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Immunology

Delineation of putative mechanisms involved in antibody-mediated clearance of rabies virus from the central nervous system

(virus neutralization/transcription)

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ABSTRACT **The *in vitro* biological activities of several rabies virus-neutralizing monoclonal antibodies (mAbs) were compared with their ability to prevent a lethal rabies virus encephalomyelitis. The protective activity of a particular mAb *in vivo* did not correlate with its virus-neutralizing activity *in vitro*; rather it was related to the mAb's ability to inhibit virus spread from cell to cell and to restrict rabies virus RNA transcription. Since treatment of rabies virus-infected cells with virus-neutralizing mAbs results in an endocytosis of the antibody, we hypothesize that an antibody may exert its inhibitory activity even after uptake by the cell. Post-exposure treatment of rats with a mAb that inhibited both virus spread and virus RNA transcription *in vitro* resulted in viral clearance from the central nervous system and protected the animals against a lethal rabies virus infection.**

Cell-mediated immune mechanisms, in particular cell lysis by class I-restricted T cells, are believed to play a major role in the clearance of viruses from infected tissues. It has also been suggested that cytotoxic T lymphocytes contribute to recovery from rabies virus infection (1–3). Although clearance of rabies virus from the central nervous system (CNS) after adoptive transfer of rabies virus-specific T cells has been reported (4, 5), the role of cell-mediated effects in the rabies virus-infected CNS remains obscure. Since rabies virus replicates almost exclusively in neurons (6), which do not express class I antigens (7), it is difficult to determine how rabies virus could be cleared from infected neurons by $CD8^+$ cells. Moreover, $CD4^+$ T cells do not appear to be directly involved in the immunoprotection against rabies. For example, it has been shown that a synthetic peptide comprising a dominant T-cell epitope of the rabies nucleoprotein (N protein) was highly effective in inducing rabies virus-specific class II-restricted T cells *in vivo* but did not confer protection against lethal challenge infection with rabies virus (8). However, mice immunized with peptides containing both T- and B-cell determinants developed rabies virus-specific neutralizing antibody and were protected against a lethal challenge infection (9), suggesting that neutralizing antibody plays a major role in immunoprotection against rabies. The particular function of an antibody as a protective immune effector was confirmed by postexposure treatment experiments of laboratory rodents with murine or human monoclonal antibodies (mAbs) directed against rabies virus glycoprotein (10, 11). The protective role of antibody against viral infection of the CNS was also demonstrated in a SCID (severe combined immunodeficiency) mouse model of persistent alphavirus encephalomyelitis, where adoptive transfer of hyperimmune serum resulted in clearance of infectious virus and viral RNA from the CNS (12). In addition, administration of immune serum and mAbs prevented fatal T-cell-mediated immunopathology of the CNS following infection by lymphocytic choriomeningitis virus (13). The mechanisms of antibody-mediated clearance of virus infection from the CNS are not clear; however, it has been shown for several viruses that the protective activity of antiviral antibodies *in vivo* does not correlate simply with the virus-neutralizing activity *in vitro* (10, 12, 13).

In this paper the *in vitro* biological activities of several rabies virus-specific mAbs are compared with their ability to clear rabies virus from the CNS and to prevent a lethal rabies encephalomyelitis. No simple relationship was found between the protective activity of a particular mAb *in vivo* and its virus-neutralizing activity *in vitro*. Instead, protective activity correlated with the ability to prevent virus spread from cell to cell and to inhibit transcription of viral RNA.

MATERIALS AND METHODS

Viruses. The fixed rabies challenge virus standard strain CVS-11 was propagated in BHK-21 cells (14). The CVS-24 challenge virus was prepared from suckling mouse brain (15).

mAbs. Hybridomas that secrete mAbs specific for rabies glycoprotein were produced by the fusion of P3X63Ag8 or -654 myeloma cells with splenocytes of BALB/c mice immunized with several strains of rabies virus (16).

Virus Neutralization Assay. Virus-neutralizing antibody titers of serum samples were tested with a modified version of the rapid fluorescent focus inhibiting test (RFFIT) (17). The reciprocal of the highest serum dilution resulting in a 50% reduction of infected foci was considered the neutralization titer of the serum sample. Titers were normalized to international units (IU) by using the National Institutes of Health reference serum (lot R-3) as the standard.

