

Use of Mouse Anti-Rabies Monoclonal Antibodies in Postexposure Treatment of Rabies

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Abstract

Immunization of mice and hamsters with a cocktail of mouse MAbs specific for rabies virus nucleocapsid protein and glycoprotein protected animals not only when challenged with a lethal dose of rabies virus after immunization, but also in post-exposure situations. Hamsters treated with the MAb cocktail 3 h after virus inoculation were completely protected from lethal rabies virus infection, and 80% of the animals survived when the MAb cocktail was given 36 h after virus challenge. The potential usefulness of this MAb cocktail for the postexposure treatment of human rabies is discussed.

Introduction

Rabies remains a significant human disease throughout the developing world; for example, ~ 500,000 persons (1) under antirabies treatment, and in India some 40,000–50,000 people are reported to die of rabies each year (2).

Effective postexposure treatment of rabies as recommended by the World Health Organization includes the prompt use of human or equine anti-rabies immunoglobulins (HRIG or ERIG, respectively),¹ together with the administration of rabies vaccine. Animal experiments have demonstrated that treatment with vaccine alone does not prevent lethal rabies virus infection in postexposure situations (3, 4). Anti-rabies antibodies appear to be an essential component in the treatment of rabies; however, the precise role of such antibodies in postexposure treatment is unclear. Possible mechanisms whereby rabies virus-neutralizing antibodies (VNA), solely directed against the rabies virus glycoprotein (G protein), exert their effect, include neutralization of extracellular rabies virus, complement-mediated lysis of rabies virus-infected cells, and antibody-dependent cellular cytotoxicity (5). In addition, other antibodies, including those directed against the internal nucleocapsid protein (RNP) of rabies virus, are capable of regulating the immune response of T lymphocytes (6). Anti-

bodies that recognize the G protein or RNP proteins were recently shown to play an important role in potentiating the antigen-induced proliferative response of rabies virus-specific T lymphocytes and their production of IFN- γ , shown to be involved in the protection against lethal rabies virus infection (7).

Because of the adverse effects ranging from local reactions at injection sites to anaphylaxis connected with the use of ERIG (8), only HRIG is ideally recommended for postexposure treatment of humans. However, because of the high cost of HRIG and the high incidence of human rabies exposures, there is often insufficient amounts of HRIG available to complete the full recommended postexposure treatment. Limited access to HRIG is probably a major contributing factor in the increasing number of postexposure treatment failures (9, 10). Additionally, safety aspects of human immune serum treatment must be considered, due to the potential risk of hepatitis B virus and human immunodeficiency virus contamination. In this paper, we describe the use of mouse MAbs in the postexposure treatment of rabies in mice and hamsters. It has been shown in humans that mouse MAbs, although being xenogenic, bear a low risk of severe allergic reactions (11). To obtain the identical or similar activities found in HRIG, several MAbs were selected to meet the following criteria: (a) the MAbs should be of IgG isotype, because IgM antibodies are not effective in vivo (12); and (b) the majority of these MAbs should recognize the G protein and neutralize a broad spectrum of rabies and rabies-related virus strains. These G-specific antibodies must differ in their epitope specificities to prevent the escape of neutralization-resistant variants. (c) Some MAbs should recognize the internal RNP protein of rabies virus. This cocktail, consisting of rabies G protein and RNP-specific mouse MAbs is highly effective in the postexposure treatment of rabies in infected mice and hamsters.

Methods

The challenge virus standard (CVS), Evelyn-Rokitnicki-Abelseth (ERA), and Pitman-Moore (PM) strains of fixed rabies virus as well as street rabies virus isolates and the rabies-related Duvenhage and Mokola viruses were propagated in BHK-21 cell culture monolayers as previously described (13).

MAbs. Hybridomas that secrete MAbs specific for rabies G protein and RNP were produced by the fusion of P3 \times 63 Ag8 or 654 myeloma cells with splenocytes of BALB/c mice immunized with several strains of rabies virus (14).

