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Genetic immunization of seronegative one-day-old piglets against pseudorabies induces neutralizing antibodies but not protection and is ineffective in piglets from immune dams

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Summary — Immune response inhibition by maternal antibodies is a major impediment to the vaccination of the young born to immune dams. This study explored the efficiency of genetic immunization of the neonates at bypassing this inhibition, by testing the muscular inoculation of the gD glycoprotein gene of pseudorabies virus (PRV) in piglets. Plasmid DNA (400 µg) was inoculated in four groups of one-day-old piglets, from sows vaccinated or not against PRV. Half of the groups received a booster injection on day 42. All piglets were challenged on day 115. Only piglets from non-immune sows and which received a booster injection developed a medium level of neutralizing antibodies, but they were not significantly protected against the challenge. Piglets from immune sows neither developed an antibody response nor were primed against PRV, as demonstrated by the antibodies kinetics after challenge. It can therefore be concluded that genetic immunization was inefficient at efficiently preventing the immune response inhibition by colostral antibodies.

pseudorabies virus / vaccine / genetic immunization / antibody response / protection

* Correspondence and reprints

virus de la maladie d’Aujeszky / vaccin / immunisation génétique / réponse anticorps / protection

INTRODUCTION

Pseudorabies is one of the most serious infectious disease in pigs. It is caused by an alphaherpesvirus. Vaccination of pigs against pseudorabies in areas with high prevalence rates of infection concerns both the sows and the fattening pigs. One of the main limitations of the vaccination of fattening pigs is the interference of maternal antibodies in piglets from vaccinated or infected dams. Different attempts have been made to bypass this limitation. One of these is to delay vaccination until the piglets reach 12–14 weeks of age. At this age, however, some of the piglets will have already been infected and others will still have enough maternal antibodies to prevent immunization. Immune response inhibition is observed with both inactivated and live vaccines and so cannot be related to neutralization of the replication of the vaccine strain. Nevertheless, it can be hypothesized that the binding of maternal antibodies to the antigens of the vaccine strain could mask them, in such a way that they cannot be recognized by the immune system of the vaccinate. Recently it has been shown that direct inoculation of the DNA encoding an immunogenic protein (ie, genetic immunization) induces antibody responses, cell-mediated immunity and finally protection against challenge (Ulmer et al, 1993). In addition, the expression of the antigen seems to be long lasting. This study tested the potency of genetic immunization by vaccinating neonates from immune dams on the basis of two properties of this procedure: (i) DNA cannot be a target for the maternal antibodies; (ii) potential masking of encoded cell-associated antigens could be overcome by their long lasting expression, in such a way that vaccinates could mount an immune response after the disappearance of the maternal antibodies.

The gD gene of PRV under the control of the major late promoter of adenovirus type 2 (Ad2) was chosen for this study because the same transcription unit was shown to be protective in mice (Eloit et al, 1990; Ganne et al, 1994) and pigs (Adam et al, 1994) when delivered by a replication-defective adenovirus. Glycoprotein gD is one of the most potent immunogens of the virus and is necessary for the infection of the target cells but not for cell-to-cell transfer (Peeters et al, 1993; Raugh et Mettenleiter, 1991). Most neutralizing monoclonal antibodies without complement addition
were shown to be directed against gD (Coe and Mengeling, 1990; Elloit et al, 1988; Wathen and Wathen, 1984). Vaccination of mice or pigs with purified or recombinant gD or with recombinant vectors harbouring the gD gene conferred a protection to the animals (Elloit et al, 1990; Ishii et al, 1988; Marchioli et al, 1987; Riviere et al, 1992).

Our results demonstrated that (1) piglets from non immune dams mounted a medium, albeit non protective, neutralizing antibody response after two plasmid injections; and (2) piglets from immune dams did not mount an antibody response nor were they primed against the foreign protein.

MATERIALS AND METHODS

Plasmids, viruses and cells

The construction of pMLP10-gD (formerly referenced as pMLP-gp50-6) has been described previously (Elloit et al, 1990). In this plasmid, the gD gene of the NIA3 PRV strain was placed under the control of the major late promoter (MLP) of Ad2 followed by its tripartite leader sequence. Plasmid stocks were prepared by standard maxi-preparation procedures, purified using resin columns (Qiagen) and dissolved after alcohol precipitation in phosphate-buffered saline, pH 7.2. The PRV strain used in the challenge experiments was the 75V19 strain, which had previously been passaged only three times in pig kidney primary cells as described by Vannier et al (1991). The 293 cell line was maintained as described previously (Elloit et al, 1990).

