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Differential Recognition of Altered Peptide Ligands Distinguishes Two Functionally Discordant (Arthritogenic and Nonarthritisogenic) Autoreactive T Cell Hybridoma Clones

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Intravenous injection of a cartilage proteoglycan (aggrecan)-specific Th1 hybridoma clone 5/4E8 induced joint lesions similar to those seen in either primary or adoptively transferred arthritis in BALB/c mice. A sister clone, TA20, recognizing the same peptide epitope of human aggrecan and using the same Vβ4 and Vα1 segments, failed to induce joint inflammation. This study examines the fine epitope specificities of these two clones. Both 5/4E8 and TA20 hybridomas were generated using T cells from the same arthritic animal that has been immunized with human aggrecan, and both clones recognized peptides containing a consensus GRVRVNSAY sequence. However, flanking regions outside this nonapeptide sequence region had differential impact on peptide recognition by the two clones. Similarly, when single amino acid substitutions were introduced to the consensus sequence, the 5/4E8 hybridoma showed greater flexibility in recognition, including a higher responsiveness to the corresponding self (mouse) aggrecan peptide, and produced more inflammatory cytokines (IFN-γ and TNF-α), whereas hybridoma TA20 produced IL-5 in response to either human or mouse self peptide stimulation. These results demonstrate that, within the pool of immunodominant (foreign) peptide-activated lymphocytes, marked individual differences of degeneracy exist in T cell recognition, with possible implications to autopathogenic T cell functions. The Journal of Immunology, 2003, 171: 3025–3033.

Rheumatoid arthritis (RA) is one of the most frequent human autoimmune diseases. Although the etiology of the disease is unknown, it is characterized by a Th1 dominance in inflamed synovium (1–3). Among many candidate autoantigens (4, 5), cartilage proteoglycan aggrecan is one of the major target molecules in the autoimmune inflammatory attack in RA joint lesions (6, 7). Aggrecan is a complex macromolecule consisting of a large core protein (~2200 aa) to which >100 glycosaminoglycan and oligosaccharide side chains are covalently attached (8, 9). The core protein of aggrecan is heavily degraded in inflammatory processes, which results in the loss of function of articular cartilage.

Immunization of BALB/c mice with partially deglycosylated human aggrecan induces chronic progressive polyarthritides and spondylitis (10). This proteoglycan (aggrecan)-induced arthritis (PGIA) shows many similarities to human rheumatoid arthritis, as indicated by clinical assessments, laboratory tests, x-rays, and histopathology of the peripheral joints (5, 10–12). The development of the disease is based upon the development of cross-reactive T and B cell responses between the immunizing human and self (mouse) cartilage aggrecan (9, 11).

We have generated aggrecan-specific T cell hybridomas from arthritic animals. One of these T cell hybridomas, 5/4E8, induced inflammatory processes in joints, when injected i.v. into naive BALB/c mice (13). The joint lesions were very similar to those described in either the primary or adoptively transferred forms of PGIA (10, 14, 15) as well as to those in RA (16). The 5/4E8 epitope (70 ATEGRVRVNSAYQDK) is located in G1 domain of human aggrecan molecule (9), and has been identified as one of the dominant arthritogenic T cell epitopes of aggrecan in BALB/c mice (17, 18). In an independent study, a peptide fragment derived from the G1 domain of aggrecan, containing the 5/4E8 epitope sequence, was also able to stimulate T cells from human patients with RA (6). Together, these results suggest that the 5/4E8 epitope plays a critical role in PGIA, and may be involved in RA.

A peptide screening of our T cell hybridoma library yielded another clone (TA20) with epitope specificity identical with 5/4E8 (13). Both T cell hybridomas (5/4E8 and TA20) express CD3, CD4, and TCR-αβ (13). They both secrete IL-2 and IFN-γ upon I-Aα-restricted recognition of aggrecan, and express high levels of standard and identical CD44 isoforms and other adhesion molecules, including CD28 and CD11a (LFA-1) (13, 19–21). To date, the only difference between the two clones was noted upon in vivo administration: 5/4E8 induced joint inflammation, whereas TA20 cells have never been detected in joint tissues, and this hybridoma has never induced arthritis (13).

