MICROBIAL PATHOGENICITY

Further characterisation of a monoclonal antibody reactive with *Escherichia coli* O157:H7

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Summary. Monoclonal antibody (MAb) 4E8C12 has been previously reported to recognise low mol. wt proteins from enterohaemorrhagic *Escherichia coli* (EHEC) serotypes O157:H7 and O26:H11. Crude lipopolysaccharide (LPS) preparations from proteinase K-digested bacterial suspensions reacted in Western blots with MAb 4E8C12, as did highly purified LPS from O157:H7 strains. The material recognised by this antibody was, therefore, LPS. The LPS epitope was identified by a whole-cell ELISA in several EHEC, verotoxin producing *E. coli* (VTEC) and verotoxin-negative strains in addition to *E. coli* serotypes O157:H7 and O26:H11. Acriflavine and bile salts enhanced the production or availability of the epitope at the cell surface and in culture supernates. These data indicate that the presence of the epitope did not correlate with the virulence of these organisms.

Introduction

Verotoxin-producing *Escherichia coli* (VTEC) have been defined as a genetically diverse group of bacterial pathogens that elaborate verotoxins (VTs; also known as Shiga-like toxins, SLTs) in their culture supernates.^{1,2} They are responsible for a wide spectrum of disease, including diarrhoea, haemorrhagic colitis, a haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP).^{3–6} *E. coli* O157:H7 is the most common serotype found in HUS cases, although other VTEC serotypes, particularly O26:H11 and O111:NM, have also been isolated from patients with this disease.^{1,4} A subset of VTEC strains that includes the serotypes O157:H7, O26:H11, O111:NM, O145:NM, O45:NM and O4:NM, has been designated enterohaemorrhagic *E. coli* (EHEC), on the basis of having a number of similar clinical and pathogenic features (reviewed by Karmali¹). Tesh and O'Brien have included in the EHEC all strains that cause haemorrhagic colitis.⁶ In this report, the term EHEC is used to describe those serotypes that have been associated with both haemorrhagic colitis and HUS.

There is currently a great deal of interest in elucidating the mechanisms by which EHEC produce disease, as well as the reasons why certain

strains seem to predominate in cases of haemorrhagic colitis and HUS. Most EHEC carry the *eae* genes involved in intimate adherence of both the enteropathogenic *E. coli* (EPEC) and EHEC to intestinal epithelial cells,⁷ although virulent strains of EHEC serotype O91:H21 do not, suggesting that intimate adherence may not be obligatory for virulence.⁸ The symptoms of haemorrhagic colitis and HUS are thought to result from the action of one or more VTs, members of a family of A-B type toxins closely related to Shiga toxin. VTs bind to the globotriaosylceramide (Gb₃) receptor^{9–11} that is present in the vascular endothelium of renal distal convoluted tubules and collecting ducts in adults, as well as in glomeruli from children < 2 years old.¹² An attenuated laboratory strain of *E. coli* containing plasmids encoding VTs was fully virulent in orally inoculated mice, indicating that VT production alone is the key determinant of pathogenicity in this model.¹³ However, the predominance of certain VTEC serotypes in cases of HUS suggests that additional virulence determinants may be present in HUS-associated EHEC or VTEC strains.^{1,4}

Bacterial virulence determinants that result in the predominance of *E. coli* serotype O157:H7 in human disease have not yet been characterised. Padhye and Doyle¹⁴ recently developed a monoclonal antibody (MAb), 4E8C12, that appeared to react specifically with low molecular mass determinants in outer-membrane vesicles of *E. coli* serotypes O157:H7 and O26:H11—the two serotypes associated most fre-

quently with HUS—raising the possibility that these molecules may represent virulence factors specific for these serotypes. Recent investigations in our laboratory revealed that the epitope recognised by MAb 4E8C12 is present on bacteria from several additional *E. coli* serotypes, and the availability of this epitope could be enhanced by manipulating the culture conditions or heating the bacteria.¹⁵ However, under non-enhancing conditions, the epitope was expressed consistently and at high concentrations only in *E. coli* O157:H7, suggesting that the epitope may be a virulence determinant contributing to the prevalence of this organism in human haemorrhagic colitis and HUS as well as a useful diagnostic marker.

