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Mycobacteria precipitate an SLE-like syndrome in diabetes-prone NOD mice

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SUMMARY

Non-obese diabetic (NOD) mice spontaneously develop organ-specific autoimmunity and are widely used as a model for diabetes. Aged NOD mice also exhibit some features of non-organ-specific autoimmune rheumatic disease such as anti-nuclear antibodies and late-onset haemolytic anaemia. Here, we report that a single dose of $2{\cdot}6 \times 10^7$ heat-killed bacillus Calmette-Guérin (BCG) i.v. in 8-week-old NOD mice prevented diabetes but precipitated a syndrome similar to systemic lupus erythematosus (SLE). Treated mice developed haemolytic anaemia, anti-DNA and anti-Sm anti-nuclear autoantibodies and an increased severity of sialadenitis. Perivascular lymphocytic infiltration in the kidneys and glomerular immune complex deposition were also found. The action of BCG appeared to be mediated by an adjuvant-like activity as treated mice showed a substantial increase in reticuloendothelial cell function and enhanced antigen presentation capacity.

INTRODUCTION

Mouse genes encoding vigorous immune responses to intracellular pathogens such as mycobacteria and leishmania have been mapped to the histocompatibility locus[1] (*H-2*) and to the proximal part of chromosome 1[2] (*Bcg/Lsh/Ity*). Diabetes susceptibility genes in the non-obese diabetic (NOD) mouse have also been mapped to each of these positions.[3,4] Resistance to infection with leishmania or *Mycobacterium bovis* [bacillus Calmette–Guérin (BCG)] appears to be mediated by cells of the mononuclear phagocyte lineage[5] and the *Bcg/Lsh/Ity* gene is probably a macrophage-specific nitric oxide (NO) transporter.[6] Similarly, monocyte activity is required for autoimmune destruction of pancreatic β cells in the NOD mouse[7] and the production of reactive oxygen intermediates by macrophages has been proposed as a mechanism of islet destruction.[8]

We therefore decided to study the effects of non-NOD alleles at *H-2* and *Bcg/Lsh/Ity* on the responses of NOD mice to BCG. The surprising finding that BCG-treated NOD mice became ill prompted the following studies.

MATERIALS AND METHODS

Mice

NOD mice were obtained from the breeding colony established at CRC, Northwick Park (Harrow, UK). NZB, BALB/c, C57BL/6, CBA and DBA mice were obtained from Harlan Olac (Bicester, UK).

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Abbreviations: ARD, autoimmune rheumatic disease; BCG, bacillus Calmette–Guérin; BSA, bovine serum albumin; CFU, colony-forming units; DTH, delayed-type hypersensitivity; MEM, minimum essential medium; NO, nitric oxide; NOD, non-obese diabetic; PBS, phosphate-buffered saline; SLE, systemic lupus erythematosus; SRBC, sheep red blood cells.

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BCG therapy

Thirty milligrams Evans (Langhurst, UK) live freeze-dried attenuated *Mycobacterium bovis* (BCG) vaccine [equivalent to $2{\cdot}6 \times 10^7$ colony-forming units (CFU)] was dissolved in isotonic saline and heat inactivated at 65° for 30 min. Control mice received saline alone.

Clinical tests

Random blood glucose estimations were performed by the glucose oxidase technique. Plasma bilirubin estimations were made using the bilirubin pads of Multistix (Ames, Basingstoke, UK) clinical urine analysis sticks. Haematocrit measurements were made on 75 μl aliquots of blood drawn into heparinized capillary tubes (Hawksley, Sussex, UK) and centrifuged at 1000 ***g*** for 15 min. The height of the column of packed red cells was expressed as a percentage of the total height. Blood films were prepared, smears air dried, fixed in methanol and stained for 20 min in a 10% Giemsa stain (Sigma Chemical Co., St Louis, MO). One hundred red blood cells (RBC) were counted under × 1000 magnification and the reticulocyte count expressed as a percentage of the total.

