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Studies on the *Escherichia coli* Hemolysin Antigenic Sites and Functional Sites for the Hemolysin Vaccine Development

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Hemolysin 백신 개발을 위한 요로계 감염 대장균들의 Hemolysin Antigenic Sites, Functional Sites 상동성 연구

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Abstract — This work was performed to investigate the possibility of using J96 hemolysin(Hly, Hly A) vaccine against urinary tract infecting *Escherichia coli*. Based on the known sequence of J96 hemolysin which was originally isolated from a pyelonephritis patient, ten 20-mer oligonucleotide probes were synthesized. Radioactive labelled 8 probes showed positive colony blots against most of the hemolysin producing wild type *E. coli*, while HA484 and HA661 showed 28.3, 71.7% positive blots, respectively. This result means that hemolysin genes are highly conserved. Also, 12 anti-Hly MABs(monoclonal antibodies) showed more than 90% positive immunoblots against secreted hemolysin from wild type *E. coli*. Especially, the result that MAB132 neutralized hemolysin from all of the wild type *E. coli* augments the idea that hemolysin will be effective as a vaccine.

The α-hemolysin (together with Gal-Gal pili, colicinV, resistance to serum) of *Escherichia coli* strains is one of the most important virulent determinants that cause extra-intestinal disease (1-5). Among uropathogenic *E. coli* strains that cause symptomatic renal or bladder infections, the prevalence of hemolytic isolates is between 44 to 75% (1, 2). In contrast, only 5 to 15% of normal enteric *E. coli* strains are hemolytic. There are considerable animal model data that demonstrate that hemolysin contributes to the severity of experimental pyelonephritis (6-11). These data support the importance of hemolysin in uropathogenesis.

α-Hemolysin causes cellular injury by disruption of the plasma membrane by pore formation, and if sufficient damage occurs, cell death subsequently follows (12-14). Cells that are injured include erythrocytes, leukocytes, urinary epithelial cells, neutrophils, fibroblasts and monocytes (7, 15, 16). The antigenic topology of α-hemolysin has been partially determined by the use of murine polyclonal and monoclonal antibodies (17). Monoclonal antibodies that recognize the amino terminal 2 to 160 amino acid residues exhibit the greatest *in vitro* neutralization of hemolysin. A considerable amount of data on the genetics and structure-function relationships of this toxin has been obtained within the last several years (18-22). The genetic control of α-hemolysin production, transport and secretion is complex, requiring for genes (HlyA, C, B and D) that are clustered on large plasmid or chromosome. HlyA encodes for the structural α-hemolysin. HlyB and HlyD convert the cytoplasmic α-hemolysin when it is secreted to an active form, possibly through a hydrolytic process occurring at its carboxy terminal (23, 24). The biologically active α-hemolysin encoded by chromosomal determinants has an estimated molecular weight of 110 K daltons (13, 18, 25). The amino terminal amino acid sequence has been par-

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tially determined by Edman degradation and the complete primary structure of α-hemolysin has been deduced by DNA sequencing (25). Site-specific mutagenesis that affects the first major hydrophobic domain leads to hemolytic negative mutants suggesting its crucial role (26). Development of vaccine is highly desirable in facing with increasing antibiotic resistent UTI (urinary tract infecting) *E. coli*.

Gal-Gal pili vaccine has been verified to be an effective vaccine against UTI by blocking colonization of *E. coli* onto uroepithelial cells in simian and murine urinary tract infection models (27-29). Due to their conserved serological properties, digalactoside-binding pili vaccines are considered potentially important candidates for broadly cross-protective vaccines against human *E. coli* urinary tract infections.

Combined vaccines that amplify immunity by interdictating several steps of the pathogenic sequence (i.e. colonization, proliferation, tissue damage and invasion/dissemination) are expected to provide more effective and prolonged immunity than the single component vaccine that neutralizes or prevents one pathogenic step.

When tested for the role of α-hemolysin antibody to modulate the pathogenic potential of hemolytic strains and for the efficacy of α-hemolysin in preventing pyelonephritis and renal tissue injury in the BALB/C mouse model, α-hemolysin vaccine did not protect against subsequent renal colonization by the wild-type pyelonephrogenic *E. coli* strain, however, there was significantly less renal damage in hemolysin recipients than the control group (28).

Furthermore, hemolysin recipients were significantly protected against septic death compared to lipopolysaccharide and phosphate-buffered-saline control recipient groups. Also, the combination and digalactoside-binding pili and hemolysin vaccines were protective. This study was undertaken to determine the degree of conservation of hemolysin genes corresponding to putative antigenic sites and functional sites of hemolysin by using synthesized oligonucleotides as probes, and hemolysin epitopes by using monoclonal antibodies on immunoblotting.

