Autoimmunity

The development of insulin-dependent diabetes mellitus in non-obese diabetic mice: the role of CD4+ and CD8+ T cells
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Introduction
The non-obese diabetic (NOD) mouse is a spontaneous animal model of the human autoimmune disease, insulin-dependent diabetes mellitus (IDDM) [1]. In this mouse model, mononuclear cell infiltration in the pancreas is evident in both sexes from 5 to 6 weeks of age. Disease development resulting from a progressive intra-islet infiltration and β-cell destruction occurs in many colonies, predominantly in female mice, from 30 weeks of age.

The development of IDDM in the NOD mouse is dependent on T cells and the disease can be passively transferred by a combination of CD4+ and CD8+ splenic T cells from a diabetic donor but not by either CD4+ or CD8+ T cells alone [2]. Macrophages are the only phagocytic cells detected in pancreatic lesions in the resulting diabetic animals. Macrophages seem to play a role in β-cell destruction as disease development can be inhibited by treatment of NOD mice with silica or antibodies directed towards the CR3 receptor on these cells [3], and these treatments inhibit phagocytosis or binding, respectively.

As in man, the disease in the NOD mouse has been shown to be under polygenic control with at least one gene linked to the class II region of the major histocompatibility complex (MHC). The NOD mouse has unusual features in the MHC class II region. This animal does not express I-E antigens because of a deletion in the promoter region of I-Ea and the sequence of its I-Ap is unique [4, 5]. Analysis of DQ β-chain (human homologue of murine I-αβ) sequences in man suggested that the presence of an aspartic acid at position 57 conferred resistance to the development of IDDM [6]. As the I-αβ chain of most mouse strains encodes an aspartate at position 57, whereas the NOD I-αβ has a serine at this position, it was suggested that this amino acid residue might also be of importance in determining susceptibility or resistance to IDDM in mice.

We have performed a series of experiments designed to address the role of different subpopulations of lymphocytes in the development of IDDM in the mouse model as well as the role of different MHC genes in conferring susceptibility to the development of IDDM [7]. In this paper, we describe some of the experiments which demonstrate the obligatory role of at least three separate subpopulations of mononuclear cells in the chain of events leading to IDDM.

Materials and methods

Mice
A breeding nucleus was established at the C.R.C., Northwick Park (NOD/CRC) from mice provided by Dr E. Leiter, Jackson Laboratory, Bar Harbour, U.S.A.

Antibodies
Rat monoclonal antibodies to mouse cell surface antigens were prepared as ascites fluid in LOU × DA rats pretreated with pristane. Antibodies were prepared by ammonium sulphate (14%, w/v) precipitation and the Ig content was determined by SDS/PAGE. For injection into mice the antibodies were diluted in 10 mm-sodium phosphate, 145 mm-NaCl, pH 7.0 phosphate-buffered saline (PBS). The antigenic specificities, Ig subclass and code of the antibodies used are as follows: [8] Ly2 (equivalent to CD8), IgG2b, YTS 169.4 [8] L3T4 (equivalent to CD4), IgG2b, YTS 191.1; [10] CD4, IgG2a, YTS 177.9.6.1; [9] CD7, - , YTH 3.2.6; Thy 1.2, - , YTS 154.7. YTS 177.9.6.1 binds to CD4 but does not cause depletion of the CD4 cell population. YTS 169.4 and YTS 191.1, in contrast, deplete CD8 and CD4 cells, respectively.

Isolation of T cell lines
Sublethally irradiated NOD mice were primed with spleen cells from diabetic animals. Two weeks later, spleen cells were taken from these mice and expanded by weekly restimulations with NOD islets and irradiated NOD spleen cells as co-stimulators.