Purification of MAbs. MAbs were precipitated from ascites fluid with $(\text{NH}_4)_2\text{SO}_4$ as described (15). The precipitate was dissolved in PBS dialyzed against 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5, and subjected to HPLC ion exchange chromatography using a 15- μm column (Baker Bound Abx Prep; Baker Chemical Co., Philipsburg, NJ). Chromatography conditions were similar to those previously described (16): solvent A was 25 mM MES, pH 5.6, and solvent

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1. *Abbreviations used in this paper:* CVS, challenge virus standard; ERA, Evelyn-Rokitnicki-Abelseth; ERIG, equine anti-rabies immunoglobulin; GMT, geometric mean titer; G-protein, glycoprotein; HRIG, human anti-rabies immunoglobulin; PM, Pitman-Moore; RNP, ribonucleoprotein; VNA, virus neutralizing antibody.

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B was 1 M sodium acetate, pH 7.0. A gradient of 0–70% B was run for 60 min at a flow rate of 3 ml/min at ambient temperature. The elution of proteins was monitored by UV absorbance at 280 nm. Peak fractions containing the MAb were pooled, dialyzed against PBS, and concentrated by ultrafiltration.

Preparation of F(ab')₂. F(ab')₂ fragments were prepared from purified MAbs as described (17). 15 mg of MAb in 10 ml 0.1 M sodium acetate buffer, pH 4.5, were mixed with 0.15 mg of pepsin (Millipore Corp., Bedford, MA) and incubated at 37°C for 16 h. The digest was dialyzed against PBS and subjected to chromatography on a protein A-Sepharose CL-4 B column (Pharmacia Fine Chemicals, Piscataway, NJ). No residual intact IgG was detectable in the F(ab')₂ preparation when analyzed by SDS-PAGE.

Virus neutralization assay. A modification of the rapid fluorescent focus inhibition test was performed as previously described (18). A reduction of viral titer by > 100 infective units was considered as positive virus neutralization.

In vivo protection experiments in mice. 6-wk-old female white Swiss mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) in groups of 7 or 10 were injected intramuscularly into the right gastrocnemius muscle with 0.1 ml of one of five serial dilutions (100–0.16 IU) of mouse MAbs. At 24 h after MAb treatment, all mice were inoculated intramuscularly in the left gastrocnemius muscle with 10⁶ mouse intracerebral (MIC) LD₅₀ of CVS-24 virus. Animals were observed for 3 wk and deaths were reported daily. The effective dose (ED₅₀) of MAbs was calculated as described (19).

Postexposure treatment of hamsters with mouse MAbs. 4–6-wk-old Syrian hamsters (Harlan Sprague-Dawley, Inc.) were used for all experiments. Challenge virus consisted of a 20% salivary gland suspension of a street rabies virus isolate of fox origin; dilutions were made in distilled water containing 2% horse serum. For each trial, hamsters were divided into groups of five and received 100 µl of MAbs, as follows: trial 1, hamsters received 10^{5.1} MIC LD₅₀/ml of rabies virus in the left gastrocnemius muscle, and 3 or 36 h later, 1,550 IU/kg MAbs at the same site; trial 2, hamsters received 10^{4.6} MIC LD₅₀/ml of rabies virus in the left gastrocnemius muscle, and 2 h later, 155 IU/kg MAbs at the same site.

Animals were examined daily for definitive clinical signs of rabies infection and were euthanized when in extremis by CO₂ intoxication. Survivors were followed for at least 30 d after rabies virus inoculation, at which time they were similarly euthanized. Control hamsters received 100 µl of PBS.

Results

Selection of MAbs for pre- and postexposure prophylaxis of rabies. 5 MAbs were selected from a panel of 76 specific for rabies virus G protein and RNP on the basis of: isotype, antigen, and epitope specificity, virus strain specificity, affinity, and virus-neutralizing activity (Table I). Three of the five MAbs were specific for the G protein and the other two non-neutralizing MAbs were specific for RNP. Each of the three G protein-specific antibodies recognizes a different epitope on the G protein, and all three G protein-specific MAbs neutralize in vitro all fixed rabies virus strains and most street rabies virus isolates (20). In addition, MAb 1112-1 neutralized the rabies-related strain Duvenhage, whereas MAb 523-11 neutralized Mokola virus (18).

