MICROBIAL PATHOGENICITY

Immune responsiveness in a rat model for type II diabetes (Zucker rat, fa/fa): susceptibility to *Candida albicans* infection and leucocyte function

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There is a causal relationship between obesity-associated diabetes and an increased risk of infection. The ability of obese (fa/fa) Zucker rats, a model for non-insulin-dependent diabetes mellitus (NIDDM), to clear *Candida albicans* from the circulation and tissues was compared to that of lean (Fa/fa, Fa/Fa) Zucker rat controls as a measure of immune function. The ID₅₀ necessary to establish tissue colonisation in lean Zucker rats was 1.18 log₁₀ times greater than that determined for the obese Zucker rats. Nine days after intravenous (i.v.) injection of a yeast suspension, the organs of obese rats had a 10-fold greater yeast/g organ burden than did lean rats. The kidney was determined to be the primary target organ for colonisation. Germ-tube formation by *C. albicans* occurred at a rate 1.5 times faster in serum from obese rats than in serum from lean rats. Peritoneal polymorphonuclear leucocytes, resident macrophages and thioglycollate-elicited macrophages from lean Zucker rats displayed a significantly higher ability to kill ingested yeast cells than analogous cell populations from obese Zucker rats.

Introduction

A causal association between obesity and maturity onset (type II) diabetes and an increased susceptibility to infectious agents is well recognised [1–7]. The exact mechanisms underlying the higher incidence of infections are not completely understood, although clinical studies have shown an association between hyperglycaemia and hypercholesterolaemia and reduced phagocytic capability of granulocytes [1, 5].

The obese Zucker rat (fa/fa) is an animal model for obesity-associated type II diabetes in man. This model is used currently for the study of endocrine disorders and metabolic abnormalities associated with non-insulin-dependent diabetes mellitus (NIDDM) [8, 9]. Heretofore, there has been no assessment of the immune competence of Zucker rats. Because obese Zucker rats have many of the metabolic characteristics of obesity-associated type II diabetes in man, this animal could provide an appropriate model for studying the relationship between immune dysfunction and obesity-associated type II diabetes. The aim of this study was to examine the usefulness of this model for the study of type II diabetes-associated immune

dysfunction. The immune competence of the obese Zucker rat was characterised both *in vivo* and *in vitro*.

Materials and methods

Animals

Six-week-old lean and obese male Zucker rats were purchased from Charles Rivers Laboratory Inc. or acquired from an in-house breeding facility. Lean rats (Fa/fa, Fa/Fa) and homozygous obese (fa/fa) pairs were held under identical conditions in the animal facility for 18 weeks to allow full development of the obese state in homozygous recessive animals. Both lean and obese rats were given free access to rat chow and water.

Yeast cells

A human clinical isolate of *Candida albicans* was used for all experiments. Stock cultures were stored in Sabouraud dextrose (SD; Difco) broth with glycerol 10% at –80°C. Working cultures were maintained on SD agar slants at 4°C and passaged once after 1 month. For each experiment, the *C. albicans* strain was inoculated into SD broth and incubated for 24 h at 37°C on a gyratory shaker. After incubation, the yeast cells were pelleted by centrifugation (500 g, 5°C, 15 min), washed three times with normal saline and resuspended in pyrogen-free saline at required

concentrations as determined by haemocytometer count and confirmed by standard plate viable count.

Plasma analyses

Plasma triglycerides, glucose and cholesterol concentrations were analysed with kits purchased from Sigma kit nos. 339-20, 115-A and 352-20, respectively). The concentration of HDL cholesterol was estimated by precipitating the serum with heparin and manganese chloride. The concentration of HDL cholesterol in the supernate was then determined (Sigma, kit no. 352-3) [10]. Blood glycosylated haemoglobin was determined with a kit obtained from Helena Labs (kit no. 5351; Beaumont, TX, USA).

Infectivity studies

Age-matched pairs of lean and obese rats were infected with i.v. injections of *C. albicans* suspended in 0.5 ml of pyrogen-free saline at the following doses: 8×10^2 , 4×10^3 , 2×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , 5×10^6 and 1×10^7 blastospores/rat. Three lean-obese pairs of rats were injected with doses $\geq 10^6$ blastospores. Five lean-obese pairs (five lean and five obese rats) were used for all other doses. At daily intervals, blood (0.1 ml) was removed aseptically from the tail vein of ether-anaesthetised animals and inoculated on to SD agar for plate count determination.

All animals given 1×10^7 or 5×10^6 yeasts/animal died at days 3–6. At day 9 after infection, all remaining live animals were killed; blood smears were made and stained with Wright's stain for differential blood cell counts.

