

EARLY DEVELOPMENT OF THE T CELL REPERTOIRE

In Vivo Treatment of Neonatal Mice with Anti-Ia Antibodies

Interferes with Differentiation of I-restricted

T Cells But Not K/D-restricted T Cells

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A large number of studies have indicated that the activation of T helper cells for both antibody (1-6) and cytolytic T lymphocyte (CTL)¹ (7-11)² responses is restricted by products of the I region of the major histocompatibility complex (MHC), while the activation of CTL themselves is restricted by products of the K/D regions of the MHC (12-14). However, it is less clear how and when during their developmental pathway T cells acquire their MHC-restricted self-recognition specificity. Studies with chimeras (12, 15, 16) and thymus-grafted nude mice (17, 18) have indicated that the MHC phenotype of the thymus dictates the particular MHC determinants that T cells recognize as self-recognition elements. In addition, the MHC phenotype of the extra-thymic environment has been implicated in the process that determines the restriction specificities of T cells (11, 14, 17, 19, 20). In vivo manipulation of the expression of MHC products may yield a better understanding of how the host environment (thymic or extra-thymic) determines restriction specificities of T cells. One approach to achieving such manipulation is by in vivo administration of antibodies (Ab) to MHC products. A few reports describe the effect of in vivo anti-Ia Ab (5, 21-26) but no data on actual expression of I region products in the recipients are so far available. A recent report described suppression of B lymphocyte development in mice injected from birth with monoclonal anti-I-A Ab (27), i.e., surface Ia⁺, IgM⁺, and IgD⁺ B cells did not develop in such mice.

The purpose of the present study was to determine whether such chronic administration of anti-Ia Ab into mice during their first weeks of life might influence their T cell self-recognition repertoire. We show here that chronic injection of anti-I-A^k Ab, starting in neonatal H-2^k mice, abrogates their ability to generate alloreactive and trinitrophenyl (TNP)-specific splenic CTL responses. Different mechanisms for this

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¹ *Abbreviations used in this paper:* Ab, antibody; CTL, cytotoxic T lymphocyte; Con A, concanavalin A; FMF, flow microfluorometry; FITC, fluorescein isothiocyanate; IF, immunofluorescence; IL-2, interleukin 2; MHC, major histocompatibility complex; SN, supernatant; TNP, trinitrophenyl.

² Ada M. Kruisbeek, Patricia Andrysiak, and Alfred Singer. Self-recognition of accessory cell Ia determinants is required in the generation of hapten-specific T lymphocyte responses. Manuscript submitted for publication.

defect were evaluated, and the results support the idea that T cells from anti-I-A-treated mice are unable to recognize self or allogeneic I-A determinants; in contrast, CTL from these mice are able to recognize both self and allogeneic K/D region determinants. A decrease in expression of thymic I-A antigens paralleled this functional defect. These findings are interpreted as suggesting that during early T cell differentiation, education of some if not all K/D-restricted T cells occurs on different thymic elements than the education of I-restricted T cells.

Materials and Methods

Mice. Term pregnant C3H/HeN (H-2^k), F₁(C57BL/6 × C3H)(H-2^b × H-2^k), and C57BL/6 (H-2^b) mice were obtained from the Small Animal Section, Research Service, National Institutes of Health; all adult mice (BALB/c, C57BL/10, C3H/HeN, B10.BR) were 8–12 wk old and obtained from The Jackson Laboratory, Bar Harbor, ME. B10 → B10.BR chimeras were made as described previously (4, 6) and used 8–12 wk after irradiation and bone marrow reconstitution.

Antibodies and Treatment of Mice. Culture supernatants containing monoclonal antibodies directed at the I-A^k determinant Ia 17 (10-2.16, IgG2b) (28) were passed over protein A-Sepharose columns (Pharmacia Fine Chemicals, Piscataway, NJ) and the adsorbed antibodies were eluted with 2 M potassium thiocyanate (Sigma Chemical Co., St. Louis, MO). These protein A-purified anti-I-A^k Ab were injected intraperitoneally into newborn mice according to a previously described protocol (27). Briefly, mice received three injections of 200 μg during their 1st wk of life, three injections of 400 μg each during the 2nd wk and three injections of 600 μg each during the 3rd. Control mice were untreated littermates or mice receiving the same treatment with inappropriate monoclonal Ab, i.e., C3H(H-2^k) mice treated with protein A-purified anti-I-A^b (25-9-17, IgG2a) (29) and C57BL/6(H-2^b) mice treated with anti-I-A^k (10-2.16).

