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Immunology

Induction of immunity and oral tolerance with polymorphic class II major histocompatibility complex allopeptides in the rat

MOHAMED H. SAYEGH*, SAMIA J. KHOURY†, WAYNE W. HANCOCK‡, HOWARD L. WEINER†, AND CHARLES B. CARPENTER*

*Laboratory of Immunogenetics and Transplantation, Renal Division, and †Center for Neurologic Diseases, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; and ‡Department of Immunology and Pathology, Monash Medical School, Prahran, Victoria 3181, Australia

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ABSTRACT **We studied the immunogenicity and tolerogenicity of class II major histocompatibility complex (MHC) allopeptides in the rat. Inbred LEW ($RT1^l$) rats, used as responders, were immunized in the foot pad with a mixture of eight synthetic class II MHC allopeptides emulsified in complete Freund's adjuvant. These sequences represent the full-length second domain of $RT1.B^u$ and $RT1.D^u$ (WF) β chains. *In vitro*, responder lymphocytes harvested from popliteal and inguinal lymph nodes of immunized animals exhibited significant proliferation to the MHC allopeptide mixture. In addition, these responder lymphocytes had significantly increased proliferation to allogeneic WF ($RT1^u$) stimulator cells, when compared to naive controls in the standard one-way mixed lymphocyte response. *In vivo*, peptide-immunized LEW animals were challenged in the ear 2 weeks after immunization with the allopeptide mixture, the individual allopeptide sequences, or allogeneic WF splenocytes. When compared to controls, these animals had significant delayed-type hypersensitivity responses to the allopeptide mixture, to the β-pleated sheet allopeptide sequences, and to allogeneic WF splenocytes but not to the α-helix allopeptide sequences, to syngeneic LEW splenocytes, or to third party allogeneic BN splenocytes. Oral administration of the allopeptide mixture to LEW responder rats daily for 5 days before immunization effected significant reduction of delayed-type hypersensitivity responses both to the allopeptide mixture and to allogeneic splenocytes. This reduction was antigen-specific, since there was no reduction of delayed-type hypersensitivity responses to mycobacterium tuberculosis. These data demonstrate that lymphocytes from animals immunized with polymorphic class II MHC allopeptides can recognize and proliferate to the same amino acid sequences on allogeneic cell surface MHC molecules. In addition, oral administration of these peptides down-regulates the systemic cell-mediated immune response in a specific fashion. Synthetic MHC allopeptides should allow the study of alloimmunity *in vivo*, including induction of immune tolerance.**

Recent work with synthetic peptides representing portions of the polymorphic regions of mouse and human class I and II major histocompatibility complex (MHC) molecules indicates that they can be bound to MHC molecules and elicit a T-cell response *in vitro* (1–5). There is no information on the induction of immunity or tolerance by administration of synthetic MHC peptides *in vivo*. The oral route of administration of antigens has been shown to induce immune hyporesponsiveness (6). In alloimmunity, we have recently reported (7) that oral administration of allogeneic splenocytes to inbred rats down-regulates the systemic cell-mediated immune response *in vitro* and *in vivo*. We now report on the immunogenicity and tolerogenicity of orally administered synthetic MHC allopeptides in the rat.

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MATERIALS AND METHODS

Animals. LEW, WF, and BN rats, 8–10 weeks old, were obtained from Harlan–Sprague–Dawley or were bred in our own animal facility.

Allopeptides. We selected the RT1.B β and RT1.D β domains of $RT1^u$ (WF) and synthesized four overlapping peptides of 20–25 amino acids (residues 1–25, 20–44, 39–64, and 68–92 for RT1.B and residues 1–25, 20–44, 39–64, and 60–84 for RT1.D) for each locus, by using published sequences of the class II β chain (8). Fig. 1 shows these polymorphic sequences aligned with those of the β chains of $RT1^l$ (LEW). Peptides that were used for *in vitro* proliferation assays were purified by HPLC yielding >95% purity as determined by amino acid analysis.

