and allogeneic effect) separated by time (7 d), and to dissect more fully the stages of maturation and differentiation in the Ts pathway.

Specificity of Suppressor Cells. To evaluate whether ligand-activated presuppressor cells become nonspecific when influenced by allogeneic effects, the following studies

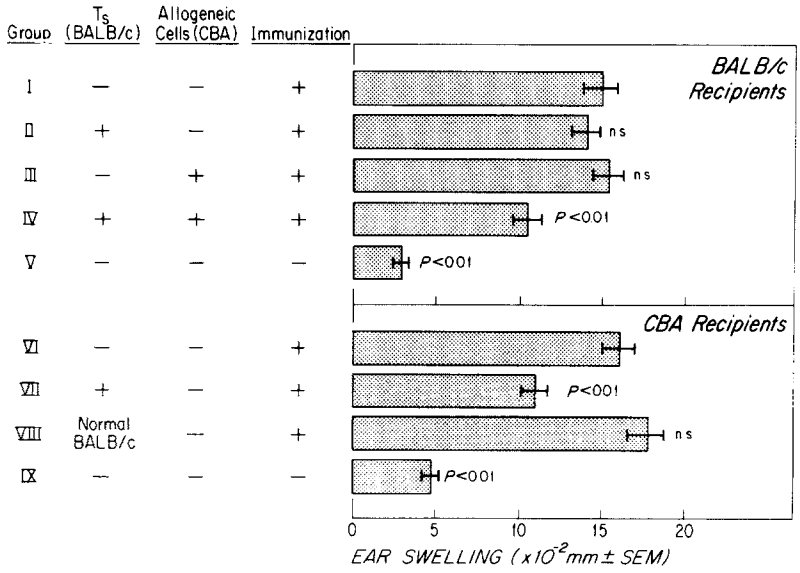


FIG. 2. Allogeneic effects on the generation of suppressor cells. 5×10^7 0.01 mM TNBS-derivatized BALB/c cells were injected intravenously into syngeneic recipients. 7 d later, 5×10^7 spleen cells from these groups of mice were transferred into syngeneic or allogeneic (CBA/J) recipients, which were then immunized epicutaneously with PCl. Some syngeneic recipients also received 3×10^7 CBA/J cells. 5 d later, the separate groups were challenged on the ear with PCl, and T cell-dependent CS reactivity was determined 24 h later. Normal BALB/c refers to donors that did not receive derivatized cells.

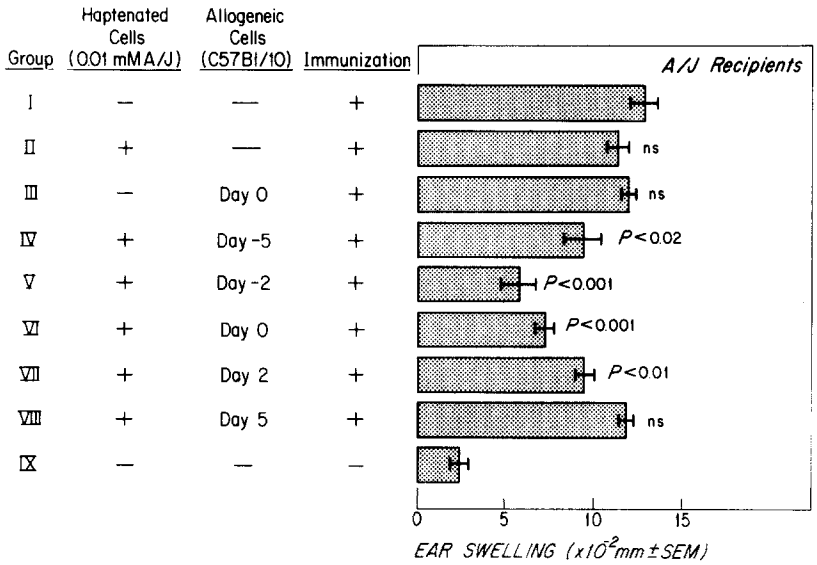


FIG. 3. Kinetics of allogeneic effect with respect to Ts induction. 5×10^7 0.01 mM TNBS-derivatized A/J cells were injected intravenously into syngeneic recipients, which were immunized epicutaneously with PCl on the same day. At various times before or after immunization, these animals also received 3×10^7 allogeneic (C57BL/6) cells intravenously. 5 d after immunization the separate groups were challenged on the ear with PCl, and T cell-dependent CS reactivity was determined 24 h later.

