Antibody Protects against Lethal Infection with the Neurally Spreading Reovirus Type 3 (Dearing)

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The mammalian reoviruses have provided a valuable model for studying the pathogenesis of viral infections of the central nervous system (CNS). We have used this model to study the effect of antibody on disease produced by the neurally spreading reovirus type 3 (Dearing) (T3). Polyclonal and monoclonal antibodies protect mice from fatal infection with T3 after either footpad or intracerebral virus challenge. Protection occurs with monoclonal antibodies directed against the viral cell attachment protein sigma 1, and with polyclonal antisera without T3 sigma 1 binding activity. In vivo protection occurs with both neutralizing and nonneutralizing monoclonal antibodies. Antibody-mediated protection does not require serum complement and, under specific circumstances, can occur via Fc-independent mechanisms. Antibody can protect mice when transferred up to 5 days after intracerebral challenge and up to 7 days after footpad challenge, times when high titers of virus are present in the CNS. Thus, antibody mediated protection against this neurally spreading virus does not require neutralizing antibody or serum complement and occurs even in the face of established CNS infection.

Infection of neonatal mice with mammalian reoviruses has provided a useful model for studying viral pathogenesis. Some of the functions of the proteins encoded by individual viral genes at specific stages in reovirus pathogenesis have been identified (56, 61). For example, following footpad (FP) inoculation with reovirus type 3 Dearing (hereafter designated T3), virus replicates in skeletal muscle and then spreads via nerves to the central nervous system (CNS) (63; K. L. Tyler and B. N. Fields, unpublished data). The capacity of T3 to spread via nerves is determined by the viral S1 double-stranded RNA segment and occurs via the microtubule-associated pathway of fast axonal transport (63). Section of the nerve innervating the site of inoculation protects mice against FP challenge with T3 (63). This indicates that after inoculation via this route, neural spread of T3 is essential for lethal infection and that, in this setting, hematogenous spread does not contribute significantly to T3 pathogenesis. Once virus reaches the CNS, discrete populations of neural cells are infected, producing a lethal meningoencephalomyelitis (37, 62, 70). The viral S1 gene plays a major role in determining the cell and tissue tropism of reoviruses in the CNS (60, 71, 73), as well as the pattern of clearance of virus from the bloodstream in infected animals (64).

Despite the increasing body of knowledge that has accumulated concerning viral pathogenesis in general and reovirus pathogenesis in particular, little is known about the specific mechanisms by which individual components of the host immune response (for example, immunoglobulin) influence the outcome of defined stages in viral pathogenesis. In addition, while antibody is protective against infection with a variety of viruses, the importance of antibody directed

against specific viral determinants, the role of complement, and the role of the Fc portion of immunoglobulin are less clear. We chose to examine the effect of antibody on infection with T3 because the pathogenesis of infection with this virus following FP and intracerebral (IC) inoculation into neonatal mice is well understood. This system therefore provides an opportunity to define specific stages during infection with a neurotropic virus at which antibody can influence disease development.

In this article, we show that polyclonal and monoclonal antibodies (MAbs) protect neonatal mice against fatal infection with T3. This protection can be mediated by antibody directed against both the neutralization (NT) and hemagglutination-inhibition (HI) epitopes of the viral sigma 1 protein and by antibodies directed against virion proteins other than sigma 1. Protection can occur even after substantial amounts of virus are present in the CNS. Antibody-mediated protection does not require the presence of serum complement and, under certain circumstances, can occur without the participation of the Fc portion of immunoglobulin.

MATERIALS AND METHODS

Animal inoculations and determination of LD₅₀. Experiments were performed with 1-day-old NIH Swiss [NIH(s)] mice born of pregnant mothers obtained from the National Cancer Institute (Frederick, Md.). IC and FP inoculations were performed by injecting 10 µl of virus diluted in gel saline (137 mM NaCl, 0.2 mM CaCl₂, 0.8 mM MgCl₂, 19 mM H₂BO₃, 0.1 mM Na₂B₄O₇, 0.3% [wt/vol] gelatin [Fisher, Medford, Mass.]) into either the right cerebral hemisphere (60) or the left hind limb FP (63). Antibodies were diluted in pyrogen-free normal saline (Travenol, Deerfield, Ill.) and injected intraperitoneally (i.p.) in 100-µl volumes. Unless otherwise noted, mice received 200 µg of purified antibodies. A 30-gauge needle and either a Hamilton or a tuberculin syringe were used for all injections. Cobra venom factor

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(CVF) was injected either undiluted (25- μ l volume) or diluted 1:1 with phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4) in 50- μ l volumes. For mixing experiments, 50 μ l of virus stock plus an equal volume of either PBS or antibody in PBS was injected (total, 100 μ l).

The 50% lethal dose (LD_{50}) of T3 was determined by injecting litters of 1-day-old mice, containing at least 7 animals per litter, with serial dilutions of virus and monitoring mortality for at least 30 days. The LD_{50} was calculated by the method of Reed and Muench (52).

Virus and virus purification. Reovirus T3 and reovirus type 1 Lang (T1) were from laboratory stocks. The T3 $\times$ T1 reassortants designated 3HA1 and 1HA3 have been previously described (73). 3HA1 contains nine gene segments derived from T3 and the S1 gene segment of T1. 1HA3 contains nine gene segments derived from T1 and the S1 gene segment of T3. Viruses were doubly plaque purified and then passaged twice on the L929 mouse fibroblast cell line (L cells). Second-passage (P2) virus was freeze-thawed (-70°C and 37°C) three times and titered before use in *in vivo* experiments. Purified virus was used for radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunization of rabbits, and determination of the T3 FP LD_{50} ($10^{9.3}$ PFU per mouse dose only).

