IMMUNISATION AND IMMUNOTHERAPY

Adjuvant effect of anti-idiotypic antibodies to Yersinia pestis lipopolysaccharide

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Rabbit anti-idiotypic antibodies (anti-Id-ab) against Yersinia pestis lipopolysaccharide (LPS) were obtained with monoclonal immunoglobulins. Their complementary character to the original antigen was confirmed by immunohistochemical analysis and ELISA and gel precipitation tests. The anti-Id-ab were shown to possess all essential properties of Ab2β subtype. Both in-vitro and in-vivo experiments demonstrated a pronounced adjuvant activity of anti-Id-ab without the toxic effect characteristic of Y. pestis LPS. Combined immunisation with anti-Id-ab plus the FI capsular antigen led to a significant increase in the protective immune response against experimental acute challenge with virulent Y. pestis.

Introduction

Immunity to plague is correlated with the presence of antibody to the capsular antigen (Fraction I antigen, FI) [1] and immunisation with FI induces protection against the disease in animal models [2–4]. Although FI is a protective antigen, studies have shown that it is not required for virulence in mice, raising the possibility that it is not an ideal vaccine candidate [5, 6]. At the same time, immunisation with FI in the presence of adjuvants provides a high degree of protection [7]. The lipopolysaccharides (LPS) of many gram-negative bacteria demonstrate pronounced adjuvant properties [8–10]; however, immunomodulating activity of Yersinia pestis LPS has not been shown. Its high toxicity discourages its application as a component of a plague vaccine [11, 12]. Furthermore, attempts to detoxify the LPS by acid or alkali treatment and other methods were of limited success [13]. Anti-idiotypic antibodies (anti-Id-ab) against Y. pestis LPS offer a convenient model for studying the biological properties of the original antigen. Anti-Id-ab provides a protein equivalent of any bacterial antigen and such mimicry of immunogenic polysaccharide and lipid epitopes makes it possible to present their antigenic determinants to the immune cells and to obtain effective immunity [14–17]. The aims of this study were to obtain anti-Id-ab against Y. pestis LPS by the use of monoclonal antibodies (MAbs), to establish their specific complementarity to the original antigen, to study their antigenic and adjuvant properties in combined immunisation with FI and anti-Id-ab against Y. pestis LPS and to compare their protective properties with those of the native antigen.

Materials and methods

Preparation of anti-Id-ab and anti-anti-Id-ab

Murine BALB/c MAbs (IgG1) against Y. pestis LPS secreted by the Yp.A6.Sp. hybridoma were used to induce anti-Id-ab [18]. Rabbits were given five intravenous injections of 1 mg of MAbs separated by 3-day intervals and were exsanguinated 7 days after the last dose. Immunoglobulins were isolated from the serum with ammonium sulphate 45% saturated solution, followed by purification through Sephadex G-50. Anti-anti-Id-ab were raised in six groups of 10 inbred CBA and C57/Bl 6–8-week-old female mice given three intraperitoneal doses of 1 mg of MAbs separated by 3-day intervals and were exsanguinated 7 days after the last dose. Immunoglobulins were isolated from the serum with ammonium sulphate 45% saturated solution, followed by purification through Sephadex G-50. Anti-anti-Id-ab were raised in six groups of 10 inbred CBA and C57/Bl 6–8-week-old female mice given three intraperitoneal doses of 1 mg of MAbs separated by 3-day intervals and were exsanguinated 7 days after the last dose. Immunoglobulins were isolated from the serum with ammonium sulphate 45% saturated solution, followed by purification through Sephadex G-50.

Bacterial strains

The strains used included 110 Y. pestis strains isolated from natural plague foci in the Community of Independent States (CIS), Mongolia, China, Vietnam, Pakistan, India, Africa and South America, and 15 Y.
plasmids

Indirect double antibody sandwich ELISA. used to study the adjuvant properties of anti-Id-ab in vars I-VI), 12 I:

plates (96-well) were sensitised with (per well) 100 pl

FI preparation

FI was isolated from supernatant fluid of Y. pestis

Immunodiffusion tests

Immunodiffusion in gel was performed according to Ouchterlony [21] with Y. pestis LPS, MAbs against

Enzyme-linked immunosorbent assay (ELISA)

Several modifications of ELISA were used for serological comparison of anti-Id-ab and Y. pestis LPS.

