Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

18. A series of degenerate oligonucleotides were synthesized that correspond to the coding or noncoding strands for segments of the tryptic peptide sequences. The oligonucleotides contained nondegenerate 5′ ends; the end of each coding strand oligonucleotide had an Eco RI restriction site, while the end of each noncoding strand oligonucleotide contained a Sal I restriction site. Each coding strand oligonucleotide was combined with each noncoding oligonucleotide in individual PCR reactions with cDNA from Rat 1 cells as template; the PCR reactions and the preparation of the genomic and cDNA templates were performed as described (*27*) [A. Bothwell, G. Yancopoulos, F. Alt, *Methods for Cloning and Analysis of Eukaryotic Genes* (Jones and Bartlett, Boston, MA, 1990)]. The amplified product obtained with the QYIGEG coding oligonucleotide and the DLKPSN noncoding oligonucleotide (designated QYDL) was isolated on a Sephadex G-50 spin column, digested with Eco RI and Sal I, gel-purified with 2% Nusieve (FMC Bioproducts), and subcloned into the pGEM4Z vector (Promega).
19. Plaques (600,000) from a rat brain cDNA library constructed in the Lambda Zap II vector (Stratagene) were screened with the subcloned QYDL PCR product as the probe, radiolabeled with a PCR-based protocol (*27*). Hybridization conditions have been described (*27*); after hybridization the library filters were washed first at low stringency [2× SSC (20 mM sodium citrate, pH 7.0, 0.15 M NaCl), 0.1% SDS, 60°C] and then at high stringency (0.2× SSC, 0.1% SDS, 60°C).
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Recognition of a Peptide Antigen by Heat Shock–Reactive γδ T Lymphocytes

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Small synthetic peptides that correspond to different portions of the 65-kilodalton mycobacterial heat shock protein (Hsp65) were used to identify a putative antigenic epitope for γδ cells. Weaker γδ responses to the equivalent portion of the autologous homolog, mouse Hsp63, were also seen. The stimulatory epitope overlaps with an epitope recognized by arthritogenic αβ T cell clones. The data suggest that γδ cells have a role in autoimmune disorders and imply that these cells recognize ligands by a mechanism similar to that of αβ T lymphocytes, that is, in the form of small processed protein fragments bound to antigen-presenting molecules.

LYMPHOCYTES BEARING THE αβ T cell receptor (TCR) recognize foreign antigens either in the form of small, processed protein fragments or as "superantigens" (*1*). The nature of the ligands recognized by γδ cells is not known, although structural similarities between αβ and γδ TCRs, and the demonstration of γδ cell responses to major histocompatibility complex (MHC) class I and class I like alloantigens, including Qa, TL, and CD1, suggest that γδ and αβ ligands are similar (*2, 3*). The smaller receptor repertoire of γδ cells implies, however, that fewer ligands are recognized. For these reasons and because of the frequent association of γδ cells with epithelia, it has been suggested that γδ cells may act as a first line of defense (*4*) and respond to stress signals of surrounding tissues (*5*) rather than recognizing diverse foreign antigens directly.

Some γδ cells respond to mycobacterial antigens (*6–9*). Many of these can be stimulated with purified protein derivative (PPD) of *Mycobacterium tuberculosis*, containing the presumably denatured mycobacterial 65-kD heat shock protein (Hsp65), and with purified recombinant Hsp65 (*6–8*). Mycobacterial Hsp65 is known to be an immunodominant antigen (*10*) that contains many stimulatory epitopes for B cells and αβ T cells. The αβ T cell epitopes have been defined with synthetic peptides correspond-

