INHIBITION OF CELLULAR DNA SYNTHESIS BY REOVIRUS OCCURS THROUGH A RECEPTOR-LINKED SIGNALING PATHWAY THAT IS MIMICKED BY ANTIIDIOTYPIC, ANTIRECEPTOR ANTIBODY

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The interaction of virus with attachment sites on target cells is an important component in determining viral tropism and pathogenicity. Demonstration of the binding of several mammalian viruses, as for example HIV (1), to membrane proteins that perform critical physiological functions has focused attention on the more immediate and direct consequences of virus-receptor interactions. The mammalian reoviruses provide a particularly useful model for examining the means by which viruses can utilize specific membrane proteins as vectors of both selective attachment and pathogenesis.

The three reovirus serotypes display distinct patterns of tropism that are controlled in part by interaction of the virus outer capsid protein σ1, which also serves as the hemagglutinin (HA)[1], with apparently unique cellular attachment proteins (2–4). Gene segment reassortants of reovirus have been used in studies of central nervous system tropism to demonstrate that viruses that bear the serotype 3 HA (HA3) bind neurons causing an often fatal neonatal encephalomyelitis while those expressing serotype 1 HA (HA1) bind ependymal cells resulting in hydrocephalus (5). The cellular attachment protein for type 3 virus is a 67-kD glycoprotein that bears structural and antigenic similarity to mammalian β adrenergic receptors (4, 6). These studies have been aided by the isolation of mAb antireovirus type 3 receptor antibody that was selected on the basis of idiotypic mimicry of the HA3 (7). This antibody competes with type 3 virus and binds to the 67-kD receptor glycoprotein (4). The theoretical rationale of this approach has been recently reviewed (8).

The serotype of reovirus HA has also been directly linked to perturbation of host cell physiology. Infection of mouse L cells by reovirus type 3 results in potent inhibition of host cell DNA synthesis (9). Inhibition is first detected at 8–10 h postinfection before any cytopathic effects (10). By 24 h, DNA synthesis in infected cells is only 15–25% of controls (9). Inhibition is manifest as a blockade of multifocal initiation

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[1] *Abbreviations used in this paper:* HA, hemagglutinin; PFU, plaque-forming unit.

of new DNA chain synthesis on replication units resulting in an accumulation of cells in G_1 phase (11–14). In contrast to these observations, the binding of type 1 virus to this same cell line has little or no effect on DNA synthesis (9). Using molecular reassortants, Sharpe and Fields (9) have shown that inhibition of DNA synthesis is dependent on the presence of HA3. The mechanism of DNA synthesis inhibition in response to HA3 binding remains in question. Studies using either inactivated virus or viral capsid components have yielded conflicting results (15, 16). The possible mechanism for these effects included a direct, receptor-mediated effect on DNA synthesis after virus (HA3) binding, or intracellular effects of HA3 protein or S1 transcripts on synthetic machinery.

To more fully examine the mechanism of reovirus inhibition of DNA synthesis and to gain a better understanding of reovirus pathogenicity toward cells of the central nervous system, we have studied the effects of reovirus and antireceptor antibody on the rat neuroblastoma cell line B104.G4. The addition of reovirus type 3 and HA3 containing virus reassortants caused potent inhibition of DNA synthesis in B104.G4 cells. Inhibition was observed using replication-defective virus and when intracellular virus processing, but not binding, was blocked by lysosomotrophic agents. Antireceptor antibody mimicked intact virus in the inhibition of DNA synthesis. Inhibition was dependent upon the presence of intact antibody or when monovalent F(ab) fragments were crosslinked by anti-Ig. The inclusion of β adrenergic agonists and antagonists had no effect on DNA synthesis in the presence or absence of virus. Therefore, inhibition of cellular DNA synthesis by reovirus type 3 occurs through a direct, receptor-triggered event that involves microaggregation but not intracellular processing of receptor molecules. These results also suggest that naturally occurring antiidiotypic, antireovirus receptor antibodies can have potent autoimmune effects in the central nervous system.

Materials and Methods

Tissue Culture. The neuroblastoma lines NB41A3 and Neuro 2A were obtained from the American Type Culture Collection (Rockville, MD). Neuroblastoma B104.G4 was derived from parental B104 cells (a gift of Dr. Jeff Drebin, Johns Hopkins University, Baltimore, MD) by selection of isolated clones for acute sensitivity to reovirus inhibition of DNA synthesis. All cells were grown at 37°C in 5% CO_2 in monolayer using DMEM (KC Biological, Inc., Lenexa, KS) supplemented with 10% FCS, 1% pen-strep-fungizone mixture (M. A. Bioproducts, Walkersville, MD), and 100 μg/ml gentamycin sulfate (M. A. Bioproducts). mAb hybridoma lines 87.92.6 and 61.21.18 were maintained in RPMI 1640 (KC Biological, Inc.) supplemented as above.

Virus. Reovirus type 3 (Dearing), 1 (Lang), 1HA3, and 3HA1 were obtained from Dr. Virginia Hinshaw (Harvard University, Boston, MA). Virus was grown on murine L-929 cells and purified by CsCl gradients as previously described (17, 18). Serotype analysis was performed using a panel of mAbs and by polyacrylamide gel analysis of the dsRNA (17). The infectious titer of virus was determined by plaque analysis (plaque-forming units [PFU]) on L-929 monolayers. Preparation of UV-inactivated virus was performed at 11 ergs/s/mm^2 as described by Shaw and Cox (19). Inactivation was verified by titer analysis on L-929 cells. Binding of inactive virus was verified by immunostaining as described below.