We now demonstrate that although the two clones respond to the same linear peptide determinant of aggrecan, and both hybridomas express identical CDR1, CDR2, and CDR3 segments of both Vα1

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; LN, lymph node; PGIA, proteoglycan (aggrecan)-induced arthritis.

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and Vβ4 chains, they recognize different patterns of synthetic peptide analogues, and respond with the secretion of different cytokines. As the parent lymphocytes of both arthritogenic (5/4E8) and nonarthritogenic (TA20) clones have been derived from the same animal, the in vivo coexistence of parental T cells with differences in T cell signaling via the same TCR structure has a possible implication in autopathogenic T cell functions. As supported by our results, a high permissivity of peptide binding by a given TCR could significantly enhance the chance of the T cell to be stimulated by both foreign peptide ligands and self epitopes, thus creating a molecular basis for autoimmunity.

Materials and Methods

T cell hybridomas, epitope specificities, and synthetic peptides

The T cell hybridomas 5/4E8 and TA20 were obtained following the fusion of in vitro aggrecan-stimulated splenic lymphocytes from an arthritic mouse with BW5147.G1.1 thymoma cells (American Type Culture Collection (ATCC), Manassas, VA), as described (13). These clones recognized aggrecan of human and bovine origin, but failed to respond to dog, sheep, rat, or mouse aggrecans (21). Based upon sequence homologies of aggrecan among the species (22–25), the epitopes of these two T cell hybridomas were located in the G1 domain (9). Peptides spanning the G1 domain including the consensus 5/4E8 epitope (GRVRVNSAY) with either 1-mer offset or amino acid substitutions (altered peptide ligand), were synthesized by Chiron Mimotopes (Raleigh, NC) using the pin method. Synthetic peptides were dissolved in DMSO, diluted with acidified PBS (pH 5.6) to a final stock solution of 10 ng/ml, aliquoted, and stored at −20°C until use.

Detection of peptide-specific IL-2 production by the T cell hybridomas

The 5/4E8 and TA20 hybridomas were tested by culturing of 2 × 10⁴ hybridoma cells with 2 × 10⁵ irradiated (14,000 rad, cesium sources; J. L. Shepard & Associates, San Fernando, CA) A20 B lymphoma cells (ATCC) or freshly isolated peritoneal macrophages (5 × 10⁵) in the presence or absence of synthetic peptides in 200 μl medium/well in triplicate wells of 96-well tissue culture plates (Corning Costar, Corning, NY). Culture medium for cytokine assays was collected from multiple wells. The cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (HyClone, Logan, UT) for 24 h in the absence or presence of various concentrations of peptides. Supernatants (100 μl) were then transferred to wells containing 5 × 10⁵ CTLL-2 cells (ATCC) in 100 μl DMEM supplemented with 10% FCS. CTLL-2 cells were cultured with the supernatants for 24 h, pulsed with 0.5 μCi [3H]thymidine/well (sp. act. 2 Ci/mmol; PerkinElmer, Boston, MA), and harvested after 14 h. The incorporated radioactivity was counted, and the results were expressed as Δcpm (calculated by subtracting the counts of nonsimulated cells from the counts of stimulated cultures). All experiments were repeated three or more times.

Peptide immunizations and detection of lymph node (LN) cell proliferative responses

For priming of LN cells, female BALB/c mice (Charles River Laboratories, Kingston Colony, Kingston, NY) were injected s.c. on the dorsal aspect of the hind paws with 50 μg of synthetic peptide in CFA (Difco Laboratories, Detroit, MI) in 50 μl total volume. Popliteal LN cells were harvested 9 days later and cultured (3 × 10⁵ cells/well) in DMEM supplemented with 1.5% FCS in the presence of serial concentrations of synthetic peptides in 96-well tissue culture plates. Cells were cultured for 72 h (cytokine assays) or 96 h (proliferation), and then 0.5 μCi of [3H]thymidine was added to each well. Cells were harvested 14 h later, radioactivity was counted, and the results were expressed as stimulation index, which is the ratio of cpm measured in peptide-stimulated cultures relative to the cpm measured in nonsimulated cultures. Media collected at 72 h were assayed for cytokine release using CTLL-2 bioassay (as described above) or measuring IFN-γ, IL-4, IL-5, IL-6, IL-10, and TNF-α by ELISA (R&D Systems, Minneapolis, MN, or BD PharMingen, San Diego, CA).

