RECOGNITION OF A MYCOBACTERIA-SPECIFIC EPITOPE IN THE 65-kD HEAT-SHOCK PROTEIN BY SYNOVIAL FLUID-DERIVED T CELL CLONES

By J. S. HILL GASTON,* PAUL F. LIFE,* PETER J. JENNER, M. JOSEPH COLSTON,† AND PAUL A. BACON*

From the *Department of Rheumatology, University of Birmingham, Birmingham; and †Laboratory for Leprosy and Mycobacterial Research, National Institute for Medical Research, Mill Hill, London, United Kingdom

T cell–mediated responses to mycobacterial antigens (Ags)1 have been implicated in the pathogenesis of inflammatory arthritis both in experimental animals and in man (1–5). In adjuvant arthritis (AA) in rats, it has been clearly established that the disease can be initiated by T cell lines and clones specific for the 65-kD mycobacterial heat-shock protein (HSP) (4); the epitope recognized by the arthritogenic T cell clone has been localized to amino acids 180–188. Animals can also be rendered resistant to the induction of AA by prior immunization either with 65 kD–specific T cell clones or with the 65-kD Ag itself (4, 5). Interestingly, previous challenge with mycobacterial 65-kD Ag is also effective in preventing the arthritis induced by streptococcal cell walls or by pristane (6, 7). A role for the 65-kD Ag is not confined therefore to arthritis induced by mycobacterial Ags.

That these observations in animal models of arthritis may be relevant to arthritis in man has been suggested by the finding that T cells from synovial fluid frequently respond to the 65-kD Ag (8–10). These responses are more marked in synovial fluid than in peripheral blood. Recently T cell clones that use either α/β or γ/δ receptors in their recognition of the 65-kD Ag have also been isolated from the joint (11), while elevated titres of antibody to the 65kD Ag have also been reported in rheumatoid arthritis (12, 13).

The mechanisms whereby immune responses to the 65-kD Ag result in arthritis are not known. A homology noted between the 65-kD antigenic epitope implicated in AA and the link protein of cartilage proteoglycan raised the possibility that 65 kD–specific T cells might crossreact with this autoantigen (4). However, further studies have shown that the homologous peptide from link protein does not stimulate the arthritogenic T cell clone (14). Attention has also been focused on the fact that the

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Address correspondence to Dr. J. S. Hill Gaston, Department of Rheumatology, University of Birmingham, Birmingham, United Kingdom.

1 Abbreviations used in this paper: AA, adjuvant arthritis; Ags, antigens; CM, complete medium; dpm, disintegrations per minute; HS, human serum; HSP, heat-shock protein; PPD, purified protein derivative of tuberculin; SI, stimulation index; SF, synovial fluid.

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65-kDa Ag is highly conserved, not only among bacteria (15), but also in eukaryotes (16), so that some T cells might respond to epitopes common to both the bacterial and eukaryotic Ag. Since HSPs may be produced at sites of inflammation (17), T cells initially induced by bacterial Ags could conceivably mediate autoimmune disease.

In this paper we describe human 65 kD-specific T cell clones, isolated from the synovial fluid of a patient with an acute, self-limiting inflammatory arthritis. Using synthetic peptides, these were shown to respond to an epitope that is mycobacteria specific.

Materials and Methods

**Patient.** Synovial fluid (SF) was aspirated from the knee of a 53-yr-old white man who had developed acute lower limb inflammatory arthritis 2 wk previously. The arthritis was preceded by a florid conjunctivitis and low back pain. In view of these clinical features a diagnosis of reactive arthritis (18) was made, but there was no history of preceding gastrointestinal or genito-urinary infection and no serological evidence of preceding infection by Salmonella, Campylobacter, Yersinia, or Chlamydia. The arthritis resolved rapidly over the following month and has not recurred. The patient's HLA type was A1, B8, DR3.

**Proliferation Assays.** SF mononuclear cells (SFM) and PBMC were isolated and tested for their proliferative responses to mitogens and bacterial Ags as previously described in detail (9, 19). Briefly, 5 × 10^6 SFM or PBMC were suspended in RPMI 1640 medium containing 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (complete medium [CM]), supplemented with 10% FCS and 1% human serum (HS), and cultured for 6 d in triplicate 0.2 ml U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) at 5 × 10^4 cells/well.[^H]Thymidine incorporation (disintegrations per minute [dpm]) was measured during the last 18 h of culture. The stimulation index (SI) was defined as[^H]thymidine incorporation in the presence of Ag divided by[^H]thymidine incorporation in the presence of medium alone.

