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THE EFFECT OF NEUROTOXIN ON RABIES VIRUS BINDING TO MOUSE NEUROBLASTOMA CELLS

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Summary. - Mouse neuroblastoma cells were exposed to alpha bungarotoxin, a neurotoxin known to inhibit rabies virus binding to the nicotinic acetylcholine receptor located at the neuromuscular junction in muscle tissue. The total amount of ^{3}H-CVS virus that bound to neurotoxin treated cells was separated into specific and non-specific binding using a cold competition assay. Comparison of untreated and neurotoxin treated cells demonstrated that the majority of cell-associated virus in untreated cells was of a specific nature whereas the majority of the cell-associated virus in neurotoxin treated cells was due to non-specific binding.

Key words: Rhabdovirus; challenge virus standard; alpha bungarotoxin; acetylcholine receptor

Introduction

Rabies virus is a highly neurotropic virus that is considered almost 100 % fatal once clinical signs develop in humans and animals. It has been reported that the binding site for rabies virus in the neuromuscular junctions is the acetylcholine receptor (AChR), (Lentz *et al.*, 1982). Alpha bungarotoxin (BTX), a snake venom neurotoxin from the Formosan banded krait, *Bungarus multicinctus* has been used extensively to determine rabies virus binding studies in AChR rich tissues. The amino acid sequence of the toxic loop of BTX and the G protein of rabies virus (the attachment protein) have a high degree of sequence similarity and subsequently both compete for attachment sites on the AChR found in *Torpedo sp.* (Lentz *et al.*, 1986, 1987; Bracci *et al.*, 1988). Thus BTX has been substituted for rabies virus in competition assays to determine the identity of the cell receptor for rabies virus in muscle cells. The effect that BTX has on the attachment of rabies virus to neuronal type cells has not been evaluated. This study compared the *in vitro* effects of BTX on specific and non-specific binding of rabies virus to a neuronal cell line derived from A/J (H-2) mice.

Materials and Methods

Cells and virus. Mouse neuroblastoma cells (MNA) from the cell line of A/J (H-2) (Wiktor *et al.*, 1977) were grown in Eagles minimum essential medium supplemented with 10 % foetal calf serum (FCS), 2 % L-glutamine, and 2 % minimum essential medium vitamins (EMEM). MNA cells were planted in 96 well plates at a concentration of 20,000 cells/well and allowed to monolayer overnight. All experimental manipulations investigating viral binding were conducted at 4^0C in order to prevent attached virus from penetrating the cell membrane. The challenge virus standard (CVS) strain of rabies virus was used throughout this study. CVS was grown in MNA cells in EMEM where 0.2 % bovine serum albumin was used to replace the 10 % FCS. Virus was pelleted by ultracentrifugation, the pellet was resuspended in a small amount of physiological saline (PBS) and purified in a 30–50 % sucrose gradient. CVS was labeled by the addition of 370 kBq/ml of ^{3}H leucine to leucine-deficient growth media when the virus was initially inoculated onto MNA cells. ^{3}H-CVS was purified using the same methodology as described above.

Alpha bungarotoxin. Alpha bungarotoxin, a neurotoxin produced by the Formosan banded krait snake was purchased from Sigma Chemical Company (St. Louis, Missouri).

Viral protein binding to MNA cells. The following experimental procedure was used to calculate viral binding in untreated and BTX treated MNA cells. The only difference between experimental treatments was that BTX treated cells were exposed to 1 μg of BTX during the overnight incubation. MNA cells were seeded in 96 well plates at a concentration of 20,000 cells/well and allowed to monolayer overnight. Cells were placed on ice at 4^0C, the EMEM was removed, and the monolayer was washed with cold PBS. Total binding of ^{3}H-CVS was determined by incubating MNA cells with 12.5 μg of ^{3}H-CVS and non-specific binding was estimated by incubating MNA cells with 1000 μg of unlabeled CVS and 12.5 μg of ^{3}H-CVS. At 30, 60, 120, and 180 minutes the excess viral inoculum was removed, the monolayer was washed with cold PBS and 0.05 ml of 0.1 N NaOH was added to remove the cells and cell-associated viral protein from the plate. Samples containing excess inoculum, wash, and MNA cells with cell-associated virus were placed in vials containing 4 ml of Ready Safe Scintillation Cocktail and counted in a LS1701 Liquid Scintillation Counter. The scintillation fluid and the Liquid Scintillation Counter System were purchased from Beckman Instruments (Fullerton, California).