Measurement of DNA Synthesis. B104.G5 cells were seeded into 96-well plates (Costar, Cambridge, MA) at 5×10^4 cells/well in 200 μl. After a minimum of 12 h, virus was added at 30 PFU/cell for 60 min at 37°C. Cells were then washed into fresh DMEM with supplements and recultured for the times indicated. Cells were pulsed with [^{3}H-methyl]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) for the final 6 h of culture, after which reac-

tions were stopped by the addition of trypsin-versene (M. A. Bioproducts) and freezing at −20°C for a minimum of 60 min. Cells were thawed and the DNA was collected onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). Filter discs were analyzed by scintillation counting. For drug inhibition studies cells were incubated with either monensin, chloroquine, ammonium chloride, or methylamine (Sigma Chemical Co., St. Louis, MO) at the concentrations indicated in the text for 60 min before, coincident with, and subsequent to virus addition. No effect of these agents on the binding of reovirus was detected at any of the concentrations used. Toxicity was assessed by the exclusion of trypan blue dye as judged by microscopic analysis, and by measurement of the level of DNA and protein synthesis using radiolabeled precursors.

Measurement of Virus Binding and Macromolecule Synthesis. Virus binding experiments were conducted by immunofluorescence staining and analysis on a flow cytofluorimeter. Cells were harvested from monolayer using buffered EDTA (Versene; M. A. Bioproducts), then washed three times in FACS medium (20 mM sodium phosphate [pH 7.4], 150 mM NaCl, 2% horse serum, and 0.2% sodium azide). 10^5 cells were incubated with virus at 50 PFU/cell for 45 min at 4°C, washed extensively, and bound virus was detected by the addition of 2 μg avidin-coupled antivirus antibody in 100 μl for 45 min at 4°C. Avidin antibody was prepared using highly purified (>98% by gel analysis) rabbit antireovirus Ig that was biotinylated and then pre-mixed with a 1:20 dilution of avidin-fluorescein (Becton Dickinson & Co., Mountain View, CA). High titer antireovirus type 3 Ig was prepared by immunization with type 3 but shows substantial crossreactivity to type 1 virus as determined by both immunoprecipitation and fluorescence binding analysis on infected L-929 cells (not shown). Cells were washed free of unbound label and 10^4 cells/sample were analyzed on a FACS analyzer (Becton Dickinson & Co.).

As an indication of virus replication at early time points (<24 h), virus-specific macromolecule synthesis was determined by immunoprecipitation analysis of radiolabeled viral proteins from infected cell extracts. B104.G4 cells were seeded onto 6-well culture plates (Costar) at 10^6 cells/well. After a minumum of 12 h, virus was added at 30 PFU/cell for 60 min at 37°C. Cells were washed and recultured in supplemented DMEM for 18 h. At this time cells were again washed and fed with methionine-free DMEM (KC Biological, Inc.), and then incubated with 100 μCi/ml [^{35}S]methionine (950 Ci/mmol; New England Nuclear). Cells were harvested for immunoprecipitation after 6 h by the addition of 500 μl of detergent lysis solution (1% Triton X-100, 0.5% NP-40, 10 mM iodoacetamide, 2 mM PMSF, 150 mM NaCl, and 20 mM sodium phosphate [pH 7.2]). Samples were incubated on ice for 30 min then clarified by centrifugation at 35,000 *g* for 30 min. Reovirus protein synthesis was determined by immunoprecipitation in the presence of purified polyclonal antireovirus antibody conjugated to sepharose beads (40 μl of a 1 mg/ml bead suspension) for 45 min at 4°C. Immunoprecipitates were harvested by centrifugation and washed extensively in detergent lysis solution containing 0.5 M NaCl before measurement of counts bound by liquid scintillation counting.

Antibody Isolation and Binding Analysis. mAb 87.92.6 (murine IgM,k) and 61.21.18 (murine IgM,k), and polyclonal rabbit antireovirus receptor were purified by affinity chromatography as previously described (20). mAb OX-18 (murine anti-rat MHC) was purchased from Bioproducts for Science (Indianapolis, IN) and purified as above. Purified rabbit anti-mouse Ig was a gift of Dr. John Monroe (University of Pennsylvania, Philadelphia, PA). Antibody purity was verified by silver stain of samples run on SDS-PAGE. Iodination of purified antibodies (25 μg/reaction) was performed according to Hunter (21) in the presence of chloramine T and 250 μCi Na^{125}I (New England Nuclear). Labeled proteins were separated from free label by passage over a mixed bed of Sephadex G50, DEAE cellulose, and Amberlite IRA-400 (Sigma Chemical Co.). sp act of labeled mAb was 1.3×10^6 cpm/μg and >97% of label was precipitated by 10% TCA. Binding studies were performed in 50-μl aliquots containing 10^6 cells in PBS with 0.5% bovine albumin and 0.2% NaN_3 as described (22). Labeled mAb was mixed with cold ligand to achieve a final concentration of 10^{-7}–10^{-11} M and incubated at 4°C for 60 min. Bound label was separated from free by centrifugation through phthalate oils (23) and cell-associated counts determined by gamma counting. Results were analyzed according to the methods of Scatchard (24) using the criteria of Klotz (25).

