Studies on Experimental Obese Mice Part I

Studies on Hypothalamic Lesion in Mice Induced by Goldthioglucose

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INTRODUCTION

Brecher and Waxler,⁴⁾ performing the experiments of the toxicity of goldthioglucose (GTG), accidentally found the development of obesity in some mice surviving the treatment with LD-50 of the agent. Mayer et al.¹⁴⁾ found among these animals hypothalamic lesions, especially at the nucl. ventromedialis and the adjacent area, and concluded that the development of obesity was caused by these hypothalamic lesions. The following several experiments were undertaken to clarify why the GTG treatment produces lesions localized only to the hypothalamus.

EXPERIMENTAL MATERIALS AND METHODS

The animals used in these experiments were dd/S stock mice supplied from the Animal Center in Kyoto University or those offsprings bred and maintained in our laboratory. These animals were fed Oriental Co.'s laboratory chows, NMF, and water ad libitum and were carefully maintained under as constant conditions as possible.

1. Experiments on the determination of the localization of cerebral lesions.

i) Acute cerebral lesions caused by GTG treatment.

Two groups of dd/S mice of about 50 days of age, each of which consisted of 10 animals were intraperitoneally injected with 1.0 and 0.5 mg./gm. of body weight of GTG (Schering Co.'s Solganal B oleosum) respectively, and were killed by decapitation at 3 days after the treatment. The portion of the brain made by frontal sections, anteriorly at a point 2 mm. anterior to the optic chiasm, and posteriorly along the anterior margin of the cerebral peduncles, were fixed in 10% formalin and embedded in paraffin. One out of each 5 serial frontal sections was taken for hematoxylin eosin staining.

ii) GTG-trypan blue double treatment.

Ten dd/S mice of 50 days of age were treated with intraperitoneal injection of 0.5 mg./gm. of body weight of GTG, and 4 hours later of 2 c.c. of 2% trypan blue in physiological sodium chloride solution. They were treated with the same amount of trypan blue for 7 days successively at 24 hour interval. On the 8th day they were sacrificed

by decapitation. The similar portion of the brain as in i) was fixed in a solution consisting of 10 parts of formalin and 90 parts of 95% alcohol, embedded in paraffin, serially cut at $7\ \mu$ in thickness in frontal sections and stained with nuclear fast red.

iii) Cerebral lesions in GTG-induced obese mice.

Three groups of dd/S male mice of 20, 40 and 120 days of age, each of which consisted of 50 animals, were injected with 0.5 mg./gm. of body weight of GTG intraperitoneally. Every 10 animals were placed in a single cage and maintained under the same conditions. Those animals weighing over 40 gm. at 7 months of age were regarded as obese mice (GTG obese mice), and used for histological examination of the brain and other experiments in the part II in the present paper. The brain was fixed immediately after decapitation in Zenker's solution, and then the similar portion of the brain as in i) and ii) was serially sectioned $4\ \mu$ in thickness in frontal sections. Mallory's phosphotungstic acid hematoxylin staining for glial fibers and hematoxylin eosin staining were used.

iv) Histological demonstration of gold.

Three dd/S mice of 90 days of age treated with intraperitoneal injection of 0.5 mg./gm. of body weight of GTG were sacrificed at 24 hours after the treatment. The brains were fixed in absolute alcohol. Paraffin embedded tissues were cut at $4\ \mu$ in thickness in frontal serial sections. These sections were used for the histochemical demonstration of gold by Okamoto's indirect method.

2. Experiments on blood brain barrier.

i) Vital staining with trypan blue.

Five dd/S mice of 20 days of age and other 5 of 90 days were treated with intraperitoneal injection of 0.1 c.c. and 0.2 c.c. of 2% trypan blue in physiological sodium chloride solution respectively for 7 days successively (these were approximately same doses if calculated as per unit of weight). They were killed by decapitation on the 8th day, and submitted to the study described in 1. ii).

ii) Radioautography with I^{131} .