Flow Microfluorometry (FMF). FMF analysis of the anti-Ia-treated mice was performed as described (30) using a fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Sunnyvale, CA). Reagents used were monoclonal Ab (culture supernatants) to I-A^k (10-2.16) (28), I-A^b (25-9.17) (29), H-2K^k (11-4.1) (31) and H-2D^k (15-1-5) (31), in combination with fluorescein-conjugated, Fc fragment-specific, F(ab')₂-goat anti-mouse IgG (FITC-F(ab')₂-goat anti-mouse IgG-Fc) (Cappel Laboratories, Cochranville, PA). Preparation of cells and staining procedures were as described (30). Briefly, 1 × 10⁶ thymus or spleen cells were incubated at 4°C for 45 min with anti-I-A or anti-H-2K/D reagents; they were then washed twice by centrifugation, incubated at 4°C for 45 min with saturating amounts of FITC-F(ab')₂-goat anti-mouse IgG-Fc, washed twice again, resuspended and analyzed for fluorescence. Lyt-marker analysis was performed with directly fluoresceinated nonallele-specific rat anti-mouse Lyt-1 and Lyt-2 monoclonal Ab (Becton, Dickinson & Co., Sunnyvale, CA). All procedures were performed in Hanks' balanced salt solution containing 0.1% bovine serum albumin and 0.1% NaN₃. Briefly, data were collected on 5 × 10⁴ viable cells, as determined by forward light scatter intensity, and analyzed for minimum numbers of positive cells by integration of immunofluorescence (IF) profiles to determine the percentage of positive cells above a selected level of IF intensity (30). Data are displayed as percentages of positive cells above the fluoresceinated reagent background or (when direct staining is used) above unstained background values.

Generation of CTL Responses. 4 × 10⁶ responder spleen cells or thymocytes were cultured with 2–4 × 10⁶ 1,500 rad irradiated splenic stimulator cells in 2 ml of Dulbecco's modified Eagle's medium, supplemented as described (16, 17). TNP modification was performed with 10 mM trinitrobenzene sulfonate according to Shearer et al. (13). After 5 d, specific lysis was determined in a 4-h ⁵¹Cr-release assay, using concanavalin A (Con A)-induced splenic blasts as target cells. Specific ⁵¹Cr-release = [(experimental – spontaneous release)/(maximum – spontaneous release)] × 100. Data shown represent the means of triplicate determinations using 5 × 10³ target cells (SD < 5%; maximum release range from 1,500 to 5,000 cpm; spontaneous release range from 6 to 19%) and are representative of at least four experiments. Lysis of unmodified target cells by TNP-specific CTL was <2% and therefore not indicated in the results.

Preparation of Con A Supernatants. In some experiments, Con A supernatants (Con A-SN) were

used as an exogenous source of T helper cell factors. Con A-SN were prepared from BALB/c mouse spleen cells as described (16) and tested for the presence of IL-2 activity and the absence of mitogenic activity after addition of 0.2 M α -methyl-D-mannoside as described (16). The Con A-SN were used at a 10–25% (vol/vol) concentration, whichever was optimal for the particular preparation.

Test for Accessory Cell Depletion and Function. Spleen cells were depleted of adherent cells by passage over G-10 Sephadex columns as previously reported (4), with the modification of incubating the spleen cells on the columns for 30 min at 37°C. This procedure markedly reduces the percentage of latex-ingesting cells (4) and, when applied to both responder and stimulator cell populations, completely abrogates CTL responses to TNP self and alloantigens (32). The CTL response of such G-10 passed spleen cells can be restored by a radiation-resistant, non-T, non-B, glass-adherent, Ia-positive, latex-ingesting cell population (32, 33) present in normal spleen.

Results

In Vitro Effects of Monoclonal Anti-I-A^k Ab on TNP-Specific CTL Responses. The aim of the present study was to investigate the effect of in vivo anti-I-A treatment on CTL responses. To illustrate that there indeed is a role for I-A-recognizing T cells in the generation of TNP-specific CTL-responses, the effect of monoclonal anti-I-A^k Ab on the in vitro generation of TNP-specific CTL responses was first studied. In Fig. 1A, it can be seen that CTL responses generated in vitro in the presence of monoclonal 10-2.16 (anti-I-A^k) Ab are inhibited in a specific fashion, i.e., H-2^k-bearing spleen cells are blocked but H-2^b-bearing spleen cells are not. In addition, this inhibition is due to inhibition of I-A^k recognition by self-Ia-specific responder T cells, as H-2^b