Immunoprecipitations

The 293 cells were transfected by calcium phosphate precipitation with pMLP10-gD (10 µg) or infected with the Kojnock strain of PRV (10 tissue culture infectious dose 50% (TCID50)/cell). Twenty four hours later, the medium was discarded and replaced by fresh medium containing 10 µCi/mL of 14C-glucosamine (ICN). Twenty four hours later, cells were lysed and precipitated with a pool of three monoclonal antibodies directed against gD (Elloit et al, 1988) as previously described (Elloit et al, 1990).

Mouse experiment

Six OF1 mice (18 g of weight) were inoculated with 100 µg of pMLP10-gD twice, four weeks apart, by a muscular route (femoral muscle). Serum samples were taken at regular intervals after the first injection and pooled.

Pig experiment (see table I)

One-day-old piglets from dams vaccinated or not against PRV with an inactivated adjuvanted vaccine (Geskypur, Rhône-Mérieux) three times during gestation were inoculated with 400 µg of plasmid by the muscular route. Half of the animals were inoculated once more under the same conditions at six weeks of age. Two different isolated experimental units were used for the animals from immune or non-immune dams, but the pigs which had received one or two injections were housed in the same units. A group of three pigs was kept as control in a third unit. All the animals were challenged when they were 16 weeks old, at approximately 80 kg of weight, with 2 mL in each nostril of the 75V19 strain of PRV, which titrated 10^6.9 TCID50/mL on the day of challenge. Clinical signs and rectal temperatures were recorded daily and each pig was weighed weekly. Clinical protection was assessed mainly by the duration of hyperthermia and by growth performances as previously reported (Adam et al, 1994; Stellmann et al, 1989) in comparison to those of the control group (three pigs).

Isolation of PRV from nasal swabs

Nasal swabs were taken from the vaccinated and control pigs from day 0 (D0) before the challenge to day 10 (D10), and PRV was titrated as previously described (Vannier et al, 1991). Titres were expressed as TCID50 per 100 mg of mucus.

Titration of antibodies

For the titration of PRV antibodies, a micro-neutralization test using a one-hour contact between serum and virus without adding complement, was used as described previously (Vannier et al, 1991). Antibodies against PRV were also titrated at the time of the challenge by an ELISA with the same protocol, using purified envelope proteins of PRV as the antigen (Adam et al, 1994). Titres
were expressed as the last dilution which gave a corrected absorbance superior to that of the negative controls plus 0.100. Specific gD antibodies were titrated with the same protocol, using extracts from cells infected with a baculovirus expressing gD (a generous gift from Dr M Banks, Central Veterinary Laboratory, New Haw, UK). Each sample was tested in parallel with a control antigen (extract from mock-infected cells) and the results were given as the difference of absorbance between the two wells.

RESULTS

Expression of gD in cells

293 cells were transfected with pMLP10-gD and the expression products were immunoprecipitated with a pool of gD-specific monoclonal antibodies (fig 1). The expected band at 62 kDa was evidenced, roughly similar to that seen in 293 cells infected with the Kojnok strain of PRV. The slight difference between molecular weights was probably dependent on the parental strain, as the gD gene cloned in pMLP10-gD was isolated from the PRV NIA3 strain.

Mice inoculated with pMLP10-gD developed specific antibodies

Serum samples taken at day 7 demonstrated a significant antibody response (fig 2). After a second injection made at day 30, the antibody titres showed a clear booster effect in all five of the six mice which previously possessed a low antibody titre.

Pig experiments

The pig assay was designed to test immunization schemes which would be practicable in the field. In this end, it was decided to avoid multiple inoculations. For a first assay (results not shown), antibody responses were tested as a function of the dose inoculated. One-day-old piglets born from non-immune dams were inoculated once, either with 200 or 400 μg of plasmid. Low levels of gD-specific antibodies were evidenced at the end of the fattening period in two out of five pigs inoculated with 400 μg of plasmid.