Direct double antibody sandwich ELISA. ELISA plates (96-well) were sensitised (per well) with 100 µl of anti-

Y. pestis LPS MAbs in phosphate-buffered saline (PBS) at 10 µg of protein/ml and incubated overnight at 4°C.

The wells were washed three times with washing buffer (PBS containing Tween-20 0.05%). The wells were

Indirect double antibody sandwich ELISA. ELISA plates (96-well) were sensitised with (per well) 100 µl of equine anti-Y. pestis IgG, rabbit and guinea-pig IgG against Y. pestis LPS at equal protein concentrations of 10 µg/ml. The following procedures were performed as described above.

Indirect ELISA. The plates were sensitised with (per

well) 100 µl of normal mouse IgG at 10 µg/ml protein

concentration. Then, dilutions containing anti-Id-ab 1.0 mg–0.5 ng/ml were added. Peroxidase-labelled

anti-rabbit immunoglobulins were used as detection conjugate. The wells were washed and blocked and

screened as described above.

Indirect ELISA was also used for studying the specificity of anti-anti-Id-ab. The plates were sensitised with (per well) 100 µl of suspension containing 1 × 10⁹ cells of different bacterial strains in 1 ml of PBS. Then, anti-anti-Id-ab diluted 1 in 100 in PBS was added. Peroxidase-labelled anti-mouse IgG was used as a detection conjugate.

Immunohistochemical method

To elucidate LPS-specific antigenic determinants in the spleen, the test group of 60 female BALB/c mice

was immunised with the anti-Id-ab described above, while the control group of these mice received five

intrapерitoneal 2.5-mg doses of normal rabbit immunoglobulins with 14-day intervals between the inoculations. At 14 days after the fifth immunisation, the spleen was excised and fixed in formaldehyde 10% solution. Paraffin-embedded sections cut with a microtome were prepared after additional fixation in cold

ethanol 80%. For the immunohistochemical studies the sections were treated with fluorescent MAbs against

Y. pestis LPS diluted 1 in 64 to reveal LPS-specific antigenic determinants and with fluorescent anti-rabbit

guinea-pig immunoglobulins diluted 1 in 8 to reveal rabbit immunoglobulins for control. The specimens

were examined by fluorescence microscopy (ML-2, Lomo, Russia).

Assessment of in-vitro activity of anti-Id-ab and LPS

The biological activity of anti-Id-ab against Y. pestis LPS and of the antigen itself was studied in vitro with a

B-cell hybridoma, Yp.Fl.B₂.D₃.Sp., which secreted MAbs to the FI of the plague bacteria [22]. Rabbit

anti-mouse immunoglobulins were used to control for the specificity of anti-Id-ab activity. After sterilisation

through nitrocellulose membranes (0.22 µm pore diameter) 1.0–0.001-mg preparations were inoculated into

48-well Costar culture plates containing 5 × 10⁴ hybridoma cells/well. The cells were incubated in RPMI-1640 medium with fetal calf serum 15% at 37°C and CO₂ 5% in air. The number of cells was evaluated on the sixth day with a haemocytometer. Each preparation was inoculated into 24 wells.

CBA and C57/B1 mice (n = 102) were used to study the anti-Id-ab adjuvant properties in vivo. The mice

were divided into three groups of 34 and immunised with 50 µg of: (i) FI only; (ii) FI + RAM, combined immunisation with FI and rabbit anti-mouse immunoglobulins; (iii) FI + anti-Id-ab, combined immunisation
with FI and anti-idiotypic antibodies against \textit{Y. pestis} LPS. Unimmunised mice were used as a control. At 14 days after the last immunisation, all mice received an acute subcutaneous challenge with 50 LD50 of virulent \textit{Y. pestis} strain 231. The efficiency of immunisation was estimated from the proportion of dead mice.

