Pulmonary Tuberculosis in Spontaneously Diabetic Goto Kakizaki Rats

ISAMU SUGAWARA, HIROYUKI YAMADA and SATORU MIZUNO

Mycobacterial Reference Center, The Research Institute of Tuberculosis, Japan
Anti-Tuberculosis Association, Tokyo, Japan

SUGAWARA, I., YAMADA, H. and MIZUNO, S. Pulmonary Tuberculosis in Spontaneously Diabetic Goto Kakizaki Rats. Tohoku J. Exp. Med., 2004, 204 (2), 135-145 — As a clinical association is thought to exist between diabetes and tuberculosis, this study was set up to examine whether GK/Jcl diabetic rats are more susceptible to Mycobacterium tuberculosis infection than non-diabetic rats. GK/Jcl diabetic rats were infected aerially with M. tuberculosis and their capacity to control mycobacterial growth, granuloma formation, cytokine secretion by alveolar macrophages and nitric oxide (NO) production was examined. The rats developed large granulomas but not necrotic lesions in their lungs, liver or spleen. This is consistent with a significant increase in number of colony-forming units of M. tuberculosis in the lungs ($p < 0.01$). Expression levels of interferon-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-12 mRNA were lower in GK/Jcl diabetic rats than those in control Wistar rats. Alveolar macrophages from GK/Jcl rats secreted less TNF-α and IL-12, and produced less NO compared with those from Wistar rats. No significant difference was observed between phagocytosis of tubercle bacilli by alveolar macrophages from GK/Jcl or Wistar rats. These data show that there is a close association between experimental tuberculosis and diabetes in animals, and that alveolar macrophages from GK/Jcl diabetic rats are not fully activated by M. tuberculosis infection. ——— GK/Jcl diabetic rat; type II diabetes mellitus; tuberculosis; cytokine

© 2004 Tohoku University Medical Press

Patients with diabetes mellitus (DM) appear to be at high risk of developing tuberculosis. In a previous study, Root obtained chest radiographs from consecutive diabetic patients who attended the Joslin Clinic between 1927 and 1930 (Root 1934), and reported that the incidence rate of tuberculosis was 2.8% among 1373 hospitalized patients, 2.45% among 10 000 diabetic patients and 1.6% in 750 juvenile diabetics. Banyai (1931) reported that the incidence of tuberculosis among diabetics was 2.6% and that it was three times greater than the estimated incidence of tuberculosis in the United States during the same time period. A survey from Philadelphia in 1946 demonstrated that the incidence of tuberculosis was nearly two-fold higher in patients with DM
Tuberculosis in Spontaneously Diabetic Rats

This background led to the current study on the pathophysiology of pulmonary tuberculosis in type II diabetic rats. Our results suggest that type II diabetic rats are more susceptible to Mycobacterium tuberculosis infection than non-diabetic rats.

MATERIALS AND METHODS

Animals

Six-week-old female GK/Jcl rats (Wistar rat background) and sex- and age-matched control Wistar rats were purchased from Japan Charles-River Co. (Tokyo) (Goto et al. 1976). The urinary glucose level was (+) with a simplified DM examination paper then. All rats were housed in a biosafety level 3 facility and given rat chow and water ad libitum after aerosol infection with M. tuberculosis Kurono strain. The degree of severity of DM in the GK/Jcl rats was evaluated by assessing urinary glucose levels with a simplified DM examination paper. Urinary glucose levels were between (+) and (+++).

Experimental infections

The Kurono strain of M. tuberculosis (ATCC 358121) was grown in Middlebrook 7H9 broth for 2 weeks, and then filtered with a sterile acro-disc syringe filter with a pore size of 5.0 μm. Aliquots of the bacterial filtrate were stored at −80°C until use. The rats were infected via the airborne route by placing them in an exposure chamber in a Glas-Col aerosol generator (Glas-Col, Inc., Terre Haute, IN, USA) (Sugawara et al. 1999; Yamada et al. 2001). The nebulizer compartment was filled with 5 ml of a suspension containing 5×10^6 colony-forming units (cfu) of Kurono tubercle bacilli so that approximately 300 bacteria might be deposited in the lungs of each animal. Inhalation infection experiments were carried out twice. Permission to experiments on animals was given by the Animal Experiment Committee in the Research Institute of Tuberculosis.
**cfu assay**