Three dd/S mice of about 90 days of age were injected intraperitoneally with a dose of $20\ \mu\text{Ci}$. of NaI^{131} dissolved in thiosulfuric sodium solution and diluted in physiological sodium chloride solution. Twenty four hours later, the blood in the brain was washed out by perfusing the heart blood with physiological sodium chloride solution. The brain was fixed in absolute alcohol. One out of each 10 frontal serial sections cut at $5\ \mu$ in thickness was submitted for radioautography using the stripping method (Fuji film for radioautography was used).

iii) Correlationship between P^{32} permeability of the cerebral blood vessels and the animal's age.

Five dd/S mice of 20 days of age and other 5 of 40 days and 9 of 120 days were injected intraperitoneally with $5\ \mu\text{Ci}$. of $\text{NaHP}^{32}\text{O}_4$ dissolved in dilute hydrochloride and diluted by physiological sodium chloride solution. Twenty four hours later, they were

sacrificed by decapitation. The blood collected from the heart and the macerated brain tissue were placed in the counting plates, weighed and dried by infrared lamp. The radioactivities of these materials were counted by Geiger-Müller counter.

$$\frac{\text{Measured counts} - \text{natural back ground}}{\text{Weight}} \text{ counts/min./mg. of the blood and the brain tissue}$$

were calculated (they were designated as n and n'). $n'/n \times 100\%$ of each animals were regarded as the permeability rate of the cerebral blood vessels.

RESULTS

1. Experiments on the determination of the localization of cerebral lesions.

i) Acute cerebral lesions caused by GTG treatment.

The cerebral lesions in the mice treated with GTG and sacrificed on the 3rd day after the treatment were found primarily in the hypothalamus.

In the mice treated with 1.0 mg./gm. of body weight of GTG, the lesions were found in the ventromedian portion of hypothalamic nuclei which correspond to the tuber cinereum. That is, posteriorly the nucl. mammillaris medialis et lateralis and the nucl. premammillaris showed moderate changes. More anteriorly the most severe changes were in the nucl. arcuatus, from which moderate changes extended dorso-laterally to the nucl. ventromedialis. And mild changes were observed in the nucl. dorsomedialis. Further anteriorly, mild changes were found in the nucl. suprachiasmaticus and in some animals in the area anterior. However, the cerebral lesions were not always distributed as described above, but in some animals the lesions were limited to the vicinity of the nucl. arcuatus.

In the other group of mice treated with 0.5 mg./gm. of body weight of GTG, lesions were always found in the nucl. arcuatus and extended to the adjacent nuclei as in the mice treated with 1.0 mg./gm. of body weight of GTG, although the changes were more localized and milder in this group.

In these cerebral lesions, the tissue showed generalized edema, and there were congestion of the small blood vessels and small foci of hemorrhage. Microgliosis was also noted, and some of microglia took the form of gitter cells and existed around the scattered small foci of necrosis. Ganglion cells were degenerated or lost near the lesions (Fig. 1 and 2). Besides these severe changes in the hypothalamus, there were dilatation and congestion of almost all blood vessels in the pia mater and also in the parenchyma of the brain, though less intense.

ii) GTG-trypan blue double treatment.

During the trypan blue treatment after GTG injection, 3 out of 10 mice died. The cerebral lesions of the remaining 7 mice, consisted of multiple foci of necrosis stained pale blue surrounded by microglia and macrophages with phagocytized blue granules, and further surrounded by a zone stained faintly blue (Fig. 3 and 4). Concerning the distribution of the blue stained tissue, it was most prominent near the nucl. arcuatus,

and also noted at the ventromedian portion of the nucl. ventromedialis. The distribution of the lesions was similar to that in the preceding experiment. However, because of the clear colour contrast, the lesions could be seen much more easily. Besides the cerebral lesions, blue granules were observed in macrophages in the pia mater and also in the epithelial cells of choroid plexus. Other cerebral parenchyma could not be vitally stained.

iii) Cerebral lesions in GTG obese mice.

Among 50 mice treated with GTG at 20 days after birth, 6 died in the first 2 or 3 days after the GTG treatment, and 21 of the remaining became obese. Among other 50 mice treated at 40 days after birth, 3 died and 13 developed obesity. Two mice died and 8 became obese among 50 mice injected at 120 days after birth. The incidence of the development of obesity among the mice injected at 20, 40 and 120 days after birth were 34, 26 and 16% respectively.

