Induction of hypoglycaemia in Japanese encephalitis virus infection: the role of T lymphocytes

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SUMMARY

We report here development of hypoglycaemia in the convalescent phase of Japanese encephalitis virus (JEV) infection in mice by the induction of antigen-specific Ly1\(^2\) T cells in the spleen which mediate hypoglycaemia through the generation of soluble T cell hypoglycaemic factor (TCHF). The TCHF acted in a dose-dependent manner and was found to be trypsin-sensitive and thermolabile. It was purified on Superose-12 high performance liquid chromatography (HPLC) gel filtration column and purified protein migrated as a \(25\)-kD band on SDS–PAGE. The JEV-induced hypoglycaemia coincided with an increased circulating glucagon level, without any alterations in blood insulin and growth hormone concentrations. These effects were mimicked by TCHF. These results indicate that JEV-primed T lymphocytes mediate hypoglycaemia through the production of a soluble hypoglycaemic factor.

Keywords hypersglycaemia Japanese encephalitis virus T cell factor T suppressor cell glucagon

INTRODUCTION

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus, responsible for epidemic encephalitis in South-East Asia [1]. Fatality rates are between 21% and 40%. Following entry into the host system, JEV replicates in various organs and generates an inflammatory response [2] with brain congestion, haemorrhage and neuronal degeneration [3]. Clinical manifestations of the disease are fever, headache, vomiting leading to convulsions and altered sensorium. Disturbance of protein, fluid and carbohydrate metabolism has been detected in large numbers of JE patients [4]. The histopathology of JE has been studied in great detail [5], while little is known regarding biochemical alteration during infection. Our earlier studies revealed a significant fall in serum iron levels in JEV infection, both in humans [6] and in mice [7], caused by the action of macrophage-derived factor, which blocks the release of iron from splenic reticuloendothelial cells [7]. We recently showed leakage of plasma protein and infectious JEV in urine [8].

Marked hypoglycaemia (blood glucose 54–70 mg%) is noticed during JEV infection [4], but its triggering mechanism is not known. Hypoglycaemia could be dangerous because glucose is the primary energy substrate of the brain and its absence may result in deranged functions and tissue damage, and could lead to death [9].

Hypoglycaemia has been reported in few viral infections. Samson et al. [10] have reported recurrent hypoglycaemia during viral hepatitis, which is associated with hyperinsulinaemia, while Oldstone et al. [11] have observed that lymphocytic choriomeningitis virus perturbs the endocrine functions without any change in serum cortisol, or insulin levels. Hypoglycaemia with hyperinsulinaemia have been reported in \textit{Plasmodium falciparum} malaria [12,13]. Considering that the mechanism of production of hypoglycaemia during JEV infection is not known, the present study was undertaken to examine this phenomenon in great detail.

MATERIALS AND METHODS

Animals and virus used

The study was performed on inbred Swiss albino mice aged 5–6 weeks, obtained from the mouse colony maintained in this Department, and the inbred BALB/c mice were purchased from the Central Drug Research Institute, Lucknow. JEV strain 78668A was propagated in suckling mouse brain and was used as infected mouse brain suspension, as described earlier [14]. The virus titre was \(10^{6.2}\) LD\(_{50}\)/ml. This produced uniform sickness and 100% mortality by day 6 when given intracerebrally in mice, but intraperitoneal (i.p.) inoculation produced no clinically evident disease.

Priming of mice

The mice were given a single i.p. dose of 0.3 ml of \(10^{2}\) LD\(_{50}\) of JEV, while control mice were inoculated with 0.3 ml of 10% normal mouse brain suspension. They were fed \textit{ad libitum} on the standard commercial laboratory chow and water, and were fasted for 18 h, before bleeding.
Hypoglycaemia in JEV

