Immunoglobulin (IgG) and (IgM) Antibody Responses to Rabies Vaccine

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SUMMARY

IgM and IgG neutralizing antibody responses were estimated in the sera of rabbits and mice immunized with different doses of Semple, duck embryo and cell culture rabies vaccines. IgM synthesis was prolonged in animals immunized with multiple doses of either Semple or duck embryo vaccines but not those immunized with cell culture vaccine.

Mice were passively protected by IgG antibody against subsequent challenge but not by IgM of equivalent neutralizing titre. Mice challenged when the antibody response was solely IgM were not protected if the transition to IgG synthesis was prevented by cyclophosphamide treatment.

INTRODUCTION

The antibody responses of man and animals to many bacterial and virus antigens are associated with the major immunoglobulin classes IgM and IgG. In primary immunization, neutralizing activity in serum is usually associated first with transient IgM synthesis and later with a more durable and quantitatively greater IgG response. By contrast, the response to secondary immunization is predominantly IgG. (Pike, 1967; Cho et al. 1972; Spiegelburg, 1974).

This pattern of immunoglobulin synthesis depends, however, upon the physicochemical nature of the antigen and the amount, route and timing of its administration. IgM responses may be prolonged in some cases by the continued presence of antigen. This occurs with polysaccharide complexes which are not metabolized and eliminated (Britten & Möller, 1968; Miranda, 1972). It also occurs with some virus antigens if the stimulus is maintained (Svehag & Mandel, 1963; Uhr & Finkelstein, 1963, 1967; Monath, 1971). These observations have particular significance in the post-exposure treatment of rabies.

Until quite recently, multiple doses of rabies vaccine were administered in attempts to ensure an immune response within the incubation period of the disease which in man is usually between 20 and 60 days (Hattwick & Gregg, 1975). This time-honoured concept was questioned by Rubin and co-workers (1971) who found that in subjects receiving repeated daily doses of duck embryo vaccine, transition to IgG synthesis was delayed and the IgM response was prolonged. Further evidence for this sequence of events was reported by Grandien & Espmark (1974). IgG antibodies enter the tissues but the diffusion of IgM is restricted and it largely remains in the intact circulation (Janeway et al. 1967; Spiegelburg, 1974; Mims, 1976). It was argued therefore that IgM antibody would be of limited value in rabies where neural rather than viraemic spread is important in pathogenesis (Baer, 1975; Murphy, 1977).
In this investigation IgM and IgG responses to several rabies vaccines were studied in rabbits and mice and the protective value of IgM and IgG neutralizing antibodies were compared in vivo by passive transfer and by selective immunosuppression.

METHODS

Viruses. Stocks of standard challenge virus (CVS), Flury low egg passage (LEP) and Pasteur (PV11) strains of rabies virus adapted to growth in cultures of BHK cells or passaged in animal brain were grown in their respective substrates and stored at $-160^\circ$C.

Vaccines. (i) Semple-type rabies vaccine prepared in rabbit brain was obtained from current stocks at the Lister Institute. Its potency value was > 6-0 estimated by the method of Habel (1973). (ii) Duck embryo vaccine (Eli Lilly & Co.) had a Habel potency of 4-0. (iii) Vaccines prepared in human diploid cells (Institute Merieux, Lyon) had antigenic values of 2-1 and 6-8 estimated by the N.I.H. test (Seligman, 1973). (iv) Experimental (BHK) vaccines were prepared from the Flury LEP strain of virus grown in BHK-21 cells and were obtained either through the courtesy of Miss J. Crick, A.V.R.I., Pirbright, Surrey, or made in our own laboratories. They were inactivated either with acetylethylhenimine or $\beta$-propiolactone and had Habel potencies of > 5-8.

Animals. New Zealand White rabbits and T.O. mice were used with initial weights of 1·5 to 2·0 kg and 11 to 14 g respectively.