Purification followed published procedures (19) with minor modifications. L cells were suspended at 10^7 cells per ml in the Joklik modification of Eagle minimal essential medium (MEM) containing 5% fetal calf serum (FCS) (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine, 1 U of penicillin per ml, and 1 μ g of streptomycin (Irvine Scientific, Santa Ana, Calif.) per ml (completed MEM). P2 virus was added to obtain a multiplicity of infection of 5 to 10 and allowed to attach to cells at 37°C for 1 h. The infected cells were diluted to 5×10^5 cells per ml in completed MEM and incubated in spinner culture for 72 h at 37°C . Virus was released from infected cells by sonication and Freon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) extraction. The aqueous phase from the Freon extraction was layered onto a preformed cesium chloride gradient (density, 1.25 to 1.45 g/cm^3), and centrifuged overnight, using a SW28.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 26,000 rpm. The virion band was harvested and dialyzed extensively against buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM MgCl_2), and then stored at 4°C . Virus concentration was estimated on the basis of the optical density measurements at 260 nm (57).

Virus assay. All tissue samples were collected into 1.0 ml of gel saline and stored at -70°C prior to assay. Brain samples were collected by extruding the brain through the foramen magnum. Samples were freeze-thawed (-70°C and 37°C) three times prior to sonication for 15 to 30 s, using a microtip probe at ca. 50% maximal setting (W225R Cell Disruptor; Heat Systems-Ultrasonics Inc., Plainview, N.Y.). Virus was quantitated by plaque assay as previously described (60). Briefly, monolayers of L cells were infected in duplicate with serial 10-fold dilutions of sample. After viral attachment (37°C , 1 h), cells were overlaid with a 50%–50% (vol/vol) mixture of completed 2 $\times$ medium 199 (medium 199 Earle salts [Irvine Scientific, Santa Ana, Calif.], 2.5% FCS, 4 mM L-glutamine, 2 U of penicillin per ml, 2 μ g of streptomycin per ml) and 2% Bacto-Agar (Difco Laboratories, Detroit, Mich.) dissolved in deionized distilled water. Monolayers were overlaid a second time at 3 days after inoculation. A final overlay with medium 199 containing 1% agar and 0.04% neutral red (Fisher Scientific Co., Pittsburgh,

Pa.) was performed 6 days after inoculation. Plaques were counted 12 to 24 h after the neutral red overlay. The viral titers are reported as plaque-forming units per milliliter of original sample.

Production and purification of antibodies. Antisera were produced in rabbits screened for the absence of anti-reovirus NT antibody. Rabbits were immunized with 5 mg of purified reovirus in complete Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.), and then rechallenged with 5 mg of purified T1 or T3 in incomplete Freund adjuvant 3 and 6 weeks later. Animals were bled prior to the second immunization (first bleed) or 1 to 2 weeks after the second and third immunization (second and third bleeds). Sera were collected, heat fixed (56°C , 30 min), diluted with an equal volume of PBS, and then frozen at -20°C . This serum is referred to as anti-T3 or anti-T1 serum. Third-bleed serum was used in protection experiments. Ascites containing MABs (C1 and F7) was generated in pristane-primed BALB/c mice (National Cancer Institute, Frederick, Md.). Ascites was collected, clarified, heat fixed at 56°C , and frozen at -70°C . Cell lines producing MABs to the T3 sigma 1 protein (G5 [7]) and the T1 sigma 1 protein (5C6; S. P. Lynn and B. N. Fields, unpublished data) were grown to confluence in roller bottles containing McCoy medium supplemented with 5% FCS, 2 mM L-glutamine, 1 U of penicillin per ml, and 1 μ g of streptomycin per ml. Cells were removed by sedimentation, and 0.2% (wt/vol) sodium azide was added. Affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) was used to purify antibodies as previously described (67). Culture supernatant or serum was filtered and loaded on a protein A-Sepharose column preeluted with 0.2 M glycine (pH 2.5). Culture supernatant (3 to 4 liters) was loaded per 5 ml of protein A-Sepharose, and the column was washed with PBS. Immunoglobulin G (IgG) was eluted, using 0.2 M glycine (pH 2.5) by collection into one-fifth of the fraction volume of 1.0 M Tris buffer (pH 8.0). IgG was dialyzed against PBS, filter sterilized (0.22- μ m-pore-size filter; Millex-GV; Millipore Co., Bedford, Mass.), and frozen at -70°C . Antibodies were not refrozen after thawing. F(ab')_2 T3 IgG was prepared by dialyzing protein A-purified T3 IgG (18 mg) extensively against 50 mM NaCl–120 mM acetate (pH 4.0) and then adding 3 mg of porcine stomach pepsin (Worthington Diagnostics, Freehold, N.J.) per 100 mg of IgG. The IgG-pepsin mixture was filtered through a 0.22- μ m-pore-size filter and incubated at 37°C for 16 h. The digested IgG was dialyzed against PBS and then loaded on a protein A column, with a measured capacity of 36 mg of intact IgG, to remove any undigested IgG. After further dialysis against PBS, the F(ab')_2 T3 IgG was filter sterilized and stored at -70°C .

Virus NT assay. For NT assays, 4,000 PFU of T3 in 0.5 ml of gel saline was mixed with an equal volume of antibody diluted in gel saline. Virus plus antibody and control samples were incubated at 37°C for 1 h. The titers of duplicate 100- μ l samples were then determined. NT titers are reported as the highest dilution of serum, or the lowest concentration of IgG, which reduced the number of plaques detected by $\geq 80\%$ compared with virus plus gel saline (Table 1). For final determination of the NT titer of the anti-T1 and anti-T3 antisera, serial twofold dilutions were made over the range between 1/3,000 and 1/96,000. The NT titer of G5 IgG was determined by using serial twofold dilutions beginning at 10 μ g/ml, and those of both T3 IgG and its F(ab')_2 fragment were determined by using serial threefold dilutions beginning at 300 μ g/ml. The NT activity of anti-T3 serum was removed

TABLE 1. Anti-T3 plaque reduction NT titers

Antibody	NT titer ^a	Concn (μ g/ml) ^b
NRS	<1:5	
Anti-T3 serum	1:12,000	
Anti-T1 serum	1:12,000	
C1 ascites	<1:10	
NRS IgG		>1,000
T3 IgG		1-10
F(ab') ₂ T3 IgG		<1
5C6 IgG		>1,000
G5 IgG		<0.03

^a Expressed as the highest dilution of the test antisera required to neutralize at least 80% of viral plaques.

