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Mapping of T-Cell Epitopes of Flagellar Antigen *d* of *Salmonella muenchen*

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Regions of the antigen *d* flagellin of *Salmonella muenchen* capable of causing the proliferation of repolymerized *d* flagellin-primed T cells were delineated by using dodecameric peptides. Three areas causing substantial DNA replication were identified, together with five areas eliciting less response. On the basis of known salmonellar flagellin structure, the major areas were located in highly conserved regions of the molecule.

The flagellar filaments of bacteria of the genus *Salmonella* are polymers of a single protein, termed "flagellin" (4), and the large number of known flagellar antigens (2) is a reflection of the wide variety of flagellins produced by the group. These proteins consist of extremely conserved terminal regions with variable central areas (10). It has been suggested (7) that parts of the variable regions could be replaced with medically important epitopes so that flagellin would act as a carrier in a possible vaccine. A range of known epitopes has been introduced into flagellin with limited success in terms of inducing an immune response against the included foreign area (8). To produce a useful anamnestic response, a vaccine is required to elicit both B- and T-cell memory cells and should include some or all of the B-cell and T-cell epitopes of the infectious agent against which protection is desired. As expected, the natural B-cell epitopes of the *d* flagellin have been shown to be located in the variable region of the molecule (5). However, the position of the T-cell epitopes has not been predicted from immunological or clinical studies and is dependent on both the processing of the antigen and the available epitope receptors (3). Here, we describe investigations to determine the normal T-cell epitopes of the *d* flagellin of *Salmonella muenchen* using the recently available cleavable pin peptide technology (1). This flagellin was selected because it has been used extensively in B-cell epitope substitution studies (8).

Progressive dodecameric peptides, based upon the amino acid sequence of *S. muenchen d* flagellin, were synthesized on polyethylene pins and cleaved off after side chain deprotection and acylation of terminal amino groups using materials and protocols purchased as a cleavable peptides kit (Chiron Mimotopes, Clayton, Australia). Randomly selected products were assayed for total amino acid content and concentration using acid hydrolysis and a Pico-Tag system (Waters Inc., Bedford, Mass.). Analyses were performed on a fee basis by A. Fowler, UCLA School of Medicine, Los Angeles, Calif. Yields of 250 to 400 nmol/ml were routine. It should be noted that the mechanism used to cleave the synthesized peptides from the solid support pins is based on diketopiperazine formation resulting in a modified C terminus (cyclic Lys-Pro) on each synthesized peptide. It has been shown (9) that such modification has no detrimental effect on T-cell recognition. Hydrolyzed peptides contained

TABLE 1. Dodecameric peptides causing proliferation of *d* flagellin-primed T cells

Amino acid residues generating reactive dodecapeptides	Dodecapeptides synthesized	Primed T-cell response
28–33	IERLSSGLRINS	High
	ERLSSGLRINSA	
	RLSSGLRINSAK	
	LSSGLRINSAKD	
	SSGLRINSAKDD	
	SGLRINSAKDDA	
39–45	SAKDDAAGQAIA	High
	AKDDAAGQAIAN	
	KDDAAGQAIANR	
	DDAAGQAIANRF	
	DAAGQAIANRFT	
	AAGQAIANRFTA	
	AGQAIANRFTAN	
77–81	TEGALNEINNNL	Moderate
	EGALNEINNNLQ	
	GALNEINNNLQR	
	ALNEINNNLQRV	
	LNEINNNLQRVR	
87–91	NLQRVRELAVQS	Moderate
	LQRVRELAVQSA	
	QRVRELAVQSAN	
	RVRELAVQSANG	
	VRELAVQSANGT	
127–131	GQTQFNGVKVLA	Moderate
	QTQFNGVKVLAQ	
	TQFNGVKVLAQD	
	QFNGVKVLAQDN	
	FNGVKVLAQDNT	
154–157	TIDIDLKEISSK	High
	IDIDLKEISSKT	
	DIDLKEISSKTL	
	IDLKEISSKTLG	
290–293	GVDTTTVAAQLA	Low
	VDTTTVAAQLAA	
	DTTTVAAQLAAA	
	TTTVAAQLAAAG	
416–420	ENPLQKIDAALA	Low
	NPLQKIDAALAQ	
	PLQKIDAALAQV	
	LQKIDAALAQVD	
	QKIDAAGAQVDT	

