

Differential effect of hyperglycaemia on the immune response in an experimental model of diabetes in BALB/cByJ and C57Bl/6J mice: participation of oxidative stress

M. R. Rubinstein, A. M. Genaro and
M. R. Wald

Centro de Estudios Farmacológicos y Botánicos
(CEFYBO), Consejo Nacional de Investigaciones
Científicas y Técnicas (CONICET), Primera
Cátedra de Farmacología, Facultad de Medicina,
Universidad de Buenos Aires (UBA), Buenos
Aires, Argentina

Summary

Diabetes is associated with an increased risk of death from infectious disease. Hyperglycaemia has been identified as the main factor contributing to the development of diseases associated with diabetes mellitus. However, experimental evidence indicates individual susceptibility to develop complications of diabetes. In this context, the aim of this work was to study the immune response in a streptozotocin-induced type 1 diabetes in two mouse strains: BALB/cByJ and C57Bl/6J. The participation of hyperglycaemia and oxidative stress was also analysed. Diabetic BALB/cByJ mice showed a decrease in both the *in-vivo* and *in-vitro* immune responses, whereas diabetic C57Bl/6J mice had higher blood glucose but exhibited no impairment of the immune response. The influence of hyperglycaemia over the immune response was evaluated by preincubation of lymphocytes from normal mice in a high glucose-containing medium. T and B cells from BALB/cByJ mice showed a decrease in cell viability and mitogen-stimulated proliferation and an increase in apoptosis induction. An increase in oxidative stress was implicated in this deleterious effect. These parameters were not affected in the T and B lymphocytes from C57Bl/6J mice. In conclusion, BALB/cByJ mice were sensitive to the deleterious effect of hyperglycaemia, while C57Bl/6J were resistant. Although an extrapolation of these results to clinical conditions must be handled with caution, these results highlight the need to contemplate the genetic background to establish models to study the deleterious effect of diabetes in order to understand phenotypical variations that are of clinical importance in the treatment of patients.

Keywords: BALB/cByJ and C57Bl/6J mice, diabetes, high glucose, immune response, oxidative stress

Accepted for publication 22 October 2012
Correspondence: M. R. Wald, Paraguay 2155
piso 15, CABA, Buenos Aires, Argentina, CP:
1121ABG.
E-mail: miriamwald@yahoo.com.ar

Introduction

Diabetes is widely believed to predispose to serious infections. Evidence from clinical studies supports a causal relation between diabetes and infections [1–3]. Individuals with diabetes might be at a higher risk of moderate or severe infection-related morbidity caused by altered defence mechanisms [4–7]. However, while some researchers have shown that infections are more prevalent and have a higher case fatality rate among patients with diabetes than among the general population [8,9], others have not observed such an association [10]. Despite the clinical importance of this

problem, the mechanisms linking diabetes and immunosuppression are not well defined.

Hyperglycaemia has been identified as the main factor contributing to the development of diseases associated with diabetes mellitus [11–13]. Concerning the risk of infection, there are substantial clinical data supporting the hypothesis that hyperglycaemia influences the susceptibility to, and the outcome of, infectious disease hospitalization [14–17].

Various pathophysiological and biochemical mechanisms have been proposed to explain the adverse effects of hyperglycaemia. Basic and clinical studies have provided new insights into the role of oxidative stress, suggesting it as a

key factor in the pathogenesis of diabetes complications [18,19]. Evidence indicates that hyperglycaemia results in an excess of reactive oxygen species (ROS) production, creating a state of oxidative stress [20–22].

In a previous report, we have analysed the immune response in an animal model of diabetes and the direct effect of hyperglycaemia on T and B lymphocyte reactivity. We found that diabetes induces an early decrease in immunoglobulin (Ig)G levels in the secondary immune response [23]. Preincubation of lymph node and spleen cells in a high glucose-containing medium leads to a significant time- and dose-dependent decrease in T and B cell proliferation associated with an increase in oxidative stress [23].

Inbred mice have been used widely as human disease models. BALB/cByJ (BALB/c) and C57Bl/6J (C57) inbred mice are useful models to study the effects of genetic differences in the pathogenesis of many diseases. These two mouse strains have differences in both innate [24] and acquired immunity [25], and have been used extensively to investigate the immunopathogenesis of several intracellular infections [26–28]. It has also been reported that both strains show different metabolic responses to a high-fat diet [29]. Concerning diabetes, the two strains show inherited differences in the susceptibility to the effects of streptozotocin in the development of hyperglycaemia: C57 are more susceptible than BALB/c mice [30].

In this context, the aim of this work was to study the immune response in diabetic mice of the BALB/c and C57 strains. In particular, we analysed the effect of hyperglycaemia and oxidative stress in lymphocyte reactivity. For this purpose, we used the multiple low doses of streptozotocin (MLD-STZ) animal model. Both mouse strains display chronic stable hyperglycaemia without a requirement for insulin therapy and have a lifespan similar to that of normal mice.

Materials and methods

Animals

Inbred female BALB/cByJ and C57Bl/6J mice were purchased from Facultad de Veterinaria, Universidad de Buenos Aires, Argentina. Sixty-day-old mice weighing between 20 and 25 g at the beginning of the experiments were used. Mice were maintained on a 12-h light/dark cycle in a room with controlled temperature (18–22°C). Animals of each strain were divided randomly into two experimental groups of 54 animals each. Additional groups of 25 mice of each strain were used to analyse the *in-vitro* effect of hyperglycaemia. Animals were handled in accordance with the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, US National Research (National Institutes of Health). Experimental protocols were approved by the Institutional Committee for use and care of Laboratory

Animal (CICUAL, Facultad de Medicina, Universidad de Buenos Aires, Argentina).

Experimental diabetes

To induce the diabetic state, one daily dose of streptozotocin (STZ, 40 mg/kg; Sigma Aldrich Co., St Louis, MO, USA) was administered intraperitoneally to the mice for 5 consecutive days [23,31]. Blood glucose measurements were performed with a One Touch Ultra test strip glucometer (Lifescan; Johnson-Johnson Co., Milpitas, CA, USA; useful range, 0.02–0.6 g%) in blood samples obtained from mice through a small skin incision at the tip of the tail. Glycaemia was determined after 2 h of fasting. Mice injected with the vehicle at the same time were used as the respective controls.

Immunizations

Sheep red blood cells (SRBC) were used as immunogens to evaluate the T cell-dependent humoral response and lipopolysaccharide (LPS) (Sigma Aldrich Co.) was used to determine the T cell-independent humoral response. For the SRBC response, mice were immunized intraperitoneally on day 0 and boosted on day 11 with 0.2 ml of 4% SBRC in saline. Blood samples were collected for antibody determination on day 10 (primary response) and day 18 (secondary response). For LPS, each mouse received an intraperitoneally injection of 10 µg LPS and blood samples were collected on day 10. Mice injected with the vehicles were used as controls.