Rabies Virus Cell-Spread Inhibition Assay. Mouse neuroblastoma cells (NA, clone 1300) were cultured in 24-well plates. CVS-11 virus (200 μl) was added to the confluent monolayer at a multiplicity of infectivity (moi) of 0.1 and cells were incubated for 2 hr at 37°C. The virus inoculum was removed and culture medium (1 ml) containing various concentrations of mAb was added to each well. Seventy-two hours after infection, the cells were fixed in 80% acetone and the percentage of infected cells was determined by the direct fluorescent staining technique (17).

Determination of mAb Internalization by Rabies Virus-Infected NA Cells. NA cell monolayers grown on round coverslips in 24-well plates were infected with CVS-11 virus

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Abbreviations: mAb, monoclonal antibody; CNS, central nervous system; moi, multiplicity of infectivity; IU, international unit(s); $MICLD_{50}$, 50% mouse intracerebral lethal dose(s); RT–PCR, reverse transcriptase–polymerase chain reaction.

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at a moi of >1.0 and were incubated for 24 hr at 37°C. To adsorb the mAb, cells were incubated at 4°C with 1 ml of culture medium containing 5 IU of mAb 1112-1, 523-11, or 509-6 for 1 hr. The cells were then washed three times with cold (4°C) culture medium. To determine internalization of mAb, some of the cultures were replenished with 0.5 ml of warm (37°C) culture medium and incubated at 37°C for 2 hr. After incubation at 4°C or 37°C cells were fixed with cold (4°C) 50% acetone in absolute methanol for 5 min. To remove bound antibody from the surface of infected cells, some coverslips were treated with 0.25 M sodium acetate (pH 4.0) for 2 min before fixation. The adsorbed or internalized antibody was visualized by a fluorescent staining technique using a fluorescein-labeled goat anti-mouse antibody (Organon Teknika, West Chester, PA).

Rabies Virus RNA Transcription Inhibition Assay. Confluent NA cell monolayers in 3.5-cm tissue culture dishes were infected with CVS-11 virus at a moi of 1.0. One hour after infection the virus inoculum was removed and the cell cultures were replenished with 4 ml of culture medium containing mAb (5 IU/ml). At various times after infection, RNA was extracted from the cells. RNA extraction and Northern blot hybridization were performed as described (18). Total RNA was denatured with 10 mM sodium phosphate buffer, pH 7.4/50% (vol/vol) formamide at 65°C for 15 min and electrophoresed in a 1.2% agarose gel, containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer (pH 7.4). RNA was transferred and covalently fixed onto nylon membranes (Fisher). The rabies virus cDNA probe (19) was labeled with ^{32}P by using the multiprime DNA labeling system (Amersham) (specific activity, 10^9 cpm/mg). As a control, actin mRNA was hybridized with a ^{32}P-labeled probe for the actin gene (5'-GCTCCCCGGGCCGTCTTCCCCTCCA-3').

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR) and Southern Blot Analysis of Rabies Virus mRNA of N-Protein Gene in Rat Brain. RNA was extracted from rat brains as described (18). RT–PCR and Southern blot analysis were performed using the N-protein gene primers of rabies virus (20, 21).

Animals. *Mice. In vivo* experiments were carried out in 6- to 8-week-old female outbred ICR mice (Harlan–Sprague–Dawley). Five glycoprotein-specific mAbs (509-9, 194-2, 248-8, 523-11, 1112-1) at four concentrations (10, 2, 0.4, and 0.08 IU/0.1 ml) were administered to groups of 10 mice by inoculation of 0.1 ml into the gastrocnemius muscle. Twenty-four hours later, mice were inoculated intramuscularly with 0.1 ml containing 10^6 $MICLD_{50}$ (50% mouse intracerebral lethal doses) of CVS-24 virus. Animals were observed for 3 weeks after challenge; illness and deaths were recorded daily.

Rats. Six-week-old female Lewis rats (Harlan–Sprague–Dawley) were inoculated intranasally with 0.03 ml containing 2.4×10^5 $MICLD_{50}$ units of CVS-24 virus. One, 2, 4, 8, 12, and 24 hr after infection, groups of five rats were treated i.m. with 0.1 ml containing 30 IU of mAb 1112-1. Five rats were not treated with mAb and served as a control group. Animals were observed for 30 days after challenge; illness and deaths were recorded daily.