Vaccination schedules. Groups of 2 to 4 rabbits were inoculated subcutaneously (s.c.) with 2·0 ml Semple vaccine: (a) daily for 14 days; (b) with six doses given on days 0, 3, 5, 7, 9 and 14; or (c) with two doses given on days 0 and 14. Similar groups of rabbits were inoculated s.c. with 14 daily doses of 0·5 ml of duck embryo vaccine (DEV). Further groups of rabbits were inoculated with human diploid cell strain (HDCS) vaccine given either as $4 \times 0.1$ ml intradermal (i.d.) doses on day 0 or as $4 \times 0.25$ ml intramuscular (i.m.) doses on days 0, 3, 7 and 14.

DEV and BHK vaccines were diluted 10-fold and 0.2 ml were given intraperitoneally (i.p.) to groups of 70 mice; the mice were inoculated daily for 14 days. Rabbits were bled before, during and after immunization on days 0, 3, 7, 10, 14, 21, 28. Groups of 10 mice were killed and bled at the same intervals. Each days bleedings from a group were pooled and tested for rabies neutralizing antibody.

Preparation of rabies IgM and IgG antibodies in mice. Mice are passively protected against subsequent rabies infection by approx. 4 International units (i.u.) of homologous antirabies IgG (Baer & Yager, 1977; G. S. Turner, unpublished data). To compare the protective value of antirabies IgM with that of IgG, attempts were made to prepare sera in which this amount of neutralizing activity was associated solely or predominantly with IgM class antibody.

Groups of mice were inoculated i.p. with 1 to 3, 0.2 ml doses of Semple, DEV or BHK vaccine. The vaccines were administered either alone or emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories) or with aluminium hydroxide ('Alhydrogel', Superphos, Denmark).

Other groups were inoculated with BHK or HDCS vaccine either during or after treatment with agents reported to enhance or prolong the IgM responses of mice to other antigens. (Jenkin et al. 1965; Sahier & Schwartz, 1965; Blinkoff, 1966; Rager-Zisman & Allison, 1973). These included 0·5 ml of Thorotrast (20% thorium oxide in 20% dextran); colchicine (BDH Ltd.) 2 mg/kg; 6-mercaptopurine (Sigma Chemical Corp.) 10 mg/kg daily, or cyclophosphamide monohydrate (Koch Light & Co.) 6 mg/20 g: all were administered i.p.
Further groups of mice were inoculated i.v. with 0.2 ml of either a BHK vaccine concentrated 20-fold or undiluted HDCS vaccine. Mice from all groups were killed and bled either 4 to 6, or 10 to 14 days after immunization. Antibody associated with IgM and IgG was assayed in their pooled sera.

**Antibody assays and 2-mercaptoethanol (2ME) treatment of sera.** Serum samples were diluted twofold and mixed with an equal volume of phosphate-buffered saline (PBS) or 2ME (0.2 M in PBS). Both samples were kept at room temperature for 18 h and then titrated for rabies antibody by the serum neutralization test in mice (Atanasiu, 1973). Residual 2ME was not removed; at the concentration used it neither affected the mice nor the challenge virus.

**Characterization of mouse antirabies IgM and IgG.** The separation of 7S and 19S antibodies from mouse sera by centrifuging through density gradients was tried but in our hands the globulins were more conveniently fractionated by gel filtration on Sephadex G200 columns (Killander, 1964; Williams & Chase, 1968). Column fractions were concentrated to the original serum volume with Centriflo membrane cones (Amicon Corp. Lexington, U.S.A.) and assayed for antibody. The globulins were identified from their elution patterns, by 2ME susceptibility and by precipitin tests with antisera to mouse IgM and IgG (Cappell Laboratories, Downington, U.S.A.). The identity of some samples of IgM was also verified in the analytical ultracentrifuge.

**Passive protection tests in mice.** Groups of 5 to 15 mice were inoculated intraperitoneally with 1 ml amounts of serum containing 0.5, 1.0 or 4 i.u. of neutralizing antibody identified as either IgM or IgG; sera were diluted when necessary with normal mouse serum. Control mice received similar amounts of normal mouse serum. Twenty-four hours after passive immunization all mice were challenged with 10 to 20 i.m. LD50 CVS virus.

