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Short Communication

Differential Effects of Oral Versus Intrathymic Administration of Polymorphic Major Histocompatibility Complex Class II Peptides on Mononuclear and Endothelial Cell Activation and Cytokine Expression during a Delayed-Type Hypersensitivity Response

Wayne W. Hancock,* Samia J. Khoury,† Charles B. Carpenter,‡ and Mohamed H. Sayegh‡

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

Oral and intrathymic exposure to antigen can each induce systemic antigen-specific immune tolerance, but the mechanisms have not been well defined. We studied the effects that the route of exposure to antigen has on the mechanisms of tolerance* in vivo *using synthetic polymorphic class II major histocompatibility complex (MHC) peptides in a skin delayed-type hypersensitivity (DTH) response model. Lewis rats were immunized by injection in the footpad with synthetic peptides (RT1.B^u and/or RT1.D^u) derived from the hypervariable domain of MHC class II β chain of the Wistar-Furth rat in complete Freund's adjuvant and challenged 2 weeks later by injection in the ear with the MHC peptides. An "oral" group received the peptide mixture by gavage (100 μg/day for 5 days) 3 days before immunization, and an "intrathymic" group received a single intrathymic injection of 100 μg of peptides 48 hours before immunization. Oral therapy reduced the DTH response to 23 ± 7%, and intrathymic exposure reduced the DTH response to 26 ± 6% ($P < 0.001$) as compared with control DTH responses of unmodified Lewis animals. Immunohistological evaluation of DTH skin lesions showed that oral and intrathymic therapy each decreased mononuclear cell infiltration, fibrin deposition, and endothelial activation when compared with that seen in control rats. In contrast, while both protocols markedly reduced interleukin (IL-2) and interferon-γ expression, they had differing effects on local expression of IL-4, transforming growth factor-β, IL-2R, and CD45RC (a possible discriminant between Th1 and Th2 cells in rats). Oral therapy was associated with increased expression of IL-4 and preservation of transforming growth factor-β expression by residual IL-2R$^+$, CD45RC$^-$ mononuclear and endothelial cells, whereas thymic exposure suppressed essentially all features of immune activation including IL-2R induction and cytokine expression. Our data a) document the detailed pattern of cytokine expression and mononuclear and endothelial cell activation markers during DTH responses and b) confirm that oral tolerance is associated with im-

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Address reprint requests to Dr. W. W. Hancock, Department of Pathology and Immunology, Monash Medical School, Alfred Hospital, Commercial Road, Prahran, Victoria 3181, Australia.

mune deviation to a predominance of Th2 cell function, whereas intrathymic tolerance may be mediated by T-cell anergy or clonal deletion. (Am J Pathol 1994, 144:1149–1158)

Delayed-type hypersensitivity (DTH) is a form of cell-mediated immunity important to the defense against intracellular bacteria and other microbial agents. In DTH, antigen-activated $CD4^+$ T cells are postulated to secrete cytokines that recruit and activate monocytes from the blood to the site of antigen challenge, leading to antigen elimination that results from three sequential processes.[1] First, $CD4^+$ T cells recognize peptide sequences of an antigen after its internalization, processing, and expression on the membrane, in conjunction with self class II major histocompatibility complex (MHC), by antigen-presenting cells. Next, upon activation, a subset of $CD4^+$ T cells termed "Th1 cells" are believed to synthesize and secrete cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ), allowing proliferation of T cells and activation of macrophages and endothelial and other cell types, respectively. Finally, an inflammatory response occurs during which cytokine-activated endothelial cells express adhesion molecules, monocytes are recruited and locally activated, and these monocytes clear the foreign antigen. Many details of this model of DTH remain uncertain, including which of the several candidate antigen-presenting cells of the skin initiate DTH, where this interaction occurs, the precise nature and extent of cytokine production required for an effective DTH, and the basis for different morphological features of a DTH response in various species. However, basic studies using monoclonal antibodies (mAbs) have demonstrated a predominance of macrophages and $CD4^+$ T cells within site of DTH in human volunteers,[2] and the quantitative assessment of a DTH reaction remains a key *in vivo* test of cell-mediated immunity.[3]

Our interest in studying the DTH response arose for several reasons. In recent years, in addition to its role in host anti-microbial responses, DTH has been suggested as a key effector mechanism of allograft rejection, as reviewed by Hall.[4] In addition, we have recently reported models of tolerance induction to alloantigen by oral[5–7] or intrathymic[8] administration of synthetic MHC class II allopeptides. In our initial studies, the immunogenicity of various peptide sequences were assessed through their ability to elicit a DTH response.[6] The current study examined the effect of the route of administration of MHC class II allopeptides on the subsequent development of a DTH response and assessed the nature of the cell infiltrate and expression of cytokines and activation molecules by dermal endothelial cells and mononuclear cells (MNC). These data provide new insights into DTH responses and the differing mechanisms through which oral or acquired thymic tolerance may arise.

