INFECTION AND IMMUNITY, Oct. 1972, p. 513-517
Copyright © 1972 American Society for Microbiology
Vol. 6, No. 4
Printed in U.S.A.

Immunization of Mice Against Encephalomyocarditis Virus

II. Intraperitoneal and Respiratory Immunization with Ultraviolet-Inactivated Vaccine: Effect of *Bordetella pertussis* Extract on the Immune Response

W. J. C. BOGAERTS AND B. J. DURVILLE-VAN DER OORD

Medical Biological Laboratory TNO, Department of Microbiology, 139, Lange Kleiweg, Rijswijk (ZH), The Netherlands

Received for publication 28 March 1972

Mice were immunized by intraperitoneal (ip) or respiratory administration of ultraviolet-inactivated virus alone or with *Bordetella pertussis* extract (BPE) as an adjuvant. The effect of immunization was tested by determination of antibody titers and by survival of a lethal challenge with 200 LD_{50} of a virulent (large-plaque variant) strain of EMC virus. For plain vaccine the ip 50% effective dose (ED_{50}) was 37 hemagglutination units (HAU; ca. 4×10^6 plaque-forming unit equivalents); with adjuvant the ip ED_{50} was reduced to 20 HAU. After respiratory immunization by intratracheal injection, an ED_{50} value of 100 HAU was found, which was not affected by BPE. After ip vaccination the primary immune response was enhanced by BPE, but the challenge response, measured 3 weeks after challenge, was unaffected. Respiratory immunization induced a primary response which was not influenced by BPE, but here the challenge response was enhanced by the adjuvant. After secondary treatment (challenge or booster vaccination) serum antibodies and protection against challenge persisted for at least 1 year.

Respiratory immunization with encephalomyocarditis (EMC) virus vaccines has been studied by several authors. Akers and co-workers (1, 9) investigated respiratory immunization with an avirulent EMC virus strain; oral vaccination with live EMC virus was reported by Friedman and Maenza (4), and comparative studies on oral immunization with live vaccine and intraperitoneal (ip) immunization with Formalin-inactivated virus were described by Lawrence and Collins (7).

For true respiratory immunization the vaccine should be delivered into the lower respiratory tract. Since aerosol immunization in our system required large amounts of antigen, other methods of application had to be used. After intranasal application an unknown fraction of the inoculum reaches the lungs, another fraction is trapped in the nasopharyngeal cavity, and by far the largest fraction is swallowed down into the stomach. After intratracheal inoculation most of the inoculum is retained in the lungs, and only a small fraction is transported from the lungs over the glottis. Therefore intratracheal injection was chosen as the method of administration of inactivated virus vaccine by the respiratory route.

From studies on immunization of mice with tetanus toxoid it appeared that the adjuvant activity of *Bordetella pertussis* extract (BPE) was maximal when antigen and adjuvant were given by the respiratory route. The 50% effective dose (ED_{50}) was lowered 100-fold (Gerbrandy, *manuscript in preparation*). After subcutaneous or ip administration the reduction of the ED_{50} value was much less. The purpose of our studies was to evaluate the immunogenicity of our ultraviolet (UV)-inactivated vaccine (2) given by the respiratory and by the ip routes. The antibody response and the protection against a lethal ip challenge were investigated after immunization with plain vaccine and vaccine in combination with BPE.

MATERIALS AND METHODS

Vaccine. UV-inactivated vaccine was prepared from suspensions of EMC virus (small plaque variant) grown in L cell suspension culture as described previously (2). The virus was precipitated by polyethylene glycol, resuspended in phosphate-buffered 0.15 M NaCl solution (PBS; *p*H 7.2), extracted with Genetron, further purified by exclusion chromatography on Sephadex G-200 or on porous glass, and inactivated by UV light in a dynamic system. The

vaccine was concentrated by ultracentrifugation and stored frozen in liquid nitrogen.

Adjuvant. Sodium deoxycholate BPE (7) containing 128×10^9 cell equivalents/ml was kindly supplied by H. Cohen (National Institute for Public Health, Bilthoven, The Netherlands). Simultaneously with the vaccine 2.5×10^8 cell equivalents of BPE were given.

Immunization of mice. Male F-1 hybrids (CBA × C57Black), aged 8 weeks and weighing 20 to 25 g, were divided into groups of 10 by using random permutation tables. For each dilution of antigen one group was used. In each experiment control groups receiving PBS or PBS with BPE were included.

