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Use of Monoclonal Antibodies in the Study of Common Antigens of Gram-Negative Bacteria

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I. INTRODUCTION

Monoclonal antibodies, when used in the study of the immunogenic properties of bacterial cell surface antigens, have several obvious advantages over polyclonal sera including availability, specificity, and reproducibility. These features of monoclonal antibodies make them the best possible reagents for studying the serology and taxonomy of bacteria. Such reagents should help in the diagnosis of difficult-to-identify organisms such as *Legionella* (10) and may also find use as

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therapeutic agents against bacterial infections (8). In this chapter we will briefly review studies from our laboratory with regard to the use of monoclonal antibodies in bacterial serology and taxonomy.

Bacterial cell surface (outer membrane) macromolecules are the primary targets of immunological comparisons of gram-negative bacteria in serologic and taxonomic studies. The outer membranes of gram-negative bacteria contain two classes of molecules of immunological importance, proteins and lipopolysaccharides (LPS) (9, 14, 20). Bacterial LPS has been the subject of intensive structural and immunological studies in recent years. Some of the immunological functions of LPS include strong antigenicity (11), a role in resistance to phagocytosis (16,18), and in resistance to the action of serum (19), mitogenic action on B cells (22), and general endotoxicity in animals (1). Structural studies on LPS indicate a tripartite structure (15) in which the hydrophobic membrane-associated lipid A region is covalently attached to the rough core oligosaccharide region which is often but not always capped with an O-antigenic side chain. These O side chains are polymeric structures of varying lengths, with a repeating tri- to pentasaccharide unit. The O-antigen can constitute the major antigenic structure of a gram-negative cell, and thus it often determines serologic specificity (3,11).

II. BACKGROUND

One of the more important tasks of clinical and bacteriological laboratories is the classification of bacteria. Identification of the taxonomic group of a bacterium allows a rapid prediction of the properties of that bacterium by simple extrapolation to other previously studied organisms of the same species. In the clinical setting, rapid diagnosis of the species causing infection can profoundly influence the strategy and outcome of the therapy. For example, although they nominally belong to the family Pseudomonadaceae, three species known to cause human infections, *Pseudomonas aeruginosa, P. cepacia,* and *P. maltophilia,* are now considered taxonomically distinct (5). Thus, therapeutic strategies effective for *P. aeruginosa* lung infections in cystic fibrosis patients, for example, will not necessarily be effective for *P. cepacia* infections of cystic fibrosis patients.

For the bacteriologist these comparisons are equally important. He is able to predict certain similarities in biosynthetic pathways and transport mechanisms, etc., of *Azotobacter vinelandii* and *Pseudomonas fluorescens* on the basis of their taxonomic interrelationship (5); in contrast, the aforementioned three taxonomically different Pseudomonadaceae species might be expected to have quite different properties.

In addition to classification of bacteria into large subdivisions, e.g., species or genera, there is considerable value in subdivision of species of bacteria into

smaller groups. For example, the serologic subdivision of bacteria allows epidemiological studies to be made, and often the source of epidemic outbreak of disease can be traced by this method.

A number of methods are currently used in the subdivision of bacteria into groups, including DNA homology, RNA homology, fatty acid analyses, and biotyping [see, e.g., (5)]. In this review we discuss the potential use of monoclonal antibodies against cell surface macromolecules in bacterial serology and taxonomy.

III. RESULTS AND DISCUSSION

A. Use of Monoclonal Antibodies as Serologic Aids

Pseudomonas aeruginosa has been classified on the basis of differences in lipopolysaccharide (LPS) O-antigenic side chains into the 17 serotypes of the International Antigen Typing Scheme (3,11). One major problem associated with such serotyping schemes is illustrated by *P. aeruginosa* isolates from cystic fibrosis patients. These strains are often difficult to serotype, since they either fail to agglutinate with any typing sera, self-agglutinate, or polyagglutinate with more than one O-antigen typing sera. These abnormal typing patterns, occurring in up to 60% of cystic fibrosis isolates (21,23), correlate with deficiencies in the amounts of O-antigen in the LPS of these strains (6). Such observations bring into question the broad application of O-antigen-based antisera for serotyping of *P. aeruginosa* and other gram-negative bacteria, especially in epidemiological, diagnostic, or clinical identification studies.

