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Interleukin-8 as a Macrophage-Derived Mediator of Angiogenesis

Alisa E. Koch,* Peter J. Polverini, Steven L. Kunkel, Lisa A. Harlow, Luisa A. DiPietro, Victor M. Elner, Susan G. Elner, Robert M. Strieter

Angiogenic factors produced by monocytes-macrophages are involved in the pathogenesis of chronic inflammatory disorders characterized by persistent angiogenesis. The possibility was tested that interleukin-8 (IL-8), which is a cytokine that is chemotactic for lymphocytes and neutrophils, is also angiogenic. Human recombinant IL-8 was potently angiogenic when implanted in the rat cornea and induced proliferation and chemotaxis of human umbilical vein endothelial cells. Angiogenic activity present in the conditioned media of inflamed human rheumatoid synovial tissue macrophages or lipopolysaccharide-stimulated blood monocytes was equally blocked by antibodies to either IL-8 or tumor necrosis factor–α. An IL-8 antisense oligonucleotide specifically blocked the production of monocyte-induced angiogenic activity. These data suggest a function for macrophage-derived IL-8 in angiogenesis-dependent disorders such as rheumatoid arthritis, tumor growth, and wound repair.

Angiogenesis is important in wound healing, tumor growth, and inflammatory disorders such as rheumatoid arthritis, and activated monocyte-macrophages are key angiogenesis effector cells in these settings (*1*, *2*). Although angiogenesis is critical to the initiation and progression of angioproliferative disease processes, the mediators responsible for this angiogenic activity remain to be precisely defined. IL-8 is a cytokine that has chemotactic activity selectively for neutrophils and lymphocytes at nanomolar and picomolar concentrations, respectively (*3*). This cytokine may be involved in leukocyte-vascular endothelial interactions such as the invasion of neutrophils though a vessel wall model via β_2 integrin attachment (*4*). Moreover, we and others have implicated IL-8 in angiogenic disease states such as psoriasis and rheumatoid arthritis (*5*). We have shown that rheumatoid synovial tissue macrophages are a major source of angiogenic mediators and IL-8 in the inflamed rheumatoid synovial milieu (*6*, *7*). IL-8 binds to heparin, a characteristic of many angiogenic cytokines such as acidic and basic fibroblast growth factor (aFGF and bFGF, respectively) and vascular endothelial growth factor (*1*). To test the hypothesis that IL-8 is angiogenic and to determine its contribution to monocyte-macrophage–associated angiogenic activity, we examined the ability of IL-8 to mediate angiogenesis in in vitro and in vivo models and compared its activity to that of tumor necrosis factor–α (TNF-α), a known macrophage-associated angiogenic mediator in murine systems (*8*).

First, we showed that recombinant human IL-8 stimulated chemotaxis of human umbilical vein endothelial cells (HUVECs) (Fig. 1A). IL-8 was chemotactic at a concentration of 0.125 nM. Chemotaxis toward IL-8 at a concentration of 1.25 nM was comparable to chemotaxis toward recombinant human bFGF at a concentration of 6 nM. Similarly, bFGF induced a doubling in the number of HUVECs (proliferation), whereas IL-8 at concentrations above 1.25 pM induced a similar response (Fig. 1B). We next determined whether IL-8 was angiogenic in vivo. IL-8 was incorporated into Hydron (Interferon Sciences) pellets and implanted in the normally avascular rat cornea (*9*). Amounts as small as 10 ng induced a corneal angiogenic response (ten out of ten positive corneas) with no evidence of nonspecific inflammation (Fig. 2, A and B).

We then determined whether IL-8 contributed to the angiogenic activity liberated by normal human monocytes activated in vitro or by macrophages isolated from diseased human synovial tissues (*10*), which are presumably activated in the inflamed joint milieu (Table 1). Antibodies to TNF-α and IL-8 (anti–TNF-α and anti–IL-8, respectively) reduced the chemotactic activity for HUVECs present in conditioned medium of lipopolysaccharide (LPS)-stimulated blood monocytes by 37% ± 9% (SE) and 34% ± 7%, respectively. Similar results were found when conditioned medium from inflammatory rheumatoid synovial tissue macrophages was incubated with anti–TNF-α or anti–IL-8: the chemotactic activity was reduced by 55 ± 1% and 39 ± 2%, respectively.