RESULTS

Comparison of *in Vitro* Neutralizing Activity and *in Vivo* Protective Ability of mAbs. Five rabies glycoprotein-specific mAbs, varying in isotype and antigenic site specificity, were used to compare the protective activity of serial antibody dilutions, when administered 24 hr prior to rabies virus inoculation of mice. Although the mAbs were calibrated to the same range of potency *in vitro* by their ability to neutralize rabies virus (IU/ml), there was no apparent effect on survivorship by the administration of any concentration of two mAbs, 194-2 (IgM) and 248-8 (IgA), compared with control mice, which succumbed to rabies infection within 21 days (Table 1). The effective dose (ED_{50}) of a third mAb, 509-6 (IgG2a), that was able to protect at least 50% of the mice from rabies was in excess of 10 IU/ml. In contrast, the ED_{50} of mAb 523-11 (IgG2a) was ≈2.0 IU/ml, whereas that of mAb 1112-1 (IgG1) was estimated at <0.08 IU/ml.

Table 1. Passive rabies protection of mice with rabies glycoprotein-specific mAbs

mAb, IU/ml	Survivorship by mAb treatment*				
	1112-1	523-11	509-6	194-2	248-8
10	10/10	8/10	3/10	0/10	0/10
2	10/10	6/10	1/10	0/10	1/10
0.4	9/10	0/10	2/10	1/10	1/10
0.08	8/10	1/10	0/10	0/10	0/10

mAbs were administered intramuscularly, and 24 hr later the mice were challenged intramuscularly with 10^6 $MICLD_{50}$ of the CVS-26 strain of rabies virus.
*For controls (no mAb), 0/8.

Inhibition of Rabies Virus Cellular Spread by mAbs. Thirteen rabies glycoprotein-specific mAbs, varying in isotype and viral epitope specificity, were compared for their ability to inhibit rabies virus spread in monolayers of NA cell cultures (Table 2). The concentration of mAb needed to inhibit virus spread from cell to cell varied considerably. For example, the amount of mAb that was necessary to protect 50% or more of cells from rabies virus infection was in excess of 10 IU/ml for four mAbs (231-2, 220-8, 240-3, and 194-2). The three latter mAbs were all members of the IgM class. The ED_{50} values of eight mAbs (509-6, 714-3, 101-2, 523-11, 162-3, 248-8, 507-1, and 1116-1) were ≥2.0 IU/ml. In contrast, the ED_{50} of mAb 1112-1 was ≤0.4 IU/ml; some virus spread inhibition was still observed at a concentration of 0.08 IU/ml.

Inhibition of Rabies Virus RNA Transcription by mAbs. To determine the effect of mAb on the transcription of viral RNA, NA cells were infected with a high moi (≥1 plaque-forming unit per cell) with rabies virus. Two hours after infection, five rabies glycoprotein-specific mAbs were added separately to the cells at a concentration of 5 IU/ml. Twenty-four hours after infection, total RNA was extracted and rabies N mRNA was determined by Northern blot analysis. Rabies virus RNA transcription was markedly inhibited only by mAb 1112-1 (Fig. 1*A*). The inhibitory effect of mAb 1112-1

Table 2. Virus-spread inhibition by mAbs directed against rabies glycoprotein

mAb	Isotype	Antigenic site	% infected cells			
			10	2	0.04	0.08
509-6	γ2a	I	29	80	100	100
719-3	γ2a	IIc	22	63	100	100
101-1	γ2a	IIb	38	85	100	100
523-11	γ2b	IIb	29	78	100	100
162-3	γ2b	IIb	20	45	95	100
231-2	γ2b	IIb	45	100	100	100
248-8	a	IIIa	23	95	100	100
507-1	γ1	IIIb	8	68	100	100
1116-1	γ1	IIb	21	73	100	100
1112-1	γ1	IIc	13	17	50	73
220-8	μ	IIa	100	100	100	100
240-3	μ	IIc	85	100	100	100
194-2	μ	IIa	65	100	100	100

Confluent monolayers of NA cells were infected with the CVS-11 strain of rabies virus at a moi of 0.1. Two hours after infection, cells were treated with mAb at 0.08, 0.04, 2, or 10 IU/ml, and 72 hr after infection the percentage of infected cells was determined by a direct fluorescent staining technique.