For ip immunization the vaccine was given in 0.5 ml of PBS. For respiratory immunization the vaccine was given by intratracheal injection (Karelse, *manuscript in preparation*). Under general anesthesia with 85 mg of sodium hexobarbital per kg and 10 mg of atropine sulfate per kg, the trachea of the animal was exposed through a midline cervical incision; under visual inspection a blunted cannula was brought via the mouth into the trachea and moved up to the bifurcation. Ten microliters of vaccine in PBS was delivered by a Hamilton PB 600 repeating dispenser connected to the cannula. The skin was sutured with one or two stitches of Perlon (Pfrimmer no. 4/0). Protection was evaluated by ip injection of 2,000 plaque-forming units (PFU) of EMC virus (large plaque variant) 21 days after immunization. This dose corresponds to 200 ip 50% lethal doses (LD_{50}) (*unpublished data*); no control mice survived this challenge. In each experiment the challenge virus was titrated on monolayers of L cells, and the actual dose was calculated. After challenge mice were observed for at least 4 weeks for death or protective immunity.

Antibody determinations. Twenty days after immunization and 21 days after challenge the mice were bled. Bleeding was done with a capillary pipette placed in the retroorbital plexus. Serum was separated and frozen within 24 hr. Individual mice were ear-marked so that each could be followed serially. All sera from one experiment were assayed on the same day by the hemagglutination inhibition (HAI) test.

HAI test. To serial twofold dilutions of 0.25 ml of serum, 0.25 ml of antigen containing 2 hemagglutination units (HAU) of EMC virus and 0.25 ml of sheep red blood cell suspension (2.5×10^7 cells/ml) were added. All dilutions were made in PBS-gelatin (2). After standing overnight the hemagglutination patterns were determined. The last serum dilution completely inhibiting hemagglutination was taken to be the antibody end point. The titer, expressed as hemagglutination-inhibiting units per milliliter, (HAIU/ml) is defined as the reciprocal of this value. From the individual serum titers for each group of mice the mean and standard error of the mean were calculated.

Statistics. From survival data, protective ED_{50} values were calculated by the probit analysis (3), using a program for the PDP-8-I computer written by M. Wijnans.

Passive immunization. Neutralizing antiserum to EMC virus was prepared in syngeneic mice by ip immunization. The primary dose consisted of 1,000 HAU of vaccine in 0.5 ml of PBS. After 3 weeks a secondary dose (100 HAU) was given. The animals were bled 2 weeks after the last injection. The titer of the antiserum was 1,024 HAIU/ml. Dilutions were made in PBS, and 0.25 ml was injected intravenously into each mouse. From the serum volume (1.0 ml) determined in mice of the same age and weight by the Evans blue method, the antibody titer in the recipients was calculated. In a control group receiving undiluted antiserum the measured antibody titer corresponded to its expected value: 200 HAIU/ml. One hour later the animals were challenged ip with 200 LD_{50} of large-plaque-forming virus.

RESULTS

Protection by antiserum. Six groups of 10 mice received amounts of antiserum giving rise to blood titers ranging from 0.8 to 205 HAIU. The ED_{50} appeared to be 1.0 HAIU, i.e., the minimal amount of antibody that can be detected by the HAI technique. Thus, small amounts of antiserum injected intravenously provided protection against ip challenge (Fig. 1). All control mice receiving PBS intravenously died.

Intraperitoneal immunization—effect of BPE: (i) survival. Five groups of 10 mice were treated by ip injection with doses ranging from 5 to 5,500 HAU; five other groups received the vaccine in combination with BPE. The protective effect of the vaccination is shown in Fig. 2. The ED_{50} appeared to be 37 HAU, corresponding to 4×10^6 PFU equivalents of inactivated virus. For the combination of vaccine and adjuvant, the ED_{50} value was significantly lower: 20 HAU. Administration of BPE enhanced the protective