Therefore, MAb 4E8C12 may be a good reagent for the specific detection of virulence factors in EHEC strains, especially O157:H7 and O26:H11.¹⁶⁻¹⁸ The main objectives of this study were to establish the nature of the antigen recognised by MAb 4E8C12, to determine if its presence is correlated with virulence, and to investigate further the effect of culture conditions on the expression of this antigen.

Materials and methods

Bacterial strains

The principal bacterial strains used in this study are given in table I. Additional strains not included in table I were used for the analyses summarised in table II. Clinical isolates of *E. coli* serotypes associated with haemorrhagic colitis or HUS were obtained from Dr M. A. Karmali, Hospital for Sick Children, Toronto, Ontario (HSC strains). All other *E. coli* strains used

Table II. *E. coli* strains carrying the 4E8C12 epitope in whole-cell ELISA assays after growth in mTSB + BS

<i>E. coli</i> group	Serotype	Number of strains	
		4E8C12 positive	Tested
EHEC	O26:H11	14	14
	O91:H21	1	1
	O111:NM	4	10
	O111:H8	2	7
	O111:H11	5	5
	O113:H21	0	6
	O157:H7	16	16
	O157:NM	2	2
VTEC	O18:H7	0	2
	O26:NM	1	1
	O46:H38	1	1
	O103:H2	1	1
	O117:H4	0	5
	O121:H19	0	3
	OR:H2	1	1
	OR:H7	0	1
	O?:H21	1	1
Other	O55:H7 (VT-ve)	1	1
	O128:H2 (VT-ve)	1	1

One or two isolates of each of the following non-VTEC serotypes were tested and found to be negative for the 4E8C12 epitope: O2:H7, O18:H7, O18:NM, O111:H21, O118:H30, O127:H21 and O157:H45. This list includes the strains analysed in more detail in table III and fig. 2.

were from the culture collection of the Health of Animals Laboratory, Agriculture and Agri-Food Canada, Guelph (HAL strains). The O and H serotypes of all strains were re-tested before use. The presence of VT gene sequences was confirmed by PCR with Cangene primers ES149 and ES151¹⁹ (Cangene Corp., Ontario, Canada). VT1, VT2 and VTe gene

Table I. *E. coli* strains used in this study

Strain designation	Serotype	Source	Presence of <i>eaeA</i> gene	Toxin type
HAL EC 910004	O46:H38	Meat: boneless beef	—	VT1, VT2
HAL EC 910026	O157:H7	Cow: rectal swab	+	VT1, VT2
HAL EC 910037	O103:H2	Beef cow: rectal swab	—	VT1
HAL EC 910078	O26:H11	Veal calf: rectal swab	+	VT1
HAL EC 910111	O111:H11	Calf: rectal swab	—	VT1
HAL EC 910115	O111:H8	Calf: rectal swab	+	VT1
HSC 2	O26:H11	Human: HUS	+	VT1
HSC 3	O18:H7	Human: HUS	—	—*
HSC 4	O121:H19	Human: HUS	+	VT2
HSC 6	O117:H4	Human: HUS	—	VT2
HSC 7	O111:H8	Human: HUS	+	VT1
HSC 8	O117:H4	Human: HUS	—	VT2
HSC 9	O111:H8	Human: HUS	+	VT1, VT2
HSC 16	OR:H7	Human: HUS	+	VT1, VT2
HSC 18	O113:H21	Human: HUS	—	VT2
HSC 19	O117:H4	Human: HUS	—	VT2
HSC 20	O118:H30	Human: HUS	—	VT2
HSC 23	O91:H21	Human: HUS	—	VT2
HSC 24	O111:NM	Human: HUS	+	VT1
HSC 27	O111:H8	Human: HUS	+	VT1, VT2
HSC 30	O157:H7	Human: HUS	+	VT2
ATCC 25922		American Type Culture Collection	—	—

*No VT genes were detected; if originally present, they may have become lost upon storage or subculture.

sequences were detected with VT-specific oligonucleotide primers²⁰⁻²² in a multiplex PCR protocol, and *eaeA* gene sequence were detected according to the method of Louie *et al.*²³

