Antigenic mimicry of *Clostridium chauvoei* flagella by polyclonal anti-idiotypic antibodies

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Summary. Polyclonal rabbit anti-idiotypic (anti-Id) antibodies against two monoclonal antibodies (MAbs) specific for the flagella of *Clostridium chauvoei* were produced, purified and characterised. Lack of cross-reactivity with heterologous MAbs indicated that the anti-Id antibodies were highly specific. The surface-exposed epitopes of the flagellar filament recognised with protective MAb were further distinguished by the anti-Id antibodies. Moreover, each anti-Id antibody inhibited the binding of its related MAb to flagellar antigens in a competitive enzyme-linked immunosorbent assay, suggesting that the anti-Id antibodies bore an internal image of the flagellar antigens. The survival rate of mice was increased to nearly twice that of controls by immunisation with anti-Id 41, which had been produced with a protective MAb; in contrast, anti-Id 114, produced with a non-protective MAb, failed to immunise. The results suggest that an anti-Id antibody containing an internal image of *C. chauvoei* flagella might be used as a vaccine.

Introduction

Clostridium chauvoei causes blackleg, a fatal disease of cattle, sheep and other ruminants associated with spore-contaminated soil. Formalin-treated whole cultures are currently used for vaccination. In contrast to immunity against other clostridial diseases, in which toxoids serve as highly efficacious vaccines, immunity against *C. chauvoei* infection depends to a considerable extent on flagellar antigens.^{1–4} Purified flagella of *C. chauvoei* were protective in mice, and the immunogenicity of non-flagellate mutants was 100-fold less than that of the flagellate parent strain. However, many points remain to be clarified as to the nature of the protective flagellar antigens.

Recently, we obtained five monoclonal antibodies (MAbs) directed against the flagella of *C. chauvoei*.⁵ Amongst them, three were protective and recognised surface-exposed and conformational epitopes on the flagellar filament; the remaining two were non-protective, recognising only internal epitopes.^{5,6}

In this study, anti-idiotypic (anti-Id) antibodies against two anti-flagellar MAbs were prepared. The flagellar epitopes were examined and anti-Id antibodies were tested for their ability to induce protective immunity in mice.

Materials and methods

Monoclonal antibodies

The production and characterisation of MAbs

against the flagella of *C. chauvoei* was described previously.^{5,6} Briefly, three MAbs (Mo-41, Mo-62 and Mo-90) showed passive protective effects and recognised surface-exposed flagellar epitopes. In contrast, non-protective MAb Mo-114 recognised an internal flagellar epitope. MAbs Mo-41 and Mo-114 were chosen to generate anti-Id antibodies, and immunoglobulin G (IgG) was purified from ascitic fluid by affinity chromatography on protein G-Sepharose (Pharmacia LKB Biotechnology, Tokyo, Japan). MAbs 1-G (IgG₃) for *Haemophilus paragallinarum*,⁷ and MG-1 (IgG₁) for *Mycoplasma gallisepticum* (unpublished observations) were also purified and used as controls in an enzyme-linked immunosorbent assay (ELISA).

Analysis of MAb purity

Ascitic fluid and purified immunoglobulin were analysed by SDS-PAGE according to Laemmli.⁸ Proteins were stained with Coomassie Brilliant Blue R-250. Purity was further evaluated by Western blot assays⁹ with horseradish peroxidase (HRPO)-conjugated anti-mouse IgG goat serum (BioRad Laboratories, Tokyo, Japan).

Production of anti-Id antibodies

Japanese white rabbits were given two subcutaneous injections of 500–700 µg of IgG protein of each MAb emulsified in an equal amount of Freund's complete adjuvant at an interval of 3 weeks and then given three or four booster doses of 100–200 µg of IgG protein of each MAb, intravenously, at intervals of 2 weeks.