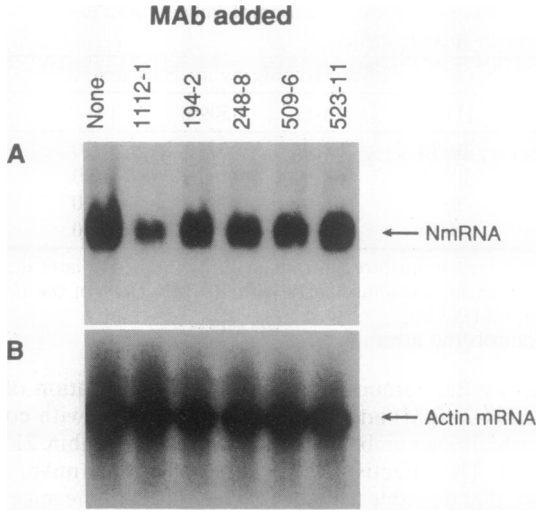

FIG. 1. Northern blot analysis of rabies N mRNA (*A*) and actin mRNA (*B*) isolated from rabies virus-infected NA cells. Two hours after infection, mAbs were added to the cells at a concentration of 5 IU/ml, and 24 hr after infection, total RNA was extracted.

on virus RNA transcription was most pronounced 18 hr after infection (Fig. 2). Furthermore, inhibition of virus RNA transcription was observed even when mAb 1112-1 was added as late as 4 hr after infection (Fig. 3). Northern blot analysis of actin mRNA revealed no difference between mAb-treated and untreated rabies virus-infected NA cells (Fig. 1*B*).

Uptake of mAb by Rabies-Infected NA Cells. To investigate the possibility that the inhibition of virus spread from cell to cell and the inhibition of virus RNA synthesis after treatment with mAb was due to an endocytosis of the antibody, infected and uninfected NA cells were treated with different mAbs at 4°C, or 4°C followed by 37°C; the antibody was then localized by indirect immunofluorescence. After incubation at 4°C, mAb 1112-1 was detected only on infected cells (Fig. 4*A*) but not on uninfected NA cells (data not shown); treatment of those cells with 0.25 M sodium acetate (pH 4) resulted in an almost complete removal of the antibody (Fig. 4*B*), indicating that the antibody was located on the cell surface. However, when the cells were shifted from 4°C to 37°C for 2 hr, the antibody could not be removed by the acid wash. This temperature-shift experiment indicated that the antibody was taken up by the infected NA cells. Some of the antibody was

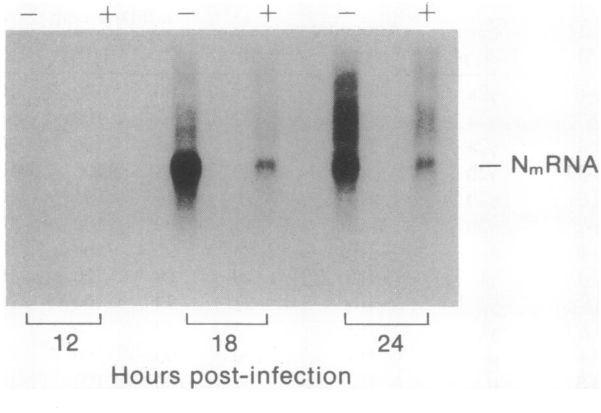

FIG. 2. Inhibition of rabies virus RNA transcription by mAb 1112-1. NA cells were infected with CVS-11 virus at a moi of >1. Two hours after infection the cells were treated with mAb 1112-1 (5 IU/ml). Total RNA was extracted from mAb-treated (+) and untreated (−) cells 12, 18, and 24 hr postinfection and rabies N mRNA was analyzed by Northern blot hybridization.

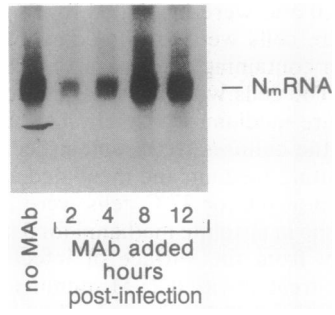

FIG. 3. Time dependence of antibody-mediated inhibition of rabies virus RNA transcription. NA cells were infected with CVS-11 virus at a moi of >1. Two, 4, 8, and 12 hr later, mAb 1112-1 was added to the cells at 5 IU/ml. Total RNA was extracted 24 hr postinfection and rabies N mRNA was analyzed by Northern blot hybridization.

found to be associated with intracellular vesicles (probably endosomes), while in other cells the antibody was uniformly distributed in the cytoplasm (Fig. 4*E*). Endocytosis of antibody by rabies virus-infected NA cells was also observed with other rabies glycoprotein-specific mAbs (e.g., 523-11, 509-6, and 194-2; data not shown). While cell surface staining of these antibodies showed similar intensity, intracellular staining revealed that the quantity of mAb 1112-1 was much higher, as evidenced by the intensity of fluorescence after acid treatment (data not shown).

