

## Heat-shock protein 65 as a $\beta$ cell antigen of insulin-dependent diabetes

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The primary  $\beta$ -cell antigen of insulin-dependent diabetes is thought to be a protein with a molecular weight of approximately 64 kD. Hyperthermic incubation and cytokines such as interleukin 1 $\beta$ ,  $\gamma$  interferon, and tumour necrosis factor induce synthesis of 64 kD protein by insulinoma cells. By western blot techniques, cross-reactivity was found between this 64 kD protein and monoclonal antibodies directed against *Mycobacterium tuberculosis* heat-shock protein 65, but not with antibodies directed against a similar epitope of *M leprae* heat-shock protein 65. Binding of *M tuberculosis* heat-shock protein 65 antibodies to interleukin-1 $\beta$ -treated cells was inhibited by prior addition of serum from insulin-dependent diabetic patients which contained antibodies to 64 kD  $\beta$ -cell antigen. It is suggested that heat-shock protein 65 may be the 64 kD  $\beta$ -cell antigen and that autoreactivity to an epitope of heat-shock protein 65 may confer susceptibility to insulin-dependent diabetes mellitus.

*Lancet* 1990; **336**: 583-85.

### Introduction

The primary  $\beta$ -cell antigen of insulin-dependent diabetes mellitus (IDDM) is thought to be a dimeric glycoprotein of an approximate molecular weight of 64 kD,<sup>1</sup> found not only in man but also in BB rats<sup>2</sup> and NOD mice.<sup>3</sup> Antibodies against this antigen have been described in patients with newly diagnosed IDDM and several years before the onset of IDDM,<sup>4</sup> and in both animal models. The human antibody against the 64 kD protein cross-reacts with the corresponding rat protein, which indicates a common epitope or structural homology.<sup>5</sup> Could this characteristic be used to identify the  $\beta$ -cell antigen? Heat-shock proteins (HSPs) show a very high degree of interspecies sequence homology, with a 40% homology even between primitive micro-organisms and vertebrates. HSPs are produced in response to many forms of cellular stress including viral infection, cytokines, and temperature change,<sup>6,7</sup> and have been implicated in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, in which a causal role for an epitope similar to *Mycobacterium tuberculosis* HSP 65 has been suggested.<sup>8,9</sup> Such an autoimmune response may be caused by molecular mimicry between mycobacterial and human HSPs.<sup>10</sup> We investigated whether a similar mechanism could occur in the pathogenesis of IDDM.

### Methods

Rat insulinoma  $\beta$  cells were established in cultures and incubated at 37°C in RPMI 16/40 (Imperial Laboratories, Andover, UK) with 10% (v/v) fetal calf serum, 0.1 mol/l HEPES, and 20 000 U/ml penicillin. 10 U/ml interleukin 1 $\beta$ ,  $2.5 \times 10^{-4}$  mmol/l tumour necrosis factor, and 10 U/ml  $\gamma$  interferon (Sigma, Poole, UK) were added to subcultures. Heat-shock preparations were produced by

heating subcultures in a waterbath at 42°C for 1 h; cells were allowed to recover at 37°C for 8 h before lysis or indirect immunofluorescence.

Before autoradiography cells were cultured in methionine-free medium to which was added <sup>35</sup>S-labelled methionine. Cytokines were added and heat-shock preparations produced as above; control samples were obtained by identical culture of rat insulinoma cells except for the addition of cytokines or heat shock. Cells were lysed in buffer that contained 15% glycerol, 2% sodium dodecylsulphate (SDS), and 75 mmol/l "tris" hydrochloride (pH 6.8). Samples were reduced by addition of 5% mercaptoethanol at 37°C for 2 h and boiled for 2 min after addition of 0.1 mmol/l dithiothreitol. Lysates were immediately cooled on ice and centrifuged at 10 000 rpm for 10 min to remove insoluble material. Trace quantities of bromophenol blue were added before electrophoresis on a 7.5% SDS homogeneous gel ('Phast', Pharmacia, Milton Keynes, UK) and stained with silver nitrate. Dried gels were autoradiographed for 7 days at -70°C on 'Hyperfilm- $\beta$ max' (Amersham International, Amersham, UK) suitable for detection of <sup>35</sup>S $\beta$ -particle emission; cell lysate aliquots were corrected for total radioactivity content before electrophoresis.

