Effectivity of Alternative Adjuvants in Comparison to Freund’s Complete Adjuvant

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Summary
In order to replace the use of mammals for the production of hyperimmune sera and to replace Freund’s complete adjuvant (FCA) with its undesirable side effects, the production and immunological features of IgY were investigated systematically. An important advantage of IgY is that large amounts can easily be extracted from the yolk of immunised laying hens.

With special regard to animal welfare, several biocompatible adjuvants (ABM system, Gerbu adjuvant, TiterMax) were tested with FCA for their usage and effectiveness in the immunisation of chickens. Under appropriate immunisation schedules which strictly avoid intramuscular administration, IgY antibodies with very high titres and a strong binding capacity (avidity) comparable to rabbit hyperimmune sera can be obtained with minimal adjuvant side effects.

Abbreviations: ABTS, DDA, PAP

Keywords: chicken, adjuvant, FCA, TiterMax, ABM, Gerbu

1 Introduction
Phylogenetically, IgY being the standard immunoglobulin of oviparous species constitutes the basic form from which the mammalian immunoglobulin classes IgG and IgE developed (Warr G. W. et al., 1985). As a result of numerous studies (Schade R. et al., 1994, Akita E. M. and S. Nakai, 1993, Gassmann M. P. et al., 1990), production of IgY from eggs without infliction of pain and suffering on the animals involved does not constitute any longer a problem of basic methodological nature. To achieve a broad acceptance of IgY, characterisation and convincing documentation of the immunological properties of this immunoglobulin are required. This will include data on the potential for production of large amounts as well as parameters such as agglutination and precipitation characteristics, specificity, titre and avidity.

The advantage of the production of egg yolk antibodies in terms of animal welfare will be seen when a non-destructive as well as effective immunisation can be performed which is the only remaining operation on the animal. For this purpose, knowledge must be improved about the effects of different immunisation schedules and adjuvants. Likewise, FCA which is still the adjuvant most frequently used in chickens is favoured due to its effectivity and low costs. Considerable side-effects, however, occur with FCA. New generation alternative adjuvants used in an analogous way are often more tissue tolerable, but still less effective. Their potential will become evident only in combination with adapted immunisation schemes. It is therefore the aim of the current investigation to optimise the effectiveness of adjuvants used in chickens, with emphasis on tolerability and effectivity. The objective is the production of antisera in large amounts with high specificity, high titres, and a high avidity that equal in their characteristics those of analogous antisera produced in rabbits.

2 Animals, materials, and methods

2.1 Animals
Hens of White Leghorn line and chinchilla bastard rabbits were immunised.

2.2 Antigens
The immunisation was performed with fox IgG, purified by affinity chromatography. For primary immunisation, 1 mg each and for booster injections, 0.5 mg each were used.

2.3 Adjuvants
The following adjuvants were applied for immunisation: ABM-N, ABM-S (Linaris, Bettingen), Freund’s Complete Adjuvant, FCA (Difco, Detroit, USA), Gerbu Adjuvant (Gerbu Biotechnik, Gaiberg) and TiterMax (Serva, Heidelberg).
2.4 Immunisation

I. To compare the different adjuvants, immunisation was performed by the s.c. route using antigen (fox IgG) and adjuvant, according to the scheme below. In contrast, adjuvant-free antigen solutions were injected by the i.v. route. II. Additional groups of chickens were used for comparison of effectivenegg and tolerability of FCA, TiterMax and Gerbu Adjuvans if applied by the s.c. or the i.m. route (Schwarzkopf, 1994).

III. A group of rabbits was treated in the same way as chicken group 3 (FCA*i.v.), to compare the immune response of mammals with that of chickens. This scheme is considered common in the production of species-specific antisera in rabbits.

Purification of IgY

IgY was extracted from the egg yolk by means of salt precipitation (Schwarzkopf, 1994).