Protection of Rats Against Lethal Rabies Infection. Following intranasal inoculation, rabies virus rapidly invaded brain tissues (Fig. 5). Analysis by RT–PCR revealed the presence of rabies virus-specific RNA in the olfactory bulb and cerebral cortex within 6 and 12 hr postinfection, respectively. However, treatment of animals with 30 IU of mAb 1112-1 from 1 to 24 hr postinfection resulted in a drastic decrease in mortality (Table 3). Even when the mAb was administered 24 hr later, 80% of the animals survived a challenge in which all control rats succumbed. Neither virus nor virus-specific RNA could be detected when survivors were euthanatized 30 days later.

DISCUSSION

The results of mouse protection experiments described herein demonstrate that administration of rabies glycoprotein-specific mAbs of the IgG class, but not of the IgM or IgA class, can protect mice against a subsequent challenge infection with rabies virus. However, as previously shown (10), the IgG class mAbs differ considerably in their protective activity *in vivo*; further, there is no correlation between the antibodies' virus-neutralizing activity *in vitro* and their protective activity *in vivo*. This discrepancy between *in vivo* and *in vitro* activities of particular mAbs is not unique for rabies but has been described for several other virus infections (12, 13). It was recently shown that antibody-mediated clearance of alphavirus infection is not related to a particular IgG subclass (12). Our results also indicate that mAbs of both IgG1 and IgG2a have protective activity. In contrast, protection against infection by lymphocytic choriomeningitis virus demonstrated that antibodies of the IgG2a subclass are essential in protection (22).

The discrepancy between *in vivo* and *in vitro* activities of rabies virus-neutralizing mAbs not only is an intriguing biological phenomenon but also has important practical implications for the potential use of mAbs in the postexposure prophylaxis of human rabies. Since the virus-neutralizing activity *in vitro* does not appear to be a reliable indicator for *in vivo* protection, it is necessary to delineate more relevant parameters that can be used for biological testing and standardization of therapeutic mAbs. Therefore, *in vitro* activi-