Fig 1. Expression of gD in cells. Immunoprecipitation of 14C-glucosamine-labelled 293 cells extracts transfected with pMLP-gD (A) or infected with the Kojnok strain of PRV (B) with a pool of monoclonal antibodies directed against gD.
Table 1. Pig trial: antibody responses and protection.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccination sow</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vaccination one-day-old piglets</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>No of piglets</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Antibody titre at D42 a</td>
<td>2-8-8-6</td>
<td>2-8-4-4</td>
<td>N-N-N-N</td>
<td>N-N-N-N</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>≥1755-632-1496-565</td>
<td>≥1728-846-735-1361</td>
<td>0-1-0-21</td>
<td>19-55-11-20</td>
<td></td>
</tr>
<tr>
<td>Boost at D42</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Antibody titre at challenge (D115) a</td>
<td>N-N-N-N</td>
<td>N-N-N-N</td>
<td>N-N-N-N</td>
<td>4-4-2-4</td>
<td>N-N-N</td>
</tr>
<tr>
<td>Antibody titre 7 days after challenge (D122) a</td>
<td>N-N-N-N</td>
<td>N-N-N-2</td>
<td>N-N-N-dead</td>
<td>512-1024-512-512</td>
<td>N-N-2</td>
</tr>
<tr>
<td>Clinical protection c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Peak of excretion of challenge strain a</td>
<td>3.9, 2.6, 2.3, 2.2 (2.8)</td>
<td>3.9, 2.6, 2.3, 2.2 (2.8)</td>
<td>2.8, 2.5, 2.3, 4.0 (2.9)</td>
<td>0, 2.4, 3.4, 2.2 (2)</td>
<td>3.8, 4.3, 3.5 (3.9)</td>
</tr>
<tr>
<td>Duration of excretion of challenge strain d</td>
<td>5, 4, 5 (5)</td>
<td>5, 5, 6, 5 (5)</td>
<td>6, 7, 5 dead (6)</td>
<td>0, 4, 4, 4 (3)</td>
<td>6, 6, 6 (6)</td>
</tr>
</tbody>
</table>

a Antibody results at D42, D115 and D122 are given as both the neutralizing titre (first line) and as absorbances recorded with an ELISA using baculovirus-expressed gD as antigen at a serum dilution of 1/50 (second line). In this case results are given as the difference between absorbance of test and mock-infected wells. b For more details about growth performances and hyperthermia, see figures 3 and 4. c Peak of excretion (log_{10} TCID_{50} per 100 mg mucus) for each pig (mean of the values). d Duration of excretion in days (mean of the values). In the cells depicting antibody titres or peak and duration of excretion of the challenge strain, the results are arranged in the same sequence relative to the reference of the pigs, in such a way that the consecutive results of each pig can be retrieved.
On the basis of these results, a protocol was designed including matched groups of pigs which either received a second injection or not on day 42 (D42) (see table I). Piglets from non-immune dams vaccinated only once at birth (group 3) developed no antibody response that was detectable by ELISA or by neutralization assay. On the other hand, corresponding piglets which received a booster injection at D42 developed medium levels of gD-specific antibodies that neutralized virus infectivity in vitro (group 4). In contrast, piglets from immune dams which received (group 2) or not (group 1) a booster injection on D42 did not develop an antibody response. In comparison with groups 3 and 4, it was obvious that the antibody titres detected on D42 in groups 1 and 2 were colostral antibodies.

All pigs were challenged on D115 and antibodies were titrated seven days later (D122) to look for an anamnestic antibody response. Upon comparison with the control animals (group 5), it is clear that only group 4 pigs (born from seronegative sows and vaccinated twice) developed such an anamnestic response. It was especially interesting to observe that piglets from seropositive sows which received a two-injection scheme (group 2) developed a primary response after the challenge, very similar to that of the control group. Growth performances were recorded after the challenge (fig 3), together with body temperatures (fig 4) and virus excretion (table I). These characteristics were similar between the control and the four tested groups, ie, no group of pigs was significantly protected against the challenge.

**DISCUSSION**

One of the main limitations of vaccination concerns the vaccination of the young born to immune dams. In all species, the existence of maternal antibodies is a strong limitation which impairs the development of an immune response in the young. As far as pets are concerned, in the case of a high risk of infection, it is often recommended to vaccinate the young several times between four and twelve weeks of age, so that an active immune response can develop as soon as possible after the disappearance of colostral antibodies. For practical and economic reasons, this is obviously not practicable in farm animals. It was recently shown that direct inoculation of plasmid-encoding immunogenic proteins was able to induce a long-term presentation of the antigen to the immune system. Neverthe-
less, it appeared that such an immune response developed slowly (Ulmer et al., 1993). It is therefore necessary to inoculate the DNA long before an active immunity is needed. Based on these observations, experiments were designed which were based on the inoculation of one-day-old piglets, with or without a booster at the age of six weeks.