Despite the broad range in virus neutralizing activity, of the three G protein-specific MAbs, (1,667, 61, and 8,242 IU/mg for MAbs 509-6, 1112-1, and 523-11, respectively), the specific virus-neutralizing activity of each of these MAbs was superior to that of HRIG (1.13 IU/mg protein).

The RNP-specific MAbs 802-1 and 502-2 recognize different epitopes on the N protein but do not have neutralizing activity in vitro (20). The MAb 502-2 reacts with all known rabies and rabies-related virus strains (20), and MAb 802-1 recognizes most rabies strains (18).

When all five MAbs were used as a mixture, the specific virus-neutralizing activity of the cocktail was 1,376 IU/mg protein.

Protective activity of MAbs in mice. The prophylactic efficacy of individual MAbs was determined. Various dilutions of the MAbs were inoculated intramuscularly into mice, followed 24 h later by a lethal dose of the CVS-24 strain of rabies virus. Whereas pretreatment of mice with the RNP-specific MAbs had no effect on survivorship (data not shown), administration of the G protein-specific MAbs 24 h before challenge effectively prevented a lethal rabies virus infection (Table II). 2 IU of MAb 509-6 and 2.36 IU of MAb 523-11 were necessary to protect 50% of mice. Although the in vitro virus-neutralizing

Table I. Characteristics of MAbs Used in this Study

MAb No.	Isotype	Antigen and epitope specificity	Specific virus-neutralizing activity IU/mg	Virus strain specificity
509-6	γ2a	G site I	1,667	Neutralization of all fixed and street rabies virus strains except some virus isolates from bats
1112-1	γ1	G site IIc	61	Neutralization of all fixed and street rabies virus strains and the rabies-related Duvenhage strains 1–6
523-11	γ2a	G site IIb	8,242	Neutralization of all fixed and street rabies virus strains except some street virus isolates from bats and foxes, neutralization of rabies-related Mokola 3
802-1 g	γ2b	N site II	0	Binding to all fixed and street rabies virus strains and some rabies-related viruses
502-2	γ2a	N site III	0	Binding to all rabies and rabies-related viruses
MAb cocktail*		G, N	1,376	ND

* The MAb cocktail represents a mixture of 112.5 IU/ml MAb 509-6, 10 IU/ml of MAb 1112-1, 800 IU/ml MAb 523-11, 0.04 mg/ml MAb 802-1, and 0.6 mg/ml MAb 502-2

Table II. Passive Protection of ICR Mice with G Protein-specific MABs

MAB dose	Mortality*			
	MAB 1112-1	MAB 509-6	MAB 523-11	F(ab') ₂ 523-11
IU				
100	—	—	0/10	2/11
20	—	1/10	2/11	7/10
4	0/10	3/10	8/10	10/10
0.8	1/10	7/10	10/10	10/10
0.16	0/10	—	—	—
0.032	2/10	—	—	—
ED ₅₀	<0.03	2.0	2.36	34.00

* Mice were challenged intramuscularly with 5×10^6 MIC LD₅₀ of CVS 11 24 h after MAB treatment.

activity of MAB 1112-1 was low, its protective activity in vivo was extremely high; as little as 0.03 IU of this MAB were sufficient to prevent a lethal rabies virus infection in 50% of the animals. The basis of the discrepancy between in vivo and in vitro activity of MAB 1112-1 is not clear, but it is likely that the mechanisms by which MABs act in vivo are very complex and involve more than neutralization of extracellular virus. The ED₅₀ using F(ab')₂ fragments of MAB 523-11 was 34.0 IU compared with 2.36 IU for the intact MAB, suggesting the importance of the Fc-containing region for in vivo protection.