F(ab) fragments of mAb were prepared by the procedure of Bidlack and Mabie (26) at pH 8.1 using trypsin-TPCK. Fragments were purified by sequential affinity and molecular sieve chromatography. mAbs were coupled to CnBr-activated sepharose beads at a density of 1 mg/ml according to the instructions supplied by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ).

Results

Serospecificity of Reovirus Binding and DNA Inhibition in Neuroblastoma Cells. To establish an in vitro model for evaluating the effects of reovirus within the central nervous system, we have investigated the binding patterns and physiological consequences of virus infection in a panel of rodent neuroblastoma cell lines, including Neuro-2A, NB41A3, and B104.G4. The binding of reovirus serotypes 1 and 3 to these lines was investigated using microfluorometric analysis. Cells were incubated with virus, as indicated in Fig. 1, followed by the addition of biotin antivirus antibody/avidin-fluorescein complex and detection using a fluorescence-activated flow cytometer. In agreement with previous analysis of infectivity patterns in vivo (5), each of the three neuronal lines tested displayed a selective binding of type 3 reovirus over type 1. No increase in antibody binding was detected in type 1 virus-treated cells as compared with controls conducted in the absence of virus or using an irrelevant antivirus (antimeasles) antibody. The highest level of type 3 binding was detected on the B104.G4 line. Previous studies using antireceptor antibody showed that the parental B104 line contained 78,000 type 3 receptors per cell (4).

Analysis of the pathogenetic effects of reovirus type 3 on B104.G4 cells was initiated by examining virus-induced DNA inhibition. The addition of reovirus type 3 or the recombinant virus 1HA3, which contained the type 3 HA on the background of a type 1 virus, resulted in inhibition of B104.G4 DNA synthesis (Fig. 2). By 48 h after infection, levels of synthesis were 22% of control (type 1) values. The timing of inhibition lagged behind that described in murine L cells that were maximally inhibited by 24 h (9). The addition of type 1 virus or the recombinant 3HA1, which contained the type 1 HA on a type 3 background, had no effect on DNA synthesis relative to untreated cells (not shown). Type 3 virus, which was UV irradiated so as to abolish its infectivity, but not binding, still blocked DNA synthesis.

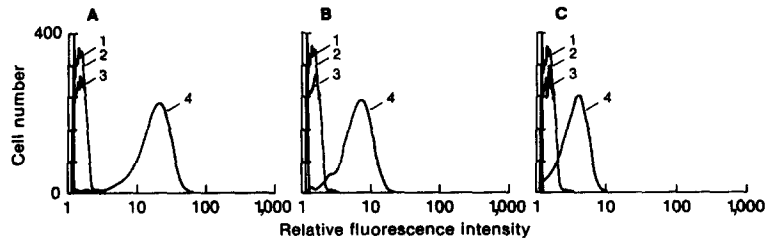

FIGURE 1. Binding of reovirus serotypes to neuroblastoma lines. The relative degree of binding of reovirus serotypes to neuroblastoma lines B104.G4 (*A*), NB41A3 (*B*), and Neuro 2A (*C*) was examined by microfluorometric analysis. Cells were first incubated with either buffer (*1*), reovirus type 1 (*2*), or reovirus type 3 (*3* and *4*). The level of virus binding was then determined by the addition of either polyclonal antireovirus 1-3 (*1*, *2*, and *4*) or control, antimeasles (*3*) antibody. Both antibody preparations were bound to biotin and indirectly coupled to avidin-fluorescein. The amount of binding was measured on a fluorescence-activated flow cytometer. Results are expressed as cell number vs. relative fluorescence intensity.

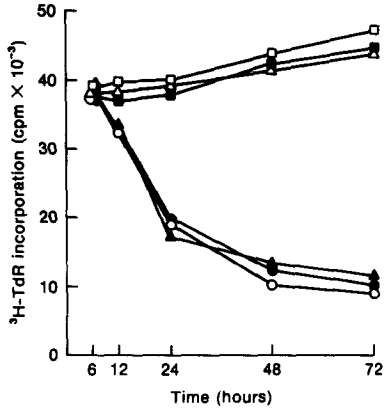

FIGURE 2. Effect of reovirus serotype on inhibition of DNA synthesis in B104.G4 cells. The effects of reovirus on B104.G4 DNA synthesis was investigated using the viruses: type 3 (○), type 1 (△), recombinant 1HA3 (●), recombinant 3HA1 (■), UV-inactivated type 3 virus (▲), or type 3 virus in the presence of neutralizing antibody (□). B104.G4 cells were seeded in subconfluent monolayers in microtiter wells at 5 × 10^4 cells in 200 μl. Cells were incubated with 30 PFU/cell of virus, or an equivalent particle number of UV-inactivated virus, for 60 min at 37°C to allow virus attachment. Cells were washed into fresh medium and incubated for the times indicated. To measure DNA synthesis cells were pulsed with 1 μCi/well of [^{3}H]thymidine for the last 6 h of culture, and DNA was harvested on glass fiber filter discs and counted using a scintillation cocktail.

In contrast, addition of high-titer neutralizing antibody abolished the type 3 effect. Therefore, these observations confirm that inhibition of DNA synthesis in neuronal cells is HA3 linked and is independent from reovirus replication.