P.T.A.H. preparations of the brains of these obese mice revealed proliferation of blue stained glial fibers near the nucl. arcuatus (Fig. 5). However, in the nucl. ventromedialis and other hypothalamic nuclei gliosis was slight or non-existent. The superficial layer of the cerebral cortex showed generalized mild gliosis.

Fig. 6. Schema of hypothalamic lesions of the GTG-treated mice. AHA; Area anterior hypothalami. ARH; Nucl. arcuatus hypothalami. BST; Nucl. proprius striae terminalis. CA; Commissura anterior. CI; Capsula interna. CO; Chiasma opticum. DMH; Nucl. dorsomedialis hypothalami. FX; Fornix. GP; Globus pallidus. LHA; Area lateralis hypothalami. MFB; Fasciculus medialis telencephali. ML; Nucl. mam-millaris lateralis. MT; Tr. mamillo-thalamicus. OT; Tr. opticus. PH; Nucl. posterior hypothalami. PMD; Nucl. premammillaris dorsalis. PMV; Nucl. premammillaris ventralis. POA; Area preoptica. PVH; Nucl. paraventricularis hypothalami. RE; Nucl. reuniens thalami. ZI; Zona incerta.

Ganglion cells were decreased in number in the nucl. arcuatus, nucl. ventromedialis and nucl. premammillaris ventralis etc. Remaining ganglion cells showed atrophy and degeneration. Mild atrophy and degeneration of ganglion cells were recognized in the nucl. dorsomedialis, nucl. mammillaris medialis et lateralis, nucl. suprachiasmaticus etc. of younger mice.

Above mentioned acute and chronic hypothalamic lesions by GTG administrations were summarized in Fig. 6. As shown in the figure, though the ventromedian portion of the hypothalamus was extensively affected, it was the nucl. arcuatus that was affected constantly and most severely. Besides, the nucl. ventromedialis always showed changes though somewhat variable in degree.

iv) Histochemical demonstration of gold.

Okamoto et al.'s direct method for demonstration of gold showed negative reaction. However, if pretreated with chlorine gas, extremely fine orange granules were demonstrated in some of phagocytes of the pia mater, endothelial cells of blood vessels and epithelial cells of plexus chorioideus under highly magnification with oil immersion. No positive granules were found in any part of parenchymal brain tissue.

2. Experiments on blood brain barrier.

i) Vital staining with trypan blue.

Whole organs in the mice treated with trypan blue were stained blue. On the

Table 1. P³² permeability rate of cerebral blood vessels

Animal number & Sex	Days after birth	Body weight gm	Permeability rate %	Mean %
P 1 ♂	19	7.4	92.8	92.4 ±4.2
P 2 ♂	19	7.5	90.4	
P 3 ♂	19	7.3	97.3	
P 4 ♀	20	7.1	94.1	
P 5 ♀	20	7.0	87.6	
P 6 ♂	38	17.0	72.3	75.6 ±5.8
P 7 ♂	38	18.2	69.6	
P 8 ♂	38	17.4	73.1	
P 9 ♀	39	15.7	83.7	
P 10 ♀	39	15.5	79.3	
P 11 ♂	118	30.2	41.9	51.8 ±9.1
P 12 ♂	118	28.9	49.2	
P 13 ♂	118	27.8	59.8	
P 14 ♂	118	30.2	66.1	
P 15 ♀	125	25.3	41.5	
P 16 ♀	125	26.1	43.3	
P 17 ♀	125	24.8	59.3	
P 18 ♀	125	23.9	57.8	
P 19 ♀	121	24.7	47.0	

other hand, the brain was not stained macroscopically except in the tuber cinereum. Compared the mice treated at 20 days after birth with those of 90 days, the tuber cinereum of the former took bluish colour more deeply. Histologically as well there was a tendency that the vital-staining reaction was more intense in the former. Phagocytes in the pia mater, epithelial cells of the plexus chorioideus and endothelial cells of blood vessels were vitally stained. However, in parenchyma of the brain, extremely small amount of fine granular pigments were recognized only around the blood vessels in the nucl. arcuatus. Scattered very fine blue granules were also found in the nucl. ventromedialis and nucl. premammillaris ventralis etc.

ii) Radioautography with I^{131} .