Materials and Methods

Animals

LEW rats, 8 to 10 weeks old, were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) or were bred in our own animal facility.

MHC Allopeptides

We selected the RT1.Bβ and RT1.Dβ domains of $RT1^u$ (WF) and synthesized four overlapping peptides of 20 to 25 amino acids (residues 1–25, 20–44, 39–64, 68–92 for RT1.B, and residues 1–25, 20–44, 39–64, 60–84 for RT1.D) for each locus, using published sequences of the class II β-chain.[9] Peptides were dissolved in sterile phosphate-buffered saline at 1 mg/ml. Oral or intrathymic administration of synthetic peptides was performed as previously described.[6,8]

Antibodies

Murine mAbs were obtained from Sera-Lab (Accurate Chemicals, Westbury, NY) unless specified, and additional antibodies were produced by us or obtained from the investigators listed. mAbs were directed against all rat leukocytes (CD45, OX-1), T cells (CD5, OX-19; T cell receptor-α/β chains, R73), T cell subsets (CD4, BWH-4; CD8, OX-8; CD45RC, OX-22, putative marker of Th1 cells), B cells (CD45R, OX-33), natural killer cells (3.2.3), mononuclear phagocytes (ED-1, ED-2), and neutrophils (courtesy of Dr. F. Sendo, Yamagata, Japan). Activation of mononuclear, endothelial, or parenchymal cells was assessed using mAbs to class II antigens (OX-3), p55 chain of the IL-2R (CD25, ART-18, courtesy of Dr. T. Diamantstein, Berlin, Germany), transferrin receptor (CD71, OX-26), intercellular adhesion molecule (ICAM-1, CD54, 1A29), and LFA-1 (CD11a/CD18, WT-1) (Seikagaku Corp., Rockville, MD), rabbit antibodies to tissue factor (American Diagnostica, Greenwich, CT) and thrombomodulin (courtesy of Dr. H. Salem, Melbourne, Australia), and by labeling for the cytokines IL-1β (Genzyme Corp., Boston, MA); IL-2 (1D10); IL-4, IL-6, and IL-7 (Genzyme); IFN-γ (DB-10, courtesy of Dr. P. van der Meide, Rijswijk, Holland); TNF-α

(Genzyme), transforming growth factor-β (TGF-β) (British Biotechnology, Abingdon, UK), platelet-derived growth factors (PDGF-AA, PDGF-BB) (Genzyme) and prostaglandin-E_2 (PGE_2, Sigma Chemical Co., St. Louis, MO). We purchased polyclonal antibodies to rat, immunoglobulin A (IgA), IgG, IgM, C3 and fibrin (ICN, Irvine, CA); rat Ig-absorbed goat anti-mouse Ig (Sigma); and rabbit anti-goat Ig, goat peroxidase-antiperoxidase (PAP), swine anti-rabbit Ig, and rabbit PAP (Dako, Carpinteria, CA). Details of these mAbs and their isotype-matched controls, plus purified rabbit or goat Ig controls and their use in immunohistological studies of rat tissues were recently described.[7,10,11]

DTH Responses

Responder LEW rats (n = 5/group) were immunized by subcutaneous injection in the foot pad with 100 μg of a mixture of 4 RT1.B^u (50 μg) and 4 RT1.D^u (50 μg) peptides (12.5 μg/peptide) in complete Freund's adjuvant (CFA). Animals were challenged subcutaneously 2 weeks later in one ear with 10 μg of the peptide mixture, and in the other ear with *Mycobacterium tuberculosis* alone. A blinded observer measured DTH responses with a micrometer caliper (Mitutoyo, Paramus, NJ) 48 hours after challenge; results were calculated as Δ ear thickness before and after the challenge ($\times 10^{-2}$ inches). Statistical analysis of data was assessed using the Student *t*-test. Percent reduction was calculated as:

$$\frac{\text{Experimental} - \text{Control } \Delta \text{ ear thickness}}{\text{Control } \Delta \text{ ear thickness}}$$