Purification of hypoglycaemic factor

T cell hypoglycaemic factor (TCHF) was prepared from JEV-infected mouse spleen. Briefly, mice were given 0.3 ml of 10^2 LD_50 of JEV intraperitoneally. On day 20 post-infection the spleens were collected and the splenic T cells were separated by a panning procedure as described above. Cells were washed three times with Hanks' balanced salt solution (HBSS), viability was checked and the purity was checked by treating the cells with anti-mouse IgG antibody (1:100 diluted in 0.05 M Tris) and then layered with 2-ME and antibiotics was layered in glass Petri dishes and incubated in moist air with 5% CO_2 at 37°C for 2 h. The supernatant with non-adherent cells was collected. A macrophage-enriched subpopulation was obtained by collecting the glass-adherent cells. About 97% of these cells phagocytosed latex particles and were considered as macrophages. Enriched subpopulations of T and B lymphocytes were obtained by a panning procedure from glass-non-adherent cells [15]. Briefly, 1.5 x 15 cm polystyrene Petri dishes were coated with anti-mouse IgG antibody (1:100 diluted in 0.05 M Tris) and then layered with 1 x 10^8 glass-non-adherent cells in MEM–HEPES for 70 min. Thereafter, the supernatant with non-adherent cells was collected. Of these cells, >95% consisted of T lymphocytes, as judged by treatment with anti-Thy1.2 antisera. The bound cells were gently scraped out using a rubber-tipped glass rod, washed with PBS, and the purity was checked by treating the cells with anti-mouse IgG antibody and complement. Of this cell population, >94% was considered B lymphocytes.

Assay of blood glucose

Blood glucose was assayed using Haemo-Glukotest strips (Boehringer, Mannheim, Germany) which were read in a glucometer for quantitative measurement and expressed as mg/dl. Before this, mice were fasted for 18 h but had free access to water. Blood samples were repeatedly taken from the same mice (n = 8–10). Data were statistically analysed by Student’s t-test and mean ± s.e.m. was calculated.

Preparation of anti-TCHF antisera

The anti-TCHF antibody was prepared in mice. Purified TCHF (100 µg) emulsified in Freund’s complete adjuvant was injected intramuscularly in the inner side of the flank, and the same dose was repeated at 3 week intervals with Freund’s incomplete adjuvant. Then three intradermal injections at weekly intervals were given without any adjuvant, followed by an intravenous injection 4 days before bleeding. The serum was collected, inactivated at 56°C for 30 min. The optimal dilution of antibody which abrogated the hypoglycaemic response was determined and sera were stored at −70°C.

RESULTS

Production of hypoglycaemia during JEV infection

The fasting plasma glucose levels of inbred Swiss albino mice were measured on alternate days after priming with 0.3 ml of 10^2 LD_50 of JEV intraperitoneally. As presented in Fig. 1, the mice developed a transient decline in their blood glucose from day 18 p.i. The maximum decrease was observed between days 20 and 22 p.i. The mean fasting plasma glucose concentration of 58 ± 8 mg/dl in JEV-infected mice was significantly lower (P < 0.001) than the mean observed in control (121 ± 14 mg/dl). These findings show the development of hypoglycaemia in the convalescent phase of JEV infection. There was no significant difference in fasting blood glucose in the initial stage of infection compared with controls.

In an attempt to investigate the effect of strain variation in production of JEV-induced hypoglycaemia, blood glucose levels in inbred BALB/c mice were measured on different days following i.p. inoculation of JEV. Findings presented in Fig. 1 show a fall in blood glucose level from day 18 following JEV infection, similar to that observed in the Swiss albino strain of mice.

In all further experiments Swiss albino mice were used.

Hormonal alterations during JEV infection

The findings presented above show a significant fall in blood glucose levels of mice during JEV infection. We investigated...
whether this could be due to alteration of synthesis of carbohydrate regulating hormones, e.g. insulin, glucagon or growth hormone. Findings summarized in Fig. 2 show no change in circulating insulin (mean value 226 ± 27.3 μU/ml on day 20 p.i. versus 219.87 ± 21.2 μU/ml in controls) and growth hormone levels (mean value 0.55 ± 0.03 pg/ml on day 20 p.i. versus 0.56 ± 0.07 pg/ml in controls) throughout the study period. The glucagon levels showed a transient rise from day 18 p.i. onwards and the increased levels were maintained up to day 20 after JEV inoculation (262 ± 19.8 pg/ml versus 177.28 ± 14.87 pg/ml in control) (Fig. 2).