**Antigens.** Purified protein derivative of tuberculin (PPD) was obtained from Statens Serum Institut, Copenhagen, Denmark. Escherichia coli GroEL (20) was a kind gift of Dr. Douglas Young, Hammersmith Hospital, London, UK. The recombinant 65-kD Ag of Mycobacterium leprae was prepared as described in reference 21. Suspensions of bacteria associated with reactive arthritis were sterilized by heat or irradiation before use in proliferation assays and soluble Salmonella Ags prepared using 0.1% SDS (SA/SDS) as previously described (10).

**Synthetic Peptides.** A library consisting of 106 overlapping peptides, of between 15 and 19 amino acids in length, was synthesized using a simultaneous multiple-peptide solid-phase synthetic method (22) using a polyamide resin (23) and FMOC chemistry. Completed peptides were extracted from the resin using trifluoroacetic acid and suitable scavengers, and isolated by solvent evaporation and precipitation with methanol and diethylether. Purity was checked by aminoacid analysis, and by HPLC.

**T Cell Cloning.** T cell blasts were harvested from 6-d cultures of SFM stimulated with the 65-kD Ag and cloned by limiting dilution in 0.2 ml wells using 4 × 10^4 irradiated (3,000 rad) autologous (auto)PBMC as APC, 100 U/ml rIL-2 (24) (Cetus Corp., Emeryville, CA), and 20 μg/ml 65 kD, in CM supplemented with 5% HS, 1% nonessential amino acids, and 1% sodium pyruvate. Growing colonies were transferred to fresh wells on day 13 and restimulated with 10^5 irradiated allogeneic PBMC, 1 μg/ml purified PHA (Wellcome, Beckenham, UK), and 100 U/ml IL-2 in CM supplemented as for cloning. Expansion was continued by twice weekly feeding with IL-2 and restimulation every 10-14 d with allogeneic (allo)PBMC and PHA. Clones were only tested in proliferation assays at least 7 d after restimulation. The phenotype of clones was determined by staining with mAbs and flow cytometry.

**Determination of the Antigenic Specificity of Clones.** Clones were screened for responses to the 65-kD Ag by removing an aliquot of cells, washing in fresh medium, and coculture with 10^5 irradiated auto-PBMC with or without the 65-kD Ag (10 μg/ml) for 72 h.[^H]Thymidine incorporation was measured as above. In subsequent proliferation assays 10^4 T cell/well were used. In addition to PBMC, auto- and allo-EBV-transformed lymphoblastoid cells (EBLCL)
were used as APC. These were cultured overnight in CM containing 5% HS with or without Ag, and then irradiated (6,000 rad) before culture with the T cells. Soluble Ag was also included in these assays. In assays using synthetic peptides, the peptide was applied to nitrocellulose (nc) discs (Hybond-C; Amersham Corp., Amersham, UK), which were then dried under vacuum, sterilized by irradiation, and placed directly into culture wells containing the T cell clone and APC (cf. reference 25). SIs were calculated as described above.

The effect of mAbs specific for HLA Ags on T cell proliferation was tested by culturing the appropriate mAb dilution with APC for 30 min before the addition of T cells. mAb W6/32 (HLA-A,B,C-specific) and RFBI (HLA-DR-specific) were obtained from Seralab (Crawley, Sussex, UK) and used as ascites; L243 (American Type Culture Collection, Rockville, MD), an HLA-DR-specific mAb, was used as hybridoma supernatant.

The cytotoxicity of clones was assessed by measuring $^{51}$Cr release from autologous Ag-pulsed macrophages after 16 h of coculture with T cells at an E/T ratio of 5:1, as described in detail elsewhere (26).