Determination of MHC-binding residues

A double-layer biotin/avidin/FITC technique was applied to detect MHC-binding peptides. 1-A2-expressing A20 lymphoma cells (1 × 10⁴) were incubated with 100 μg of biotinylated synthetic peptides (with altered sequences) for 2 h at 37°C in 100 μl DMEM. Subsequently, the cells were washed with PBS containing 1% BSA and then incubated with 10 μg avidin-FITC (BD PharMingen) for 20 min at room temperature. After washing, the cells were incubated with 10 μg of biotin-conjugated anti-avidin mAb (Sigma-Aldrich) in 100 μl of 1% BSA in PBS (pH 7.4) for 20 min at room temperature. This was followed by washing and incubating the cells with 20 μl avidin-FITC (BD PharMingen). Finally, the cells were washed and analyzed by FACSCalibur (BD Biosciences, San Jose, CA). Results are expressed as geometric mean values calculated by CellQuest Software (BD Biosciences) using the following formula:

\[
\text{geometric mean} = 10 \times \exp \left( \frac{\sum \log (X_i)}{n} \right)
\]

where \(X_i\) is the channel value for the \(i\)th event and \(n\) is the number of events used in the calculation.

Serial dilutions of nonbiotinylated peptides with identical sequence were used for competitive inhibition, or nonbiotinylated anti-avidin IgG were used as specificity controls.

Abs

mAbs to mouse cell surface markers including costimulatory markers were purchased from BD Biosciences or R&D Systems. These were anti-mouse CD154 (anti-CD40L; clone TRAP1), hamster anti-mouse CD28 (clone 37.51), and rat anti-mouse CD53 (clone OX-79), which were used at 12.5, 25, and 50 μg/ml concentration in the presence or absence of synthetic peptides. Isotype-specific mouse, hamster, and rat IgGs were used at the same concentrations. Culture conditions were the same as described for IL-2 production. Cytokines were measured by capture ELISAs from BD PharMingen or R&D Systems.

Statistical analysis

Statistical analysis was performed using SPSS v10.0.5 statistical software (SPSS, Chicago, IL). As the results of all in vitro tests showed normal distribution, Student’s two-sample \(t\) test was used for comparison of results from two groups. Significance level was set at \(p < 0.05\).

Results

We have shown earlier that the arthritogenic T cell hybridoma clone 5/4E8 recognized an epitope represented in three overlapping peptides (P67–81, P70–84, and P73–87) of the G1 domain of aggrecan (9). To determine the fine epitope specificity of the seemingly identical, however nonarthritogenic TA20 clone, and to further characterize the epitope recognition of the two T cell hybridomas, a set of overlapping peptides with 1-mer offset (from P64–78 to P76–90) was used. The responses of the 5/4E8 and TA20 clones are shown in Fig. 1. Both clones recognized all synthetic peptides containing the GRVRVNSAY consensus sequence, suggesting that they both were specific for the same epitope of aggrecan. The absence of either the N-terminal Gly (P74–88) or the C-terminal Tyr (P66–80) completely abolished the response of both hybridomas (Fig. 1). Quantitative differences in peptide recognition, however, were clearly detectable, especially at low peptide concentrations. Although the 5/4E8 clone responded to all overlapping peptides with comparable levels of IL-2 secretion, TA20 cells showed decreased responses to peptides P71/73–85/87 used at lower concentrations (Fig. 1).