Radioactivity was found in the plexus chorioideus and hypothalamus. The nucl. arcuatus was the part with the strongest radioactivity in the hypothalamus. The activity decreased in the degree gradually towards the more dorsal and lateral portion (Fig. 7). The pituitary stalk also showed positive reaction.

iii) Correlationship between P^{32} permeability of the cerebral blood vessels and the animal's age.

The rates of P^{32} permeability of the cerebral blood vessels of 3 groups of mice which consisted of the animals of 20, 40 and 120 days of age were shown in Table 1. The mean permeability rate was 92.4 ± 4.2 , 76.5 ± 5.8 and $51.8 \pm 9.1\%$ respectively.

DISCUSSION

The appearance of the stereotaxic apparatus made it possible to destroy and stimulate selectively any desired nucleus. Hetherington et al.¹⁰⁾ found that the destruction of hypothalamus caused the development of obesity. Hetherington¹¹⁾ also demonstrated that the hypothalamic destruction of hypophysectomized rat also caused the development of obesity, and concluded that the development of obesity had no relationship to the pituitary. It was also found that the destruction of hypothalamus especially the ventrolateral portion of the nucl. ventromedialis caused hyperphagia and obesity.

On the other hand, the fact that the destruction of a part of hypothalamus caused the decrease or disappearance of appetite was found by Hetherington and Ranson.¹⁰⁾ Anand et al.^{1,2)} located the center to be in the area lateralis hypothalami; they⁵⁾ could increase the animal's appetite by electrically stimulating this area. Two chemical agents, hypertonic sodium chloride¹³⁾ and adrenergic agents⁹⁾ are known to increase the appetite when injected directly into the area lateralis hypothalami.

Epstein⁷⁾ reported that the injection of procain and hypertonic sodium chloride into the nucl. ventromedialis caused the increase and decrease of appetite respectively, and on the other hand, the injection of sodium chloride and procain into the lateral portion of hypothalamus caused the decrease and increase of appetite respectively.

Based on these findings, it is the generally accepted view that the area lateralis hypothalami is the fascilitating center of appetite and that the nucl. ventromedialis is

the satiety or inhibiting center of appetite.

Brecher et al.⁴⁾ accidentally found the fact that the injection of GTG caused the development of obesity in mice. It was clearly demonstrated by Mayer et al.¹⁴⁾ that this development of obesity was caused by the destruction of hypothalamus especially the nucl. ventromedialis made by GTG treatment.

Mayer¹⁵⁾ proposed the glucostatic theory concerning the regulation mechanism of appetite. That is, the arterio-venous difference of or utilizing rate of blood glucose in the glucoreceptor which is located in the hypothalamus regulates appetite. Mayer et al.¹⁴⁾ suggested that the nucl. ventromedialis should be regarded as the glucoreceptor on the following bases. First, GTG which has a glucose radicle as its component destroys selectively the nucl. ventromedialis. Nevertheless other chemically similar compounds which have gold but not glucose radicle do not give any specific effect on this nucleus. Second, this nucleus is the center of appetite. Third, there is a report by Larsson¹³⁾ that this portion of appetite center concentrates much more radioglucose than other portions. It may be concluded from this hypothesis of Mayer that GTG which has glucose radicle is concentrated selectively to the nucl. ventromedialis because this is the glucoreceptor, and that the destruction of this portion takes place as a result.

Contrally to this hypothesis of Mayer, Perry and Liebelt¹⁷⁾ proposed that the destruction caused by GTG is due to the inborn error of the blood brain barrier in this portion, on the basis that the cerebral lesions by GTG were not localized to the nucl. ventromedialis but extended to other cerebral portions. As mentioned already in this paper, the cerebral lesions by GTG, if only hypothalamus is concerned, were not limited to the nucl. ventromedialis which has been regarded as the glucoreceptor by Mayer, but showed wider distribution as shown in Fig. 6. Although the distribution varied to some extent according to the age of the animals or the dose of GTG, the main lesion was in and around the nucl. arcuatus which was affected without fail. The destruction of the nucl. ventromedialis and other neighboring nuclei in the hypothalamus were milder than that of the nucl. arcuatus. The cerebral lesions by GTG were widely distributed, and did not correspond with the portion which has been regarded as the glucoreceptor. Therefore it is difficult to conclude that the nucl. ventromedialis being the glucoreceptor is playing the most important role in causing the cerebral lesions.