Direct Coombs' test

Ten microlitres of packed cells was resuspended in 5 ml phosphate-buffered saline (PBS) with 0·3% bovine serum albumin (BSA), washed and resuspended in 1 ml of the same solution. Triplicates of 100 μl aliquots were placed in 96-well round-bottom plates (Nunc, Roskilde, Denmark), vortexed and incubated at 37° for 1 hr. Wells were then assessed for false positive results. Ten microlitres of 10 mg/ml polyclonal goat anti-mouse IgG (Sigma) or isotype-specific polyclonal goat

anti-mouse IgM, IgG1, IgG2a or IgG2b (Southern Biotechnology Associates, Birmingham, AL) were added, plates vortexed and incubated at 37° for a further 2 hr. Wells in which the cells collected in a button at the bottom were recorded as negative, while those in which the cells remained spread over the surface of the well were recorded as positive.

Assessment of anti-nuclear antibodies

Sera diluted in PBS (starting concentration 1:100) were incubated on HEp-2 slides (Inova Diagnostics, San Diego, CA) at room temperature for 30 min. Reactivity was detected with 1:50 fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse immunoglobulin (Serotec, Oxford, UK) and examined on an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany). Sera from MRL. *lpr-lpr* and BALB/c mice were used as controls. Autoantibodies to immunoaffinity-purified rabbit thymus La and Sm antigens were detected as described previously[9] and the method was modified for detection of autoantibodies against ultra-pure (caesium chloride gradient purified) calf thymus DNA (Sigma) and recombinant human Ro60 and Ro52 (kind gifts of Dr G. Pruijn, University of Nijmegen, the Netherlands).

Histology

Tissues were fixed in 10% phosphate-buffered formalin, paraffin embedded, sectioned and stained with haematoxylin and eosin. Pancreata were scored as previously described.[10] Fresh tissues were embedded in OCT Compound (BDH, Poole, UK) and snap frozen. Six micrometre cryostat (Slee Technik, Mainz, Germany) sections were fixed for 10 min in acetone, rehydrated, blocked with 10% fetal calf serum (FCS) and stained with FITC-conjugated polyclonal goat anti-mouse C3c (Nordic Immunology, Tilberg, the Netherlands) at 1:25.

RESULTS

Diabetes

Injection i.v. of $2{\cdot}6 \times 10^7$ heat-killed BCG organisms at 8 weeks of age prevented spontaneous diabetes (Fig. 1a). The severity of insulitis in mice killed 3 months after injection was significantly reduced from 81 ± 29% (mean ± SD) to 36 ± 37% ($P < 0{\cdot}002$, rank sum test). This effect was not seen in mice injected at 10 weeks (data not shown).

Anaemia

Anaemia occurred in half of BCG-treated mice between 2 and 3 months after injection (Fig. 1b). Haematocrits of the control group 3 months after injection were 51 ± 4% (mean ± SD) while those of the treated group were 40 ± 5%. Ten of 13 treated mice had haematocrits less than 2 SD (i.e. probability of haematocrit occurring by chance < 0·05 on a one-tailed test of a normal distribution) below the mean for the untreated group ($P < 0{\cdot}0001$, Fisher's exact test).

Anaemic mice had bilirubinaemia and reticulocytosis. The reticulocyte counts in the control group were 1·6 ± 1·3% while those in the treated group were 6·3 ± 6·3%. Six of 13 treated mice had reticulocyte counts greater than 2 SD above the mean for the untreated group ($P < 0{\cdot}02$, Fisher's exact test).