Materials and Methods

Strains used

E. coli strains isolated by Peter O'Hanley (Stanford University, U.S.A.) from voided urines of patients with asymptomatic bacteriuria, bladder infection, pyelonephritis or from feces of normal people were kindly provided for us. In each strain, A strains were from women with acute pyelonephritis or bladder infection, B strains from normal people, C strains from women with chronic UTI, P strains from male with acute UTI, L strains from UTI patients above 35 years old living in Washington, R strains from homosexuals with acute UTI, S strains from UTI patients below 35 years old, J96 is a strain isolated from a pyelonephritis women and studied in most detail. J96 belongs to O4, K6 serotypes and produces hemolysin, colicinV, F-13 type Gal-Gal pili, resistence to normal serum. C1212 produces hemolysin and $F7_2$ type Gal-Gal pili (3). GEJ800 is a recombinant strain that contains hemolysin operon cloned into pBR322 in a VCS host strain (17).

Assay for hemolytic activity

For the production of hemolysin on the solid agar medium, formation of clear hemolysis zones were checked on sheep red blood cell-agar or human red blood cell-agar plates. For the liquid assay of hemolytic activity, same procedure as published before was used (17).

Oligonucleotide labelling with polynucleotide kinase

150 ng of synthesized 20-mer oligonucleotides and 10 unit of kinase was mixed with 6 μl of 5× forward reaction buffer (BRL). 10 μCi[r-^{32}P] ATP was added. Distilled water was added to adjust the reaction volume to 30 μl. After 30 min incubation at room temperature, the temperature was raised to 37℃ and incubation was continued for 30 more minutes.

Purification of labelled oligomers

Sep-Pak column was washed with 20 ml acetonitrile and then with 20 ml distilled water. Labelled oligomers were poured on the washed Sep-Pak column. The column-bound oligomers were eluted

with 20 m*l* of 50% acetonitrile, dried and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 7.5).

Preparation of colony filters

The optical density of overnight grown *E. coli* cells was adjusted to be 1.0 unit. 25 μ*l* of these strains were dotted onto nitrocellulose filter (Bio-Rad) using Bio-Rad Dot Apparatus. Blotted nitrocellulose filters were overlaid onto TSA (2% bacto tryptone, 0.5% NaCl, 2.5% Na_2HPO_4, 2.0% bacto agar) plates and incubated for 3~4 hours at 37℃. Maniatis method was used for cell lysis on the nitrocellulose filter for colony blotting (31).

Hybridization

Susan's method was used for hybridization (32). Prehybridization solution contained 6× SSC, 5× Denhardt's, 10 mM EDTA (pH 7.5), 0.5% SDS and heat-treated (95℃, 10 min) yeast tRNA (100 μg/m*l*). After 8 hours incubation at 65℃, prehybridization solution was discarded and hybridization solution was added. Hybridization solution contained 6× NET, 5× Denhardt's, 0.1% SDS, heat-treated yeast-tRNA (250 μg/m*l*) and $0.5\sim1.0\times10^7$ cpm of radiolabelled oligomer probes. The temperature for hybridization was calculated using following equation. $T_H=T_D-5℃$, $T_D=2℃\times$ number of A-T base pairs$+4℃\times$ number of G-C base pairs. After 36 hours incubation, nitrocellulose filters were washed with 6× NET, 0.5% SDS 4 times at room temp., 8 min each time with gentle agitation. Final wash was done at T_H for 5 min. Washed and dried nitrocellulose filters were exposed for 2 days and developed.

Immunoblotting of wild type hemolysin using MAB probes

Like described above, colonies were grown on nitrocellulose filter. Hemolysin secreted during growth was adsorbed on nitrocellulose filter. Cells were washed away from nitrocellulose filter with blocking buffer by agitating gently for one hour. After 1 hour, 0.1 μg of each MAB was added and incubated for 90 min. Free MABs were washed away with PBS-Brij 3 times, 5 min each time. 1:1,000 diluted alkaline-phosphatase conjugated anti-mouse IgG was added and incubated for 1 hour. Then the filter was washed three times with PBS-Brij. The Bio-Rad alkaline-phosphatase substrate kit was used for color development.

Hemolysin neutralization assay

This procedure was described before (17).

