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Effects of L-Glutamate on Viabilities of Cultured Diploid Skin Fibroblasts and Lymphocytes

Increased Toxicity not Observed in Huntington's Disease

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SUMMARY

The toxicity of L-glutamic acid toward cultured fibroblasts and lymphocytes from age-matched, sex-matched controls, patients with Huntington's disease (HD) and patients with other diseases has been studied. In an initial non-blinded study, L-glutamic acid (15 mM) exerted a significant toxic effect on HD fibroblasts, decreasing viability by approximately 60% after 48 h exposure. The magnitude of the toxic effect on HD fibroblasts as a group was significantly different from the mean effect on the normal control group ($P < 0.003$) and non-HD control group ($P < 0.004$). However, there was variability in the sensitivity of a given fibroblast culture to L-glutamate. This toxic effect was also seen in several normal control and non-HD control fibroblasts. In a second blinded study using cultured fibroblasts from HD patients and age-matched, sex-matched controls, we were unable to distinguish between HD and control cultures.

No difference in L-glutamate toxicity was observed between control and HD lymphocytes in short-term cultures.

We conclude that the toxicity of L-glutamate is not specific for HD cells and that this experimental approach will be of little value in identifying cells from patients with HD.

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INTRODUCTION

L-Glutamate is likely to be an excitatory neurotransmitter in brain. It has also been implicated as a potential neurotoxin, since injection of high concentrations into the striatum of animals has led to degeneration of neurons (Olney 1978). L-Glutamate is present normally in brain up to 20 mM, and it has been suggested that homeostatic mechanisms controlling glutamate levels in specific cells are impaired in patients with Huntington's disease (HD). This might involve modified membrane transport of glutamate or yet undefined receptor-mediated processes. Such abnormalities have not been documented in post-mortem brain tissue or in cells from peripheral tissues (Comings et al. 1981) from Huntington's patients. The report of Gray et al. (1980) showed that L-glutamate decreased viability of cultured diploid skin fibroblasts from patients with Huntington's disease by greater than 90%. Since this finding seemed to be specific for L-glutamate it warrants further investigation. It has implications with regard to mechanisms underlying degenerative changes in HD and raises the possibility that this might be the much sought after means for early identification of individuals with this disease.

Therefore, we undertook a study of L-glutamate toxicity in skin fibroblasts and lymphocytes from several HD patients and compared these individuals to normal controls and patients exhibiting other diseases.

MATERIALS AND METHODS

Fibroblast studies

In study 1, skin fibroblasts from 21 individuals, divided into eight groups, were studied. Each group was initially age- and sex-matched and contained one or more normal controls, and patients with HD and/or patients with medical illnesses other than Huntington's disease (myotonic muscular dystrophy, adult onset diabetes mellitus, episodic ataxia and nonspecific disease of the basal ganglia). One female was used as a normal control in two different groups. The mean age ($\pm$ SD) of normal controls, HD and non-HD disease controls were 41.3 $\pm$ 3.1, 41.9 $\pm$ 3.7 and 42.8 $\pm$ 5.9 years, respectively. These groups were either in- or out-patients of the Seattle or American Lake V.A. Medical Centers. All HD patients had adult onset of progressive chorea and dementia with a positive family history in more than one generation and radiologic or pathologic evidence of caudate atrophy in some family member.

Study 2 followed a blinded protocol and used different individuals than in study 1. Fibroblasts were obtained as described below from normal control (mean age 44.0 $\pm$ 5.2) and HD patients (mean age 43.6 $\pm$ 4.4). Cultures were then age-matched into HD + control sets and renumbered as shown in Table 1 by one of us (T.B.). Viability–toxicity studies were then carried out as described below and predictions were