Several conclusions can be drawn from our results. First, it was obvious that more than one inoculation was required to induce a significant amount of antibodies. Only one out of six mice developed a high level of antibodies one month after a single injection of 100 μg of DNA. All the five remaining mice showed a clear booster effect after a second injection. This was also observed in the pigs, in which a unique injection at birth induced no antibody response (table I, group 3). On the other hand, all the piglets that were given a booster injection six weeks after the first developed a medium antibody response (table I, group 4). This response lasted at least till the age of challenge (i.e., 70 days after the booster inoculation). These results are similar to those previously published by other teams who generally used a multiple injection protocol (Coney et al., 1994; Ulmer et al., 1994).

The second conclusion which can be tentatively drawn from these results was that the antibody response in group 4 could not be paralleled with protection. The antibody response in this group can be compared to that obtained in previous experiments, in which the same standardized seroneutralization test was used (Vannier et al., 1991). Although the neutralizing titres obtained in group 4 were similar to those obtained with

Fig 3. Growth performances in pigs. Growth performances are depicted from birth to D135. The challenge was performed at D115.
some conventional live non-adjuvanted vaccines (Vannier et al, 1991), the protection was clearly not comparable. These results can also be compared to those recently obtained in pigs (Adam et al, 1994). In this experiment, the same gD gene with identical regulatory sequences was cloned in a replication-defective adenovirus and administered to pigs after formulation in oil adjuvants. Higher antibody responses were observed and a clear protection was recorded. It can therefore be concluded that the gD gene product is sufficient to induce a protection in pigs.

There are two possible hypotheses as to why genetic immunization was unsuccessful. In the first hypothesis, the reason for the failure was only quantitative, i.e., a higher level of expression of the protein would have been sufficient to obtain a protective response. If this hypothesis is true, the use of stronger promoters may bypass this limitation. The second hypothesis relies on the fact that the inoculation of naked DNA and replication-defective adenoviruses introduced foreign genes into different types of cells which were not as efficient as antigen-presenting cells (APC). Until now, to our knowledge, only muscle cells have been shown to be transduced after inoculation of DNA in saline buffer, a property thought to be linked to the presence of T-tubules (Danko and Wolf, 1994). The adenovirus, however, is able to introduce foreign genes into a very large range of cell types, including typical antigen-presenting cells such as dendritic and macrophage cells (Hadadda et al, 1993). It is possible, then, that differential targeting of the gD gene could account for the different levels of protection. Although

**Fig 4.** Rectal temperatures in pigs. Rectal temperatures are shown from D112 to D135. The challenge was performed at D115.
Genetic immunization was shown to elicit not only an antibody response but also cytotoxic T cell responses, it remains possible that such induced responses were too low to confer sufficient immunity against a strong challenge. Due to the low number of MHC I antigens on muscle cells, it is possible that the CTL response, though detectable in certain experiments (Coney et al., 1994; Ulmer et al., 1994; Lowrie et al., 1994), was insufficient to provide resistance in our model. In addition, it was recently shown that genetic immunization induces preferentially a Th1-type response and only a weak or undetectable Th2-type response (Xu and Liew, 1994). This may also explain our results.

Finally, the most striking conclusion emerging from this experiment was the complete inhibition of antibody responses in piglets born to immune dams (Table I, groups 1 and 2). In fact, no evidence of antibodies was seen even after a booster injection was made in the presence of low levels of maternal antibodies. Moreover, these piglets did not show any anamnestic response after challenge, even when using a sensitive ELISA specific to gD antibodies. One explanation could be that a down-regulation of the immune response accounts for the lack of recognition of foreign antigens in the presence of specific antibodies. If confirmed, such an explanation would be a major impediment for the vaccination of the young. Another hypothesis is that maternal antibodies efficiently prevent the recognition of gD at the membrane of transduced cells, by a mechanism involving the masking of antigenic sites. Nevertheless, this does not account for the lack of seroconversion after maternal antibodies have disappeared. Such a result was hoped for with the postulate of a long-lasting presentation of gD. It is possible that muscle cells expressing gD and coated by maternal gD antibodies were cleared by killer (K) cells, and replaced by newly differentiated muscle cells in new-born animals. Direct targeting of foreign genes in APC by using replication-defective adenoviruses should circumvent this deprivation. Such experiments are currently being conducted in both mice and pigs.

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