Neutralization of IL-8 in the LPS-activated monocyte-conditioned medium resulted in either diminution or complete

A. E. Koch, Department of Medicine, Section of Arthritis and Connective Tissue Diseases, Northwestern University Medical School, Ward Building 3-315, Chicago, IL 60611, and the Veterans Administration Lakeside Medical Center, Chicago, IL 60611.
P. J. Polverini and S. L. Kunkel, Department of Pathology, University of Michigan, Ann Arbor, MI 48109.
L. A. Harlow, Department of Medicine, Section of Arthritis and Connective Tissue Diseases, Northwestern University, Chicago, IL 60611.
L. A. DiPietro, Department of Surgery, Loyola University Medical Center, Maywood, IL 60153.
V. M. Elner and S. G. Elner, Departments of Pathology and Ophthalmology, University of Michigan, Ann Arbor, MI 48109.
R. M. Strieter, Department of Medicine, Pulmonary Division, University of Michigan, Ann Arbor, MI 48109.
*To whom correspondence should be addressed.

abrogation of the angiogenic response (zero out of eight positive corneas) (Fig. 2C). Similarly, neutralization of TNF-α resulted in a reduced or markedly suppressed corneal response (zero out of eight positive corneas). Incubations with control nonimmune serum had no effect on the angiogenic activity present in the conditioned medium (13 out of 13 positive corneas). These results indicated that TNF-α and IL-8 are responsible for the majority of the monocyte-macrophage–associated angiogenic activity elaborated by human monocytes activated in vitro and by human macrophages obtained from an inflammatory disease site. The neutralization of the in vivo monocyte-associated angiogenic activity by either TNF-α or IL-8 suggests that these two mediators may be complementary in the induction of the in vivo angiogenic response.

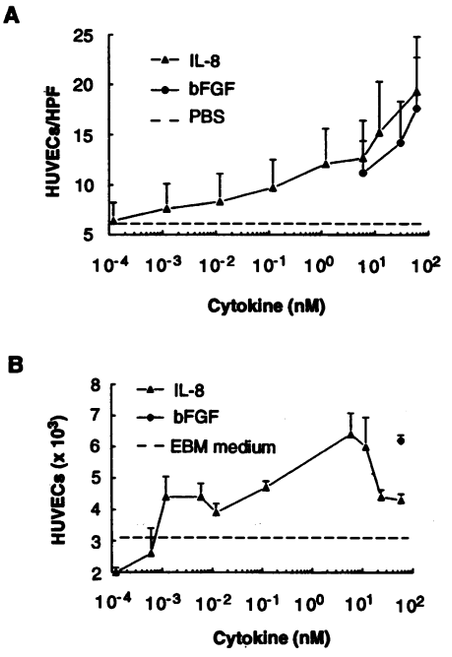

Fig. 1. (**A**) Chemotaxis of HUVECs (Clonetics, San Diego, California) was performed in 48-well blind-well chemotaxis chambers fitted with membranes (8-μm pore size) (Neuro Probe, Cabin John, Maryland) (*7*). HUVECs [2 × 10^4 cells in 25 μl of RPMI + 0.1% fetal calf serum] were added to the bottom wells of the chemotaxis chambers. The chambers were inverted and incubated in a humidified incubator gassed with 5% CO_2 at 37° for 2 hours, which allowed the HUVECs to attach to the membranes. The chambers were inverted, and phosphate-buffered saline (PBS) with or without recombinant human IL-8 (Peprotech, Rocky Hill, New Jersey) or bFGF (R&D Systems, Minneapolis, Minnesota) was added to the top half of the chambers. The chambers were incubated as before for 2 hours, and the membranes were fixed in methanol and stained with Leukostat (Fischer Scientific, Itasca, Illinois). The number of cells that had migrated through the filter were counted per 12 high-power fields. Results are expressed as the mean number of cells per high-power field (HPF) ± SE for quadruplicate determinations. Results represent one of four experiments. (**B**) Proliferation of HUVECs. Proliferation was performed as described (*15*). In brief, HUVECs were plated at 1 × 10^4 cells per 0.5 ml of endothelial basal medium (EBM)–umbilical vein (EBM) (Clonetics) + 10% fetal calf serum in each well of 24-well plates. The next day, the cells were washed, and recombinant human IL-8 or bFGF (60 nM, a concentration shown to induce proliferation of HUVECs) in EBM + 1% fetal calf serum was added for 72 hours. Cells were trypsinized and counted in a Coulter counter (Coulter, Hialeah, Florida). Triplicate determinations of each test group were performed. Results shown represent one of three experiments.