^b Expressed as the lowest concentration of the test antisera required to neutralize at least 80% of viral plaques.

by passage over a protein A-Sepharose column (data not shown).

RIA. A two-layer solid-phase RIA was used to assess specificity of antibody preparations (65). Purified virus in PBS with 0.02% (wt/vol) NaN_3 (PBS-A) (50 μ l at 50 μ g/ml = ca. 5.7×10^{11} particles per ml [57]) was added to 96-well U-bottom flexible plates (MicroTest III, Falcon, Becton Dickinson Labware, Oxnard, Calif.). Plates were incubated at 4°C overnight, washed with PBS-A, and then incubated for several hours at room temperature with 100 μ l of PBS-A per well, containing 10 mg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) (RIA-block) per ml. Fifty microliters of serial 0.5 $\log_{10}$ dilutions of antibody (starting at 30 μ g/ml) or sera in RIA-block were then plated in duplicate on virus-coated or control wells. After incubation at 4°C overnight, plates were washed with PBS-A and the second-layer antibody was added in RIA-block. For assay of mouse anti-reovirus MAbs, the second antibody was ^{125}I -rabbit anti-mouse immunoglobulin F(ab')₂ fragment (Pel-Freez, Rogers, Ark.). For assay of rabbit anti-reovirus antibodies, ^{125}I -mouse anti-rabbit immunoglobulin heavy and light chains (Accurate Chemical, Westbury, N.Y.) was used. After several hours at room temperature, plates were washed in PBS-A and individual wells were counted in a gamma counter. Reagents were iodinated by using chloramine T (23). One hundred microliters of protein (1.0 mg/ml) was incubated with 2.0 mCi of sodium ^{125}I (Amersham Corp., Arlington Heights, Ill.) and 30 μ l of freshly prepared chloramine T (5 mg/ml) (Sigma Chemical Co., St. Louis, Mo.). After 15 min on ice, the reaction was stopped by adding 30 μ l of freshly prepared $\text{Na}_2\text{S}_2\text{O}_5$ (Sigma Chemical Co., St. Louis, Mo.). Unbound iodine was separated from labeled protein on a G-25 column (Pharmacia Diagnostics, Piscataway, N.J.), and the labeled reagent was stored in RIA-block at 4°C.

The amount of antibody present in serum at various times after passive transfer of T3 IgG or F(ab')₂ T3 IgG was determined by RIA. Serum from neonatal mice injected with either T3 IgG or F(ab')₂ T3 IgG was collected by adding 1 drop of blood (20 to 50 μ l) to 200 μ l of PBS and allowing the blood to clot. Samples were spun in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and frozen at -20°C prior to assay by RIA.

Immunoprecipitations. L cells (10^7 in 10 ml) were plated overnight in completed MEM in tissue culture dishes (100 mm by 20 mm; Falcon Labware, Becton Dickinson and Co., Lincoln Park, N.J.). Each dish was infected with 2×10^8 PFU of virus in 2 ml of gel saline. Virus was allowed to adsorb to cells for 1 h at 37°C, and then dishes were

incubated for 12 h at 37°C with 5% CO_2 in 10 ml of completed MEM. Infected monolayers were washed in methionine and serum-free MEM and incubated for 15 min with 1.0 mCi of [^{35}S]methionine in 10 ml of methionine and serum-free MEM. After 15 min, 0.2 ml of FCS was added and plates were then incubated at 37°C for an additional 30 min. Monolayers were washed in completed MEM, and adherent cells were scraped off, pelleted, and then suspended in 2 ml of buffer [0.25 M NaCl, 5 mM MgCl_2 , 10 mM Tris (pH 7.4), and 0.5% (wt/vol) Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) detergent] per plate. After 30 min on ice, nuclei were pelleted and the supernatant fluid containing labeled cytoplasmic proteins was centrifuged at $100,000 \times g$ for 3 h.

Labeled cell lysates were cleared by adding 50 μ l of preimmune rabbit serum and 150 μ l of mouse anti-rabbit immunoglobulin heavy and light chains (1.0 mg/ml) to 1.0 ml of lysate. After overnight incubation at 4°C, the lysate was spun in a Microfuge for 10 min and the supernatant was decanted for use in specific immunoprecipitations. Cleared lysate and immune serum (100 μ l each) were mixed and then incubated at 4°C for 4 h; 20 μ l of mouse anti-rabbit immunoglobulin heavy and light chains (1.0 mg/ml) was added, and this mixture was incubated at 4°C overnight and then pelleted for 10 min in a Microfuge. Pellets were then washed five times in cold PBS, suspended in 100 μ l of water, and mixed with 100 μ l of 2 $\times$ Laemmli sample buffer (125 mM Tris hydrochloride [pH 6.8], 4% sodium dodecyl sulfate [wt/vol], 10% 2-mercaptoethanol, 20% glycerol, 0.002% bromophenol blue [wt/vol]) prior to electrophoresis on 10% polyacrylamide gels. A sample of 1.8×10^4 cpm was run in each lane of the gel. Gels were fixed, impregnated with Enlightening (Dupont, NEN Research Products, Boston, Mass.), dried, and autoradiographed. Purified [^{35}S]methionine-labeled virus, kindly provided by Tricia Lucia-Jandris, was run as a standard to identify viral bands.