To assess yeast colonisation of the organs, each animal was anaesthetised with ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively) before being killed. Spleen, heart, lung, liver and kidney were removed aseptically, weighed, and dissociated in the presence of phosphate-buffered saline (PBS, pH 7.2) in Dounce homogenisers. One-ml samples of organ homogenates and dilutions of the homogenates were plated directly in duplicate on to SD agar and the number of organisms/g of organ tissue was determined by standard plate count. Representative colonies were confirmed as *C. albicans* by microscopic observation, biochemical profile (API Yeast-Ident System, Analytab Products, Plainview, NY, USA), colonial appearance on SD agar and germ-tube formation in serum.

Histology

The kidneys from infected animals not used for ID50 determinations or organ colonisation experiments were fixed in formalin, cut in half and processed by established paraffin infiltration and embedding techniques. They were then serially sectioned at 5- μ m intervals and stained with Grocott's application of

Gomori's methenamine silver stain for fungi followed by haematoxylin and eosin [11]. Each section was coded, examined microscopically and scored for the presence of yeasts, hyphae and inflammatory cells. After analysis, the code was broken and data were analysed.

Germ-tube assay

C. albicans was grown in SD broth for 24 h at 37°C. Yeast were pelleted (500 g, 10°C, 15 min) then suspended in 0.3 ml of serum (10^6 blastospores/ml) from either lean or obese Zucker rats. At various time intervals, the number of cells with germ tubes/ml was determined microscopically by haemocytometer count. The rate of germ-tube elongation for 50 cells with germ tubes was determined microscopically with an eyepiece micrometer. All assays were done in triplicate and repeated three times.

Phagocytosis assay

Resident peritoneal macrophages were obtained after lavage of the peritoneal cavity with 30–40 ml of PBS. Thioglycollate-elicited macrophages were obtained 4 days after i.p. injection of 10 ml of 4% aged (6 months) thioglycollate broth. Polymorphonuclear leucocytes (PMNLs) were obtained 18 h after i.p. injection of 10 ml of a 4% aged (6 months) thioglycollate broth. In each case, peritoneal washes (30–40 ml PBS) were collected through an abdominal incision and the cells were pelleted by centrifugation (200 g, 10 min). The cell pellet was suspended in cold RPMI media (Gibco Laboratories, Grand Island, NY, USA) to a concentration of $c. (2-4) \times 10^6$ cells/ml, as determined by haemocytometer count, and layered on to 22×40 mm glass coverslips (0.5 ml/coverslip). The various cell populations were allowed to adhere for 45 min (37°C, CO₂ 5% in air) after which the monolayers were washed in warm (37°C) PBS and used immediately. More than 95% of the adherent cell populations identified as macrophages reacted positively by direct fluorescent staining with mouse-anti-rat monoclonal antibody (MAb) specific for rat macrophages and monocytes (MCA 341; Serotec, Inc). More than 90% of the adherent cells obtained 18 h after thioglycollate injection were confirmed as PMNLs by morphological and histological characterisation and a negative reaction with the MCA 341 MAb.

The neutral red technique was used to microscopically determine the ability of the various cell populations obtained from obese and lean Zucker rats to phagocytose and kill non-opsonised *C. albicans* [12, 13]. *C. albicans* blastospores were grown in GYEP medium (glucose 1%, yeast extract 0.3% w/v, Bactopeptone 1% w/v; Difco Laboratories) to stationary growth phase. Yeast cells were harvested by centrifugation and suspended in RPMI media without serum to yield an effector:target ratio of 1:1 (2–

4×10^6 yeast/ml). At this effector:target ratio, phagocytosed *Candida* cells could still be observed. This ratio was used for all experiments. Viability was $>97\%$ as determined by trypan blue dye exclusion test. The yeast suspension (0.5 ml) was added to the adherent cell monolayers. The adherent cells were then incubated for 1 h (37°C , CO_2 5% in air). Fifteen min before the end of the 1-h incubation period, $10 \mu\text{l}$ of a neutral red solution (10 mg/ml in PBS) was added to each coverslip. The incubation period was continued for 15 min, following which the coverslips were washed in PBS and examined immediately. Fifty phagocytosing cells were scored for the number of red (dead) and colourless (viable) yeast cells. Only cells containing blastospores were counted. The phagocytic capacity = number of yeast/50 phagocytes; the candidacidal activity = number of dead yeast cells/50 phagocytes $\times 100$. The correlation of the neutral red to viability was assessed by comparing colony formation [14] with results from the neutral red assay [12]. Resident macrophages from outbred Sprague Dawley rats (Charles River) were harvested and processed and monolayers were prepared (2.5×10^6 cells/coverslip) as described above. The monolayers were then infected with *C. albicans* at a target:cell ratio of 1:1. After incubation for 1 h, the coverslips were scraped with a rubber policeman and dilutions of the cell suspension were made in distilled water. Sabouraud dextrose pour plates were made. The number of colony-forming units (cfus) was determined after incubation for 24 h at 37°C and compared with control cultures consisting of *C. albicans* incubated on coverslips without macrophages. The neutral red assay was done as described above with a portion of the same macrophage and yeast suspension used for the plate count assay.