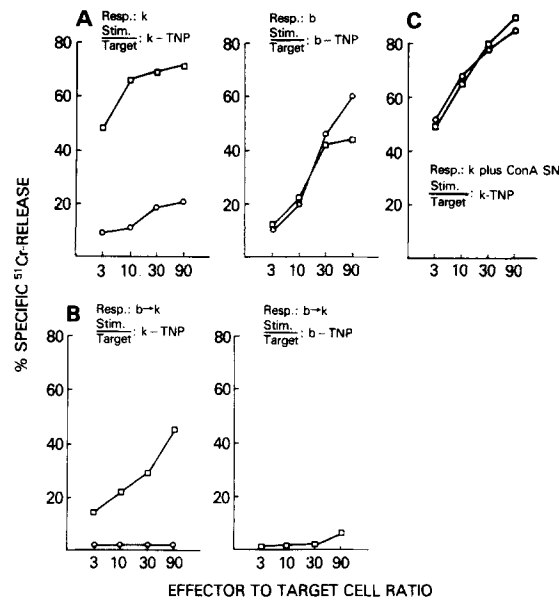


FIG. 1. In vitro inhibition of TNP-specific CTL responses by anti-I-A^k is dependent on recognition of I-A^k-encoded determinants by helper T cells. (A) Normal B10.BR (H-2^k) or B10 (H-2^b) spleen cells were cultured with TNP-self stimulators and no (□) or 5 μg/ml (○) anti-I-A^k antibody; (B) B10 → B10.BR (H-2^b → H-2^k) chimeric spleen cells were cultured with TNP-B10.BR or TNP-B10 stimulator cells and no (□) or 5 μg/ml (○) anti-I-A^k antibody; (C) normal B10.BR spleen cells were cultured with Con A-SN in the absence (□) or presence (○) of 5 μg/ml anti-I-A^k antibody and TNP-modified B10.BR stimulator cells.

spleen cells from H-2^k-restricted B10 → B10.BR allogeneic chimeras are also inhibited by anti-I-A^k when stimulated by TNP-modified H-2^k stimulators (Fig. 1B). Finally, Fig. 1C also shows that the inhibitory effect of anti-I-A Ab is reversed when cultures are supplemented with Con A-SN, supporting the notion that anti-Ia Ab do not directly interfere with the activation of CTL but rather with the activation of Ia-specific T helper cells required for the generation of CTL. A more extensive description of the *in vitro* effects of anti-I-A^k Ab on CTL-responses will be given elsewhere.² Together with our previous studies (14), these experiments suggest that T cells restricted to recognition of self-I-A determinants are required for the generation of TNP-specific CTL responses.

Splenic CTL Responses Are Abrogated in Mice Treated from Birth with Anti-I-A Ab. In an attempt to evaluate the role of Ia antigens in the development of T cell function, the expression of Ia antigens was suppressed by chronic administration of anti-Ia Ab to neonatal mice. Previous studies (27) have shown that the number of surface Ia⁺ cells in the spleens of such mice is reduced >95% as determined by FMF analysis. There was also a concomitant reduction of 80–95% in the number of surface IgM⁺ and IgD⁺ cells; the number of Thy-1⁺ cells was unaffected (27).

In the present report, the possibility was tested that the development of T cell function might also be affected by anti-Ia treatment. First, splenic CTL function was determined in anti-Ia-treated mice that had been shown by FMF analysis to exhibit the previously described suppression of splenic surface Ia⁺ cells (27). Fig. 2 shows that CTL responses to both TNP and alloantigens are significantly decreased in H-2^k mice treated with anti-I-A^k Ab. In contrast, H-2^b mice treated with anti-I-A^k exhibit normal CTL responses, indicating that the antibody needs to react with I-A antigens in the host for suppression to occur. H-2^k mice treated with protein A-purified anti-

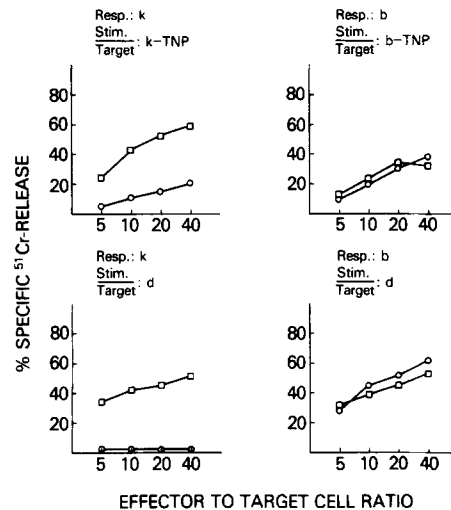


FIG. 2. In anti-I-A-treated mice (○) splenic CTL responses are abrogated in a haplotype-specific fashion. C3H/HeN (H-2^k) or C57BL/6 (H-2^b) mice were treated from birth on with protein A purified anti-I-A^k (10-2.16) Ab, and their splenic CTL responses to either TNP-self or allogeneic BALB/c (H-2^d) stimulators were determined at 3 wk of age. Data are representative of 11 separate experiments. CTL responses to TNP-self were TNP-specific, in that no lysis of unmodified target cells was observed (data not shown). Total spleen cell numbers and Thy-1⁺ cell numbers are equal in anti-Ia (○) and untreated mice (□) (27).

Ia^b monoclonal Ab also exhibit normal responses (data not shown); in further experiments, control responses were generated with spleen cells from normal untreated littermates. Thus, anti-Ia-treatment has a profound effect on the expression of alloreactive and TNP-specific CTL function. The nature of the observed decrease was next investigated.