To determine whether monocyte-associated angiogenic activity could be inhibited at the pretranslational level, we used an IL-8 antisense oligonucleotide strategy. An oligonucleotide sequence complementary to nucleotides 261 through 291 of the IL-8 gene (antisense) was synthesized as was the sense (control) sequence. Although conditioned medium from the IL-8 sense oligonucleotide–treated monocytes had no appreciable effect on the chemotaxis of HUVECs, treatment with the IL-8 antisense oligonucleotide in concentrations of 5 μM and higher inhibited up to 84% of the chemotactic activity for HUVECs (Fig. 3). Similarly, conditioned medium from blood monocytes treated with IL-8 antisense oligonucleotide elicited a markedly diminished or absent corneal response, whereas conditioned medium from IL-8 sense oligonucleotide–treated monocytes had no effect on the angiogenic response (ten out of ten and zero out of twelve positive corneas, respectively)

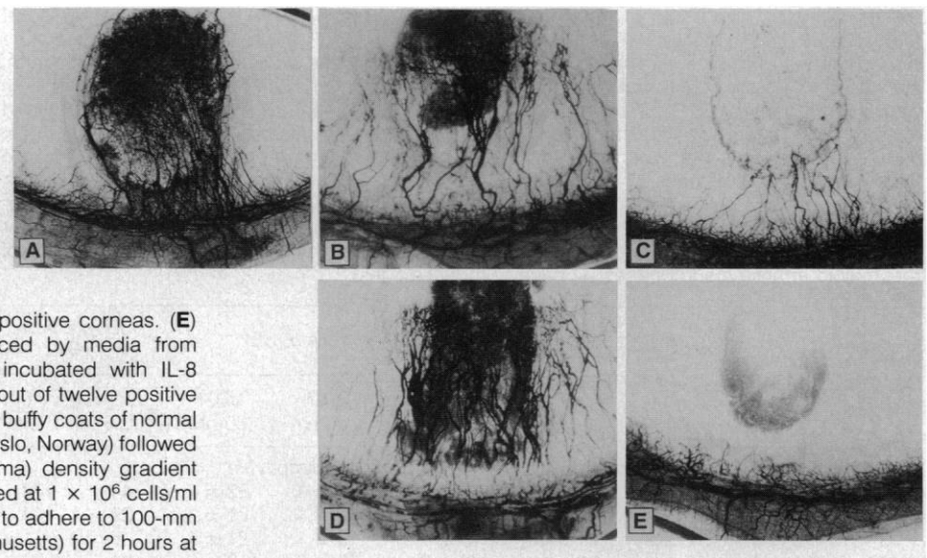

Fig. 2. (**A**) Positive angiogenic response induced by recombinant human IL-8 (100 ng). (**B**) Positive response induced by conditioned media from control LPS-stimulated human monocytes. (**C**) Markedly suppressed angiogenic response induced by conditioned media (of LPS-stimulated human monocytes) incubated with anti–IL-8. Results are representative of zero out of eight positive corneas. (**D**) Positive response induced by conditioned media from LPS-stimulated human monocytes, which were treated with IL-8 sense (control) oligonucleotide. Results are representative of ten out of ten positive corneas. (**E**) Markedly decreased angiogenic response induced by media from LPS-stimulated human monocytes, which were incubated with IL-8 antisense oligonucleotide. Results represent zero out of twelve positive corneas. Human monocytes were isolated from the buffy coats of normal donors with the use of Ficoll-Hypaque (Nycomed, Oslo, Norway) followed by Sepracell (Sepratech, Oklahoma City, Oklahoma) density gradient separation as described (*16*). Cells were suspended at 1 × 10^6 cells/ml in RPMI + 10% fetal calf serum (FCS) and allowed to adhere to 100-mm tissue-culture plates (Costar, Cambridge, Massachusetts) for 2 hours at 37°C in an incubator gassed with 5% CO_2. The medium was replaced with an equal volume of RPMI + 0.5% FCS + LPS (5 μg/ml) and collected 24 hours later. Monocyte-conditioned medium was prepared as described in Table 1. In some experiments, monocytes were incubated with LPS (5 μg/ml) and an IL-8 antisense oligonucleotide with the sequence 5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′ or sense (control) oligonucleotide from the identical region. Conditioned medium was collected, concentrated (×20), and assayed in the rat corneal bioassay as described (*13, 17*). In brief, conditioned medium (5 μl; ×20) was incorporated into an equal volume of slow-release Hydron, and 10-μl pellets were implanted into a pocket in the normally avascular rat corneal stroma. Corneas were examined daily for 7 days with a stereomicroscope and perfused with colloidal carbon at the end of the observation period to provide a permanent record of the angiogenic response (×33).