As peptides P67–81 and P73–87 containing the GRVRVNSAY sequence at either the N or C terminus achieved full stimulation of the 5/4E8 cells (Fig. 1), we concluded that no flanking amino acid residues at the N or C terminus of this nonapeptide sequence were essential for MHC-anchoring and/or TCR contact. Therefore, we next investigated whether the consensus sequence GRVRVNSAY itself was stimulatory for the two T cell hybridoma clones. The nonapeptide sequence did not elicit a significant response by either 5/4E8 or TA20 cells (Fig. 2). Although peptides bound to MHC-II can be varied in length (13–17 aa in most cases) (26–28), we assumed that the 9-aa-length core sequence might have been below the critical limit. Addition of a single Ala spacer to both ends (29) rendered the peptide stimulatory for both clones. Surprisingly, the presence of two Ala residues at both termini had differential influence on the peptide recognition by the two clones: while IL-2
production of the 5/4E8 cells was further augmented, the recognition by TA20 cells was significantly reduced (Fig. 2).

To further investigate the function of peptide-flanking sequences, which potentially can modify T cell responses (26, 30, 31), the core sequence was flanked with 3 aa of the wild-type sequence at one end and flanked with double Ala at the other end. As showed in Fig. 2, the replacement of the wild-type sequence with double Ala at the N terminus significantly reduced the response of TA20 cells, but did not influence the 5/4E8 response. In contrast, the 3 aa of the wild-type sequence at the N terminus reconstituted the response of TA20 cells (Fig. 2). In view of results summarized in Figs. 1 and 2, the presence of the wild-type sequence at the N terminus is crucial for the response of TA20 cells, but it is essentially negligible for the 5/4E8 response.

We then introduced single amino acid (Ala or Lys) substitutions within the nonapeptide consensus sequence (P1-P9) that was flanked by 3 aa of the wild-type sequence at both termini (Fig. 3). Ala substitution in P1 and P5 positions significantly reduced, and in P2, P3, and P4 positions completely abrogated the response by 5/4E8 cells, while Ala at P9 position proved to be a preferred ligand (Fig. 3A). Ala substitutions in other positions did not alter significantly the IL-2 release by 5/4E8 cells. In contrast, all Ala-substituted analogues proved to be poor stimulators for TA20 cells (Fig. 3B). Although 5/4E8 cells tolerated Lys substitutions in P1, P4, and P9 positions (Fig. 3C), the response of TA20 cells to all peptide analogues was less than 50% of the response to unmodified peptide, except Lys in P9 (Fig. 3D).

To have a better understanding of the molecular interactions among TCR, peptide, and MHC, binding of single amino acid-substituted peptide analogues to MHC class II (I-A<sup>d</sup>) of A20 APCs was tested by flow cytometry (Fig. 4). Although P2, P3, and P5 Ala- and Lys-substituted peptides were defective in eliciting a response by either of the T cell clones (Fig. 3), the MHC binding of these peptides was not reduced (Fig. 4). Therefore, amino acids at P2, P3, and P5 positions most likely represent residues involved in the interaction with TCR of both clones. P4 and P6 nonconservative amino acid substitutions abrogated MHC binding, which occurred at P7 position only with Ala substitution (Fig. 4). Ala substitution at P8 by Lys dramatically reduced, essentially abolished, the MHC binding of the peptide, whereas P8 Ala to Gly substitution partially reduced the peptide binding (Fig. 4). The Tyr substitution with either Ala or Lys in P9 position clearly enhanced peptide binding to MHC class II (Fig. 4), and this effect, especially in Lys substitutions, was also reflected by an increased response to the peptide by both clones (Fig. 3, C and D). Therefore, Tyr P9 appears to be an I-A<sup>d</sup> anchor. Although P1 substitutions dramatically reduced MHC-peptide binding (Fig. 4), the 5/4E8 response was high when the Gly at P1 was substituted with Lys (Fig. 3C).

These results in context with those shown in Fig. 3 indicate a distinct pattern of TCR contact residues for each clone, and are in agreement with the anchor positions (P1-P4-P6-(P7)-P9) proposed for binding to I-A<sup>d</sup> (32–34). It is noteworthy that peptides with P1 and P4 substitutions, despite a weak binding by MHC (Fig. 4), were recognized by the 5/4E8 cells (Fig. 3C). The P7 Ser/Ala
substitution, while strongly reducing the binding of peptide to MHC (Fig. 4), was well tolerated by 5/4E8 cells, similar to the Ala/Gly substitution at the P8 position.