Immunohistology

DTH specimens (n = 5/group) were harvested, sliced into small pieces, quick-frozen in liquid nitrogen and stored at −80 C in preparation for immunohistological studies. Cryostat sections were fixed in paraformaldehyde-lysine-periodate for demonstration of leukocytes and activation antigens or fixed in acetone for localization of cytokines and stained by a three- (polyclonal antibodies) or four-(mAbs) layer PAP method, as previously described.[7,10,11] Briefly, sections were incubated overnight with primary antibodies at 4 C followed by incubations at room temperature with bridging antibodies, PAP complexes and the substrate, diaminobenzidine, dissolved in a 1 M imidazole-0.1 M Tris buffer, pH 7.6. Labeled cells within 20 high-power fields (×400)/section/rat were assessed with the aid of an ocular grid micrometer. Cytokine and endothelial labeling was judged semi-quantitatively because of the presence of extracellular (cytokines) or continuous (endothelium) labeling, as <1%, <5%, 10–20%, 20–50%, or >75% of the cells indicated, as described.[7,10,11] The specificity of labeling was assessed using multiple controls. First, isotype-matched mAbs or purified Ig, and a control for residual endogenous peroxidase activity, were included in each experiment. Second, studies were undertaken to confirm the lack of labeling of normal ears except where appropriate, eg, endothelial labeling of thrombomodulin in normal skin, whereas positive controls for antibody labeling were provided using sections of rat allografts and rat brains after induction of experimental allergic encephalomyelitis (EAE), the data from which were previously reported.[7,10,11,12] Finally, antibody absorption with corresponding purified antigen (IL-1, TNF-α, IFN-γ, or IL-4) was undertaken before immunohistological labeling, as previously described.[10–13]

Results

Tolerogenicity of synthetic class II MHC allopeptides in vivo

We have previously reported that oral administration of synthetic 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.[6] LEW responder animals were fed 100 μg of the entire allopeptide mixture (4 RT1.D and 4 RT1.B peptides, 12.5 μg each) by gavage daily for 5 days. Three days after the last feeding, the animals were immunized with an injection of the allopeptide mixture and CFA, and DTH responses were determined 2 weeks later. Oral administration of the corresponding peptide mixture caused significant reduction of DTH responses elicited by the allopeptide mixture (77% reduction, $P = 0.001$), when compared to control animals fed PBS. This reduction was antigen-specific, as there was no reduction of DTH responses to Mycobacterium tuberculosis (present in CFA). When the more polymorphic RT1.D allopeptides, alone, were administered, significant reduction of antigen-specific DTH responses to subsequent immunization by the RT1.D peptides was observed (67% reduction, $P < 0.001$). Moreover, oral administration of the immunogenic allopeptides RT1.D1 plus RT1.D2, but not the non-immunogenic peptides RT1.D3 plus RT1.D4, resulted in significant reduction of DTH responses to subsequent immunization by the RT1.D allopeptide

Table 1. *Immunopathology of DTH Responses in Untreated Rats versus Those Receiving Oral or Intrathymic MHC Class II Peptides (RT1.B and RT1.D) Pre-DTH**

Feature	Control DTH Response†	Oral Group	Intrathymic Group
Edema, swelling	3+	1+	1+
Infiltrate	dense, MNC	minor, MNC	minor, MNC
$CD45^+$leukocytes	>150 cells/field	<20 cells/field	<10 cells/field
T cells	10–20% cells	50–75% cells	<10% cells
$CD4^+$ T cells	10–20% cells	50–75% cells	<10% cells
$CD8^+$ T cells	<1% cells	<10% cells	<1% cells
Macrophages	>75% cells	20–50% cells	>75% cells
CD45RC ($OX\text{-}22^+$)	10–20% cells	<1% cells	<1% cells
$IL\text{-}2R^+$ MNC	10–20% cells	10–20% cells	<1% cells
IL-2	10–20% cells	<1% cells	<1% cells
IL-7	10–20% cells	<1% cells	<1% cells
IFN-γ	50–75% cells, EC	<1% cells	<1% cells
TNF-α	>75% cells, EC	<5% cells, focal EC	<1% cells
IL-1β, IL-6	>50% cells, EC	<10% cells, focal EC	focal EC
TGF-β, PDGF	>75% cells, EC	25–50% cells, EC	<5% cells
IL-4	<5% cells, EC	3+, >75% cells, EC	negative
Class II MHC	3+, >75% cells, EC	<5% cells or EC	<1% cells
ICAM-1	3+, >75% cells, EC	1–2+, MNC only	1–2+, MNC only
Tissue factor	3+, >75% cells, EC	<5% cells	<1% cells
Thrombomodulin	1+, occasional dermal EC	3+, all vessels	3+, all vessels
Fibrin	3+, diffuse labeling of cells, SM and EC	1+, focal capillaries	1+, focal capillaries