At 1, 3, 5, 7 and 12 weeks (6 rats/time point) after aerosol infection, rats were anesthetized with pentobarbital sodium. The abdominal cavity was incised and exsanguination was performed by splenectomy. The left lobe of each lung and a part of the spleen tissue were weighed and used to evaluate in vivo growth of mycobacteria. The lung and spleen tissues were homogenized with a mortar and pestle, and 1 ml of sterile physiological saline was added. Ten-fold serial dilutions of homogenate (100 μl) were then cultured on 1% Ogawa’s egg medium. Colonies were counted after 4 weeks incubation at 37°C (Yamada et al. 2001).

**Histopathology**

For light microscopy, the rats were sacrificed 1, 3, 5, 7 and 12 weeks after infection. Tissue sections (5 μm) were cut from paraffin blocks containing lung, liver or spleen tissue and stained with hematoxylin and eosin or by the Ziehl-Neelsen method for acid-fast bacilli.

**RT-PCR**

Lung tissue samples were taken from infected rats 1, 3, 5, 7 and 12 weeks after aerosol infection (6 rats/time point), frozen in liquid nitrogen and stored at −85°C until use. RNA was extracted from the samples as described previously (Sugawara et al. 2001). PCR was performed with equivalent amounts of cDNA from each sample, TAKARA EX Taq and gene-specific primer sets. The primer sequences, annealing temperature and cycle number for amplification are shown in Table 1. Amplifications were performed with a DNA thermal cycler model 480 (Perkin-Elmer Cetus). Ten microliters of each PCR product were electrophoresed on a 4% agarose and NuSieve GTG (Bio-Rad) (1:3) gel and visualized by ethidium bromide staining. The same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA from the lung tissue was used as an internal control in the RT-PCR analysis. We made a densitometric analysis of electrophoretic RT-PCR using NIH image ver. 1.62. Relative densitometric ratios of various cytokines, iNOS and Nramp-1 mRNA to GAPDH mRNA as an internal control were determined (Sugawara et al. 2002).

**Cytokine assays**

Alveolar macrophages were prepared from GK/Jcl and Wistar rats to determine which of the various cytokines they secreted (interleukin (IL)-12, tumor necrosis factor (TNF)-α, IL-1β or interferon (IFN)-γ). After the rats had been anesthetized with 300 μl pentobarbital, alveolar macrophages were obtained by bronchoalveolar lavage. Briefly, the rat trachea was cannulated and 2.5 ml physiological saline was poured in. The saline was recovered using a 5 ml disposable syringe. The cells obtained consisted of more than 99% macrophages as assessed by phagocytosis of BCG Tokyo. The cell suspensions (1×10⁶ cells/well) were plated in 96-well culture plates and incubated for 24 hours at 37°C in 5% CO₂ in air, and the cells were stimulated with live M. tuberculosis Kurono strain (multiplicity of infection (moi)=10). The concentration of IL-12, TNF-α, IL-1β and IFN-γ in the culture supernatants was measured by sandwich ELISA (Biosource International Inc., Camarillo, CA, USA) (Sugawara et al. 2001). At the same time, phagocytosis of tubercle bacilli by alveolar macrophages was examined. Two hundred macrophages were counted and the percentage of macrophages ingesting tubercle bacilli was calculated.

**Alveolar macrophage nitric oxide (NO) assay**

Alveolar macrophages (1×10⁶/well) were plated in 96-well culture plates in RPMI 1640 (Sigma-Aldridge, St. Louis, MO, USA) supplemented with 10% fetal calf serum and then stimulated with M. tuberculosis Kurono strain and cultured overnight. The supernatants were collected 16 hours after culture seeding and filtering and their NO concentrations were determined by the Griess assay as described previously (Green et al. 1990; Sugawara et al. 2000).
I. Sugawara et al.

**Statistical analysis**

All values are expressed as means±S.E. and compared using Student’s t-test. For all statistical analyses, the level of statistical significance was set at \( p < 0.01 \).