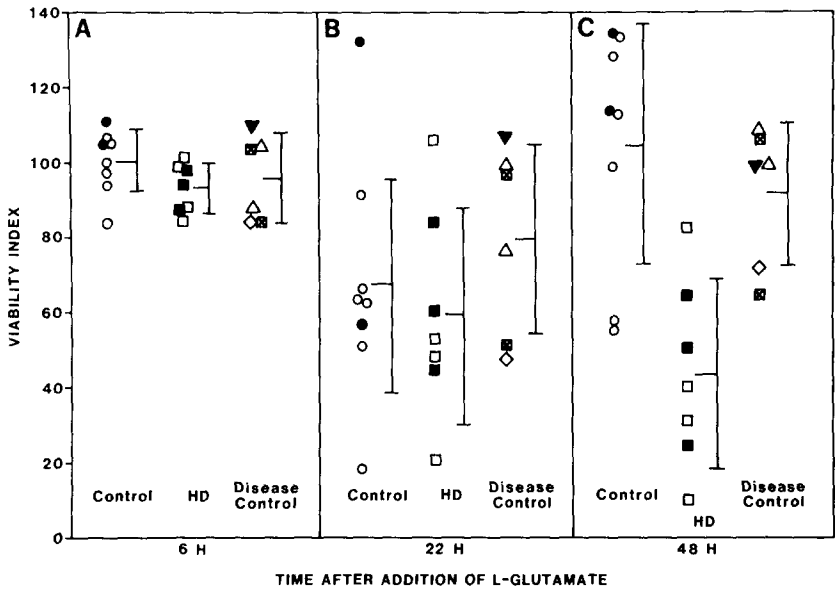

Fig. 1. Sensitivity of human diploid fibroblasts to 15 mM L-glutamate. Cultures were from study 1. Normal controls (○); Huntington's disease (□); and non-HD disease "controls": adult onset diabetes (⊠); myotonic muscular dystrophy (△); episodic ataxia (◇); nonspecific basal ganglia disease (▽). Females are designated by solid symbols. Means with SD are shown.

made about the identity of the HD cultures. The code was then broken to assess the accuracy of the predictions (Table 1).

After obtaining informed consent, all skin biopsies were obtained from the lateral aspect of the left arm, midway between the elbow and the axilla using a 4 mm skin punch after infiltration of the skin with 1% xylocaine. The skin was minced and primary outgrowth was obtained in a plastic flask in Dulbecco MEM media containing 10% fetal calf serum (FCS) and 1% penicillin–streptomycin (PS) at 36.5 °C. Cells from the primary outgrowth were released by trypsinization and were reseeded into 75 cm^2 plastic flasks, fed, and passaged until sufficient cell numbers could be obtained for storage and amino acid toxicity studies. Cells were routinely monitored and found to be free of mycoplasma. Population doublings were obtained using the procedure of Vracko et al. (1983).

Toxicity studies

For the first study, fibroblasts (passages 2–9) belonging to a given group were plated at approximately 200 000 cells/60 mm plastic dish unless stated otherwise. In the blinded study, fibroblasts at passage 3 were exclusively used. The standard medium contained Dulbecco's MEM, 20% FCS and 1% PS (3 ml/dish) at 36.5 °C. After 24 h the medium was replaced with fresh medium containing 0, 7.5, 15 or 30 mM amino acid as indicated. The final pH of these media was 7.2. Duplicate dishes were withdrawn

at 6, 22 and 48 h after addition of amino acid and were either trypsinized for cell counting (Coulter Counter) or assessed for viability using the neutral red dye uptake method (Finter 1969). Dye uptake was calculated as $A_{540\,nm}$/250000 cells. Certain indicated studies were performed using the trypan blue exclusion and/or the neutral dye uptake methods to assess cell viability. A correlation coefficient of 0.876 was obtained when cell culture viabilities varying from 5% to 100% (trypan blue exclusion method) were plotted versus $A_{540\,nm}$/250000 cells (neutral red method).

In other studies with chemically defined media, cells were initially grown up in the 10% FCS medium described above. It was observed that after attachment their proliferative capacity was not affected by substitution to a defined medium (Phillips and Cristofalo 1981; Walthall and Ham 1981) of MCDB-104 with epidermal growth factor (10 μg/ml), insulin (50 μg/ml) and transferrin (500 μg/ml).