Results

Hemolysin production of *E. coli* isolates

E. coli strains were isolated from voided urines of patients carrying UTI infections (asymptomatic bacteriuria, cystitis, pyelonephritis) or feces of normal people. These strains were assayed for the production of hemolysin on solid media using RBC-agar plates. Filtrable α-hemolysin production was checked after filtering through 0.45 μm Acrodisc membrane filters of the *E. coli* culture supernatants. The summary of the production of hemolysin on solid media and liquid assay was shown in Table 1.

Most of the strains which showed clear zones on RBC-agar plates were also positive on filtrable-hemolysin assay. C158 and S105 were positive on plate assay but were negative on liquid assay. These strains may carry only cell-bound β-hemolysin. Hemolysin titers of the strains showing hemolysis on liquid assay were variable from strain to strain.

Nucleotide sequence of the synthesized oligonucleotide probe

The information about the relationship between molecular structure and functional site of hemolysin has been steadily increased. J96 hemolysin is encoded in a bacterial chromosome. Its amino terminal partial amino acid sequence was known by Edman degradation and complete amino acid sequence was deduced from DNA sequencing (25). Whole protein is composed of 1,023 amino acids. Two transmembrane hydrophobic segments residing in R 240-R430 inferred from Kyte and Doolittle program (33) were implicated to be critical for hemolysin function. Albrecht *et al*. (26) site-direct mutagenized R313 and R314, and abolished hemolysin function.

Table 1. Summary of the presence of hemolysin production by various *E. coli* isolates

GEJ 800	J96	C1212	HB101	A105	A134	A109	A110	A111	A112	A115	A116
+/+	+/+	+/+	−/−	+/+	−/−	−/−	−/−	+/?	+/+	+/+	+/+
A154	B110	B210	B103	B131	C153	C155	C147	C152	C158	P128	P139
+/+	−/−	−/−	+/+	+/+	−/−	−/−	+/+	+/+	+/−	−/−	−/−
P104	P109	P112	P115	P117	P118	P121	P125	P127	L134	L136	L101
+/+	+/+	+/+	−/−	+/+	+/+	+/?	+/+	+/+	−/−	−/−	+/+
L103	L111	L122	L123	L135	R101	R103	R104	R105	R106	R107	R109
+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
S128	S170	S103	S104	S105	S109	S112	S117	S118	S121	S125	S129
−/−	−/−	+/−	+/+	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/?
S133	S135	S136	S137	S138	S141	S142	S144	S146	S148	S152	S160
−/−	+/+	+/+	+/+	?/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
S166	S175	S185	S194								
+/+	+/+	+/+	+/+								

Table 2. Characteristics of the oligonucleotide probes used in this experiment

Probes used	Base sequence	Correspodning amino acids	Position	Remarks
HA204	5′ AAACCCGCAATGCGGGAAAC	QTRNAGN*	41- 47	antigenic
HA224	AAAGATTATAAAGGGCAGGG	*KDYKGQG	55- 61	antigenic
HA419	TATCTGCGATTTCAGCAAGT	LSAISAS*	248-254	hydrophobic
HA477	CCGGTTTAATTGCTTCTGTT	AGLIASV*	306-312	hydrophobic
HA484	GTGACACTGGCAATTAGTCC	*VTLAISP	313-319	hydrophobic
HA507	GAGGAGTATTCACAACGATT	*EEYSQRE	336-342	hydrophobic
HA573	ACGGGGATAATTTCAGGCAT	*TGIISGI	402-408	hydrophobic
HA661	AGAAATGGAGACAAAACACT	*RNGDKTL	494-497	antigenic
HA697	ACCTTTCTGACAGCAAATCT	DLSDSKS*	532-538	antigenic
HA1100	TTTTTGATAAAGACGGCAGG	IFDKDGR*	929-935	antigenic

*Two corresponding bases of the marked amino acid are included in the synthesized oligomer.

Jameson and Wolf developed a program which can locate highly probable antigenic sites of the proteins. Using Jameson's program (34), Ji reported several strong candidates of antigenic sites (17). Based on the informations described above, we synthesized 5 oligomers corresponding to amino acid sequences of strong epitope candidates. Four probes were matched to transmembrane hydrophobic region, 1 probe to the hydrophilic region inbetween two transmembrane hydrophobic segments. Their characteristics were summarized in Table 2. Whether computer-searched epitopes will behave as real epitopes should be confirmed yet. It will be interesting to examine if MABs produced in a previous study and used as probes for this experiment can recognize the computer-searched amino acid sequences.