Production of hypoglycaemic factor in spleen during JEV infection
In order to know the mechanism(s) of hypoglycaemia in JE, efforts were made to study the role of the spleen in this phenomenon. Spleens were collected 20 days after JEV infection (i.e. coinciding with the time of maximum fall in blood glucose) and the ability of the 24 h spleen cell culture supernatant to produce hypoglycaemia by in vivo inoculation in mice was studied. Injection of 200 μg of JEV-primed spleen cell culture supernatant intravenously in mice showed significant reduction in blood glucose between days 8 and 10 after inoculation compared with control mice, which received 200 μl of normal splenic cell culture supernatant (Fig. 3).

Identification of cell type producing hypoglycaemic factor
To delineate the splenic cell type responsible for the production of hypoglycaemic factor, the splenic single-cell suspension prepared on day 20 p.i. was enriched for macrophage, T and B lymphocyte subpopulations, and individual subpopulations were cultured in saline at 37°C. The 24-h culture supernatants were collected and tested for hypoglycaemic activity by i.v. inoculation into mice. The hypoglycaemia occurred 8–10 days after inoculation of JEV-primed T lymphocyte supernatant only (P < 0.05), while macrophage and B lymphocyte culture supernatants failed to produce any significant alteration in plasma glucose compared with controls (P > 0.05) (Table 1). This shows that during JEV infection, splenic T lymphocytes secrete a factor which mediates hypoglycaemia. We named this factor T cell hypoglycaemic factor (TCHF).

Surface phenotype of T cells
The splenic T cells mediating hypoglycaemia on day 20 after JEV priming were treated with anti-Ly1.2 or anti-Ly2.1 antibody and complement as described earlier [26] before they were assayed for their ability to alter blood glucose concentration in mice. It was observed that anti-Ly1 antisera treatment of JEV-primed splenic T cells did not abrogate the hypoglycaemic activity, while treatment with anti-Ly2 antisera abrogated it completely (Table 2). Hence, the culture supernatant of Ly2+ splenic T cells collected on day 20 following JEV infection in mice was used as hypoglycaemic factor.
throughout the study. The supernatant was concentrated by freeze drying, 100 μg of the crude protein were applied to Superose 12 FPLC column and eluted with 0.1 M PBS pH 7.4. Figure 4 shows the elution profile of different proteins present in the supernatant. Each of the peaks obtained was concentrated and tested for hypoglycaemic activity, which was recovered in peak III. The corresponding fractions were concentrated, and repurified by reversed-phase high performance liquid chromatography (HPLC) on Pep-S column (Pharmacia) using 70% acetonitrile as elution solvent. The purified TCHF was run on SDS–PAGE in 10–15% linear gradient polyacrylamide gels for molecular weight determination. Silver staining of gels revealed that TCHF migrated as a single 25-kD band on SDS–PAGE (Fig. 5).

Characterization of TCHF
The sensitivity of purified TCHF was screened to proteases at different time periods. The findings presented in Table 3 show that i.v. injection of TCHF in mice incubated for 4 h with trypsin resulted in no change in hypoglycaemic activity, while prolonged incubation with the serine protease caused 98.4% loss in activity, indicating that TCHF is proteinaceous in nature. TCHF was found to be heat-sensitive, as incubation at 37°C for 3 h had no effect on hypoglycaemic activity, whereas exposure to 56°C for 1 h and to 100°C for 15 min resulted in 38.5% and 53.2% reduction in hypoglycaemic response, respectively. There was partial inactivation of its activity at acid and alkaline pH.

Effect of anti-TCHF antisera treatment
The response to TCHF was inhibited by pretreatment of mice with anti-TCHF antibody (plasma glucose 111.9 ± 7.6 mg/dl versus TCHF induced-decrease in plasma glucose 57.2 ± 4.7 mg/dl). Normal mouse serum had no effect on lowering in glucose levels mediated by TCHF (mean value 52.8 ± 8.9 mg/dl).