Results

Proliferative Responses to M. leprae 65-kD Antigen by SFM and PBMC. SFM and PBMC, isolated from a patient with acute inflammatory arthritis, were tested for their ability to respond to bacterial Ags (Fig. 1). Although the clinical picture was suggestive of reactive arthritis, unlike the majority of such patients (see reference 19) the SFM did not respond to any of the organisms tested that are known to trigger arthritis. However, a marked response to the recombinant 65-kD Ag of M. leprae was noted; this was similar in magnitude to the response to an optimal concentration of PPD (representing multiple mycobacterial Ags, including the 65-kD Ag). The proliferative responses of PBMC tested concurrently were generally low, but a significant response to PPD was recorded (SI similar to that seen using SFM; Fig. 1 b), whereas no PBMC response to the 65-kD Ag could be detected. These SFM and PBMC were tested on two occasions with the same results. To investigate in detail the marked recognition of the 65-kD Ag by the SFM, cloning studies were undertaken.

Properties of Synovial Fluid-derived 65-kD-specific T Cell Clones. 22/31 T cell clones, derived from wells seeded with 0.3–10 T cell blasts (obtained from SFM stimulated for 6 d with 65-kD Ag), were found to respond specifically to the 65-kD Ag. Two clones (from cultures seeded at 1 cell/well) were characterized in detail; by flow cytometry analysis they were both CD3+, CD4+, CD8-, TCR$\delta$+. Single rearrangements of both TCR $\beta$ and $\gamma$ chain genes, consistent with clonality, were noted when DNA from the clones was compared with that of the autologous EBLCL by Southern blotting, using both $\beta$ and $\gamma$ chain gene-specific probes (data not shown). Evidence for their antigenic specificity is given in Table I. Both clones proliferated in response to the recombinant M. leprae 65-kD Ag, and to PPD, presented by either autologous or HLA-DR3-matched PBL and EBLCL. The apparent restriction by HLA-DR was confirmed by blocking studies; an HLA-DR-specific mAb (RFBI) decreased proliferation to a much greater extent than the anti-class I mAb, W6/32 (Table II). The response to Ag was essentially abolished by RFBI whereas that to PHA and IL-2 was still evident even at the highest mAb concentration used. Another HLA-DR-specific mAb (L243) also inhibited the response to Ag by >80% (data not shown). In addition to proliferative responses, both clones were capable of lysing PPD- or 65-kD-pulsed macrophages (29 and 27% lysis of PPD-pulsed macrophages; 51 and 43% lysis of 65-kD-pulsed macrophages by clones MAW1.4 and MAW1.5, respectively).
Mapping of the Epitopes within the 65-kD Ag Recognized by Synovial T Cell Clones. A panel of 106 overlapping peptides of between 15 and 19 amino acids in length and corresponding to the complete sequence of the 65-kD Ag (27) was used for epitope mapping. The peptides were divided into 11 pools for initial screening. Table III, Exp. a, shows that using clone MAW1.4 only pool 1 (peptides 1–10) proved stimulatory; in addition, neither recombinant E. coli 65 kD (GroEL) nor soluble Ags derived from Salmonella were recognized, indicating that the epitope seen was unlikely to be found in a conserved region of the 65-kD sequence. When the peptides comprising pool 1 were tested individually (Table III, Exp. b), only peptide 1 (amino acids 1–15) was stimulatory; the partially overlapping peptide 2 (amino acids 6–22) gave only background proliferation. The sequence of peptide 1 is shown in Table
TABLE I

Specificity of Clones MAW1.4 and MAW1.5

| Exp. | Stimulus          | Proliferative response (\[^{3}H\]thymidine incorporation) | dpm |
|------|-------------------|----------------------------------------------------------|-----|
|      |                   | Clone MAW1.4 | Clone MAW1.5 | APC alone |
| 1    | Medium            | 264          | 186          |          |
|      | Auto PBMC         | 150          | 58           | 256      |
|      | Auto PBMC + 65kD  | 18,998       | 16,546       | 596      |
| 2    | Medium            | 136          | 122          |          |
|      | Auto EBLCL        | 420          | 674          | 2,979    |
|      | Auto EBLCL + 65kD | 19,781       | 16,658       | 2,769    |
| 3    | Medium            | 178          | 262          |          |
|      | MR EBLCL (DR3 +)  | 71           | 117          | 602      |
|      | MR EBLCL (DR3 +)  + PPD | 18,996 | 10,890 | 665 |
|      | SW EBLCL (DR3 -)  | -83          | 45           | 1,009    |
|      | SW EBLCL (DR3 -)  + PPD | 234      | -16          | 757      |
|      | NT EBLCL          | 147          | 238          | 103      |
|      | NT EBLCL + PPD    | 250          | 321          | 409      |