Media

The growth medium developed by Padhye and Doyle²⁴ was used with some modifications. Bacteria were grown routinely in modified Tryptic Soy Broth (mTSB) consisting of Tryptic Soy Broth (Difco) 30 g/L, K₂HPO₄ 1.5 g/L and Casamino acids (Difco) 10 g/L. Modified Tryptic Soy Broth containing acriflavine (mTSB+A) was made by adding filter-sterilised acriflavine-HCl (Sigma) to the cooled broth to give a final concentration of 10 mg/L. Bile salts no. 3 (Difco) (1.5 g/L) were included in the mTSB to make mTSB+BS, and medium with both bile salts and acriflavine was designated mTSB+A+BS. Bacteria were grown overnight at 37°C with agitation for all assays.

Preparation of material containing the 4E8C12 epitope from culture supernates

E. coli O157:H7 strain 920026 was grown in mTSB+A overnight at 37°C and harvested by centrifugation at 10200 *g*. The supernate was filtered through 0.2-µm filters to remove intact bacteria. Protein-containing material was obtained by overnight precipitation with ammonium sulphate 60% w/v followed by centrifugation at 10200 *g*. Ammonium sulphate was removed by dialysis with a 2000 mol.wt cutoff membrane against two changes of sodium phosphate-buffered physiological saline (PBS), pH 7.4 (4-L volumes), and the material was concentrated with Amicon Centriprep units having 10000, 30000 and 100000 mol.wt cutoff membranes (Amicon Inc., Beverly, MA, USA). Concentrates were analysed by gel electrophoresis and Western blotting before and after digestion with proteinase K by the method of Hitchcock and Brown.²⁵

Preparation of bacteria for gel electrophoresis

Crude LPS preparations were obtained from whole bacteria by the method of Hitchcock and Brown.²⁵ *E. coli* grown to mid-to-late log phase (OD₆₀₀ ≈ 0.9) were collected, suspended in Laemmli digestion buffer²⁶ containing 50 mM dithiothreitol, boiled for 5 min, and cooled. Proteinase K (20 µl of a 1 mg/ml solution in water) was added, and the suspensions were incubated for 90 min at 60°C. Samples of these preparations were analysed by gel electrophoresis. Highly purified *E. coli* O157:H7 LPS used for comparison were kind gifts from K. L. Dodds (Dodds LPS) and M. Perry (Perry LPS). This purified LPS was prepared by the hot phenol-water method, followed by repeated ultracentrifugation until the preparation gave a sharp peak at 460 nm in the carbocyanine dye assay and no

measurable absorption peaks in the UV region (200–310 nm).

Gel electrophoresis and Western Blotting

SDS-PAGE was performed according to the procedure of Laemmli²⁶ with a BioRad Mini PROTEAN II apparatus. Tricine-gel electrophoresis was done by the method of Lesse *et al.*²⁷ Proteins and LPS were transferred to nitrocellulose (BioRad Trans-Blot Transfer Medium; BioRad Laboratories, Richmond, CA, USA), blocked overnight at 4°C with KPL Milk Diluent/Blocking Solution Concentrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) diluted 10-fold in PBS+Tween 20 0.05% (PBST). After three 10-min washes with PBST, blots were treated with purified MA b 4E8C12 (a gift from Rebecca Durham, Organon Teknika Corporation, Rockville, MD, USA) for 1.5 h at room temperature. After three washes with PBST, blots were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (H+L; Zymed Laboratories Inc., San Francisco, CA, USA). The blots were again washed three times with PBST and developed with 4-chloro-1-naphthol. The original gels were stained with silver stain.

Whole-cell and culture supernate ELISA assays

The whole-cell ELISA was done according to the protocol of Padhye and Doyle.¹⁴ Bacteria were grown overnight at 37°C in 5 ml of each medium tested. Two 1-ml samples were taken from each culture; one was boiled for 15 min while the other was kept at room temperature for that time. These samples were centrifuged to pellet bacteria and the culture supernate was removed for further analysis as described below. Bacteria adjusted to an OD₆₄₀ of 0.535 ± 0.5 in 50 mM carbonate buffer, pH 9.6, were added to wells (100 µl/well) of 96-well Immulon 2 microtitration plates (Dynatech Laboratories Inc., Biotechnology Products, Chantilly, VA, USA), and incubated overnight at 4°C. After blocking wells with gelatin 2% w/v in PBST for 1.5 h at 37°C, plates were washed with PBST and incubated for 1.5 h at 37°C with HRP-4E8C12 antibody (from R. Durham) 100 µl/well diluted to 1 µg/ml in PBST. Tetramethylbenzidine substrate (TMB; Dimension Laboratories Inc., Mississauga, ON, Canada) 100 µl/well was added for colour development, the reaction was stopped with 0.5 N H₂SO₄, and the microtitration plates were read at 450 nm in a Bio-Tek EL 312 Microplate ELISA reader.