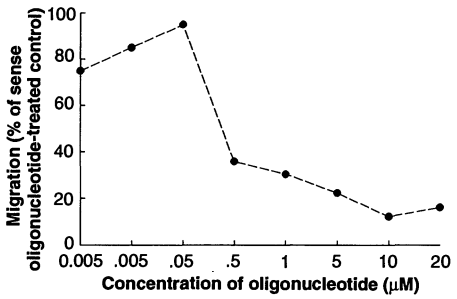

Fig. 3. Inhibition of macrophage-induced HUVEC chemotaxis by IL-8 antisense oligonucleotide. Human monocytes were isolated as described in Fig. 2. Either the IL-8 antisense or sense (control) oligonucleotide (0.005 μM to 20 μM) was added to human peripheral blood monocytes concomitant with LPS (5 μg/ml). After 24 hours, the conditioned medium was harvested and assayed for chemotactic activity for HUVECs as described in Fig. 1. Chemotactic activity for HUVECs induced by conditioned medium from IL-8 sense oligonucleotide–treated monocytes was equal to that induced by untreated monocytes. In further control experiments, either an IL-8 sense or antisense oligonucleotide was added to previously conditioned monocyte medium. No effect on the chemotactic activity for HUVECs was observed [HUVEC migration for nontreated monocytes: 6.3 ± 0.5 cells per HPF; HUVEC migration in response to monocyte-conditioned medium to which IL-8 antisense oligonucleotide (0.5 μM) had been added: 7.6 ± 0.5 cells per HPF]. Negative control migration in response to RPMI + 0.5% FCS was 2.3 ± 0.6 cells per HPF. In additional control experiments, bFGF was added to conditioned medium from IL-8 antisense–treated peripheral blood monocytes. Whereas conditioned medium from IL-8 antisense–treated monocytes resulted in minimal chemotactic activity for HUVECs (0.6 ± 0.2 cells per HPF), the addition of bFGF (60 nM) reconstituted the angiogenic response (6.8 ± 0.5 cells per HPF). bFGF-induced (60 nM) chemotaxis was 7.6 ± 0.4 cells per HPF.

(Fig. 2, D and E). To exclude the nonspecific effect of IL-8 antisense oligonucleotide, we added the antisense oligonucleotide to previously conditioned medium from blood monocytes. The addition of IL-8 antisense oligonucleotide in this setting failed to alter the effect of the monocyte-conditioned medium either on chemotactic activity for HUVECs or on angiogenic activity.

Table 1. Chemotaxis of HUVECs: Monocyte-macrophage–conditioned media were prepared as described in Fig. 2 and were used undiluted. Macrophages were obtained from human rheumatoid synovial tissues that were minced into 1-mm pieces and digested in a solution of dispase, deoxyribonuclease, and collagenase (*7, 13, 14*). The single-cell suspension was filtered through a 110-μm Nitex mesh (Tetco, Elmsford, New York) and layered on continuous Percoll gradients (Percoll, Pharmacia, New Jersey). Macrophages (density of 1.042 to 1.062 g/ml) that we previously showed to be angiogenic (*7, 14*) were harvested from the gradients and allowed to adhere to fibronectin-coated collagen gels. Residual nonmacrophage cells were removed with brief (4-min) trypsinization with the use of trypsin-EDTA (Gibco). Adherent macrophages were removed from the gels by treatment with 0.1% collagenase (Sigma). Macrophages (>90% pure) were suspended at 1×10^6 cells per milliliter in RPMI + 10% FCS and allowed to adhere to 100-mm tissue-culture dishes for 1 hour. The medium was replaced with an equal volume of RPMI + 0.5% fetal calf serum, and the cells were incubated at 37°C in an incubator gassed with 5% CO_2. Conditioned medium was collected after 24 hours. For neutralization studies, monocyte-macrophage–conditioned medium was incubated at 37°C for 30 min with polyclonal IL-8 antiserum (*6*) (1 μl of antiserum per milliliter of conditioned media), control rabbit nonimmune serum, monoclonal anti–TNF-α (Clone F12, Olympus, Lake Success, New York) (2 μg of antibody per milliliter of conditioned media), or control mouse serum. Chemotaxis of HUVECs was performed with the use of monocyte-macrophage supernatants as described in Fig. 1. $P < 0.05$ for suppression of IL-8 or TNF-α in either conditioned medium of LPS-activated peripheral blood monocytes or conditioned medium of rheumatoid arthritis macrophages by paired Student's *t* test.