Proliferation Assays. Responder LEW rats were immunized subcutaneously in the foot pad with 100 μg of the mixture of the four $RT1.B^u$ and four $RT1.D^u$ peptides (each at 12.5 μg) in complete Freund's adjuvant (CFA). Popliteal and inguinal lymph nodes were harvested 1 week after immunization and mashed through 60-gauge sterile stainless steel sieves. The recovered cells were then washed twice and resuspended into RPMI 1640 medium (Microbiological Associates), containing 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 20 μM 2-mercaptoethanol, and 5 mM Hepes. T and B cells were separated by nylon adherence as described (9). Responder unseparated LEW lymphocytes (3×10^5 cells) were cultured in 96-well flat-bottom plates (Costar) with 10–50 μg of the mixture of $RT1.B^u$, $RT1.D^u$, or both, sets of allopeptides. In other experiments, 1×10^5 nylon-wool-adherant cells, used as antigen-presenting cells, were preincubated with 10–50 μg of the $RT1.B^u$, $RT1.D^u$, or both, sets of allopeptides for 30 min at 37°C. The cells were then washed twice to remove excess peptides before adding 2×10^5 nylon-wool-nonadherent responder T cells. Negative control wells were set up with culture medium only. LEW × WF one-way mixed lymphocyte responses (MLRs) were set up by using equal numbers of responder LEW and allogeneic WF stimulator lymphocytes (prepared as described for LEW lymphocytes and irradiated with 3000 rads; 1 rad = 0.01 Gy) per well. The plates were incubated at 37°C with 5% CO_2/95% air for 4 days before they were pulse-labeled for 6 h with [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq; NEN/DuPont) and harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA). Proliferation was assayed by [^{3}H]thymidine incorporation measured by a Beckman liquid scintillation

Abbreviations: MHC, major histocompatibility complex; MLR, mixed lymphocyte response; DTH, delayed-type hypersensitivity; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis.

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                10        20        30        40        50
RT1.Bβu  QRLRRDFLVQFKPYCYFTNGTQRIRNVIRYIYNREEYLRYDSDVGEYRAV

RT1.Bβl  ......................----------Q---------------Q

                60        70        80        90
         TELGRPSAEYFNKQ*Y*LERTRAELDTVCRHNYEKTEVPTSL

         --------------*-*--Q--------------------..

                10        20        30        40        50
RT1.Dβu  RDPTPRFLGYLKPECHFYNGTNRVRLLARLIYNREEYARFDSDVGEYRAV

RT1.Dβl  ........................------Y-------T--------F---

                60        70        80        90
         TELGRPSAEYRNKQKEPMERRRATVDTYCRHNYEIFDRFL

         ----------Y-----Y--QI------A-K-D-.......
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FIG. 1. Amino acid sequences of the synthetic MHC RT1.B^u and RT1.D^u peptides aligned with those of RT1^l. Dots denote unknown sequences, dashes denote identical sequences, and asterisks denote absent sequences.

counter. Experiments were set up in quadruplicate, and results are expressed as cpm (mean ± SEM) or relative response = (experimental cpm − background cpm)/(control cpm − background cpm).

Delayed-Type Hypersensitivity (DTH) Response. LEW rats, used as responders, were immunized subcutaneously in the foot pad with 100 μg of the mixture of four RT1.B^u (50 μg) and four RT1.D^u (50 μg) peptides (12.5 μg of each peptide) in CFA. These animals were challenged subcutaneously 2 weeks later in one ear with 10 μg of the peptide mixture and in the other ear with 1×10^7 freshly prepared and irradiated (3000 rads) splenocytes from WF (RT1^u), syngeneic LEW (RT1^l), or a third party, BN (RT1^n). The DTH responses were measured with micrometer caliper (Mitutoyo, Field Tool Supply, Chicago) by a blinded observer as the change in ear thickness before and 2 days after the challenge (inches × 10^{-2}; 1 inch = 2.54 cm). Experiments were performed using five animals in each study groups. *P* values were calculated using the Student *t* test.