Supernates saved from the original 1-ml samples of bacterial culture were also tested for the presence of the MA b 4E8C12 epitope in a second ELISA. Supernates were diluted 1 in 5 in 50 mM carbonate buffer, pH 9.6, and 100-µl volumes were added to wells of Immulon 2 microtitration plates. All subsequent steps were the same as in the whole-cell assay.

Results

Identity of the antigen recognised by MAb 4E8C12

MAb 4E8C12 reacted with material of *c.* 5000–6000 Da when the material retained by the 100000 mol.wt cutoff membrane was analysed by SDS-PAGE and Western blotting (fig. 1); antibody-reactive material was found only in the fraction retained by the 100000 mol.wt membrane. Therefore, the molecule containing the 4E8C12 epitope was either part of a macromolecular complex or was aggregated, and this complex or aggregate was soluble under the conditions used for SDS-PAGE.

Proteinase K treatment did not affect the level of reactivity of the epitope with the MAb in Western blots, although the mobility of the antigen changed slightly after enzyme digestion (fig. 1). Together, these

Fig. 1. Effect of proteinase K treatment on the reactivity of MAb 4E8C12 with culture supernate from *E. coli* O157:H7 strain HAL 920026. Western blot of culture supernate from strain HAL 920026 prepared by the protocol summarised in *Materials and methods*. The positions to which the protein standards migrated are indicated by their mol. wts (kDa) on the left.

results suggested that the antigen may not be a protein. Boiled, proteinase K-treated bacterial whole cells analysed by SDS-PAGE and Western blotting contained MAb 4E8C12-reactive material (fig. 2), confirming the heat stability and resistance to protease treatment of this epitope. Two preparations of highly purified LPS from *E. coli* O157:H7 strains were also recognised by the antibody (fig. 2, lanes 12 and 13). Pure Ra and Rc LPS from *Salmonella typhimurium* did not react with MAb 4E8C12 (fig. 2, lanes 14 and 15), nor did Hitchcock and Brown preparations of *Hafnia alvei*, *Yersinia enterocolitica*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Aeromonas hydrophila* or *Citrobacter freundii* (data not shown). Samples analysed by conventional SDS-PAGE and Western blotting showed binding of MAb 4E8C12 to *E. coli* LPS cores as well as LPS molecules containing O side chains (fig. 2). When the same preparations were analysed by tricine-SDS-PAGE and Western blotting, the only reactive bands seen were two bands of low molecular mass (fig. 2). LPS containing O side chains did not appear to react with MAb 4E8C12 after tricine-SDS-PAGE.

Distribution of the antigen recognised by MAb 4E8C12

Analysis of the ability of MAb 4E8C12 to bind to antigen on whole cells or in bacterial culture supernates from a large number of strains indicated that not all of the *E. coli* serotypes tested contained antigen capable of reacting with MAb 4E8C12. While present in the O157:H7, O26:H11 and O91:H21 EHEC serotypes, the epitope was not recognised in EHEC serotype O113:H21 or in several of the other HUS-associated clinical isolates (HSC strains, fig. 2; table II). Furthermore, the epitope was present on non-EHEC strains from several serotypes (table II), including the VT-negative O55:H7 and O128:H2 strains. Serotypes O111:H8 and O111:NM were heterogenous for expression of the 4E8C12 epitope, suggesting that the production of this epitope is strain specific. Therefore, the presence or absence of the MAb 4E8C12 epitope did not correlate with the ability of the bacteria to cause disease.