Antibody titres

Quantitative enzyme-linked immunosorbent assay (ELISA) was performed to determine SRBC- and LPS-specific antibodies, as described previously [32]. Briefly, 96-well plates (MaxiSorp immunoplates; Nunc, Thermo Fisher Scientific, Langensfeld, Germany) were coated overnight with SRBC membranes (7.5 µg) or LPS (1 µg). Dilutions of sera were added and incubated for 2 h at room temperature and then with a goat immunoglobulin (Ig)G anti-mouse IgM or IgG, phosphatase alkaline-conjugated (Sigma Aldrich Co.) and p-nitrophenylphosphate (Sigma Aldrich Co.) as substrate to develop coloration that was read at 405 nm. Reactions were considered positive when optical density values were above the mean value plus 2 standard deviations (s.d.) of normal sera (sera from non-immunized vehicle-injected mice).

Cell suspensions and culture conditions

Lymphoid cell suspensions from lymph nodes (axillary, inguinal and mesenteric) or spleen were obtained, as described previously [23]. The cell culture medium used was RPMI-1640 (Invitrogen, Carlsbad, CA, USA),

containing 0.2 g% glucose (Sigma Aldrich Co.). Briefly, lymphoid organs were removed aseptically and disrupted through a 1-mm metal mesh and the resulting cell suspensions were filtered through a 10- μ m nylon mesh. After three washes in RPMI-1640 medium, cells were resuspended in RPMI-1640 supplemented with 10% of batch-tested non-stimulatory fetal bovine serum (Invitrogen), 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen). To analyse the effect of high concentrations of glucose, glucose or mannitol or anti-oxidant was added directly to the medium in the cell culture to yield the final concentrations indicated in each experiment.

Assessment of cell viability

Cell viability was estimated according to the trypan blue (Sigma Aldrich Co.) exclusion criteria. Normally, freshly obtained cell viability was higher than 95%.

Proliferation assay

Cells (1.5×10^6 living cells/ml) were settled at a final volume of 0.2 ml in 96-well flat-bottomed microtitre plates (Nunc) in triplicate aliquots and incubated at 37°C in a 5% CO₂ atmosphere. The T cell-selective mitogen concanavalin A (Con A; Sigma Aldrich Co.) and the mitogen selective for B cells, lipopolysaccharide (LPS) were used [23]. Proliferation was measured by adding 0.75 μ Ci [³H]-thymidine (specific activity: 20 Ci/mmol; Perkin-Elmer, Boston, MA, USA) for the last 18h of culture. Thymidine incorporation was measured by scintillation counting. The mean of triplicate determinations was calculated for each lectin concentration.

Detection of lymphocyte apoptosis

Apoptosis was estimated by labelling DNA and analysing chromatin morphology by measuring subdiploid DNA content by flow cytometric analysis, according to a modification of the Nicoletti method [33], as described previously [23].

Lymph node and spleen cells were harvested after 24 h incubation, washed and fixed overnight with cold 70% ethanol. The cells were then stained in the dark for 30 min at room temperature with propidium iodide (PI) (Sigma Chemical Co.) and analysed by flow cytometry using a fluorescence activated cell sorter (FACS) can flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with log amplification and FACScan research software. For each sample, 10 000 cells were acquired and analysed using the WinMDI version 2.9 program. Apoptotic nuclei appeared as a broad hypodiploid DNA peak which was easily discriminated from the narrow peak of normal (diploid) DNA content [23].

Measurement of intracellular reactive oxygen species (ROS) generation

ROS produced within lymphocytes were detected with the membrane-permeant probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma Aldrich Co.) [34]. The lymphocytes (1×10^6 living cells) were incubated in the dark with 5 μ M DCFH-DA for 15 min at 37°C. The intensity of dichlorofluorescein (DCF) fluorescence in the supernatant was measured with an excitation wavelength of 485 nm, and an emission wavelength of 530 nm (Hitachi F2000 Fluorescence Spectrophotometer; Hitachi, Tokyo, Japan). Intracellular ROS production was calculated from a DCF standard curve (1–40 nmol/ml).

Colorimetric assay for lipid peroxidation

Malondialdehyde (MDA), a marker for lipid peroxidation formed by the breakdown of polyunsaturated fatty acids, was determined [35]. Lymphocytes (2×10^7 living cells) were resuspended in 5% trichloroacetic acid and incubated with 15% trichloroacetic acid, 0.375% thiobarbituric acid (Sigma Aldrich Co.) and 0.25 N hydrochloric acid. The samples were heated for 15 min in a boiling waterbath. After cooling, the flocculent precipitate was removed by centrifugation and the absorbance in the supernatant was determined at 535 nm. A standard plot for MDA was prepared using 1,1,3,3-tetraethoxypropane (Sigma Aldrich Co.).

Measurement of the intracellular total anti-oxidant capacity (TAC)

TAC within lymphocytes was determined by using the ABTS [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)] anti-oxidant assay [36]. Briefly, acetate buffer and the lymphocyte samples (7×10^6 living cells) were mixed in 96-well plates (MaxiSorp immunoplates; Nunc) with ABTS+ solution 10 mmol/l (0.549 g of ABTS (MP Biomedicals) in acetate buffer 30 mmol/l pH 3.6 and 2 mmol/l hydrogen peroxide). Samples were read at 660 nm on a plate reader. Results were calculated using a reference curve based on the anti-oxidant Trolox (Sigma-Aldrich Co) as a standard.

Measurement of glutathione

Glutathione (GSH) analysis was achieved using the GSH reductase recycling assay as described by Tietze, modified by Baker [37,38]. Lymphocytes (10×10^7 living cells) were resuspended in 0.5% trichloroacetic acid, and for quantification of oxidized glutathione (GSSG) the samples were incubated for 1 h with 1 M 3-vinyl pyridine, a scavenger of reduced glutathione (GSH). Then, the reaction mixture [1 mM 3,3'-dithio-bis(6-nitrobenzoic acid (DTNB)),

200 U/ml glutathione reductase (GR), 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 100 mM sodium phosphate buffer pH 7.5 with 1 mM ethylenediamine tetraacetic acid (EDTA)] was added. Absorbance was recorded at 2-min intervals for 10 min at 405 nm in an ELISA plate reader (Bio-Rad, Hercules, CA, USA). Total and GSSG were determined in each sample in a 96-well microtitre plate in duplicate. The GSH was calculated as the difference between the total glutathione and GSSG.

Statistical analysis

Group differences were tested by one- or two-way analyses of variance (ANOVA), with treatment and time or treatment and strain as main factors. When the interaction was significant, simple effects analysis was carried out. *Post-hoc* comparisons were applied to compare the data between the groups with the Student–Newman–Keuls (SNK) test. For antibody titres, the Kruskal–Wallis test was performed following Conover non-parametric *post-hoc* tests. All tests were two-sided, and a *P*-value of 0.05 was considered statistically significant. Statistical and data analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and Infostat Software (Cordoba University, Argentina).