Anti-MAb sera were collected periodically throughout the immunisation schedule and analysed for anti-Id activity by ELISA.¹⁰ The sera possessing the highest idiotypic-specific activity were pooled and the anti-Id antibodies were purified.

Purification of anti-Id antibodies

The specific anti-Id antibodies were purified by sequential affinity chromatography.¹⁰ First, the IgG fraction of each rabbit serum was isolated with a protein G-Sepharose affinity column. Purified IgG was then passed through a Sepharose 4B column to which had been coupled normal mouse IgG (chromatographically purified; Zymed Laboratories, CA, USA) to remove antibodies directed against the constant region of mouse immunoglobulin, and the unbound IgG of each rabbit serum was collected and concentrated by ultrafiltration with a type B15 membrane (Amicon Corporation, MA, USA). The success of this procedure was monitored by the failure of the control MAbs, 1-G and MG-1, to bind in an ELISA to wells coated with the absorbed sera.¹⁰

ELISA

All assays were performed in 96-well micro-ELISA plates (Dynatech Laboratories, Virginia, USA). Wells were coated overnight at 4°C with antigen suspended in 100 µl of coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.5) and incubated for 3 h with 200 µl of blocking buffer (bovine serum albumin 1% in coating buffer) to saturate the protein-binding capacity of the wells. Between subsequent steps of the assay, wells were washed with physiological saline containing Tween 20 0.05%. Antibodies (HRPO-conjugated or unconjugated) were added in 100-µl volumes, the diluent being Dulbecco's phosphate-buffered saline (PBS) containing fetal calf serum 5% and Tween 20 0.05%. *o*-Phenylenediamine was used as the substrate, and colour development was produced with 100 µl of substrate solution for 30 min at room temperature in the dark. The reaction was stopped by adding 100 µl of 2 N H₂SO₄. The optical density of each well was observed with a Sjeia Auto Reader (Sanko Junyaku Co. Ltd, Tokyo, Japan) at 490 nm. The assays used are described below.

Assay of anti-Id antibody in rabbit antisera. The wells were coated with serial dilutions of unabsorbed or absorbed anti-Id sera. Homologous or control MAbs were incubated in the wells for 1 h at room temperature, and bound antibody was detected by the addition of HRPO-conjugated anti-mouse IgG goat serum for 1 h.

Idiotypic competition assay. A competitive binding assay was carried out to detect idiotypic expression on immunoglobulin molecules.¹⁰ Wells were coated with anti-Id antibodies. Dilutions of the test competitor (unconjugated MAbs) were added to the wells 2 h before the addition of HRPO-conjugated MAb. The

percentage of competition was determined by the formula $100(A-n)/(A-B)$, where A is the OD in the absence of competing antibody, B is the OD in the presence of homologous antibody, and n is the OD in the presence of competitor.⁵

Antigen binding inhibition assay. The ability of anti-Id antibodies to inhibit the binding of different anti-flagellar MAbs to *C. chauvoei* flagella was examined. HRPO-conjugated MAbs were pre-incubated with serial dilutions of anti-Id antibodies for 2 h at room temperature before addition to purified flagella-coated⁶ wells. After 1 h, the wells were washed and the percentage of competition was determined as described above.

Coupling of anti-Id antibodies to keyhole limpet haemocyanin (KLH)

Anti-Id IgG or chromatographically purified rabbit IgG (Zymed Laboratories) was conjugated chemically to KLH (Sigma).¹¹ Purified IgG (10 mg) in 2 ml of PBS was mixed with 10 mg of KLH (in 2 ml of distilled water), and then the two were conjugated to each other by the addition of 0.2 ml of glutaraldehyde 8%. After incubation for 20 min at room temperature, the reaction was stopped with 0.52 ml of 0.2 M glycine. After the addition of 4.3 ml of distilled water, the mixture was dialysed overnight at 4°C against PBS.