The *viability index* for each pair (± amino acid) was calculated from $A_{540\,nm}$ in the presence of amino acid/$A_{540\,nm}$ in the absence of amino acid × 100. Therefore, a value of 100 demonstrates that the fibroblasts have no sensitivity or change in viability in the presence of amino acid at the concentration used.

Lymphocyte studies

Heparinized blood from subjects (6 patients with HD and their age-matched, sex-matched controls from study 1) was mixed with an equal volume of phosphate buffered saline (PBS) and layered on an Isolymph column (sodium diatrizoate and Ficoll, Gallard–Schlesinger, Carle Place, NY) and after centrifugation for 30 min at 400 × *g*, the lymphocyte-rich layer was drawn off with a pipette and washed 2 times with PBS. The pellet was suspended in 5 ml of culture medium containing RPMI 1640 (GIBCO, Grand Island, NY) with 5% FCS, 1% phytohemagglutinin (PHA, Wellcome Reagents Ltd., Beckenham, U.K.), and 1% PS. Total cell yield (Coulter Counter) and viability (trypan blue dye exclusion method using a hemocytometer) were determined immediately. The neutral red dye method used with fibroblasts could not be conveniently used in experiments with lymphocytes in suspension cultures.

A volume of the cell suspension containing between 500000 and 700000 cells was inoculated into the culture tubes containing media identical to that in which the cells were suspended. The final volume of each tube after inoculation was 1.0 ml. The cultures were then incubated at 37 °C, 50% humidity and 5% CO_2.

On day 3 the cultures were exposed in triplicate to 7.5, 15 and 30 mM L-glutamate, while one triplicate was left unexposed. A solution adjusted to pH 7.2 of 60 mM L-glutamate, 5% FCS, 1% PHA and 1% PS was prepared and sterilized by filtration. A 30 mM concentration was obtained in the culture tubes by adding 1 ml of the above solution to the 1 ml of cultured cells. The 15 mM and 7.5 mM concentrations were obtained by mixing 0.5 ml and 0.25 ml of the 60 mM L-glutamate solution with 0.5 ml and 0.75 ml, respectively, of the fresh culture media, and adding this mixture to the 1 ml of fresh culture media. The final volume at day 3 in all culture tubes was 2.0 ml.

After the initial assessment of cell counts and viability on day 0, viability and cell number were reassessed on days 3 and 7 using trypan blue exclusion. The day 3 assessments were made before the addition of new media to the cultures.

Lymphocyte viability index was calculated as % viable cells in presence of L-glutamate/% viable cells in absence of L-glutamate × 100.

RESULTS

Fibroblasts

We carried out initial studies with L-glutamate, D-glutamate, L-glutamine, γ-aminobutyrate (GABA) and kainate and found that 15 and 30 mM L-glutamate was highly toxic to the HD cells and less toxic to several of the control cultures at both 22 and 48 h (data not shown). Generally, the toxicity of L-glutamate, as well as that of most of the other amino acids tested, increased as the concentration was raised to 30 mM. Overall, L-glutamate was the most toxic amino acid tested, and the toxicities of the others seemed to depend on time in culture and the specific fibroblasts studied. We also observed that D-glutamate and GABA increased the viability, especially in control cultures as evidenced by a high viability index, which was often > 100. These initial experiments confirmed the relatively high toxicity of L-glutamate, but its specificity in decreasing the viability of Huntington's cells required further study.

We elected to examine L-glutamate toxicity in 3 groups of cells derived from (a) Huntington's patients, (b) age-matched, sex-matched normal controls, and (c) age-matched, sex-matched patients with other diseases. We selected 15 mM L-glutamate for further study since this concentration seemed to be generally less toxic to control cells (data not shown) and also this was in the range described by Gray et al. (1980) as being very toxic to HD fibroblasts. All of the fibroblast cultures were examined in the absence of L-glutamate to establish the number of doublings occurring in a 2 week period. These were 5.69 ± 0.95 (S.D.), 5.47 ± 0.39 and 5.80 ± 0.94 for normal controls, HD cells and non-HD disease controls, respectively. No significant difference was observed between these 3 groups, which is in accordance with the report of Goetz et al. (1981). These authors found no differences in maximum cell density, population doublings per week or colony size, between HD and control fibroblasts suggesting that both groups have similar proliferative capacities.