Oligonucleotide hybridization

For hybridization experiment following Maniatis, kinase-labelled radioactive 20-mer oligomer probes were used to do colony blotting on nitrocellulose filter. One example of the hybridization results was shown in Fig. 1 and summary of the hybridization

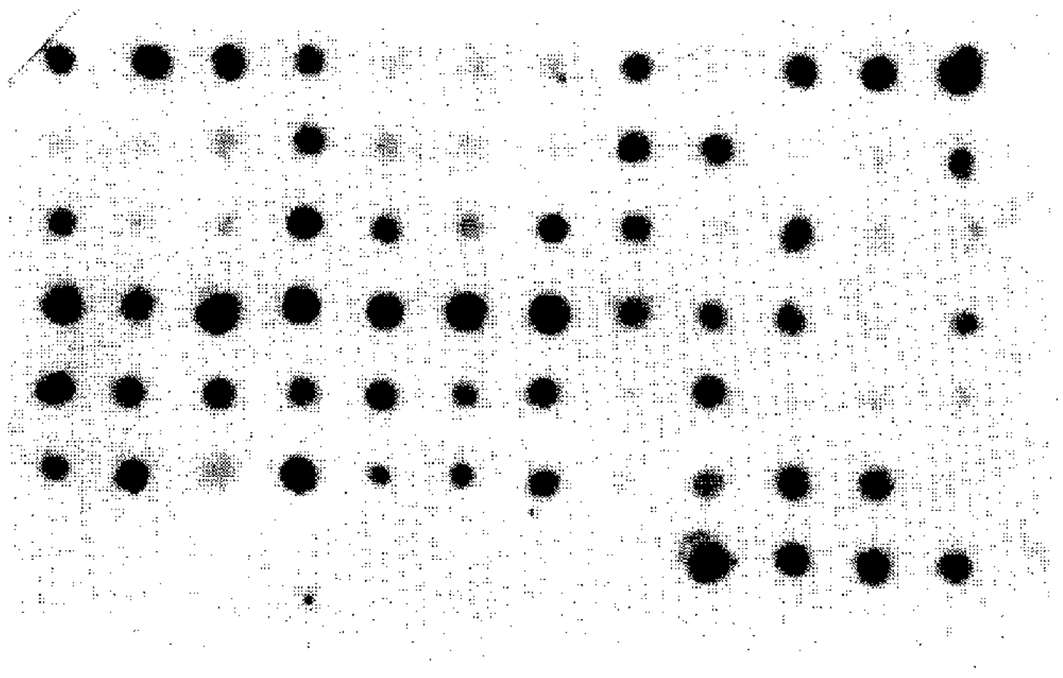

Fig. 1. A colony blot of hemolytic and nonhemolytic *E. coli* isolates probed with 20-mer oligonucleotide HA 507.

Table 3. Summary of the oligonucleotide hybridization pattern

Probes used	Hemolysin positive strains (53 +/+ strains)	Hemolysin negative strains (16 −/− strains)
HA204	50 +, 2 I, 1 −	16 −
HA224	51 +, 2 I	11 −, 5 I
HA419	51 +, 2 I	12 −, 5 I
HA477	50 +, 3 I	16 −
HA484	14 +, 1 I, 38 −	16 −
HA507	47 +, 6 I	16 −
HA573	52 +, 1 I	14 −, 2 I
HA661	33 +, 5 I, 15 −	16 −
HA697	47 +, 6 I	15 −, 1 I
HA1100	52 +, 1 I	16 −

+: positive signal, −: negative signal, I: intermediate signal

pattern using 10 different oligomer probes was shown in Table 3. Most of the probes showed negative signal against hemolysin negative strains. Probe HA224, HA419, HA573, HA697 showed intermediate signals against a few strains. For more accurate interpretation of these results, stringency of hybridization can be changed to examine further. Counting intermediate signals as positive, 8 probes showed nearly 100% positive hybridization signals against all hemolysin positive strains. HA484 showed positive signal against 28.3%, and HA661 against 71.7%, respectively. From these results, HA484 and HA661 regions are thought to be somewhat variable. HA484 corresponds to transmembrane hydrophobic region R313-R319, HA661 to epitope region of R491-R497. Many strains reacting negative against these probes still produced relatively high titers of hemolysin. This tells us that the variation of these regions can be tolerated for hemolysin function. Albrecht *et al.* reported that hemolysin activity was completely abolished by mutating hydrophobic alanine-valine (R313-R314) to hydrophilic aspartate-glutamate (26). It is likely that wild type strains reacting negative to HA484 probes may have degeneracy or changed from alanine-valine to other hydrophobic amino acids. For those strains showing clear zones on RBC-plates but negative hemolysin on liquid assay, all the probes showed no positive signals. This suggests that the nucleotide sequences of the filtrable α-hemolysin differ considerably from that of cell-bound β-hemolysin, and HA224 and HA 419 showed intermediate signal against 5 and 4 hemolysin negative strains respectively. Maybe these strains have some homologous sequence toward HA 224 and HA419.