Statistical analysis

Depending on the experimental design, data were analysed either by the paired Student's *t* test, a χ^2 , one-way and two-way ANOVA (comparison of the different phagocytic capacities) or linear regression. In some cases the data were transformed accordingly: percentages by the arcsin square root of the percentage and distribution by the square root for the statistical analyses, but the data were presented untransformed in Table 1 and Figs 1–3.

Results

Comparison of physical characteristics and blood values of lean and obese rats

Twelve lean-obese pairs of age and sex-matched uninfected Zucker rats were killed in the course of other experiments and physical data and serum were collected from these animals. The results shown in Table 1 support previous findings [8] that obese Zucker rats are hyperlipidaemic and have a tendency toward

Table 1. Physical characteristics and blood values of lean and obese Zucker rats

Characteristics	Mean (SD) results obtained with	
	Lean rats (n = 12)	Obese rats (n = 12)
Body weight (g)	496 (24.0)	904 (125.7)*
Organ weights (g)		
Spleen	0.78 (0.08)	1.06 (0.17)*
Liver	16.33 (1.31)	30.57 (3.95)*
Kidney	4.07 (0.50)	6.22 (0.98)*
Lung	2.05 (0.31)	1.49 (0.33)
Heart	1.15 (0.28)	1.46 (0.22)
Plasma values (mg/dl)		
Glucose	169 (38.2)	216 (43.6)
Glycosylated haemoglobin	10.7 (0.6)	13.9 (1.4)
Triglycerides	25.7 (15.5)	221.6 (45.5)*
Cholesterol	116.6 (15.6)	252.4 (28.7)*
HDL cholesterol	15.4 (1.4)	27.0 (1.9)

*Significantly different from lean Zucker rat controls ($p < 0.05$) by Student's *t* test.

becoming hyperglycaemic. The largest elevation in serum values was observed in triglyceride levels of obese rats, which were increased $>800\%$ above those observed in lean animals.

Organ weights for lean and obese rats were also measured (Table 1). A significant increase in the weights of spleen, liver and kidneys from obese rats was observed. Weights of heart and lungs were not significantly different from those of lean rats. No significant differences in the differential blood cell counts were observed between obese and lean animals.

Infectivity studies

In both obese and lean Zucker rats, the kidney was the primary organ colonised (Fig. 1). The obese rats were found to be more susceptible to infection with *C. albicans* at lower challenge doses than the lean rats. The lowest dose tested that resulted in colonisation of the lean rat kidney was 2×10^4 yeast/rat, compared to 8×10^2 in the obese rat. The ID₅₀ calculated for the

Fig. 1. Colonisation of the kidney from lean (○) or obese (●) Zucker rats after i.v. injection of various doses of *C. albicans* (24-h culture, saline washed, Sabouraud-dextrose broth grown yeast, 37°C). Organs were sampled on day 9 after infection. Values represent the mean and SD.

Fig. 2. Colonisation of lung, liver, spleen and heart from lean (■) and obese (▨) Zucker rats at day 9 after i.v. injection of various doses of *C. albicans* (24-h culture, saline washed, Sabouraud-dextrose broth grown yeast, 37°C), $p < 0.05$. Organs were sampled on day 9 after infection. Values represent the mean and SD.

lean rats was 1.2×10^4 cfu, a level 1.18 log₁₀ times greater than that measured for the obese rats. For both lean and obese rats no fungaemia was detected either by daily examination microscopically or by culture of the blood.

The infectious dose tested that resulted in colonisation of heart, liver and lungs of the obese rats was also lower than that necessary to establish colonisation in lean rats (2×10^4 cfu/obese rats versus 10^5 cfu/lean rat) (Fig. 2). Furthermore, the level of organ colonisation for obese rats was significantly higher than that measured for lean rats.