Inhibition of Virus Processing Does not Affect DNA Inhibition. Reoviruses are distinguished by their use of lysosomal processing as a means for uncoating the double capsid viral particle to yield an infective viral core, termed the subviral particle (27). Virus receptor complexes are first taken into phagocytic vacuoles that rapidly (30–60 min) fuse with lysosomal vesicles (28). Uncoated subviral particles enter the cytoplasm apparently by direct penetration of the lysosomal membrane and begin the replicative phase (29). The relative importance of receptor attachment vs. internalization of viral particles in the mechanism of inhibition of DNA synthesis was investigated using lysosomotrophic agents that block virus entry and/or processing in target cells (30).

A panel of drugs that disrupt lysosomal function were first screened for effects on basal DNA and protein synthesis, and the ability to block virus replication in B104.G4 cells (Fig. 3 *A*). The concentrations of drugs used are the maximal doses at which no inhibitory effects were observed on basal synthesis of macromolecules. The level of virus replication during the first 24 h of infection was determined by measuring virus-specific protein synthesis. This was achieved by immunoprecipitation of viral proteins from ^{35}S-methionine pulse-labeled cells using an antireovirus antisera. A fourfold increase in immunoprecipitable viral proteins was detected in infected vs. noninfected or antibody controls (Fig. 3 *A*). The addition of 10–15 mM NH_4Cl or 5 × 10^{-4} M chloroquine 60 min before and coincident with virus completely abrogated viral protein synthesis without effect on basal protein synthesis. In contrast to these results, at decreasing concentrations of chloroquine (10^{-5}–10^{-6} M) and the highest nontoxic concentration of monensin (10^{-6} M) and methylamine (1 mM, not shown), significant viral protein synthesis was detected. These concentrations and drugs were therefore not used in the remaining studies. The effects of ammonium chloride and chloroquine, at concentrations that blocked viral replication, on virus-induced DNA inhibition is presented in Fig. 3 *B*. In each instance agents that blocked virus replication were without effect on reovirus-directed inhibition of DNA synthesis.

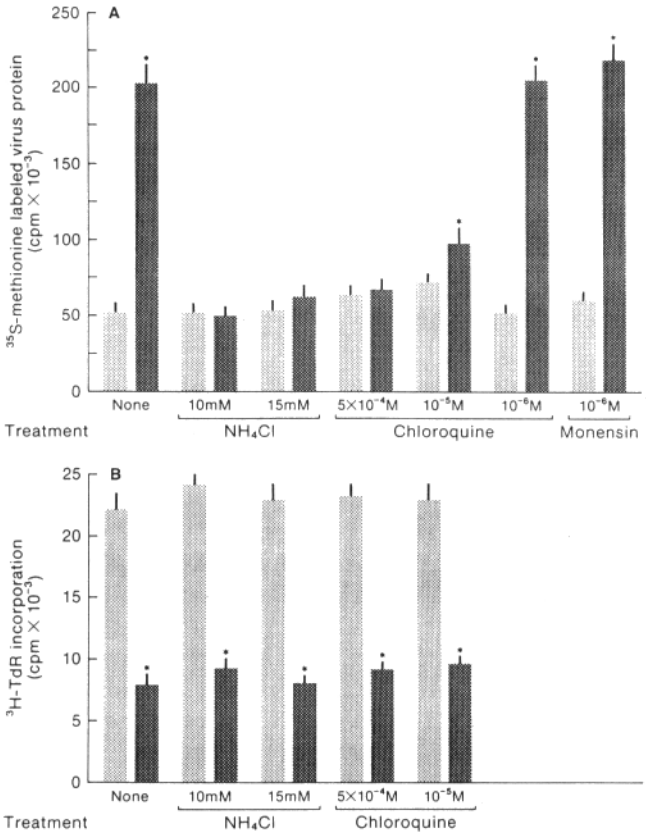

FIGURE 3. Effects of inhibitors of lysosomal processing or trafficking on virus replication and inhibition of DNA synthesis. (*A*) Effects of drugs on virus replication. Subconfluent monolayers of B104.G4 cells (10^6 cells/condition) were incubated with media or either of the compounds (ammonium chloride, chloroquine, or monensin) at the concentrations listed for 60 min before the addition of buffer control (*light bars*) or type 3 reovirus (30 PFU/cell; *dark bars*). After 60 min unbound virus was withdrawn and cells were recultured in media in the presence of these same inhibitors. Cells were incubated for 18 h and then pulsed with [^{35}S]-methionine for an additional 6 h. The level of reovirus protein synthesis was determined by immunoprecipitation of detergent-lysed cell extracts using antireovirus sepharose beads. (*B*) Effects of drugs on reovirus induced DNA inhibition. B104.G4 cells were treated with virus and drugs as described in *A* and pulsed with [^{3}H]thymidine for the final 6 h of a 24-h culture. DNA synthesis was determined by harvesting cell extracts onto glass fiber filters and measuring thymidine incorporation by scintillation counting. Results presented represent the mean ± SE of experiments performed in triplicate. (*) $p < 0.001$ by paired *t* test.

Antireceptor Antibodies Mimic Virus Inhibition of DNA Synthesis. Antireceptor antibodies that serve as internal images of the reovirus HA3 have been isolated and characterized. These antibodies were selected as anti-(anti-HA3)idiotypes (7, 31), and mimic the effects of HA3 by both immunologic and biochemical criteria. Antiidiotypes stimulate anti-HA3-specific B and T cell–mediated immune responses across species barriers (20). Antiidiotypes bind to cells in an HA3-restricted fashion, compete with intact virus for cell surface attachment, and immunoprecipitate the same 67-kD glycoprotein as type 3 virus (4, 22).