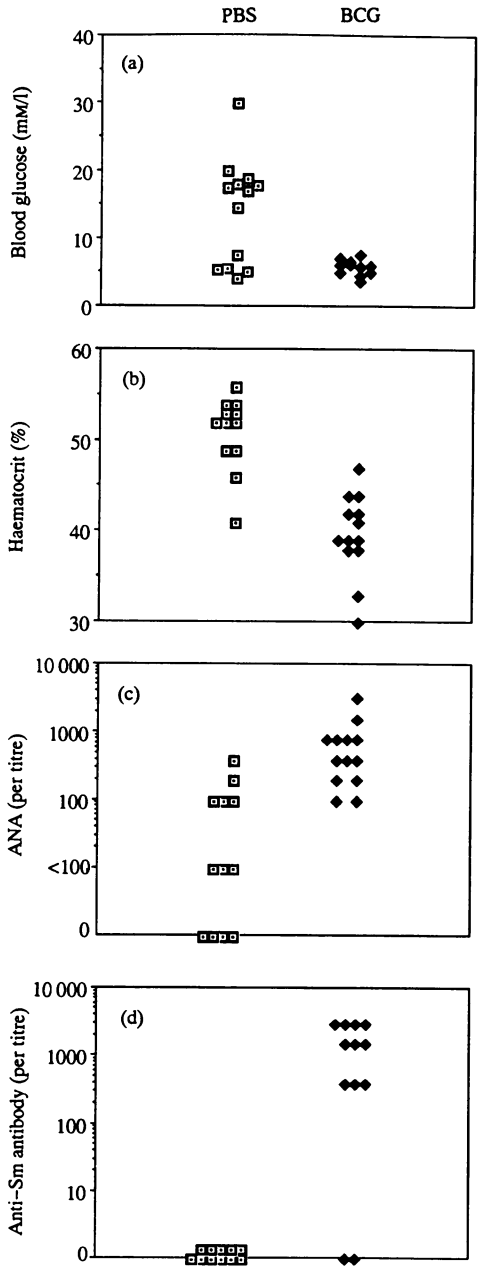

Figure 1. Scattergrams demonstrating the results of individual mice in assays examining aspects of the autoimmune responses affected by BCG treatment. Results of 13 control mice (□) and those of 13 BCG-treated mice (◆). The assays were: (a) random blood glucose estimation; (b) haematocrit; (c) titration of anti-nuclear autoantibodies; (d) titration of anti-Sm autoantibodies.

Five of 13 treated mice tested positive for the direct Coombs' test. The Coombs' antibodies were isotyped by using isotype-specific agglutinating antibodies. The primary isotype was IgG1, although two mice had some IgG2b and one, a little IgG2a.

Anti-nuclear autoantibodies

Strongly positive anti-nuclear antibodies (titre > 1:200) were demonstrated by immunofluorescence of HEp-2 cells in 2/12

control mice and 12/13 BCG-treated mice (Fig. 1c) 3 months after injection. A speckled pattern of antibody binding was suggestive of reactivity to nuclear proteins. Reactivity to DNA, Ro60, Ro52, La and Sm were determined by ELISA. Plasma with anti-nuclear activity bound DNA and Sm (Fig. 1d), but not the other nuclear proteins. The anti-Sm autoantibodies were isotyped using isotype-specific detection antibodies in an ELISA. The antibodies were mostly IgG2a and most mice also had some IgG2b and some mice a little IgG1.

Sialadenitis

Both control and BCG-treated mice had infiltration of the ductal regions of their mixed-type salivary glands. Three of 13 mice in the treated group had very severe infiltration, associated with destruction of ductules and replacement of glandular tissue, and in one case, haemorrhage. These three mice were in the group of treated mice with the highest anti-Sm antibody titres.

Renal disease

Perivascular lymphocytic infiltrates were seen in the kidneys of treated mice but were absent in control mice. Extensive glomerular antigen complex deposits were identified in treated mice by immunofluorescence of mouse C3c (Fig. 2).