Sample	Chemotactic activity of HUVECs (mean of cells ± SE)						Suppression (%)	
	Rabbit serum	Rabbit anti–IL-8	Mouse serum	Mouse anti–TNF-α	EBM medium	bFGF (60 nM)	Anti–IL-8	Anti–TNF-α
Conditioned medium of LPS-activated monocytes								
1	25 ± 1	14 ± 2	29 ± 2	20 ± 2	10 ± 1	22 ± 1	44	33
2	27 ± 2	19 ± 1	27 ± 2	15 ± 2	9 ± 1	21 ± 1	29	44
3	18 ± 2	17 ± 2	19 ± 1	13 ± 1	9 ± 1	21 ± 1	8	31
4	11 ± 1	5 ± 1	11 ± 1	2 ± 0	5 ± 0	8 ± 0	81	53
5	8 ± 1	7 ± 1	8 ± 1	8 ± 1	5 ± 0	8 ± 0	0	17
6	22 ± 1	11 ± 1	17 ± 0	12 ± 1	9 ± 1	7 ± 4	50	31
Conditioned medium of rheumatoid synovial tissue macrophages								
1	12 ± 1	7 ± 1	14 ± 1	6 ± 1	4 ± 1	14 ± 1	41	58
2	12 ± 1	6 ± 1	13 ± 2	6 ± 3	1 ± 0	8 ± 0	45	52
3	14 ± 1	9 ± 1	16 ± 1	8 ± 1	4 ± 1	9 ± 0	32	53
4	17 ± 1	10 ± 1	16 ± 1	7 ± 1	4 ± 1	9 ± 0	37	56

These results indicate that IL-8 in amounts of approximately 10 ng can function as a mediator of angiogenesis. This amount of IL-8 compares with amounts reported for the induction of corneal angiogenic activity by TNF-α, aFGF, bFGF, angiogenin, angiotropin, and vascular endothelial growth factor (*1, 8*). IL-8 belongs to the chemotactic gene superfamily that includes murine inflammatory peptide–2, platelet factor–4, platelet basic protein and its cleavage products connective tissue–activating peptide–III, β-thromboglobulin, and neutrophil-activating peptide–2), interferon-gamma–inducible protein, growth-regulated gene product, and epithelial neutrophil activating peptide (*11*). Interestingly, a member of this family, platelet factor–4, has been reported to be a potent inhibitor of angiogenesis (*12*). Although the predominant biological activities of IL-8 to date have included both chemotactic and activating activity for neutrophils and chemotactic activity for lymphocytes, IL-8 shares sequence homology with several known growth proteins that are believed to be involved in inflammation and cell proliferation, which suggests alternative biological actions for this molecule (*11*).

In this study, we have shown that the majority of the angiogenic activity present in the conditioned media of monocytes-macrophages activated in vitro or obtained from rheumatoid synovial tissue was neutralizable with anti–IL-8 or anti–TNF-α. Moreover, monocyte-associated angiogenic activity in vivo and in vitro was significantly inhibited by in vitro treatment of mononuclear phagocytes with an antisense oligonucleotide to IL-8. Our results suggest a function for a previously undescribed macrophage-derived angiogenic mediator that can be implicated in the pathogenesis of inflammatory diseases such as rheumatoid arthritis. In addition, the ability of IL-8 antisense oligonucleotide to inhibit macrophage-induced angiogenesis provides possible avenues to attenuate the angiogenic response found not only in inflammation, but also in tumor growth and other conditions characterized by persistent neovascularization.

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10. All human samples were obtained with Northwestern University Institutional Review Board Approval.
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Invariant Chain Peptides in Most HLA-DR Molecules of an Antigen-Processing Mutant

Alessandro Sette,* Stephanie Ceman, Ralph T. Kubo, Kazuyasu Sakaguchi, Ettore Appella, Donald F. Hunt, Theresa A. Davis, Hanspeter Michel, Jeffrey Shabanowitz, Richard Rudersdorf, Howard M. Grey, Robert DeMars

Class II major histocompatibility complexes bind peptides in an endosome-like compartment. When the class II null cell line 721.174 was transfected with class II DR3 genes, DR molecules were produced in normal amounts. However, the DR molecules were abnormally conformed and unstable because deletion of an antigen-processing gene had impaired intracellular formation of most class II–peptide complexes. Yet, 70 percent of the DR molecules still bore peptides, 80 percent of which were 21– to 24–amino acid fragments of the class II–associated invariant chain. These peptides were rare on DR3 from control cells. Thus, a defect in the main antigen-processing pathway revealed a process in which DR molecules bind long peptides derived from proteins present in the same compartment.