The effects of polyclonal and mAb antireceptor antibody on DNA synthesis in B104.G4 cells is presented in Fig. 4. The addition of purified rabbit polyclonal or murine monoclonal (mAb 87.92.6) antireceptor antibody at 100 μg/ml induced potent inhibition of DNA synthesis. The kinetics of this effect were indistinguishable from that induced by intact reovirus type 3. Significant differences in DNA synthesis relative to controls was detected at 24 ($p < 0.005$), 48, and 72 ($p < 0.001$) h.

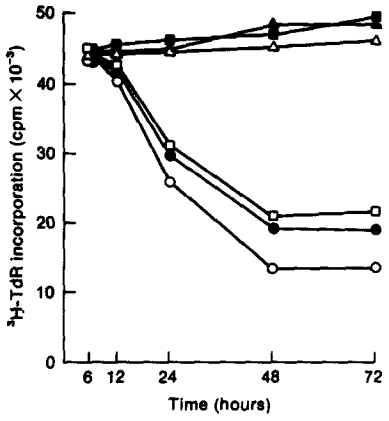

FIGURE 4. Inhibition of DNA synthesis by antireceptor antibody. The effects of antibody on DNA synthesis in B104.G4 cells was determined as described in Fig. 2. Antibody or virus was first incubated with cells for 60 min at 37°C then washed and recultured for the times indicated. Cells were pulsed with [^{3}H]thymidine for 6 h before assay. Reovirus type 3 (○) and recombinant virus 3HA1 (■) were added at 30 PFU/cell. Polyclonal rabbit antireovirus receptor (●), mAb 87.92.6 antireceptor (□), mAb 61.21.18 isotype control (△) and mAb OX-18 anti-MHC (▲) were purified and incubated at 100 μg/ml.

Controls included virus 3HA1, the isotype-matched nonbinding mAb 61.21.18, and mAb OX-18 an anti-rat MHC binding antibody. These results confirm the hypothesis that engagement and/or perturbation of virus receptors and not virus-encoded function is the key event in inhibition of DNA synthesis by reovirus type 3. Interestingly, the level of inhibition with antibody was significantly less than that seen with virus at the 48- and 72-h time points. Preliminary data indicate that antibody-treated cells begin to recover from inhibition after 2–3 d. This is in marked contrast to virus-infected B104.G4 cells that undergo viral cytolysis at 3–5 d. These observations point out a potentially important consequence of the generation of antiidiotypic, antireceptor antibody in the course of normal antivirus immune responses.

The concentration dependence of mAb antireceptor antibody on the inhibition of DNA synthesis in B104.G4 cells is sigmoidal (Fig. 5 *A*). This in part reflects the concentration dependence of mAb binding, which is also presented in Fig. 5 *A*. mAb antireceptor showed linear and saturable binding to B104.G4 over an ~3-log dose range of 5×10^{-9}–1×10^{-7} M mAb. The apparent K_d was 2×10^{-9} M as determined by Scatchard analysis of binding data (not shown) in good agreement with previously published results on other receptor-positive lines (4, 22). Comparison of the dose dependence of antireceptor antibody inhibition to that of antibody binding provides a clue to the mechanism of the inhibitory process. Inhibition of DNA synthesis was not detected below a concentration of 2.5×10^{-9} M antibody (Fig. 5 *A*), yet at this concentration, 43% of virus receptors were already occupied. A comparison of the extent of DNA inhibition vs. receptor occupancy (percent of receptor bound by mAb), shown in Fig. 5 *B*, illustrates that inhibition occurred over a high concentration and narrow range of bound antibody, e.g., 10% inhibition was seen at 50% receptor occupancy, and 50% inhibition was seen at 70% occupancy. These data suggest that inhibition results when a critical threshold of signal is attained (antibody agonist) or lost (antibody antagonist), and/or that the simultaneous engagement of multiple receptors is required for signal transmission.

Receptor Aggregation Is Required for Inhibition of DNA Synthesis. Requirements for aggregation of multiple receptors by antireceptor antibody were directly assessed by examining DNA synthesis in the presence of monovalent F(ab) fragments of antireceptor mAb and of secondary crosslinking agents such as anti-Ig on intact mAb

