Immunization of Mice with Live Attenuated Encephalomyocarditis Virus: Local Immunity and Survival

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Mice were vaccinated with an attenuated encephalomyocarditis (EMC) virus strain by the intraperitoneal (i.p.) route and by various ways of respiratory administration: aerosol exposure and intratracheal (i.t.) and intranasal (i.n.) instillation. A linear relationship was found between vaccine dose and the resulting serum antibody titer. The effectiveness of the vaccine was determined by measuring the 50% protective doses (ED$_{50}$ values) after a lethal challenge with live virulent virus given by the i.p. route. For all three methods of respiratory immunization essentially the same ED$_{50}$ value was found, about 200 plaque-forming units (PFU), but i.p. immunization was less effective, the ED$_{50}$ value being about 600 PFU. To investigate the protective effect of local immunity, mice were vaccinated i.p. or i.n. and challenged by the i.n. route. The same ED$_{50}$ values were found as after i.p. challenge, indicating that the degree of protection afforded by the vaccine depends only on the route of vaccination and not on the route of challenge. This means that protection depends largely on systemic immunity and that local immunity plays only a minor role in this system. The results are discussed in relation to the feasibility of respiratory immunization against animal viruses.

Although many studies have been reported on respiratory immunization with live virus vaccines, the effectiveness of parenteral and respiratory immunization was not always compared. In nearly all cases only one method of respiratory immunization was used, either aerosol exposure (19) or intranasal (i.n.) instillation (5, 16, 21, 27, 28).

Immunization of mice against encephalomyocarditis (EMC) virus was one of the models used for respiratory vaccination. Aerosol immunization with the attenuated A-37 Mongo virus strain has been reported by Prato and Akers (19). Peroral immunization with a tissue culture strain of EMC virus (13) was shown to protect mice against infection with a fully virulent EMC virus strain. In a previous communication (4) we reported the respiratory and intraperitoneal (i.p.) immunization of mice with a killed EMC virus vaccine.

In experiments with inactivated vaccine (4) it was found that Bordetella pertussis extract (BPE) exerted an adjuvant action when given together with the vaccine. From preliminary experiments it appeared, however, that BPE given simultaneously with the live vaccine impaired the antibody response against EMC virus, but BPE, given 3 days after vaccination no longer depressed antibody formation. It was therefore decided that all immunization experiments with live vaccine should be carried out without adjuvant.

In this study we will compare the effectiveness of immunization of mice with an attenuated EMC virus strain by the peritoneal and by several variants of the respiratory route: i.n. instillation, intratracheal (i.t.) injection, and aerosol exposure. After i.n. and i.p. immunization, immunity was evaluated by i.n. and i.p. challenge to test whether local immune reactions play an important role.

MATERIALS AND METHODS

Vaccine. Titrations of suspensions of the small-plaque-forming variant (SFP) of EMC virus (3) revealed the presence of minute plaques. The plaque diameter of the SFP variant is approximately 2.5 mm, whereas that of the minute-plaque-forming (MPF) variant appeared to be less than 0.5 mm. Better, observable plaques were obtained after 4 days of incubation.

Twenty MPF clones were isolated, grown in L929...
cells, and tested for immunogenicity and absence of lethality for mice after i.p. injection of 10^4 and 10^6 PFU per animal. All clones showed good immunogenic properties; the variant causing no mortality and paralysis was used as a model of a live EMC virus vaccine.

The vaccine stock was stored in liquid nitrogen. In each experiment the titer was assayed by plaque titration in L cells (3).

**Mice.** Male F1 hybrids (C57 Bl n × CBA n ), aged 8 weeks, were used throughout this study.

**Inoculation techniques.** For i.p. injection, 0.5 ml of vaccine diluted in phosphate-buffered saline was given to each mouse. For i.t. injection, 10 μl of vaccine dilution was delivered into the trachea under general anaesthesia (4). For i.n. instillation, 50 μl of vaccine dilution was administered into the nostrils of mice under light ether anesthesia by use of an Eppendorf micro-pipette. For aerosol exposure in a Henderson apparatus (1), mice received the vaccine by the respiratory route since only mouth and nose were exposed to the aerosol. The exposure time was 15 min; the relative humidity was 60 to 70%. The aerosol was sampled by means of an all-glass impinger (1), and the inhaled vaccine dose was estimated assuming a mean respiratory volume for mice of 25 ml/min (14).

**Serum collection and antibody determinations.** Twenty days after immunization and 3 weeks after challenge the mice were bled. Hemagglutination inhibition tests were carried out as described previously (4).

**Statistics.** From survival data mean effective doses (ED_{50}) were calculated by the method of probit analysis (12). Geometric mean antibody titers were calculated from serum titers of individual mice.

