Studies on immune response and protective capacity of rabies vaccine in Indian dogs

R. JAYAKUMAR, P. RAMADASS and N. RAGHAVAN *

Summary: Mongrel dogs were inoculated once daily for seven days with 2 ml of a vaccine prepared from 5% suspension of sheep brain inactivated with propiolactone. Their humoral immune response was monitored by enzyme immunoassay at intervals during the year following vaccination. IgM was formed during the early immune response, which lasted for up to 6 weeks, but subsequently IgG became dominant. A local strain of rabies street virus was used for challenge infection by inoculation into the masseter muscle. All vaccinated dogs withstood challenge infection one year after vaccination, while all unvaccinated dogs died from rabies. The fluorescent antibody test, rapid rabies enzyme immunodiagnostic technique, indirect peroxidase technique and counter immunoelectrophoresis all proved to be highly specific and reliable. The correlation between immune response and protection is discussed.

KEYWORDS: Bioassay - Dog - Immune response - Rabies - Serological techniques - Vaccines.

INTRODUCTION

Rabies is prevalent in man and animals in all parts of India, and occurs throughout the year. A recent World Health Organisation report states that each year approximately three million people undergo rabies post-exposure treatment, using more than 35,000 litres of vaccine. Human deaths due to rabies in India are estimated to be 12,000-20,000 each year. The available information on human rabies in India indicates that stray dogs play a primary role in maintaining and spreading rabies among animals and man. In view of increasing costs of post-exposure treatment in man, the elimination of dog rabies has become even more important in developing countries.

The first betapropiolactone-inactivated rabies vaccine was prepared from sheep brain and suckling mouse brain by French research workers in 1968 in Algeria. They found that this type of vaccine was free from residual virulent virus and the encephalitogenic factor present in phenolised rabies vaccine prepared from adult sheep brain (8). Further modifications and improvements in the preparation of betapropiolactone-inactivated sheep brain vaccine were carried out by another group of French veterinary scientists at Nancy, who also improved the potency tests used...

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in the case of vaccines for domestic as well as wild animals. They confirmed that betapropiolactone-inactivated sheep brain vaccine was suitable for domesticated animals, especially in tropical countries (2, 4).

About 22 institutions are engaged in producing sheep brain rabies vaccines for treatment and prophylaxis in man and animals in India. These centres do not assess the potency of the sheep brain vaccine in dogs by challenge experiments. The best method of studying the protective value is to test the vaccine in the species in which it will eventually be used. To obtain satisfactory results, it is necessary to immunise the target species with the vaccine, followed by challenge infection with street virus from the same geographical region, using an adequate number of vaccinated and unvaccinated dogs (1, 5).

MATERIALS AND METHODS

Experimental dogs

Forty-five stray mongrel pups aged 3-6 months were used in this study. Only pups with no detectable rabies antibody were selected.

Vaccine

Rabies vaccine prepared from 5% sheep brain suspension, inactivated with betapropiolactone, was obtained from the Pasteur Institute of India, Coonoor, Tamil Nadu. The potency of the test vaccine was 1.40 by the National Institute of Health (NIH) test. Thirty dogs were selected at random, each of which was inoculated subcutaneously with 2 ml of the vaccine daily for seven days. Another 15 dogs were maintained as unvaccinated controls.

Serology

Serum samples from all vaccinated and unvaccinated dogs were collected at 0, 7, 14, 21 and 28 days after vaccination and subsequently once a month up to 12 months. Antibody assay was performed by means of enzyme immunoassay (EIA) kits (Pasteur Institute Production, Paris, France) using 96-well microplates according to the technique described elsewhere (3). Any value above the negative value from unvaccinated dogs was taken as positive.

Immunoglobulins IgG and IgM: separation by gel filtration

Sephadex G-200 superfine (Pharmacia, Sweden) was used with Tris-HCl-NaCl buffer, pH 8.0, 0.1M. Three peaks were obtained with dog serum on Sephadex G-200 gel filtration. The ascending half of the first peak and the second and third peak fractions were pooled separately and concentrated by using polyethylene glycol (Carbwax, Loba). Its purity was checked by immunoelectrophoresis against anti-dog immune serum prepared in rabbits (10). Usually, the ascending half of the first peak contained pure IgM devoid of IgG, while the ascending half of the second peak contained IgG without IgM.