Hormonal study after TCHF inoculation
Glucagon, insulin and growth hormone were measured by RIA on different days after injection of 200 μg of purified TCHF (Fig. 6). Serum glucagon levels rose gradually after injection with peak titres on day 8, and fell sharply by day 14 p.i. Serum insulin and growth hormone levels, however, remained almost the same throughout the study period. This indicates that the changes in circulating concentration of glucagon during JEV infection could be attributed to the secretion of TCHF.
Further studies have suggested that this immunosuppression leads to evasion of anti-viral immune mechanisms, resulting in persistence of virus in T cells [20]. Our present study shows that time of production of hypoglycaemia following JEV infection coincides with the appearance of suppressor cells.

The TCHF was purified by HPLC gel filtration on Superose 12 column and was found to be thermolabile and partially sensitive to acid and alkaline pH. On the basis of protease sensitivity it appears to be a polypeptide. The purified TCHF was shown to have an approximate mol wt of 25 kD. We have not come across any description of release of hypoglycaemic factor during JEV infection.

A frequent feature of most of the viral infections is hyperglycaemia. Mice infected with reovirus [21], encephalomyocarditis virus [22], coxsackie B4 and mumps [23] and scrapie virus [24] show increase in blood sugar levels. However, virus-induced hypoglycaemia is rare. Despite extensive study, the factors responsible for the reduction of blood glucose are unknown. Fulminating hepatitis with recurrent hypoglycaemia has been reported [10]. In lymphocyte choriomeningitis virus (LCMV) infection in mice, perturbation of endocrine function resulting in altered synthesis of growth hormone is reported to be responsible for hypoglycaemia [11]. As a corollary to this, canine distemper virus-induced fall in blood sugar is known to occur due to a reduction in norepinephrine and dopamine concentrations [25]. Alternatively, cytokine released during the immune response could also mediate hypoglycaemia. Rey & Besedovsky [26] have reported that low doses of IL-1 can affect glucose metabolism by increasing blood insulin levels, which may in turn serve to satisfy the enhanced metabolic demands during infection.

Sufficient evidence now exists regarding the role of insulin-like growth factors (IGF)-I and II in the regulation of blood glucose. In general, they are considered to be anabolic hormones, and the changes in glucose metabolism by IGFs could be attributed to decreased hepatic glucose production, or secretion of factors that enhance glucose uptake [27].

Hypoglycaemia may be considered to be a consequence of the catabolic phase of carbohydrate metabolism coupled with secretion of counter regulatory hormones such as insulin, glucagon, epinephrine, cortisol and growth hormone [9]. In the present study we tried to find out whether JEV infection or TCHF results in alteration of circulating levels of hormones, thus contributing towards hypoglycaemia. We found that there occurred a rise in circulating glucagon levels coinciding with the production of hypoglycaemia. No change in growth hormone or insulin concentrations was observed at any time after JEV or TCHF inoculation. No infectious JEV virus from the pancreas at any time following infection was isolated, suggesting thereby that a direct effect of the virus on pancreas is unlikely. Thus, this study suggests that hypoglycaemia during JEV infection may be attributed to the production of TCHF, which mimics the effects of JEV. At the present juncture, however, its mechanism is not clear. Studies are in progress to confirm these results.

![Fig. 5. SDS–PAGE of purified T cell hypoglycaemic factor (TCHF). Lane 1 shows molecular weight markers. Lane 2 shows position of TCHF (arrow).](Image)

![Fig. 6. Blood insulin (■; μU/ml), glucagon (○; pg/ml) and growth hormone (●; ng/ml) levels in T cell hypoglycaemic factor (TCHF)-inoculated and control (C) mice.](Image)
underway to investigate whether TCHF induces a fall in blood glucose by eliciting an over-utilization of glucose in the muscle, or by increasing the hepatic glucose uptake by measuring C14-glucose incorporation in organ explants.

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