Results

Glucose determination

Hyperglycaemia has been identified as the main factor contributing, through either direct or indirect mechanisms, to diabetic pathogenesis by producing biochemical and metabolic alterations that lead to both functional and structural alterations. Therefore, we determined glycaemia in both BALB/c and C57 mice until 6 months of diabetes induction. As shown in Fig. 1, diabetic mice displayed an increase in blood glucose concentrations, but C57 showed higher levels of glucose than BALB/c mice (0.16–0.3 g% *versus* 0.15–0.2 g%) (RM ANOVA; main effects: strain $F_{(3,44)} = 48.25$, $P < 0.0001$). However, survival without insulin administration was similar in both strains.

Antibody production after *in-vivo* immunization

To investigate if changes in the humoral response are present in diabetic mice, we examined the antibody production after immunization with SRBC (a T cell-dependent antigen) and LPS (a T cell-independent antigen) at early (15 days and 1 month) and late (6 months) periods of diabetes induction. To analyse the primary immune response, specific antibody titres of the IgM type were evaluated. In BALB/c mice, IgM production after LPS and SRBC immunization was not significantly different between control and diabetic animals after 15 days and 1 month of diabetes

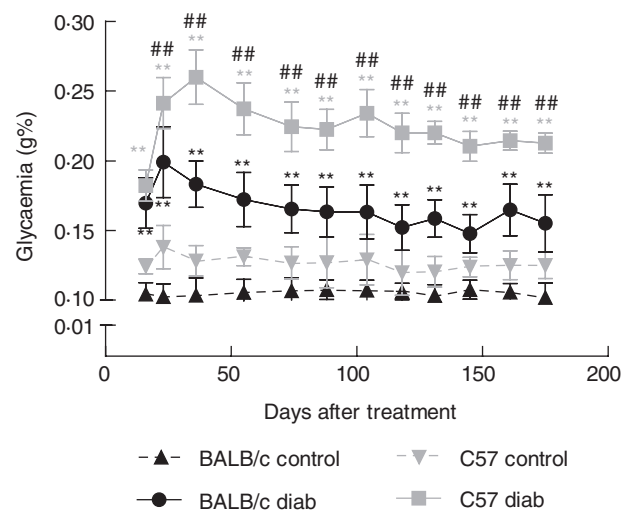


Fig. 1. Glycaemia values in BALB/c and C57 normal and diabetic mice. Average glycaemia values *versus* days after streptozotocin treatment in BALB/c and C57 mice. Data shown are the mean \pm standard error of the mean of 12 animals in each group. Statistical significance was determined with two-way repeated-measures analysis of variance with a 4×12 design followed by Student–Newman–Keuls (SNK) post-test. ** $P < 0.01$ with respect to control mice; ## $P < 0.01$ with respect to BALB/c diabetic mice.

induction, whereas a significant decrease was observed after 6 months of diabetes induction (Fig. 2a,b). However, during the secondary response, titres of anti-SRBC IgG were lower in animals with diabetes than in controls after 1 month of diabetes induction (Fig. 2c). In contrast, diabetic C57 mice displayed a decrease in the production of anti-SRBC IgG only after 6 months of diabetes induction (Fig. 2a–c) (Kruskal–Wallis test IgM LPS: BALB/c $P = 0.0045$; C57 P not significant; IgM GRc: BALB/c $P = 0.0093$; C57 P not significant; IgG GRc: BALB/c $P = 0.0084$; C57 $P = 0.0273$).

Lymphocyte reactivity

To investigate if *in-vivo* antibody production is correlated with *in-vitro* lymphocyte reactivity, we evaluated the mitogen-induced T and B cell proliferation. Due to the well-known lymphoid profile, cell suspension from lymph nodes was used for the stimulation of the T selective mitogen ConA, while spleen lymphocyte suspensions were used to evaluate LPS (B cell selective) mitogen effect. The maximal proliferation was reached at 1 μ g/ml of ConA and 25 μ g/ml of LPS for both control and diabetic mice at all times tested (data not shown). The maximal mitogen-induced T and B lymphocyte proliferation was altered in BALB/c diabetic animals 6 months after diabetes induction (Fig. 3) (simple effect analysis, 6 months, T proliferative response: $P < 0.0001$; B proliferative response: $P < 0.0001$). However, we found no differences for B and T lymphocyte reactivity in C57 mice at any of the times tested.