RESULTS

Immunogenicity of Class II MHC Allopeptides Assessed *in Vitro*. To test the immunogenicity of the synthetic RT1.B and RT1.D allopeptides, lymphocytes harvested from responder LEW animals immunized with the mixture of eight allopeptides 1 week earlier were compared to naive controls for their ability to proliferate to the allopeptides in a standard 96-h proliferation assay. As shown in Fig. 2*A*, whereas naive lymphocytes had only minimal proliferation, immunized animals exhibited significant proliferation to the allopeptide mixture and, separately, to allopeptides of RT1.B (four peptides) and RT1.D (four peptides). In addition, when compared to naive controls, responder lymphocytes from immunized animals exhibited significantly increased proliferation to allogeneic WF stimulator cells in the standard one-way MLR (relative response = 2.65 ± 0.2; $n = 6$). To test whether syngeneic antigen-presenting cells can bind and present MHC allopeptides, nylon-wool-adherant LEW lymph node cells were preincubated with the entire allopeptide mixture or with the RT1.B or RT1.D allopeptides separately. After washing, responder T cells were added to the cultures. Fig. 2*B* show that T cells from immunized animals proliferate to syngeneic antigen-presenting cells that had been preincubated with the MHC allopeptides.

These data demonstrate that the synthetic class II MHC allopeptides are immunogenic *in vivo*, as assessed by lymphocyte proliferation *in vitro*. Furthermore, lymphocytes

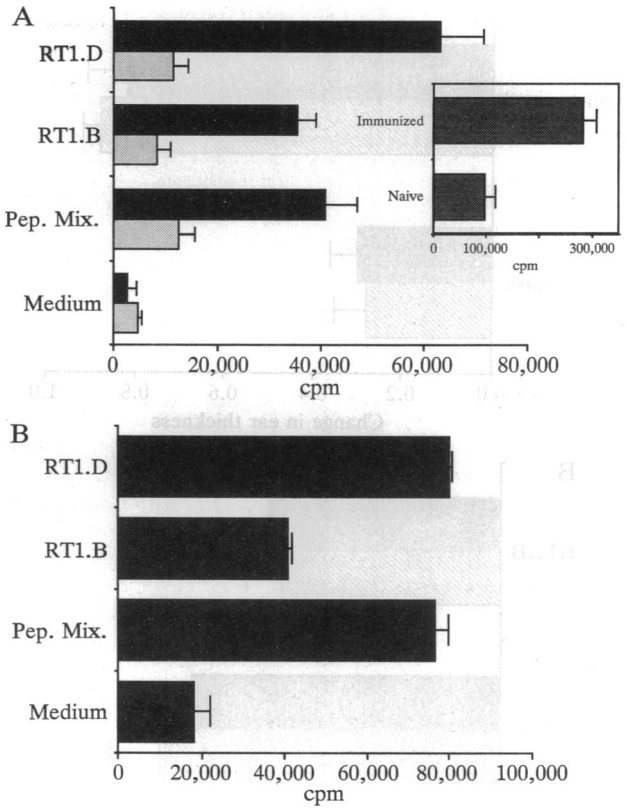

FIG. 2. (*A*) Direct proliferation of lymphocytes harvested from LEW rats, naive (shaded bars) and immunized with the entire allopeptide mixture (solid bars), that were incubated with the entire peptide mixture (bars Pep.Mix) or RT1.B or RT1.D allopeptides. Bars represent cpm (mean ± SEM) of a representative experiment performed in quadruplicate (five experiments). (*Inset*) Concomitant LEW × WF MLR for the experiment shown. The calculated relative response for the MLR was 2.65 ± 0.2 ($n = 6$). (*B*) Proliferation of nylon-wool-non-adherant mononuclear cells harvested from immunized (with the entire allopeptide mixture) LEW animals to the entire peptide mixture (Pep.Mix) or RT1.B or RT1.D allopeptides presented by syngeneic nylon-wool-adherant cells. Bars represent cpm (mean ± SEM) of a representative experiment performed in quadruplicate (four experiments).

from animals immunized with these allopeptides proliferate more vigorously to allogeneic cell surface MHC molecules.