**RESULTS**

**Mycobacterial burden in the lung and spleen tissue of GK/Jcl rats**

When GK/Jcl and Wistar rats were infected via the airborne route with the Kurono strain of *M. tuberculosis* \((5\times10^6)\), all rats survived until the date of sacrifice (84 days after infection). GK/Jcl and Wistar rats were sacrificed at weekly intervals for organ cfu assay and the number of mycobacteria was expressed as cfu/whole organ (lung or spleen). As shown in Fig. 1, the number of mycobacterial colonies in the lungs increased from 1 week post-infection onwards, and there was a significant difference in the lung cfu count of GK/Jcl and Wistar rats 5, 7 and 12 weeks post-infection \((p<0.01)\). No colonies were found in the spleen of GK/Jcl or Wistar rats during the first week, but there was a significant difference at 3 weeks on-
wards (p<0.01).

**Histopathology of infection**

When $5 \times 10^6$ cfu of Kurono strain was given to the rats via the airborne route, larger granulomas were observed in the lungs of GK/Jcl rats than in the Wistar controls (Fig. 2A). No Langhans-like multinucleated giant cells were found in the granulomatous lesions. No necrotic lesions were present in these granulomas. The pulmonary granulomas merged with the surrounding granulomas with time and foamy epithelioid macrophages were more prominent (Fig. 2D). There was a statistically significant difference in the size of the granulomas between GK/Jcl and Wistar rats (p<0.01). Although no tubercle bacilli were noted in the pulmonary granulomas of Wistar rats, acid-fast tubercle bacilli were more prominent in the granulomas of GK/Jcl rats (Fig. 2B). Small granulomas were recognized in the spleen and liver of GK/Jcl rats. Conversely, the granulomas of Wistar rats were discrete and isolated (Fig. 2C).

**PCR analysis**

In order to examine the major cytokine, iNOS and natural resistance-associated macrophage protein (Nramp-1) mRNA expression profiles of GK/Jcl rats, mRNA expression levels in the lungs of GK/Jcl and Wistar rats were examined by RT-PCR. There was no significant expression of cytokine mRNA and iNOS mRNA in the GK/Jcl and Wistar rats when aerosol inhalation without tubercle bacilli was carried out. IFN-$\gamma$ mRNA was expressed more weakly in the lungs of GK/Jcl rats at 3 and 5 weeks post-infection. TNF-$\alpha$ mRNA was also expressed more weakly in GK/Jcl rats 3 and 5 weeks post-infection (Fig. 3). IL-12 p40 mRNA was expressed more weakly in GK/Jcl rats than in Wistar rats 3, 5 and 7 weeks post-infection. Tumor growth factor (TGF)-$\beta$, IL-1$\beta$, IL-2, IL-18 and iNOS mRNA was expressed strongly in both GK/Jcl and Wistar rats, but there was no statistically significant difference in mRNA expression between them. IL-4 and IL-10 mRNA was expressed very weakly and there was no statistically significant difference in mRNA expression between them. Nramp-1 mRNA was expressed more strongly in the lungs of GK/Jcl rats than in the lungs of Wistar rats 5, 7 and 12 weeks post-infection.
Cytokine assays

The in vitro cytokine capability of rat alveolar macrophages from GK/Jcl rats was investigated after stimulation with *M. tuberculosis* Kurono strain. Three hundred alveolar macrophages were counted for phagocyrosis capacity. Alveolar macrophages from GK/Jcl and Wistar rats phagocyted tubercle bacilli equally (99% vs. 99%) and there was no significant difference in phagocytosis capacity between them. As shown in Table 2, the alveolar macrophages from Wistar rats secreted significantly higher levels of TNF-α (725±12 pg/ml) and IL-12 (200±18 pg/ml) than those from GK/Jcl rats (305±9 and 130±11 pg/ml) (*p*<0.01). However, there was no significant difference in secretion capacity of IFN-γ (50±4 vs. 55±3 pg/ml) and IL-1β (80±4 vs. 85±8 pg/ml).

NO assay

NO levels in the culture supernatants of alveolar macrophages were determined with the Griess reagent and reference to a standard NaNO₂ curve. The levels of NO produced by unstimulated alveolar macrophages from both GK/Jcl and Wistar rats were <20 μM. However, when the alveolar macrophages were stimulated overnight with Kurono strain (moi=10), NO levels increased to 50±3 μM (Wistar control rats) and 30±2 μM (GK/Jcl rats). The difference in NO secretion capacity between GK/Jcl and Wistar rats was statistically significant (*p*<0.01) (Table 3).