We have extensively characterized three LPS-specific monoclonal antibodies derived from hybridomas isolated from the fusion of NS1 myeloma cells and spleen cells from BALB/c mice immunized with *P. aeruginosa* strain H103 or AK1012 (a rough derivative lacking O-antigenic side chains) outer membranes. It should be noted that in our experience it is rather easy to isolate hybridomas secreting LPS-specific monoclonal antibodies, and we have isolated hundreds in the past 3 years, although most of these have not been extensively characterized. Our primary method of identification of LPS-specific monoclonal antibodies has been to screen hybridoma supernatants by enzyme-linked immunosorbent assay (ELISA) using purified strain H103 LPS (7). These are further classified as LPS rough core or O-antigen specific using strain AK1012 LPS (lacking O-antigen) as an antigen in ELISAs. The value of LPS as a monoclonal antibody screening tool is that it is rather easy to purify free of other cellular components (4). In contrast, the converse, isolation of outer membrane proteins free of LPS, is extremely difficult, if not impossible.

Two rough core-specific monoclonal antibodies were characterized (6,13)

TABLE I

Cross-Reaction with Monoclonal Antibodies Specific for Outer Membrane Antigens

Taxonomic grouping based on rRNA homology[a]	Bacterial outer membrane antigen	Reaction using monoclonal antibodies to: Outer membrane proteins H2 (MA1–6)	F (MA4–4)	F (MA5–8)	Lipopolysaccharide: O-antigen (MA1–8)	Rough core (MA3–5, MA3–6)	Lipid A (5E4, 8A1)
P. fluorescens	*P. aeruginosa* serotype 5	+	+	+	+	+	+
	P. aeruginosa serotypes 7, 8, 10, 14, 16	+	+	+	–	+	+
	P. aeruginosa other serotypes	+	+	+	–	–	+
	P. aeruginosa rough	+	+	+	–	–	+
	P. aeruginosa protein F deficient	+	–	–	+	+	+
	P. putida	+	+	–	–	ND[b]	+
	P. syringae	+	+	–	–	ND	ND
	P. chlororaphis	+	–	–	–	ND	ND
	P. aureofaciens	+	–	–	–	ND	ND
	P. stutzeri	+	–	–	–	ND	ND
	P. fluorescens	+	–	–	–	ND	+
	P. anguilliseptica	+	–	–	–	ND	+
Azotobacter	*Azotobacter vinelandii*	+	–	–	–	ND	+
Xanthomonas	*P. maltophilia*	–	–	–	–	ND	ND

	P. aeruginosa protein F deficient	+	−	−	+	+	+
	P. putida	+	+	−	−	ND[b]	+
	P. syringae	+	+	−	−	ND	ND
	P. chlororaphis	+	−	−	−	ND	ND
	P. aureofaciens	+	−	−	−	ND	ND
	P. stutzeri	+	−	−	−	ND	ND
	P. fluorescens	+	−	−	−	ND	+
	P. anguilliseptica	+	−	−	−	ND	+
Azotobacter	*Azotobacter vinelandii*	+	−	−	−	ND	+
Xanthomonas	*P. maltophilia*	−	−	−	−	ND	+
P. acidovorans	*P. acidovorans*	−	−	−	−	ND	ND
P. solanacearum	*P. solanacearum*	−	−	−	−	ND	ND
	P. cepacia	−	−	−	−	ND	+
Enterobacteriaceae	*Escherichia coli*	−	−	−	−	ND	+
	Salmonella typhimurium	−	−	−	−	ND	+
	Edwardsiella tarda	−	−	−	−	ND	+
	Yersinia pestis	ND	ND	ND	ND	ND	+
Vibrionaceae	*Vibrio cholera*	ND	ND	ND	−	ND	+
	Vibrio anguillarum	−	−	−	−	−	+
	Aeromonas salmonicida	−	ND	ND	−	ND	+
	Aeromonas hydrophila	−	−	−	−	ND	+
Rhizobiaceae	*Agrobacterium tumefaciens*	ND	ND	ND	−	ND	+

[a] Based on the data and discussion of De Vos and De Ley (5). *Pseudomonas anguilliseptica* is placed in this group on the basis of whole-cell fatty acid analysis and immunological cross-reactions.