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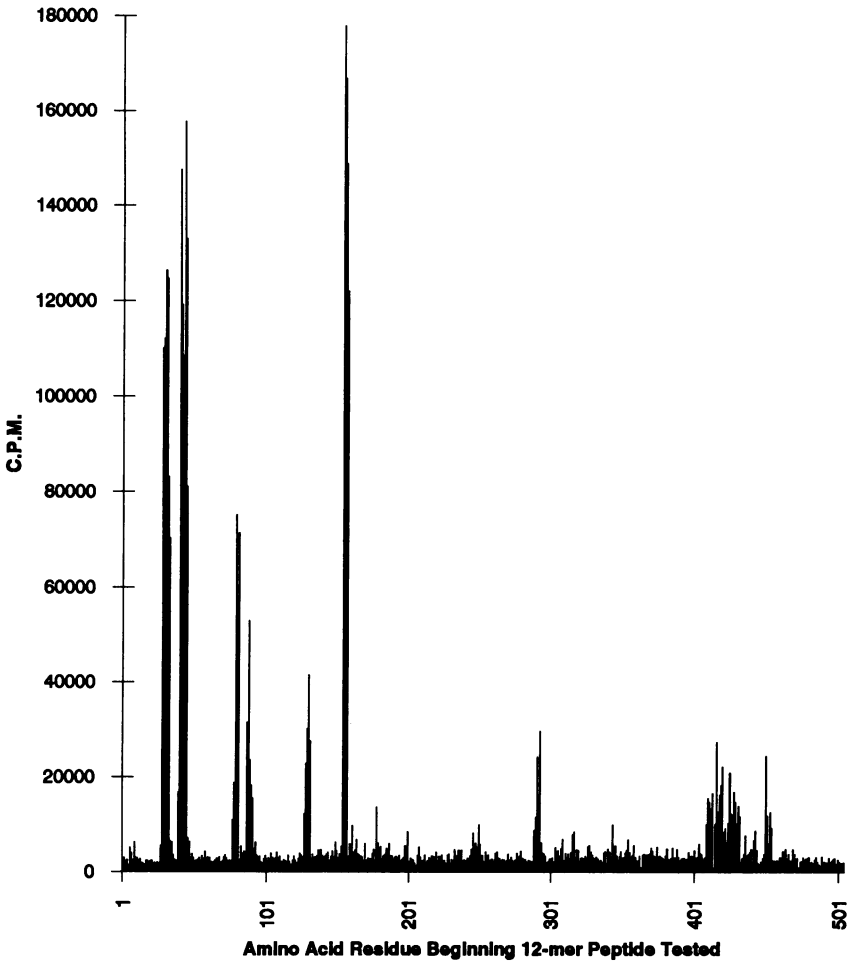

FIG. 1. Results of T-cell epitope analysis by the method of Gamon et al. (3) using peptides synthesized on the basis of the sequence of the *d* flagellin of *S. muenchen* (10). All were dodecameric peptides from the linear sequence. Twelve mice were immunized with repolymerized flagellin as described in the text, and their popliteal lymph nodes were harvested and pooled after preparation of single-cell suspensions. Cells were tested in duplicate wells against each peptide. Mice immunized with complete Freund's adjuvant alone did not produce T cells capable of responding to stimulation with these peptides.