\textbf{Statistical analysis}

Statistical analysis of the results of assays for anti-Id-ab adjuvant activity \textit{in vitro} and \textit{in vivo} was performed according to Ashmarin and Vorob’ov [23].

\textbf{Results}

Anti-Id-ab produced a single well-defined precipitation line with MAbs to \textit{Y. pestis} LPS diluted 1 in 64 in the immunodiffusion gel precipitation assay. The original antigen, LPS at 1 mg/ml, diluted 1 in 128, gave a precipitation reaction with specific MAbs. Anti-Id-ab reacted with normal mouse IgG at titres of 4–8.

In the direct sandwich variant of ELISA test, anti-Id-ab detected MAbs against \textit{Y. pestis} LPS down to 12 ng/ml and normal mouse immunoglobulins down to 2 \(\mu\)g/ml in indirect ELISA. Anti-Id-ab to LPS and \textit{Y. pestis} LPS were determined in a similar concentration, 2 ng/ml, in indirect double antibody sandwich ELISA with rabbit, equine and guinea-pig IgG against \textit{Y. pestis} LPS. Non-specific cross-reactivity among the immunologically active components used in ELISA was excluded.

MAbs against \textit{Y. pestis} LPS (Ab1) identified all strains of \textit{Y. pestis} with different plasmid profiles grown at 28°C or 37°C as well as 30% of \textit{Y. pseudotuberculosis} strains [24]. Anti-anti-Id-ab also responded to all \textit{Y. pestis} strains and the same 30% of \textit{Y. pseudotuberculosis} strains, but gave no reaction with \textit{Y. enterocolitica} or other Enterobacteriaceae strains (Table 1).

The immune sera of all the mice immunised with anti-Id-ab irrespective of the dose reacted specifically with \textit{Y. pestis} LPS (Fig. 1). The highest antibody titre, 64 000, was registered after the mice had been immunised with 0.1 mg of anti-Id-ab. This dose also induced antibody formation at titres of 8000–16 000. Other concentrations of the preparation led to the release of antibodies active at dilutions from 1 in 640 (2.5 mg) to 1 in 400 (0.5, 5.0, 10.0 mg). The highest specific activity (titre 128 000) was observed with immune sera from the animals that had received 1.0 mg of anti-Id-ab.

Fluorescence microscopy with FITC-labelled antibodies against normal rabbit immunoglobulins in the spleens of control mice revealed many antibody-containing cells (lymphoblasts and plasmacytes) with brightly fluorescent cytoplasm. No antibodies to rabbit immunoglobulins were found in test animals. On the other hand, when fluorescent MAbs against \textit{Y. pestis} LPS were used, antigen-containing cells specific for \textit{Y. pestis} LPS were registered in this group of mice.

\begin{table}[h]
\centering
\caption{Comparison of specific properties of anti-anti-Id-ab and MAbs against \textit{Y. pestis} LPS}
\begin{tabular}{llll}
\hline
\textbf{Bacterial species} & \textbf{Number of strains} & \textbf{Number of strains identified in indirect ELISA by} \\
& & \textbf{Anti-anti-Id-ab} & \textbf{MAbs} \\
\hline
\textit{Y. pestis} & 110 & 110 & 110 \\
\textit{Y. pseudotuberculosis} & 31 & 9 & 9 \\
\textit{Y. enterocolitica} & 13 & 0 & 0 \\
Other micro-organisms* & 21 & 0 & 0 \\
\hline
\end{tabular}
\end{table}