The sensitivity of the whole population of fibroblasts, considered without regard to pairing of controls, HD or non-HD disease patients is shown in Fig. 1. The major effect of L-glutamate was clearly seen only after 48 h. After 6 and 22 h in the presence of 15 mM L-glutamate no significant differences were observed in sensitivities between the 3 groups of fibroblasts (Fig. 1, panels A and B) and a great deal of scatter is seen in the data.

After 48 h in 15 mM L-glutamate differences in the 3 populations of cells as a whole were found (Fig. 1, panel C). The sensitivity of the HD population had a mean viability index of 43.3 compared to the normal control group mean of 104.5 and these were significantly different ($P < 0.003$) but significant overlap in the data is seen (Fig. 1, panel C). Similarly, the HD group and the non-HD disease controls (mean = 91.4) were significantly different ($P < 0.004$), but no significant differences were observed between the normal control and non-HD disease control populations.

When the sensitivities of fibroblasts from patients with HD and non-HD diseases

TABLE 1

BLINDED STUDY OF EFFECT OF 15 mM L-GLUTAMATE ON VIABILITY OF HUMAN DIPLOID FIBROBLASTS

Fibroblast culture	Age donor	% Viability[a]	Viability index[b]	HD culture	
				Predicted	Actual
1	50	63.1	54.1	×	×
2	52	86.7	75.4		
3	46	73.5	66.2		
4	45	52.1	44.8	×	×
5	48	51.8	49.2		×
6	47	6.4	5.5	×	
7	35	43.9	41.7		
8	37	1.7	1.6	×	×
9	41	3.3	2.8	×	×
10	42	11.0	9.9		
11	43	28.6	26.0	×	
12	42	48.8	43.4		×
13	43	13.1	11.5	×	
14	42	92.5	82.3		×

[a] Viability in all cultures in the absence of 15 mM L-glutamate was 95–100% when measured by trypan blue exclusion.

[b] Viability index was calculated as described in Figure 1 from determination of neutral red dye uptake on cells grown for 48 h in the presence or absence of 15 mM L-glutamate. The neutral red dye uptake studies were done concurrently with the trypan blue exclusion experiments.

were matched with the preselected, concurrently studied normal controls after 48 h in L-glutamate these differences were also apparent (data not shown) and the mean difference between paired HD and controls was significantly different ($P < 0.01$). Similarly the controls and non-HD control pairs differed with a $P < 0.04$, but these differences seem marginally significant. L-Glutamate (15 mM) was more toxic to the HD fibroblasts in 6 out of 7 control/HD pairs studied at the 48-h time point. However, L-glutamate seemed also to be more toxic to the non-HD disease controls than normal controls in 5 out of 6 pairs studied.

The mean viability indices of control fibroblasts at 22 h are clearly lower than at 48 h, whereas the mean indices of HD fibroblasts do not significantly differ at these 2 times. The large difference between control and HD populations (Fig. 1, Panel C) is reflected in this change in L-glutamate sensitivity between the 22–48 h time period for the controls.

Possible criticisms of this study are that the skin biopsies were obtained at different times and the passage numbers of the fibroblasts were not exactly matched for the toxicity studies. Also, fairly high variability in results was observed for any given culture when reassayed at different times and the study was not blinded. In view of the

results and these potential problems, we elected to perform a second (blinded) study on a new group of fibroblast cultures obtained from 7 male HD patients and age-matched and sex-matched normal controls. Details are given in the Materials and Methods section. In order to more closely follow the protocol of Gray et al. (1980), we measured % viability (trypan blue exclusion) as well as viability index of the cells. One of us (W.L.S.) predicted which member of a pair was the HD patient, based on the premise that the HD fibroblasts had lower viability than the corresponding control. As shown in Table 1, the outcome was essentially random. Only 4 of the 7 predictions were correct. In this study, we found no reproducible indication that HD fibroblasts were less viable than age-matched sex-matched controls in the presence of 15 mM L-glutamate.