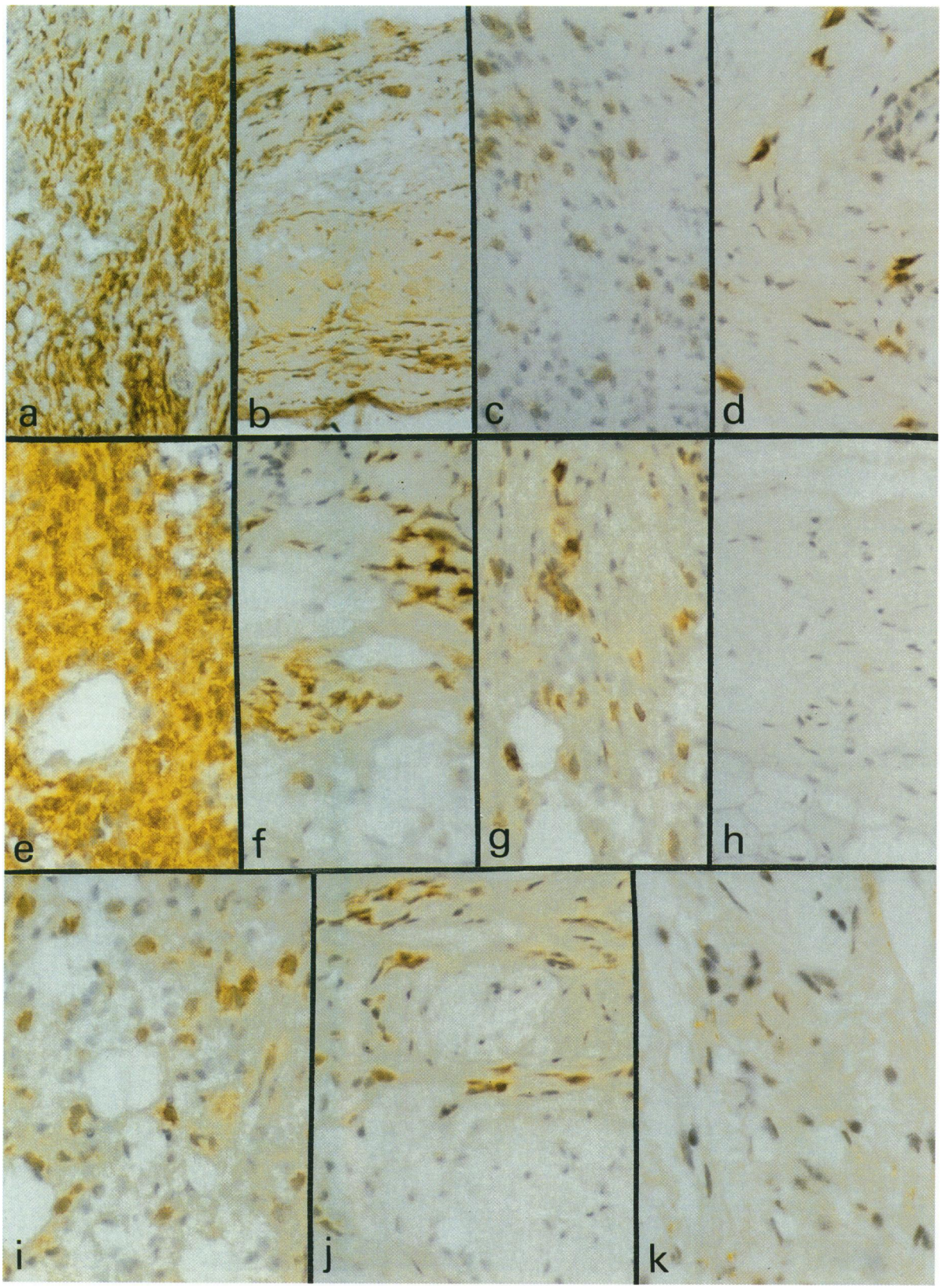
* Semi-quantitative assessment of infiltrating cells and vascular smooth muscle (*SM*) *and endothelial* (*EC*) *labeling within cryostat sections of DTH specimens* (×400).