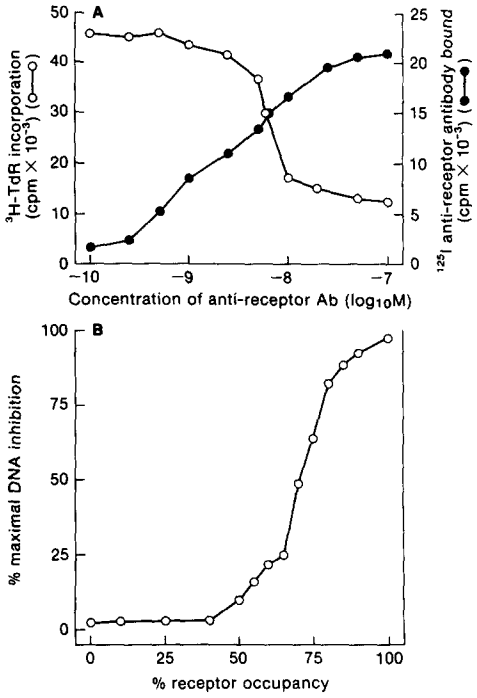

FIGURE 5. Comparison of the extent of antibody binding to the level of DNA inhibition. (*A*) mAb binding and DNA inhibition concentration dependence. The dose dependence of DNA inhibition at 48 h by mAb antireceptor antibody was conducted as described in Fig. 4 in the presence of antibody concentrations of 10^{-10}–10^{-7} M (O). mAb binding analysis was conducted by incubation of 10^6 B104.G4 cells with increasing concentrations of ^{125}I-labeled mAb in 50 μl PBS/0.5% bovine albumin/0.2% sodium azide. After 60 min at 4°C, cells were washed free of unbound label by centrifugation through phathlate oils and bound mAb was determined in a gamma counter. Values presented (●) were corrected for nonspecific binding, which was measured in the presence of a 100-fold excess of unlabeled mAb. (*B*) Plot of receptor occupancy vs. extent of DNA inhibition. The percentage of maximal DNA inhibition by mAb antireceptor observed at 48 h was calculated based on 74% inhibition at 10^{-7} M mAb. The percentage of receptor occupancy by mAb was calculated based on 100% occupancy at an mAb concentration of 10^{-7} M.

and F(ab) fragments. Results presented in Fig. 6 show that the addition of 10^{-8} M antireceptor mAb induced significant DNA inhibition while mAb at 10^{-9} M had no effect. The addition of 10 μg rabbit anti-mouse Ig to these cultures had potent effects. Inhibition was augmented in cultures containing 10^{-8} M mAb to a level equivalent to that seen upon virus addition. Interestingly, the addition of anti-Ig also stimulated maximal inhibition in cultures with 10^{-9} M mAb, where no inhibition was previously observed despite 41% receptor occupancy.

Reciprocal analysis of these effects was conducted using monovalent F(ab) fragments of antireceptor mAb. As shown in Fig. 6, F(ab) fragments had no effect on DNA synthesis even when administered at 10 times (10^{-7} M) the intact antibody concentration. Binding of these fragments to cells at >70% receptor occupancy was verified by radiolabeled binding analysis as described previously (data not shown). The addition of anti-Ig to these cultures resulted in maximal inhibition of DNA synthesis. Similar results were obtained when F(ab) fragments were rendered multivalent by conjugation at high density to sepharose beads. Controls conducted using whole or F(ab) fragments of the cell surface binding mAb OX-18 uniformly had no effect, even when incubated in the presence of anti-Ig. These results confirm that mAb binding alone does not initiate the inhibitory process and that aggregation of multiple virus receptor proteins is required.

Estimation of the role played by accumulation of receptor second messengers is more obtuse. An analysis of the kinetics of antibody receptor binding and signal transmission is presented in Fig. 7. In this experiment antibody was first bound to cells in the cold at 5×10^{-8} M, which saturates virus receptors. Cells were then

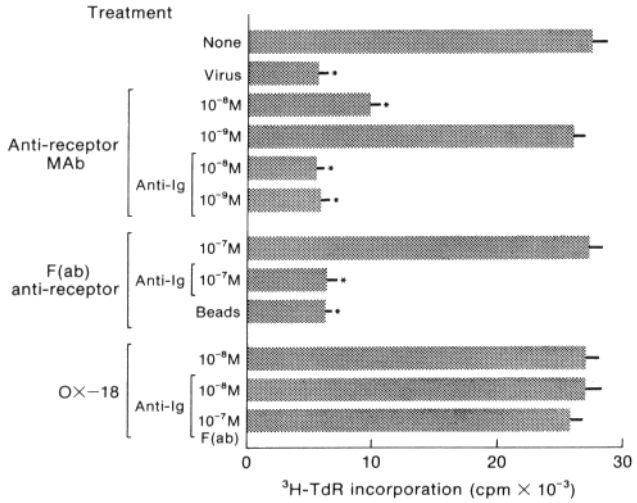

FIGURE 6. Requirements for virus receptor aggregation in the inhibition of DNA synthesis. Assays for DNA synthesis were conducted essentially as described in Fig. 4. Reovirus type 3 was added at 30 PFU/cell as described previously. Whole mAb or F(ab) fragments were added to cells at the concentrations listed for 60 min at 4°C. Where noted, rabbit anti-mouse Ig (10 μg/well) was included for an additional 60 min. F(ab)-sepharose bead conjugates were included in some wells at a concentration of 20 μl of a 1-mg F(ab)/ml bead suspension. After virus or antibody treatment, cells were washed and recultured in DMEM with supplements at 37°C for standard thymidine incorporation assay at 48 h. Data are presented as the mean ± SE of experiments performed in triplicate. (*) $p <$ 0.001.

shifted to 37°C and incubated for the times indicated. At these points bound antibody was removed by rapid (1 min) washing in DMEM containing 25 mM sodium acetate (pH 4.0). Cells were quickly washed into fresh media and recultured for 48 h at which time the level of DNA synthesis was measured. Under these conditions >90% of bound mAb is removed from the cell surface without toxic effects on cells. As shown in Fig. 7, maximal inhibition was observed when antibody remained bound for at least 30–60 min at 37°C. Inclusion of mAb for longer periods, up to the entire length of the assay (48 h), failed to augment the inhibitory effects of suboptimal concentrations of mAb. The same pattern was observed when 5×10^{-9} M antibody was bound despite the fact that only 45% inhibition was obtained. When mAb-cell