The specific antirabies immunoglobulin classes IgG and IgM from vaccinated dogs were assayed by solid-phase enzyme-linked immunosorbent assay (ELISA) in 96-well
microplates coated with 100 µl of rabies vaccine prepared in human diploid-cell cultures and diluted 1:32 (Mérieux, France) per well (20). The method used to titrate the specific antirabies immunoglobulin classes is described elsewhere (11). Any value above the negative value from unvaccinated dogs was taken as positive.

**Challenge experiments**

The immunity of 18 randomly selected vaccinated dogs was assessed 12 months after vaccination by injecting into masseter muscle 1 ml of a suspension of local street rabies virus containing $10^{3.83}$ intracerebral MLD$_{50}$ per 0.03 ml. Five unvaccinated dogs were included as controls. The challenged dogs were observed for 60 days. Surviving dogs were killed after two months. Cerebrospinal fluid (CSF), brain and salivary glands were collected and the last two were examined for the presence of specific viral antigens. CSF was tested for specific antibodies by ELISA. The specificity, sensitivity and reliability of the above-mentioned tests were assessed and compared statistically according to the method described by Schwabe et al. (18).

**Demonstration of viral infectivity**

Five unvaccinated dogs were infected intracisternally with 0.2 ml of the street rabies virus suspension. They were then kept under observation to verify the behaviour of the street rabies virus in susceptible dogs. The infection was confirmed by the presence of specific antigen in the brain and salivary glands from dogs which died, as demonstrated by Negri body staining, fluorescent antibody technique (FAT) (9), indirect peroxidase technique (IPT) (13), rapid rabies enzyme immunodiagnosis (RREID) (15), and counterimmunoelectrophoresis test (CIEPT) (7, 16).

**RESULTS**

Antibody titration was carried out by using the Pasteur Institute rabies EIA kit. As specified by the manufacturer, every test was performed by using a known positive and a known negative dog serum sample for comparison with the optical density of the serum under test. The optical density was utilised in statistical analysis and interpretation of the test. The mean optical density (OD) values obtained with serum samples from vaccinated dogs at various times are presented in Figure 1. Antibody titres rose gradually from the first week to six months, and then declined gradually. Statistical analysis showed that there was no significant increase in ELISA OD values ($P < 0.01$) between one and four weeks after vaccination. Values for the first and second months after challenge were significantly higher than the pre-challenge values ($P > 0.01$).

The mean OD values of IgM and IgG antibody classes at different intervals are illustrated in Figure 2. The peak value of IgM was reached by the second month and thereafter it declined. In the case of IgG, the peak value was detected by the sixth month and thereafter steadily declined.

The infectivity of the challenge virus was demonstrated in susceptible unvaccinated dogs. All five dogs died from the infection (Table I). One year after vaccination, 18 vaccinated and five unvaccinated dogs were challenged with the street rabies virus. All 18 vaccinated dogs withstood the virulent challenge, whereas all five unvaccinated
**FIG. 1**

Mean optical density of ELISA antibody in vaccinated dogs at different postvaccinal periods (with standard error)

**FIG. 2**

Mean optical density of Sephadex G-200 fractions of dog serum immunoglobulins 19S and 7S by ELISA
dogs died from rabies. During the observation period all the vaccinated group were clinically normal. The development and course of rabies infection in control dogs are presented in Table II. The results of various diagnostic tests employed to detect the presence of the specific rabies virus antigens in the brain and salivary gland materials are presented in Table III. Their sensitivity, specificity and diagnosability

**TABLE I**

*Response of susceptible dogs to virus inoculation*

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Challenge virus</th>
<th>Dose and route</th>
<th>Incubation period</th>
<th>Course of the disease</th>
<th>Form of the disease</th>
<th>Confirmation of rabies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negri FAT IPT RREID CIEP</td>
</tr>
<tr>
<td>B₁</td>
<td>Street virus 10³,₈₃ MLD₅₀/0.03 ml</td>
<td>0.2 ml intra-cisternal</td>
<td>8 days</td>
<td>3 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>B₂</td>
<td>12 days</td>
<td>3 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₃</td>
<td>10 days</td>
<td>3 days</td>
<td>Furious</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₄</td>
<td>13 days</td>
<td>3 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₅</td>
<td>11 days</td>
<td>4 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FAT: Fluorescent antibody technique
IPT: Indirect peroxidase technique
RREID: Rapid rabies enzyme immunodiagnosis
CIEP: Counterimmunoelectrophoresis