ELISA. A capture ELISA using G5 IgG to attach expressed T3 sigma 1 protein to plates was used to assess the sigma 1 antibody activity of immune sera. The construction of the S1 expression vector and the method for expressing T3 sigma 1 have been previously described (49). Flat-bottom Immulon 2 plates (96 wells each; Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 10 μ g of purified G5 IgG or 5C6 IgG per well in 0.1 M Na_2CO_3 (pH 9.6) (coating buffer) at 4°C overnight. Plates were blocked overnight at 4°C with coating buffer containing 3% bovine serum albumin. Plates were washed once with 0.15 M NaCl plus 0.05% Tween 20 (ELISA wash), coated (30 min, 37°C) with 50 μ g of *Escherichia coli* JM105 bacterial lysate with or without expressed T3 sigma 1 per well, and then washed three times with ELISA wash. Bacteria were lysed with 3 mg of lysozyme per ml in 50 mM Tris hydrochloride-5 mM EDTA (pH 7.5), and lysate from bacteria containing the pKK233-2 vector alone was used as a control. Plates coated with G5 IgG, but not 5C6 IgG, bound sigma 1 in lysate from bacteria containing the pKK233-2 expression vector with the S1 cDNA insert (pKKRV3S1). Serial 10-fold dilutions (100 μ l, 1/10 to 1/100,000) of normal rabbit serum (NRS), anti-T3 serum, or anti-T1 serum in ELISA buffer (0.15 M NaCl, 0.001 M EDTA, 0.05 M Tris hydrochloride, 0.05% Tween 20) were then added to wells of sigma 1-coated or control plates. Plates were incubated (37°C, 30 min), washed three times with ELISA wash, and then overlaid with 100 μ l of a 1/400 dilution (46.5 μ g/ml) of peroxidase-conjugated anti-rabbit IgG (Organon Teknica, West Chester, Pa.). Plates were incubated (37°C, 30 min) prior to five washes with ELISA wash and the addition of 100 μ l of substrate solution

[10 ml of 0.05 M citric acid (pH 4.0) containing 40 μ l of 2% H_2O_2 and 40 μ l of a 27.5-mg/ml solution of 2,2'-azinobis(3-ethylbenz-thiazoline sulfonic acid) per well. Plates were incubated (room temperature, 10 min), and the reaction was stopped by adding 100 μ l of 0.2 M H_3PO_4 per well. The optical density at 570 nm was then determined by using an ELISA reader.

Decomplementation of mice and assay of C3. Mice were decomplemented by using CVF (*Naja haje* [Egyptian] cobra, Diamedix Corporation, Miami, Fla.). CVF was suspended, frozen at -70°C , and used immediately after thawing. CVF was inactivated by heating (80°C , 1 h). Decomplementation was assessed by measuring C3 in plasma collected from neonatal mice by adding a single drop (20 to 50 μ l) of blood to 200 μ l of PBS containing 10 mM EDTA. After being spun in a Microfuge, plasma was frozen at -20°C prior to assay for C3. The amount of C3 was measured in a single-layer solid-phase RIA as previously described (66). Goat anti-mouse C3 (Organon Teknika, West Chester, Pa.) was iodinated as described above. The amount of protein in each sample was equalized by using the optical density at 280 nm. Samples were serially diluted in PBS (0.5 $\log_{10}$ steps), and 50 μ l was added in duplicate to flexible 96-well plates. Plates were incubated (overnight at 4°C or 2 h at room temperature) and washed with PBS-A, and then 100 μ l of RIA-block was added per well. After 1 h at room temperature, plates were washed in PBS-A and 50 μ l of ^{125}I -goat anti-mouse C3 was added per well. Plates were incubated at room temperature for 1 h and washed in PBS-A, and then individual wells were counted. All RIAs included normal adult and neonatal plasma as positive controls and CVF-treated adult and neonatal plasma as negative controls.

The specificity of the RIA for C3 was assessed using Western blot (immunoblot) analysis of proteins in normal and CVF-treated plasma. Samples of normal adult and neonatal plasma and of adult and neonatal plasma from mice treated 24 h before with 2.5 U of CVF were electrophoresed in 7.5% polyacrylamide gels under reducing conditions (20 mM iodoacetamide). Purified mouse C3, kindly provided by Winnie Wong (Department of Medicine, Brigham & Women's Hospital and Harvard Medical School), was used as a standard. Protein was transferred to nitrocellulose paper and then probed with ^{125}I -goat anti-mouse C3 and autoradiographed.

RESULTS

Antibody protects mice from lethal infection with reovirus T3. We first identified a challenge dose of virus that was invariably lethal in untreated mice. The IC LD_{50} for T3 in 1- to 2-day-old CD-1 mice is 10^1 PFU per mouse (28, 58). Both the inoculation technique and the strain of mice [NIH(s)] used in this study differ from those used previously. We therefore reexamined the mortality and mean day of death of 1-day-old NIH(s) mice injected IC with T3. The IC LD_{50} of T3 was 10^1 PFU per mouse, and essentially 100% of mice injected with inocula greater than or equal to 10^2 PFU per mouse died. Mice inoculated IC with 10^1 PFU of T3 died after a mean of 11 days. The mean day of death was earlier with higher IC inocula, reaching a mean of 7 days at the highest dose tested (10^5 PFU per mouse).

We used 139 mice to determine the lethality of T3 following FP inoculation. An average of 14 mice were inoculated at each of 10 different doses of T3 ranging from $10^{2.9}$ to $10^{9.3}$ PFU per mouse (Fig. 1). By the method of Reed and Muench (52), the FP LD_{50} of T3 was calculated to be $10^{4.8}$ PFU per

FIG. 1. Lethality of T3 following FP inoculation. One-day-old NIH(s) mice were challenged with the indicated doses of T3 in the hind limb FP. Mice living for at least 30 days were counted as survivors. ●, Actual percent mortality at each viral dose. The line is a curve through the mortality values calculated by the method of Reed and Muench (52).

mouse. The mean day of death declined as the viral inoculum increased, falling from 15 ± 3.5 days (at $10^{4.2}$ PFU per mouse) to 10.4 ± 0.5 days (at $10^{9.3}$ PFU per mouse).