To determine whether the time period between MAB treatment and virus inoculation influenced the protective activity of the antibodies, mice were pretreated with the MAB cocktail at different times before introduction of rabies virus. The ED₅₀ of the MAB cocktail was 0.8 IU when administered 24 h before challenge, but increased to 2.36 when the MABs were given 2 h before challenge. Moreover, when the MAB cocktail was injected 2 h after inoculation, 42.8 IU were necessary to protect 50% of the mice (Table III).

Effect of site of virus challenge on MAB efficacy. Groups of mice were treated with dilutions of the MAB cocktail and 24 h later inoculated intracerebrally, or intrafootpad with a lethal dose of rabies virus. Treatment of mice with MABs had vir-

Table III. Passive Protection of ICR Mice with MAB Cocktail

MAB dose	Mortality		
	MAB cocktail 24 h before challenge	MAB cocktail 2 h before challenge	MAB cocktail 2 h after challenge
IU			
100.0	0/10	0/7	3/7
20.0	0/10	1/10	8/10
4.0	0/10	4/10	8/10
0.8	5/10	7/10	9/10
0.16	10/10	—	—
ED ₅₀	0.80	2.36	42.80

Mice were challenged intramuscularly with 5×10^6 MIC LD₅₀ of CVS 24.

Table IV. VNA Titers and Mortality Rates after MAB Treatment or Immunization with BPL-inactivated ERA Virus

MAB treatment*		Immunization with EPL-ERA†	
VNA titer‡ (GMT, n = 7)	Mortality	VNA titer‡ (GMT, n = 7)	Mortality
3,992 (2,430–7,290)	6/7	768 (90–3,125)	1/7

* Mice were treated with 200 IU of MAB cocktail and 24 h later, challenged intracerebrally with 5×10^4 MIC LD₅₀ of CVS 11.

† Mice were immunized with 2 µg of BPL-inactivated ERA virus on day 0 and 7 and challenged on day 14 intracerebrally with 5×10^4 MICLD₅₀ of CVS 11.

‡ Mice were bled 2 h before challenge.

tually no effect on mortality after intracerebral inoculation; even when 200 IU of MAB were administered 24 h before intracerebral inoculation only one of seven mice survived. The ineffectiveness of MAB treatment against an intracerebral virus inoculation is puzzling because mice that received 200 IU of MABs had a circulating VNA geometric mean titer (GMT) of 3,992 24 h after injection but were not protected, whereas mice immunized with β-propiolactone inactivated rabies virus had a circulating VNA GMT of only 767 but survived intracerebral inoculation (Table IV). When mice were inoculated intrafootpad, 4 IU of MAB cocktail administered 24 h before challenge were necessary to protect 50% of mice (not shown). Thus, the ED₅₀ of MABs against intrafootpad challenge is five times higher than that of the same MABs against intramuscular challenge.

Efficacy of MABs in the postexposure treatment of rabies in hamsters. Because most pathogenic rabies virus strains have a very short incubation time in mice (onset of clinical signs is 5–6 d after infection), it is difficult to perform postexposure treatment experiments in mice. The incubation period in hamsters is much longer (10–14 d), allowing the use of these animals as an alternative model for post-exposure treatment experiments. Five hamsters inoculated intramuscularly with rabies street virus were treated 2, 3, and 36 h after infection intramuscularly with 1,550 or 150 IU/kg of the MAB cocktail. The data in Table V indicate that all hamsters receiving MAB 2 or 3 h after challenge survived, whereas four of the five un-

Table V. Postexposure Rabies Treatment of Syrian Hamsters with Mouse MAB Cocktail

MAB dose	Time of treatment after virus challenge	Challenge virus dilution‡	Morbidity
IU/Kg	h		
1,550	3	1:200	0/5
1,550	36	1:200	1/5
150	2	1:1,000	0/5
0	—	1:200	4/5
0	—	1:1,000	4/5

* MAB were administered intramuscularly.