10^4 T cells were cocultured with 10^5 irradiated autoPBMC (1,500 rad) or 10^4 auto- or allo-EBLCL (6,000 rad) in CM supplemented with 5–10% HS. Ag concentrations were 20 μg/ml and 7.5 μg/ml for 65 kD in Exp. 1 and 2 respectively, and 10 μg/ml for PPD. Before irradiation, EBLCL were cultured overnight either in CM-5% HS alone, or with 10 μg/ml 65 kD or 20 μg/ml PPD. \[^{3}H\]Thymidine incorporation by triplicate (Exp. 2 and 3) or duplicate (Exp. 1) cultures was measured after 3 d. The results are shown as dpm after subtraction of the \[^{3}H\]thymidine incorporation by the corresponding APC when cultured alone; the latter is shown in parentheses.

IV. Two other clones (including MAW1.5) tested in parallel with MAW1.4 gave identical results.

To determine whether these three clones were typical of all those obtained, further 65-kD-responsive clones were tested for their ability to respond to peptides. The results for 11 clones are summarized in Table V. Again pool 1 stimulated responses by the majority of clones tested, but the clearest results were obtained using peptide 1 alone. All 11 of these clones, and indeed all 15 of the 65 kD Ag–specific clones tested responded to this peptide. Control autologous clones that did not respond to the 65-kD Ag also failed to recognize peptide 1 (data not shown). The differences in the results obtained when using pool 1 and peptide 1 may well reflect inhibition by peptides that can bind to DR3 but that do not contain the T cell epitope. Peptide 9 was weakly stimulatory for one clone (SI = 14.4); however, since the corresponding SI for peptide 1 was 163, the significance of this weak response to peptide 9 is uncertain.

Dominance of the Response to Peptide 1 in a Polyclonal T Cell Population. PBMC, obtained 3 mo after resolution of the arthritis, were tested for their proliferative responses. These PBMC, in marked contrast to those tested earlier, showed a significant response to the 65-kD Ag (Fig. 2). Furthermore, peptide 1 elicited a response similar to that seen using the whole 65-kD Ag molecule, suggesting that the epitope identified by using SFM-derived clones was also dominant in the response mounted by the peripheral T cell population.
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Table II

| Inhibition of the 65 kD-specific Proliferative Response of Clone MAW1.4 by mAbs |
| Proliferative response ([3H]thymidine incorporation) at antibody concentration of: |
| None | 1:10,000 | 1:1,000 | 1:100 |
|---|---|---|---|
| (a) RFB1 (anti-class II) | | | |
| T + APC + 65 kD | 26,452 | 19,462 | 13,575 | 1,242 |
| T + APC + IL2/PHA | 26,960 | 21,290 | 20,993 | 14,020 |
| (b) W6/32 (anti-class I) | | | |
| T + APC + 65 kD | 21,791 | 25,669 | 23,875 | ND |
| T + APC + IL2/PHA | 23,510 | 21,578 | 15,270 | ND |

10^4 MAW1.4 T cells were cocultured with 4 x 10^4 allogeneic irradiated PBMC (3,000 rad) from DR3+ donor MR (see Table I), and either 10 μg/ml 65-kD Ag or 1 μg/ml PHA + 50 U/ml IL-2. Appropriate dilutions of mAbs RFB1 and W6/32 were added to the PBMC that were incubated at 37°C for 30 min before the addition of T cells. [3H]thymidine incorporation was measured after 3 d. All cultures were performed in triplicate. T cells cultured with the APC in the absence of Ag, or with IL-2 in the absence of PHA and APC, gave 291 and 1534 dpm, respectively; APC cultured alone gave 138 dpm.