*See Materials and methods.
A comparative study of the LPS and anti-Id-ab against *Y. pestis* LPS with regard to their biological activity showed *Y. pestis* LPS to be highly toxic for B-cell hybridoma (Table 2). This preparation added to the wells to give final concentrations of 5–100 µg/ml inhibited cell proliferation, in contrast to those without LPS (p < 0.05). The indices of proliferation were 0.26 (SEM 0.01) × 10⁶ cells/ml and 0.10 (SEM 0.01) × 10⁶ cells/ml, respectively. Larger LPS concentrations resulted in the complete death of hybridoma cells. The presence of anti-Id-ab 5–50 pg/ml in the culture medium stimulated inhibited cell proliferation, in contrast to those without (SEM 0.01). LPS (p < 0.001); anti-Id-ab 0.1–1.0 mg/ml increased growth rate 10–12-fold, (3.59 SEM 0.04–4.20 SEM 0.03) × 10⁶ cells/ml (p < 0.001). The number of cells after the addition of analogous concentrations of normal rabbit anti-mouse immunoglobulins did not exceed the control values (0.33 SEM 0.01–0.37 SEM 0.01) × 10⁶ cells/ml (p > 0.05).

The antibody response after three subsequent immunisations of CBA and C57/Bl mice with *Y. pestis* capsular antigen (FI) and 50 µg of anti-Id-ab was studied (Fig. 1). The mean specific antibody titre in mice treated with FI only was 1000. When this antigen was inoculated simultaneously with anti-Id-ab, the titre increased to 16000. In the control group of mice, immunised with FI and rabbit anti-mouse immunoglobulins, the levels of anti-capsular antibodies were almost the same as those in the animals immunised with the capsular antigen alone.

The results of an acute challenge with 50 LD50 of virulent *Y. pestis* strain 231 in three groups of mice – (i) immunised with FI only; (ii) immunised with FI + RAM; (iii) immunised with FI + anti-Id-ab – are shown in Table 3. Immunisation of mice with FI alone protected 47.0 SEM 8.6% of animals. The same level of protection was observed in the group of mice immunised with FI + RAM. FI + anti-Id-ab immunisation led to a significant increase in survival of mice challenged with 50 LD50 of *Y. pestis* strain 231. In this group, the number of deaths was half that in the group of mice inoculated with FI alone or FI plus normal rabbit anti-mouse immunoglobulins (p < 0.01). The highest level of protection among the mice immunised with FI + anti-Id-ab correlated with the highest specific antibody titre to FI.

**Discussion**

According to Jerne [25] the immune system is a set of Id (Ab1) and anti-Id (Ab2) that interact with each other. At present Ab2α, Ab2β, Ab2γ and Ab2ε are known. Only a certain type of anti-idiotypic antibodies (Ab2β) has the property of bearing the internal image of an original antigen [26, 27]. Such antibodies have been used successfully for induction of highly protective antibody responses against parasites, bacteria and viruses [28–31]. It is known that the LPS of gram-negative bacteria may increase T- and B-cell proliferation [8–10], but no information about the immunomodulating properties of *Y. pestis* LPS has been reported. The use of the relevant anti-Id-ab may help to reveal

<p>| Table 2. Effect of anti-Id-ab and <em>Y. pestis</em> LPS on B-cell hybridoma proliferation in vitro |
| Dose of preparation (µg/ml) | Mean (SEM) hybridoma cell concentrations (10⁶/ml of culture medium) after addition of |</p>
<table>
<thead>
<tr>
<th></th>
<th>LPS</th>
<th>p value</th>
<th>anti-Id-ab</th>
<th>p value</th>
<th>RAM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.30 (0.04)</td>
<td>&gt;0.05</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.26 (0.01)</td>
<td>&lt;0.05</td>
<td>1.74 (0.02)</td>
<td>&lt;0.001</td>
<td>0.33 (0.01)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>10</td>
<td>0.26 (0.01)</td>
<td>&lt;0.05</td>
<td>1.85 (0.02)</td>
<td>&lt;0.001</td>
<td>0.34 (0.02)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>25</td>
<td>0.22 (0.01)</td>
<td>&lt;0.05</td>
<td>2.51 (0.04)</td>
<td>&lt;0.001</td>
<td>0.34 (0.02)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>50</td>
<td>0.22 (0.01)</td>
<td>&lt;0.05</td>
<td>2.98 (0.04)</td>
<td>&lt;0.001</td>
<td>0.33 (0.01)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>100</td>
<td>0.10 (0.01)</td>
<td>&lt;0.01</td>
<td>3.59 (0.04)</td>
<td>&lt;0.001</td>
<td>0.36 (0.02)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>200</td>
<td>0.36 (0.03)</td>
<td>&lt;0.001</td>
<td>0.37 (0.01)</td>
<td>&gt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>0.19 (0.04)</td>
<td>&lt;0.001</td>
<td>0.35 (0.01)</td>
<td>&gt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.42 (0.03)</td>
<td>&lt;0.001</td>
<td>0.35 (0.01)</td>
<td>&gt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (no preparation added)</td>
<td>0.35 (0.01)</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not done.

