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Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis

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Adjuvant arthritis (AA) is a chronic disease inducible in rats by immunization with an antigen of *Mycobacterium tuberculosis*[1]. After the isolation of arthritogenic T-cell lines[2] and clones[3], it became possible to demonstrate that the critical *M. tuberculosis* antigen contained an epitope cross-reactive with a self-antigen in joint cartilage[4–6]. Like AA rats, patients suffering from rheumatoid arthritis demonstrated specific T-lymphocyte reactivity to the *M. tuberculosis* fraction containing the cross-reactive epitope[7]. To characterize the critical *M. tuberculosis* epitope we used AA T-cell clones to screen mycobacterial antigens expressed in *Escherichia coli* and genetically engineered truncated proteins and synthetic peptides. The AA T-cell clones recognized an epitope formed by the amino acids at positions 180–188 in the sequence of a *Mycobacterium bovis* BCG antigen[8]. Administration of this antigen to rats induced resistance to subsequent attempts to produce AA.

Some rat strains develop an autoimmune-like arthritis after immunization with *M. tuberculosis* (Mt) (ref. 1), but AA can sometimes be induced without using Mt (ref. 9). To investigate the relation between AA and immunization with Mt, we raised lines of arthritogenic anti-Mt T cells from Lewis rats immunized to induce AA (ref. 2). Clone A2b of one such line produced arthritis in heavily irradiated (750R) Lewis rats[3], and recognized both an Mt antigen and a fraction of cartilage proteoglycan probably associated with the core protein[4]. An acetone-precipitable fraction of Mt containing the epitope that is cross-reactive with cartilage was recognized not only by AA T lymphocytes, but also by T lymphocytes from patients with rheumatoid arthritis[7]. Another clone, A2c, functioned as a supressor-inducer T cell[5,10]. Rats treated with A2c acquired resistance to AA (ref. 5).

We have used clones A2b and A2c to probe individual mycobacterial antigens expressed in *E. coli* recombinants. Table 1 shows the responses of anti-Mt clones A2b and A2c, and of control clone C1a, responsive to ovalbumin. It can be seen that both clones A2b and A2c responded not only to whole Mt, but also to an *M. bovis* BCG antigen of relative molecular mass (M_r) ~65,000 (65K) and expressed by *E. coli.* A control protein

Table 1 Proliferative response of T-cell clones A2b and A2c to the 65K *M. bovis* BCG protein

	Mt	65K protein	*E. coli* control	Ovalbumin	Concanavalin A
A2b	180 (±21)	500 (±64)	2.9 (±0.4)	—	430 (±41)
A2c	304 (±18)	516 (±44)	1.5 (±0.2)	—	390 (±28)
C1a	—	1.5 (±0.1)	1.2 (±0.4)	45 (±5.1)	64 (±6.3)

Proliferative response of T-cell clones A2b, A2c and C1a in the presence of heat-killed *M. tuberculosis* H37Ra (Mt) (Difco), purified 65K *M. bovis* BCG recombinant protein[8], and a control protein fraction (10 μg ml^{-1}) purified from non-recombinant *E. coli*, ovalbumin (20 μg ml^{-1}) and concanavalin A (2.5 μg ml^{-1}). Cloning and maintenance conditions of T cells have been described previously[3]. Proliferative responses were measured by 16 h [^{3}H]thymidine incorporation in cells (2×10^4 well) cultured for 4 d in the presence of irradiated (1,500 R) syngeneic thymocytes (2×10^6 per well) as accessory cells. The counts per minute (c.p.m.) for [^{3}H]thymidine incorporation were measured in triplicate test cultures and divided by the mean of triplicate cultures without antigen. The means of the resulting ratios are shown as stimulation index (SI, c.p.m. test per c.p.m. control without antigen) ± 1 standard deviation. Culture medium was DMEM (Gibco) supplemented with 1% rat serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The 65K recombinant protein was prepared from the 65K over-producing *E. coli* host strain 1046, which carries the lambda repressor CI857-encoding plasmid pCI857 and the 65K protein-encoding plasmid pRIB1000 (ref. 12). The control *E. coli* K12 was obtained from strain 1046, carrying pCI857 and the cloning vector pPLc 236 (ref. 12).