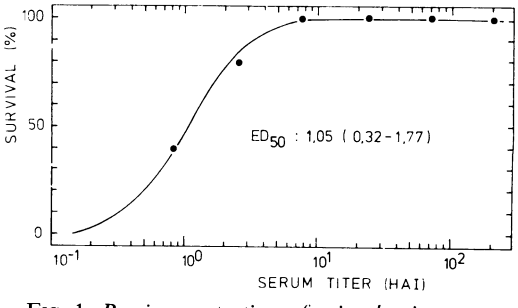

FIG. 1. *Passive protection of mice by intravenous administration of antiserum against ip challenge. To 60 mice 0.25-ml samples of antiserum dilutions were given 1 hr before ip challenge. The dose response line was calculated by the probit method. The ED_{50} value and, between parentheses, its 95% confidence limits are indicated. The effectiveness of the protection appears from the low ED_{50} value.*

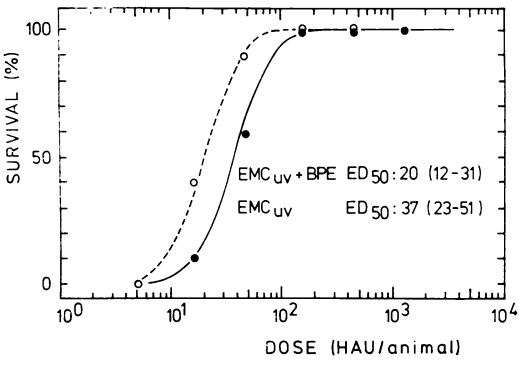

FIG. 2. *Protection of mice by ip immunization with inactivated encephalomyocarditis (EMC) virus vaccine; effect of Bordetella pertussis extract (BPE) on survival. Three weeks after vaccination the animals were challenged ip with 200 LD_{50} of live large-plaque-forming virus. Each point represents the survival rate of a group of 10 mice. The dose response line was calculated by the probit method. The ED_{50} values with 95% confidence limits are given. ●, EMC_{uv}; ○, EMC_{uv} + BPE.*

effect of the vaccine by a factor of 1.8. In a second experiment these values appeared to be 29 and 7 HAU, so here the value of the relative adjuvant effect is 4.1. All control mice died.

(ii) **Antibody response.** Antibody titers were measured in sera collected 1 day before challenge (primary response) and 21 days after challenge (challenge response). After treatment with vaccine and adjuvant, the animals produced antibody titers which were significantly higher than those produced by animals treated with vaccine only. No antibody titers were found in sera of mice treated with PBS alone or with BPE. A second experiment led to similar results. Enhancement of antibody production by BPE is in accordance with enhanced survival of the challenge. Antibody responses as a function of immunizing dose are shown in Fig. 3. Three weeks after challenge, about the same titer is found in all animals regardless of primary antigen dose and BPE treatment.

Intratracheal immunization—effect of BPE: (i) survival. By intratracheal injection 110 mice received 2 to 6,000 HAU of EMC virus vaccine. For each dose half of the animals (10/20) were treated simultaneously with adjuvant. The survival ratio as a function of the immunizing dose is shown in Fig. 4. The ED_{50} values were 101 and 107, respectively, for animals treated with vaccine and with vaccine in combination with adjuvant. BPE treatment did not enhance the protective effect of respiratory immunization with inactivated EMC virus vaccine. A second experiment led to similar results.

(ii) **Antibody response.** Figure 5 shows the mean serum antibody titers of the same groups as in Fig. 4. A linear relationship exists between primary antibody response and vaccine dose. BPE failed to enhance the primary antibody response but strongly affected the challenge response; both results are in contrast with those obtained after ip vaccination. Control mice receiving PBS alone or in combination with BPE by intratracheal injection showed no antibody response and died after challenge.

Persistence of immunity. After primary immunization antibody titers were maximal after ca. 15 days and remained stable for at least 3 weeks. Thereafter they decreased gradually, and after 1 year they were low or absent. However, if a booster dose (100 HAU) was given at that time, a typical secondary response was evoked. A mean titer of 500 HAIU was reached, much