Because the kidney was observed to be the primary target organ for *C. albicans* colonisation (Figs 1 and 2), kidneys from rats infected with 2×10^4 yeast were examined histologically. The pathology of kidneys from both lean and obese rats showed an acute inflammatory response, mostly occurring in the corticomedullary and medullary portions, regardless of fungal morphological form observed. At the time of histological assessment (day 9 after infection) no significant differences were observed in the degree of inflammation in kidneys from lean and obese rats. The most common fungal form seen (day 9 after infection) was hyphae with rare budding yeasts. Occasional rare foci of chronic inflammation also were encountered in a few specimens.

Germ-tube formation

The influences of serum from lean or obese rats on adherence and germ-tube formation were determined. The rate of germ-tube formation was significantly ($p < 0.01$) enhanced (1.5 times) in serum from obese

rats than that measured for serum from lean rats (Fig. 3). Germ-tube formation by 24-h-old yeast cells in the presence of serum from obese rats was significantly accelerated, reaching a maximum of 100% at least 30 min before maximal germ-tube formation occurred in yeasts incubated in the presence of serum from lean rats. The rate of hyphal elongation in the presence of obese rat serum proceeded at a rate significantly faster (2.3 times, $p < 0.01$) than that measured in lean rat serum. Hyphal elongation proceeded at a rate of $43.0 \mu\text{m/h}$ versus $18.7 \mu\text{m/h}$ in yeast incubated in serum from obese and lean rats, respectively. The effect of obese rat serum on both germ-tube formation and elongation was concentration dependent. In 50% or 10% obese rat serum in RPMI or Sabouraud-dextrose broth, the rate of germ-tube formation and elongation was not significantly different from that measured for the lean serum control. Serum from either obese rats or lean rats did not significantly

Fig. 3. Germ-tube formation in 24-h *C. albicans* blastospores by incubation in whole serum from obese (▼) or lean (▽) Zucker rats. A total of 300 cells was observed at random. Values represent the mean and SD.

affect rate of growth of *C. albicans* as determined both by standard plate counts and haemocytometer counts.

Phagocytosis and killing

Phagocytosis and leucocyte cidal activity were determined by direct microscopic examination *via* the neutral red assay. Preliminary studies comparing this procedure with standard plating techniques showed the two techniques to be comparable (data not shown), as has been reported elsewhere [12]. The results in Table 2 indicated that the degree to which peritoneal PMNLs, resident macrophages and elicited macrophages from obese and lean animals engulfed non-opsonised *C. albicans* blastospores was not significantly different; although engulfment, i.e., number of yeast/macrophage, by the elicited macrophages tended to be higher. However, by the neutral red assay, all cell populations from obese Zucker rats displayed a significant decrease ($p < 0.001$) in their ability to kill phagocytosed yeast cells as compared to the analogous cell populations from lean Zucker rats.

Discussion

Animal models previously used to study immunocompetence in diabetes mellitus correlate better with insulin-dependent diabetes mellitus (type I) rather than with non-insulin-dependent diabetes mellitus (NIDDM) [15–23]. These models include animals with a genotype for spontaneously occurring diabetes or animals which have had either streptozotocin- or alloxan-induced diabetes [17, 19, 21, 22]. The C57B-1/6J ob/ob mouse has been used in some studies on immune responsiveness in NIDDM [21]; however, interpretation of immunological studies is confounded by a disproportionately smaller than normal, for the size of animal, spleen and thymus, which contain fewer mononuclear and Thy 1.2-positive lymphocytes, respectively [17, 19, 21]. The differences in immune cell numbers alone could account for observed immunocompromise in the ob/ob mouse, in the absence of the obese state. Furthermore, the organ:body ratio of ob/ob mice is incongruent with the findings of organomegaly in obese human patients [10, 12, 14]. Taken together,

these findings make the differences in immune status in the obese/lean mouse model difficult to interpret.

The obese Zucker rat (fa/fa) is an animal model more representative of obesity-associated type II diabetes in humans than those models studied previously. This model is used currently for the study of endocrine disorders and metabolic abnormalities associated with type II diabetes [8, 9]. Obese Zucker rats are hyperlipidaemic, grossly obese, hyperinsulinaemic, insulin resistant and not prone to ketosis [8]. These animals are not always hyperglycaemic but do exhibit an abnormal glucose tolerance similar to that observed for man [9]. Obese Zucker rats also exhibit organomegaly, a feature in common with obese human patients and in contrast to the ob/ob mouse model [8, 15, 20]. In most human patients with type II diabetes, diet affects insulin levels and food restriction is a major modality for management of the disease [5]. Hyperinsulinaemia can also be controlled by food restriction in the Zucker rat [8, 9]. In the ob/ob mouse, hyperinsulinaemia appears to be a result of a genetic defect in the pancreas and is not remedied by food restriction [24].