To ensure that survival of antibody-treated mice was due to antibody-mediated protection, we used $100\times LD_{50}$ in protection experiments (either 10^3 PFU per mouse IC or $10^{6.8}$ PFU per mouse FP; Fig. 1). These doses typically produce death in $>90\%$ of untreated animals at a mean of 10 days after IC and 12 to 13 days after FP challenge.

We used RIA, ELISA, NT assays, and immunoprecipitation to characterize antibody used in protection experiments

FIG. 2. Anti-T3 binding activity of polyclonal antireovirus antisera. Antibody activity was assessed by RIA with purified T3 as antigen. ^{125}I -mouse anti-rabbit immunoglobulin (MARG) was used as the second antibody. Inset shows the results of immunoprecipitation of [^{35}S]methionine-labeled T3 viral proteins by NRS (lane A), anti-T3 serum (lane B), and anti-T1 serum (lane C). Lane D contains [^{35}S]methionine-labeled purified T3. The location of the lambda, mu, and sigma proteins are noted. The arrow indicates the location of the sigma 1 protein.

FIG. 3. Specificity of the G5 anti-T3 sigma 1 MAb. The antigenic specificity of G5 IgG was assessed by RIA. Purified T3, T1, the T1 $\times$ T3 reassortants 3HA1 and 1HA3, and PBS containing 10 mg of bovine serum albumin per ml were used as antigens. Iodinated F(ab')₂ rabbit anti-mouse immunoglobulin (RAMG) was used as the second antibody.

(Fig. 2 and 3 and Table 1). Anti-T3 serum, T3 IgG, and anti-T1 serum all have significant anti-T3 antibody activity (Fig. 2) and high plaque reduction NT titers (Table 1). In contrast NRS, NRS IgG, and preimmune serum have no significant anti-T3 antibody activity (Fig. 2) or NT capacity (Table 1). Anti-T3 serum showed significant binding to purified T1, indicating that there are many shared epitopes between T1 and T3 (data not shown).

The extent of cross-reactivity between anti-T1 and anti-T3 serum was confirmed by immunoprecipitation which showed precipitation of most T3 proteins by anti-T1 serum (inset, Fig. 2). Anti-T1 serum did not precipitate the T3 sigma 1 protein even when undiluted serum was used (inset, Fig. 2). We confirmed this result by using a capture ELISA in which expressed T3 sigma 1 protein was bound to ELISA plates coated with G5 IgG (data not shown). The binding of NRS and anti-T1 serum was identical and did not vary between T3 sigma 1-coated and control plates. In contrast, anti-T3 serum showed significantly greater binding to T3 sigma 1-coated plates than to control plates. There was no significant binding of anti-T1 serum to the T3 sigma 1 protein under conditions which detected binding by anti-T3 serum at a 1/100,000 dilution. Thus, anti-T1 serum contains, at most, 0.001% of the anti-T3 sigma 1 activity of anti-T3 serum.

Since our rabbit anti-T1 serum does have T3 NT activity (Table 1), the results of the immunoprecipitation and ELISA studies confirm that there are antibodies directed against proteins other than sigma 1 that are capable of neutralizing virus (26, 72). We used third-bleed sera from rabbits immunized with large amounts of purified virus in complete Freund adjuvant. This may result in an increased amount of heterologous (non-type-specific) NT activity.

The antigenic specificity of G5 IgG for the T3 sigma 1 protein was assessed by RIA using purified T3, T1, 1HA3, and 3HA1 as antigens (Fig. 3). G5 IgG showed >300-fold more binding to virus bearing the T3 sigma 1 protein (T3 and 1HA3) than to virus bearing the T1 sigma 1 protein (T1 and 3HA1). Similarly, 5C6 shows binding to the T1 sigma 1 protein but not to the T3 sigma 1 protein (data not shown; S. P. Lynn and B. N. Fields, unpublished). G5 IgG had significant NT activity (7; Table 1), whereas 5C6 IgG had no

detectable anti-T3 NT activity (Table 1). Ascites containing the C1 monoclonal antibody did not have significant NT activity (7; Table 1).

We next assessed the ability of antibody passively transferred i.p. to protect mice against T3 infection via the FP route (Fig. 4). Thirteen percent (9 of 68) of mice receiving normal saline and six percent (1 of 18) of mice receiving NRS survived T3 infection. Anti-T3 serum protected 100% of mice (10 of 10) from lethal FP challenge with T3 (Fig. 4). Similar results were obtained after direct IC challenge with T3. Two percent (1 of 54) of mice receiving either normal saline or NRS survived IC challenge, while ninety percent of mice (9 of 10) receiving anti-T3 serum were protected.

We wished to determine whether the protective effect of immune serum was due to specific IgG. We therefore tested

FIG. 4. Survival of T3-challenged mice after passive transfer of antibody. Litters of 1-day-old NIH(s) mice were inoculated IC or in the hind limb FP with 100 $\times$ LD₅₀ of T3. At 24 h later, mice received antibody i.p. Mice living for at least 30 days after infection were counted as survivors. Fractions represent the number of surviving mice divided by the number of injected mice.

FIG. 5. Protective capacity of the non-NT anti-T3 sigma 1 MAb C1. Litters of 1-day-old NIH(s) mice were inoculated IC or in the hind limb FP with $100\times$ LD₅₀ of T3. Undiluted ascitic fluid containing the C1 MAb was given i.p. 24 h after virus challenge. Mice were monitored daily for deaths.

the capacity of protein A-purified T3 IgG to protect mice against T3 infection. Ninety-one percent (10 of 11) of mice receiving T3 IgG after IC viral challenge and seventy-eight percent (29 of 37) of mice receiving T3 IgG after FP challenge survived (Fig. 4). In contrast, only 6% (1 of 17) mice challenged with T3 IC and 13% (1 of 8) of mice challenged with T3 in the FP survived after passive transfer of NRS IgG. Non-IgG components of anti-T3 serum were not protective (data not shown).