Fig. 2. Histopathology of infected lung tissue. Rats were sacrificed 5 and 12 weeks after infection, and formalin-fixed sections were stained with hematoxylin and eosin (A, C and D) and Ziehl-Neelsen stain for acid-fast bacilli (→) (B). (A) Pulmonary tissue from a GK/Jcl rat infected with the Kurono strain (5 weeks after infection) (magnification, ×100). (B) Pulmonary tissue from a GK/Jcl rat infected with the Kurono strain (magnification, ×600). (C) Pulmonary tissue from a Wistar rat infected with the Kurono strain (5 weeks after infection) (magnification, ×100). (D) Pulmonary tissue from a GK/Jcl rat infected with the Kurono strain (12 weeks after infection) (magnification, ×100).
Fig. 3. The in vivo expression profiles of mRNA for IFN-γ (A), TNF-α (B), TGF-β (C), IL-1β (D), IL-2 (E), IL-4 (F), IL-10 (G), IL-12p40 (H), IL-18 (I), iNOS (J) or Nramp-1 (K) in Kurono strain-infected rats. Lung tissue of GK/Jcl and Wistar rats (six rats/time point) was removed 1, 3, 5, 7 and 12 weeks after aerosol infection. Inhalation infection experiments were performed twice. GAPDH gene primer sets were used as internal controls. Relative densitometric ratios of cytokines, iNOS and Nramp-1 mRNA to GAPDH mRNA were determined by using NIH image ver. 1.62.

••••••, wild Wistar rats. ○○○○○○, GK/Jcl rats.
DISCUSSION

In this study, large granulomas were induced in GK/Jcl rats after aerosol infection with *M. tuberculosis*. The number of cfu in lung tissue taken from GK/Jcl rats was significantly higher than that in Wistar control rats (at 5 weeks onwards, p<0.01). Similar findings were observed when the experiments were repeated.

Although central necrosis was not recognized in the granulomas of GK/Jcl rats, larger granulomas were induced in GK/Jcl rats after infection with *M. tuberculosis*. Urinary glucose levels were (+) to (+++) in the GK/Jcl rats used. Impaired insulin secretion has been reported in GK/Jcl diabetic rats (Kimura et al. 1982). Impaired glucose tolerance may be associated with active tuberculosis. Although there is no experimental report on the relationship between hyperglycemia and tuberculosis, there are a few clinical papers available. The levels of urinary glucose (++++ after *M. tuberculosis* infection did not increase significantly in GK/Jcl and wild-type Wistar rats. Hyperglycemia is common in patients with tuberculosis, and persons who have no prior history of DM may present with glucose intolerance at the time of diagnosis. In a study of 506 patients with active pulmonary tuberculosis, nine had a history of DM, 25 were found to be newly diabetic and 82 had impaired glucose tolerance test results (Mugusi et al. 1990). Additional clinical studies have supported the association between active pulmonary infection and glucose intolerance, noting that impaired glucose tolerance resolved with efficacious antituberculosis treatment (Gulbus et al. 1987; Oluboyo et al. 1990). This suggests that, like other serious infections, active tuberculosis is associated with hyperglycemia. Our laboratory findings have provided substantial evidence to support this clinical observation.