[b] Not determined.

(Table I). Antibody MA3-5 interacted with the LPS of serotypes 5 (the serotype of strain H103), 7, 8, 10, 14, and 16 of the International Antigen Typing Scheme of *P. aeruginosa* (17 serotypes), whereas MA3-6 interacted only with serotypes 5, 8, 10, and 16. Each of these monoclonal antibodies interacted with only 1 of the 16 *P. aeruginosa* isolates from patients with cystic fibrosis tested; MA3-5 interacted with strain CF1452 and MA3-6 with strain CF4522 (6). This provides the first definitive evidence for LPS rough-core heterogeneity in *P. aeruginosa*. Furthermore, the high specificity of MA3-5 and MA3-6 indicates the possibility that some of the typing sera in the larger typing schemes for *P. aeruginosa* may be directed against LPS rough-core determinants.

We also characterized an O-antigen-specific monoclonal antibody, MA1-8. This antibody is relatively specific for serotype 5 of the International Antigen Typing Scheme for *P. aeruginosa*. However, we did observe slight cross-reaction with a serotype 17 strain (7), an observation made previously with polyclonal sera (11). Thus, even at the monoclonal antibody level, antigenic cross-reactions between strains of different serotypes are not avoided.

These studies point out some of the difficulties that may be experienced in using monoclonal antibodies directed against antigenic sites on LPS. In contrast, *Tam et al.* (20) have clearly demonstrated the potential of monoclonal antibodies against outer membrane protein I in typing *Neisseria gonorrhoeae*.

B. Monoclonal Antibodies to Lipid A: A Common Antigen in Gram-Negative Bacteria

Chemical characterization of the lipid A portions of the LPS of diverse bacteria has indicated that lipid A is a strongly conserved structure (17). Consistent with this, lipid A bears the general name "endotoxin" and lipid A's from different bacteria have similar effects on host cells (1).

We have demonstrated, in studies using monoclonal antibodies reactive against purified lipid A from *Escherichia coli* J-5 and *P. aeruginosa* PA01 (12), extensive antigenic cross-reactivity among outer membranes and LPS preparations from 36 *P. aeruginosa* strains and 22 other gram-negative bacteria from the families Vibronaceae, Enterobacteriaceae, Rhizobiaceae, and Pseudomonadaceae. These monoclonal antibodies, 5E4, 8A1, 1D4, and 6B2, were isolated by Centocor, Malvern, Pennsylvania, from a mouse immunized with whole cells from *E. coli* strain J-5. They showed no cross-reaction by ELISA or by the Western electroblot procedure with gram-positive organisms.

One of these monoclonal antibodies, 5E4, interacted with a similar fast-migrating band corresponding to the rough LPS from *P. aeruginosa* strains of all 17 serotype strains and 14 clinical isolates from cystic fibrosis patients. This monoclonal antibody also interacted with outer membranes or LPS from many other gram-negative bacteria, including *P. putida, P. fluorescens, P. anguilliseptica,*

P. cepacia, Edwardsiella tarda, Agrobacterium tumefaciens, Vibrio anguillarum, Vibrio cholera, E. coli, Salmonella typhimurium, Azotobacter vinelandii, Aeromonas hydrophilia, and *Aeromonas salmonicida* strains (Table I). In addition, the three other monoclonal antibodies, 8A1, 1D4, and 6B2, interacted with between 84 and 97% of all strains of gram-negative bacteria tested.

This extensive cross-reactivity of the lipid A-specific monoclonal antibodies explains in part the protective effects of antisera to *E. coli* J5 organisms against bacteremia caused by other gram-negative bacteria (2). The strong conservation of these antigenic determinants throughout gram-negative bacteria suggests that lipid A has an important role in these bacteria and may indicate a common evolutionary lipid A molecule.