Our next question concerned the recognition of the homologous murine self sequence, which has two amino acid mismatches at P2 and P8 positions (ATEGQVRVNSIYQDR), when compared with the human sequence (ATEGQRVNYASAYQDK). At 1 \mu g/ml peptide concentration, we could hardly detect any reactivity to the self peptide (Fig. 5). Increasing the peptide concentration by one order of magnitude restored the response of 5/4E8 cells, while TA20 cells were activated only in the presence of 100 \mu g/ml synthetic peptide. For comparison, the far right columns of Fig. 5 show the responses of T cell hybridomas to 1 \mu g/ml peptide corresponding to the human sequence.

We also tested the immunogenicity of synthetic peptides in BALB/c mice (Fig. 6). The highest LN cell proliferation rate was measured in human peptide P70–84-primed mice (Fig. 6A; first group of columns). Immunization with the peptide having the consensus 9-aa sequence (GRVVRVNSAY), flanked by either a single (data not shown) or double Ala, resulted in significantly smaller cell proliferation upon in vitro challenge (9 days postimmunization) than the wild-type peptide (Fig. 6A). Although the P2 Ala-substituted peptide was not recognized by either 5/4E8 or TA20 clones (Fig. 3, A and B), we were able to detect a proliferative response after injection of BALB/c mice with this peptide variant (Fig. 6, third group).

It was of importance to determine whether cell response to the peptide with the murine sequence had an autoreactive character. T cells from human (wild-type) consensus sequence-primed mice responded to the corresponding self sequence (ATEGQRVTVNINYQDK) (data not shown) and the human wild-type P70–84 peptide (Fig. 6A, first group of columns) in a similar manner. Moreover, a weak but definite cell response was detected in self peptide-primed BALB/c mice (Fig. 6, A and B, last groups of columns). This observation indicated that T cells recognized this sequence as an autoepitope. Immune tolerance to self peptide was evidently incomplete in BALB/c mice and the parent cells of the two T cell hybridomas derived from a T cell pool with incomplete tolerance.

The differences between 5/4E8 and TA20 in IL-2 production were evident when the same substituted (altered) core peptides were used for in vitro stimulation of the two T cell hybridomas (Fig. 3), and could be the consequences of differences between the TCR binding sites. Sequencing of the \alpha- and \beta-chains revealed that both hybridomas expressed the CDR3 segments of \alpha1 (...CAA(KANYGNEKI)TFG...Ja) and \beta4 (...CAS(SQDQVQDTQ)YFG...Jb) chains, in an identical manner.