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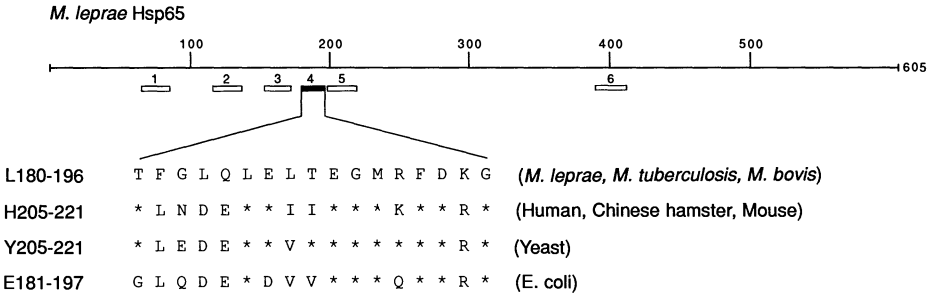

Fig. 1. Linear map of *M. leprae* Hsp65 (*13*) and synthetic heat shock peptides. The six synthetic peptides shown (*12*) and nine further peptides listed in Fig. 2 (*11, 12*) were used to map a putative stimulatory epitope for γδ cells. Also shown are the sequences of newly synthesized peptides, corresponding to the same epitope in homologous proteins of other species (*13, 19*). Peptides are designated by a capital letter to indicate the species origin of the protein sequence (L for *M. leprae*, *M. tuberculosis*, and *M. bovis*; H for human, Chinese hamster, and mouse; Y for yeast; and E for *E. coli*) and by the position numbers of the NH_2-terminal and COOH-terminal amino acids (*13*). New peptides were synthesized according to the solid-phase methods generally described by Merrifield *et al.* (*22*) and detailed by Buus *et al.* (*23*). To verify primary structures, we purified peptides by high-performance liquid chromatography (HPLC) and analyzed for amino acid compositions and sequences (Applied Biosystems model 470A gas-phase sequencer). The predicted protein sequence of a Hsp65 homolog in mice was derived by polymerase chain reaction amplification and sequencing of DNA complementary to mouse mRNA, with the use of primers corresponding to short stretches of gene sequence identical in Chinese hamsters and humans (*19*).

ing to portions of the molecule (*11, 12*). Using a collection of PPD-reactive γδ hybridomas [isolated without antigen selection from newborn mouse thymus and spleen (*6*)] and an in vitro stimulation assay, we tested such Hsp65 peptides for their ability to serve as antigens for γδ cells.

In a set of six nonoverlapping peptides that stimulate αβ T cell responses (*12*) (Fig. 1), one [L180–196, corresponding to amino acids 180 to 196 of *M. leprae* Hsp65 and identical to *M. tuberculosis* and *M. bovis* Hsp65 in this region (*13, 14*)] strongly stimulated several PPD-reactive γδ cells, although most responded weakly. Nine additional peptides, either overlapping with or proximal to peptide L180–196, were added to the test panel (Fig. 2). Stimulation patterns of the two hybridomas that had the strongest responses (BNT-19.8.7 and BNT-9.12.18, both expressing $V_\delta 6.3$ and $V_\gamma 1$) (*15*) and a PPD-nonreactive cell (33BTE-140.9, expressing $V_\delta 1$ and $V_\gamma 6$) (*16*) are shown. The two PPD responders were both stimulated by L180–196, whereas 33BTE-140.9 was not stimulated by any of the peptides. BNT-19.8.7 also responded strongly to a slightly shorter peptide, L181–195. Since BNT-19.8.7 and BNT-9.12.18 differ in the junctional sequences of both γ and δ chains (*15*), it remains unclear whether only one or both receptor chains determine fine specificities.