Failure to Generate CTL in Anti-I-A-treated Mice Is Not Due to Demonstrable Suppression, to Carry-over of Blocking Antibodies, or Solely to the Absence of a Functional Accessory Cell. Several mechanisms may be proposed for the abrogation of CTL function expression in anti-I-A-treated mice. First, the induction of a suppressor cell might account for the observed decrease. Second, carry-over of anti-I-A Ab into the cultures might be responsible for the observed decrease. In either case, it might be anticipated that spleen cells from anti-I-A-treated mice, when mixed with spleen cells from normal mice, would suppress responses of the normal spleen cells. The mixing experiment shown in Fig. 3 gives no indication for such a suppressive effect of spleen cells from anti-I-A-treated mice. A mixture of equal numbers of normal and anti-I-A-treated spleen cells generated alloreactive and TNP-self-reactive CTL responses comparable to those obtained with normal spleen cells alone (Fig. 3). Thus, although failure to demonstrate suppression does not provide conclusive evidence against it, these experiments make induction of suppressor cells or carry-over of anti-I-A Ab a less likely explanation for the observed abrogation of CTL responses in anti-I-A-treated mice.

We next investigated whether a lack of functional responder accessory cells might account for the observed defect. Anti-I-A treatment can be expected to not only reduce Ia⁺ B cells, but to also affect other Ia⁺ cells, such as accessory cells, required for the generation of CTL responses (32, 33). Supplementation of spleen cells from anti-I-A-treated mice with a source of normal accessory cells should then reconstitute the defect. The results shown in Fig. 4 indicate that, even when irradiated normal spleen cells are added to responder cells from anti-I-A-treated mice, the latter still are incapable of generating a normal CTL response to either allogeneic or TNP-modified stimulator cells. It might be noted that irradiated normal spleen cells are functionally competent accessory cells for reconstituting the CTL responses of spleen cells depleted of accessory cells by G10-passage (see Fig. 6). Thus, lack of a responder accessory cell in spleen cells from anti-I-A-treated mice is not sufficient to account for the observed defect.

The Decreased Splenic CTL Responses of Anti-I-A-treated Mice Is Not Due to a Defect in

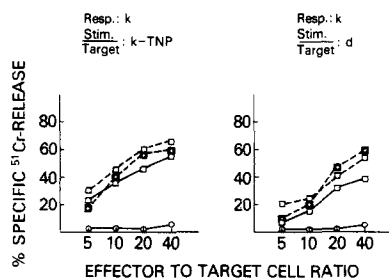


FIG. 3. The reduction in CTL responses from anti-I-A-treated mice is not mediated by suppression. Spleen cells from untreated H-2^k mice (4×10^6 /culture) (—□—) were mixed with equal numbers of either normal syngeneic spleen cells (—□—) or syngeneic spleen cells from anti-I-A^k-treated (4×10^6 /culture) mice (■), and stimulated with TNP-modified H-2^k stimulator cells (left) or allogeneic H-2^d stimulator cells (right). (○), anti-I-A-treated responders (4×10^6 /culture).

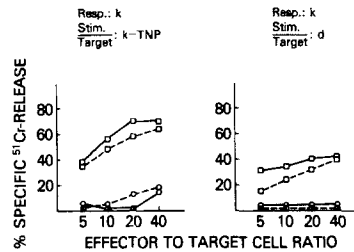


FIG. 4. The decrease in CTL response from anti-I-A-treated mice is not due to the absence of a responder accessory cell. Spleen cells from either normal (—□—) or anti-I-A^k-treated (---○---) H-2^k mice were supplemented with 2×10^6 2,000 rad-irradiated normal spleen cells (—□—, —○—, respectively) that can provide accessory cell function for accessory cell-depleted reduced CTL responses (see Fig. 6), and tested for their ability to generate TNP-self (*left*) or anti-H-2^d allogeneic CTL responses (*right*).

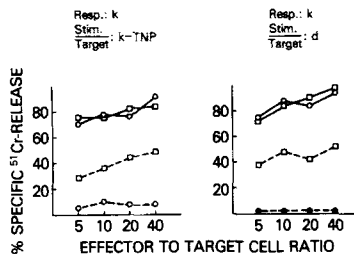


FIG. 5. The decrease of CTL responses from anti-I-A-treated mice can be restored by addition of Con A-SN. Spleen cells from either normal (□) or anti-I-A^k (○) treated H-2^k mice were cultured in the presence (—) or absence (---) of Con A-SN and stimulated with TNP-modified H-2^k (*left*) or allogeneic H-2^d (*right*) stimulator cells.

CTL Competence, But to a Defect in I-A-recognizing T Cell Function. Generation of CTL responses is dependent on activation of both CTL precursor cells and T helper cells (7-10, 28). Consequently, it was necessary to investigate whether the anti-I-A treatment affected CTL responses directly by reducing the CTL precursors or indirectly by affecting T helper cell function, or both. CTL responses can be made independent of endogenous T helper cell activation by addition of a source of T helper cell factors (34) such as those contained in Con A-SN. It can be seen in Fig. 5 that Con A-SN completely restored the anti-TNP and anti-allo-CTL responses of spleen cells from anti-I-A-treated mice. The response of normal spleen cells was also enhanced by Con A-SN (Fig. 5), but normal and anti-I-A-treated spleen cells reached the same elevated level of CTL responsiveness in the presence of Con A-SN. The target cell specificity of the response obtained in the presence of Con A-SN was the same as for cultures without Con A-SN (data not shown). Thus, these data suggest that the CTL precursors themselves are unaffected by treatment of mice with anti-I-A antibodies; rather it is likely that it is T cells that normally are responsible for production of T helper factors, such as provided by Con A-SN, that are defective in these mice.