Mouse protection test

Female 4-week-old BALB/c mice were each immunised intraperitoneally with 100 µg of anti-Id IgG or KLH-conjugated anti-Id IgG emulsified in an equal amount of Freund's complete adjuvant and then given six booster injections containing 50 µg at 2- or 3-week intervals. Three weeks after the final injection, mice were challenged intramuscularly with 2.3×10^8 spores of *C. chauvoei* strain Okinawa (100 MLD) suspended in 0.25 ml of calcium chloride solution 3%, and the survival rate was monitored for 5 days.¹

Results

Purification of MAbs

MAbs were purified from ascitic fluid by affinity chromatography, and monitored by SDS-PAGE and Western blot assays. After treatment with 2-mercaptoethanol, only two bands, corresponding to heavy and light chains of mouse IgG, were observed (data not shown). The purified MAbs were used as immunogens to generate the anti-Id antibodies.

Production and purification of anti-Id antibodies

Sera exhibiting high idiotypic-specific activity (maximum dilution of sera showing an OD₄₉₀ of 0.3 in ELISA was 10⁴–10⁵) were pooled and subjected to anti-Id antibody purification by sequential immuno-

Fig. 1. Detection of anti-Id antibodies in serum from a rabbit immunised with MAbs Mo-41 (a and b) and Mo-114 (c and d), before (a and c) and after (b and d) absorption with normal mouse IgG. Curves show the binding of homologous MAbs (○), irrelevant MAbs of same isotype (△), diluent buffer (□).

Fig. 2. Competitive inhibition of HRPO-conjugated MAbs Mo-41 (a) and Mo-114 (b) to corresponding anti-Id antibodies in the presence of excess concentrations of unconjugated MAbs: Mo-41 (○); Mo-62 (△); Mo-90 (□); Mo-114 (●).

Fig. 3. Competitive inhibition of HRPO-conjugated MAbs Mo-41 (○), Mo-62 (△), Mo-90 (□) and Mo-114 (●) to flagella of *C. chauvoei* in the presence of excess concentrations of anti-Id 41 (a) and anti-Id 114 (b) antibodies.

affinity chromatography. Fig. 1 shows the reactivity of unabsorbed and absorbed sera to homologous and irrelevant MAbs of the same isotype. Before absorption with normal mouse IgG, the antiserum to MAbs reacted equally well with either homologous or irrelevant MAbs, suggesting a strong reaction to common determinants. In contrast, absorbed sera reacted only with the homologous MAb, suggesting that the antibodies against constant-region determinants of mouse immunoglobulin, that were detected by binding of an irrelevant MAb, were almost completely removed by absorption on a normal mouse IgG column.

Idiotypic competition assay

To demonstrate the specificity of anti-Id antibody and to determine whether cross-reactive idiotypes existed among the various MAbs, each anti-Id antibody preparation was titrated against homologous and heterologous MAb in an ELISA. Fig. 2 shows the percentage inhibition curves of diluted competitor. Each MAb was blocked only by homologous antibody, showing that each anti-Id antibody was specific for its homologous MAb.

Antigen-binding inhibition assay

Anti-Id antibodies were tested for their ability to inhibit the binding of anti-flagellar MAbs to flagellar antigens. Fig. 3 shows the results of the inhibition ELISA. Each anti-Id antibody inhibited the binding of the corresponding MAb to flagellar antigens.

Table. Effect of immunisation with anti-Id antibodies in mice

Immunogen	KLH-coupled	Survivors in group (/n) of mice	
		Subgroup	Total
Anti-Id 41	Yes	3/7	7/14†
	No	4/7*	
Anti-Id 114	Yes	2/5	2/11
	No	0/6	
Normal rabbit IgG	Yes	1/5	3/11
	No	2/6	
None		0/10	

*Significantly different ($p < 0.05$) from non-immunised group by Fisher's exact test.

†Significantly different ($p < 0.01$) from non-immunised group by Fisher's exact test.