**Experimental design.** Groups of 10 mice were immunized by graded doses of vaccine. In each experiment 10 control mice were given phosphate-buffered saline instead of vaccine. Protection was evaluated by i.p. injection of 200 mean lethal doses or by (LD_{50}) i.n. instillation of 1,000 LD_{50} of large-plaque-forming (LPF) EMC virus 21 days after immunization. None of the control mice survived challenge. Mice were observed for at least 4 weeks after challenge.

**RESULTS**

**Development of serum antibody titers after i.p. immunization.** Forty mice received 2.5 × 10^4 plaque-forming units (PFU) of live MPF virus by i.p. injection. At various time intervals, groups of four animals were sacrificed and bled by heart puncture. The sera were stored at −20°C and later assayed simultaneously for antibodies. The development of these titers is shown in Fig. 1. After 3 days no antibodies are detectable, after 5 days the antibody level is sufficiently high so that all animals would be protected, the protective ED_{50} being 1.0 hemagglutination inhibiting unit (HAIU)/ml as described previously (4). After 2 weeks, maximum antibody titers are reached. After a period of 9 months, the residual titers are approximately 100 HAIU/ml, so that all animals would still survive challenge. Immunization of mice with MPF virus thus appears to be possible; the protective ED_{50} being much less than 2.5 × 10^4 PFU/animal.

**ED_{50} determinations: intraperitoneal immunization.** For i.p. immunization experiments, five groups of 10 mice were used. Figure 2A shows the relationship between vaccine dose and survival after i.p. challenge. The calculated regression line is plotted together with the percentage of survival. From three experiments the combined ED_{50} value was calculated and appeared to be 607 PFU; its 95% confidence limits are 422 and 884 PFU.

**Respiratory immunization: aerosol treatment.** Five groups of 10 mice were exposed to aerosols of MPF EMC virus. The survival after i.p. challenge as a function of vaccine dose is shown in Fig. 2B. The ED_{50} corresponds to approximately 2,300 PFU of virus, which is roughly four times as much as the i.p. ED_{50}.

**Intratracheal injection.** To five groups of 10 mice the vaccine was administered by i.t. instillation. The ED_{50} value appeared to be approximately 200 PFU and is lower than its i.p. counterpart. A second experiment yielded similar results. Figure 2C shows the protective effect in relation to the antigen dose.

**Intranasal instillation.** Five groups of 10 mice received the vaccine by i.n. instillation. Figure 2E shows the survival rates after i.p. challenge. The ED_{50} value is low, 140 PFU/animal.
Protection against intranasal challenge.

To investigate whether local immune reactions play an important role in protection against respiratory EMC virus infections, five groups of 10 mice were immunized by i.p. injection and five other groups were immunized by i.n. instillation. All animals were challenged with 1,000 i.n. LD₅₀; the i.n. LD₅₀ was measured in a previous experiment and appeared to be 548 PFU of LPF virus. The survival after i.n. challenge is shown in Fig. 2D and F. After i.p. immunization an ED₅₀ value of 740 is found, which is not significantly different from the value of 607 found for mice challenged by the i.p. route. After i.n. immunization the ED₅₀ for mice challenged intranasally is 188, as compared to 140 for mice challenged intraperitoneally. After i.n. immunization a systemic and possibly also a local form of immunity will develop. Protection against an i.p. challenge will only be afforded by the systemic component of this immunity, but after i.n. challenge local and systemic immunity will cooperate. If local immunity plays an important role against respiratory EMC virus infection, a lower ED₅₀ value will be found after i.n. than after i.p. challenge. If, on the other hand, local immunity plays only a minor part, the same ED₅₀ values will be found. It can be concluded that, after immunization with live vaccine, local immunity cannot play an important role in protection against respiratory EMC virus infection.

Antibody response. The relationship between mean antibody titers of the groups and vaccine dose are shown for i.p. immunization in
Fig. 3 and for i.n. immunization in Fig. 4. The secondary responses after both i.n. and i.p. challenge are also indicated. The titers are low in comparison with those shown in Fig. 1 where a much higher vaccine dose was used.

A vaccine dose equal to the $ED_{50}$ value will evoke a serum antibody titer of about 5 HAIU after i.n. and also after i.p. administration. In both cases, i.n. and i.p. challenge will lead to 50% survival. This means that, in all combinations, the survival rate depends on the serum antibody titer and is not enhanced by local defense mechanisms, which could be active after i.n. immunization followed by i.n. challenge.

**DISCUSSION**

From the slope of the dose response curves (Fig. 3 and 4), which is about 45°, it appears that the serum titer of a group depends on the injected dose of live virus vaccine. This implies that the effective antigenic mass of virus antigen produced by the host is roughly proportional to the vaccine dose, i.e., the same number of multiplication cycles has taken place until virus reproduction was stopped by the appearance of antibodies in the blood.

