Evaluation of local toxicity after repeated intranasal vaccination of guinea-pigs

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Abstract

In intranasal vaccination it is important that the adjuvant does not have any toxic effect on the sensitive nasal mucosa. In this study a histological and clinical evaluation of the effects of two different adjuvants in a vaccine containing detoxified diphtheria (DT) and tetanus toxoid (TT) in guinea pigs was done. The guinea pigs were divided in four groups and treated daily for 14 days with different formulations. Group 1 with saline, Groups 2 and 3 with the vaccines in a non-ionic surfactant formulation containing glycerides and Group 4 with tetraethyleneglycol formulation containing glycofurol. The guinea pigs in Groups 1, 2 and 4 were sacrificed on day 15 and Group 3, 1 week later and the tissues processed for histological examination. The animals remained healthy during the treatment and minor clinical signs, such as nose-blowing, decreased with time. The histological appearance, including the development of lymphoid tissue, was comparable in all groups. A specific toxic effect on the nasal mucosa by the different vaccine and adjuvant formulations was not observed.

Keywords: Intranasal; Local toxicity; Nasal; Toxicology; Vaccination

1. Introduction

The respiratory system is the route of entry for a variety of airborne materials. Inhaled foreign substances with the capacity to immunize the host appear to be trapped in the mucous layer of the airways and transported towards the pharynx by ciliary motion unless an adjuvant is added, which may stimulate lymphatic absorption or phagocytosis by macrophages which enter the lymphatics and induce the immunological response. Unfortunately, many adjuvants seem to be slightly irritant, causing unpleasantness, fever, inflammation and even granulomas after injection (Stewart-Tull, 1989a). Significant expansion has occurred in the field of adjuvant research during the last decade. It has required a great deal of effort to find adjuvant substances which do not have adverse bio-
logical properties, especially for sensitive areas such as the nasal cavity. The assessment of irritation of the nasal mucosa by histological examination is necessary for evaluation of possible toxic effects on the mucosa and the response of local lymphoid tissue to the vaccination. A candidate adjuvant substance should be non-toxic, non-pyrogenic, well tolerated and preferably biodegradable (Stewart-Tull, 1989b).

Injectable adjuvants have been studied extensively, whereas few studies have dealt with mucosal adjuvants. Some parenteral adjuvants have been used mucosally such as Freund's complete adjuvant (Taubman et al., 1983) and Avridin (Anderson and Reynolds, 1979). Recently, a number of different compounds have been found to have adjuvant properties when applied to mucosal surfaces together with antigen, as reviewed by Walker (1994) and Elson and Bertzbauch (1994). These included cholera toxin (Lycke et al., 1992) and cholera toxin B subunit (Gizurarson et al., 1992), lipophilic immune stimulating complexes (ISCOMs) containing saponin (Lovgren et al. 1990) and the RV adjuvant, a non-ionic bioadhesive surfactant containing glycerides (Gizurarson and Heron, 1994). The objective of this study was to evaluate the effect, both histological and clinical, of intranasal vaccination with RV adjuvant, which would indicate whether the adjuvant effect was augmented by damage to the mucosal lining. For comparison, 0.9% sodium chloride solution and a non-toxic formulation having similar characteristics (viscosity, bioadhesivity) containing glycofurol and tetraethyleneglycol, which is both viscous and bioadhesive (Bechgaard et al., 1995) were used. Clinical symptoms were monitored during the experiment. Histological examination was performed on material obtained at the end of the experiment.

2. Materials and methods

2.1. Chemicals and test articles

Detoxified diphtheria (DT) and tetanus toxoids (TT) were provided by Statens Seruminstitut (Denmark). Isotonic sodium chloride solution was commercially available from Lyfjaverslun Islands (Iceland), tetraethyleneglycol was commercially available from Fluka Chemical Co. (Switzerland), glycofurolum was provided by Hoffman-La Roche (Switzerland) and RV adjuvant was provided by Lyfjathroun hf. All chemicals were of analytical grade.

The following three test substances were used: Control, pure isotonic sodium chloride solution; RV, 80 Lf diphtheria and 80 Lf tetanus toxoids were dissolved in 1 ml RV adjuvant formulation (isotonic saline containing 36% polysorbate 20 and 10% caprylic/capric glyceride) and TG by dissolving 5% glycofurol in tetraethyleneglycol (Bechgaard et al., 1995).

2.2. Animals

Forty female albino guinea pigs, 7–9 weeks old, weighing 463 ± 16 g, were obtained from Statens Seruminstitut's own stock. The animals were healthy and had never been used in experiments before, they had no signs of inflammation in the respiratory tract prior the experiment. They were randomized into four groups of 10 animals, housed in cages, two in each, at 21.5 ± 1.5°C, relative humidity of 50–82% and with 10 h light per day. Drinking water and food were given ad libitum. Prior to the experiment the animals were allowed to acclimatize for 7 days. Five days before the experiment was begun, they were taken out of their cages once a day and held up as they were to be dosed, to accustom them to dosing.