Sex and strain comparisons

Three months after BCG injection, 8/15 female NOD mice had positive direct Coombs' tests, but only 1/7 male NOD mice tested positive. Ten of 15 treated female NOD mice had anti-nuclear autoantibodies detected by HEp-2 binding, while 4/7 treated male NOD mice tested positive. Three months after BCG injection, 0/8 BALB/c, 0/3 C57BL/6, 0/3 CBA, 0/3 DBA and 3/3 NZB female mice tested positive for the direct Coombs' test. Two uninjected female NZB mice also tested positive—indicating that the Coombs' antibodies detected in the treated mice probably represented the onset of spontaneous disease in this strain. One untreated NZB mouse also had anti-nuclear autoantibodies. None of the mice from the other strains (either treated or untreated) had any antinuclear antibodies.

DISCUSSION

Hypergammaglobulinaemia and thymocytotoxic autoantibodies are typical features of rheumatic autoimmune disease in NZB and NZB × NZW mice and have been previously reported in NOD mice.[11] Similarly, anti-nuclear autoantibodies have been described,[12] and we have found that senescent NOD mice develop Coombs' positive, haemolytic anaemia.[13] Despite these features of systemic autoimmunity, NOD mice do not spontaneously develop the renal lesions associated with SLE.

Prevention of diabetes by administration of BCG to NOD mice has been previously described by Harada, Kishimoto & Makino[14] who reported prevention of diabetes and insulitis by infection with 4×10^7 CFU BCG. In this case, protection was mediated by splenic macrophages.[15] Similar effects on diabetes were noted when NOD mice were injected with complete Freund's adjuvant (CFA) which is an emulsion of killed BCG in mineral oil.[16] Here, we have found that although treatment of NOD mice with BCG inhibited organ-specific autoimmunity, it precipitated rheumatic autoimmune responses typical of SLE: haemolytic anaemia, hypergammaglobulinaemia, increased sialadenitis, anti-nuclear antibodies and glomerular immune complex deposits.

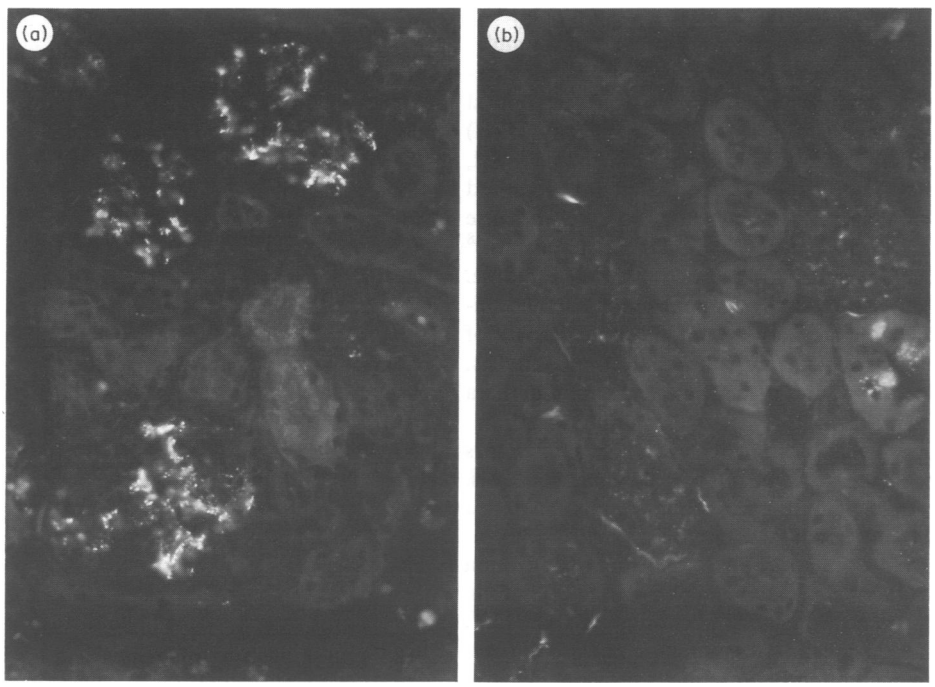

Figure 2. Typical appearance of immunofluorescence of immune complexes deposited in renal glomeruli of a NOD mouse 3 months after BCG injection (a) and the appearance of a PBS-injected control (b).