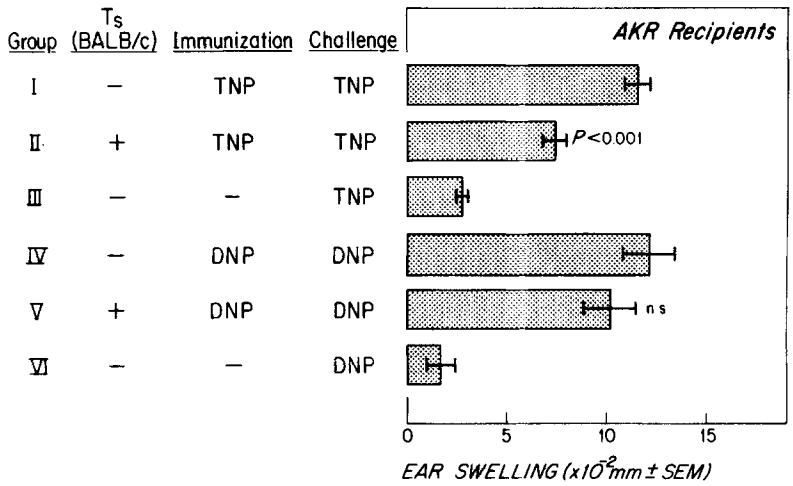


FIG. 4. Antigenic specificity of suppression. 5 × 10^7 0.01 mM TNBS-derivatized BALB/c cells were injected intravenously into syngeneic recipients. 7 d later, 5 × 10^7 spleen cells from these groups of mice were transferred into allogeneic (AKR/J) recipients, which were then immunized epicutaneously with PCl or DNFB. 5 d later, the separate groups were challenged on the ear with PCl or DNFB, and T cell-dependent CS reactivity was determined 24 h later.

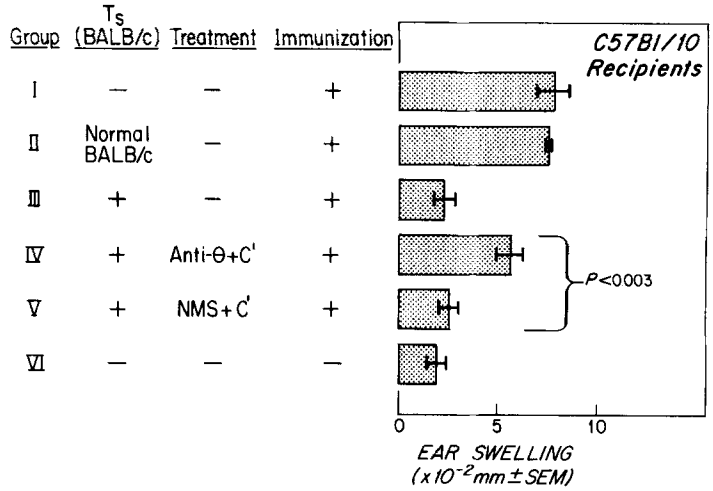


FIG. 5. T cell nature of suppression. 5 × 10^7 0.01 mM TNBS-derivatized BALB/c cells were injected intravenously into syngeneic recipients. 7 d later, 5 × 10^7 spleen cells from these groups of mice were transferred into allogeneic (C57BL/6) recipients after no treatment or after treatment with normal mouse serum or a monoclonal anti-Thy 1.2 plus complement. The mice were immunized epicutaneously with PCl and 5 d later were challenged on the ear with PCl; T cell-dependent CS reactivity was determined 24 h later. Normal BALB/c refers to donors that did not receive derivatized cells.

were performed. 0.01 mM TNP-derivatized BALB/c cells were used to induce pre-Ts in syngeneic recipients. 7 d later, these cells were transferred into AKR recipients which were then immunized epicutaneously with PCl or DNFB and challenged 5 d later with the homologous hapten. As depicted in Fig. 4, such ligand-activated and allogeneic effect-influenced presuppresor cells function in an antigen-specific manner.