Defective T helper cell function could be due to either a lack of T helper cells or to functional incompetence of the T helper cells that are present. Results from an analysis of the Lyt-phenotype distribution of spleen cells from anti-I-A-treated mice support the latter possibility, as there is no significant difference between the number of Lyt-1⁺2⁻ cells in spleen cells from normal and from anti-I-A-treated mice: both contain ~6% Lyt-1⁺2⁻ cells, as determined with monoclonal rat anti-mouse Lyt-1 and

Lyt-2 typing reagents (data not shown). The relative number of Lyt-positive cells is very low in such young mice (<3 wk of age), but the data nonetheless allow the conclusion that cells with the Lyt phenotype of helper T cells are present to the same extent in the spleens of anti-I-A-treated mice. Thus, it is more likely that the defect in T helper cell function in anti-I-A-treated mice is not due to absence of T helper cells, but rather a consequence of a defect in the ability of T helper cells to attain competence.

Effect of In Vivo Anti-I-A^k Treatment on Splenic Accessory Cell Function. A defect in T helper cell function, as suggested above, could be due to either a direct effect of anti-I-A treatment on T helper cell precursors, or reflect an effect on another cell type that is necessary for the generation of T helper cell function. A direct effect on T cells seems unlikely, as 10-2.16 antibodies do not react with T cells (28). The most likely other cell type to be affected by anti-I-A treatment is the Ia-bearing accessory cell (4, 6, 33) involved in presenting antigens to T helper cells. We have already excluded the possibility that the defect in CTL responses of anti-I-A-treated mice is solely due to defective accessory cell function in the responder cell population, but these experiments were performed under conditions in which the stimulator cells contained normal accessory cells. Thus, the possibility remained that accessory cell function as well as T helper cell function were affected by the in vivo anti-I-A treatment. Indeed, if Ia-bearing accessory cells are involved in the education of T cells recognizing self-Ia (35), a defect in accessory cell function might conceivably be the reason defective development of the Ia-specific T cell repertoire occurs in these mice. We therefore evaluated accessory cell function in the spleens of anti-I-A-treated mice by testing their ability to reconstitute reduced CTL responses from responder and stimulator spleen cells that had been depleted of accessory cells by G10 passage (32, 33). The data shown in Fig. 6 indicate that G10 passage leads to an almost complete abrogation of splenic TNP-specific CTL responses that can be reconstituted by addition of irradiated normal spleen cells. However, spleen cells from anti-I-A-treated mice did not reconstitute the reduced response of these G10-passed cells. Previous data (32, 33) indicated that the reconstituting ability of spleen cells lies in a non-T, non-B, I-A/E⁺

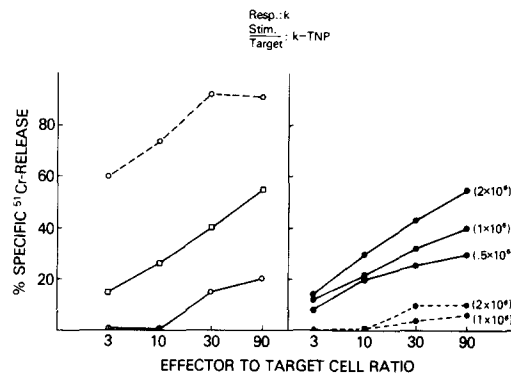


FIG. 6. Spleen cells from anti-I-A^k-treated H-2^k mice cannot function as accessory cells for CTL responses generated with macrophage-depleted spleen cells. Responder and stimulator spleen cells from normal H-2^k mice (□) were G10-passed (—○—) and their defective TNP-reactive CTL responses were reconstituted with irradiated spleen cells from either normal (—●—) or anti-I-A^k-treated (—○—) mice or with Con A-SN (—○—). Numbers in parentheses indicate numbers of spleen cells added.

radiation-resistant, phagocytic, adherent cell. Thus, the present data demonstrate that anti-I-A-treated mice lack functional accessory cells, in addition to lacking Ia-specific T cells.