- , only dead hybridoma cells.

<p>| Table 3. Adjuvant properties of anti-idiotypic antibodies against <em>Y. pestis</em> LPS in mice infected with a virulent <em>Y. pestis</em> strain |</p>
<table>
<thead>
<tr>
<th>Antigens used in immunisation</th>
<th>Number of mice</th>
<th>Challenge dose (LD50)</th>
<th>Number (%) of dead mice</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI only</td>
<td>34</td>
<td>50</td>
<td>16 (47.0 SEM 8.6)</td>
<td></td>
</tr>
<tr>
<td>FI + RAM</td>
<td>34</td>
<td>50</td>
<td>15 (44.1 SEM 8.5)</td>
<td></td>
</tr>
<tr>
<td>FI + anti-Id-ab</td>
<td>34</td>
<td>50</td>
<td>8 (23.5 SEM 4.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Control (unimmunised)</td>
<td>10</td>
<td>50</td>
<td>10 (100)</td>
<td></td>
</tr>
</tbody>
</table>
such properties of this antigen. In the current study, rabbit anti-Id-ab against \textit{Y. pestis} LPS were obtained by the use of MAbs. The pool of rabbit immunoglobulins contained antibodies to both the non-specific moiety of mouse immunoglobulins and the LPS idiotype; however, anti-Id-ab against LPS predominated. Several methods were used to define their subtype and complementary character to the antigen. The results of ELISA cross-reactions and the immunodiffusion gel precipitation test provided evidence for anti-Id-ab complementarity to the original antigen.

As the inhibition of binding of the Id to the antigen is a characteristic of both \textit{Ab2β} (internal image) and \textit{Ab2γ}, \textit{Ab2β} and \textit{Ab2γ} were distinguished by their ability to bind Id in xenogenic antisera specific for \textit{Y. pestis} LPS (equine, rabbit and guinea-pig). \textit{Ab2β} are able to recognise \textit{Ab1} produced in animals of other species, whereas \textit{Ab2γ} are not. In the current study, anti-Id-ab and the antigen itself, \textit{Y. pestis} LPS, were shown to react with equine, rabbit and guinea-pig antisera against \textit{Y. pestis} LPS in equal concentration. This suggests that the anti-Id-ab obtained belonged to \textit{Ab2β}.

One of the most significant properties of \textit{Ab2β} is their ability to induce the synthesis of anti-anti-Id-ab which demonstrate the same activity as \textit{Ab1}. The anti-Id-ab used in the current study for immunisation of mice produced immune murine sera that contained high levels of anti-anti-Id-ab that recognised the original antigen, \textit{Y. pestis} LPS. The specific activity of mouse anti-anti-Id-ab was dependent on the immunising dose of the antigen. High anti-anti-Id-ab titre in the animals treated with anti-Id-ab are likely to reflect powerful antigenic activity of \textit{Y. pestis} LPS, because only a strong antigen can induce the production of specific antibodies against its idiotype. Comparison of specific properties of MAbs against \textit{Y. pestis} LPS (\textit{Ab1}) and anti-anti-Id-ab showed their complete coincidence. Both antibodies identified \textit{Y. pestis} strains with different plasmid profiles as well as strains of \textit{Y. pseudotuberculosis} and did not react with other species of Enterobacteriaceae. This strongly suggests that the anti-Id-ab obtained were of \textit{Ab2β} subtype.