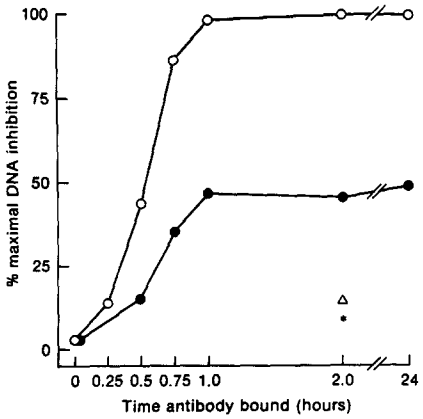

FIGURE 7. Determination of the requirements for mAb-induced DNA inhibition. B104.G4 cells were preincubated with either 5×10^{-8} M (○) or 5×10^{-9} M (●) mAb for 60 min at 4°C. At t = 0, cells were shifted to 37°C. At the times indicated cells were washed briefly in pH 4.0 buffer to remove bound antibody and then recultured for up to 48 h. As controls, cells were left at 4°C (△) or in the presence of 0.2% sodium azide (*) for the first 2 h after preincubation and then subjected to acid washing. Thymidine incorporation assays were conducted as described in Fig. 4.

conjugates were left at 4°C or in the presence of the metabolic inhibitor sodium azide for an additional 2 h after binding, there were no inhibitory effects. Two facts can be deduced from this data. First, the induction of DNA inhibition by antireceptor antibody is an energy-dependent process; and second, if a threshold of signal is required, it occurs within the first 30–60 min after binding at 37°C. An additive signal was not generated by increasing the incubation time of mAb bound at suboptimal concentrations. These observations are consistent with a receptor aggregation signaling model.

Involvement of β Adrenergic Receptors in Inhibition of DNA Synthesis. The biochemical and immunological similarities of the mammalian reovirus type 3 and β adrenergic receptor families suggest the hypothesis that reovirus type 3 might utilize β adrenergic or β adrenergic-like signaling pathways as pathogenetic vectors. To test this hypothesis we have investigated the capacity of virus and antireceptor antibody to act as β agonists, and the effects of well-established β agonists and antagonists on DNA synthesis in B104.G4. As shown in Fig. 8 *A*, the stimulation of cyclic AMP accumulation in B104.G4 cells occured rapidly (15–30 s) in response to the β adrenergic agonist (−)isoproterenol. In contrast, the continuous presence for 60 min of type 3 reovirus or antireceptor antibody at concentrations that inhibit DNA synthesis had no effect on cyclic AMP levels. Experiments performed in the presence of 1 mM 3-isobutyl-1-methyl xanthine, a potent inhibitor of cyclic AMP phosphodiesterase, gave similar results (not shown).

The capacity of β agonists to affect DNA synthesis of B104.G4 cells is presented in Fig. 8 *B*. The addition of (−)isoproterenol at 10^{-8}–10^{-5} M had no effect on DNA synthesis or on the inhibition of DNA synthesis induced by virus or mAb. Inhibition of DNA synthesis at concentrations of (−)isoproterenol $>10^{-4}$ M was the result of toxicity as determined microscopically by dye exclusion. Similar results were obtained in the presence of the β agonists (−)epinephrine, (−)norepinephrine, and

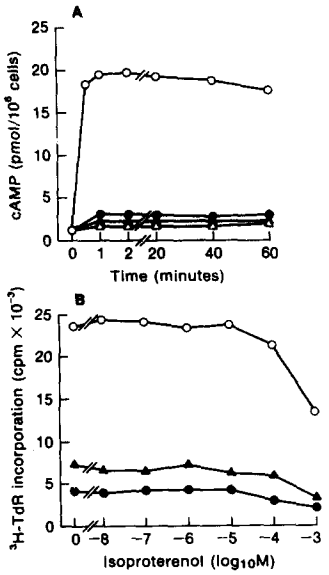

FIGURE 8. Role of β adrenergic receptors in inhibition of DNA synthesis. (*A*) Accumulation of cyclic AMP in response to virus receptor binding. Cyclic AMP formation in intact B104.G4 cells was measured in the presence of either 10^{-6} M (−) isoproterenol (○), 30 PFU/cell reovirus type 3 (●), 10^{-7} M mAb antireceptor (▲), or buffer (△) by RIA. (*B*) Effect of β agonists on DNA synthesis in B104.G4. The effect of the agonist (−) isoproterenol at concentrations of 10^{-8}- 10^{-3} M on DNA synthesis either alone (○), or in the presence of 30 PFU/cell reovirus type 3 (●) or 10^{-7} M mAb (▲) antireceptor was measured as described in Fig. 4.

(+)isoproterenol, and the β antagonist (−)alprenolol (data not shown). These results confirm that activation of classical β adrenergic pathways is not involved in the mechanism of virus-mediated inhibition of DNA synthesis.

Discussion

The mammalian reoviruses provide a well-studied example of the complex patterns and varied means by which viruses exert pathology. The ease of generating reovirus reassortants that may differ in a single genomic segment has enabled correlation of gene function to viral growth and interaction with host. These studies have demonstrated that the σ1 outer capsid protein plays a crucial role in the interaction of reovirus at the organismal and cellular levels. σ1 serves as the reovirus HA (32), it is the primary factor in directing host immune responses (33–35), it specifies tissue and cell tropism (4, 36), and affects neurovirulence (5, 36). At the cellular level it determines the interaction of virus particles with microtubules (37) and regulates cellular DNA synthesis (9).