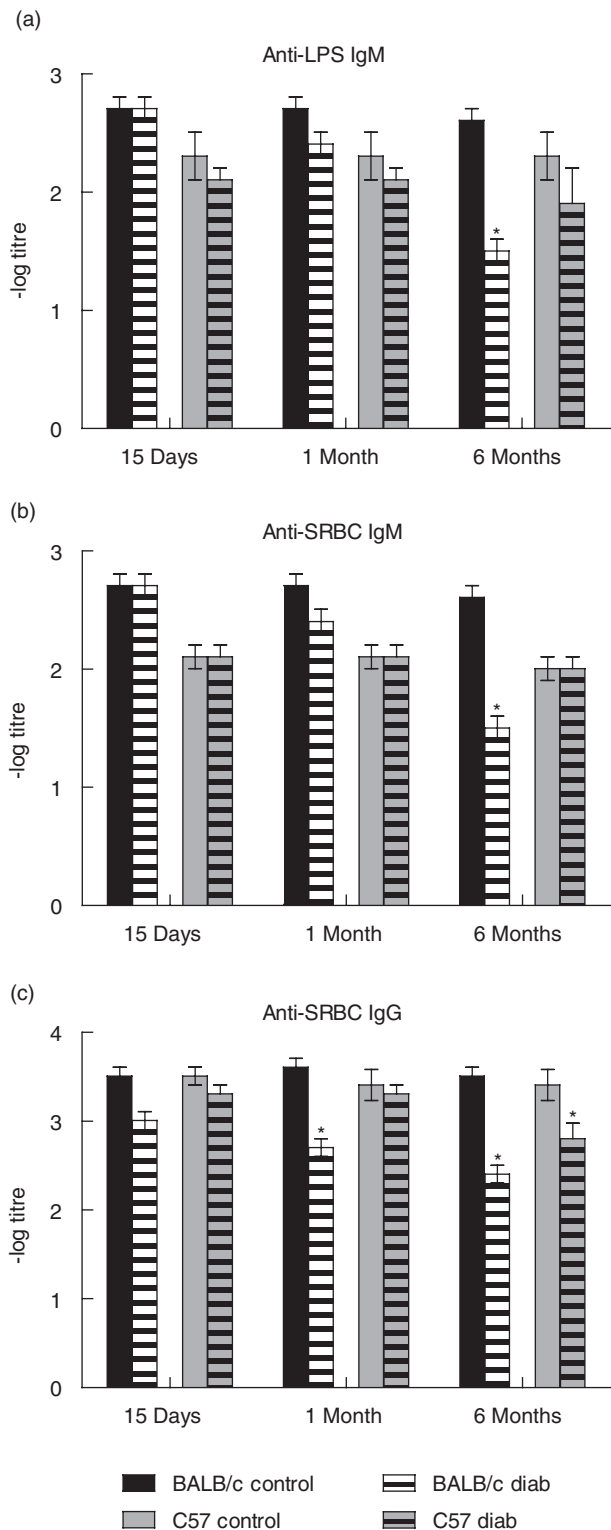


Fig. 2. Antibody production following sheep red blood cells (SRBC) and lipopolysaccharide (LPS) immunization in BALB/c and C57 control and diabetic mice. Antibody titres in controls and at 15 days, 1 month and 6 months after diabetes induction in BALB/c and C57 mice. (a) Anti-LPS immunoglobulin (Ig)M production. (b) Anti-SRBC IgM production. (c) Anti-SRBC IgG production. Data shown are the mean \pm standard error of the mean of three animals in each group. Statistical significance was determined with the Kruskal–Wallis test followed by Conover post-test. * $P < 0.05$ with respect to control mice.

of hyperglycaemia on the immune response. Lymph node and spleen lymphocytes isolated from normal BALB/c and C57 mice were exposed to media containing increasing concentrations of glucose for 24 h. After these treatments, viability, apoptosis and mitogen-induced proliferative response were determined. As seen in Fig. 4, after 24 h of culture with high glucose concentrations, T and B lymphocytes from BALB/c mice showed a significant decrease in mitogen-stimulated proliferation (Fig. 4a,b, simple effect analysis, T and B proliferative response: $P < 0.0001$ for each one) and cell viability (Fig. 4c,d, simple effect analysis, T and B proliferative response: $P < 0.0001$ for each one) and an increase in apoptosis induction (Fig. 4e, T lymphocytes: $P = 0.0061$; B lymphocytes: $P = 0.0028$). Interestingly, these parameters were not affected in T and B lymphocytes from normal C57 mice exposed to 0.5 and 1 g% of glucose; however, a significant decrease was found with 2 g% of glucose (Fig. 4). Unlike D-glucose, 0.5 and 1 g% of mannitol did not change T and B cell proliferation, viability or apoptosis in BALB/c lymphocytes when compared with controls, but with 2 g% of mannitol a significant alteration of these parameters was observed (Table in Fig. 4). Similar results were observed in C57 mice. These findings suggest that the effect of 0.5 and 1 g% of glucose, but not of 2 g%, is not the result of high osmolarity. Thus, the following studies were performed with 0.5 and 1 g% of glucose.

Oxidative stress participation in hyperglycaemia effects

Oxidative stress in lymph node and spleen lymphocytes was analysed by the detection of ROS and by the measurement of lipid peroxidation performed by MDA production. After 24 h incubation with high glucose, T and B lymphocytes from BALB/c but not from C57 mice showed an increase in ROS production (simple effect analysis, T lymphocytes and B lymphocytes: BALB/c $P < 0.0001$; C57 P not significant for each one) and lipid peroxidation (MDA production: simple effect analysis, T lymphocytes: BALB/c $P = 0.0026$; C57 P not significant; B lymphocytes: BALB/c $P = 0.0002$; C57 P not significant) in comparison with control cells (Fig. 5). When the same concentration of mannitol was used, no enhancement of oxidative stress was observed (data not shown).

Effect of high glucose on *in-vitro* lymphocyte proliferation

In order to gain insight into the potential mechanisms involved in this alteration, we then evaluated the influence

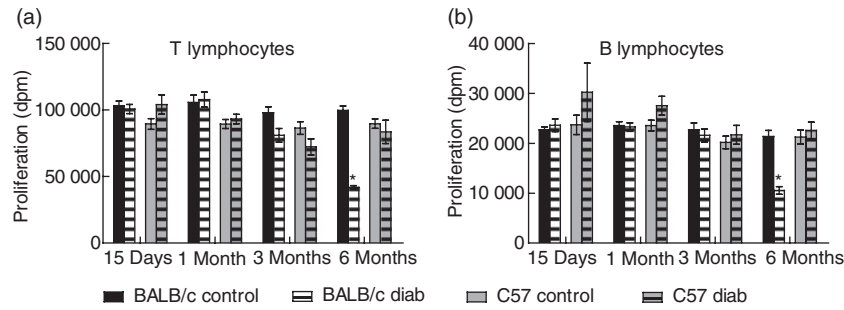


Fig. 3. Mitogen-induced proliferative response in T and B cells from controls and at 15 days, 1, 3 and 6 months of diabetes in BALB/c and C57 mice. (a) T lymphocytes stimulated with 1 $\mu\text{g/ml}$ of concanavalin A. (b) B lymphocytes stimulated with 25 $\mu\text{g/ml}$ of lipopolysaccharide (LPS). Results shown are the mean \pm standard error of the mean of three independent experiments performed in triplicate. Statistical significance was determined with two-way analysis of variance with a 4×4 design followed by Student–Newman–Keuls (SNK) post-test. * $P < 0.05$ with respect to cells from control mice.

To corroborate if the decrease in T and B cell reactivity of lymphocytes from BALB/c mice in the presence of high glucose was due to an increase in oxidative stress, we incubated the cells in the presence of N-acetylcysteine (NAC) (5 mM). Figure 6 shows that the presence of NAC prevented the effect of high glucose on proliferation, viability

and apoptosis in both T and B lymphocytes from BALB/c mice (proliferation, T and B lymphocytes, main effects: glucose \times anti-oxidant interaction $P = 0.0009$ and $P < 0.0001$, respectively; viability, T and B lymphocytes, main effects: glucose \times anti-oxidant interaction $P = 0.0139$ and $P < 0.0001$, respectively; apoptosis: T and B