Effect of In Vivo Anti-I-A^k Treatment on Splenic CTL Function in (H-2^b × H-2^k)F₁ Mice. If T helper cell function in anti-I-A^k-treated mice is defective because developing T helper cells have not "learned" to recognize I-A^k, the question arises whether the I-A^k antigen expression is simply blocked by anti-I-A^k antibodies or whether those cells expressing I-A^k and responsible for "teaching" are absent from the environment. To address this question, (H-2^b × H-2^k)F₁ mice were treated with anti-I-A^k and their splenic CTL responses to H-2^b-TNP, H-2^k-TNP, and H-2^d-allogeneic stimulator cells were determined. If the anti-I-A^k treatment had only blocked I-A^k antigen expression in vivo, it would be expected that in (H-2^b × H-2^k)F₁ mice, I-A^b-specific T helper cells would be unaffected by treatment with anti-I-A^k antibodies. However, it can be seen in Fig. 7 that both k-TNP, b-TNP and allogeneic responses were all abrogated by anti-I-A^k treatment, suggesting that the anti-I-A^k treatment has actually removed or functionally inactivated the cells that educate both I-A^b- and I-A^k-reactive T cells. Nonetheless, this result could also be explained by steric hindrance if the distribution of I-A^b determinants on F₁ cells is such that binding by antibody to I-A^k is sufficient to block recognition of I-A^b determinants. However, because in vitro blocking of I-A^k recognition sites on F₁ cells leaves the other parental I-A determinants undisturbed (6),² it is more likely that the in vivo anti-I-A^k treatment has either removed or inactivated the F₁ cells involved in educating T cells to recognize both I-A^k and I-A^b MHC determinants as self.

The defects exhibited by in vivo anti-I-A^k-treated F₁ mice are also in other ways comparable to those observed in parental H-2K^k mice, i.e., the defective CTL responses were restored by addition of Con A-SN (Fig. 7), and mixing equal numbers of untreated and anti-I-A-treated spleen cells did not lead to suppression of the response of normal spleen cells (data not shown).

Effect of In Vivo Anti-I-A Treatment on I-A Antigen Expression in the Thymus. The above data show that the defect in T helper cell function in anti-I-A-treated mice coincides with a defect in Ia-bearing accessory cell function. It is tempting to speculate that defective T helper cell function is a secondary consequence of defective accessory cell function. If the absence of Ia-positive accessory cells has interfered with the development of Ia-restricted T helper cell function, the thymus would be the most likely site for that interference to have occurred, as the thymus is one of the sites where the

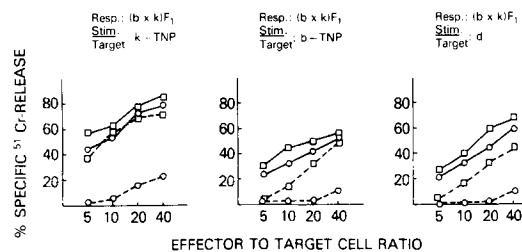


FIG. 7. Spleen cells from (H-2^b × H-2^k)F₁ mice treated with anti-I-A^k express decreased TNP-specific and alloreactive CTL responses that can be reconstituted with Con A-SN. Spleen cells from either normal (□) or anti-I-A^k-treated (○) (H-2^b × H-2^k)F₁ mice were cultured with (—) or without (- -) Con A-SN and with either TNP-modified cells of each parental haplotype or allogeneic (H-2^d) stimulator cells.

TABLE I
In Vivo Anti-I-A^k Treatment of Neonatal H-2^k Mice Leads to a Decrease in Expression of I-A Antigens in Both the Thymus and the Spleen

Cells	Percent of cells positive with reagents specific for:	
	I-A ^{k*}	K ^k /D ^{k‡}
Thymocytes from control mice [§]	20.9 ± 2.4	29.4 ± 2.0
Thymocytes from anti-I-A-treated mice	6.9 ± 2.3	24.2 ± 2.9
Spleen cells from control mice	23.9 ± 2.6	66.1 ± 4.0
Spleen cells from anti-I-A-treated mice	3.1 ± 1.7	68.0 ± 4.3

Data are expressed as means ± SE of 11 separate experiments. Values obtained with fluorescent reagent only have already been subtracted.

* Monoclonal anti-I-A^k antibody (clone 10-2.16).

‡ Monoclonal anti-H-2K^k and anti-H-2D^k antibodies (mixture of clones 11-4-1 and 15-1-5).

§ Control and anti-I-A-treated mice were 3 wk old.

TABLE II
In (H-2^b × H-2^k)F₁ Mice, In Vivo Treatment with Anti-I-A^k of Neonates Leads to a Decrease in Expression of Both I-A^k and I-A^b in the Thymus and the Spleen

Cells	Percent of cells positive with reagents specific for:	
	I-A ^{k*}	I-A ^{b‡}
Thymocytes from control mice [§]	19.1 ± 1.9	18.8 ± 1.5
Thymocytes from anti-I-A-treated mice	6.8 ± 1.7	7.8 ± 1.5
Spleen cells from control mice	27.1 ± 3.4	23.3 ± 3.1
Spleen cells from anti-I-A-treated mice	2.6 ± 1.5	1.2 ± 1.3

Data are expressed as means ± SE of four separate experiments. Values obtained with fluorescent reagent only have already been subtracted.

* Monoclonal anti-I-A^k antibody (clone 10-2.16).

‡ Monoclonal anti-I-A^b antibody (clone 25-9-17).

§ Control and anti-I-A-treated mice were 3 wk old.