T Cell Nature of Suppression. It was experimentally determined that such suppres-

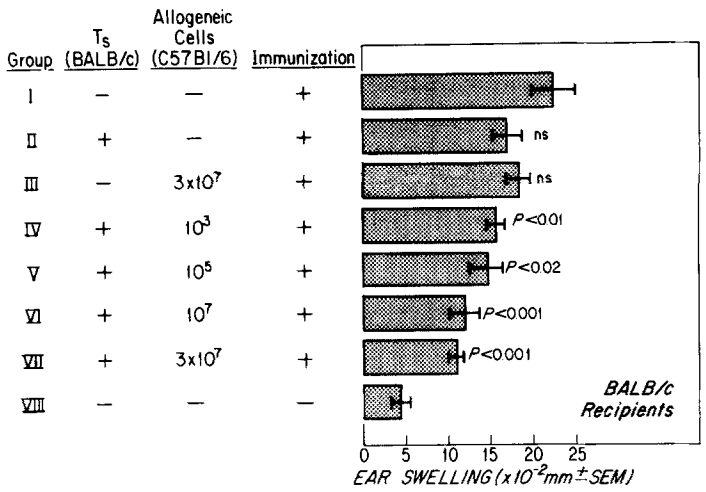


FIG. 6. Dose dependence of allogeneic effect. 5×10^7 0.01 mM TNBS-derivatized BALB/c cells were injected intravenously into syngeneic recipients. 7 d later, 5×10^7 spleen cells from these groups of mice were transferred into syngeneic recipients along with varying numbers (10^3–3×10^7) of allogeneic (C57BL/6) cells and then immunized epicutaneously with PCl. 5 d later the separate groups were challenged on the ear with PCl, and T cell-dependent CS reactivity was determined 24 h later.

sion was mediated by thymus-derived cells by inducing presuppressors in BALB/c mice and treating aliquots of these cells in vitro with monoclonal anti-Thy 1.2 and complement or normal serum and complement before transfer to allogeneic C57BL/10 recipients. As is depicted in Fig. 5, antigen-specific T cells are responsible for the observed effects. These results do not, however, exclude a role for B cells and/or macrophages which could be crucial for idiotypic signals and antigen-presenting function, respectively.

Dose Dependence of Allogeneic Effects. We next evaluated whether the allogeneic signals required to trigger preTs→Ts differentiation or activation were dependent upon the dose of alloantigenic stimuli. We used constant numbers of pre-Ts induced in BALB/c which were transferred into syngeneic recipients at the same time as varying numbers of allogeneic cells (Fig. 6). The observed dose dependence of the resultant suppression clearly reflects the requirement of defined allogeneic effects to activate or cause the differentiation of pre-Ts to Ts. We have continued to use 3×10^7 allogeneic cells for those experiments in which the Ts and recipient are syngeneic.

It should be noted here that because all these experiments involve some sort of allogeneic transfer we do see some variability in responses that we attribute to antigen-nonspecific allogeneic effects. Thus, in about 10–15% of our experiments, the allogeneic controls are unacceptably suppressed, masking specific suppression by Ts. This variability has been reported by others (13).

Mapping of the Direction of the Allogeneic Effect. In all of the above-mentioned studies, allogeneic effects were bidirectional between the pre-Ts and the allogeneic cells or allogeneic recipients. To determine which effect was sufficient for activating pre-Ts, the following sets of experiments were undertaken. As shown in Fig. 7A, pre-Ts were induced in C57BL/6 mice and then transferred into (C57BL/6 × DBA/2)F_1 mice. In this case, suppression did not occur. However, if B10.D2 allogeneic cells were added