To determine whether differences in the production of proinflammatory vs anti-inflammatory cytokines accounted for the arthritogenic and nonarthritogenic behavior of 5/4E8 and TA20 cells, respectively, we compared the cytokine profile in the presence of: 1) human or mouse aggrecan (full-length Ag); 2) wild-type human and mouse (self) peptides (P70–84); and 3) peptides with either Ala or Lys substitution. A20 B lymphoma cells or peritoneal macrophages were used as APCs. Table I summarizes the most important findings of these experiments. Although both hybridomas were Th1 type with dominant IFN-\gamma and low levels of IL-4 production (13), the ratio of IFN-\gamma to IL-4 increased disproportionally when either peptides (Table I) or aggrecan (data not shown) were presented by macrophages, particularly to 5/4E8 cells. Moreover, while 5/4E8 cells did not produce measurable amounts of IL-5 in any condition, TA20 hybridoma cells secreted large amounts of this anti-inflammatory cytokine in the presence of mouse or human aggrecans (data not shown), and mouse (self) or human wild-type peptides (Table I). Peptide-induced IL-5 production by TA20 cells (Table I) seemed to be independent of the weak IL-2 production in the presence of self (mouse P70–84) peptide (Fig. 5), and could not be changed by Abs to costimulatory molecules, including CD40L and CD28, or to CD53 (data not shown).
Proteoglycan (aggrecan) Ag or peptide-induced TNF-α secretion was 2- to 3-fold greater in 5/4E8 than TA20 hybridoma cells (Table I). TNF-α levels appeared to be independent of the type of APCs, and neither blocking Ab to costimulatory molecule CD40L nor the stimulatory mAb to CD28 could significantly change TNF-α release in peptide-stimulated cultures. These data suggested that TNF-α was secreted primarily by the T cell hybridomas, but the TNF-α production by T cells was independent of the interactions via costimulatory molecules. In contrast to TNF-α, IL-6 and IL-10 were produced only in cultures in which the peptide was presented by macrophages (Table I), indicating that the primary source of these cytokines in vivo could be the macrophage. Peptide-stimulated 5/4E8-macrophage cultures contained 3 times more IL-6 than TA20-macrophage cultures, while the IL-10 content was comparable in both cultures (Table I). Production of both IL-6 and IL-10 was 2- to 3-fold greater in the presence of anti-CD28 Abs, but was almost completely blocked by anti-CD40L Ab (Table I). As IL-6 has been consistently detected at highly increased levels in RA, and IL-6 deficiency prevents or ameliorates some forms of experimental arthritis (35, 36), the overproduction of IL-6 may be relevant to pathological changes induced by joint-infiltrating 5/4E8 cells in our hybridoma-induced arthritis model. Thus, it may contribute to the observed cartilage destruction by augmenting aggrecanase-dependent proteoglycan catabolism in articular cartilage (37). Consistent with the in vitro results (Table I), self peptide-primed LN cells produced large amounts of cytokines (Fig. 6B), whereas this self peptide-induced cytokine production was significantly lower than those induced by heterologous (human) peptide. These results together suggest that 5/4E8 hybridoma represents a more flexible T cell population than TA20 cells in response to the self peptide sequence, but essentially both T cell populations should exist in proteoglycan-immunized mice.
Peptides with amino acid substitutions within the TCR binding site (P2, P3, and P5) failed to induce any cytokine release, indicating that specific T cell signals are required even for the production of cytokines (IL-6 or IL-10) by macrophages. In contrast, hybridomas stimulated with peptides altered at the MHC binding site (P4, P6, P7, or P8), although partially or completely lost IL-2 (Fig. 3), IFN-γ, or IL-4 (data not shown) secretion, the IL-5, IL-10, and TNF-α levels were similar to wild-type peptide-stimulated cultures (Table I). Thus, while a single amino acid substitution at the MHC binding site may greatly diminish the conventional T cell response (measured by IL-2, IFN-γ, or IL-4 production), production of other cytokines may remain less influenced, or unaffected. Moreover, while an altered peptide (e.g., substituted at P6 or P7) may fail to provoke appropriate signals emanating from the TCR, T cells appear to be able to transmit signals to APCs via costimulatory interaction.

**Discussion**

This study was prompted by the puzzling difference in the arthritogenic properties of two Th1 hybridomas (13) that recognized the same proteoglycan (aggrecan) peptide epitope and exhibited identical Vα1 and Vβ4 TCR sequences. Using a panel of synthetic human (50 μg peptide/ml) cartilage aggrecan, and mouse-specific synthetic peptide (ATEGQYRVSAYQDK) at 100–150 μg/ml gave highly comparable results; thus cytokine productions in the presence of the human sequence are shown only, except for TNF-α when the mouse peptide was even more stimulatory for 5/4E8 cells than the wild-type human peptide.
two clones. The peptide ligands, we compared the fluence of flanking regions of the core epitope on the degree of peptide-specific responses (Fig. 2). Similarly, a profound influence of flanking residues on epitope recognition has been demonstrated earlier in several studies (26, 27, 30, 31). Although the consensus nonpeptide epitope flanked with either 0- to 6-aa-long natural sequences or 1–2 Ala spacers at both ends induced uniformly high responses from 5/4E8 cells, these combinations in the flanking sequences proved to be suboptimal for the TA20 clone. The poor response by TA20 hybridoma to the peptide with no flank on the N terminus of the core sequence seems to identify TA20 as a clone of peptide-flanking residue-dependent cells (31). Similarly, we found marked differences in the response by the two clones when single amino acid substitutions were introduced into the shared nonapeptide sequence. Interestingly, several substituted peptide analogues were more stimulatory than the core sequence; some of them seemed to be even more preferential ligands for 5/4E8 cells than the native (wild-type) core epitope.