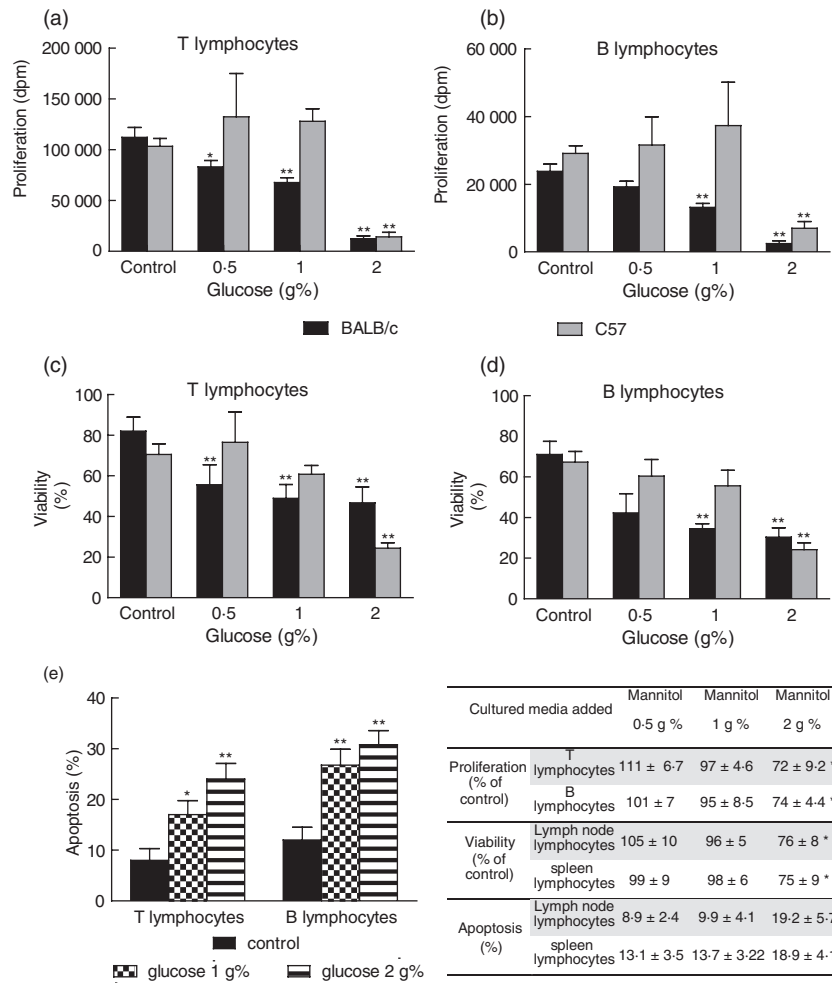


Fig. 4. Effect of high concentrations of glucose and mannitol on proliferation, viability and apoptosis of T and B normal lymphocytes. T lymphocytes (a,c) or B lymphocytes (b,d) from BALB/c and C57 mice were incubated for 24 h in RPMI-1640 either with or without (control) addition of glucose in the culture medium. Proliferation (a,b) and cell viability (c,d) were determined. (e) BALB/c lymphocyte apoptosis. Table: effect of high concentrations of mannitol on T and B cells from BALB/c mice. Results shown are the mean \pm standard error of the mean of five independent experiments performed in triplicate for proliferation and viability and of three independent experiments performed in triplicate for apoptosis. Statistical significance was determined with two-way analysis of variance with a 2×4 design, followed by Student–Newman–Keuls (SNK) post-test for proliferation and viability and with one-way analysis of variance for three factors for apoptosis and for mannitol effect. * $P < 0.05$ and ** $P < 0.01$ with respect to control values (standard medium alone).

Fig. 5. Effect of high concentrations of glucose on oxidative stress. Reactive oxygen species (ROS) production and lipid peroxidation. Lymph node (a,c) and spleen (b,d) lymphocytes from BALB/c and C57 mice were incubated for 24 h in RPMI-1640 either with or without (control) addition of glucose in the culture medium. ROS (a,b) or malondialdehyde (MDA) production (as a measure of lipid peroxidation) (c,d) were determined. Data shown are the mean \pm standard error of the mean of five independent experiments performed in duplicate. Statistical significance was determined with two-way analysis of variance with a 2×3 design followed by Student–Newman–Keuls (SNK) post-test. $**P < 0.01$ with respect to control values.

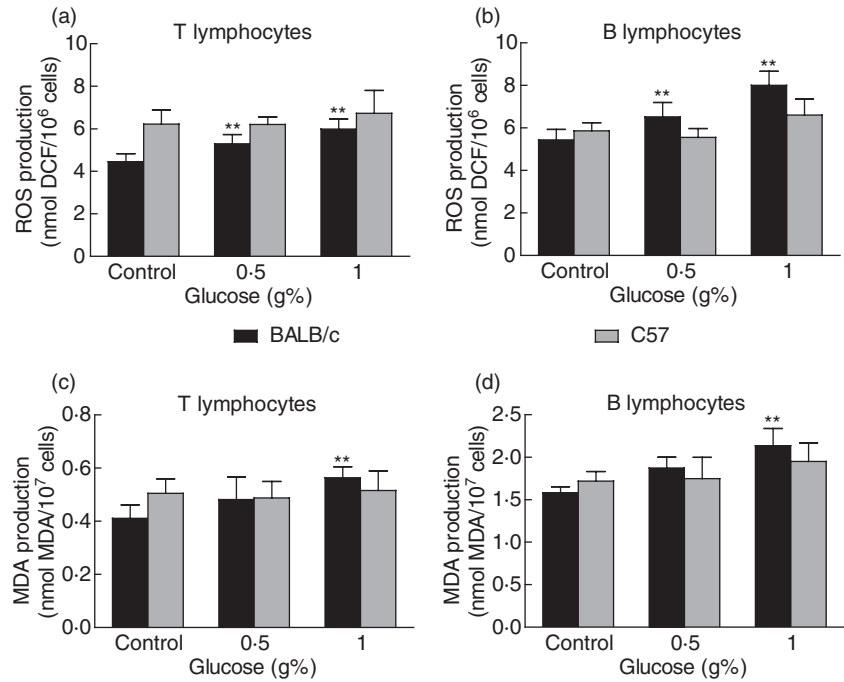
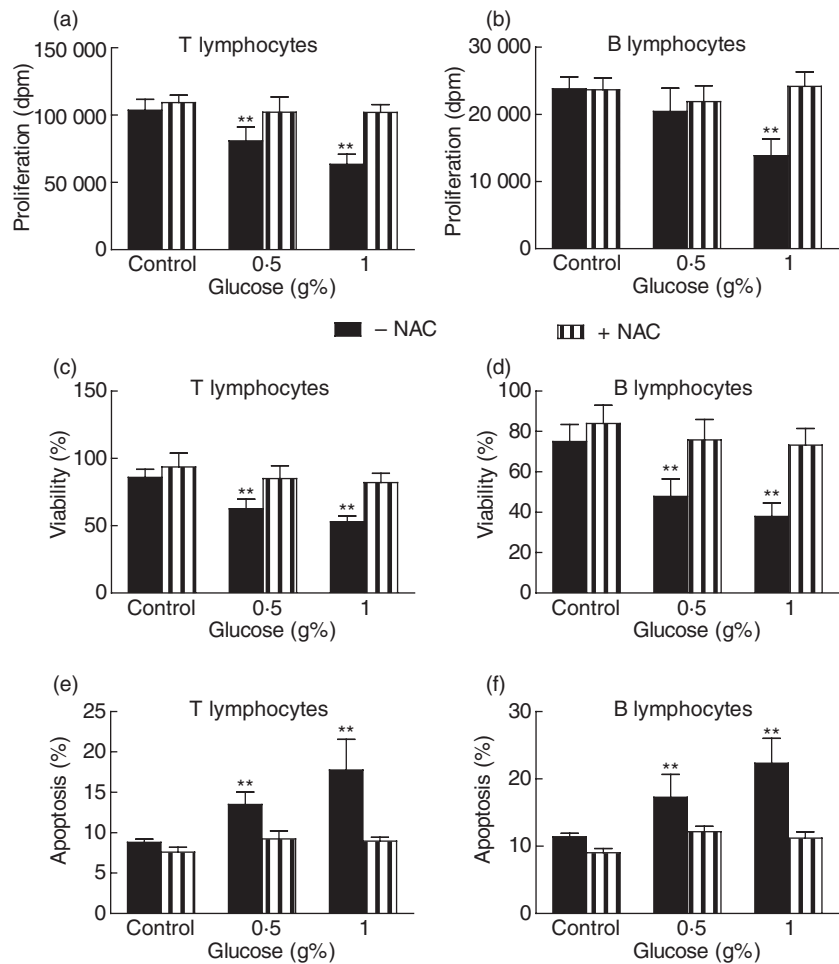