From antibody measurements it appears that, in groups showing 50% survival, the serum titer of the group, determined by extrapolation from the dose-response curve, is approximately 1 to 5 HAIU. This corresponds to the serum titer causing 50% protection against i.p. challenge in a passive immunization test (4). As blood samples were collected 1 day before challenge, it is almost certain that survival of an i.p. challenge depends on circulating antibodies elicited by the immunization procedure.

In immunizations with live vaccine it is probable that differences in $ED_{50}$ values for different routes only represent a difference in probability to reach a suitable target cell to start a successful replicative cycle. The probability of replication seems higher in the respiratory tract ($ED_{50}$ 140, 188) than in the peritoneum ($ED_{50}$ 607). The high $ED_{50}$ (2,325) for aerosol immunization could be explained by the low fraction reaching the lung. Experiments with labeled tetanus toxoid (J. L. F. Gerbrandy, manuscript in preparation) have shown a lung retention of 10% of the inhaled dose. Assuming the same retention for vaccine virus, a lung dose of 250 PFU would represent the $ED_{50}$, i.e., comparable to the $ED_{50}$ i.p. and i.t., although it should be realized that the deposition of a number of individual aerosol particles on the
mucous escalator in the bronchial tree is different from flooding with a virus suspension. If the aerosol ED₅₀ was due to lung retention only, this would imply, however, that the large fraction of the aerosol dose retained in the upper respiratory tract would not contribute to the ED₅₀, i.e., the nose is not a primary multiplication site. This is the more peculiar as a large part of the i.n. ED₅₀ (140, 188 PFU) is swallowed and a minor part reaches the lung. Compared with the i.t. ED₅₀ (188 PFU), this fraction would be too low for immunizing 50% of the animals. The swallowed fraction is probably ineffective: after administration of large amounts of LPF EMC virus by a stomach tube, the animals neither died nor developed antibody titers (unpublished results).

Considering all of this, a study of the primary multiplication site appears necessary for the interpretation of the data obtained.

By comparing Fig. 2A, D, E, and F, it appears that the degree of protection against a lethal infection depends only on the method of immunization and not on the challenge route. The protective ED₅₀ values are the same, irrespective of whether vaccination and challenge were given by the same or by different routes: no additional protection is provided by local immune reactions.

After respiratory immunization with live EMC virus vaccine, lower ED₅₀ values are obtained than after i.p. immunization. With inactivated vaccine (4) the situation is reversed, the i.p. route is more effective than the respiratory route. It follows that, after i.p. administration of a fixed antigen dose, antibody production is higher than after respiratory vaccination, but reproduction of vaccine virus is better in the respiratory tract. The same conclusion can be drawn from the antibody response data.

Local production of secretory immunoglobulin (Ig) A antibodies is frequently used as an argument in favor of respiratory immunization. Indeed Fazekas de St. Groth (11) has shown that protection against influenza virus infection correlated better with the development of a local immune response than with circulating antibody titers in the blood. This means that circulating antibodies alone are not sufficient for total protection. However, this does not hold true for all respiratory immunization-respiratory infection systems. In the case of adenovirus, serum antibodies provide protection against virus infection and illness (7). For picornaviruses the situation is more complex. For rhinovirus, resistance to illness is based on local antibodies (17, 18), and for poliovirus a systemic immunity will provide full protection although local immune mechanisms are able to suppress virus growth in the respiratory and alimentary tract. Prato and Akers (19) have shown that aerosol immunization induces resistance against a lethal respiratory challenge with EMC virus, but it was not determined if local immunity played a substantial role in this protection. From our results it may be concluded that protection against respiratory EMC virus infection with virulent EMC virus is not confined to the respiratory system because the brain and the myocardium are the target organs. The use of respiratory immunization to evoke a local immune reaction seems to be only necessary against infections which are limited to the respiratory tract (influenza and rhinovirus) because local IgA antibody titers are better correlated with resistance against this type of infection. When other sites are affected (adenovirus), circulating antibodies are sufficient for protection (7). The results with EMC virus support this view.

When comparing the merits of respiratory and parenteral immunization, the effectiveness of the procedure and the occurrence of overt and hidden side reactions must be assessed. Although respiratory immunization with live vaccine results in a significantly lower ED₅₀ value, this difference is small, and both methods evoke a long-lasting immunity, protecting the mice for years against a lethal challenge with virulent EMC virus. Sometimes side reactions occur which can be attributed to the appearance of SPF variants, which arise by mutation from the vaccine strain. In rare occasions even paralysis of the limbs may occur, but this is only seen after i.p. and never after respiratory immunization. This is in accordance with the results from infectivity studies with the three EMC virus variants administered by various routes which will be published later.

**LITERATURE CITED**


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