Immunogenicity of Class II MHC Allopeptides Assessed by DTH *in Vivo*. LEW animals that were immunized with the entire allopeptide mixture and CFA had significant DTH responses both to the allopeptides and to freshly prepared allogeneic WF splenocytes (Fig. 3*A*). These DTH responses were not due to nonspecific immunization by CFA and were antigen-specific, since the immunized animals had no DTH responses to syngeneic LEW (change in ear thickness, 0.22 ± 0.07 × 10^{-2} inch vs. 0.67 ± 0.06 × 10^{-2} inch for the peptides; $P < 0.001$; $n = 5$ in each group) or allogeneic third party BN splenocytes (change in ear thickness, 0.12 ± 0.06 × 10^{-2} inch vs. 0.67 ± 0.06 × 10^{-2} inch for the peptides; $P < 0.001$; $n = 5$ in each group). In addition, immunization with RT1.B or RT1.D allopeptides separately resulted in significant DTH responses both to the respective allopeptide mixtures and to allogeneic WF splenocytes (Fig. 3*B*). These data further demonstrate that the synthetic class II MHC allopeptides are immunogenic *in vivo* and that lymphocytes from animals immunized with these allopeptides can respond to polymorphic amino acid sequences on, or derived from, allogeneic cell surface MHC molecules.

Tolerogenicity of Orally Administered Class II MHC Allopeptides. We have recently shown that oral administration

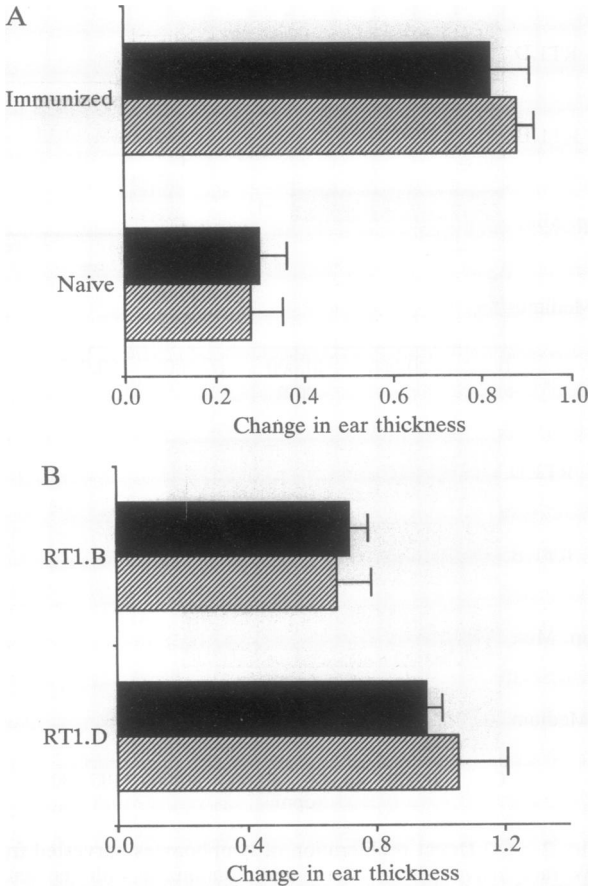

FIG. 3. (*A*) DTH responses of LEW rats, naive and immunized with the entire allopeptide mixture (five experiments), challenged with the peptide mixture (hatched bars) or WF splenocytes (solid bars). Bars represent the change in ear thickness in inches × 10^2 (mean ± SEM) of a representative experiment ($n = 5$ in each group). (*B*) DTH responses of RT1.B-immunized (four experiments) or RT1.D-immunized (five experiments) animals challenged with the respective allopeptides (hatched bars) or WF splenocytes (solid bars). Bars represent the change in ear thickness in inches × 10^2 (mean ± SEM) of a representative experiment ($n = 5$ animals in each group).