Addition of 1–2 Ala spacers to the core epitope dramatically improved the responding capabilities of these two clones, but in a different nature. To explain this difference, one has to consider the fact that the addition of Ala flanks can alter the conformation of the peptide (38). Consequently, the core epitope could adopt slightly different three-dimensional structures in constructs with different flanking residues. It is likely that the interaction among members of the MHC-peptide-TCR three-party system permits some conformational flexibility without altering the specificity (39–41). Therefore, the same GRVRVNSAY core peptide flanked with one or two alanines, or the wild-type sequence at the corresponding position, might induce a defined conformation, which then could be accommodated slightly differently between the MHC and TCR molecules in the two hybridoma cells. This slight change in conformation (especially at the N terminus) resulted in the altered T cell response, but did not change the specificity of the hybridomas. The negative effect of double Ala flanks on recognition by TA20 cells resembles hindrance observed by us and others in several experimental systems (26, 27).

Together, these results indicate that: 1) residues both outside (flanking region) and inside the core epitope (consensus sequence) have a differential impact on the interaction with TCR molecules; 2) Ag recognition by the two phenotypically identical aggrecan-specific T cell clones was degenerate; and 3) differences in this degeneracy distinguished the two clones. In recent years, it has become clear that TCR recognition of peptide is promiscuous. In other words, a single TCR can recognize a large continuum of altered ligands, and reciprocally, the same peptide-MHC complex can be recognized by several different TCRs (40–46). Expression libraries, positional scanning libraries, and one-bead-one-peptide library screening strategies clearly proved that the same TCR can interact with a whole repertoire of peptides (41, 47, 48).

As a conclusion, an epitope-specific T cell repertoire may represent a spectrum of T cell clones with varying degrees of specificity/degeneracy in peptide recognition. It has been hypothesized that T cell clones with degenerate peptide recognition are more frequently engaged in an immune response and are more likely to participate in autoimmunity due to cross-reactivity between a microbial and a self peptide (49). This study provides some evidence for this hypothesis, as we show that autopathogenicity of an arthritogenic Th1 clone is associated with highly degenerate peptide recognition. The 5/4E8 T cell hybridoma, which is more flexible in peptide recognition, i.e., tolerates alterations either in the flanking or the core sequence better than TA20 cells, is arthritogenic in BALB/c mice, while the TA20 hybridoma is not (13). As these two T cell hybridomas are phenotypically identical, carrying the same TCR (9), a highly flexible self epitope recognition by 5/4E8 cells, in context with appropriate APCs in the synovium, may contribute to the initiation of autoimmune/inflammatory reactions in the joint. Our findings are strongly supported by a recent study that isolated multireactive T cell clones from inflamed rheumatoid synovium, several of which recognized more than one peptide structure (46, 50). Also, promiscuous epitope recognition has been recently implicated in the pathomechanism of multiple sclerosis and lupus (51, 52).

In this study, we found that peptides that bound poorly to the MHC (e.g., P4, P6, or P7) could still elicit high responses from the 5/4E8 cells, as indicated by release of high amounts of IL-2 (Fig. 3), or TNF-α (Table I), as demonstrated in other systems (53–56). The differences in replacement sensitivities are presumably due to different three-dimensional positions of the peptides in the MHC-TCR sandwich. The common feature of changes caused by Ala substitutions in stimulatory activity of the two clones is that Arg in P2 and P4 and Val in P3 and P5 and Gly in P1 positions are crucial for maintaining at least partial activity. Ala substitutions in other positions are more acceptable for 5/4E8 than for TA20 cells. This could be interpreted as a consequence of the fact that residues flanking the N terminus of the core epitope are firmly locked in the MHC-peptide-TCR complex, while the C-terminal part has more, but clone-dependent, conformational freedom in the space provided by the MHC/TCR (26). Changes (Ala substitution) in this part of the molecule have essentially no effect on stimulation of the 5/4E8 clone, but lead to a pronounced decrease in the case of TA20. It is an attractive speculation that perhaps the space for flexibility is larger in one complex (5/4E8 clone) than in the other (TA20). X-ray data on both complexes could provide a more exact explanation for the finding.