**TABLE II**

*Development and course of rabies in unvaccinated dogs*

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Challenge virus</th>
<th>Dose and route</th>
<th>Incubation period</th>
<th>Course of the disease</th>
<th>Form of the disease</th>
<th>Confirmation of rabies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negri FAT IPT RREID CIEP</td>
</tr>
<tr>
<td>C₁</td>
<td>Street virus 10³,₈₃ MLD₅₀/0.03 ml</td>
<td>1.0 ml intra-masseter</td>
<td>16 days</td>
<td>2 days</td>
<td>Furious</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>C₂</td>
<td>34 days</td>
<td>4 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₃</td>
<td>18 days</td>
<td>3 days</td>
<td>Furious</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₄</td>
<td>33 days</td>
<td>4 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₅</td>
<td>30 days</td>
<td>3 days</td>
<td>Paralytic</td>
<td>+ + + + +</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FAT: Fluorescent antibody technique
IPT: Indirect peroxidase technique
RREID: Rapid rabies enzyme immunodiagnosis
CIEP: Counterimmunoelectrophoresis
Results of tests for virus-specific antigen in the brain of dogs

<table>
<thead>
<tr>
<th>Samples tested</th>
<th>Number of specimens tested</th>
<th>Direct FAT</th>
<th></th>
<th>RREID</th>
<th></th>
<th>IPT</th>
<th></th>
<th>CIEP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Street virus inoculated control dogs</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>15</td>
<td>100</td>
<td>15</td>
<td>100</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Field materials</td>
<td>13</td>
<td>13</td>
<td>100</td>
<td>13</td>
<td>100</td>
<td>11</td>
<td>84.61</td>
<td>7</td>
<td>53.85</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>28</td>
<td>100</td>
<td>28</td>
<td>100</td>
<td>26</td>
<td>92.85</td>
<td>22</td>
<td>78.57</td>
</tr>
<tr>
<td>Normal dog brains</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

FAT: Fluorescent antibody technique
RREID: Rapid rabies enzyme immunodiagnosis
IPT: Indirect peroxidase technique
CIEP: Counterimmunoelectrophoresis

were assessed. In case of samples from unvaccinated dogs challenged with virulent street virus, there was complete agreement between all four tests. In case of field samples, there was good correlation between direct FAT and RREID. The IPT and CIEP tests were comparatively less sensitive. Brain samples from vaccinated dogs challenged with virulent rabies virus gave consistent negative results with all the above tests. The sensitivity of the direct FAT and RREID was again higher than that of IPT and CIEP. The latter was the least sensitive. However, there was no difference in specificity and reliability of the above tests.

DISCUSSION

We immunised a batch of stray dogs with betapropiolactone-inactivated rabies vaccine (with some unvaccinated controls) to study their humoral immune response and patterns of immunoglobulins present in serum for a period of 12 months, which is the duration of immunity stated by the manufacturer. After 12 months, vaccinated and unvaccinated dogs were inoculated with a local street virulent rabies virus to assess the potency of the vaccine.

It is reported that interferon and cell-mediated immunity are important in protecting animals and man from rabies. However, there is universal agreement that virus neutralising antibody is the key to successful prophylaxis both before and after infection (12). Most published literature on rabies antibody assay pertains to cell culture vaccines and other types of vaccines used in countries other than India. There are no reports on the use of ELISA for antibody assay in dogs vaccinated with
inactivated vaccine prepared from sheep brain. Earlier reports indicated that the ELISA test results showed a good correlation with serum neutralisation titres in mice (19). The immune responses as revealed by ELISA in the present study seemed to be satisfactory.

In rabies infection, viraemia either does not occur or is not important in pathogenesis. It is presumably in tissue along the peripheral nerves that serum neutralising antibody exerts its protective effect, rather than within the circulation (14). IgM antibodies do not leave the circulation, whereas IgG goes into tissues. Therefore, IgG classes are the serum neutralising antibodies which are more effective and more important in the prophylaxis of rabies (21). In the present study, IgG persisted for up to 12 months in vaccinated animals, showing that the immune response offered by the present schedule of vaccination was satisfactory. The findings largely agree with those of earlier workers (17).