**RESULTS**

**IgM and IgG responses**

Mercaptanol sensitive, rabies neutralizing antibody was detectable in rabbits 7 days after the inoculation of the first of 14 daily doses of Semple vaccine. Antibody titres increased for the next 14 days and although the proportion of 2ME sensitive antibody (IgM) declined sharply it nevertheless accounted for approximately 50% of the total antibody found in samples taken for the next 25 days (Fig. 1 a). The responses to 6 doses of Semple vaccine spread over the same immunization period were also detectable after 7 days. Although antibody titres were lower and declined more rapidly, a persistent IgM response was again observed (Fig. 1 b). When only two doses of Semple vaccine were inoculated on days 0 and 14, no antibody response was detected until the 14th day and was associated only with IgG (Fig. 1 c). The antibody response of rabbits to 14 daily doses of DEV was first detectable after 4 days and although the decay of the IgM component was more rapid than that observed after immunization with Semple vaccine, it remained detectable for 14 to 21 days after immunization began (Fig. 1 d).

By contrast rapid decay rates of the IgM response were observed in rabbits inoculated either intradermally with HDCS vaccine on day 0, or with 4 intramuscular doses given on days 0, 3, 7 and 14 (Fig. 1 e and f). Similar differences in immunoglobulin response to DEV and cell culture vaccines were also demonstrated in mice: IgM antibody was again detectable for 14 to 21 days in mice immunized with 14 daily doses of DEV but declined within 7 to 10 days in those immunized with a similar number of doses of BHK vaccine (Fig. 2 a and b).
Fig. 1. Antibody responses of rabbits inoculated s.c. with Semple vaccine: (a) 14 x 2 ml doses days 0 to 13; (b) 6 x 2 ml doses, days 0, 3, 5, 7, 9 and 14; or (c) 2 x 2 ml doses days 0 and 14; (d) D.E.V. 14 x 0.5 ml doses, days 0 to 13; (e) 4 x 0.1 ml doses i.d. HDCS vaccine day 0; (f) 4 x 0.25 ml doses HDCS vaccine i.m. days 0, 3, 7 and 14. O---O, Titre of untreated serum; •---•, titre of 2ME treated serum; △—△, % reduction in titre after 2ME treatment.
Immunoglobulin responses to rabies vaccine

Fig. 2. Antibody responses of mice: (a) to 14×0.2 ml i.p. doses of DEV days 0 to 13; (b) 14×0.2 ml i.p. doses of BHK vaccine days 0 to 13. ○--○, Titre of untreated serum; □--□, titre of 2ME treated serum; △--△, % reduction in titre after 2ME treatment.

Fig. 3. Neutralizing activity and mercaptanol sensitivity of globulins fractionated on Sephadex G200. Serum taken (a) 14 days and (b) 4 days after immunization with a single i.v. dose of BHK or HDCS vaccine. Samples under the peaks were pooled and concentrated to the original volume of serum. Neutralizing antibody: □, before; □, after 2ME treatment.

Production of IgM antibody and its comparison with IgG in passive immunization

Antibody associated with IgG was readily obtained from mice 10 to 14 days after immunization with cell-culture vaccine (Fig. 3 a). Attempts to increase the IgM response to rabies antigen by the adjuvants tested, or by pre-treatment of mice with thorotrast, colchicine or 6 mercaptopurine were unsuccessful. These agents either completely suppressed antibody responses or failed to influence the normal transition from minimal amounts of IgM to much higher titres of IgG.

No antibody was detected in the sera of mice treated with cyclophosphamide either before,
Fig. 4. Effect of time of cyclophosphamide treatment on antibody responses of mice to 1 ml BHK vaccine given i.p. on day 0. ● — ●, No cyclophosphamide. Cyclophosphamide 6 mg/20 g i.p.: ○ — ○, 3 days; △ — △, 4 days; □ — □, 5 days after vaccine.