2.3. Procedures

The experiments were designed according to the toxicological section in the Nordic Guidelines. Unanesthetized animals were dosed daily for 14 days. They were held in a supine position and 10 μl (Gizurarson, 1990) of the test compound was injected into the left nasal cavity with an Eppendorf pipette. After the administration they were kept on their back for 1 min. As it has a septal window, the right nasal cavity could not serve as a control. The maximal volume to be administered per nostril to guinea pigs was 25 μl. If this volume is exceeded, there is a high risk of dose loss, either anteriorly or into the oesophagus.

The animals were divided into four groups. Group 1 received saline, Groups 2 and 3 received 12 μl DT and 12 μl TT in RV, and Group 4 received the TG solution (control).
2.4. Clinical observation

The animals were observed daily during the experiment with special emphasis on symptoms of the respiratory system. The following signs, at and up to 30 min following dosing were recorded: (1) sneezing, (2) blocking of the nose with one foreleg, (3) kicking with the legs, (4) attempting to blow out the vehicle. Their weights were recorded daily and other symptoms noted.

2.5. Histology

The animals in Groups 1, 2 and 4 were sacrificed immediately after the experiment, on day 15, but Group 3 was sacrificed 1 week later. In the case of irritation it would be important to evaluate the recovery rate. They were anaesthetized by intraperitoneal injection of hypnorm/diazepam and bled by puncture of both jugular vein and the

Fig. 1. Schematic illustration of the guinea pig nasal cavity, showing the reference points for making cross sections of the nose (a–d). (1) Nostrils, (2) dorsal nasal conchae, (3) ventral nasal conchae, (4) endoturbinates, (5) bulbous olfactoryis and thereafter the cerebrum, (6) lingua, and (7) incisors.

Fig. 2. Transverse section through Fig. 1a–d (s, nasal septum; d, dorsal nasal conchae; v, ventral nasal conchae; c, cavum nasi; r, rostral recess; n, nasolacrimal duct; m, maxillary sinus; etho, ectoturbinates; endo, endoturbinates).
carotid artery. The entire nose was removed, gently
flushed with 10% buffered formalin (KLV, Den-
mark) via the pharyngeal duct (Young, 1986) and
fixed in 10% formalin for 4 weeks at room tem-
perature.

After fixation, the samples were decalcified with
5% nitric acid (Merck Schuchard, Germany) for 19
days at room temperature. The medium was
changed once during the period. After decalcifica-
tion, four tissue slices were taken at the following
levels (Fig. 1): (1) immediately posterior to the
upper incisor teeth, (2) at the incisive papilla or the
anterior nasal cavity, (3) premolar or middle part
of the nasal cavity, and (4) at the middle of the first
molar teeth or posterior part of the nasal cavity
(Fig. 2).

The nasal tissues were processed in a conven-
tional manner, embedded in paraffin with anterior
face downward, sectioned at 5–6 µm and stained
with haematoxylin and eosin. The samples were
renumbered randomly and evaluated blindly.

The following histological parameters were
assessed quantitatively in following manner: (a)
submucosal infiltration was recorded as +, (b)
lymphoid follicles were graded 1–5, (c) epithelial
damage was graded 1–3 and (d) exudate in the
nasal cavity, i.e. mucus (cellular), was graded 1–3.

2.6. Calculations and statistical analysis

For histological evaluation, the average for each
parameter in each group was calculated by adding
the grades and pluses, respectively and dividing by
the number of animals in each group. The results
were analyzed by Student's t-test and regression
analysis; probability (P) values <0.05 were con-
sidered significant.

3. Results

3.1. Clinical symptoms

The total and average frequency of all com-
plaints noted during and immediately after dosing
were not significantly different between the groups
(Table 1). Some trends were, however, noticed in-
ssofar as the group receiving saline did react more

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Frequency</th>
<th>S.D.</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.39</td>
<td>0.21</td>
<td>54</td>
</tr>
<tr>
<td>RV formulation</td>
<td>0.21</td>
<td>0.12</td>
<td>30</td>
</tr>
<tr>
<td>TG formulation</td>
<td>0.26</td>
<td>0.12</td>
<td>36</td>
</tr>
</tbody>
</table>

Fig. 3. The average frequency of following complaints, during and up to 30 min after intranasal administration of saline, RV and
TG formulations: (1) sneezing, (2) blocking of the nose with one foreleg, (3) kicking with the legs, and (4) attempting to blow out
the vehicle.
frequently than the other groups to the dosing. As shown in Fig. 3, which shows the frequency of various complaints, this difference was mainly due to the more frequent attempts of the saline-dosed animals to try to blow out the vehicle, whereas the groups treated with the vaccine or control formulations showed more of a tendency to try to block the nose. However, the differences in the frequency of the complaints decreased with time (Fig. 4).

The animals did not show any other clinical symptoms and gained weight during the experimental period (Fig. 5).