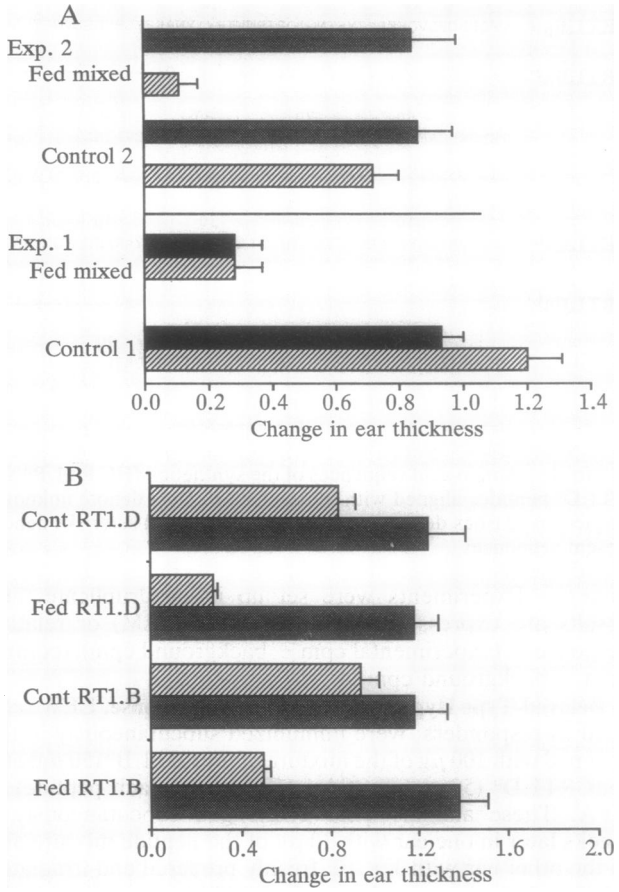

FIG. 4. (*A*) Reduction of DTH responses by oral administration of the entire allopeptide mixture. In experiment 1, animals were immunized with the entire allopeptide mixture and challenged with the peptide mixture (hatched bars) or WF splenocytes (solid bars). In experiment 2, animals were immunized with the entire allopeptide mixture and challenged with the peptide mixture (hatched bars) or mycobacterium tuberculosis (shaded bars). Bars represent the change in ear thickness in inches × 10^2 (mean ± SEM; $n = 5$ animals in each group) of control (Control 1 and 2) and peptide-fed (Fed mixed) animals. (*B*) Reduction of DTH responses by oral administration of RT1.B or RT1.D allopeptides. Animals were immunized with RT1.B or RT1.D and challenged with the respective allopeptides (hatched bars) or mycobacterium tuberculosis (shaded bars). Bars represent the change in ear thickness in inches × 10^2 (mean ± SEM; $n = 5$ animals in each group) of control (Cont RT1.B and Cont RT1.D) and peptide-fed (Fed RT1.B and Fed RT1.D) animals (two experiments).

of allogeneic splenocytes effected significant antigen-specific reduction (48% reduction; $P = 0.026$) of DTH responses in the rat (7). Since we now show that synthetic class II MHC allopeptides are immunogenic *in vivo*, we also studied their ability to induce immune hyporesponsiveness after oral administration. LEW responder animals were fed 100 μg of the entire allopeptide mixture (eight peptides, each at 12.5 μg) or 50 μg of RT1.B or RT1.D, by gavage daily for 5 days. Three days after the last feeding the animals were immunized with the allopeptide mixture and DTH responses were determined 2 weeks later. In Fig. 4*A*, experiment 1 shows that animals fed all eight peptides had significantly marked reduction of DTH responses to the same allopeptide mixture (77% reduction; $P = 0.001$) as well as to WF splenocytes (70% reduction; $P = 0.003$), when compared to unfed controls. This reduction was antigen-specific since there was no reduction of DTH responses to mycobacterium tuberculosis (the antigen present in CFA) (Fig. 4*A*, experiment 2). When either RT1.B or RT1.D allopeptides were fed separately (Fig. 4*B*), significant reduction of antigen-specific DTH responses was effected [RT1.B, 47% ($P = 0.001$); RT1.D, 67% ($P < 0.001$)]. In addition, oral administration of either allopeptide mixture resulted in significant reduction of DTH responses to allogeneic WF splenocytes (RT1.B, 42%; RT1.D, 48%; $P < 0.05$; $n = 5$ in each group; data not shown). These data indicate that oral administration of polymorphic class II MHC allopeptides down-regulates the systemic cell-mediated response to subsequent immunization and that this down-regulation is specific to the orally administered antigens. *In vitro*, cervical lymph node cells harvested 3 days after the last feeding from naive animals that received the oral allopeptide mixture had marked reduction of MLR proliferation to WF stimulator cells as compared to naive controls (73% reduction; $n = 3$; $P < 0.001$; data not shown).