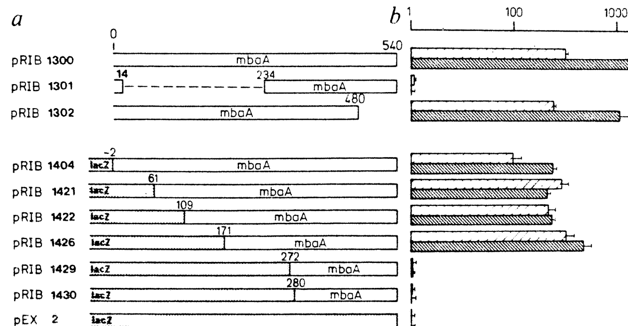

Fig. 1 *a*, Map of deletion mutants of the gene encoding the 65K protein fused to *lacZ* and truncated derivatives of mbaA expressed in the plasmids indicated. *b*, Proliferation of T lymphocyte clones A2b (upper, light shaded bars) and A2c (lower, dark shaded bars) in response to the *M. bovis* BCG 65K protein (mbaA), truncated derivatives and fusion proteins with *β*-galactosidase.
Methods. Plasmid pRIB1300 is an *Eco*RI-*Bst*MII deletion mutant of plasmid pRIB1011 (ref. 8). Plasmid pRIB1301 is an in-frame *Xho*I deletion derivative of pRIB1300, and pRIB1302 is an *Acc*I-*Sal*I deletion mutant of pRIB1300. Plasmid pRIB1300 expressed the native mbaA protein composed of 540 amino-acid residues. The deletion mutants pRIB1301 and pRIB1302 express truncated proteins. In the plasmids pRIB1404, pRIB1421, pRIB1422, pRIB1426, pRIB1429 and pRIB1430, the carboxy-terminal part of *lacZ* is fused with parts of the 65K protein-coding gene[19]. The number of the residue from the amino-terminal of mbaA is indicated for the coding gene fused to *lacZ*. In pRIB1404, *lacZ* is fused to the *M. bovis* DNA 7 base pairs upstream of the mbaA start codon and therefore the fusion protein contains two extra residues. All *lacZ* fusions were constructed using the *lacZ*-containing expression vector pEX2 (ref. 20). Proliferative responses of T-cell clones A2b and A2c in the presence of various mutant proteins were measured as in Table 1. Mean stimulation indexes ± 1 standard deviation (SI ± 1 s.d.) are given. Antigens were prepared as follows. *E. coli* K12 M1070 (ref. 8) carrying the plasmids pRIB1300, pRIB1301 or pRIB1302 was used to produce the 65K protein or truncated derivatives. Induced cultures were resuspended in 100 mM Tris, 5 mM EDTA, *p*H 8 and sonicated by 8 pulses of 30 s at 80 Watts. The resulting lysates contained the 65K protein up to 30% (pRIB1300), or truncated derivatives up to 5% (pRIB1301 or pRIB1302) of the total protein content. Final total protein concentration used during T-cell stimulation was 1 μg ml^{-1}. *E. coli* K12 M1070 carrying the plasmid pEX2 or derivatives was used to induce the various *β*-galactosidase fusion proteins[20]. Induced cells were resuspended in 100 mM Tris, 10 mM EDTA, *p*H 8.0 containing lysozyme 0.1 mg ml^{-1}, freeze-thawed once and ultrasonicated by three pulses of 30 s at 70 Watts. About 40% of the total protein in the resulting lysates consisted of fusion protein. Final total protein concentration used during T-cell stimulation was 0.5 μg ml^{-1}.

Peptide	Sequence	T-cell response SI(±1 s.d.)		
	171 181 191 GVITVEESNTFGLQLELTEGMRFDKGYISG	A2b	A2c	Z1a
153–171	<-----+	<1	=1	ND
174–192	+-----------------+	**16 (±2)**	**11 (±2)**	<1
180–196	+---------------+	**33 (±5)**	**120 (±8)**	<1
180–188	+-------+	**47(±4)**	**58(±3)**	<1
183–196	+------------+	9.2 (±0.3)	2.9(±0.8)	<1
185–196	+----------+	<1	<1	ND
190–200	+----------+	<1	<1	ND
197–218	+------<	<1	<1	=1
Mt		180(±21)	304(±18)	<1
BP		=1	<1	162 (±18)

Fig. 2 T-cell epitope mapping with synthetic peptides of the 65K *M. bovis* BCG protein. The amino-acid sequence of the protein is shown for the area critical for T-cell recognition by arthritis clones A2b and A2c. The amino-acid sequence is predicted from the nucleotide sequence[12,21]. The underlined and bold-printed sequence is the region essential for T-cell recognition.

Methods. Peptides 180–196, 180–188, 183–196, 185–196 and 190–200 were prepared by solid-phase techniques[22] using a Labortec SP640 peptide synthesizer. Boc amino acids were coupled as the preformed symmetric anhydrides (hydroxybenzotriazole esters for arginine, asparagine and glutamine). *N,N*-Diisopropylcarbodiimide was the activating agent. Peptides were deprotected and cleaved from the resin with trifluoromethanesulphonic acid in TCA in the presence of thioanisole. After separation from the resin by filtration, the peptides were precipitated and washed with dry ether. Finally, peptides were desalted on G10 Sephadex in 5% acetic acid and lyophilized. Peptides 153–171 (ref. 23) and 174–192 and 197–218 were gifts. T-cell responses were determined in the standard lymphocyte proliferative assay described in Table 1. Mean SI ± 1 s.d. are given. Data are shown for responses at a peptide concentration of 1 μg ml^{-1}. BP (basic protein of myelin) and Mt were used at 10 μg ml^{-1}. ND, not determined.

fraction from *E. coli* not transfected with mycobacterial DNA did not stimulate A2b or A2c. Control clone C1a responded to ovalbumin but not to any of the Mt or *E. coli* antigens. All three clones responded to the mitogen concanavalin A. Clones A2b and A2c did not respond to other cloned mycobacterial antigens of 12K, 18K, 28K or 34K (not shown)[11].