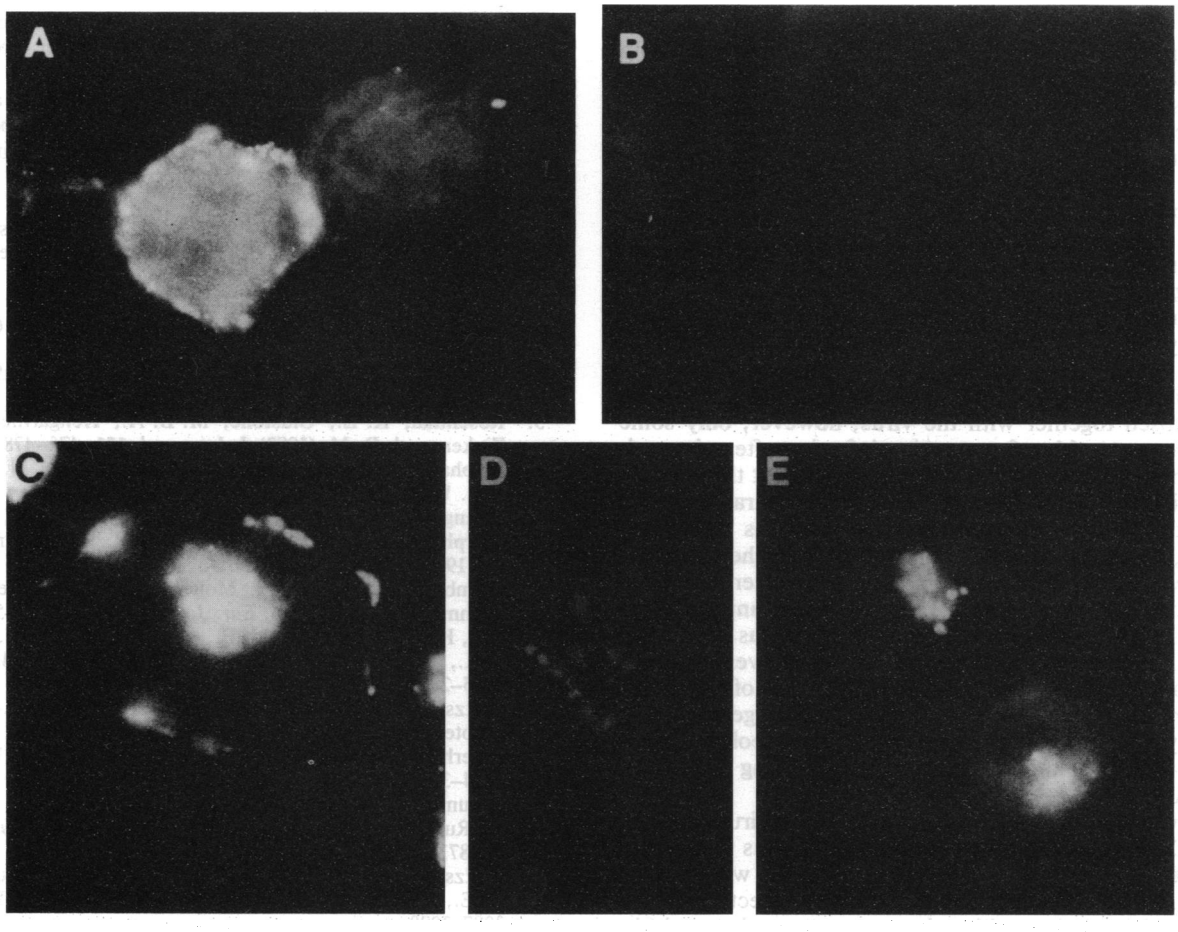

FIG. 4. Adsorption and internalization of mAb 1112-1 by rabies virus-infected NA cells. NA cells were infected with CVS-11 virus at a moi of 1. Twenty-four hours after infection the cells were incubated with mAb 1112-1 either for 1 hr at 4°C (*A* and *B*) or for 1 hr at 4°C followed by 2 hr at 37°C (*C–E*). To remove adsorbed antibody, cells that had been incubated with the mAb were treated with 0.25 M sodium acetate (pH 4) for 2 min (*B*, *D*, and *E*). Adsorbed or internalized mAb was detected by fluorescent staining techniques as described in *Materials and Methods*.

ties of virus-neutralizing mAbs were compared with the protective activity *in vivo*.

Rabies virus has the ability to spread in cell monolayer cultures from cell to cell in the presence of virus-neutralizing antibody (23); the potential effect of several virus-neutralizing mAbs on the rate of virus spread in an NA cell monolayer was determined. This experiment revealed that different mAbs vary considerably in their ability to inhibit virus spread from cell to cell. While most of the mAbs inhibited virus spread only at a relatively high virus-neutralizing activity (10 or 2 IU/ml), virus-spread inhibition by mAb 1112-1 was observed even at a concentration of 0.08 IU/ml. Thus, the high protective activity of mAb 1112-1 *in vivo* correlates with its ability to inhibit virus spread from cell to cell *in vitro*.

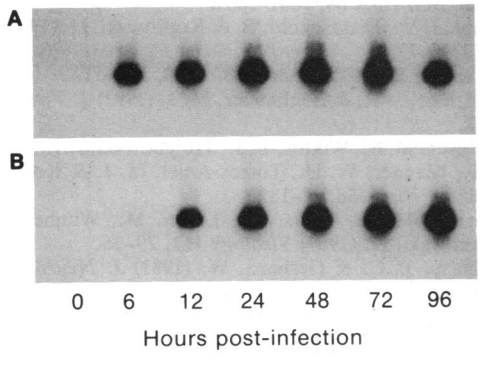

FIG. 5. Appearance of rabies virus RNA in the olfactory bulb (*A*) and midbrain (*B*) after intranasal inoculation of rabies virus. Rats were infected intranasally with 2.4×10^5 $MICLD_{50}$ of CVS-24 virus. At various times after infection, total RNA was extracted from olfactory bulb or midbrain and 1 μg of RNA was subjected to RT–PCR. The amplified cDNA was analyzed as described in *Materials and Methods*.