Mouse protection test

The ability of anti-Id antibodies to generate protective immunity against *C. chauvoei* was examined. Anti-Id 41, anti-Id 114, and normal rabbit IgG as control were each coupled to KLH, and antibodies with or without KLH were used as immunogens. The protective effects of the anti-Id antibodies are shown in the table. The survival rate of the group immunised with anti-Id 41 (50.0%) was approximately twice that of the control (immunised with normal rabbit IgG; 27.3%), and significantly higher ($p < 0.01$) than that of the non-immunised group (0%). In contrast, the survival rate of the group immunised with anti-Id 114 (18.2%) was lower than that of the control. No

significant differences occurred between the KLH-coupled and non-coupled groups.

Discussion

Antibodies carry idiotypes, which are regions in or near the antigen recognition sites. Idiotypes are themselves capable of acting as antigens and of stimulating further antibody production, as explained by the idiotypic network theory of Jerne.¹² Accordingly, anti-Id antibodies that carry an internal image of an antigen epitope (Ab2 antibodies) can be used as vaccines.¹³ Such vaccines, which obviate the need to inactivate cultures, have been shown to induce protective immunity in animals that have not been intentionally exposed to the native antigen epitope.^{10, 11, 14–18} Previously, we produced MABs that reacted with the flagella of *C. chauvoei*.⁵ Of these, protective MAB Mo-41 and non-protective MAB Mo-114 were chosen to produce anti-Id antibodies, and investigated for their ability to immunise mice.

In idiotypic competition assay, in which the specific or cross-reactive nature of the idiotypic detected by each anti-Id antibody was examined, MABs were blocked only by homologous antibody and there was no cross-inhibition, although reciprocal competitions had been observed among MABs Mo-41, Mo-62 and Mo-90 when purified flagella were used as competing antigen.⁶ This may be because of steric hindrance of binding to a nearby epitope.¹⁹ The results suggested that the anti-Id antibodies were highly specific for unique variable region determinants. The surface-exposed epitopes of flagellar filament recognised with the protective MABs were further distinguished by the anti-Id antibodies.

Each anti-Id antibody inhibited the binding of its corresponding MAB to flagellar antigens in an antigen-binding inhibition assay, and this competitive binding to the original antigen was one of the criteria used to distinguish internal-image from non-internal-image

anti-Id antibodies.¹³ The results suggested that the anti-Id antibodies bore an internal image of flagellar antigens of *C. chauvoei*.

In the mouse protection test, the survival rate of mice was increased by immunisation with anti-Id 41, which had been produced with a protective MAB; anti-Id 114, which had been produced by a non-protective MAB, failed to immunise. Percival *et al.*¹⁵ pointed out that protective anti-Id antibodies have generally originated from surface epitopes. Likewise, our previous results showed that protective MABs recognised the surface epitopes on the flagella of *C. chauvoei*.⁶

The survival rate of mice given anti-Id 114 or normal rabbit IgG was higher than that of non-immunised mice, and this phenomenon was observed repeatedly (data not shown). One possible explanation is that excessive immunisation evoked a non-specific immunity, such as that resulting from macrophage activation.

In most cases, monoclonal or polyclonal xenogeneic anti-Id vaccines require to be given with adjuvant or coupled to a potent carrier molecule.²⁰ The immunogenicity of the anti-Id antibodies in the present study was weak, necessitating the use of both adjuvant (Freund's complete) and repeated injections. Percival *et al.*¹⁵ showed that anti-Id antibody increased in immunogenicity when coupled with KLH. However, in the present study KLH coupling made no significant difference.

The results suggest that an internal-image anti-Id antibody will induce protective immunity and that immunity to a single flagellar epitope is sufficient to give at least some protection against *C. chauvoei* challenge.

As already shown²⁰ a single anti-Id antibody can immunise against an infectious organism, but different epitopes may be instrumental in stimulating the various aspects of immunity required for effective protection. It is possible that vaccines composed of a cocktail of the appropriate anti-Id antibodies may represent a practical approach to vaccination.

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