Based on these observations, we can conclude that the two Th1-type hybridomas with identical TCR sequence and core protein peptide recognition sites are able to produce fundamentally different set and amounts of cytokines when these T cells are stimulated with the same peptide presented by either the same or different APCs. Thus, pro- and anti-inflammatory cytokine secretions mostly depend on the type of APC transmitting costimulatory signals to even suboptimally activated T cells. Therefore, while the primary responsiveness of a T cell is determined by a specific peptide sequence, both T cells and APCs (B cells, macrophages, dendritic cells) exhibit flexibility in responding to a highly specific or a less specific signal.

Initially, we hypothesized that the TCR of 5/4E8 cells might have an enhanced affinity for MHC-II molecules, which could compensate for the reduced peptide-MHC-II binding. Normally, thymic selection is expected to delete T cells that have high affinities for the MHC (regardless of its peptide load), and thus are likely to be self reactive (57). We speculate that the parent cell of the 5/4E8 hybridoma could represent a T cell that, despite high MHC-binding affinity, escaped thymic TCR-peptide-MHC avidity selection.

Neither of the two T cell hybridomas responded to mouse aggrecan upon natural processing (22), but they both recognized human aggrecan (used for immunization). Both mouse (ATEGQVRVNSIYQDK) and human (ATEGVRVNSAYQDK) synthetic peptide sequences were, however, recognized by both 5/4E8 and TA20 cells (Fig. 5), and both peptides primed LN cells. Therefore, this study identified the mouse sequence-specific peptide as a cryptic self-determinant. An increasing body of evidence supports the notion that poor presentation of a cryptic epitope may
result from several competitive interactions with other determinants of the same molecule (58). A dominant epitope(s) gains access to most MHC/TCR molecules; thus, upon priming with the whole molecule, polyclonal T cell activation will be directed predominantly to this determinant(s). Immunizing mice with mouse aggrecan and using P70–84 as a recall Ag, oligoclonal responsiveness to the cryptic aggrecan peptide could remain hidden in the bulk polyclonal response. Consequently, our failure to detect P70–84-specific polyclonal stimulation does not rule out that P70–84 was presented at least by a minor proportion of MHC molecules. We hypothesize that in vivo this cryptic epitope of mouse aggrecan could also occupy the peptide-binding groove of some pericellular/synovial endothelial cells (as a peptide fragment of a molecule taken up by endocytosis from the tissue environment). Local recognition by circulating self epitope-reactive 5/4E8 cells could thus contribute to extravasation of these cells similar to what has been shown for synovium for autoreactive T lymphocytes (59–61).

Although the P2/Ala-substituted peptide variant was not recognized by either of the two hybridoma clones, this peptide (like the mouse analogue) successfully primed naive mice (Fig. 6). Thus, a single amino acid in a T cell epitope could elicit cross-reactive autoreactive T cell populations that could be heterogeneous in TCR structure. The specificity of these cells can be best described as partially overlapping sets of epitopes. Such populations might therefore include cells with different functional properties or different activation pathways (62, 63), and one of the functions could result from several competitive interactions with other determinants of the same molecule. Recognition of a self peptide is considered to be a critical issue in autoimmunity. Therefore, we should consider that differential homing and recognition of a self epitope do not necessarily represent independent features of the two clones. Tissue-specific homing has been recently hypothesized to be, in part, dependent on a cell’s recognition of the cognate MHC/peptide complexes presented by local endothelial cells (59–61). Recruitment of cells expressing Ag-specific TCR has also been demonstrated at sites of Ag expression (64). Thus, we consider that recognition of the locally presented aggrecan self epitope could possibly contribute to the periarthritic/arthritic trafficking of the 5/4E8 cells. Although both hybridomas recognized the mouse peptide sequence, it seemed to be a suboptimal ligand for these clones in comparison with the corresponding human peptide. Within the range of sensitivity tested, T cell hybridoma 5/4E8, however, has recognized the self aggrecan sequence at much lower concentrations than the TA20 cells, and this low threshold sensitivity could be sufficient for initiation of inflammatory processes in the joints. Whether arthritis induction by 5/4E8 cells results merely from the enhanced recognition of the murine self peptide (as part of degeneracy), accompanied by high IFN-γ, IL-6, and TNF-α production, remains to be established.

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