Cell lysate aliquots run on 7.5% SDS 1.5 mm slab gels were used for western blot analysis. Molecular weight markers (Pharmacia) were used to identify the position of proteins which were transferred onto 0.2 micropore nitrocellulose in a Hoffer protein transfer unit for 20 min. Transferred proteins were blocked by use of a solution of 5% dried milk protein for 1 h. Mouse monoclonal primary antibodies were then added at a dilution of 1 in 1000. Bound antibodies were detected with second antibodies that incorporated a biotinylated moiety and complexes were visualised with an alkaline phosphatase label (Vector Laboratories, Peterborough, UK). The primary antibodies used included mouse monoclonal antibodies against *M tuberculosis* HSP 70 and 65, and *M leprae* HSP 65. Recombinant HSP standards (*M tuberculosis* HSP 70 and 65, *M leprae* HSP 65) were prepared in lysis buffer under reducing conditions and run in parallel with cell lysate preparations in western blots.

Rat insulinoma cell subcultures were studied by indirect immunofluorescence after incubation with or without addition of 10 U/ml interleukin 1 $\beta$  or after heat shock to 42°C for 1 h. Cells were dislodged from culture flasks in calcium-free and magnesium-free Hanks' balanced salt solution (Imperial). Cells were then cytospun onto glass slides after repeat washing, fixed in acetone, blocked with mouse serum, and stained with mouse monoclonal primary antibodies as above. An avidin-biotin detection system (Amersham) with a fluorescein label was used to identify the presence of bound antibodies.

Blood was obtained from 6 newly diagnosed insulin-dependent diabetic patients from the Royal Infirmary, Edinburgh (courtesy of Dr B. M. Frier) and from 6 age-matched controls who had no family history of IDDM. Serum was frozen and stored at -20°C until used. Serum from 2 patients known to possess 64 kD antibodies was kindly supplied by Dr S. Baekkeskov (Hagedorn Institute, Gentofte, Denmark).

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## Results

Fig 1 shows an autoradiograph of rat insulinoma cell lysates ( $^{35}\text{S}$ -methionine) cultured in the absence of interleukin  $1\beta$  (control, lane A), with interleukin  $1\beta$  (lane B), and after heat shock (lane C). The 64 kD protein (arrowed) was present in control cells but increased by interleukin  $1\beta$  and by heat shock. Similar results were obtained after addition of tumour necrosis factor or  $\gamma$  interferon (data not shown).

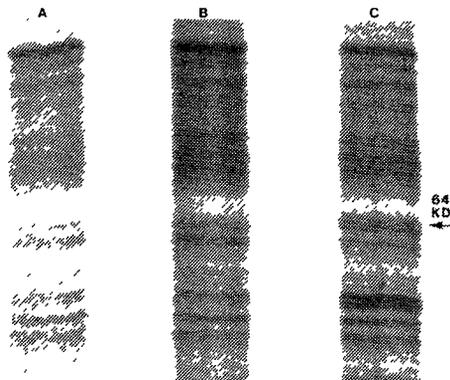


Fig 1—Autoradiograph of rat insulinoma cell lysates from control culture (A), culture with interleukin  $1\beta$  (B), and after heat shock (lane C).

Arrow illustrates position of 64 kD protein

A western blot of cell lysates of control rat insulinoma cells (lane a) and cells cultured with interleukin  $1\beta$  (lane b), labelled with monoclonal antibody against *M tuberculosis* HSP 65, is shown in fig 2. Binding of antibody against the 64 kD protein is present in control rat insulinoma cells, with increased reactivity after culture with interleukin  $1\beta$ . A protein with the same antibody reactivity and molecular weight was also induced by heat shock (data not shown). Fig 3a shows binding of *M tuberculosis* HSP 65 antibodies to control rat insulinoma cells detected by indirect immunofluorescence; fig 3b shows immunofluorescence of the same antibody against interleukin- $1\beta$ -treated rat insulinoma cells.