Peptide L180–196 also stimulates arthritogenic T cell clones in rats (*17*). The sequence of Hsp65 is evolutionarily conserved, and this particular peptide overlaps partially with a nonconserved (amino acids 176 to 188) and partially with a highly conserved (amino acids 189 to 203) region of the protein (*13*). The core epitope recognized by arthritogenic αβ T cells has been mapped to the nonconserved portion (amino acids 180 to 188) (*17*). We might expect that the conserved portion would be the core epitope for the PPD-reactive γδ hybridomas because our previous experiments showed a TCR-dependent "spontaneous"

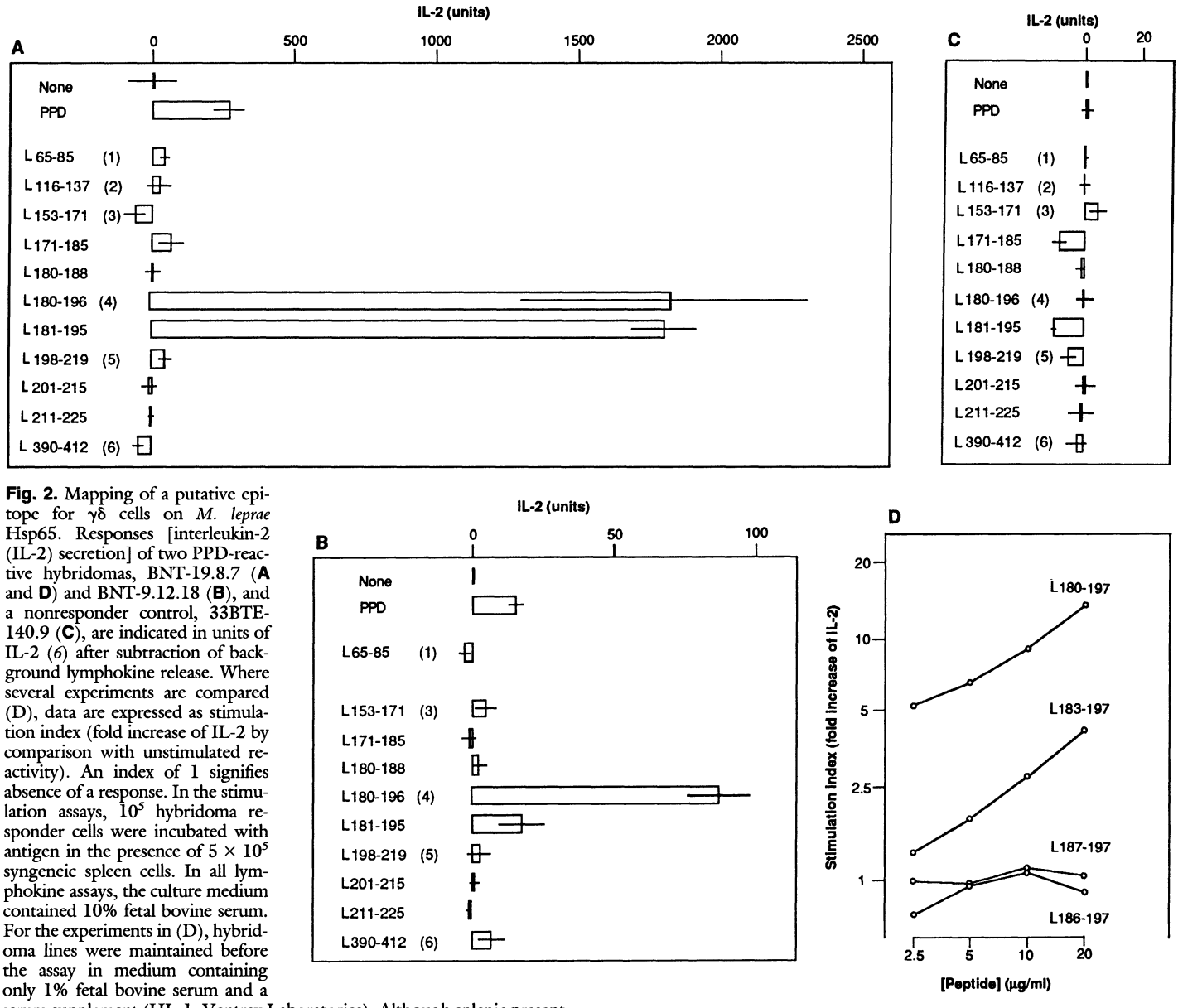

Fig. 2. Mapping of a putative epitope for γδ cells on *M. leprae* Hsp65. Responses [interleukin-2 (IL-2) secretion] of two PPD-reactive hybridomas, BNT-19.8.7 (**A** and **D**) and BNT-9.12.18 (**B**), and a nonresponder control, 33BTE-140.9 (**C**), are indicated in units of IL-2 (*6*) after subtraction of background lymphokine release. Where several experiments are compared (D), data are expressed as stimulation index (fold increase of IL-2 by comparison with unstimulated reactivity). An index of 1 signifies absence of a response. In the stimulation assays, 10^5 hybridoma responder cells were incubated with antigen in the presence of 5×10^5 syngeneic spleen cells. In all lymphokine assays, the culture medium contained 10% fetal bovine serum. For the experiments in (D), hybridoma lines were maintained before the assay in medium containing only 1% fetal bovine serum and a serum supplement (HL-1, Ventrex Laboratories). Although splenic presenter cells are not required, they have been found to increase antigen-specific responses (*6*). PPD [prepared from the culture filtrate proteins of *M. tuberculosis* H37Rv (*24*)] and peptides were used at 20 μg/ml. Splenocytes alone do not respond to the antigens during the period of the stimulation assay (20 hours) (*6*). The ability of the hybridomas to secrete IL-2 was ascertained in every experiment by nonspecific stimulation with plate-bound, cross-linking monoclonal antibody (MAb) to CD3 as described (*6*). IL-2 release was visualized in a modified HT-2/MTT assay (*25*), measured with a BIOTEK enzyme-linked immunosorbent assay (ELISA) reader, and analyzed (*6*). Bar graphs represent the mean ± SEM of three determinations.