These results clearly indicate that polyclonal anti-T3 serum can protect mice against lethal challenge with T3 and that this protection is mediated by IgG.

Epitopes of T3 against which protective antibodies are directed. We next sought to identify the viral determinants against which our protective antibody was directed. The S1 gene, which encodes the outer capsid protein sigma 1 and the nonstructural protein sigma 1 ns, determines the CNS tropism and route of spread of reovirus in the infected host (60, 63, 71, 73). We therefore began by testing the capacity of an affinity-purified NT MAb against the T3 sigma 1 protein (G5) to protect mice from challenge with T3. Ninety-four percent (46 of 49) of mice receiving G5 IgG were protected from either FP or IC challenge with T3 (Fig. 4). Only 11% (2 of 18) of mice receiving an anti-T1 sigma 1-neutralizing MAb (5C6) survived T3 challenge. Thus, antibody directed against the NT epitope of the T3 sigma 1 protein protects against lethal infection with T3.

To determine whether antibodies directed against epitopes other than the NT epitope of sigma 1 could protect mice against T3 infection, we tested the protective capacity of a non-NT, hemagglutination-inhibiting (HI), anti-sigma 1 MAb (C1) and of an antibody with low NT and high HI activity (F7) (7). One hundred percent of mice (18 of 18) receiving C1 at 24 h after IC challenge and eighty-nine percent of mice (8 of 9) receiving C1 after FP challenge with T3 survived (Fig. 5). One hundred percent (34 of 34) of mice receiving F7 within 48 h of either FP or IC challenge also survived. Control ascites was not protective under the same conditions (data not shown). These results indicate that anti-sigma 1 antibodies directed against either the NT epitope (G5), the HI epitope (C1), or both sites (F7) are capable of mediating protection against T3 infection.

We next sought to determine whether antibody directed against proteins other than sigma 1 could be protective. Our RIA, ELISA, and immunoprecipitation studies indicate that

FIG. 6. Relationship between the dose of antibody transferred and survival of T3-challenged mice. Litters of 1-day-old NIH(s) mice were challenged either in the hind limb FP or IC with $100\times$ LD₅₀ of T3. At 24 h later, mice received the indicated dose of G5 IgG i.p. Mice living for at least 30 days after viral inoculation were counted as survivors.

anti-T1 serum has significant amounts of cross-reacting antibody against many T3 virion proteins but no detectable antibody against the T3 sigma 1 protein (Results; Fig. 2 inset, column C). We therefore used this antiserum to test whether antibody binding T3 proteins other than sigma 1 could protect mice. One hundred percent of mice (9 of 9) challenged with T3 in the FP and ninety-four percent of mice (17 of 18) challenged IC survived after receiving anti-T1 serum (Fig. 4). These results show that antibody directed against T3 viral proteins other than sigma 1 are capable of protecting mice against lethal challenge with T3.

More antibody is required for protection from IC than FP challenge with T3. We injected mice IP with various amounts of either G5 IgG or anti-T3 serum at 24 h after FP or IC challenge with T3. Decreasing the amount of antibody administered resulted in a progressive increase in mortality after FP or IC challenge. The data shown in Fig. 6 indicates that a dose of approximately 5 μ g of G5 IgG per mouse will protect 50% of mice from FP challenge with T3. The dose of G5 IgG required to protect 50% of mice from IC challenge with T3 was approximately 37 μ g per mouse (Fig. 6). A dose of 200 μ g per mouse protected >90% of mice against either FP or IC challenge with T3. Thus, nearly eight times more antibody is required to protect mice against IC than against FP challenge with T3.

Serum complement is not required for G5 function in vivo. We used CVF to achieve prolonged depletion of serum C3 in neonatal mice. CVF forms a stable C3 convertase which degrades C3 and depletes the terminal complement components (10, 29, 43, 68, 69). We found that a single i.p. injection of 2.5 U of CVF removed 99% of C3 antigen from the serum of neonatal mice for at least 24 h as measured by RIA (data not shown). When mice were given six daily injections of 2.5 U of CVF, no intact C3 alpha chain was observed on Western blots of pooled plasma samples at any time point (data not shown). Heat-inactivated CVF did not deplete complement.

We next assessed the ability of G5 IgG to protect deplemented mice from infection with T3. Mice received six

FIG. 7. Effect of antibody on titers of T3 in brain in the presence or absence of serum complement. One-day-old NIH(s) mice were inoculated in the FP with $100 \times \text{LD}_{50}$ of T3. Antibody +, Mice received G5 IgG IP 24 h later; antibody -, mice received IP saline. Serum complement +, Mice received daily IP injections of either heat-inactivated CVF or no injections. Serum complement -, mice received daily IP injections of CVF. At 7 days postinfection, the titers of virus in brains were determined. Titters are shown as the mean $\log_{10}$ plaque-forming units per milliliter plus standard deviation.

daily injections of either CVF (complement depleted) or heat-inactivated CVF (complement sufficient) starting on the day of birth. One-day-old mice were challenged via the FP with virus and passively transferred with antibody 24 h later. On day 7, the titers of the virus in brains were determined (Fig. 7). We assessed protection by measuring brain titers of virus, rather than by survival, because of the difficulty in maintaining mice complement deficient for the time period required for protection studies. In the absence of antibody treatment, T3 titers in the brain exceeded 10^7 PFU. Decomplementation did not significantly alter the level of T3 infection. Antibody, in either the presence or absence of serum complement, reduced brain titers by approximately 100,000-fold. Thus, complement depletion did not affect the ability of G5 IgG to decrease virus titer in brain tissue after FP challenge with T3.