It was reported that antibody can mediate clearance of alphavirus infection from neurons by restricting viral gene expression (12). Similarly, treatment of NA cells with mAb 2 hr postinfection with rabies virus can influence the transcription of virus RNA. However, of the five mAbs used in this experiment, only mAb 1112-1 markedly inhibited rabies virus RNA transcription, suggesting that the high protective ac-

Table 3. Postexposure treatment of rats with mAb 1112-1 after intranasal infection with challenge virus strain CVS-24

Time of mAb treatment, hr postinfection	Survivorship* (day of death)	Virus neutralization titer,† IU/ml
1	4/5 (14)	45, 0, 0, 0
2	4/5 (15)	45, 30, 0, 0
4	3/5 (14, 16)	1215, 15, 15
9	5/5	1215, 135, 90, 15, 15
12	5/5	1215, 45, 0, 0, 0
24	4/5 (20)	810, 270, 135, 90

Rats were challenged intranasally with 2.4×10^5 $MICLD_{50}$ of the CVS-24 strain of rabies virus. mAbs were administered intramuscularly at indicated times after infection.
*For controls (no mAb), 0/5 (day 8).
†Thirty days postinfection.

tivity of this mAb is related to its ability to restrict rabies virus gene expression. Although rabies virus is internalized within some 20 min (23), inhibition of virus RNA transcription was still observed when the mAb was added as late as 4 hr postinfection, indicating that the antibody can still exert its inhibitory function after virions have already penetrated the cell.

One possible explanation for antibody-mediated inhibition of virus RNA transcription may be that antibody can exert its inhibitory activity after it is taken up by infected cells. Although the uptake of rabies glycoprotein-specific antibodies by rabies virus-infected cells is predictable, the fate of the antibody after endocytosis is largely unknown. It was previously shown that after treatment of rabies virus-infected cells with various antibodies, all neutralizing mAbs were endocytosed together with the virus; however, only some mAbs were capable of preventing infection after virus adsorption to the cells (24). It was concluded that these mAbs block virus infection by an inhibition of the intraendosomal acid-catalyzed fusion step that leads to virus uncoating. Clearly, several mechanisms are involved in the antibody-mediated inhibition of a virus infection; however, sufficient information is lacking that can fully explain the uniqueness of mAb 1112-1 in respect to its ability to inhibit virus spread and virus RNA transcription *in vitro* and to prevent a lethal encephalomyelitis *in vivo*. The higher intensity of intracellular fluorescent staining of mAb 1112-1 may suggest that this antibody possesses a greater resistance to proteolytic degradation, which could be one of the distinguishing features of this mAb.

To investigate whether antibody can inhibit virus infection in the CNS or clear virus from infected nervous tissue, rats were infected intranasally with rabies virus and were treated with mAb 1112-1 at various times after infection. After intranasal inoculation, rabies virus genome was detected 6 hr postinfection in the olfactory bulb and 12 hr postinfection in the midbrain. One hundred percent and 80% of animals that were treated with mAb at 12 and 24 hr postinfection, respectively, remained clinically normal with no signs of rabies virus infection, nor could viral RNA be detected in the brains of surviving animals 30 days later. In contrast, all untreated animals succumbed to rabies virus infection. This experiment demonstrates that antibody can mediate clearance of rabies virus from infected nervous tissue, thereby preventing death from a lethal rabies virus encephalomyelitis.

The mechanisms by which antibody clears rabies virus from the CNS remain largely unresolved. For example, it is still puzzling how antibody can pass the brain barrier to reach infected neurons. Possibly, rabies virus infection of the CNS may result in a breakdown of the blood/cerebrospinal fluid barrier, as has been described for mice that were infected intracerebrally with a neurotropic influenza virus (25). However, anti-rabies antibody was detected in the CNS only in late stages of the disease (data not shown), when antibody treatment is ineffective. No indication of neurolysis was found by microscopic examination of brains of rabies virus-infected rats that were treated with antibody (data not shown). This observation is consistent with findings by Levine *et al.* (12) which suggested that antibody-mediated clearance of alphavirus from the CNS occurs through a mechanism distinct from antibody-dependent cell-mediated cytotoxicity or complement-dependent lysis. It appears that antibody can abrogate virus infection even after CNS invasion, by inhibiting virus spread from cell to cell and by blocking virus RNA transcription and replication. In addition to antibody, other factors such as cytokines, neuropeptides, and neurotransmitters that are induced during virus infection may also contribute to the clearance process.

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