Antigenicity of the 64 kD rat insulinoma cell protein in IDDM was confirmed by the presence of specific antibody to that protein in the serum of 4 of 6 newly diagnosed insulin-dependent diabetic patients but in 0 of 6 non-diabetic controls with no family history of insulin-dependent diabetes. Binding of *M tuberculosis* HSP 65 antibodies against interleukin- $1\beta$ -treated rat insulinoma

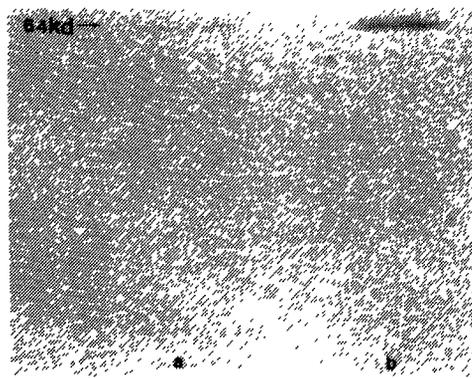


Fig 2—Western blot of control cell lysate (a) and interleukin- $1\beta$ -treated cell lysate (b) after addition of monoclonal antibody against *Mycobacterium tuberculosis* HSP 65.

Antibody binding visualised by alkaline phosphatase stain.

cells was inhibited by prior addition of serum from the 2 patients known to have antibodies against 64 kD protein.

## Discussion

The known cross-reactivity between rat  $\beta$ -cell and human 64 kD antibodies,<sup>5</sup> and the conservation in sequence between rat and human heat-shock proteins, support the view that rat islet cell preparations are valid models of human  $\beta$ -cell antigenicity. Our findings indicate that the 64 kD  $\beta$ -cell protein in IDDM can be induced by cytokines and by heat shock, and may thus be a heat-shock protein. Moreover, the 64 kD  $\beta$ -cell protein shows cross-reactivity with antibodies directed against *M tuberculosis* HSP 65. In addition, serum known to contain 64 kD antibodies inhibited the binding of *M tuberculosis* HSP 65 antibodies to interleukin- $1\beta$ -treated rat insulinoma cells.

The mycobacterial heat-shock proteins that we tested show a high degree of sequence homology and differ in structure by only two aminoacids within the common epitopes recognised by these monoclonal antibodies (D. Young, personal communication). Thus the target 64 kD  $\beta$ -cell antigen may have structural similarities with the *M tuberculosis* HSP 65 at a region between aminoacids 200 and 230—a region recognised by the monoclonal antibodies against *M tuberculosis* HSP 65 but not by those against *M leprae* HSP 65. We do not propose that the cross-reactivity of *M tuberculosis* heat-shock protein antibodies indicates a pathogenic connection between tuberculosis and insulin-dependent diabetes—it is more likely that an epitope