Table 1: Immunisation scheme

<table>
<thead>
<tr>
<th>Chickens (group)</th>
<th>Primary immunisation (s.c.)</th>
<th>Day</th>
<th>Booster immunisation (s.c.)</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FCA</td>
<td>0</td>
<td>FCA</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Gerbu adjuvant</td>
<td>0</td>
<td>Gerbu adjuvant</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>FCA</td>
<td>0</td>
<td>Ag without adjuvant</td>
<td>18/19/20</td>
</tr>
<tr>
<td>4</td>
<td>Control (Ag without adjuvant)</td>
<td>0</td>
<td>Control (Ag without adjuvant)</td>
<td>14/21/28/49</td>
</tr>
<tr>
<td>5</td>
<td>ABM-N</td>
<td>0</td>
<td>ABM-S</td>
<td>21/36/49</td>
</tr>
<tr>
<td>6</td>
<td>TiterMax</td>
<td>0</td>
<td>TiterMax</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 2: Pathological changes after s.c. and i.m. application of three adjuvants

<table>
<thead>
<tr>
<th>Type of change</th>
<th>FCA</th>
<th>TiterMax</th>
<th>Gerbu adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application</td>
<td>s.c.</td>
<td>i.m.</td>
<td>s.c.</td>
</tr>
<tr>
<td>Abscess</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microabscess</td>
<td>X</td>
<td>X, X*</td>
<td></td>
</tr>
<tr>
<td>0.5 x 0.5 x 0.3 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 x 0.5 x 1.5 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 x 1.5 x 2.5 cm</td>
<td>X, X*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 x 2.0 x 3.5 cm</td>
<td>X*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degeneration of muscle</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X: subcutaneous abscesses; X*: abscess localized between muscles or fascia

2.5 Determination of titre

An indirect ELISA was performed to determine the titre directly from the egg yolk diluted with double-distilled water. Microtiter plates were coated with the respective target antigen and then washed several times. After this dilutions of egg yolk were added in series from 1:32 to 1:32 000. This was followed by additional washing cycles and a final incubation with a peroxida-se-labelled rabbit anti-chicken antibody (Nordic, Tilburg, Netherlands) and developed with ABTS®.

2.6 Determination of avidity

Ammonium sulfate-precipitated IgY (2 ml/egg) was used to perform the slightly modified method of MacDonald et al. (1985). The antibody binding capacity was determined in the presence of increasing concentrations of ammonium thiocyanate. The avidity index (AI) is defined by the molality of ammonium thiocyanate solution required to dissociate the antigen-antibody complex and thus to decrease the initial light absorbance in the ELISA by 50%.

3 Results

3.1 Extraction of IgY

Depending on the method of extraction, 60 – 110 mg of total IgY, containing 1 – 3% of antigen-specific antibody, may be obtained from one egg. The ammonium sulfate method combines the advantages of high environmental compatibility (absence of solvents) with simple and low-cost performance. For this reason, it was chosen as the standard method for onward studies.

3.2 Side-effects of adjuvants

Table 2 provides tissue changes as a response of the application of different adjuvants. The size of abscesses correlated with the applied volume of oil-containing adjuvants. Abscesses also occurred after s.c. injection. Subcutaneous abscesses, however, are embedded in the connective tissue, and are therefore mechanically more flexible than intramuscular abscesses. Generally, application of oil-containing adjuvants is accompanied by abscess formation. Tissue tolerability of the app-
lied adjuvant always decreased with an increasing content of non-metabolisable oil. The rate of side-effects was lowest for the Gerbu adjuvant which does not contain oil but only a lipophilic substance, DDA.

Histological examination of the pathological changes listed in table 2 revealed similar results for chickens immunised with FCA and with TiterMax (s.c. and i.m.). After intramuscular application of adjuvant and antigen abscesses developed together with diffuse interstitial infiltrates, containing heterophil granulocytes and oil cysts. Also large muscular degenerations were found. With the PAP technique, the presence of mycobacterial antigen could be demonstrated in all abscesses caused by FCA.