Discussion

Interest in the role of the 65-kD heat-shock protein in joint inflammation stems in part from its implication in the pathogenesis of AA (3, 4) and other forms of experimental arthritis (6), coupled with evidence that immune responses to this same Ag may be prominent in human subjects with arthritis, particularly within the inflamed joint (2, 8–10). In AA, an arthritogenic clone was shown to respond to the epitope formed by amino acids 180–188. In this report we have mapped the antigenic epitope recognized by a series of SF-derived T cell clones from a patient with an acute inflammatory arthritis. In all cases the response was to an epitope contained within amino acids 1–15 but not 6–22. A minimal peptide has not been established for these clones, but the epitope is likely to be identical to that previously described for clones from a DR3+ leprosy patient (amino acids 2–12) (28, 29). It is of interest that this epitope is the only one so far described for DR3-restricted 65-kD antigen-specific T cells. In the present case (a DR3 homozygous individual), postarthritogenic patients also gave a marked response to the peptide, suggesting that this single epitope dominates the T cell response to the 65-kD Ag, and thus to mycobacterial Ags, since the response to the 65-kD Ag was a substantial proportion of the response to all the mycobacterial Ags represented within PPD. It is likely that more than one 65 kD-derived peptide is capable of binding to DR3, so that the recognition of a single peptide implies that the T cell repertoire for the 65-kD Ag is markedly restricted in DR3+ individuals. Despite this, DR3 is associated with immunoresponsiveness to mycobacterial Ags (30), and it is relatively rare for lepromatous leprosy (i.e., T cell nonresponder) patients to be DR3+ (31). DR3-bearing haplotypes are also strongly associated with autoimmune diseases (32) (though not arthritis), but whether there is any relationship between the dominant response to a single epitope in the mycobacterial 65-kD Ag and susceptibility to autoimmunity remains to be established.
TABLE III
Proliferative Responses of Synovial Clone MAWJ.4 to Synthetic Peptides Corresponding to the M. leprae 65-kD Ag

| Stimulus | Exp. a* | Exp. b1 |
|----------|---------|---------|
| PBSnc    | 171     | PBSnc  | 228 |
| 65kDnc   | 17,555  | 65kDnc | 15,504 |
| Pool 1   | 4,408   | Peptide 1 | 11,809 |
| Pool 2   | 190     | Peptide 2 | 322 |
| Pool 3   | 193     | Peptide 3 | 117 |
| Pool 4   | 793     | Peptide 4 | 142 |
| Pool 5   | 137     | Peptide 5 | 294 |
| Pool 6   | 131     | Peptide 6 | 218 |
| Pool 7   | 309     | Peptide 7 | 100 |
| Pool 8   | 190     | Peptide 8 | 167 |
| Pool 9   | 193     | Peptide 9 | 129 |
| Pool 10  | 234     | Peptide 10 | 110 |
| Pool 11  | 170     | Pool 1 | 1,189 |
| Pool 1-11| 258     | Pool 3 | 95 |
| Medium   | 137     | Pool 5 | 102 |
| 65kD     | 29,522  | Pool 11 | 115 |
| GroEL    | 137     | Medium | 307 |
| SA/SDS   | 487     | 65kD | 27,948 |
| PPD      | 32,110  | GroEL | 259 |
|          |         | SA/SDS | 446 |
|          |         | PPD | 18,849 |

10^4 T cells were cocultured with 5 x 10^4 irradiated autoPBMC (3,000 rad). The Ags 65 kD, GroEL, SA/SDS, and PPD were all used at a final concentration of 10 μg/ml. 65 kD and pooled or individual peptides (4 μl per fraction, using Ags at 1 ng/ml) were applied to nc as described in Materials and Methods resulting in a final concentration of 20 μg/ml for 65 kD and individual peptides and 2 μg/ml for each peptide constituent of the pools. [3H]Thymidine incorporation by triplicate cultures was measured after 3 d. APC cultured alone gave 162 dpm.

* Exp. a, pooled peptides.
† Exp. b, individual peptides from pool 1.
§ PBS or antigen applied to nitrocellulose as described in Materials and Methods.

In AA, the 65 kD-specific clone has been shown directly to be able to initiate disease (1,5); the involvement of the human 65 kD-specific clones in the pathogenesis of joint inflammation must remain more conjectural. Nevertheless, the marked response to the 65-kD Ag in SF, which was not reflected in PB during acute inflammation, is striking. This response was of a similar magnitude to the response to PPD; in contrast, when a response to the 65-kD Ag appeared in PB it accounted for ~50% of the response to PPD. This suggests that there may have been some localization of 65 kD-specific T cells within the synovial compartment during acute inflammation. Synovial T cells show a surface phenotype associated with memory cells (33), but whether all memory T cells are recruited equally to a site of inflammation is not known.