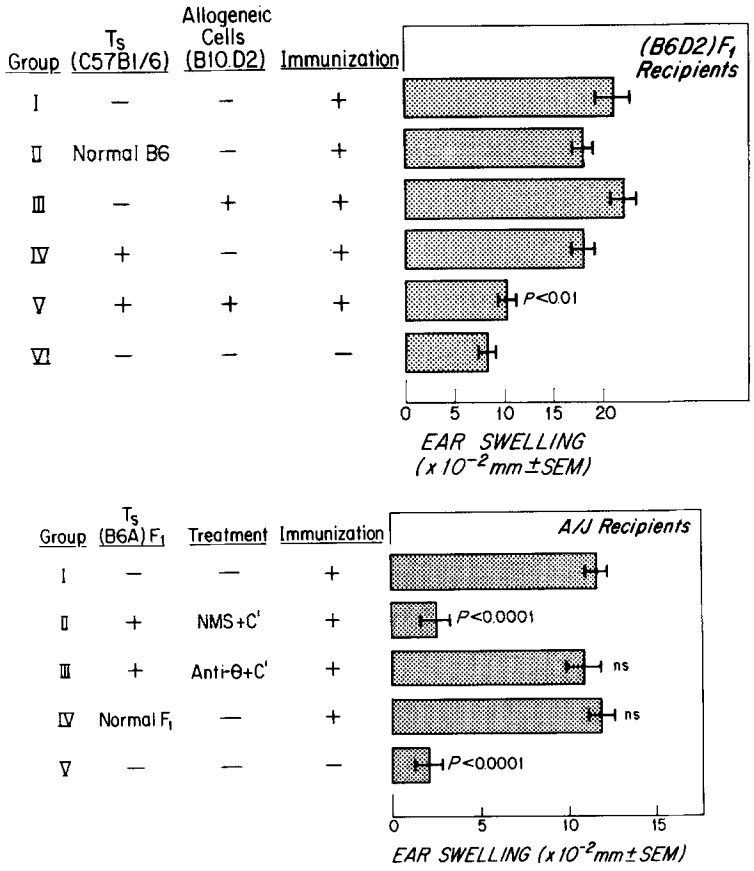
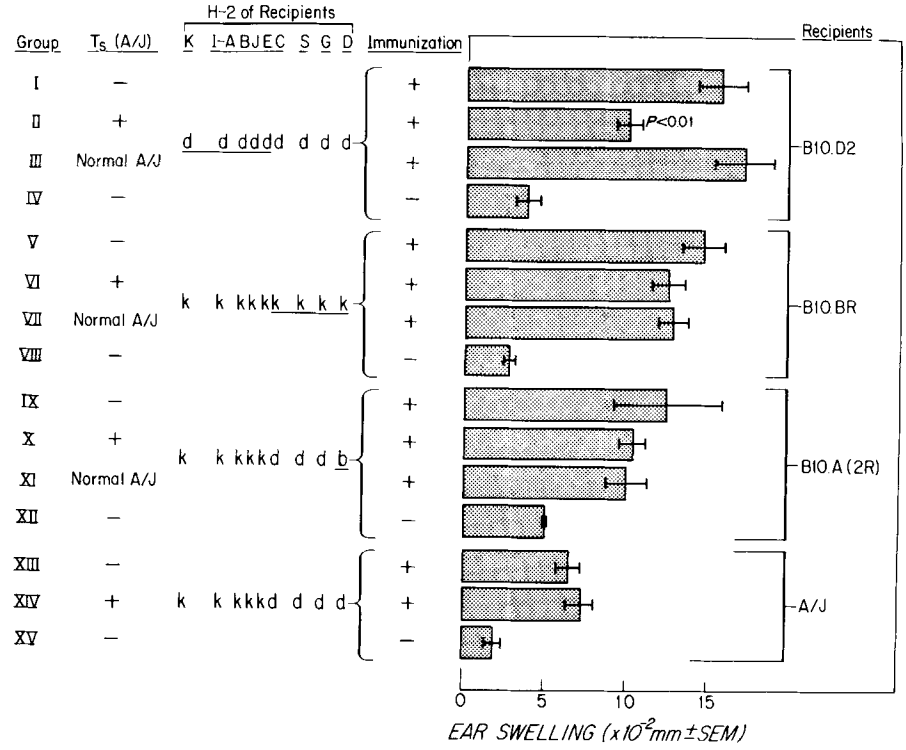