Specificity of Immunogenicity and Tolerogenicity of Class II MHC Allopeptides. We asked the question whether, in addition to polymorphism, the native location of the allopeptide, β-pleat vs. α-helix, may be an important determinant of immunogenicity and tolerogenicity *in vivo*. To answer this question, we studied the immunogenicity and tolerogenicity of the individual allopeptide fragments. LEW rats, used as responders, were immunized subcutaneously in the foot pad with 12.5 μg of each of the four RT1.D (1–25, 20–44, 39–64, and 60–84) and four RT1.B (1–25, 20–44, 39–64, and 68–92)

allopeptide fragments and CFA. DTH responses were then determined for each of the peptide fragments. As seen in Fig. 5, only the first (1–25) and second (20–44) fragments, which correspond to the β-pleat of both RT1.B and RT1.D, were immunogenic. Oral administration of 25 μg of the immunogenic allopeptide sequences RT1.D1 plus RT1.D2 (12.5 μg each) but not RT1.D3 plus RT1.D4 resulted in significant reduction of DTH response to the RT1.D allopeptide mixture [75% reduction ($P = 0.005$) vs. 14% reduction (P not significant); $n = 5$ in each group]. These observations, in addition to showing that the native location of the allopeptide (β-pleat vs. α-helix) is an important determinant of immunogenicity and tolerogenicity, also provide negative peptide controls for the observed specificity of immunogenicity and tolerogenicity.

DISCUSSION

The availability of sequence data for the variable domains of MHC molecules (8) has made it possible to synthesize peptides representing various portions of the native cell surface molecules and to use these peptides for study of immunogenicity and tolerogenicity. Our data show that rat polymorphic class IIβ MHC allopeptides are immunogenic *in vivo* as assessed by lymphocyte proliferation *in vitro* and by DTH responses *in vivo*. Moreover, when administered orally, these MHC allopeptides are tolerogenic; they induce a state of immune hyporesponsiveness that is antigen-specific. Our data also show that, in addition to polymorphism, the native location of the allopeptide, β-pleat vs. α-helix, appears to be an important determinant of immunogenicity and tolerogenicity in this strain combination. These observations probably represent the ability of LEW antigen-presenting cells to bind the β-pleat allopeptide fragments. The α-helix allopeptides serve as negative controls. Although autologous sequences could also be used for these studies, Benichou *et al.* (1) in the mouse show that self tolerance may not develop to autologous β-pleat sequences. They screened five autologous class II mouse MHC peptides and showed that two β-pleat fragments can bind to self MHC molecules and are immunogenic. Moreover, neonatal tolerance could be induced after intraperitoneal injection of an immunogenic peptide. Similar *in vitro* immunogenicity data in humans have been presented by Liu and Sucia-Foca (10) using allopeptide fragments derived from the first domain of HLA-DRB1*0101; only a β-pleat fragment was immunogenic in the example studied.