Table 1. Effect of route of inoculation on early antibody response of mice to BHK rabies vaccine

<table>
<thead>
<tr>
<th>Route and dose</th>
<th>Antibody (i.u./ml) after 4 days</th>
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<tbody>
<tr>
<td>i.v. 0.2 ml</td>
<td>Whole serum 2ME-treated serum</td>
</tr>
<tr>
<td>i.p. 0.2 ml</td>
<td>2.2, 3.5, 5.6* 0.13, 0.2, 0.3</td>
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* Pooled bleedings from groups of 5 mice.

or 3 to 4 days after immunization with 1 ml of BHK vaccine i.p. When cyclophosphamide treatment was delayed until the 5th day after immunization mercaptanol-sensitive antibody was detectable 2 days later but declined to almost undetectable levels after 14 days. Normal responses were observed in control mice similarly immunized without cyclophosphamide treatment (Fig. 4).

Substantial antibody titres were observed in mice 4 days after i.v. inoculation of BHK or HDCS vaccine. More than 95% of this antibody was destroyed by 2ME treatment and was identified as IgM (Fig. 3b). By contrast, mice inoculated i.p. with similar doses of the same vaccine had barely detectable responses (Table 1).

Passively administered rabies antibody associated with IgG showed a dose-related increase in protection up to the expected median protective dose (PD₅₀) of 4.0 i.u. Equivalent amounts of IgM afforded no protection (Table 2).

IgM responses and immunosuppression with cyclophosphamide

The foregoing results showed that substantial amounts of IgM antibody appeared 4 days after an intravenous dose of vaccine (Table 1), and that further antibody synthesis was arrested by cyclophosphamide treatment (Fig. 4). It appeared possible therefore that confirmatory evidence of any protection by actively induced IgM might be obtained by challenging mice immediately after this early response and preventing the transition of antibody synthesis to IgG by administering cyclophosphamide at the same time.
Table 2. Passive protection tests with homologous antirabies IgM and IgG sera in mice

<table>
<thead>
<tr>
<th>Amount given</th>
<th>% survivors* treated with</th>
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<tr>
<td>i.u.</td>
<td>IgG</td>
</tr>
<tr>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>1.0</td>
<td>22</td>
</tr>
<tr>
<td>4.0</td>
<td>48</td>
</tr>
<tr>
<td>o†</td>
<td>0</td>
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* Groups of 5 to 15 mice inoculated i.p. with 1 ml amounts of sera – IgG was diluted in normal mouse serum.
† o, control mice given normal mouse serum only. All mice challenged with 10 to 20 i.m. LD₅₀ CVS virus 24 h after administering serum.

Fig. 5. Effect of cyclophosphamide on survival and further antibody synthesis of mice challenged i.m. 4 days after immunization with a single i.v. dose of BHK vaccine. (a) No cyclophosphamide; (b) unimmunized control mice; (c) cyclophosphamide (6 mg/20 g) at time of challenge; (d) cyclophosphamide at time of challenge and 3 days later. Times of challenge (Ch) and cyclophosphamide (Cy) administration are arrowed. □—□, Surviving mice (%); ■—■, control mice treated with cyclophosphamide; ○—○, neutralizing antibody (i.u./ml); ●—●, neutralizing antibody after 2ME treatment. Percentage survival was calculated from groups of 10 to 20 mice. Antibody was estimated in pooled sera of 3 to 5 mice. The challenge dose was 10 to 20 i.m. LD₅₀.
Ninety per cent of mice were protected against intramuscular challenge with CVS virus 4 days after immunization with a single intravenous dose of BHK vaccine. The sera of mice killed and bled at this time contained 3 to 5 i.u./ml of neutralizing antibody almost entirely associated with IgM. In unchallenged mice of the same group transition from IgM to IgG occurred between 7 and 10 days and mice that survived challenge for 21 days had antibody titres of 80 i.u./ml (Fig. 5a). Only 10% of unimmunized control mice survived challenge (Fig. 5b).