Summary of the immunoblot pattern using 12 anti-HlyA MABs

13 anti-HlyA MABs were produced in the previous work. These MABs were used to analyze the antigenic topology of α-hemolysin on Western-blot using CNBr-cleaved hemolysin fragments (17). And also the degree of neutralization of hemolysis by these MABs was reported. Five MABs (MAB132, MAB145, MAB326, MAB417, MAB915) recognized CNBr II(R2-R160). Seven MABs (MAB151, MAB424, MAB559, MAB659, MAB703, MAB942, MAB943) recognized CNBr V (R425-R892). One MAB (MAB 835) recognized CNBr VI (R893-R1023). No MAB was produced which can recognize CNBr III (R161-R416) where transmembrane hydrophobic segments reside. Out of 13, 12 MABs were used in this study as immunoblotting probes against secreted and nitrocelluose-bound hemolysins during growth. All the MAB probes showed positive immunoblots against more than 90% of the hemolysin positive strains. In cases where the signal was negative against hemolysin positive strains, longer incubation of those strains on nitrocellulose filter gave positive immunodots. Therefore the negative result of the immu-

Table 4. Summary of the immunoblot pattern using 12 anti-HlyA MABs

Probes used	# of positive blots againt hemolysin positive strains (37 +/+ strains)		# of positive blots againt hemolysin negative strains (15 −/− strains)	
	1st trial	2nd trial	1st trial	2nd trial
MAB132	36	34	1	0
MAB145	29	34	1	0
MAB151	36	35	3	1
MAB326	36	35	0	0
MAB417	34	34	0	0
MAB424	30	33	0	0
MAB559	30	35	2	0
MAB659	32	35	0	0
MAB703	33	34	0	0
MAB835	34	34	0	0
MAB915	36	35	0	0
MAB942	36	35	0	0

Table 5. Hemolysin inhibition by MAB132

	+559 +132			+559 +132			+559 +132	
P127	32	20	A112	32	12	A105	64	16
R106	32	20	A154	32	12	A115	32	6
S142	12	8	B103	24	10	B131	16	4
S185	64	48	L103	16	6	C147	16	4
S194	32	24	L123	64	26	L101	8	2
			P125	12	4	L122	64	4
			R109	8	4	L135	16	4
			S109	64	24	P104	32	8
			S118	10	4	P109	32	8
			S121	16	6	P112	32	8
			S160	12	4	R101	16	4
						R105	32	8
						S104	32	8
						S125	64	16
						S135	32	8
						S144	32	8
						S146	64	4
						S160	16	4
						S175	64	16
						J96	64	16
						GEJ800	1024	256

3/4-1/2 reduction 1/2-1/3 reduction 1/4 reduction

noblots against some of the hemolysin positive strains were in most cases due to low levels of hemolysin production from low-titer strains. Conclusively, J96 hemolysin epitopes were well conserved in most of the wild type hemolysin positive strains. This result is consistent with the oligonucleotide hybridization pattern.

Neutralization of wild type hemolysins by MAB 132

MAB132 was shown to neutralize J96 hemolysin most strongly and MAB559 didn't show any neutralization. In this experiment, neutralizing ability of MAB132 against the hemolysins produced from various wild type *E. coli* strains was tested. As shown in Table 5, MAB132 neutralized all of the wild type hemolysins giving from 3/4 reduction to 1/4 reduction of the hemolysin titers. This result shows that anti-J96 HlyA MAB132 can neutralize against all hemolysins at somewhat various degree.