Effect of culture conditions and heating on detection of the 4E8C12 LPS epitope

Seven of 12 *E. coli* strains gave positive reactions for the 4E8C12 epitope when bacteria were grown in modified TSB broth, although the amount of cell-associated, antibody-reactive LPS varied markedly among strains (table III). Addition of acriflavine to the growth medium caused an increase in yield of the 4E8C12-reactive LPS epitope associated with cells and in the supernate, although strains HAL 910078 (O26:H11), HAL 910111 (O111:H11) and HSC 16 (OR:H7) were unable to grow when acriflavine was present. Bile salts enhanced the release of the epitope

Fig. 2. Reactivity of MAb 4E8C12 with LPS from *E. coli* strains. Bacteria were grown in mTSB + BS. LPS preparations (8 μ l/well except where noted) were analysed by conventional SDS-PAGE in polyacrylamide 16% separating gels (A) followed by Western blotting (B) or by tricine-SDS-PAGE (C) followed by Western blotting (D). Lane 1, HSC 3 (O18:H7); 2, HSC 4 (O121:H19); 3, HSC 9 (O111:H8); 4, HSC 27 (O111:H8); 5, HSC 19 (O117:H4); 6, HSC 20 (O118:H30); 7, HSC 2 (O26:H11); 8, HSC 30 (O157:H7); 9, HSC 23 (O91:H21); 10, HSC 7 (O111:H8); 11, HSC 18 (O113:H21); 12, purified *E. coli* O157:H7 LPS from K. L. Dodds (15 μ g); 13, purified *E. coli* O157:H7 LPS from M. Perry (15 μ g); 14, pure *Salmonella* Ra LPS (4 μ g); 15, pure *Salmonella* Rc LPS (4 μ g).

Table III. Effect of medium composition on the reactivity* of MAb 4E8C12 with cells and culture supernates of various *E. coli* serotypes

Serotype	Source	Strain	mTSB		mTSB+A		mTSB+BS		mTSB+A+BS	
			Cells	Supernate	Cells	Supernate	Cells	Supernate	Cells	Supernate
O157:H7	HAL	920026	++	+	+++	++	++	+++	+++	+++
	HSC	30	++	+	++++	+++	++++	++++	++++	++++
O26:H11	HAL	910078	++++	+	NG	NG	++	++	NG	NG
O111:H8	HAL	910115	+	—	+	—	+	+/-	+	+
	HSC	9	—	—	—	—	—	—	+	—
O111:H11	HAL	910111	+++	—	NG	NG	++++	+	NG	NG
O111:NM	HSC	24	—	—	—	—	—	—	—	—
O46:H38	HAL	910004	++	—	+++	+	++	++++	++	++++
O103:H2	HAL	910037	+	—	++	+	+	+	++	++++
OR:H7	HSC	16	—	—	NG	NG	—	—	NG	NG
O117:H4	HSC	8	—	—	—	—	—	—	—	—
	HSC	6	—	—	—	—	—	—	—	—

NG, no growth.

*Scores (— to +++) for reactivity were based on ELISA OD₄₅₀ values: —, 0.0–0.196; +/-, 0.197–0.3; +, 0.3–1.0; ++, 1.0–2.0; +++, 2.0–3.0; +++, > 3.0. The mean of the negative control plus 3 SD was OD₄₅₀ 0.196.

into the medium, and the combination of bile salts and acriflavine had an additive effect on expression of both cell-associated and released 4E8C12-reactive material.

Boiling the bacteria increased the MAb 4E8C12 reactivity on whole cells and in culture supernates (data not shown). Some *E. coli* strains (HSC 9 and HSC 24) that did not initially express the 4E8C12 epitope on the cell surface in unheated medium became positive for the surface-associated epitope in whole-cell ELISA assays after boiling (data not shown). LPS from strain HSC 9 prepared by boiling and proteinase K digestion did not react in Western blots with MAb 4E8C12 (fig. 2). This indicates that the process of boiling did not in itself cause the expression of the 4E8C12 epitope on LPS and suggests that the molecule containing the epitope may be sequestered in an intracellular compartment for subsequent addition to LPS.