† Results from animals immunized with peptides and challenged with mycobacteria were identical to those of the baseline control response and are not shown.

mixture (75% reduction, $P = 0.005$, *versus* 14% reduction, P not significant).[6]

We have recently demonstrated that intrathymic administration of donor synthetic class II MHC allopeptides can induce specific systemic unresponsiveness to renal allografts in the rat,[8] although the mechanisms mediating acquired thymic tolerance remain uncertain. We used the same RT1.B and RT1.D allopeptides to determine whether thymic recognition of MHC allopeptides can down-regulate the systemic cell-mediated immune response to subsequent immunization. LEW responder animals received a single intrathymic injection of 100 μg of RT1.B plus RT1.D allopeptides 48 hours before immunization with the same allopeptide mixture. DTH responses were determined 2 weeks later. Animals that received the intrathymic injection of the allopeptides had significant reduction of DTH responses elicited by the RT1.B plus RT1.D mixture (60% reduction, $P = 0.0005$), when compared with PBS-injected controls. When the RT1.D allopeptide mixture was injected intrathymically, significant reduction of DTH responses to subsequent immunization by the RT1.D mixture was observed (63% reduction, $P < 0.008$). In addition, intrathymic injection of the immunogenic allopeptides RT1.D1 plus RT1.D2, but not the non-immunogenic peptides RT1.D3 plus RT1.D4, resulted in significant reduction of DTH responses to subsequent immunization by the RT1.D allopeptide mixture (74% reduction, $P < 0.0001$, versus 26% reduction, P value not significant), similar to what we have previously reported in the oral tolerance model.[6]

Figure 1. *Immunoperoxidase localization of leukocytes within rat DTH samples. In both Figures 1 and 2, data from unmodified DTH (left) are contrasted with those of hosts receiving oral peptide therapy (right), except in cases where the results seen following intrathymic administration are significantly different from that of oral administration, when all three are illustrated. The epidermis is toward the top of each panel but, in the control group, only dermal features are seen at this magnification because of the extent of edema and swelling.* **a**, **b**: *CD45 (Leukocyte-common antigen).* **a** *shows a low power of the extensive dermal MNC infiltrate in an unmodified recipient; note the massive swelling and edema of the ear, which renders only a portion of the dermis visible, as compared with* **b** *taken at the same magnification, which shows the ear of an animal fed the peptide mixture prior to subcutaneous immunization and challenge in this site 2 weeks later. As indicated by the cross-section of the entire ear fitting within the optical field, allopeptide feeding resulted in a marked decrease in leukocyte infiltration and local tissue swelling.* **c**, **d**: *T cell receptor-α/β. Panel* **c** *shows how T cells contributed about 10% of the dermal infiltrate in control DTH samples. By contrast, panel* **d** *shows how peptide feeding decreased T-cell infiltration by >50% based upon assessment of the numbers of residual T cells/field. Subset analysis (not shown) indicated that >80% of T cells in both control and fed hosts were $CD4^+$ cells, consistent with a Th cell function and previous studies of classical DTH responses.* **e**, **f**: *Macrophages. The vast majority of the infiltrate in control rats were ED-1^+ macrophages* (**e**), *whereas feeding* (**f**) *markedly decreased (by >80%) macrophage accumulation.* **g**, **h**: *High-density $CD45RC^+$ ($OX\text{-}22^+$). Control tissue* (**g**) *showed consistent staining of ~10% of MNC for the OX-22 antigen; labeled cells were similar in their size and distribution to those labeled using the T cell markers. Functional studies by others have shown that $OX\text{-}22^+$ cells are associated with production of the cytokines IL-2 and IFN-γ; hence, high-density OX-22 antigen expression is suggestive of a Th1 phenotype. Tissues from "fed" rats* (**h**) *only minimal, low-density labeling of residual MNC for OX-22.* **i**, **j**, **k**: *CD25 (IL-2R, p55). Approximately 10% of dermal MNC in unmodified recipients were $IL\text{-}2R^+$* (**i**), *as were infiltrates in animals receiving oral peptide therapy* **j**, *whereas essentially no $IL\text{-}2R^+$ cells were seen in rats given intrathymic allopeptides* (**k**). *(Immunoperoxidase labeling of cryostat sections, hematoxylin counterstain, ×400, except for* **a** *and* **b** *which are ×100).*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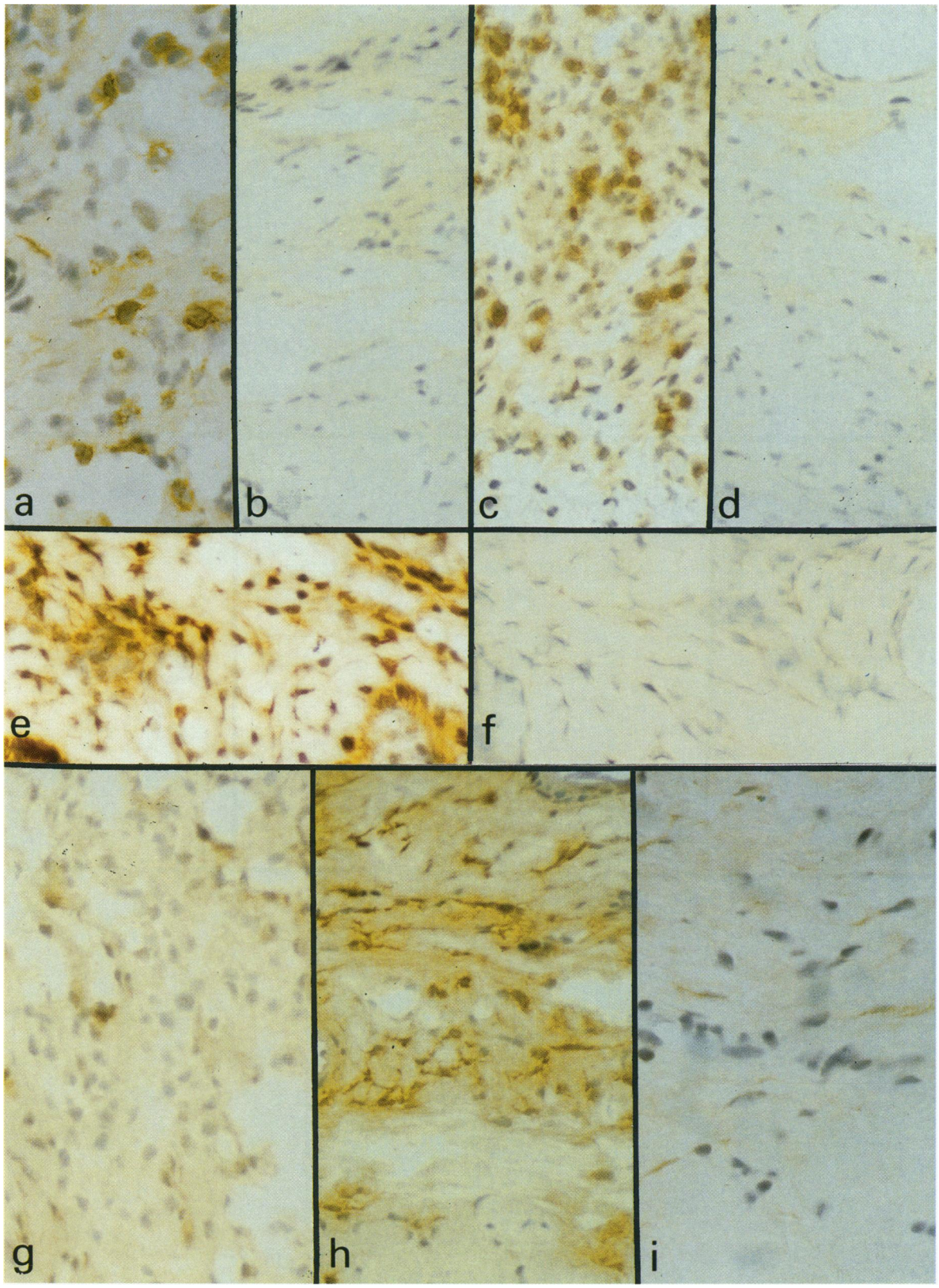
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Similar protocols of intraperitoneal administration of the immunogenic and the non-immunogenic RT1.D peptides did not effect reduction of DTH responses to subsequent immunization with the RT1.D allopeptide mixture (data not shown). These observations indicate that oral or intrathymic, but not systemic, administration of the immunodominant epitopes of class II MHC allopeptides is tolerogenic *in vivo*.