reactivity by the hybridomas in the absence of any deliberately added antigen, suggesting that these γδ cells recognized both the prokaryotic antigen and its eukaryotic homolog (*6*). To determine which portion of the sequence was recognized, we tried to stimulate the stronger responder cell (BNT-19.8.7) with shorter peptides (Fig. 2, A and D) and found that L180–188 was not stimulatory. A peptide truncated by three amino acids at the nonconserved NH_2-terminus (L183–197) weakly stimulated BNT-19.8.7. A similar peptide, covering amino acids 183–196, also weakly stimulates the arthritogenic T cell clones (*17*). Peptides that were further truncated (L186–197 and L187–197) did not stimulate the γδ hybridoma; similarly, a peptide covering amino acids 185 to 196 was not recognized by the arthritogenic T cell clones (*17*). These data suggest that the epitopes for mouse γδ hybridomas and for rat arthritogenic T cell clones are overlapping but not identical, because the nonconserved portion of the sequence, amino acids 180 to 188, is required but not sufficient for stimulation of the γδ hybridomas. Alternatively, it is possible that the truncated peptides failed because they were not presented efficiently, or were subject to degradation during the assay.

To define the putative γδ epitope further, we synthesized peptides of equal length corresponding to the same region (amino acids 180 to 196 of *M. leprae* (Hsp65) in homologous proteins of several distant species (Fig. 1) (*13*). These peptides differ from the mycobacterial sequence mostly at the NH_2-terminal end, but also in some of the 12 highly conserved COOH-terminal positions. In stimulation experiments with these peptides (Fig. 3), we used hybridomas adapted to grow in medium containing only 1% fetal bovine serum. Under normal assay conditions [10% fetal bovine serum (*6*)], these cells show reduced spontaneous reactivity and thus allow the detection of smaller responses (*18*). To identify the murine homolog of the stimulatory mycobacterial sequence, we amplified and sequenced mouse mRNA encoding murine Hsp63 (*19*). The mouse gene encodes in this region of the protein an amino acid sequence identical to previously published sequences of the Chinese hamster and human Hsp65 homologs (*13*). A peptide synthesized according to this sequence stimulated both γδ hybridomas tested (Fig. 3). Two other peptides, corresponding to the equivalent region of the Hsp65 homologs in yeast and *Escherichia coli* (*13*), also stimulated BNT-19.8.7. Responses to these peptides were much weaker, however, than to the mycobacterial peptide. BNT-9.12.18 did not recognize the *E. coli*