Fig. 6. N-acetylcysteine (NAC) action on the effect of high glucose on mitogen-induced proliferative response, viability and apoptosis in T and B cells from normal BALB/c mice. Lymph node (a,c,e) and spleen lymphocytes (b,d,f) from BALB/c mice were incubated for 24 h in RPMI-1640 either with or without (control) the addition of glucose or NAC in the culture medium. Proliferation (a,b), cell viability (c,d) and apoptosis (e,f) were determined. Results shown are the mean \pm standard error of the mean of five independent experiments performed in triplicate for proliferation and viability and of three independent experiments performed in triplicate for apoptosis. Statistical significance was determined with two-way analysis of variance with a 2×3 design followed by simple effects analysis. $**P < 0.01$ with respect to control values.



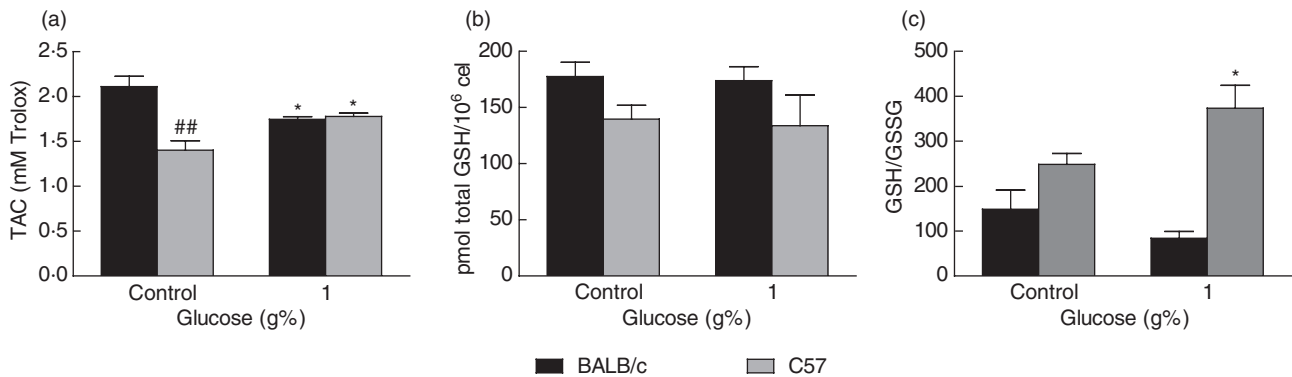


Fig. 7. Effect of high concentrations of glucose on total anti-oxidant capacity (TAC), total glutathione (GSH) levels and reduced glutathione (GSH) : oxidized glutathione (GSSG) ratio. Lymph node lymphocytes from BALB/c and C57 mice were incubated for 24 h in RPMI-1640 either with or without (control) addition of glucose in the culture medium. TAC (a), total GSH (b) and GSH : GSSG ratio (c) were determined. Data shown are the mean \pm standard error of the mean of five independent experiments performed in duplicate. Statistical significance was determined with two-way analysis of variance with a 2×2 design followed by simple effects analysis for TAC and GSH : GSSG ratio and followed by Student–Newman–Keuls (SNK) post-test for total GSH. * $P < 0.05$ with respect to the control values; ## $P < 0.01$ with respect to BALB/c mice.

lymphocytes, main effects: glucose \times anti-oxidant interaction $P = 0.0062$ and $P = 0.0161$, respectively).

Studies of the anti-oxidant mechanisms

To investigate the mechanisms that protect lymphocytes from diabetic C57 mice against high glucose-induced production of free radicals, we studied the anti-oxidant mechanisms. Due to the chemical diversity and co-operative nature of anti-oxidants, we measured the TAC of lymphocytes in the two strains of mice. As can be seen in Fig. 7a, after 24 h incubation with high glucose, lymph node lymphocytes from BALB/c mice diminished TAC. A different response was observed with lymph node lymphocytes from C57 where TAC has an increment in the presence of 1 g% of glucose (main effects: glucose \times strain interaction $P = 0.002$). However, C57 lymphocytes have a lower basal TAC than BALB/c mice.

Because glutathione (GSH) – an abundant intracellular tripeptide – has been implicated as an important regulator of lymphocyte proliferation [39], we studied the total GSH levels and the ratio of reduced (GSH) and oxidized (GSSG) glutathione. No change in total glutathione levels was observed following high glucose treatment of lymph node lymphocytes (Fig. 7b). However, the GSH : GSSG ratio decreased in BALB/c lymphocytes cultured in the presence of 1 g% of glucose, while an increment was observed in those from the C57 mice (Fig. 7c) (main effects: glucose \times strain interaction $P = 0.0219$).

Discussion

In this study we have shown that two genetically different strains of mice (BALB/c and C57) have different responses to MLD-STZ-induced diabetes. BALB/c mice showed a

decrease in the *in-vivo* and *in-vitro* immune response. In contrast, C57Bl/6J mice, despite having a higher increase in glycaemia, exhibited no impairment of the immune response. This suggests the importance of the different sensitivity to the deleterious effects of high glucose and oxidative stress.

In agreement with our previous results [23], diabetic BALB/c mice displayed altered humoral immune responses, the T cell response being the most affected. It has been demonstrated that the production of primary antibodies to T cell-dependent antigens (hepatitis A viral antigen, diphtheria toxoid) but not to a T cell-independent polysaccharide (pneumococcal polysaccharide) is reduced in type 1 diabetes patients and that additional booster immunization can overcome the defect [40]. This decreased immune response appears to be due to a functional impairment at the level of antigen-presenting cells and T cells [41]. Similarly, in diabetic mice, it has been shown that a depression in primary and secondary immune responses is due to altered T cell function and phagocytic activity in macrophages [42]. Furthermore, Sakowicz-Burkiewicz *et al.* [43] have described that T cells isolated from the spleen of diabetic rats proliferate less than cells isolated from normal rats when stimulated with mitogen or anti-CD3 or anti-CD28 antibodies.