3.2. Histological examination

There was a slight variation in the number of cells, mainly mononuclear, in the submucosa. In general, a discrete increase in mononuclear cells was observed, which was rarely beneath the squamous epithelium lining the nasal cavity in plane 1, but more frequently in planes 2–4 (Fig. 6a), beneath the columnar partly ciliated epithelium or olfactory epithelium. There was no significant difference in the frequency of these discrete increases in submucosal mononuclear cells between the various groups, i.e. those given vaccine, control formulation or saline (Table 2). The same applies to the occurrence of lymphoid follicles in...
Table 2
Quantitative assessment: average gradings of histological parameters

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Submucosa</th>
<th>Exudate</th>
<th>Epithelial damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mononuclear cells</td>
<td>Lymphoid follicles</td>
<td>Mucus</td>
</tr>
<tr>
<td>Saline</td>
<td>23.3 ± 11.7</td>
<td>10.6 ± 3.0</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>RV formulation</td>
<td>20.0 ± 14.8</td>
<td>10.7 ± 2.6</td>
<td>0.5 ± 1.0</td>
</tr>
<tr>
<td>TG formulation</td>
<td>18.7 ± 12.4</td>
<td>13.9 ± 4.8</td>
<td>0.6 ± 0.8</td>
</tr>
</tbody>
</table>

Fig. 6. Dorsal nasal conchae with maximal diffuse infiltration of mononuclear cells in the submucosa. Similar intensity in RV adjuvant (a) and control (b) (H&E, ×240). Nasopharyngeal duct with well developed lymphoid tissue with formation of lymphoid follicles similar in RV adjuvant (c) and saline (d) groups (H&E, ×96).
Table 3
Quantitative assessment: average gradings of histological parameters observed at different time points in vaccine dosed animals

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Submucosa</th>
<th>Exudate</th>
<th>Epithelial damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mononuclear cells</td>
<td>Lymphoid follicles</td>
<td>Mucus</td>
</tr>
<tr>
<td>RV formulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediately after dosing</td>
<td>20.0 ± 14.8</td>
<td>10.7 ± 2.6</td>
<td>0.5 ± 1.0</td>
</tr>
<tr>
<td>One week after dosing</td>
<td>20.9 ± 13.5</td>
<td>11.9 ± 6.0</td>
<td>1.0 ± 1.4</td>
</tr>
</tbody>
</table>

the submucosa, which were regularly present either individually or in clusters. The lymphoid follicles, especially the clusters, were most pronounced ventrally in planes 3 and 4 (Fig. 6b). The solitary follicles were common especially on the septum and around the nasolacrimal duct, but were occasionally found in the ethmoid, both underneath the epithelial lining of the wall and the turbinals (Fig. 6c). In several animals, signs of acute inflammation, which were in general mild, were noted. This consisted of mucus and cellular exudate in the nasal cavity and localized infiltration in the submucosa (Fig. 6d). In the cellular exudate and submucosal infiltrates, neutrophil leukocytes were prominent, sometimes with considerable admixture of eosinophil leukocytes. The epithelial lining was generally intact except for some vacuolization and sometimes desquamation, especially in the ethmoid (Fig. 6e), in animals showing signs of acute inflammation these acute inflammatory changes were of comparable frequency in the control and vaccine groups. No significant difference was noted between the vaccine groups sacrificed immediately or 1 week after vaccine dosing (Table 3).

4. Discussion

The animals did not show any ill-effects of the treatment. They thrived and showed a weight gain during the experimental period comparable to the controls. The less frequent attempts to try to blow out the vaccine and control formulations than the saline may be due to their higher viscosity and bioadhesiveness, making it more difficult to remove the vaccine and control formulations with a single blow. Interestingly, the animals got accustomed to the administration, which was seen by decreases in complaints with time.

The histological features observed apparently fall within a normal variation. The mild rhinitis, which was apparently non-specific, or in some cases with an allergic component (as indicated by the admixture of eosinophils), was obviously not due to a toxic effect of the vaccine, as no significant difference was seen between the vaccine and control groups. There were no signs of epithelial necrosis. The slight sublatent variation in submucosal lymphoid tissue may be due to some variation in the plane of sections. In contrast to the findings of Langermann et al. (1994) who, in an experiment with intranasal vaccination of BCG in mice, found highly organized lymphoid tissue in the nasal submucosa of vaccinated but not in control mice, we did not detect any difference between vaccinated and controls. Thus, there was no difference in the extent of submucosal lymphoid tissue or the occurrence of active lymphoid follicles between vaccinated and control guinea pigs. This apparent discrepancy is possibly due to the difference in the length of the experiment, 2 weeks in our case versus 6 weeks in their experiment. One has also to consider that we did not keep the experimental animals in a specially protected environment. Thus, they were certainly exposed to airborne irritants or agents as indicated by the occurrence of non-specific rhinitis in both vaccinated and control groups.

When animals are vaccinated with the RV adjuvant, a significant immunological response is seen (Gizurarson et al., 1995). This indicates that the vaccines have been absorbed from the nasal cavity.
Method of administering a biologically active substance.

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