In 1885, Ehrlich⁶⁾ discovered that intravenous injection of coerulein-s, an acidic dye, caused vital-staining of all organs except for the brain. Goldmann⁸⁾ performed systemic experiments and stated that the existence of blood brain barrier kept the brain from vital-staining. However, it has been known in the normal brain that mesodermal component of meninx, blood vessels, and ectodermal components such as endothelial cells of the pia mater, epithelial cells of the plexus chorioideus and some of ependymal cells are subjected to vital-staining.¹²⁾ Besides, the tuber cinereum, however, is vitally stained as an exception in spite of being the cerebral parenchyma. In the experiment on vital-staining with trypan blue in this paper, very small amount of pigment granules were

found in the neighborhood of the infundibulum. Bakay³⁾ regards the hypothalamus as an "area of normally increased permeability."

Radioautograph also clearly demonstrates the distribution of permeability. My experiment revealed that the cerebral part which corresponds to the nucl. arcuatus is the area of the highest permeability. This result accords with Wsniowski et al.'s¹⁹⁾ result of the experiments in cats.

It is my opinion that the specific localization of cerebral lesions by GTG can be explained on the basis of blood brain barrier. As mentioned above, GTG-induced cerebral lesions were more extensive among younger mice, and these mice showed the higher incidence of the development of obesity. These facts can be explained by the fact that the younger mice have weaker blood brain barrier as known by the permeability experiment with P^{32} . It is probably true as mentioned by Mayer¹⁴⁾ that the existence of glucose radicle in GTG increases the permeability of this substance.

The GTG-trypan blue double treatment in this study was performed for the purpose of making color contrast between the injured and intact normal portions of the brain tissue. This color contrast was made by the following mechanism; GTG-induced lesion causes the destruction of blood brain barrier, this in turn increases the localized permeability of the blood vessels and permits the vital-staining of the destroyed cerebral portion. By this method minute lesion can be demonstrated clearly. Histochemically no significant amount of gold was not demonstrated in the cerebral parenchyma. According to Swartz,¹⁸⁾ the amount of gold was also at the minimum demonstrable border by chemical method.

SUMMARY

A study was undertaken to clarify the mechanism by which the specific localization of the hypothalamic lesions takes place by injection of goldthioglucose (GTG) which causes the development of obesity in mice.

1. A new method for demonstration of the cerebral lesion caused by GTG, GTG-trypan blue double treatment, was established.

2. From the histological results obtained by above-mentioned method, and stained by hematoxylin and eosin and phosphotungstic acid hematoxylin, the severest lesion produced by GTG was in the nucleus arcuatus. Some changes, though variable in degree, were always recognized in the nucleus ventromedialis.

3. The results of trypan blue vital-staining and radioautography with I^{131} indicated that the area of most increased permeability within the brain was the neighboring portion of the nucleus arcuatus.

4. There was a tendency that the incidence of development of obesity by GTG treatment was higher among younger mice.

5. The experiments with P^{32} showed a tendency that younger mice have the increased permeability within the brain.

On the basis of above-mentioned evidences, the following conclusion can be drawn: there is entirely intimate correlation between the specific localization of hypothalamic lesion caused by GTG and the specificity of the hypothalamic blood brain barrier in mice.

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Fig. 1. Hypothalamic lesion of the GTG-treated mouse. Three days after treatment (H.E. $\times 20$)

Fig. 2. Detail of previous figure, showing small hemorrhage, edema and microgliosis (H.E. $\times 100$)

Fig. 3. Hypothalamus of the mouse the mouse treated with GTG and trypan blue vital-staining (Trypan blue vital-staining and nuclear fast red $\times 20$)

Fig. 4. Detail of previous figure, showing accumulation of macrophages with trypan blue granules (Same as previous figure. $\times 400$)

Fig. 5. Gliosis in the hypothalamus of a GTG-induced obese mouse (P.T.A.H. $\times 100$)

Fig. 7. I^{131} -radioautograph of hypothalamus ($\times 20$)