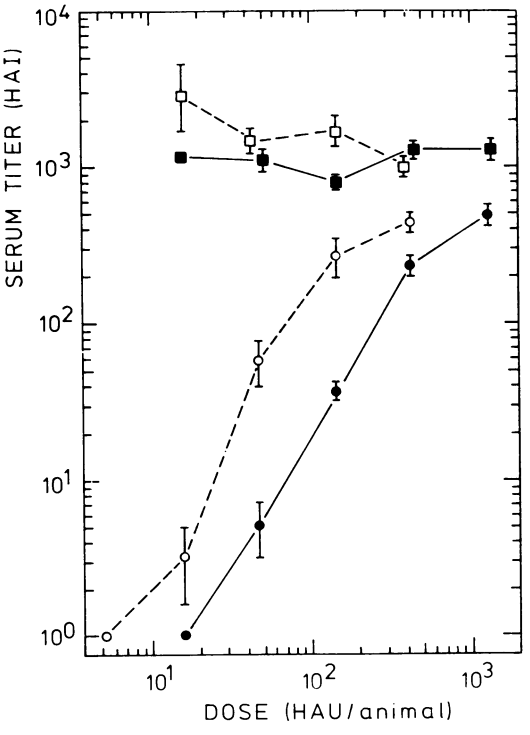

FIG. 3. *Antibody titers after ip immunization with inactivated encephalomyocarditis virus vaccine; effect of Bordetella pertussis extract on the immune response. Presented are the mean titers and standard error of the mean 3 weeks after immunization (primary response) and 3 weeks after challenge (challenge response). Circles indicate the primary response, and squares the challenge response. Closed symbols indicate titers of animals treated with vaccine only, and open symbols titers of animals treated with vaccine and adjuvant.*

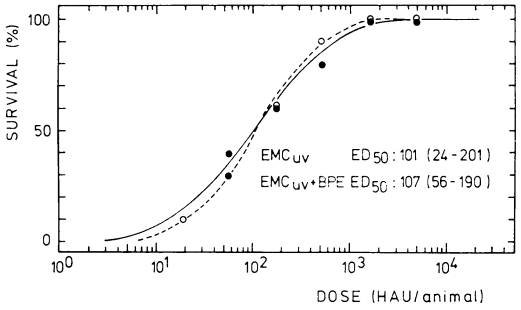

FIG. 4. *Protection by intratracheal immunization of mice with inactivated EMC virus vaccine; effect of BPE on survival. Symbols as in Fig. 2.*

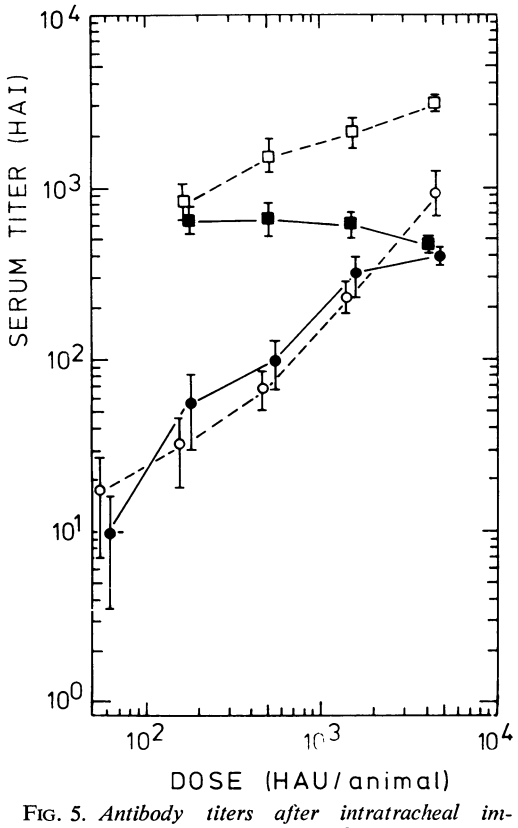

FIG. 5. *Antibody titers after intratracheal immunization with inactivated EMC virus vaccine; effect of BPE on the immune response. Symbols as in Fig. 3.*

higher than after the primary immunization with 50 HAIU. If a secondary treatment (challenge with 200 LD_{50} or booster with 100 HAU) was given 3 weeks after primary immunization, the animals showed a typical secondary response. Antibody production was short and rapid. Titers were maximal after 1 week, decreased rapidly until the level of the primary response was attained, and remained practically constant for months. After 1 year titers were measured that were able to protect all mice against lethal challenge. A booster treatment with 100 HAU provoked hyperimmune titers (1,000 to 3,000 HAIU).