When mice of the same immunized group were challenged and treated with cyclophosphamide 4 days after i.v. immunization, 60% survived. In these cyclophosphamide-treated mice there was a decline in the titre of antibody which persisted as IgM and showed no transition to IgG for the next 10 days. However, detectable amounts of IgG antibody were found in the sera of mice surviving challenge (Fig. 5c).

In further groups of similarly immunized mice, cyclophosphamide was administered both at the time of challenge and again 3 days later. In these mice IgM antibody showed a rapid decline and no further antibody was detectable. Only 10% of mice survived, a rate no different from unimmunized control mice (Fig. 5d). These findings again show that although IgM antibody was present at the time of challenge it afforded no protection.

**DISCUSSION**

Previous reports of the classes of antirabies immunoglobulin produced by animals in response to live or inactivated virus have been largely confined to the dynamics of their formation and their serological reactivity *in vitro* (Fujisaki et al. 1968; Prochazka et al. 1972; Coe & Bell 1977). The present experiments in rabbits and mice confirm observations in man which showed that repeated doses of DEV prolonged the IgM response (Rubin et al. 1971). Even more persistent IgM responses occurred in animals immunized with either multidose or abbreviated courses of Semple vaccine. However, when animals were immunized with cell culture vaccines, neither the route of administration nor the frequency of the doses prolonged macroglobulin synthesis and rapid transition to IgG occurred. It is probable that persistent IgM responses to some rabies vaccines are related less to continued antigenic stimulation than to the purity and quantity of the antigen-factors known to influence immunoglobulin responses to other virus antigens (Svehag & Mandel, 1963; Uhr & Finkelstein, 1967).

There are relatively few reports of the type of immunoglobulin produced in human responses to the new cell-culture vaccines. Thraenhart & Kuwert (1977) reported that IgM-associated antibody was detectable at 3 to 7 days, and peak titres occurred 8 to 16 days after human subjects received the first of six doses of HDCS vaccine. Hauser and co-workers (1978) also reported that 46% of rabies antibody was present in the IgM fraction of the sera of 20 volunteers 14 days after beginning a similar course of HDCS vaccine; by 90 days, 83% of their antibody was associated with IgG.

IgM has more antigen-reactive sites than IgG and much serological evidence confirms its superior combining capacity with bacterial and cellular antigens. In those virus infections that cause viraemia, IgM no doubt has a major role. However, neither passively transferred nor actively induced antirabies IgM protected mice in the experiments reported here. These observations add validity to previous assumptions and are in agreement with current views of the pathogenesis of rabies. If IgM were produced early enough it is possible that it might neutralize any extracellular virus in contact with blood in bite wounds, it seems even more unlikely however, that any vaccine-induced early antibody would be of value systemically.
Immunoglobulin responses to rabies vaccine

The routine use of equine or human antirabies immunoglobulin has been recommended for many years (WHO 1966), but in spite of the dramatic demonstration of its value by Balthazard & co-workers (1955) the precise function of antibody administered or induced at or after the time of infection remains unclear. Recent reports suggest that passive immunization with antibody alone protects some animals but merely prolongs the incubation period in others (Baer & Cleary 1972). Others showed that animals with meagre antibody responses died earlier after challenge than unimmunized control animals (Sikes et al. 1971). There is also evidence suggesting that some antibodies are more concerned in the immunopathology of rabies rather than with protection (Tignor & Shope, 1972; Tignor et al. 1976). High titres of rabies antibody have also been demonstrated in subjects who nevertheless died of rabies (Rubin et al. 1970; Emmons et al. 1973; Maton et al. 1976).

Antibody responses may only be part of the host defence mechanism in which interferon and cell mediated immunity are involved. Whatever the role of antibody may be, the experiments reported here suggest that while IgG antibodies can significantly protect against rabies, early-appearing IgM class antibody is ineffective. These experiments also suggest that those vaccines inducing rapid IgG responses might be more effective.

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