The main biological function of class I and class II proteins encoded in the major histocompatibility complex (MHC) is to bind antigenic peptides. The peptides bind in a groove walled in by two parallel α-helical segments on top of a floor formed by a β-pleated sheet (*1*–*3*). Complexes between MHC proteins and antigenic peptides are normally produced in antigen-presenting cells as a result of antigen processing (*4*) and are then displayed on the cell surface. They can then be recognized by the antigen receptors of specific T lymphocytes and thus trigger T cell activation. Thus, any interference with the formation of MHC-antigen complexes will impair activation of peptide-specific T cells.

Studies of cell mutants (*5*) have shown that the binding of self or foreign peptides is necessary for the normal assembly, stability, intracellular trafficking, and cell surface display of class I molecules. These and other (*6*, *7*) studies have demonstrated that TAP1 and TAP2 transporter genes in the class II region of the MHC are necessary for the formation in the endoplasmic reticulum of most class I–peptide complexes. At least one gene in the vicinity of the TAP genes is needed for the intracellular production of class II–peptide complexes. Human B lymphoblastoid cell lines (LCLs) that have homozygous deletions (*8*, *9*) or presumably point mutations (*10*) in this region have the following characteristics: (i) They display normal amounts of class II heterodimers on their surfaces, but the molecules lack certain antigenic epitopes and dissociate more readily than class II molecules from normal cells in the presence of SDS (*9*, *11*). (ii) They produce molecules that are not recognized by many DR3-specific T cells (*12*). (iii) They have reduced ability to process and present epitopes derived from exogenous whole protein antigens but efficiently present epitopes from exogenously added antigenic peptides (*8*, *10*, *11*). Proteolytic cleavage of the class II–associated invariant chain normally must precede binding of peptides to class II molecules and occurs in processing-defective mutants (*13*). The antigen-processing defects in these mutants may be functionally analogous to those causing the production of empty class I molecules in TAP1- or TAP2-deficient mutants (*5*). We show here that class II DR3 molecules produced in the cell line 721.174, which has the abnormalities described above (*8*), carried less self peptide than DR3 purified from control cells. Most of the mutant-derived DR molecules carried a limited variety of peptides, which were undetected or rare on DR3 isolated from nonmutant cells. The antigen-processing defect in these cells, therefore, has revealed an alternative processing pathway.

These observations (*8*) were made with cells created by the transfection of DR α and β genes into LCL mutants 721.82 (.82) (*14*) and 721.174 (.174) (*15*), which have homozygous deletions of the DR and DQ1 α and β genes. The MHC region bounded by and including the DP1 and DQ2 loci is present in .82 but absent in .174 (*16*, *17*). This region includes the TAP1 and TAP2 transporter genes and LMP2 and LMP7 (*18*), which encode components of a multicatalytic proteinase (*5*). Because antigen processing and presentation with class II molecules apparently are normal in a mutant in which these four genes are not expressed, at least one undiscovered gene in this region is needed for antigen presentation and is deleted in .174 (*19*).

DR-expressing transfectants derived from .174 [.174(DR3)] have abnormalities associated with an impediment to intracellular antigen processing or presentation with class II molecules, but this impediment is absent in DR-expressing transfectants derived from .82 [.82(DR3)] (*8*). We thought that the impaired antigen processing in class II–expressing transfectants of .174 might result in empty DR3 molecules that would bind peptides in solution in larger quantities and at faster rates than DR3 derived from .82(DR3) and that fewer self peptides would be bound to DR3 from .174(DR3) than to DR3 produced by

A. Sette, R. T. Kubo, H. M. Grey, Cytel, 3525 John Hopkins Court, San Diego, CA 92121.
S. Ceman, R. Rudersdorf, R. DeMars, Laboratory of Genetics, University of Wisconsin, Madison, WI 53706.
K. Sakaguchi and E. Appella, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.
D. F. Hunt, T. A. Davis, H. Michel, J. Shabanowitz, Department of Chemistry, University of Virginia, Charlottesville, VA 22901.
*To whom correspondence should be addressed.