Discussion

The epitope recognised by MAb 4E8C12 is present on the core region of *E. coli* LPS molecules. The antibody reacted with highly purified, protein-free LPS from *E. coli* O157:H7 and with Hitchcock and Brown LPS preparations of *E. coli* O157:H7 and other strains. The remarkable heat and protease stability of the antigen and its presence in *E. coli* O55:H7, from which the O157:H7 serotype is derived,² are also consistent with the presence of the antigen in LPS cores. Although contrary to Padhye and Doyle's initial description of this antigen as protein,¹⁴ our results are not inconsistent with those reported by these workers. They identified two reactive bands with apparent molecular masses of 5000 and 6000 Da in Western blots of outer-membrane vesicles obtained by a combination of differential centrifugation and discontinuous sucrose density-gradient centrifugation. Material obtained in this way²⁸ contains large amounts of LPS in addition to proteins, so that the antigen(s)

recognised by the antibody could be either protein or LPS. Furthermore, the method of electrophoresis clearly influenced the results of Western blotting. After tricine-SDS-PAGE of pure or crude (Hitchcock and Brown) preparations of *E. coli* LPS, only the lowest core LPS bands were visible, replicating previous results.^{14,15} However, characteristic LPS ladders containing several O side chain repeat units were apparent in Western blots of the same material after conventional SDS-PAGE (fig. 2).

Padhye and Doyle produced MAb 4E8C12 by immunising mice with a rough strain derived from *E. coli* O157:H7, although the LPS chemotype of the immunising strain was not reported.¹⁴ Therefore, the epitope recognised by the antibody is present either in LPS cores or on lipid A, and may result from some modification of LPS cores in these strains. Lipid A and the LPS core are known to be modified by sugars such as 4-aminoarabinose and substituents such as phosphorylethanolamine.²⁹

Inclusion of acriflavine 10 mg/L in bacterial growth medium was recommended by Padhye and Doyle²⁴ for isolation of *E. coli* O157:H7 from food. We found that while acriflavine increased the amount of this epitope in many strains tested, it also inhibited the growth of some *E. coli* strains. Therefore, the use of acriflavine in enrichment medium, although selective, is not suitable for detecting all pathogenic *E. coli* in food or environmental samples. Sensitivity or resistance to acriflavine is determined by four distinct *acr* genes in *E. coli*, and addition of acriflavine to growth medium affects the levels of phospholipids in the plasma membranes of both sensitive and resistant cells, perhaps by activating enzymes involved in phospholipid synthesis after binding to membrane phospholipids.³⁰ Similarly, the increased synthesis or availability of the 4E8C12 epitope in the presence of acriflavine could result from changes at the cell surface. Alternatively, changes in cell-surface levels of the epitope could result from changes in gene expression.

When bile salts were included in the medium, an increase in the amount of the epitope in culture supernates was apparent, consistent with removal of LPS from bacterial cells. Bile acids are known to have a detergent action on bacterial cell walls that results in the release of LPS from the outer membrane³¹ and heat-labile enterotoxin from the periplasm.³² An effect of bile salts on synthesis of the epitope was not ruled out, since SDS is known to affect the synthesis of many *E. coli* proteins.³³

Earlier findings that the 4E8C12 epitope is restricted to *E. coli*^{15,24} but is not specific to *E. coli* O157:H7 and O26:H11¹⁵ were confirmed. The fact that MAb 4E8C12 recognises epitopes in several strains of *E. coli* of serotypes other than O157:H7 and O26:H11, including VT non-producing bacteria from EPEC and non-pathogenic groups, coupled with the fact that several non-O157:H7 EHEC organisms did not react with the antibody, indicates that the presence of the epitope is strain specific and not highly correlated with the virulence of EHEC strains. The presence of this epitope in a strain of serotype O55:H7, from which the O157:H7 serotype strains are clonally derived,² supports this conclusion. A practical consequence of the fact that many strains carry the epitope for MAb

4E8C12 is that assays for *E. coli* O157:H7 with this antibody may have an increased rate of false positive results, although assay modifications may overcome this limitation.¹⁵ Whether the presence of this epitope in serotypes other than O157:H7 influences serological diagnosis of *E. coli* O157:H7 infections with LPS from *E. coli* O157:H7 as antigen remains to be determined.

In summary, the expression of the 4E8C12 LPS core epitope is very complex, regulated by components in the growth medium, and distinct from the expression of EHEC virulence determinants. The chemical nature of the LPS substitution creating the epitope remains to be determined.

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