MHC specificity of T cells is determined (12, 15, 18). It was therefore of interest to test whether the anti-I-A treatment resulted in changes in MHC-antigen expression of thymocytes. It should be noted that the Ia determinants that thymocytes express are predominantly those that they have acquired passively from Ia-positive cells present in the thymus microenvironment (30). Table I shows that thymocytes from anti-I-A^k-treated H-2^k mice exhibit a marked decrease in I-A^k antigen expression, while the expression of H-2K^k and H-2D^k antigens is not significantly affected. To address the possibility that the decrease in number of I-A^k-positive thymocytes might be due to blocking of Ia^k determinants on thymocytes by the in vivo administered anti-Ia^k-antibodies, thymocytes and spleen cells from anti-I-A^k-treated (H-2^b × H-2^k)F₁ mice were also assayed for their I-A antigen expression. It can be seen in Table II that in these F₁ mice both I-A^k and I-A^b antigen expression were comparably reduced, directly demonstrating that the in vivo anti-Ia^k treatment has removed, rather than blocked, Ia-bearing cells from the thymus and spleen of these mice. Thus, the absence of competent Ia-specific T cells in in vivo anti-Ia-treated mice correlated with the absence of Ia⁺ cells in their spleens and with diminished expression of Ia antigens in their thymi.

TABLE III
Decrease in Splenic CTL Responses in Anti-I-A^k-Treated H-2^k Mice Coincides with Reduced I-A^k Antigen Expression in the Thymus and Not with Suppressed Splenic I-A^k Antigen Expression

Responder spleen cells from:	Percent of I-A ^k -positive cells in:*		Percent specific ⁵¹ Cr-release at effector target ratio of 20:1 [‡]	
	Thymus	Spleen	k-TNP [§]	d [¶]
<i>Experiment 1[†]</i>				
Control mice	23.4	30.0	44.2	45.4
Anti-I-A ^k -treated mice	17.8	0.1	40.3	51.1
<i>Experiment 2^{**}</i>				
Control mice	24.0	24.4	49.7	51.6
Anti-I-A ^k -treated mice	6.3	1.5	6.2	10.0

* Percent of cells staining with monoclonal anti-I-A^k (10-2.16); values obtained with fluorescent reagent only have already been subtracted.

[‡] Means of triplicate determinations.

[§] Stimulator and target cells: TNP-modified C3H spleen (H-2^k).

[¶] Stimulator and target cells: BALB/c (H-2^d).

[†] Individual experiment with hardly any thymic I-A^k reduction.

^{**} Individual experiment with usual thymic I-A^k reduction.

To determine whether the absence of competent Ia-specific T cells in these experimental mice correlated with diminished Ia expression in the thymus or with diminished Ia expression in the spleen, it would be necessary to assess T cell function in anti-Ia-treated animals whose peripheral and thymic Ia antigen expression were differentially suppressed. In the course of the present studies, one group of experimental animals existed whose thymic I-A antigen expression was not markedly diminished, even though their peripheral I-A antigen expression was profoundly suppressed. It can be seen in Table III, experiment 1, that in this group of experimental mice, T cell function was normal. Thus, the defective development of Ia-specific T cells in this experimental system coincides with suppression of intrathymic I-A expression (as in Table III, experiment 2) and not with suppression of peripheral Ia expression.

Discussion

It is assumed that H-2-restricted T cell specificities are the result of confrontation with the appropriate H-2 determinants during early T cell differentiation (7-9, 11, 12, 14-18, 35). However, little information is available concerning the mechanisms by which precursor T cells are affected by interaction with H-2 determinants or the characteristics of the cell types presenting H-2 determinants to T cells. In the present study, we approached these questions by attempting to modify the presentation of I-A determinants during early T cell development.

To this end, monoclonal Ab to I-A^k were injected into neonatal mice for a period of 3 wk. Previous studies indicated (27) that such treatment abrogates the development of surface Ia⁺ B cells. We speculated that development of other, nonlymphoid Ia⁺ cell types, such as those involved in T cell education (7, 12, 35) might also be affected by anti-I-A treatment. It could be anticipated that such an alteration of the environment would have a profound effect on the specificity of the developing MHC-specific repertoire of T cells. To test this possibility, allo- and TNP-self-specific CTL responses in such anti-I-A-treated mice were analyzed at the end of the in vivo treatment

period. It was observed that splenic CTL responses were almost completely abrogated by *in vivo* treatment with monoclonal anti-I-A^k Ab, and that such abrogation required Ia molecules expressed by the host which the Ab could recognize. Development of suppressor cells, carry-over of blocking Ab, lack of responder accessory cells, or defective CTL function were not responsible for the observed defect. The defect was due to the absence of competent Ia-specific T helper cells. In addition, anti-I-A-treated mice exhibit reduced I-A antigen expression in the thymus and defective Ia-bearing accessory cell function in the spleen. It was found that the absence of competent Ia-specific T helper cells correlated better with reduced I-A antigen expression in the thymus than it did with reduced I-A antigen expression in the periphery. The most straightforward interpretation of these data is that, for development of Ia-specific helper T cells to occur, precursor T cells need to interact with Ia-antigens in the thymus, and treatment with anti-Ia Ab has interfered with this development. Finally, the mechanism of this interference by anti-I-A Ab was shown to be due to the actual removal or functional inactivation of those Ia-positive elements responsible for the education of I-A-recognizing T cells.