F(ab')₂ T3 IgG can inactivate T3 in vivo. To assess the role of the Fc portion of IgG in antibody-mediated protection against T3, we tested the protective capacity of F(ab')₂ fragments of T3 IgG. While both T3 IgG and F(ab')₂ T3 IgG are active in NT assays (Table 1), a single dose (60 μg per mouse) of F(ab')₂ did not protect mice from death after FP infection with T3 (9 of 9 mice died). An equal dose of T3 IgG protected 67% (6 of 9) of mice. However, when we measured the amount of antibody present in the serum of mice after IP injections of intact T3 IgG and F(ab')₂ T3 IgG by RIA, we found that the half-life of F(ab')₂ was less than 24 h, whereas that of intact T3 was >3 days (data not shown). Even when daily injections of F(ab')₂ were given to neonatal mice, there was no protection against T3 infection and no effect on viral titers in CNS, and levels of F(ab')₂ T3 IgG were significantly lower than antibody levels after a single dose of intact IgG (data not shown). Thus, the failure of F(ab')₂ to protect mice may have resulted from inadequate antibody concentrations in the serum or CNS.

FIG. 8. Relationship between viral titer in the brain at different times after FP infection and the capacity of antibody to protect. One-day-old NIH(s) mice were inoculated in the hind limb FP with $100 \times \text{LD}_{50}$ of T3. At the indicated times after infection, mice received either G5 IgG or polyclonal anti-T3 serum and their subsequent percent survival was ascertained. In parallel experiments, the titers of T3 in the brains of untreated mice were determined at various times after infection. The bar graph shows the titer plus standard deviation of T3 in the brain at the indicated times after infection.

While the short half-life of F(ab')₂ T3 IgG prevented assessment of the role of Fc under our usual experimental conditions, we still wished to assess the importance of an intact Fc in vivo. We therefore studied whether F(ab')₂ T3 IgG bound to T3 in vitro was functional in vivo by mixing virus and antibody prior to infection. Equal volumes of T3 viral stock (5×10^7 to 5×10^8 PFU/ml) and either T3 IgG or F(ab')₂ T3 IgG (600 $\mu\text{g}/\text{ml}$) were mixed in vitro. After incubation at 37°C for 60 min, mixtures were inoculated (0.1 ml) i.p. into neonatal mice. One hundred percent of mice receiving the T3-T3 IgG mixture survived (19 of 19), while eighty percent of mice receiving the T3-F(ab')₂ T3 IgG mixture survived (16 of 20). In contrast, only 9% of mice receiving T3-saline lived. Thus, when F(ab')₂ T3 IgG is allowed to combine with virus in vitro, it is capable of preventing lethal infection after i.p. inoculation.

Antibody protects against T3 infection even after substantial amounts of virus have reached the CNS. We examined the effect of progressively delaying antibody treatment after IC and FP viral challenge. Even 6 to 7 days following FP inoculation, G5 IgG protects 50% of mice (Fig. 8). After IC challenge with T3, the 50% protection time point occurs after 5 days (Fig. 9). Similar results were obtained when undiluted anti-T3 serum was used. To assess the extent of CNS infection at times when antibody can still act therapeutically, we measured the amount of virus at various times after FP or IC infection (Fig. 8 and 9). At the 50% protection point following FP inoculation, the viral titer in the brain was $7.8 \log_{10}$ PFU per brain (day 7). The viral titer in the brain at the 50% protection point for IC inoculation was $7.1 \log_{10}$ PFU per brain (day 5). These results show that antibody can protect 50% of animals even after massive CNS viral infection is established.

To confirm these results, we challenged mice with increasing doses of T3 IC and then treated them 24 h later with G5 IgG. Thirty-one mice were inoculated IC with between $4.9 \log_{10}$ PFU and $6.9 \log_{10}$ PFU of T3. At the highest dose tested ($6.9 \log_{10}$ PFU), 71% (5 of 7) of mice survived when given G5 IgG. This experiment shows that antibody protects

FIG. 9. Relationship between viral titer in the brain at different times after IC infection and the capacity of antibody to protect. One-day-old NIH(s) mice were inoculated IC with $100 \times LD_{50}$ of T3. At the indicated times after infection, mice received either G5 IgG or polyclonal anti-T3 serum and their subsequent percent survival was ascertained. In parallel experiments, the titers of T3 in the brains of untreated mice were determined at various times after infection. The bar graph shows the titer plus standard deviation of T3 in the brain at the indicated times after infection.

mice even after IC challenge with 1,000,000 times the T3 IC LD_{50} .

DISCUSSION

It has long been known that antibody protects animals from many lethal viruses. Antibody protects against both neurally spreading viruses (12, 30, 34, 35, 41, 42, 48), and viruses that spread via the bloodstream (3, 38, 39, 45, 46). Despite this, little is conclusively known about the stages in viral pathogenesis at which antibody acts or about the mechanisms by which this occurs. The mammalian reoviruses are useful to analyze these issues since a number of steps in reovirus pathogenesis have been mapped to specific viral genes, and the effect of antibodies can be studied. We feel that a better understanding of these issues is important to understanding viral pathogenesis and the immune response to viruses and for vaccine design and assessment.

Lack of a correlation between neutralization and protection. The mechanism of antibody-mediated protection in vivo may be related to measurable in vitro properties of antibody. An anti-reovirus MAb with NT activity protected against reovirus T3 (Fig. 4). However, a non-NT MAb (C1) and a MAb with only low NT capacity (F7) also protected mice from T3 (Fig. 5). A similar lack of correlation between in vitro NT and protection has been observed among virtually all classes of viruses (4-6, 20, 25, 33, 39, 44, 51, 54, 59, 74). Another property of antibodies against certain viruses is HI. The anti-reovirus G5 MAb protected mice but has no HI activity (7), showing that HI is not required for protection against T3. A correlation between HI and protection has been suggested for measles (20, 50) but has not been seen with yellow fever virus (YFV) (9, 53).

Thus, as a general principle, it is impossible to predict the in vivo protective capacity of an antibody from its in vitro properties. One explanation for this is that in vitro assays do not measure antibody functions relevant to protection in the host. For example, MAbs which do not reduce YFV plaque formation in Vero cells, and hence are considered non-NT,

are able to inhibit the growth of YFV in a mouse neural cell line (5). A similar situation could exist in the case of non-NT anti-T3 MAbs. Alternatively, protection could involve components present in vivo such as serum complement or Fc receptor-bearing cells.