Discussion

This project was carried out to examine the homology of the antigenic and functional sites among wild type *E. coli* strains isolated from UTI infected people or from normal people. Also the capability of neutralization of the anti-J96 HylA MAB132 was investigated for the evaluation of using hemolysin as a cross-immunity vaccine. Many studies have undertaken to analyze the functional sites of the

J96 hemolysin. Complete amino acid sequence was deduced from the Sanger dideoxysequencing of the cloned hemolysin gene (25). Antigenic sites and functional sites analysis using anti-Hly A MABs has been undertaken (17). Based on those efforts, Kyte and Doolittle's hydropathy profile (33) and Jameson's antigenecity program (34), ten kinds of 20 mer-oligonucleotide probes were synthesized. Albrecht *et al.* (26) showed that hemolysin R299-R327 transmembrane hydrophobic region is essential for hemolysin function. In this study four 20 mer-oligonucleotides corresponding to the most hydrophobic region and one 20 mer-oligonucleotide corresponding to the hydrophilic region inbetween two transmembrane hydrophobic region were synthesized. Also five oligomers corresponding to the computer-searched strong antigenic sites on Jameson program. These probes were used for hybridization on colony blotting against various *E. coli* strains. Eight oligomers showed positive signals against more than 90% of the hemolysin positive strains. This is the first extensive investigation of the homology between hemolysin positive strains. Extraintestinal infecting *E. coli* isolated from human encodes for hemolysin in chromosome in human (19, 20, 22). Majority of the hemolysin positive strains infecting animals contain hemolysin gene in extrachromosomal plasmid DNA (10, 18). Hess *et al.* found that chromosome-encoded hemolysin gene and plasmid-encoded one share high homology (35). Also *E. coli* hemolysin gene does have significant homology with *Proteus vulgaris* hemolysin gene, *Morganella morganii* hemolysin gene, and *Pasteurella haemolytica* leukotoxin (36-38). The conservation of hemolysin gene studied from the oligoncleotide probing was reconfirmed by the immunoblotting. All 12 MABs showed more than 90% positive immunoblotting against hemolysin positive strains. Ferdinand *et al.* produced 3 anti-Hly A MABs. On immunoblotting they showed that 2 anti-HlyA MABs recognized most of the hemolysin positive strains they used but 1 MAB recognized only a few strains (39). We think our experiment is more comprehensive. Hemolysin itself is weak in protecting UTI infection but can give a significant protection from cell damage. Gal-Gal pili vaccine gives good protection from infection. There are various serotypes of Gal-Gal pili, but four serotypes comprise 95% of the UTI strains and they share a common antigenic epitope (27). As nucleotide and amino acid sequence of Gal-Gal pili were known, putative strong epitopes were inferred by using computer analysis. Chosen synthetic peptide vaccine R5-12 protected from infection of 7 strains out of 8 strains (27). Also Gal-Gal whole pili vaccine obtained from HU 847, which is a recombinant of HB101 having J96 Gal-Gal pili gene cloned, gave protection from most of the wild type *E. coli* (40). The fact that J96 Gal-Gal pili cross-protected the attack from various *E. coli* strains and the conservation of hemolysin functional sites and antigenic sites shown in this study suggests that combination vaccine containing Gal-Gal pili and hemolysin together would amplify immunity by interdicting several steps of pathogenic sequence (i.e. colonization, proliferation, tissue damage and invasion) and the problem of antigenic drift.

Now Gal-Gal pili and hemolysin can be purified in a high yield because these two proteins can be obtained from genetic-engineered recombinants.

요 약

요로계 감염에 대처함에 있어 hemolysin과 Gal-Gal pili의 multicomponent 백신 개발 가능성을 조사하기 위하여 본 연구를 수행하였다. 요로계 감염 환자로부터 분리되어 그의 hemolysin gene의 염기배열과 아미노산 배열이 밝혀진 대장균 J96 균주의 hemolysin에 대하여 10곳에 해당하는 20 mer-oligonucleotide를 합성하였다. 이들 probe를 사용하여 요로계 감염 환자들로부터 분리한 wild type 대장균들의 DNA에 대하여 hemolysin gene의 상동성을 조사하였는 바 8개의 probe는 거의 모든 hemolysin 생산 wild type에 대하여 positive hybridization signal을 보여주었고 HA484는 28.3%, HA661은 71.7%의 positive signal을 나타내었다. 이는 요로계 감염 대장균들의 hemolysin gene의 상동성이 매우 높은 것을 의미한다. 또한 J96 hemolysin에 대한 12개의 MAB은 모두 wild type 대장균들이 분비하는 90% 이상의 균주에 대하여 양성 immunoblotting을 나타내었다. 특히 J96 hemolysin의 functional site를 가장 강력

하게 block할 수 있는 monoclonal antibody MAB132가 모든 wild type으로부터 분리된 hemolysin의 function을 중화시킬 수 있는 결과는 J96의 cloned hemolysin product를 vaccine으로 사용할 수 있는 가능성을 증대시키는 결과라고 할 수 있다.

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