FIG. 7. Direction of the allogeneic effect. 5×10^7 0.01 mM TNBS-derivatized C57BL/6 (A) (top) or (C57BL/6 × A/J)F_1 (B) (bottom) cells were injected intravenously into syngeneic recipients. 7 d later 5×10^7 spleen cells from these groups of mice were transferred into semisyngeneic (C57BL/6 × DBA/2)F_1 (A) or parental A/J (B) recipients. In A, 3×10^7 allogeneic (B10.D2) cells were also transferred to some groups. Recipients were then immunized epicutaneously with PCl and 5 d later challenged on the ear with PCl; T cell-dependent CS reactivity was determined 24 h later. B also shows the results of another monoclonal anti-Thy 1.2 experiment. Normal B6 and normal F_1 refer to donors that did not receive derivatized cells.

simultaneously with the B6 pre-Ts, then suppression was observed. This experiment demonstrates that the allogeneic effect must at least be directed against the pre-Ts. Nevertheless, bidirectional allogeneic influences could account for the suppression. These experiments were then extended to evaluate the impact of F_1 pre-Ts given to a parental recipient as shown in Fig. 7B. (C57BL/6 × A/J)F_1 pre-Ts administered to A/J recipients resulted in suppression. These experiments unequivocally demonstrate that an allogeneic effect directed solely against the pre-Ts is necessary and sufficient to activate or differentiate such cells into those capable of suppressing T cell immunity.

Gene Regions Responsible for Allogeneic Effects That Influence Pre-Ts Function. Mapping of the region(s) of the H-2 complex that are responsible for the allogeneic effect we have observed is shown in Fig. 8. Panel A maps the region to the left side of H-2 and panel B maps the effect precisely to the I-J subregion. It should be noted that even though the A/J recipients that received B10.A(5R) cells alone were slightly nonspe-



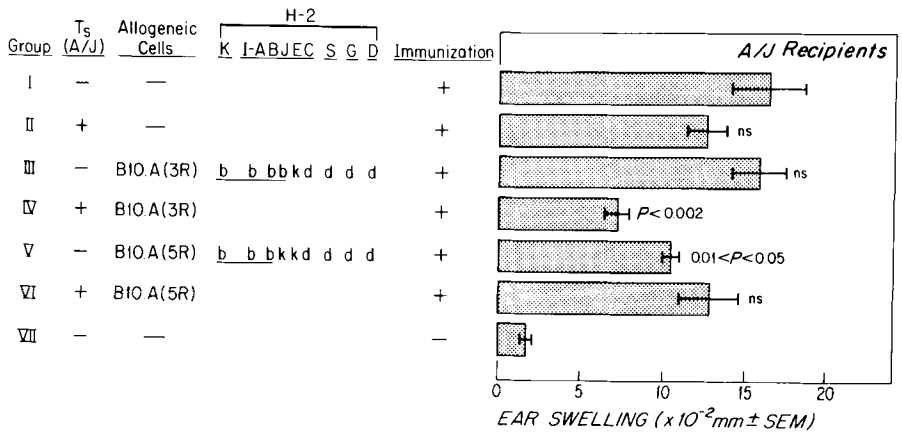


FIG. 8. Genetics of allogeneic effect. (A) (top) 5×10^7 0.01 mM TNBS-derivatized A/J cells were injected intravenously into syngeneic recipients. 7 d later 5×10^7 spleen cells from these groups of mice or from normal mice were transferred into B10.D2, B10.BR, B10.A(2R), and A/J recipients. Recipients were immunized epicutaneously with TNCB, and 5 d later challenged on the ear with PCl; T cell-dependent CS reactivity was measured 24 h later. (B) (bottom) 5×10^7 0.01 mM TNBS-derivatized A/J cells were injected intravenously into syngeneic recipients. 7 d later, 5×10^7 spleen cells from these groups of mice were transferred into A/J recipients along with 3×10^7 B10.A(3R) or B10.A(5R) cells. Recipients were immunized and challenged as in A.

cifically suppressed, those that received A/J Ts plus B10.A(5R) cells were not at all suppressed. Since A/J and B10.BR differ at the Mls locus, the data also rule out a contribution to the allogeneic effect by Mls differences in this particular strain combination. In the course of these studies, we used strains mostly of the b, d, and k haplotypes and were able to provide a suitable allogeneic, activational signal in all six possible stimulator-responder strain combinations.