To identify the epitopes more precisely, we tested the responses of clones A2b and A2c to fragments of the 65K protein which were obtained either from deletion mutants of the gene for the protein, or from deletion mutants of the gene after fusion with the β-galactosidase gene or by synthesis of peptide regions included in the 65K protein. Figure 1 shows a map of the deletion mutants, the parts of the 65K coding gene fused to *lacZ*, and the stimulation of clones A2b and A2c by the expressed proteins. It can be seen that both clones responded to protein fragments that lacked the amino acids up to residue 171 (see pRIB1426) but deletions extending to amino acid 234 (pRIB1301) showed no antigenic activity, indicating that the epitopes were probably located between amino-acid residues 171–234. A deletion of the 60 carboxy-terminal amino acids (pRIB1302) did not abolish activity.

Synthetic peptides based on the sequence of the 65K *M. bovis* BCG protein were tested for stimulation of both T-cell clones. Figure 2 shows the amino-acid sequence of the 65K *M. bovis* BCG protein[12] from residue 171–200 and of the informative synthetic peptides. Note that both clones A2b and A2c responded to peptides 174–192, 180–196 and 180–188, whereas Z1a, the control clone with a specificity for myelin, did not. The responses of A2b and A2c to peptide 183–196 were significantly lower than to 180–196 and 180–188, and were negative in the case of 185–196. Therefore, the critical epitope for both clones resides in the 180–188 sequence.

An amino acid-homology search showed some similarity between the 180–188 sequence and the link protein of rat proteoglycan[13], with four of the nine amino acids identical. No similarity was observed with published sequences of the core protein of chicken and rat proteolgycan[14,15].

Thus arthritogenic clone A2b and protective clone A2c both recognize a single nonapeptide epitope of the 65K protein of mycobacteria. It was important to establish whether active immunization with the 65K antigen would activate A2b-like T lymphocytes and induce AA, or if it would activate A2c-like T suppressor-inducers and induce resistance to AA. Unlike immunization with whole mycobacteria, the administration of the 65K antigen emulsified in oil did not induce AA (Table 2), but the immunized rats did show resistance to a subsequent attempt to induce AA by immunization to whole Mt in oil (Table 2). Thus the engineered 65K antigen, although recognizable *in vitro* by the arthritogenic T lymphocyte clone A2b, induced resistance to AA, presumably by favouring the emergence of A2c-like (suppressor-inducer) T cells. Indeed, A2b cells treated to cause aggregation of membrane components were found, similarly to A2c cells, to induce anti-idiotypic immunity and remission of established AA (ref 16).

A study of a small number of patients with rheumatoid arthritis showed that T-cell recognition of the 65K antigen correlated with T-cell recognition of the acetone-precipitable fraction of whole Mt (ref. 7 and R. R. P. de Vries, personal communication). Therefore, the 65K antigen could be a specific target for T-lymphocyte recognition in such patients. A possible function for the 65K protein has been suggested[17] after extensive homology was found with a sequence encoded by an essential *E. coli* gene involved in messenger processing. It appears that the 65K antigen is a heat-shock protein (D. B. Young, personal communication), related to homologous proteins in higher eukaryotes as well as in bacteria. Self-epitopes present in stress proteins could be a necessary by-product of their phylogenetic conservation. Antigens cross reactive with the 65K *M. bovis* BCG protein are present in some mycobacteria[8] and this antigen is also related to another shared by more than 50 different

Table 2 65K protein of *M. bovis* BCG protects against AA

Primary immunization		Secondary AA challenge (after 35 d) with Mt in oil	
Immunizing agent	Arthritis incidence	Arthritis incidence	Clinical grade
Saline	0/8	8/8	Severe
65K protein	0/8	1/8	Very mild
E. coli control protein	0/7	6/7	Severe

Groups of 7 or 8 Lewis rats were treated by intraperitoneal inoculation of saline, 65K protein (50 μg) or *E. coli* control protein (weight equivalent to *E. coli* content of 50 μg of 65K protein) in oil. After 35 d the susceptibility to induction of AA was tested by challenging the rats intracutaneously at the base of the tail with 1 mg heat-killed Mt in oil. Incidence of arthritis was checked by daily inspection of the rat joints and confirmed by histological examination.

bacterial species, including *Klebsiella, Shigella, Salmonella, Yersinia* and *Campylobacter,* all suspected of being involved in human arthritis[18]. Possibly humans could be exposed to the 180–188 epitope, or to a cross-reactive epitope associated with different environmental bacteria under conditions which might influence their susceptibility to autoimmune arthritis. If the 180–188 epitope is involved in the pathophysiology of arthritis, a purified peptide containing the epitope might induce therapeutic suppression of the disease process in the same way as the 65K antigen in AA.

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