However, our results indicate that IgG production in BALB/c mice is impaired after 1 month of diabetes induction, while the proliferation of T and B cells to mitogens is affected after 6 months of a maintained mild diabetic state. These differences between *in-vivo* and *in-vitro* results may be a consequence of the abnormal metabolic environment present in these animals. Hyperglycaemia has been identified as the main factor contributing, either through direct or indirect mechanisms, to diabetic pathogenesis by producing biochemical and metabolic alterations that lead to

both functional and structural alterations [11–13,44]. In fact, in a previous work, we have observed a negative correlation between plasma glucose levels and T and B cell proliferation [45]. Diabetic BALB/c mice exposed to chronic mild stress had a sustained increase in blood glucose levels. In these animals, a decrease in T and B cell proliferation was observed earlier, after 3 and 6 weeks under stress exposure, respectively [45]. We have also reported previously that *in-vitro* high glucose levels can alter the reactivity of both normal T and B lymphocytes in a time- and concentration-dependent interaction manner [23]. In addition, we have observed that the presence of high glucose in lymph node and spleen lymphocyte cultures decreases cell viability with a higher percentage of apoptotic cells. We have also found that an increase in oxidative stress, in particular an excess of reactive oxygen species (ROS) production, would be implicated in these deleterious effects [23]. Under normal conditions, these toxic species are produced by cellular metabolism and neutralized by endogenous anti-oxidant defences. However, in adverse conditions, such as hyperglycaemia, cellular defences might be insufficient, leading to damage of cellular components [46]. Glutathione (GSH, L-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol in mammalian cells and is one of the major cellular defences against ROS and free radicals [47]. GSH is essential for cell survival and has been implicated as an important regulator of T cell function and proliferation [39]. Furthermore, GSH depletion is necessary for lymphocyte apoptosis [47]. N-acetylcysteine (NAC) is a small thiol compound that leads to increased glutathione levels [39]. In this study we found that co-incubation with the anti-oxidant NAC prevented the deleterious effects of high glucose.

Interestingly, diabetic C57 mice showed impaired IgG production only after 6 months of diabetes induction. These results indicate that although diabetic C57 mice have higher plasma glucose levels than BALB/c mice, the immune response is less affected. In addition, lymphocyte reactivity was not altered at this time. In accordance with these findings, proliferation, viability and apoptosis were not altered by high glucose incubation. Moreover, we did not find an increase in oxidative stress under this condition. To assess the involvement of anti-oxidant mechanisms, we determined the TAC of lymphocytes. In the presence of high glucose, BALB/c lymphocytes show a decrease in TAC; however, an increment was observed in those from C57. The same pattern of response was observed for the GSH : GSSG ratio. Changes in the intracellular GSH : GSSG balance are considered major determinants in the redox status/cell signalling [48]. Taking these results into account, it can be postulated that the increase in the GSH : GSSG ratio observed in lymphocytes from C57 mice may constitute a mechanism of protection against the deleterious effects of oxidative stress induced by high glucose. Consistent with this observation, an up-regulation of anti-oxidant

enzymes was reported as an adaptation to oxidative stress during the formation of acquired bleomycin resistance in a cell line [49]. However, the involvement of other anti-oxidant mechanisms that would protect C57 lymphocytes cannot be ruled out. Further studies are necessary to elucidate the precise biochemical and molecular mechanisms involved.

In conclusion, BALB/cByJ mice are more sensitive to the deleterious effect of hyperglycaemia on the immune response than C57Bl/6J mice. Qi *et al.* [50] characterized the severity of diabetic nephropathy in six inbred mouse strains, including C57Bl/6J, DBA/2J, FVB/NJ, MRL/MpJ, A/J and KK/HIJ mice, by inducing diabetes by multiple low-dose streptozotocin injection and found that KK/HIJ mice seemed to be relatively resistant to STZ-induced hyperglycaemia with lower levels of blood glucose. Without insulin supplementation, most diabetic mice survived for more than 25 weeks. After that, the mortality increased in most strains studied, especially in DBA/2J, KK/HIJ and A/J mice. In contrast, C57Bl/6J mice seemed to tolerate persistent hyperglycaemia well, with a group of diabetic C57Bl/6J mice surviving for longer than 45 weeks despite fasting glucose levels of 0.3–0.6 g%. With respect to renal injury, DBA/2J and KK/HIJ mice developed significantly more albuminuria than C57Bl/6J, MRL/MpJ and A/J mice. These results highlight the need to contemplate the genetic background in order to establish models to study the deleterious effect of diabetes. These results also open the possibility of using mouse models to map genes for susceptibility to different diabetic complications. Nowadays, individual variability is an important challenge in current clinical practice and phenotypic variations are of clinical importance in the treatment of patients. Knowledge of the genetic determinants of the disease pathogenesis is not always available. Although the extrapolation of these results to clinical conditions should be handled with caution, it is important to emphasize the importance of conducting studies with large samples that can provide better estimates of the magnitude of the genetic contributions to major individual variability, and deliver solid results to decision-making in clinical practice, both preventive and therapeutic.

Acknowledgements

This study was supported by grants from CONICET, PIP 11220080100281. The authors wish to thank Daniel Gonzalez and Marcela Marquez for their valuable help in the animal care, María Rosa Gonzalez Murano for technical support and Patricia Fernandez for secretarial assistance. We thank LifeScan Argentina for supplying One Touch Ultra strips for the glucometer.

Disclosure

The authors declare that there are no conflicts of interest.