† Hamsters were challenged intramuscularly with a suspension of dog salivary gland infected with the NYC strain of rabies virus.

treated control animals succumbed to rabies infection. In hamsters treated with the MAb cocktail 36 h after infection, all but one of the five animals survived.

Discussion

Rabies virus-specific antibodies represent important factors in protective immunity against rabies. Rabies VNA have been considered the primary basis of effective protection against death in animals and humans from street rabies virus infection (3, 4). Therefore, antirabies serum as an adjunct to vaccination is an essential component in the prophylaxis of rabies after exposure. Because of the potential risks associated with the use of immune sera derived from animals and the high costs of HRIG, we have tested the usefulness of mouse anti-rabies MAbs. To obtain an activity spectrum comparable to that found in HRIG, we selected five MAbs, three of which recognize different epitopes on the rabies virus G protein and neutralize rabies and rabies-related virus strains in vitro. We also included two RNP-specific MAbs in our MAb cocktail because in addition to VNA, nonneutralizing antibodies elicited by the RNP contribute to the protective immune response against a rabies virus infection (7). These RNP-specific antibodies may act in part by promoting the attachment of viral RNP via Fc receptors to phagocytic cells, which are then stimulated by the infecting virus to produce monokines (e.g., IFN) that can inhibit viral replication (7).

To determine the therapeutic effect against a lethal rabies virus infection, we treated mice with individual MAbs and the MAb cocktail. The results of these protection experiments in mice revealed that the individual G protein-specific MAbs, as well as the MAb cocktail, are highly effective when administered before virus challenge. The individual antibodies differ significantly in their protective activity. Moreover, there is apparently no correlation between in vitro neutralizing activity and the protective activity in vivo, suggesting that mechanisms other than neutralization of extracellular virus are mediated by these antibodies. The observation that the protective activity of F(ab')₂ fragments is > 10 times lower than that of the complete antibody supports the assumption that the protection conferred by MAbs is the result of a complex interaction of various immune effector mechanisms (6). The inconsistent correlation between the efficiency of virus neutralization in vitro by MAbs and animal protection has also been observed with foot-and-mouth disease virus (21). It was speculated that opsonization and subsequent rapid phagocytosis play a major role in the antibody-mediated immune defense against foot-and-mouth disease virus (21).

Protection experiments in hamsters demonstrated that the MAb cocktail is also highly effective in postexposure situations. Treatment of hamsters with 1,550 or 150 IU/kg MAb cocktail resulted in complete protection from lethal rabies virus infection. Even when the MAbs were administered 36 h after infection, 80% of these animals survived, whereas 80% of the untreated control animals succumbed to rabies. These protection experiments suggest that a cocktail of several mouse MAbs may be of great use for the postexposure treatment of human rabies. Such a cocktail has several advantages over hyperimmune sera: (a) The specific virus neutralizing activity and protective activity is much higher than that of HRIG; (b) based on protein content, only relatively small quantities of MAbs have to be injected; (c) the use of MAbs may be superior

for local wound treatment, where only small volumes can be inoculated at the bite site.

The disadvantages of mouse MAbs for therapeutic use in humans are such possible side effects as anaphylactic reactions and hypersensitization. However, mouse MAbs have been used extensively in the immunotherapy of patients with gastrointestinal adenocarcinoma (11), pancreatic adenocarcinoma (22), and colorectal carcinoma (23), and no or only mild and reversible (11, 24) immediate or delayed side effects, signs of toxicity, or serum sickness after the application of a single dose of mouse MAbs were observed in nonimmunosuppressed patients (11, 24, 25). Most patients tolerated even the multiple applications well (26, 27). Thus, the number and severity of side effects caused by mouse MAbs can be assumed to be low, especially because only one dose of antibody is administered during postexposure treatment of rabies, and not for subsequent exposures. These data strongly suggest that murine anti-rabies MAbs should be given serious consideration as to their use in future rabies postexposure treatment.

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