One implication of these findings is that education of at least most self K/D region-specific T cells (i.e., TNP-specific CTL precursor cells) (11, 14) occurs through interaction with different elements than the education of self-I region-specific T cells. Clearly, the anti-I-A treatment has not affected the repertoire of most K/D-recognizing T cells, as normal CTL responses were obtained when the need for T helper cell activation was bypassed by culturing responder cells in the presence of Con A-SN. Since anti-I-A treatment has removed I-A⁺ cells (either identical to accessory cells (35) or unique "teaching" cells) that are involved in T helper cell education, then precursor CTL self-K/D-recognition specificities must have arisen at least in part from interaction with other cell types. Our recent observation (11, 14) that self-K/D-recognizing T cells differentiate both intra- and extrathymically, while self-I region-specific T cells seem to differentiate exclusively intrathymically (14, 18) is consistent with this interpretation.

The present studies also showed that anti-I-A treatment abrogated alloantigen-specific CTL responses, and that Con A-SN could restore the defective response. The implication of this finding is that in the defective alloreactive CTL response the cause of the defect also lies at the helper T cell level, not at the precursor CTL level. It then needs to be explained how a defect in recognizing self-I-A antigens results in a defect in alloreactive T helper cell activation. There are several ways in which this finding can be explained. First, allo-Ia-specific T helper cells might in fact all be specific for self-I-A plus conventional antigen, so that they require intrathymic Ia education, which has been disturbed in these mice. Reports that several self-I-A plus antigen-specific T cell clones and hybridomas (36-38) were cross-reactive with allogeneic I-A determinants support this notion. Second, the generation of allo-K/D region-specific CTL responses might require presentation of the allogeneic determinants in the context of self-Ia allogeneic determinants (39, 40), but self-Ia-restricted T helper cells are incompetent in these mice. Third, those intrathymic elements which are absent in these mice might not only educate T cells to recognize self-I-A, but might, in addition, be responsible for the differentiation into functional competence of all T helper cells, regardless of their MHC specificity. For example, if all T helper cells must have a receptor for IL-1, it is conceivable that intrathymic Ia-positive elements function to

induce the expression of IL-1 receptors on developing T helper cells. Whatever the precise explanation for the failure of alloreactive T helper cells to develop in anti-I-A-treated mice, it is likely to result from the same mechanism that results in the failure of self-Ia-specific T helper cells to develop functional competence in this model.

The current studies have used treatment with a monoclonal anti-I-A^k reagent in vivo to study the functional role of I-A antigen expression during the development of the T cell repertoire. The anti-I-A treatment inhibited an I region-restricted T helper cell function, while leaving K/D region-restricted CTL function intact. Moreover, this treatment reduced expression of I region determinants intrathymically while leaving expression of K/D region products in the thymus intact. Because the thymus is one of the sites where the specificity of the T cell repertoire is being determined, these findings suggest that confrontation with I region-encoded products during their development is essential for T cells to establish their I region-restricted repertoire. Moreover, expression of K/D region-encoded products must be on different elements than that of I region products, as CTL precursors were unaffected by anti-I-A treatment. Thus, development of most of the K/D region-specific T cell repertoire must occur on Ia-negative education elements.

Summary

Monoclonal antibodies to I-A^k were injected into neonatal H-2^k mice for a period of 3 wk. The spleens of such mice are devoid of Ia-positive cells. Allo- and trinitrophenyl (TNP)-self-specific cytotoxic T lymphocyte (CTL) responses in such anti-I-A-treated mice were almost completely abrogated at the end of the 2-3 wk in vivo treatment period. Development of suppressor cells, carry-over of blocking antibodies, lack of responder accessory cells, or defective CTL function were not responsible for the observed defect. As concanavalin A supernatant could restore the defect, it is more likely that the defect is due to the absence of competent Ia-specific T helper cells. In addition, anti-I-A-treated mice exhibit reduced I-A antigen expression in the thymus and defective Ia-bearing accessory cell function in the spleen. It is postulated that, for development of Ia-specific T cells to occur, precursor T cells need to interact with Ia-encoded products in the thymus, and anti-Ia treatment interferes with this process. Finally, the mechanism of this interference was shown to be due to actual removal or functional inactivation of those I-A-positive elements responsible for the education of I-A-recognizing T cells, since in (H-2^b × H-2^k)F₁ mice, treatment with anti-I-A^k antibodies results in abrogation of CTL responses to TNP in association with both parental haplotypes, while in the thymus of these mice expression of both I-A^k and I-A^b was reduced.

We are grateful to Dr. David H. Sachs and Dr. Keiko Ozato for contributing hybridoma cell lines, and to Drs. Howard B. Dickler, Richard J. Hodes, Ronald H. Schwartz, and Ronald N. Germain for critical reading of the manuscript.

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