The challenge experiment was conducted according to guidelines of the World Health Organisation. All vaccinated dogs survived whereas all control dogs succumbed to rabies infection. The non-proliferation of rabies virus in vaccinated dogs explained the absence of specific viral antigens in the brain and salivary glands, and the absence of rabies neutralising antibodies from the cerebrospinal fluid. In the control dogs, rabies specific viral antigens were present in the brain and salivary gland tissues.

In planning experimental challenge procedures, two views require consideration. The California Department of Health Standards (6) maintains that a challenge dose should be sufficient to kill at least 50-100% of the control dogs, with a goal of 75% mortality as optimum. The theory behind this concept was that an excessive challenge inoculum might be expected to produce an anamnestic response in vaccinated animals, which would interfere with evaluation of the immunity produced by vaccination. According to the US Department of Agriculture (6), the lowest acceptable challenge dose should kill not less than 100% of the control dogs to indicate satisfactory virulence. In the present study there was 100% mortality among control dogs and thereby it was able to meet the minimum requirement of the US Department of Agriculture.

The challenge results of this study clearly showed that the present vaccine, normally used before exposure, is capable of protecting all vaccinated dogs for at least one year from the date of vaccination. However, it is necessary to carry out further experiments to find out the protective capacity of the vaccine in dogs inoculated after exposure. The infectivity of the challenge virus was ascertained by intracisternal inoculation with 1/5th of the peripheral challenge inoculum. This route of inoculation usually causes a paralytic form of the disease.

At present, the fluorescent antibody technique is the recommended method because of its high sensitivity. In the present study, there was complete agreement between all four tests used in experimentally infected dogs.

In conclusion, the authors suggest that further research be carried out with this vaccine in dogs for post-exposure treatment, and also with a reduction in the vaccination schedule from seven daily injections to a maximum of two injections on two different days. It will reduce the number of visits to the veterinary hospital, and will reduce the production cost, especially important for developing and underdeveloped countries.
ACKNOWLEDGEMENTS

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Résumé : Des chiens bâtards ont reçu l’inoculation quotidienne, pendant sept jours, de 2 ml d’un vaccin préparé à partir d’une suspension à 5 % de cerveau de mouton, inactivée par la propiolactone. Leur réponse immunitaire humorale a été suivie par des épreuves immuno-enzymatiques pratiquées à intervalles réguliers pendant l’année qui a suivi la vaccination. Les IgM se sont formées au cours de la réponse immunitaire précoce, qui a duré jusqu’à six semaines, mais par la suite les IgG sont devenues dominantes. Une souche locale du virus rabique urbain a été utilisée pour la contamination d’épreuve, effectuée par inoculation dans le muscle masséter. Tous les chiens vaccinés ont résisté à l’épreuve virulente un an après la vaccination, tandis que tous les chiens non vaccinés sont morts de rage. L’épreuve d’immunofluorescence, la technique d’immunodiagnostic rapide de la rage, la technique indirecte à la peroxydase et la contrainmuno-électrophorèse se sont toutes montrées hautement spécifiques et fiables. La corrélation entre réponse immunitaire et protection est discutée.


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ESTUDIOS SOBRE LA RESPUESTA INMUNITARIA Y EL PODER PROTECTOR DE LA VACUNA ANTIRRÁBICA EN PERROS INDIOS. – R. Jayakumar, P. Ramadass y N. Raghavan.

Resumen: Se vacunaron mestizos perros, inoculando cada día, durante 7 días, 2 ml de una vacuna preparada a partir de una suspensión al 5% de cerebro de carnero, inactivada por propiolactona. La respuesta inmunitaria humoral fue controlada por titulaciones inmuno-enzimáticas practicadas a intervalos regulares durante el año consecutivo a la vacunación. Los IgM se formaron durante la respuesta inmunitaria precoz, que duró hasta seis semanas, pero, después, los IgG resultaron dominantes. Se utilizó para la prueba virulente una cepa local del virus rábico urbano, inoculada en el músculo masetero. Todos los perros vacunados resistieron a la prueba virulenta un año después de la vacunación, mientras que los perros no vacunados murieron de rabia. La prueba de inmunofluorescencia, la técnica de inmunodiagnóstico rápido de la rabia, la técnica indirecta por peroxidasa y la contrainmunoelectroforesis resultaron altamente específicas y fiables. El artículo trata también de la correlación entre respuesta inmunitaria y protección.
REFERENCES