The results presented here indicate that the genetically obese Zucker rat is well suited for studies involving the relationship between infection and type II diabetes. The obese Zucker rat parallels human obesity-associated diabetes not only with regard to observed organomegaly, but also in regard to physiological changes, and lack of changes in the differential blood cell count (Table 1). In man, the association between obesity and maturity onset (type II) diabetes relating to a compromised immune system and an increased susceptibility to infection with *C. albicans* are well recognised [2–5, 7]. Our view is supported by the metabolic, physiological and anatomical data previously reported from this animal model. In addition, obese Zucker rats displayed: (1) a marked increase in susceptibility to systemic infection with *C. albicans* compared to their lean litter mates (Figs 1 and 2); and (2) a higher level of organ colonisation than matched lean controls (Fig. 2). These findings, taken together, strongly confirm our view that the obese Zucker rat is an appropriate model for the study of host–pathogen

Table 2. Phagocytosis and candidacidal activity of peritoneal cells from obese and lean Zucker rats

Type of rat	Phagocytes (n)	Mean (SD) number of yeasts ingested per phagocyte*	Percent (SD) of yeast cells staining with neutral red†
Lean	PMNLs (12)	2.6 (0.2)	74 (2)
	Resident macrophages (12)	2.2 (0.2)	71 (6)
	Thioglycollate-elicited macrophages (6)	2.9 (0.2)	86 (5)
Obese	PMNLs (12)	2.4 (0.2)	56 (7)
	Resident macrophages (12)	2.0 (0.5)	53 (7)
	Thioglycollate-elicited macrophages (6)	3.0 (0.3)	65 (6)

*50 phagocytic cells were examined/rat.

†Significant difference between lean and obese, $p < 0.001$ by Student's *t* test.

interactions as they relate to the metabolic changes associated with type II diabetes.

The exact mechanisms underlying the higher incidence of infectious disease in type II diabetics are not well understood [1–7, 24]. Factors that can affect the course of infection may do so by altering the immune responsiveness of the host, the virulence of the pathogen, or both. Factors that could affect establishment of infection were examined, i.e., germ-tube formation, phagocyte engulfment and killing. Initiation of germ-tube formation and elongation was enhanced by exposure to serum from obese rats (Fig. 3). Because germ-tube formation is generally considered to be an essential step in the pathogenesis of *C. albicans* infection and a fundamental component of the invasive process [25, 26], enhanced yeast-mycelial conversion could potentially augment the ability of *C. albicans* to establish infection in patients with type II diabetes. In the early stages of infection, phagocytes are able to clear and destroy most yeast phase *C. albicans*. In the presence of serum from obese rats, the more rapid dimorphic transition effectively compromised this step with the resultant increased potential of the hyphal phase to invade and cause tissue damage [25]. In addition to differences in pathogenicity associated with dimorphic transition, there are changes in the expression of antigenic determinants [27, 28]. These shifts in surface determinants between yeast and hyphal morphologies have been demonstrated to affect the recognition and processing of *C. albicans* by macrophages [29].

Both PMNLs and macrophages play an essential role in the early response to infection with *C. albicans* [30–34]. Hyperglycaemia and hyperlipidaemia have been shown to affect the phagocytic capability of granulocytes [34–37]. Assessment of the essential function of phagocytes in the animal model was accomplished with non-opsonised *C. albicans* so as to obviate the problems inherent in assessing the cellular response to antigenically distinct *C. albicans* morphological types.

A significant depression in the cidal activity of all phagocyte populations examined from obese Zucker rats was observed as compared to that measured for cells from lean rats. This included a macrophage population which, by virtue of being elicited with a sterile irritant (thioglycollate), is in an activated state (Table 2) [36]. This observed suppression of primary phagocyte function would explain, in part, the increased susceptibility of obese rats to infection with *C. albicans*. The mechanism of this suppression could be the result of a genetic abnormality affecting the oxidative killing mechanisms of phagocytes or be the result of the abnormal metabolic milieu associated with the NIDDM state. One approach to answering this question, which is currently under investigation, is a determination of the effect serum from the obese rats has on phagocytic function.

In light of the newly emerging understanding of the endocrine–immunological inter-relationship, the use of obese Zucker rats – an animal with well established endocrine abnormalities – provides great potential for gaining an understanding of the cause(s) for the increased susceptibility of type II diabetics to diseases of infectious aetiology. This study demonstrated that obese Zucker rats are more susceptible to systemic candidiasis than lean Zucker rat controls and provides an excellent model to study the relationship between obesity, diabetes and immune competence in a scientifically controlled manner; thus, the Zucker rat has the potential for becoming a very powerful model for studying the relationship between type II diabetes and ability to resist infection.

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