The dominant epitope seen by the SF T cells proved to be in a nonconserved por-
tion of the 65-kD Ag (Table IV); this was also the case for the 180–188 epitope recognized by the rat arthritogenic clone. Thus, neither rat AA, nor the present case of human arthritis, is related to T cells specific for epitopes common to the bacterial and eukaryotic (self) 65-kD Ag, although such clones have been demonstrated (34). The rat clone also responds to rat proteoglycan (4, 35), but the particular protein containing the putative crossreactive epitope has not yet been identified. The human synovial 65 kD-specific clones will now be tested for crossreactive responses to joint constituents including proteoglycan and collagen. In addition, it will be of interest to determine the TCR V region genes used to recognize 65 kD Ag-specific epitopes, since recent work in experimental encephalomyelitis has shown that recognition of myelin basic protein by encephalitis-inducing T cell clones is associated with the use of particular V region genes (36).

### Table IV

**Sequence of Stimulatory Peptide 1 from the *M. leprae* 65-kD Ag**

| Aminoacid number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|
| **M** | **A** | **K** | **T** | **I** | **A** | **Y** | **D** | **E** | **E** | **A** | **R** | **G** | **L** |

Sequence of the NH2-terminal 15 amino acids of the *M. leprae* 65-kD Ag using the single letter amino acid code. Residues conserved in both GroEL and the human 65-kD homologue are underlined.

### Table V

**Proliferative Responses of MAW Synovial T Cell Clones to Synthetic Peptides Corresponding to the *M. leprae* 65-kD Ag**

| Exp | Ag     | Mean SI | Range     |
|-----|--------|---------|-----------|
| 1   | 65kDnc | 67.5    | 29.9–141.8|
|     | SA/SDSnc | 1.0    | 0.8–1.6   |
|     | Peptide 1 | 12.1   | 5.0–22.4  |
|     | Peptide 5 | 0.7    | 0.5–0.9   |
|     | Peptide 10| 0.9    | 0.5–1.1   |
|     | Pool 1   | 4.0    | 2.1–8.6   |
|     | Pool 5   | 0.9    | 0.5–1.1   |
|     | Pool 11  | 1.1    | 0.8–1.8   |
| 2   | 65kDnc | 91.8 | 23.7–328.5|
|     | SA/SDSnc | 1.5    | 0.7–1.6   |
|     | Peptide 1 | 45.1   | 5.1–163.0 |
|     | Peptide 4 | 1.4    | 0.8–2.2   |
|     | Peptide 9| 3.9    | 1.2–14.4  |
|     | Pool 1   | 5.3    | 1.3–13.9  |
|     | Pool 5   | 1.4    | 1.0–2.2   |
|     | Pool 11  | 1.1    | 0.8–1.8   |

$10^6$ T cells were cocultured with $5 \times 10^4$ (Exp. 1) or $10^5$ (Exp. 2) DR3 + allo-PBMC (donor MR, Tables I and II) and Ags or peptides applied to nCs as described in Table III. $[^3]H$Thymidine incorporation by triplicate cultures was measured after 3 d. SIs for each Ag:clone combination were calculated by reference to the PBSnc control.
Summary

Adjuvant arthritis in rats is induced by a T cell clone specific for amino acids 180-188 of the mycobacterial 65-kD heat-shock protein, and synovial T cell responses to this same Ag have been noted in human arthritis. We have isolated 65-kD Ag–specific T cell clones from synovial fluid mononuclear cells of a patient with acute arthritis, which, unlike the corresponding PBMC, showed a marked proliferative response to the 65-kD Ag. Using synthetic peptides corresponding to the whole sequence of the 65-kD Ag, all the clones were shown to recognize an epitope present in the first NH₂-terminal peptide (amino acids 1-15), with no response to the adjacent peptide (amino acids 6-22) or to any other peptide. The complete dominance of this epitope in the response to the 65-kD Ag was shown by documenting responses to the peptide in PBMC obtained after recovery from the arthritis. This epitope, like that recognized by the rat arthritogenic T cell clone, is in a portion of the 65-kD sequence that is not conserved between bacteria and eukaryotes, so that in this case, joint inflammation could not be attributed to bacteria-induced T cell clones cross-reacting with the self 65-kD Ag.

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