BCG therapy raised the titres of anti-nuclear autoantibodies in NOD mice. The pattern of staining was speckled, indicating that reactivity was primarily directed to protein nuclear components, although anti-DNA autoantibodies, which have not been previously reported in NOD mice,[13] were also identified. In order to determine the primary nuclear autoantigen, we tested sera for reactivity against Sm, La, Ro60 and Ro52. No reactivity to La, Ro60 or Ro52 was identified. Titres $>1:400$ anti-Sm autoantibody were found in 10/12 BCG-treated mice and in 0/11 untreated mice. The fine specificity of the anti-Sm antibodies was found by immunoblotting to be the 28 000 MW polypeptide of the Sm–RNP complex (data not shown) and the primary isotype of antibody reactive to Sm to be IgG2a. Although the presence of circulating IgG anti-Sm antibody is a diagnostic criterion of SLE, it is not associated with any particular disease feature. Antibody of this specificity is found in few inbred mouse strains, being described only in the MRL.+/+ and MRL.*lpr* strains of autoimmune mice, which share many clinical features with SLE. It has been induced previously only by monoclonal anti-Sm antibody[17] or immunization with Sm antigen, but without obvious pathological sequelae.

Haemolytic anaemia is not normally seen in NOD mice before 250 days of age.[15] Half of the BCG-treated NOD mice developed haemolytic anaemia between 2 and 3 months after injection, at 150 days of age—about the time untreated NOD mice develop diabetes. The anaemia was associated with hyperbilirubinaemia, reticulocytosis and Coombs' autoantibodies, which were primarily of the IgG1 isotype. Haemolytic anaemia is a major feature of systemic autoimmunity in NZB mice and a common feature of SLE.

The most significant feature reminiscent of SLE found in BCG-treated NOD mice was the presence of immune complex deposits in renal glomeruli. Extensive complement deposition was found within glomeruli by 3 months after injection.

We considered three mechanisms of action by which BCG may induce SLE-like disease: adjuvant-like immunostimulation; cross-reactive immune responses; and specific enhancement of Th2-mediated immune responses. We therefore examined the effect of BCG therapy on delayed-type hypersensitivity (DTH) responses to sheep RBC (SRBC) and found it increased DTH, consistent with a systemic adjuvant-like activity. Generalized enhancement of Th2 responses was considered unlikely because treated mice showed features typical of Th1 responses:[18] enhanced DTH and an IgG2a anti-Sm autoantibody response. Cross-reactivity between autoantigens and mycobacterial antigens was considered unlikely because of the large variety of tissue-specific antigens involved: DNA, Sm, islet antigens, surface RBC antigens, SRBC antigens and thyroglobulin (data not shown).

Mycobacterial cell wall components are efficient activators of monocytes and macrophages and stimulate phagocytosis. We have found that BCG therapy dramatically increased reticuloendothelial cell function as well as *in vitro* antigen-presentation capacity (data not shown), resulting in activation of T-helper cells. A large proportion of splenic $CD4^+$ T cells in treated mice switched to a memory phenotype ($CD45RB^{low}$, $CD44^{high}$), while $CD8^+$ splenocytes remained unaffected (data not shown). BCG immunotherapy may provide effective activation of autoreactive T-helper cells which previously existed in a state of 'antigenic ignorance'. This mechanism of self-tolerance was previously described in a T-cell receptor transgenic model of diabetes.[19] As autoreactive B cells are dependent on T-cell help, activation of such T cells may result in B-cell activation, isotype switching and autoantibody production.

While BCG has been used relatively harmlessly in humans as a vaccine, autoimmune rheumatic complications have been reported in a minority of patients resulting from its use for immunotherapy in doses similar to those used here.[20] It may be prudent for patients with a history of organ-specific autoimmune disease or autoimmune rheumatic diseases who are candidates for BCG immunotherapy to be monitored carefully after treatment.

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