Calculation of viral binding. Viral binding was recorded in Counts Per Minute (CPM) and interpreted into μg of protein by dividing the amount of ^{3}H-CVS that bound to MNA cells by CPM/μg of ^{3}H-CVS. The amount of virus that attached to cells in the absence of unlabeled CVS was considered to be the total amount of virus that could bind to 20,000 MNA cells. Non-specific binding was estimated to be the amount of ^{3}H-CVS that bound to MNA cells in the presence of 1000 μg of unlabelled CVS. Specific binding was derived by subtracting the amount of non-specific binding from the total amount of cell-associated virus.

Results and Discussion

The total amount of ^{3}H-CVS that was able to bind to untreated MNA cells was 0.71 μg of viral protein. Saturation of receptor sites to rabies virus on MNA cells occurred at 120 minutes (Fig. 1). Samples taken after that time did not reveal any increase in viral binding. The total binding was identified as either specific or non-specific using a cold-competition assay where the unlabeled CVS competed with ^{3}H-CVS for receptor sites. Most of the cell-associated virus was attributed to specific binding. At the sampling times of 30, 60, 120, and 180 minutes, specific binding was calculated to be 35 %, 59 %, 63 % and

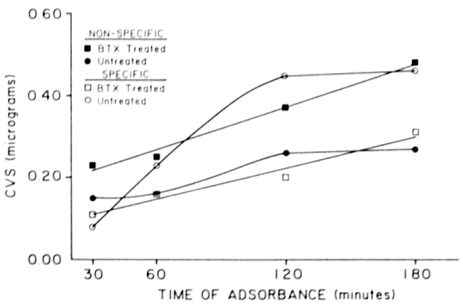

Fig. 1
Specific and non-specific binding of rabies virus to untreated and alpha-bungarotoxin treated mouse neuroblastoma cells
Cells were either left untreated or exposed to 1 μg of alpha-bungarotoxin overnight. Non-specific binding of ^{3}H-CVS corresponded to the amount of cell-associated virus that bound to cells in the presence of 1000 fold excess of unlabeled CVS. Specific binding was determined by subtracting non-specific binding from the total amount of ^{3}H-CVS that bound to 20,000 mouse neuroblastoma cells.

63 % respectively of the total amount of bound virus. Non-specific binding to untreated MNA cells progressively decreased during the sampling period.

Saturation of receptor sites to rabies virus did not occur in BTX treated MNA cells during the 180 min experimental period. At each sampling period, the total amount of viral protein that bound to BTX treated cells increased. The specific and non-specific binding trends which occured in BTX treated cells were exactly reversed to that which occurred in untreated MNA cells. When total binding was separated into specific and non-specific binding, a larger percentage was attributed to non-specific attachment. At the sampling times of 30, 60, 120, and 180 min, non-specific binding accounted for 67 %, 61 %, 65 % and 61 % respectively of the cell-associated virus. Although total binding of rabies virus to receptor sites on BTX treated MNA cells continually increased during the experiment, the percentage of cell-associated virus attributed to specific and non-specific binding remained constant.

BTX has been used successfully to compete with rabies virus for viral receptor sites in muscle tissue. The use of BTX has allowed researchers to identify the AChR as the site where the virus passes from muscle tissue into nerve tissue from which it eventually continues on to the central nervous system and brain (Lentz *et al.*, 1983; Superti et al., 1984). When BTX is incubated with MNA cells the cell membrane is altered in such a way that more non-specific than specific binding occurs. Electron miscroscopy studies have demonstrated that rabies virus enters the cell by two differrent mechanisms (Iwaski *et al.*, 1973). Binding of rabies was shown to occur in coated pit-like structures followed by entry into coated vesicles, and binding also occurred in uncoated pits. The receptor for rabies virus is also believed to be lipid associated (Superti *et al.*, 1984). These studies indicate that there is more than one cell receptor by which rabies virus enters cells. Identification of cell surface

changes that occur in MNA cells pre-exposed to BTX may reveal which membrane components are critical for rabies virus binding to specific receptor sites and which components are important in non-specific attachment.

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