the extra amino acids *beta*-alanine (which elutes in the Pico-Tag system at the position of histidine), lysine, and proline. Synthetic peptides were tested in a lymphocyte proliferation assay essentially as described elsewhere (3). Briefly, BALB/c mice were injected in the hind footpads with 50 μl of a 50% solution of Freund's complete adjuvant containing 50 μg of *S. muenchen* phase 1 repolymerized flagellin (6). After 10 days, the popliteal lymph nodes were removed and cell suspensions were prepared. These cells were cultured in 96-well plates at 5×10^5/well in HL-1 medium (Ventrex) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5 μg of gentamicin per ml. Each peptide (0.5 to 0.8 nmol) was added to duplicate wells. Proliferation was measured by the addition of 1 μCi of [^{3}H]thymidine for the last 18 h of a 5-day culture with incorporation measured by liquid scintillation counting. Results were expressed by using Microsoft Excel (Microsoft Corp., Redmond, Wash.). To prepare repolymerized flagellin for injection (6), flagella were removed by blending, purified by differential centrifugation, disaggregated to flagellin by exposure to pH 2 with removal of suspended material by centrifugation at 100,000 × *g* for 2 h, and allowed to repolymerize at pH 7.0 in ammonium sulfate.

The results obtained from a typical experiment are shown in Fig. 1. As can readily be seen, three regions of the molecule caused profuse proliferation of the primed T cells (radioactive counts of 60 to 90 times the background count), and five regions caused a more moderate proliferation (15 to 40 times the background count). The amino acid sequences of these regions are shown in Table 1.

It is interesting that the major T-cell epitopes of the flagellin tested were all located in regions I (residues 1 to 100) and II (residues 101 to 180) (10), which were highly conserved in five flagellins analyzed. As in previous analyses (10), residues were numbered from the N-terminal alanine of the native flagellin. Small changes in these regions give rise to flagellin molecules that do not yield functional flagella (6). The principles which govern the selection of regions of protein molecules as T-cell epitopes are not yet understood. Whether T-cell epitopes of medically important molecules could be placed in less inflexible parts of the flagellin remains to be determined in further experiments. However, it appears that such substitution in the natural T-cell epitopes delineated here would most likely not result in a flagellated vaccine strain. These results are presented as a preliminary study to aid in other approaches using flagellins as epitope

carriers. Other mouse strains, derived independently of BALB/c, will need to be tested before studies can be generalized to apply to other species.

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REFERENCES

1. **Bray, A. M., N. J. Maeji, R. A. Campbell, and H. M. Geysen.** 1990. Multi-pin peptide synthesis strategy for T-cell epitope analysis. *In* Proceedings of the 15th Annual Conference on Protein Structure and Function. Lorne, Australia.
2. **Ewing, W. H. (ed.).** 1985. Edwards and Ewing's identification of *Enterobacteriaceae*, 4th ed., p. 247–318. Elsevier, New York.
3. **Gamon, G., H. M. Geysen, R. J. Apple, E. Pickett, M. Palmer, A. Ametani, and E. E. Sercarz.** 1991. T cell determinant structures: cores and determinant envelopes in three mouse major histocompatibility complex haplotypes. J. Exp. Med. **173:**609–617.
4. **Joys, T. M.** 1988. The flagellar filament protein. Can. J. Microbiol. **34:**452–458.
5. **Joys, T. M., and F. Schödel.** 1991. Epitope mapping of the *d* flagellar antigen of *Salmonella muenchen*. Infect. Immun. **59:** 3330–3332.
6. **Kanto, S., H. Okino, S. Aizawa, and S. Yamaguchi.** 1991. Amino acids responsible for flagellar shape are distributed in terminal regions of flagellin. J. Mol. Biol. **219:**471–480.
7. **Newton, S. M. C., C. O. Jacob, and B. A. D. Stocker.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagella. Science **244:**70–73.
8. **Newton, S. M. C., W. C. Manning, M. Hovi, and B. A. D. Stocker.** 1990. Aromatic-dependent *Salmonella* with foreign epitope insert in flagellin as live vaccine, p. 439–445. *In* F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Vaccines 90. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Suhrbier, A., S. J. Rodda, P. C. Ho, P. Csurhes, H. Dunckley, A. Saul, H. M. Geysen, and C. M. Rzepczyk.** 1991. Role of single amino acids in the recognition of a T cell epitope. J. Immunol. **147:**2507–2513.
10. **Wei, L.-N., and T. M. Joys.** 1985. Covalent structure of three phase-1 flagellar filament proteins of *Salmonella*. J. Mol. Biol. **186:**791–803.