C. Antigenic Conservation of *Pseudomonas aeruginosa* Outer Membrane Protein H2

In *P. aeruginosa* we have demonstrated the conservation of outer membrane polypeptides among the serotype strains by three methods: conservation of protein receptors for bacteriophages, close similarity of outer membrane protein patterns on SDS–polyacrylamide gel electrophoresis, and antigenic cross-reactivity using polyclonal serum to *P. aeruginosa* PA01 strain H103 outer membranes (14). In recent studies (6,7,13), we have applied hybridoma technology to the production of highly specific monoclonal antibodies that react with specific antigenic sites (epitopes) on outer membrane proteins and have used these monoclonal antibodies to study the antigenic conservation of these epitopes among the pseudomonads and other gram-negative bacteria. A major finding from these studies was that all *P. aeruginosa* strains studied shared at least two separate conserved outer membrane antigenic sites, on proteins F and H2, regardless of source, serotype, colony morphology (i.e., mucoid or nonmucoid, rough or smooth), or serum susceptibility.

Monoclonal antibody MA1-6 (7) interacted specifically with the major outer membrane lipoprotein H2 (Fig. 1). The antigenic site was present on lipoprotein H2 from the 17 serotype strains (Fig. 1) and 28 of the 30 cystic fibrosis *P. aeruginosa* strains examined (Fig. 2). The outer membranes from the two exceptional strains (Fig. 2, lanes 7 and 8) showed no protein H2 on SDS–polyacrylamide gels stained with Coomassie Blue. In the double-antibody blot (Fig. 2), a monoclonal antibody, MA4-4, specific for protein F showed a labeled band that comigrated with purified protein F in all the outer membranes of the cystic fibrosis isolates. Monoclonal MA1-6 showed no interaction with protein F (lane F, Fig. 2).

Cross-reactivity of the monoclonal antibody MA1-6 was also seen with another outer membrane protein of similar molecular weight as lipoprotein H2 of *P.*

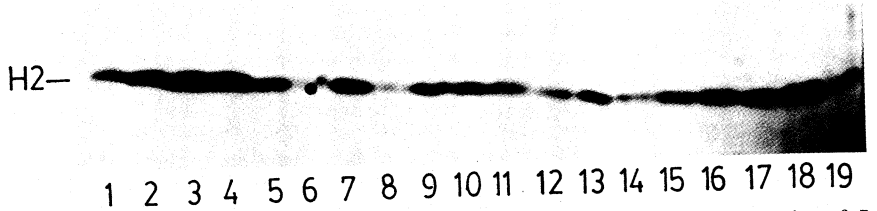

Fig. 1. Western electrophoretic blots of outer membranes of the serotyping strains of *P. aeruginosa* after treatment with monoclonal antibody MA1-6. The blot was made by electrophoretic transfer of separated outer membrane proteins from SDS–polyacrylamide gels onto nitrocellulose paper. These electrophoretic blots were treated with the monoclonal antibodies followed by a goat anti-mouse alkaline phosphatase conjugated antibody and addition of the substrate (Napthol AS MX phosphoric acid and Fast Red TR salt). Outer membrane samples were lane 1, wild-type H103; lane 2, serotype 1; lane 3, serotype 2; lane 4, serotype 3; lane 5, serotype 4; lane 6, serotype 5; lane 7, serotype 6; lane 8, serotype 7; lane 9, serotype 8; lane 10, serotype 9; lane 11, serotype 10; lane 12, serotype 11; lane 13, serotype 12; lane 14, serotype 13; lane 15, serotype 14; lane 16, serotype 15; lane 17, serotype 16; lane 18, serotype 17; lane 19, purified lipoprotein H2. only one band was labeled. Only the relevant portion of the blot is shown, no other bands appeared.

aeruginosa in Western immunoblots from *P. chlororaphis, P. fluorescens, P. putida,* and *P. syringae,* with a protein of higher molecular weight in *P. stutzeri, P. pseudomallei, P. anguilliseptica,* and *A. vinelandii,* and with a protein of slightly lower molecular weight in *P. aureofaciens* (13a). There was no cross-reactivity with the outer membranes of *E. coli, P. acidovorans, P. maltophilia,* or *S. typhimurium* [Hancock *et al.* (7) and unpublished observations].

These results confirm taxonomic data from rRNA homology studies suggesting *A. vinelandii* is taxonomically related to the fluorescent pseudomonads, while *P. acidovorans* and *P. maltophilia* are not closely related (5). We have also shown antigenic cross-reactivity, using polyclonal sera, between *P. fluorescens, P. putida,* and *P. anguilliseptica,* despite the fact that *P. anguilliseptica* is nonfluorescent. We feel that this monoclonal antibody, MA1-6, may be valuable for determining taxonomic relationships between the *P. fluorescens* branch (5) of the Pseudomonadaceae.