Histology and Immunohistology

Examination of ear biopsies from the control DTH group showed massive tissue swelling and a dense MNC infiltrate into the subcutaneous tissue and dermis, with particularly marked leukocyte accumulation around dilated small veins and venules of the superficial and deep dermal plexi, comparable to classical morphological studies of human DTH lesions.[14] Cryostat sections of DTH specimens were used for immunohistological evaluation of leukocytes, cytokines and activation antigens. No labeling was observed in sections incubated with isotype-matched mAbs or control Ig, or with specific mAbs after their absorption with corresponding cytokines. Results are summarized in Table 1, and selected markers are illustrated in Figures 1 and 2. No deposition of Ig or C3 was detected.

Control DTH responses in unmodified rats showed massive edema and dense interstitial and perileukocyte fibrin deposition in association with widespread cellular labeling for tissue factor and markedly decreased thrombomodulin expression by endothelial cells. Dermal infiltrates (Figure 1) consisted predominantly of macrophages plus smaller numbers of $CD45RC^+$, $IL\text{-}2R^+$, $CD4^+$ T cells, and the majority of cells (>75%) showed dense expression of class II and ICAM-1 antigens. Leukocytic infiltrates were associated with dense expression of the pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α (Figure 2), as well as lesser expression of IL-1 and IL-6, whereas labeling for TGF-β and PDGF was diffusely distributed.