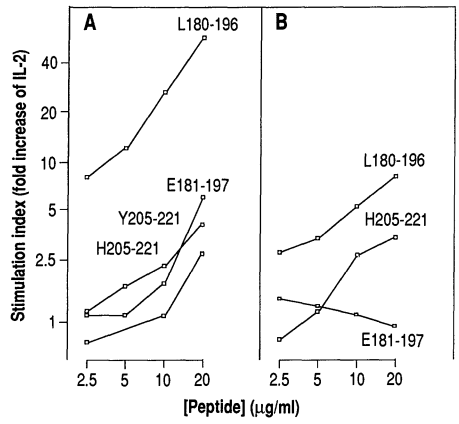

Fig. 3. Responses to homologous peptides of different species. Hybridomas BNT-19.8.7 (**A**) and BNT-9.12.18 (**B**) were stimulated with peptides corresponding to equivalent portions of Hsp65 homologous protein in *M. leprae*, *E. coli*, yeast, and mice (*13*). The amino acid sequences of these peptides are shown in Fig. 1. The data are in each case representative examples of at least three similar experiments. Hybridomas were stimulated and IL-2 secretion determined as described for Fig. 2D. Data are expressed as in Fig. 2D.

peptide, perhaps because it differs from the mycobacterial sequence at the NH_2-terminal position, which seems to be more significant for this hybridoma (Fig. 2), whereas the autologous peptide does not. Overall, the data support the concept that the conserved part of the sequence is required for stimulation of γδ cells (*6, 20*) and thus provide evidence for the suspected cross-reactive response of γδ cells with autologous hsp's. Nonconserved, species-specific amino acids, however, appear to influence the magnitude of the response, and the superior stimulation by the mycobacterial sequence may explain why mycobacterial antigens have been implicated in triggering self-specific responses (*7, 17*).

Under conditions close to maximal stimulation (20 μg of peptide per milliliter, 200 μg of PPD per milliliter), many γδ hybridomas (but not BNT-19.8.7 and BNT-9.12.18) responded better to PPD than to the peptide antigen L180–196 (*21*). It is possible that these cells have higher affinities for other, unknown antigens and only fortuitously cross-react with recombinant Hsp65 and hsp peptides. It is more likely that the peptides tested may not cover the entire stimulatory epitope for all Hsp65-reactive clones or they may be presented less efficiently than naturally processed protein fragments. Also, autologous antigens, if indeed responsible for the spontaneous reactivity of the hybridomas (*6*), might be better competitors for binding sites than short synthetic peptide antigens.

In summary, our data show that γδ T cell hybridomas can specifically respond to small synthetic heat shock peptides. This ability to recognize peptides suggests that γδ cells, like αβ T lymphocytes, recognize processed fragments of naturally occurring proteins. Since antigen-pulsed cells are stimulatory (*18*), the peptides are probably presented on the cell surface. The nature of possible presenting molecules remains unclear, with the exception that these molecules appear to be nonpolymorphic and not MHC class II (*6*). In addition, our data confirm the notion that γδ cells recognize not only heterologous but also autologous stress proteins (*5, 6, 20*). A role for γδ cells in rheumatoid arthritis has been suggested (*7*) and is supported by our finding that γδ cells and arthritogenic αβ T cell clones recognize overlapping hsp sequences. The αβ T cell responses to this region of the mycobacterial hsp (amino acids 180 to 196) have been found in all species studied (*10*). Thus, unique properties may make this peptide more suitable for T cell stimulation than others.

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