Discussion

We have been studying how MHC-derived molecules and antigen may influence the induction and specificity of Ts to contact sensitivity. Others have shown (3), and we have confirmed (12), that haptenated lymphoid cells injected intravenously generate antigen-specific Ts whose specificity for antigen plus H-2 is regulated by the genotype of the haptenated donor cell. Thus, dinitrophenyl (DNP)-conjugated cells, when injected into a syngeneic strain, will generate Ts that may be transferred to any other strain regardless of H-2 type. Miller (14) has obtained evidence that this is due to polyclonal expansion of Ts subsets specific for DNP plus various H-2 specificities. In contrast, intravenous injection of haptenated cells into an allogeneic strain generates Ts that may be successfully transferred only to strains bearing the same H-2 type as the original haptenated inoculum. However, in the TNP system we showed (12) that this apparent H-2 restriction was dependent on antigen density: derivatization of lymphoid cells with higher concentrations of hapten resulted in a tolerogen capable of inducing unrestricted Ts in allogeneic strains. Hence, lymphoid cells haptenated with 1 mM TNBS could induce allogeneically restricted Ts, whereas 10 mM derivatized cells induced unrestricted Ts. The implications of this finding for antigen reprocessing, critical haptenization of cell surface determinants, and/or changes in receptor affinity and specificity of the Ts were discussed at that time.

We have continued our studies of the effect of hapten density on the induction and functional specificity of Ts. The present results show that a suboptimal dose of antigen (0.01 mM TNBS-derivatized lymphoid cells) is able to induce a T cell which requires a second, activational signal to fully express its suppressive potential. This second signal can be provided by an I-J allogeneic effect directed solely against the induced T cell. The allogeneic effect has traditionally been viewed as an activational or differentiation signal which, although delivered in a nonphysiologic manner (across allogeneic barriers), is a reflection of normal physiologic processes and signals in the intact animal (13, 15, 16). In the best-studied allogeneic effect systems, it has been shown that an allogeneic Ly 1^+ T cell responds to stimulator alloantigens and produces a factor which bears those antigens and binds to and is genetically restricted to help B cells syngeneic to the stimulators (17, 18). How the allogeneic effect factor (AEF) works and what constitutes the molecule are unknown. However, in view of our presently described experiments, and the recent data of Delovitch et al. (19), we would like to suggest that the factor bears a binding site (idiotype) for the stimulator molecule(s). Such an idiotypic specificity would explain the genetic restriction of the factor. If the AEF is polyvalent, then an explanation is also provided for why the factor both bears alloantigen and is genetically restricted to that alloantigen. This implies that alloantigen may not be necessary for the functional activity of AEF. The AEF may also bear its own "Fc" portion which endows the molecule with the ability to provide a differentiation signal once it has bound its target. Thus, in the experiments

defined herein, AEF binds to I-J region-related molecules on pre-Ts. The binding of the specialized AEF molecules to such a site on a ligand-activated pre-Ts results in the activation or further differentiation into functional antigen-specific Ts. One interesting implication of this line of thought is that any properly focused triggering event may provide a sufficient second signal for these suboptimally induced Ts. Thus antisera (e.g., anti-I-J) without complement might provide an appropriate second signal. This may explain the results of Conlon et al. (20) in which an Mls allogeneic effect provided a potent second signal for tolerance induction to DNP CS.