DTH tissues from peptide-"fed" rats showed a >75% reduction in cell infiltration and edema. Interestingly, residual cells were largely Th2-like ($CD45RC^-$, $CD4^+$ T cells), but with residual expression of IL-2R comparable to that of the control group (Table 1, Figure 1). Sections from animals orally given peptides showed preservation of expression by endothelial cells of the anticoagulant molecule thrombomodulin, only very limited staining for the inducible procoagulant protein tissue factor, markedly reduced fibrin deposition, and a distinctly different pattern of cytokine expression from that of the control group (Table 1, Figure 2). Fed animals lacked IL-2, IFN-γ or TNF-α and showed markedly reduced staining for IL-1β or IL-6 within ear biopsies, with labeling for TGF-β and PDGF still apparent. However, this group consistently showed strong labeling of residual dermal MNC and endothelium for IL-4 (Figure 2).

DTH specimens from rats injected intrathymically with the corresponding class II peptide mixture showed suppression of cellular infiltration comparable to those of the peptide-fed group (Table 1), but differed from the "oral" group in that tissue samples largely lacked MNC or endothelial activation, or cytokine expression of any type, as shown by lack of labeling for IL-2R, TGF-β, or IL-4 (Figures 1, 2).

Discussion

We have analyzed aspects of cytokine expression and mononuclear and endothelial activation within tissues of rat DTH responses as a baseline for comparing the effects of tolerogenic protocols involving suppression of a DTH response through the novel approaches of oral or intrathymic antigen administration. Relatively little is known of the inflammatory and immune processes underlying a DTH response, although both $CD4^+$ T cells and macrophages are essential components of this cell-mediated immune response. Classic descriptions of the morphology of DTH reactions[15,16] reported the key features of an MNC infiltrate, increased vascular permeability leading to dermal and epidermal edema and endothelial cell swelling and vascular dilation, and extensive extravascular fibrin deposition. More recent studies with mAbs have demonstrated the predominance of $CD4^+$ T cells and macrophages among the MNC in

Figure 2. *Immunoperoxidase localization of selected cytokines within rat DTH samples. As with Figure 1, data from unmodified DTH (left) are contrasted with those of hosts receiving oral peptide therapy (right), except in cases where the results seen following intrathymic administration are significantly different from that of oral administration, when all three are illustrated.* **a, b:** *IL-2. Dermal infiltrates in control rats were associated with considerable IL-2 expression. Labeling was detected in and, in some cases at a lesser intensity, between infiltrating MNC* (**a**), *consistent with the presence of activated (Th1) T cells. Fed animals showed an essentially complete absence of staining for IL-2* (**b**). **c, d:** *IFN-γ. Large numbers of IFN-γ⁺ mononuclear cells (10–20% of dermal MNC) were detected within tissues from control rats* (**c**), *again consistent with the presence of Th1 cells. Fed animals showed markedly reduced or no staining for IFN-γ* (**d**). **e, f:** *TNF-α. Almost every cell within tissues of control animals showed dense labeling for TNF-α* (**e**), *whereas, apart from occasional cells of skin adnexa, feeding was associated with almost complete abolition of staining for TNF-α* (**f**). **g, h, i:** *IL-4. Only minor, weak staining for IL-4 was detected within tissues from control rats* (**g**). *Fed animals, by contrast, showed considerable labeling of dermal and subdermal mononuclear cells and capillaries and venules for IL-4* (**h**), *consistent with the concept that residual MNC cells were largely Th2 cells,* (**i**) *whereas DTH tissues from animal receiving intrathymic peptide administration showed only basal or no IL-4 expression. (Immunoperoxidase labeling of cryostat sections, hematoxylin counterstain, ×400).*

filtrate.[2] In addition, analysis of DTH lesions in patients with active *versus* quiescent forms of leprosy, using polymerase chain reaction to assess mRNA expression, showed prominent IL-1β, TNF-α, IL-2, and IFN-γ mRNA, and concomitant decreases in expression of mRNA for IL-4, IL-5, and IL-10 during active disease.[17] To our knowledge, however, this is the first study to dissect the immunopathology of DTH lesions, and particularly those elicited by synthetic peptides, at the protein level, and to document the correlations between cytokine expression and activation of macrophages and host endothelial cells *in situ*.