Lack of a role for complement and Fc. Serum complement was not found to be required for inhibition of reovirus T3 growth by an NT MAb (Fig. 7). The classical pathway of complement activation is not required for protection since $F(ab')_2$ T3 IgG protected against T3. Complement plays a role in the host response to some viral infections (27, 42), and a role for antibody-dependent, complement-mediated lysis of infected cells or alteration in viral clearance has been proposed as a mechanism of in vivo protection. Antibody-dependent, complement-mediated lysis of cells expressing viral proteins could provide a mechanism for protection by MAbs against nonstructural virus proteins or by non-NT MAbs (4, 22, 39, 53, 54, 59). In agreement with our studies, complement-independent antibody-mediated protection has been described with Venezuelan encephalitis virus (39), herpes simplex virus (1, 40, 47), and rabies virus (42). Furthermore, there is no correlation between the capacity of antibodies to mediate antibody-dependent, complement-mediated lysis in vitro and their ability to protect mice from viruses, including herpes simplex virus (33, 51), Sindbis virus (59) and YFV (5). We conclude that complement is not required for protection by NT antibody against T3 reovirus and that no definitive evidence exists for an in vivo role for complement in antibody-mediated protection in other viral systems.

A role for Fc receptor-bearing cells in viral clearance or in antibody-dependent cell-mediated cytotoxicity has been proposed for several viruses. Our studies show that when the short half-life and poor access of $F(ab')_2$ to serum are bypassed, protection did not require the Fc portion of IgG. Similar results have been obtained with herpes simplex virus (40, 47), and Semliki Forest virus (4). In several studies, the absence of a protective effect of $F(ab')_2$ has been used as evidence for the importance of the Fc portion of IgG (4, 11, 36, 39). These studies are flawed by the lack of documentation of serum $F(ab')_2$ concentrations. Thus, while the Fc portion of antibody may play an important role in the tissue distribution and half-life of antibody, there is no definitive evidence for a more direct requirement for the Fc portion of IgG in antibody-mediated protection in vivo.

Viral determinants against which protective antibody is directed. A further possible mechanism for protection is inhibition of viral functions by antibody binding to critical determinants on the virus or the surface of virus-infected cells. MAbs directed against the T3 sigma 1 protein protected mice from T3. The S1 gene, coding for the sigma 1 structural protein, determines T3 tropism in the CNS (60, 71, 73) and the capacity of T3 to spread neurally (63). Antibody may be acting by inhibiting sigma 1 function and thus altering viral tropism and/or preventing neural spread of T3. In fact, neural spread of T3 is inhibited by antibody (K. L. Tyler, H. W. Virgin IV, R. Bassel-Duby, and B. N. Fields, manuscript in preparation). Antibody to proteins other than sigma 1 also protected in our system (Fig. 2 and 4), showing that binding to sigma 1 and inhibition of sigma 1 functions are not required for protection. Protection by non-sigma 1 antibody may reflect inhibition of specific in vivo functions mediated by other viral proteins.

The fact that many protective antibodies bind proteins on the surface of virions has suggested that binding to the viral surface is important in vivo (1, 14, 51). In our studies, MAbs

to the T3 outer capsid were protective. Antibodies directed against nonsurface proteins, such as the nucleoprotein of murine hepatitis virus (44) and nonstructural proteins of YFV and Venezuelan encephalitis virus (22, 39, 53), are also protective. Similarly, with Sindbis virus, non-NT antibodies to cryptic epitopes present on infected cell surfaces and present in, but not exposed on, intact virions are protective (54, 55). Thus, expression of an epitope on the surface of a virus is not required for protection *in vivo*. It is possible that expression of T3 determinants on the surface of infected neural cells could serve as a target for protective antibody.

Amount of antibody required for protection. Approximately eight times more antibody was required to protect mice from IC than from FP infection with reovirus T3 (Fig. 6). Similarly, more antibody is required to protect against IC compared with peripheral (e.g., oral, intramuscular) challenge with polio virus (2, 46). The presence of the blood-brain barrier may explain this observation. In uninfected animals with an intact blood-brain barrier, antibody detected in CSF is derived from serum. However, CSF antibody levels are typically less than 0.4% of coincident serum values (15). The transfer of antibody into the CNS does not depend on the Fc fragment of antibody and occurs as well with heterologous antibody as with homologous antibody (24). The development of meningoencephalitis, as occurs with T3 infection of the CNS, results in profound disruption of the blood-brain barrier and a corresponding marked increase in the CSF/serum antibody ratio. The requirement for increased amounts of antibody to protect against IC compared with FP challenge may reflect the need for serum antibody concentrations sufficiently high to allow protective levels of antibody in CSF and brain tissues.

Site(s) of antibody action. The capacity of antibody to protect against established CNS infection has been described with arboviruses (5, 8), poliovirus (34, 35), and herpes simplex virus (41) but has not been universally observed (12, 14, 32, 41). Antibody may act extracellularly at sites including the neuromuscular junction and synapses or at other sites within the CNS parenchyma. Alternatively, antibody may act intracellularly to inhibit critical steps in the viral life cycle (13, 17, 18, 21, 31). Recent studies show that neurons pinocytose and transport antibody into the CNS (16, 75), suggesting that intraneuronal antibody may be active in the CNS.

The studies described in this article show that antibody protects mice from the neurally spreading T3 reovirus. Antibody can act both early in the course of T3 infection and after substantial amounts of virus have entered the CNS (Fig. 8 and 9). The fact that antibody protected against established CNS infection indicates that antibody inhibits steps in reovirus pathogenesis subsequent to viral entry into the CNS. Studies to be described elsewhere show that antibody acts at multiple steps in T3 pathogenesis, including blockade of viral entry into the CNS and prevention of spread within the CNS (Tyler et al., in preparation).

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