References

- 1 Peleg AY, Weerathna T, McCarthy JS, Davis TM. Common infections in diabetes: pathogenesis, management and relationship to glycaemic control. *Diabetes Metab Res Rev* 2007; **23**:3–13.
- 2 Joshi N, Caputo GM, Weitekamp MR, Karchmer AW. Infections in patients with diabetes mellitus. *N Engl J Med* 1999; **341**:1906–12.
- 3 Pozzilli P, Leslie RD. Infections and diabetes: mechanisms and prospects for prevention. *Diabet Med* 1994; **11**:935–41.
- 4 Shah BR, Hux JE. Quantifying the risk of infectious diseases for people with diabetes. *Diabetes Care* 2003; **26**:510–3.
- 5 Boyko EJ, Fihn SD, Scholes D, Abraham L, Monsey B. Risk of urinary tract infection and asymptomatic bacteriuria among diabetic and nondiabetic postmenopausal women. *Am J Epidemiol* 2005; **161**:557–64.
- 6 Muller LM, Gorter KJ, Hak E *et al.* Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clin Infect Dis* 2005; **41**:281–8.
- 7 Bertoni AG, Saydah S, Brancati FL. Diabetes and the risk of infection-related mortality in the US. *Diabetes Care* 2001; **24**:1044–9.
- 8 Marrie TJ. Bacteraemic pneumococcal pneumonia: a continuously evolving disease. *J Infect* 1992; **24**:247–55.
- 9 Watanakunakorn C, Bailey TA. Adult bacteremic pneumococcal pneumonia in a community teaching hospital, 1992–1996. A detailed analysis of 108 cases. *Arch Intern Med* 1997; **157**:1965–71.
- 10 Thomsen RW, Hundborg HH, Lervang HH, Johnsen SP, Sorensen HT, Schonheyder HC. Diabetes and outcome of community-acquired pneumococcal bacteremia: a 10-year population-based cohort study. *Diabetes Care* 2004; **27**:70–6.
- 11 The Diabetes Control and Complications Trial Research Group (DCCT). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**:977–86.
- 12 The Diabetes Control and Complications Trial Research Group (DCCT). Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med* 2005; **353**:2643–53.
- 13 UK Prospective Diabetes Study Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 1998; **352**:854–65.
- 14 McMahon MM, Bistrian BR. Host defenses and susceptibility to infection in patients with diabetes mellitus. *Infect Dis Clin North Am* 1995; **9**:1–9.
- 15 Benfield T, Jensen JS, Nordestgaard BG. Influence of diabetes and hyperglycaemia on infectious disease hospitalisation and outcome. *Diabetologia* 2007; **50**:549–54.
- 16 Butler SO, Btaiche IF, Alaniz C. Relationship between hyperglycemia and infection in critically ill patients. *Pharmacotherapy* 2005; **25**:963–76.
- 17 Latham R, Lancaster AD, Covington JF, Pirollo JS, Thomas CS. The association of diabetes and glucose control with surgical-site infections among cardiothoracic surgery patients. *Infect Control Hosp Epidemiol* 2001; **22**:607–12.
- 18 Baynes JW. Role of oxidative stress in the development of complications in diabetes. *Diabetes* 1991; **40**:405–12.
- 19 Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ, Packer L. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 2001; **17**:189–212.
- 20 Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; **414**:813–20.
- 21 Ceriello A, Bortolotti N, Crescentini A *et al.* Antioxidant defenses are reduced during the oral glucose tolerance test in normal and non-insulin-dependent diabetic subjects. *Eur J Clin Invest* 1998; **28**:329–33.
- 22 Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr Rev* 2004; **25**:612–28.
- 23 Rubinstein R, Genaro AM, Motta A, Cremaschi G, Wald MR. Impaired immune responses in streptozotocin-induced type I diabetes in mice. Involvement of high glucose. *Clin Exp Immunol* 2008; **154**:235–46.
- 24 Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock* 2004; **22**:460–66.
- 25 Guinazú N, Pellegrini A, Giordanengo L *et al.* Immune response to a major *Trypanosoma cruzi* antigen, cruzipain, is differentially modulated in C57BL/6BL/6 and BALB/c mice. *Microbes Infect* 2004; **6**:1250–58.
- 26 Ulett GC, Ketheesan N, Hirst RG. Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent burkholderia pseudomalle. *Infect Immun* 2000; **68**:2034–42.
- 27 Roggero E, Pérez AR, Bottasso OA, Besedovsky HO, Del Rey A. Neuroendocrine-immunology of experimental Chagas' disease. *Ann NY Acad Sci* 2009; **1153**:264–71.
- 28 Autenrieth IB, Beer M, Bohn E, Kaufmann SH, Heesemann J. Immune responses to *Yersinia enterocolitica* in susceptible BALB/c and resistant C57BL/6 mice: an essential role for gamma interferon. *Infect Immun* 1994; **62**:2590–9.
- 29 Nishikawa S, Yasoshima A, Doi K, Nakayama H, Uetsuka K. Involvement of sex, strain and age factors in high fat diet-induced obesity in C57BL/6J and BALB/cA mice. *Exp Anim* 2007; **56**:263–72.
- 30 Herold KC, Baumann E, Vezys V, Buckingham F. Expression and immune response to islet antigens following treatment with low doses of streptozotocin in H-2d mice. *J Autoimmun* 1997; **10**:17–25.
- 31 Like AA, Rossini AA. Streptozotocin-induced pancreatic insulinitis: new model of diabetes mellitus. *Science* 1976; **193**:415–17.
- 32 Silberman DM, Wald MR, Genaro AM. Acute and chronic stress exert opposing effects on antibody responses associated with changes in stress hormone regulation of T-lymphocyte reactivity. *J Neuroimmunol* 2003; **144**:53–60.
- 33 Nicolletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; **139**:271–9.
- 34 Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 1983; **130**:1910–7.
- 35 Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1979; **52**:302–10.
- 36 Erel O. A novel automated direct measurement method for total

- antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 2004; **37**:277–85.
- 37 Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal Biochem* 1968; **27**:502–22.
- 38 Baker MA, Cerniglia GJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal Biochem* 1990; **90**:360–5.
- 39 Hadzic T, Li L, Cheng N, Walsh SA, Spitz DR, Knudson CM. The role of low molecular weight thiols in T lymphocyte proliferation and IL-2 secretion. *J Immunol* 2005; **175**:7965–72.
- 40 Eibl N, Spatz M, Fischer GF *et al.* Impaired primary immune response in type-1 diabetes: results from a controlled vaccination study. *Clin Immunol* 2002; **103**:249–59.
- 41 Spatz M, Eibl N, Hink S *et al.* Impaired primary immune response in type-1 diabetes. Functional impairment at the level of APCs and T-cells. *Cell Immunol* 2003; **221**:15–26.
- 42 Saiki O, Negoro S, Tsuyuguchi I, Yamamura Y. Depressed immunological defence mechanisms in mice with experimentally induced diabetes. *Infect Immun* 1980; **28**:127–31.
- 43 Sakowicz-Burkiewicz M, Kocbuch K, Grden M, Szutowicz A, Pawelczyk T. Diabetes-induced decrease of adenosine kinase expression impairs the proliferation potential of diabetic rat T lymphocytes. *Immunology* 2006; **118**:402–12.
- 44 Stratton IM, Adler AI, Neil HA *et al.* on behalf of the UK Prospective Diabetes Study Group. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* 2000; **321**:405–12.
- 45 Rubinstein MR, Cremaschi GA, Oliveri LM, Gerez EN, Wald MR, Genaro AM. Possible involvement of stress hormones and hyperglycaemia in chronic mild stress-induced impairment of immune functions in diabetic mice. *Stress* 2010; **13**:384–91.
- 46 Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med* 1995; **18**:775–94.
- 47 Franco R, Cidlowski JA. Apoptosis and glutathione: beyond an antioxidant. *Cell Death Differ* 2009; **16**:1303–14.
- 48 Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* 2006; **8**:1865–79.
- 49 Yen HC, Li SH, Majima HJ *et al.* Up-regulation of antioxidant enzymes and coenzyme Q(10) in a human oral cancer cell line with acquired bleomycin resistance. *Free Radic Res* 2011; **45**:707–16.
- 50 Qi Z, Fujita H, Jin J *et al.* Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes* 2005; **54**:2628–37.