Macrophage activation was defined operatively in terms of expression of class II antigens, tissue factor, and cytokines such as TNF-α and IL-1β. We observed widespread and dense expression of tissue factor on intralesional macrophages, and to a lesser extent on dermal vessels, in association with extensive extravascular fibrin deposition. The key role of this procoagulant molecule in extravascular fibrin deposition in DTH responses was reviewed recently.[18] Similarly, in their review of the key features of endothelial cell activation, Pober and Cotran[19] defined two distinct phases: an early phase, which does not require protein synthesis, and a later phase, which involves gene up-regulation and protein synthesis. The latter responses, at least *in vitro*, are characterized by a loss of vascular integrity, up-regulation of cell surface adhesion molecules, cytokine secretion, and a shift from an anticoagulant to a procoagulant phenotype at the endothelial surface membrane through loss of the normally-occurring anticoagulant molecule thrombomodulin, and up-regulation of tissue factor. Comparable increased expression of the adhesion molecule ICAM-1 and tissue factor, and depression of thrombomodulin, were noted in the sections from control DTH responses in our study, again in conjunction with labeling for the cytokines TNF-α and IL-1β.

Evidence of host MNC activation was also provided by detection of IL-2, IL-7 and IFN-γ, as well as IL-2R, in the control group. Double-labeling studies to establish the precise identity of these cytokine-positive cells were not performed as part of the current studies, but based on analysis of serial sections, the size and morphology of labelled cells, the almost complete predominance of $CD4^+$ T cells over $CD8^+$ T cells, and the lack of B cells or natural killer cells, many of these latter, cytokine-positive MNC are likely to be $CD4^+$ T cells. These cells were also largely $CD45RC^+$, and lacked staining for IL-4. T cell labeling for IL-2 and IFN-γ but not IL-4, is consistent with a local Th1-predominant immune response,[20,21] and is supported by our data using an mAb CD45RC mAb (OX-22), which is a possible discriminant for Th1 *versus* Th2 cells in rats.[22–24] Previous cell transfer and *in vitro* studies showed that the $OX\text{-}22^+$ cells mediate DTH responses and produce IL-2 on antigen exposure.[22,24]

In contrast to the florid DTH responses to allopeptides seen in the control group, both oral and intrathymic administration of RT1.B and RT1.D class II MHC allopeptides (including the immunodominant epitopes of RT1.D, RT1.D1 and RT1.D2) were tolerogenic *in vivo*. Tissues from animals receiving either the oral or intrathymic allopeptides showed markedly reduced cellular infiltration, and mononuclear and endothelial cell activation, as shown by the general lack of up-regulation of class II and adhesion molecules, and maintenance of an anticoagulant state, consistent with the greatly diminished fibrin deposition in these samples. However, a significant difference in the pattern of cytokine expression was observed. Thus, although both protocols were associated with a general lack of IL-2 or IFN-γ (Th1 cell function) expression *in situ*, oral therapy appeared to induce a state of hyporesponsiveness in association with selective activation of Th2 cells ($CD45RC^-$) and expression of IL-2R and IL-4, and preservation of TGF-β expression. Intrathymic exposure, on the other hand, led to complete inhibition of T-cell activation, including lack of induction of IL-2R, IL-4, and TGF-β. These findings provide *in vivo* evidence of differences in the mechanisms of action of these two novel protocols for tolerance induction. Such differences correlate with our data from the autoimmune model EAE[10,11] in rats, where oral and intrathymic autoantigen each suppressed Th1 activation but also differed in their effects on elaboration of potentially inhibitory cytokines such as IL-4 by activated Th2 cells. In addition, in the EAE model, oral administration of antigen was associated with up-regulation of TGF-β expression in the brains of tolerized animals. Details of the complex and myriad inhibitory features of IL-4 and TGF-β on the immune response, and additional data, from several groups, which are relevant to this concept, were recently discussed.[7,10]

The pathways by which tolerance can be induced by oral or intrathymic antigen exposure are as yet only poorly understood. Oral tolerance may arise from "aberrant" antigen presentation in the gut wall, with selective activation of Th2 responses.[25,26] The mechanisms responsible for acquired thymic tolerance are the subject of intensive current investigation given the potent and profound effects of intrathymic administration of alloantigen on alloresponses in widely varying fields.[8,27–29] One of several theories for this form of tolerance rests on the demonstrated ability of ac-

tivated T cells to recirculate through the adult thymus[30] with the potential for inactivation of T cells which have been activated in the periphery, including after allostimulation.[31]

In summary, the current findings involving analysis of cytokine expression and mononuclear and endothelial cell activation provide new insights into the immune mechanisms of T cell-dependent tissue damage and how such responses may be altered by varying the route of antigen administration to exploit natural pathways for induction of immunological tolerance. These results have potential relevance to the wide variety of diseases involving DTH responses, and suggest strategies for therapeutic targeting of the host immune system.

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