DISCUSSION

In our mouse immunization tests the UV-inactivated EMC virus vaccine appeared to be highly immunogenic. Physical methods of virus inactivation like UV or gamma irradiation (5) are to be preferred above formaldehyde inactivation because they are clean and leave protein structure intact as is shown by the stability of the HA titer during inactivation (2). Formaldehyde destroys the HA activity and decreases the immunogenicity of the vaccine as is shown by the ip immunization experiments with Formalin-treated EMC virus described by Lawrence et al. (7).

From the survival data of our mouse immunization experiments, the effectiveness of the vaccine and route of administration was evaluated. The individual antibody titers allowed prediction of survival and gave insight in the development and persistence of antibodies and protection against a systemic lethal viral infection.

Intraperitoneal and respiratory immunization do not strongly differ in effectiveness as is seen from their ED_{50} values, although after respiratory immunization antibody titers are always lower than after ip immunization with inactivated EMC vaccine. After both ways of immunization a secondary treatment gives rise to about the same antibody titers, indicating that in both cases priming for a secondary response is comparable. The primary antibody response after ip immunization is enhanced by BPE, which is in accordance with the higher survival rates, but the challenge response is not affected. However, after respiratory immunization, BPE does not change the primary immune response, but it does enhance the challenge response especially in groups showing 100% survival of the challenge. This may be explained by the use of the peritoneal route for administering the challenge dose if BPE exerts its adjuvant activity only after ip immunization with EMC virus vaccine. This explanation will be tested by respiratory challenge and booster treatments after respiratory and ip immunization tests. Another explanation may be that after respiratory immunization BPE would exert its adjuvant activity mainly on the induction of immunological memory,

whereas after ip immunization it enhances primary antibody production.

The low value of the relative adjuvant effect (i.e., ED_{50} with plain vaccine, divided by the ED_{50} with vaccine and adjuvant) found after ip immunization with EMC virus (1.8 to 4.1) agrees fairly well with the value of 8.4, found after ip immunization of mice with tetanus toxoid (Gerbrandy, *manuscript in preparation*). However, the total absence of the adjuvant effect of BPE on respiratory immunization is remarkable, especially in comparison with its impressive influence on respiratory immunization with tetanus toxoid.

Protection against ip challenge with a high dose of virulent EMC virus is mainly due to circulating antibodies, as is shown by the passive immunization test. This is consistent with our data on individual antibody titers and survival in active immunization experiments. Even when a very low serum titer is found (3 to 4 HAIU), the individual responds with a high challenge titer (500 to 2,500 HAIU) and will survive a lethal challenge. In these experiments other defense mechanisms than humoral antibodies can only play a minor role.

Protection against respiratory infections is probably dependent not only on the occurrence of circulating antibodies, but also on local immunity. Therefore the protection against respiratory infections is now being investigated.

LITERATURE CITED

1. Akers, T. G., S. B. Bond, C. Papke, and W. R. Leif. 1966. Virulence and immunogenicity in mice of airborne encephalomyocarditis viruses and their infectious nucleic acids. J. Immunol. **97**:379–385.
2. Bogaerts, W. J. C., and B. J. Durville-van der Oord. 1972. Immunization of mice against encephalomyocarditis virus. I. Purification, concentration, and inactivation of EMC virus. Infect. Immunity **6**: 508–512.
3. Finney, D. J. 1947. Probit analysis. Cambridge University Press, Cambridge.
4. Friedman, R., and R. M. Maenza. 1968. Peroral infection and resistance to reinfection with encephalomyocarditis virus in the mouse. J. Infect. Dis. **118**:125–134.
5. Gruber, J. 1970. Purification, concentration, and inactivation of Venezuelan equine encephalitis virus. Appl. Microbiol. **20**:427–432.
6. Gruber, J. 1971. Immunogenicity of purified Venezuelan equine encephalitis virus inactivated by ionizing radiation. Infect. Immunity **3**:574–579.
7. Lawrence, T. G., and H. Collins. 1970. The effect of parenteral and oral immunization on encephalomyocarditis infection in mice. Proc. Soc. Exp. Biol. Med. **133**:1066–1069.
8. Nagel, J. 1971. Isolation from Bordetella pertussis of protective antigen free from toxic activity and histamine sensitizing factor. Nature (London) **214**:96–97.
9. Prato, C. M., and T. G. Akers. 1969. The immunologic response of the mouse to aerosols of an attenuated encephalomyocarditis virus strain. J. Immunol. **103**:79–86.