3.3 Comparison of intramuscular with subcutaneous application of adjuvant and antigen

For the adjuvants examined (FCA, TiterMax, Gerbu), antibody titre and avidity did not differ by route of application (data not shown).

3.4. Species comparison of chicken with rabbit

Following primary immunisation with antigen in combination with FCA and three subsequent booster injections without adjuvant in the third week, the rabbits were bled weekly and finally exsanguinated. The determination of avidity was performed both from rabbit sera and IgY. Since titres depend both on avidity and antibody concentration, titres were not shown here.

Fig. 1 includes the corresponding avidity indices for a group of chickens treated with FCA in both primary and booster immunisation.

The avidity indices of rabbit antibodies fell into the range of 3.5-4 relative units, those from the chickens immunised in an analogous way were below 2.5, even after affinity maturation beyond 28 days. However, for the second group of chickens which was immunised twice with antigen and FCA, higher values of 4-5 were finally reached but at a lower speed than in the rabbit.

3.5 Development of titre and affinity maturation after use of adjuvants

For primary and secondary immunisation FCA, TiterMax, ABM, and Gerbu were used as described. A comparative group was given FCA for primary immunisation only and adjuvant-free, aqueous antigen solution i.v. for booster injections. The control group received only intravenous injections of pure antigen at weekly intervals. Fig. 2 represents the maximal titres achieved until day 50, fig. 3 shows the corresponding avidity levels.

Two-fold application of FCA in combination with antigen supplied high titres and avidity levels exceeding the levels for the corresponding parameters in rabbits. Moderate avidity levels in combination with sufficient titres were obtained after use of TiterMax or single FCA injection combined with triplicate i.v. boosting without adjuvant. In contrast to that, titres attained with the adjuvants Gerbu and ABM applied to the same protocol, did not differ from adjuvant-free i.v. application of antigen.

4 Discussion and conclusions

FCA and all other oil-containing adjuvants that cause abscess formation should no longer be used intramuscularly. This can easily be attained because titres and avidities are identical after s.c. and i.m. application.

In chickens, the time course of affinity maturation is slower and initially behind the rabbit. The most probable reason for this consists in the narrower V-gene repertoire of birds (Warr et al.,...
This genetic disadvantage is compensated by a more active somatic diversification (hypermutation) in the chickens, finally leading to avidity levels similar to those of conventionally immunised rabbits. This requires the availability and knowledge of species-adapted immunisation schedules.

The frequently-cited assumption of a lower affinity of avian antibodies cannot be confirmed hereby. Following an extended period of affinity maturation, identical or higher avidities are reached without problems and the amount of continuously produced antibodies will exceed that of the rabbit.

The achievable titres and avidity levels are influenced by the immunisation schedule and the choice of adjuvant. Primary immunisation and booster injection in the third week in combination with ABM and Gerbu will result in titres which are even lower than those obtained by pure i.v. application of antigen. Antibodies obtained in this way are hardly suitable for further use. TiterMax is the only alternative adjuvant that produces antibodies at an acceptable level over an extended period. Disadvantages of its large-scale use consist in the low avidity as compared to FCA and the high price of TiterMax. If a period of 50 days is taken, FCA still continues to be the yardstick for producing a pronounced immune response.

Onward experiments have shown, however, that irrespective of the initial titre, sudden increases of titres were produced in chickens following late booster injections in combination with alternative adjuvants or FCA. These results are independent of the adjuvant used initially during regular immunisation. Even late i.v. administration of pure antigen produced relatively high titres. Therefore, alternative adjuvants which inflict less suffering on the animal will exert an increasing potential in the near future.

References


Acknowledgement

The author is greatly indebted to Mr. Enno Luge for his assistance in the performance of numerous procedures in the laboratory.

This report is based on a project financed by the Federal Ministry of Education, Science, Research and Technology (Project No. 0310126 B).

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