TNF-α and IFN-γ mRNA expression was
relatively low in the early-phase of mycobacterial infection (1-5 weeks post-infection). This finding may explain the larger granulomas in GK/Jcl diabetic rats because TNF-α and IFN-γ has previously been shown to be important in defense against murine tuberculosis (Cooper et al. 1993; Flynn et al. 1993; Sugawara et al. 1998; Kaneko et al. 1999). In the absence of IFN-γ, H37Rv and the Kurono strain of M. tuberculosis induced disseminated abscesses in various organs leading to eventual death. Mac-3-positive macrophages were not detected in the abscesses (Cooper et al. 1993; Flynn et al. 1993; Sugawara et al. 1998). Likewise, in the absence of TNF-α, multiple abscess formation was seen in the lungs, spleen, liver and lymph nodes, and these abscesses contained large numbers of tubercle bacilli (Kaneko et al. 1999). Furthermore, IL-12 p40 mRNA expression was low at 3, 5 and 7 weeks after infection in GK/Jcl rats. It has also been reported that IL-12 plays a major role in defense against murine tuberculosis (Cooper et al. 1997). Taken together, low expression of IFN-γ, IL-12 and TNF-α mRNA allows granulomas to grow to a large size. Further study will be required to explain why IFN-γ, TNF-α and IL-12 mRNA expressions levels are low in GK/Jcl rats. There are a few clinical papers on serum cytokine levels in DM patients (Maltezos et al. 2002; Ozer et al. 2003). The highest serum IL-1α and IFN-γ levels were found in newly diagnosed type I DM patients without diabetic ketoacidosis and the highest serum TNF-α level was observed in newly diagnosed patients with diabetic ketoacidosis. The serum TNF-α concentration is significantly elevated in non-diabetic offspring of type II diabetics (Maltezos et al. 2002). Thus, serum cytokine levels vary depending on the type and disease state of DM (ketoacidosis, diabetic coma, etc.). It should be stressed that immunologic studies in diabetic patients may not adequately assess the impact of age or the pathophysiologic differences between insulin-dependent and non-insulin-dependent diabetes. Furthermore, peripheral blood cell studies may not accurately reflect the immunologic function of alveolar macrophages. Therefore, it is meaningful to study immunologic function of rat alveolar macrophages and pulmonary tissue from diabetic animals.

Although no significant difference was noted in phagocytosis of tubercle bacilli by alveolar macrophages from GK/Jcl and Wistar rats, alveolar macrophages from GK/Jcl rats secreted less TNF-α and IL-12 and also produced less NO compared with Wistar control rats. It is known that TNF-α and IL-12 play an important role in defense against tuberculosis. NO is a well-known antituberculous substance synthesized by inducible NO synthase in macrophages (Chan et al. 1992). It is thought that alveolar macrophages from GK/Jcl rats cannot be activated fully to produce NO by stimulation with tubercle bacilli.

GK/Jcl diabetic rats are model animals of DM type II. There have been no reports on the relationship between type II DM and tuberculosis using DM animal models. On the other hand, NOD mice are model animals of type I DM and develop autoimmune diabetes. There have been a few papers on DM and mycobacterial infection (Bras and Aguas 1996; Martins and Aguas 1999). Diabetes-prone NOD mice are resistant to M. avium and the infection prevents autoimmune diabetes (Bras and Aguas 1996). In order to explain the mechanism of M. avium-induced resistance against insulin-dependent DM (type I DM) in NOD mice, studies have focussed on the role of Fas and Th1 cells, and the increase in cytotoxicity of T-cells (Martins and Aguas 1999). NOD mice are able to control M. avium infection, following a pattern similar to that observed in infected C3H mice. It is proposed that the diabetes genes of NOD mice are linked to the Nramp-1 gene that is associated with resistance to infection by mycobacteria.

In the current study, the expression level of Nramp-1 mRNA was investigated in infected GK/Jcl diabetic rats. As described previously, Nramp-1 mRNA was expressed more intensely in GK/Jcl rats than in Wistar rats. The Nramp-1/bcg gene is closely associated with resistance to infec-
tion by mycobacteria (Frehel et al. 2002). If so, it is expected that Nramp-1 mRNA expression is depressed in GK/Jcl rats. It is not known why Nramp-1 mRNA levels are higher in M. tuberculosis-infected GK/Jcl rats. Further studies using both GK/Jcl rats and NOD mice will be required to explain this observation.

In conclusion, mycobacterial infection was studied in GK/Jcl diabetic rats to investigate the basis for the high incidence of clinical tuberculosis in diabetic patients. GK/Jcl diabetic rats were highly susceptible to M. tuberculosis infection. Alveolar macrophages from GK/Jcl rats secreted less TNF-α, IL-12 and NO suggesting incomplete macrophage activation. Future studies will focus on the role of transcription factor in experimental tuberculosis in diabetic animal models since low expression of NF-κB affects the expression of TNF-α and IL-12 in NF-κB p50-deficient mice (Yamada et al. 2001).

Acknowledgements

This study was supported in part by an International Collaborative Study Grant to the chief investigator, Dr. Isamu Sugawara, from the Ministry of Health, Welfare and Labor, Japan.

References