Lymphocytes

The toxic effect of L-glutamate on cultured human lymphocytes from 6 of our groups was examined (data not shown). On average all concentrations of L-glutamate tested decreased the viability of the cells, but no significant differences existed between 7.5 and 30 mM L-glutamate. At all concentrations tested (7.5, 15 and 30 mM), no significant difference between the control and HD cells was found and a large scatter was seen in the data from patient to patient. Moreover, when the HD cells were matched to their controls, the difference between the paired groups was not significant. We attempted to correlate viability indices of fibroblasts and lymphocytes of specific groups, i.e. did the HD cells have the same relationship to the control when challenged with L-glutamate (higher or lower viability index) for both the fibroblasts and the lymphocytes? This correlation was positive in 3 out of the 6 groups, i.e. it appeared to be random.

DISCUSSION

The main finding reported here is that toxicity of L-glutamate is not higher in fibroblasts or lymphocytes from Huntington's disease patients than in controls or non-HD disease controls. Although our first (non-blinded) study suggested that L-glutamate toxicity was higher in HD fibroblasts than controls, we were unable to confirm this in a more rigorous, blinded study. These findings are generally in accordance with the conclusion of Archer and Mancall (1983) that L-glutamate toxicity of fibroblasts cannot be used as a criterion for identifying individuals having HD. In our blinded study (Table 1), we were correct in only one-half of the cases in predicting which cell line was from an individual with HD. This reinforces Comings' (1981) statement regarding the importance of performing such studies with blinded protocols.

It is of interest that the viability of several control cultures (Table 1) was severely reduced by 15 mM L-glutamate and the toxicity must be related to factor(s) other than HD. It should be noted that nearly all of the amino acids tested at 15 or 30 mM decreased the viability of fibroblasts. The order of decreasing toxicity was L-glutamate > kainic acid > L-glutamine. The fact that D-glutamate, the non-metabolized isomer, did not exhibit especially toxic effects compared to the L-amino acid suggests specificity in the toxicity of the latter.

The mechanism of the toxic effect of L-glutamate and other amino acids is clearly of interest. L-Glutamate and kainic acid are well known excitotoxins in the nervous system and it has been hypothesized that neurotoxicity properties are probably mediated by a depolarization mechanism at common receptors in neurons (Olney 1978). Kainic acid, when injected into the striatum of rats produces a model which mimics HD. However, in the nervous system kainic acid is 500 times more powerful a neurotoxin than L-glutamate, which has a toxicity similar to D-glutamate. This order of toxicity is clearly different from our observations in fibroblasts where L-glutamate is much more toxic than D-glutamate or kainic acid. In our experiments with fibroblasts, the toxicity was shown in the mM concentration range and it is difficult to compare these concentrations to the local concentration of neurotoxin in the striatum after direct injection (Olney 1978). Studies on the toxicities of L-glutamate and kainate have been carried out in mouse neuroblastoma cell cultures (Prasad et al. 1980), in primary cell cultures of cerebellum (Seil et al. 1981) and spinal cord (Brooks and Burt 1980). Viabilities are difficult to quantitate in the primary cultures, but in the study of Prasad et al. (1980), 10–15 mM L-glutamate inhibited growth by approximately 60–70%, whereas D-glutamate, kainate and other amino acids had little effect. Brooks and Burt (1980) found that muscarinic receptors in spinal cord cultures were lost in the presence of 1 mM L-glutamate or kainate, probably due to degeneration of a subpopulation of neurons.

Data on kainic acid and glutamate receptors in human fibroblasts have not been published. Other studies (Comings et al.1981) have not shown a defect in L-glutamate transport in HD fibroblasts. At the present time the mechanism of the toxicity of L-glutamate in fibroblasts is obscure, but probably is fundamentally different than the excitotoxicity observed after injection of L-glutamate or kainic acid into brain tissue. Moreover, in a recent review, Beverstock (1984) has critically reviewed studies which have utilized peripheral tissues and cells in search for a suitable diagnostic marker for HD. The idea of a general membrane defect extending to peripheral cell membranes clearly remains unproven and is not supported by the present study.

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