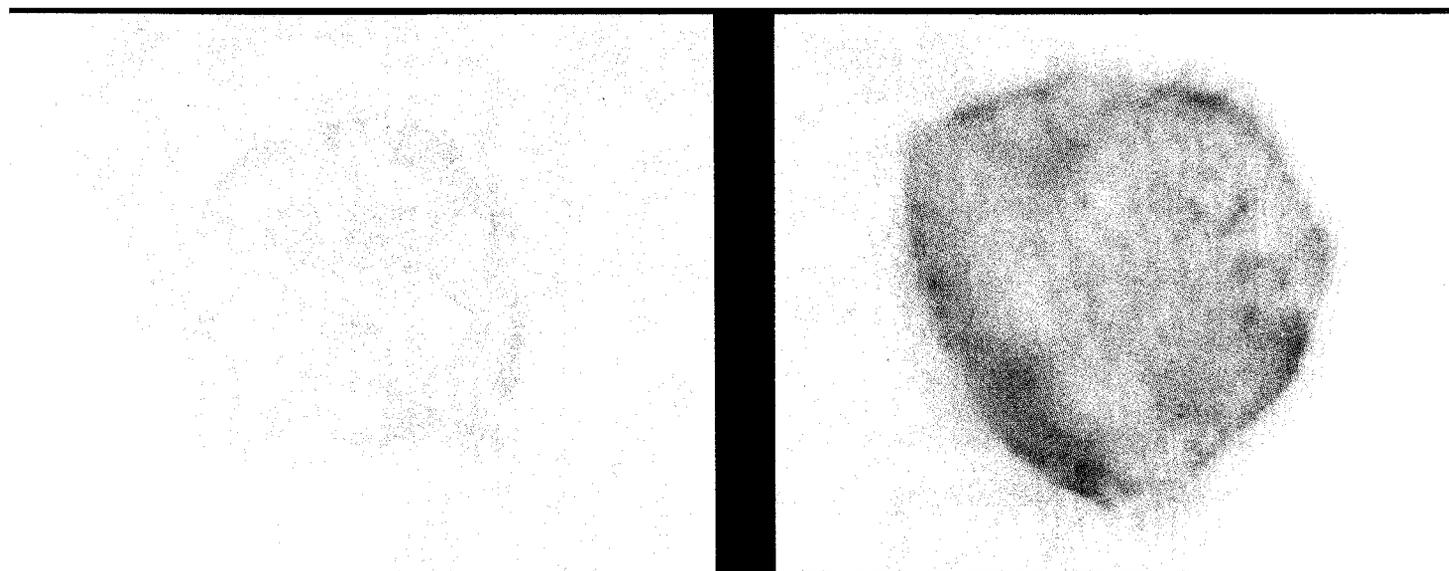


Fig 3—Indirect immunofluorescence of control (left) and interleukin- $1\beta$ -treated (right) rat insulinoma cells stained with monoclonal antibody against *M tuberculosis* HSP 65.

of heat-shock protein 65 is shared by *M tuberculosis* and man.

Elias et al<sup>11</sup> recently reported that the 65 kD mycobacterial HSP induced diabetes when given with an adjuvant to diabetes-prone NOD mice; the diabetes was preceded by the appearance of antibodies against HSP 65 and by development of T-cell clones directed against HSP 65 antigen. Elias et al<sup>11</sup> also found that previous inoculation of soluble HSP 65 to NOD mice reduced the incidence of diabetes on subsequent injection with adjuvant. The precise clinical role of immunity against stress proteins has yet to be confirmed. However, these observations may be relevant to our understanding of the aetiology of IDDM and to the search for potential preventive measures.

This research was supported by the British Diabetic Association and the UNDP/World Bank/WHO special programme for Research and Training in Tropical Diseases. G. W. D. is supported by the Arthritis and Rheumatism Council.

We thank Dr Anne Cooke (Middlesex Hospital, London, UK) for rat insulinoma  $\beta$  cells; Dr Alan Shaw of Glaxo for recombinant human interleukin-1 $\beta$ ; Dr Gunther Adolf of Boehringer Institute for recombinant human TNF- $\alpha$ ; Dr Douglas Young (MRC Tuberculosis and Related Infections Unit, Hammersmith Hospital, London) for gifts of monoclonal antibodies; Dr Jan van Embden (National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands) for recombinant mycobacterial heat-shock proteins; Dr Duncan Pepper and staff of the Scottish National Blood Transfusion Service (Edinburgh) for practical assistance; and Mrs B. Beattie, who prepared the typescript.

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## Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy

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**Human immunodeficiency type 1 isolates from 18 initially symptom-free men who were treated with zidovudine for 2 years were investigated for drug sensitivity. At the start all the men had persistent core antigenaemia; the acquired immunodeficiency syndrome developed in 6 during the study. The polymerase chain reaction was used to detect mutations at residue 215 of reverse transcriptase, a mutation associated with reduced drug sensitivity. After 2 years 16/18 isolates were mutant. However, after about 6 months of treatment the mutation was detected in only 7 isolates, 4 from individuals who subsequently had AIDS. Limited direct virus sensitivity data correlated with the genetic data. The rate of appearance of the 215 mutation seemed to correlate with CD4 counts and viral virulence.**

*Lancet* 1990; **336**: 585-90.

### Introduction

Treatment with the nucleoside analogue 3'-azido-3'-deoxythymidine (zidovudine or AZT) has been shown to extend life-expectancy and to lower the frequency and

severity of opportunistic infections in selected patients with the acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC).<sup>1</sup> Administration of zidovudine to symptom-free individuals infected with human immunodeficiency virus (HIV) also seems to reduce the occurrence of opportunistic infections.<sup>2</sup> This drug probably suppresses the replication of HIV-1 in vivo since the serum concentration of free HIV-core (p24) antigen<sup>13</sup> and the amount of infectious virus in plasma<sup>4</sup> decline during treatment. This reduction in HIV-1 replication is paralleled by a rise in the CD4-positive lymphocyte counts in

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