D. Antigenic Conservation of Porin Protein F

In studies using five different monoclonal antibodies specific for antigenic epitopes on outer membrane porin protein F of *P. aeruginosa,* we demonstrated antigenic conservation among all tested strains of *P. aeruginosa* (13). The specificity of these monoclonal antibodies for protein F was demonstrated by the absence of cross-reactivity with a protein F-deficient mutant strain H283 derived from *P. aeruginosa* PA01 strain H103 (13), and by their interaction with protein

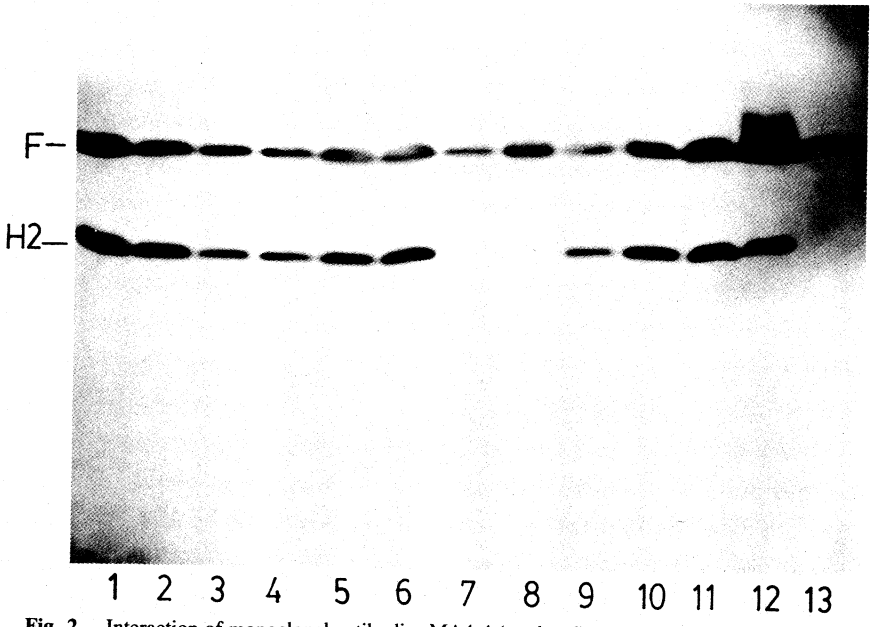

Fig. 2. Interaction of monoclonal antibodies MA4-4 (top band) and MA1-6 (bottom band) with outer membranes from six pairs of mucoid and nonmucoid cystic fibrosis *P. aeruginosa* isolates. The electrophoretic blots of the outer membranes were prepared as described for Fig. 1. The outer membrane samples were lane 1, wild-type H103; lane 2, Plm; lane 3, CFClm; lane 4, CFClnm; lane 5, CFC47m; lane 6, CFC47nm; lane 7, CFC46m; lane 8, CFC46nm; lane 9, CFC4m; lane 10, CFC4nm; lane 11, CFC6m; lane 12, CFC6nm; lane 13, purified protein F. Abbreviations: m, mucoid and nm, nonmucoid.

F on Western blots of SDS–polyacrylamide gel separated outer membrane components. Figure 3 shows the interaction of monoclonal antibody MA4-4 with outer membrane proteins isolated from the serotype strains of *P. aeruginosa*. Four of these monoclonal antibodies, MA2-10, MA4-2, MA4-4, and MA4-10, showed antigenic cross-reactivity with one *P. syringae* and two *P. putida* strains (Table I). These monoclonal antibodies failed to interact with porin protein F after reduction of its internal disulfide bond with 2-mercaptoethanol. The other monoclonal antibody, MA5-8, interacted exclusively with *P. aeruginosa* porin protein F. Monoclonal antibody MA5-8 was the only one of the five porin protein F-specific monoclonal antibodies studied which was able to interact with 2-mercaptoethanol-treated protein F, but unlike the other antibodies, it failed to interact with *P. putida* or *P. syringae* strains. None of the monoclonal antibodies interacted with the outer membranes from a variety of other gram-negative species, including strains which shared reactivity with monoclonal antibody MA1-6 (Table I).