These experiments show, as demonstrated previously (12), that the interaction of nominal antigens with MHC-encoded antigens may have a profound effect on the generation of Ts. In the present instance, suboptimal doses and/or densities of antigen prime a population of T cells which are poised to act as Ts. These cells resemble certain other Ts (3) in that they are ostensibly unrestricted once activated: Ts may suppress allogeneic or syngeneic recipients as long as a proper allogeneic effect is provided. It is difficult to understand how suboptimal haptenization results in such a poised cell. On the one hand, it may simply be a quantitative problem: a low dose of antigen is inefficient at triggering a Ts precursor but may be able to bind enough receptors to prime the cell. Alternatively, the problem may be more complex. As elegantly demonstrated by Forman et al. (21, 22), the concentration of TNBS has profound effects on the derivatization of H-2 antigens and the generation of neoantigens. Higher antigen densities (1 or 10 mM) may generate a neoantigen, or altered self molecule, which can serve as a stimulator moiety for an allogeneic effect in vivo. The AEF produced by such an allogeneic effect would bind to the neoantigen. If a Ts precursor had bound antigen to its surface via its antigen receptor, then the AEF could bind to the cell-associated neoantigen and deliver its signal to the precursor Ts through an antigen bridge. At lower antigen densities (0.01 mM), a Ts precursor could bind antigen (assuming it is specific for nominal antigen, not neoantigen), however at such low densities there is no generation of an altered self molecule, no allogeneic effect, and therefore no generation of activated Ts. Our data imply that the crucial molecule altered by TNBS treatment is I-J.

The association of an I-J allogeneic effect with suppressor cell activation is interesting in light of the association of I-J with suppressor cells and their products (6–8). The recent report of Zinkernagel (23) that an isolated I-J graft vs. host reaction may suppress the in vivo response to *Listeria* is consonant with the present data. He suggests that I-region molecules may act as receptors for differentiation signals. The provocative data of Streilein and Klein (24), and Streilein (25) on neonatal tolerance may also involve an allogeneic host-vs.-graft response. In this case, there is strong evidence for associative recognition of allogeneic I-J and H-2D determinants. A model for this system, similar to the one above, can be constructed. One cell in the suppressor circuit may recognize allogeneic I-J plus H-2D on the allograft and signal a second cell in the circuit which has bound H-2D to its surface via a specific receptor. The first cell recognizes allogeneic H-2D, or perhaps a complex of allogeneic H-2D, plus self I-J on the surface of the second cell. This model argues for a limited polymorphism of I-J determinants among different strains and supports the concept that there is an essential association between recognition of "J-ness" and activation of suppressor circuits. These models also support the notion that restriction (5, 6) or lack of restriction (7, 8) in a suppressor system is a function of how entry into the suppressor

pathway is initiated and what stage is being assayed in a particular protocol. Recently Czitrom et al. (26) have obtained data similar to Streilein's in a primary mixed lymphocyte reaction. There, an I-J difference suppresses the response to allogeneic H-2D and must be associatively linked to H-2D. This raises the possibility that I-J$^+$ Ts may functionally be antigen-presenting cells for other cells in the suppressor circuit. One caveat on these systems is that I-J is also associated with helper T cell function and not strictly suppressor cell mechanisms (27–30). It should be noted that additional I subregions may be involved in certain steps of the suppressor pathway. Sherr et al.[2] have recently found that I-A and/or I-E subregion genes on antigen-presenting cells are relevant for suppression of the reaction to protein antigens. It may be that ligand-receptor interaction requires appropriate H-2 presentation independent of the type of activation or differentiation signals described here.

Perhaps the greatest implication of our findings relates to their potential application for enhancement of allograft survival. A search for the human equivalent of I-J may prove rewarding. In fact, the association between certain HLA-DR incompatibilities and enhanced renal allograft survival is encouraging (31, 32).

Summary

Intravenous injection of 0.01 mM 2,4,6-trinitrobenzene sulfonic acid-derivatized syngeneic lymphoid cells generates a Thy-1-positive, antigen-specific suppressor cell for contact sensitivity which requires an I-J allogeneic effect to become fully activated. It is necessary and sufficient for the allogeneic effect to be directed solely against the suppressor cell, and once activated, the cell can suppress in an H-2-unrestricted fashion. The results are discussed in the framework of entry into the suppressor pathway, the allogeneic effect as a reflection of normal physiologic processes, and the importance of I-J as a receptor and signal among cells in the suppressor pathway.

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