One essential criterion for defining an anti-Id-ab as an internal image of an antigen is mimicry of its biological activity. Therefore, after the anti-Id-ab subtype was determined, the biological properties and adjuvant activity were studied. The following approaches were used: (i) anti-Id-ab influence upon B-cell hybridomas in vitro; (ii) the level of murine antibody response after combined immunisation with FI and anti-Id-ab; (iii) protection in vivo after combined immunisation with FI + anti-Id-ab in the acute challenge with a virulent strain of \textit{Y. pestis}. Addition of anti-Id-ab against \textit{Y. pestis} LPS to the growth medium increased B-cell hybridoma proliferation 5–6-fold, whereas the original antigen (LPS) was extremely toxic for these cells, killing them even in minute concentrations. This observation indicates that \textit{Y. pestis} LPS possesses the immunomodulating activity inherent in LPS of other gram-negative bacteria [8–10].

The stimulating effect of anti-Id-ab on lymphoid cells served as the basis for the in-vivo experiments. Significantly higher levels of murine antibodies (anti-anti-Id-ab) in the animals immunised with FI and anti-Id-ab conjointly, in comparison with FI-treated mice, were observed. This is important evidence of the adjuvant properties of anti-Id-ab. Immunomodulating (adjuvant) activity of anti-Id-ab was also confirmed by the results of an acute challenge. CBA and C57/Bl mice were used, as it had been shown earlier that mice of these strains are the most susceptible to plague and they are the most convenient experimental model for studying the protective properties of \textit{Y. pestis} antigens [32]. Preliminary titration showed that the optimal dose was 0.1 mg. The number of surviving mice after the combined immunisation with FI and anti-Id-ab was twice that of the groups of mice immunised without anti-Id-ab. Correlation of high titres of specific murine antibodies (anti-anti-Id-ab) with the highest level of protection in the group of mice immunised with FI + anti-Id-ab confirms the immunomodulating properties of anti-Id-ab.

The ‘in-vitro/in-vivo’ system developed for studying adjuvant properties of anti-Id-ab against \textit{Y. pestis} LPS was very convenient and allowed the identification of the anti-Id-ab as carrier of the ‘internal image’ of the original antigen. It should be emphasised that, in contrast to the highly toxic \textit{Y. pestis} LPS (LD50 for mice, 130 µg), anti-Id-ab did not have this activity, as doses of 10 mg of anti-Id-ab inoculated into the mice to induce anti-anti-Id-ab did not kill the animals. In contrast to LPS, anti-Id-ab did not contain pathogenic material and did not cause death in mice in doses 80 times higher than the toxic concentration of LPS. This strategy may be useful for increasing the immunogenicity of FI and may be applicable to other LPS [33–35]. These results provide evidence of the immunomodulating properties of anti-Id-ab.

Immunohistochemical staining with anti-Id-ab provided presumptive evidence of the tissue localisation of endotoxin (LPS)-target cells. The specific receptors for this antigen appeared to be located in the cytoplasm of plasmacytes, in the B-zones of murine spleen. These data correspond with those obtained by others [36] after immunisation with \textit{Y. pestis} LPS.

In summary, rabbit polyclonal anti-idiotypic antibodies were generated against \textit{Y. pestis} LPS by immunisation with MAbs against this antigen. These anti-Id-ab were directed predominantly against an Id and possessed the properties of \textit{Ab2β}. The observed adjuvant activities of the anti-Id-ab in a murine model demonstrated the possibility of increasing the protective immune re-
sponse against plague by combined immunisation with F1 anti-Id-ab.

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References