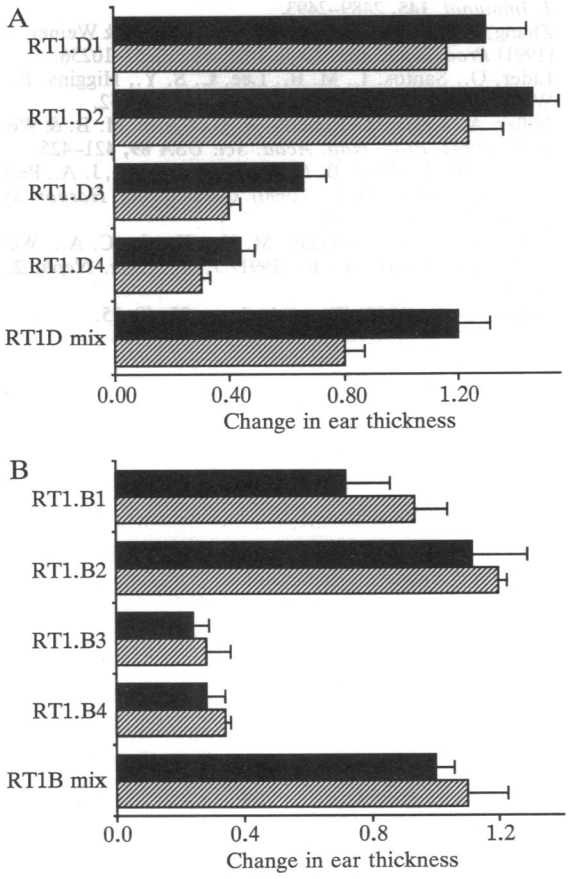

FIG. 5. DTH responses of animals immunized with the individual four fragments of RT1.B (*B*) and RT1.D (*A*) and challenged with the respective allopeptide ($n = 5$ in each group, two experiments). RT1.B1, -2, -3, and -4 correspond to the peptide sequences 1–25, 20–44, 39–64, 69–92, and RT1.D1, -2, -3, and -4 correspond to the peptide sequences 1–25, 20–44, 39–64, and 60–84, respectively, in Fig. 1. RT1.D mix and RT1.B mix refer to animals immunized with the mixture of the four allopeptides. Solid bars, cells; hatched bars, peptides. Bars represent the change in ear thickness in inches × 10^2 (mean ± SEM).

Recent work with mouse and human peptides, representing portions of the polymorphic regions of class I and II MHC molecules, indicate that exogenous allopeptides and self peptides are taken up by antigen-presenting cells *in vitro* and presented on MHC molecules, presumably by the endogenous process of pinocytosis, processing in the Golgi, and transport to the cell surface bound to an MHC molecule for recognition (1–4). Demonstration by Chen *et al.* (5) that a class I synthetic peptide can be presented on an intact class II molecule via the exogenous pathway shows that some T-cell clones recognize an alloantigen that has been processed and presented as peptides in a self-MHC binding site. Self-MHC or allo-MHC peptides may, therefore, be processed in a manner identical to any other peptide moiety, although recognition of intact MHC molecules that bind endogenous peptides may be a major route of immunization to cells or grafts (24). There are data to indicate that peptides presented on class I MHC molecules are nonomers (11) and those presented by class II molecules are 13–17 amino acids long (12). To our knowledge, there is no such data available for MHC allopeptides. Our data demonstrate that animals immunized with class II MHC allopeptides will recognize and respond to allogeneic cells *in vitro* and *in vivo*, indicating that a significant number of T-cell clones will recognize polymorphic amino acid sequences on intact cell surface MHC molecules. Alternatively, the targets could be peptides presented by allo- or self-MHC. The route of administration of MHC allopeptides and the qualitative and quantitative aspects of peptide processing and presentation could be determinants of the induction of immunity or tolerance to alloantigens.

Introduction of autoantigens into the intestinal tract will suppress the immune response in several experimental autoimmune models (6). The most extensively studied is the experimental autoimmune encephalomyelitis (EAE) (13–15). Other experimental models where oral administration of antigen results in immunologic unresponsiveness or "oral tolerance" include experimental autoimmune uveoretinitis (16), collagen-induced and adjuvant arthritis (17, 18), and diabetes in nonobese diabetic mice (19). The mechanisms mediating the tolerizing effects of oral administration of antigen have been studied in the EAE model where it is possible to adoptively transfer protection against EAE with $CD8^+$ cells from mesenteric lymph nodes and spleens of animals orally tolerized with myelin basic protein (20). More recently, Miller *et al.* (21) showed that these suppressor T cells suppress *in vitro* and *in vivo* immune responses by the release of transforming growth factor β1. Others have reported that clonal anergy may also play a role in oral

tolerance for myelin basic protein in EAE (15). There is initial evidence in EAE that synthetic peptides can induce tolerance after oral administration (13). In the mouse, intravenous cI-(12–26) peptide (amino acids 12–26 of λ repressor protein) produces long-term tolerance that does not function by a suppressor mechanism and is presumably mediated by T-cell anergy (22). In the alloimmune system, we have shown that oral administration of allogeneic splenocytes to inbred rats down-regulates the cell-mediated immune response to histocompatibility antigens and prevents sensitization by skin allografts (7). We now have data demonstrating that oral administration of allogeneic splenocytes is associated with selective inhibition of responder type 1 T helper-like cell function and that this inhibition may be mediated by inhibitory cytokines secreted by $CD4^+$ type 2 T helper-like cells (23). Our current experiments demonstrate that oral administration of class II MHC allopeptides to inbred rats induces a state of specific immunologic hyporesponsiveness; either RT1.D or RT1.B β-chain peptides produce comparable reduction of DTH response to whole spleen cells that bear both sets of incompatibilities and α-chain, RT1.H class II, and RT1.A class I differences. It seems, therefore, that induction of negative regulatory pathways may play a major role in this form of tolerance.

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