The inhibition of DNA synthesis initiated by reovirus has been carefully described. Inhibition is not related to an alteration in DNA precursor pools (19, 38), and occurs without detectable degradation of cellular DNA (11). Rather, inhibition results from a blockade of multifocal initiation of new chain DNA synthesis on replication units (12, 13). This process is unrelated to general effects on host protein synthesis (10, 11, 39). The observation that inhibition of DNA synthesis is seen in virions with type 3 HA but not those with type 1 HA suggested that the mechanism of this effect resulted from the direct action of HA3 on target cells (9). This was supported by observations that replication-defective UV-irradiated virus-blocked synthesis-infective virion cores, which lack outer capsid, uniformly failed to cause inhibition (9, 15). One conflicting result was that virus top component, empty capsid that contains no nucleic acid, did not inhibit DNA synthesis (15, 16). However, only 20% of top component bound to cells relative to intact virus (40).

The data presented in this study definitively link the processes of cellular tropism and regulation of host cell macromolecule synthesis through the actions of the viral HA on specific cellular membrane proteins. HA (HA3)-specific binding and inhibition of DNA synthesis was demonstrated on the neuroblastoma cell line B104.G4. The essential role of virus attachment as contrasted to virus replication in the inhibition of DNA synthesis was shown through the use of UV-inactivated virus and drugs that block lysosomal processing and trafficking, which are essential to virus replication. The ability of antireceptor antibodies to mimic viral inhibitory effects confirmed that this pathogenesis was not virus encoded per se. It therefore appears that reovirus type 3 binds to a physiologic membrane protein and induces the inhibition of cellular DNA synthesis by either normal or aberrant signaling through this molecule.

Several observations indicate that aggregation of multiple receptor molecules is required for inhibition to occur. Inhibition was only seen when >50% of receptors were bound by pentameric antireceptor mAb while the addition of anti-Ig to cells with <50% receptor occupancy augmented inhibition. The level of inhibition in the presence of anti-Ig was more pronounced in all conditions and was equivalent to that seen with virus. Monovalent F(ab) fragments of antireceptor antibody were ineffective, even when used at concentrations where 75–100% of surface receptors

were bound. Inhibition was restored when anti-Ig was added to bound F(ab) or when multivalent F(ab)-sepharose beads were used. Finally, binding of mAb had no effect when cells were maintained at 4°C but was effective when cells were shifted to 37°C for 30–60 min. Requirements for receptor aggregation fit nicely with previous data obtained using virus preparations. Intact reovirus virions contain 48 HA molecules/capsid (41) and therefore should engage multiple receptors upon cell contact. Virus top component shows reduced binding and does not inhibit DNA synthesis, and more pronounced inhibition is seen when virus is used at high multiplicity of infection (42).

The interesting structural association between reovirus type 3 and β adrenergic receptors may offer a clue as to how this effect is directed. Our preliminary studies, however, indicate that classical β agonist pathways are not activated by virus or antireceptor antibody. β adrenergic agonists and antagonists neither mimicked nor blocked the effects of virus or mAb on B104.G4 cells. Receptor aggregation is not associated with any known β adrenergic receptor function. Therefore, these observations suggest that any signaling via β receptors is through an aberrant pathway. Crosslinking of β receptors using anti-β receptor-specific antibody will help to resolve this issue. These results are in agreement with those recently reported by Choi and Lee (43). Their investigation failed to detect any physical or functional association of reovirus type 3 and β adrenergic receptors on A431 cells. Studies are presently underway in our laboratories to directly assess reovirus and β receptor homology at the biochemical and genetic levels.

An alternative possibility is that reovirus type 3 binds an as yet unreported β-related receptor class. Sequencing of reovirus receptor genes is essential to understanding the degree of homology. The consequences of this interaction in terms of second messengers activated are now being investigated. Preliminary data indicates that inhibition is antagonized by agonists of cyclic adenosine monophosphate and that antireceptor antibody binding enhances membrane-associated protein kinase activity.

The ability of antiidiotypic antibody to act as an internal image of virus raises the possibility that some aspects of viral pathogenesis may be mediated by the endogenous production of antiidiotypic antibodies in the course of a normal immune response. The production of antiidiotypic, antiacetylcholine receptor antibodies and myasthenia gravis–like pathology in response to immunization with the acetylcholine receptor agonist BisQ provides a working model of this process (44, 45). More detailed investigations are now being conducted in vivo to examine antiidiotype pathology in reovirus type 3–infected mice.

Summary

Mammalian reovirus type 3 binds to a 67-kD surface glycoprotein on the membrane of neuronal cells. This interaction initiates the infective reovirus cycle. The physiological function of this virus receptor is not known, however, initial studies illustrate a striking structural and antigenic homology to the β adrenergic receptor family. The earliest known pathologic effect of reovirus type 3 is an inhibition of host cell DNA synthesis within 8–10 h after virus attachment. The studies reported here demonstrate that binding and aggregation of reovirus receptor molecules provides the signal for this inhibitory process. Inhibition of DNA synthesis in the neuroblastoma cell line B104.G4 was unaffected by using replication-defective virus or

when lysosomal processing of normal virus was blocked. Inhibition was mimicked by an antiidiotypic, antireceptor mAb. Inhibition was not observed when monovalent mAb fragments were bound to receptors, but was reconstituted when these fragments were aggregated by the addition of anti-Ig. The signal for the inhibitory effect was generated within the first 60 min after mAb binding. These observations demonstrate that reovirus and antiidiotypic pathogenicity can result from the perturbation of membrane proteins that may perform normal physiological functions.

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