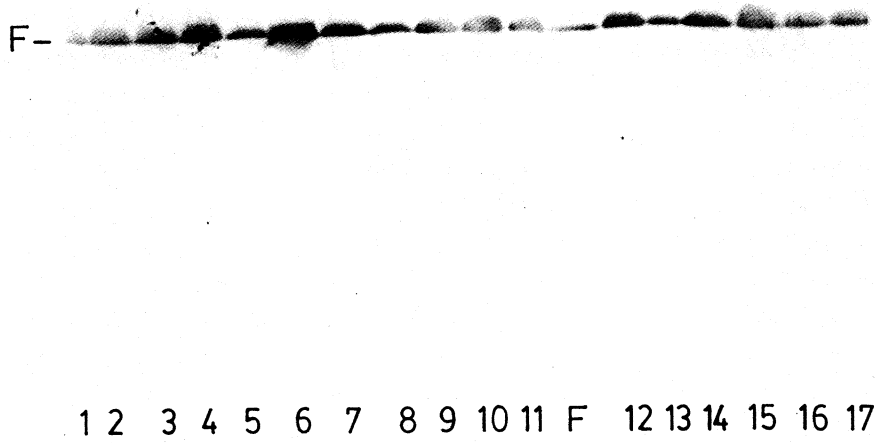

Fig. 3. Interaction of monoclonal antibody MA4-4 with outer membranes from the serotyping strains of *P. aeruginosa*. The numbers at the bottom of each lane denote the number of the serotyping strains 1–17, with purified protein F in lane F.

We have also shown cross-reactivity among the *P. aeruginosa* serotype strains with another monoclonal antibody, MA1-3. However, we have not been able to identify the specific antigenic determinant recognized by this monoclonal antibody.

IV. CONCLUSIONS AND PROSPECTS

These studies demonstrate the feasibility and application of monoclonal antibodies that are highly specific for an antigenic determinant as tools to study the immunologic and taxonomic relationships of bacterial outer membranes. Using these monoclonal antibodies, we have identified the existence of both common and variable antigenic determinants in the outer membrane protein and LPS molecules of pseudomonads and other gram-negative bacteria. The antigenic determinants on proteins were conserved only among closely related strains of bacteria, as opposed to LPS lipid A determinants which are shared by diverse gram-negative bacteria. Such monoclonal antibodies are thus potentially useful in taxonomic, epidemiological, and clinical studies. It is our feeling that a battery of similar monoclonal antibodies will make classification of bacteria considerably easier.

V. SUMMARY

Monoclonal antibodies provide excellent tools for studying the conservation of specific antigenic sites in different bacteria. For example, monoclonal antibodies specific for the rough-core or O-antigenic side chain of one *Pseudomonas aeruginosa* strain demonstrated only limited cross-reaction with other *P. aeruginosa* strains. In contrast, monoclonal antibodies specific for the lipid A portion of LPS interacted with the LPS or outer membrane of 36 *P. aeruginosa* strains and 22 other gram-negative bacteria from four different families and 16 separate genera. This demonstrates that lipid A is antigenically conserved (highly) in gram-negative bacteria. A monoclonal antibody against outer membrane lipoprotein protein H2 of *P. aeruginosa* interacted with 28 *P. aeruginosa* strains and organisms representative of eight other genera of the family Pseudomonadeceae (mostly members of the fluorescent pseudomonads), as well as *Azotobacter vinelandii,* but did not interact with pseudomonads demonstrated to be unrelated to *P. aeruginosa* by rRNA homology studies; nor did it react with bacterial strains from other gram-negative bacterial genera. A group of four monoclonal antibodies against porin protein F of *P. aeruginosa* interacted with all *P. aeruginosa* strains tested as well as with *P. putida* and *P. syringae* strains, but not with other pseudomonads. Another protein F-specific monoclonal interacted only with the *P. aeruginosa* strains. We feel that similar and more extensive studies will be of great benefit to bacterial taxonomy.

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Application
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I. INTRODUCTION

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