Abbreviations used: NOD, non-obese diabetic; IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

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Transfer of diabetes

Whole spleen cells from overtly diabetic NOD mice, usually female, were prepared as single cell suspensions in Hanks’ balanced salt solution and \(2 \times 10^7\) cells were injected intravenously into disease-free 2-3-month-old male recipients which were given 650 rads (a sub-lethal dose) earlier the same day (cobalt source). After transfer, the recipients were monitored at least weekly for the development of diabetes, the clinical onset of which was judged by the presence of glucose in the blood and urine. Urine was tested using ‘Diastix’ reagent strips (Miles Laboratories) and blood samples were tested using a Beckman glucose analyser or a Glucometer (Ames Division, Miles Laboratories). A consistent blood glucose reading of \(<10\) mm/litre coupled with a positive test by ‘Diastix’ was considered to be an indication of overt diabetes. When T cell lines were used to induce disease, \(10^7\) washed cells were injected intravenously with or without \(10^7\) diabetic donor spleen cells depleted of either CD4+ or CD8+ T cells as described above.

T cell depletions in vivo

Rat monoclonal antibodies to mouse T cell surface antigens were used to treat (a) irradiated recipients of spleen cells, or (b) diabetic donor mice.

(a) For depletion of specific T cell populations in recipients, each NOD mouse received 400 \(\mu\)g (in 200 \(\mu\)l PBS) of antibody (YTS 169.4; YTS 191.1) intravenously on day one and the same amount intraperitoneally on the following two days. Control mice were treated with either YTH 3.2.6 or with PBS alone. Treatment of recipients with the antibody YTS 177.9.6.1, which does not lead to depletion of CD4+ T cells, but binds specifically to this cell population, was as follows: NOD mice recipients were injected with YTS 177.9.6.1 (2 mg) on three consecutive days prior to transfer of spleen cells from donor diabetic NOD mice. Recipient mice were then injected with YTS 177.9.6.1 three times per week throughout the course of the experiment. The initial injection was intraventricular and all subsequent injections were intraperitoneal.

(b) Diabetic donor mice were treated with YTS 169.4 or YTS 191.1 to enrich spleen cells for either CD4+ or CD8+ T cells, respectively. Donor mice were injected with these antibodies on three consecutive days prior to sacrifice. The injection schedule was as described above for recipient mice.

Immunohistology

Frozen pancreatic tissue sections pretreated with 20% (v/v) normal mouse serum in PBS, were stained by a two-layer peroxidase technique. Mouse T cell surface components were detected by incubation with the following rat anti-mouse monoclonal antibodies, prepared as described above: anti-L3T4 (YTS 191.1); anti-Ly2 (YTS 169.4); and anti-Thy 1.2 (YTS 154.7). Sections were then incubated with goat anti-rat IgG biotin conjugate (Sera Lab) followed by avidin bound to horseradish peroxidase. Macrophage cell surface antigens were detected by an indirect immunofluorescence technique. Frozen fixed sections were preblocked with 20% (v/v) normal mouse serum and incubated with F4/80, specific for mature macrophages [11] or with Mac-1 which binds to the iC3b receptor [12]. These rat anti-mouse monoclonal antibodies were followed by goat anti-rat antibodies labelled with fluorescein isothiocyanate (Jackson Immunobio-Assays) diluted in normal mouse serum. To stain for NOD class I antigen, the rat anti-mouse monoclonal antibody M1/42.3 [13] was used. Pancreatic \(\beta\)-cells were detected by incubating sections with guinea-pig anti-porcine insulin (ICN Immunobiologicals) followed by rhodaminated goat anti-guinea-pig IgG (Cappel).

The role of T cells in the development of IDDM

When spleen cells from a diabetic donor are transferred into an irradiated recipient of the same NOD strain, IDDM is passively transferred. Monoclonal antibodies specific for CD4+ or CD8+ T cells can be employed to treat recipients of such diabetic spleen cells to assess the relative roles of these two T cell subpopulations in disease development.

(a) Treatment with anti-CD4 antibodies prevents transfer of IDDM

It can be seen from Table 1 that treatment of recipients with anti-CD4 antibodies (the non-depleting antibody YTS 177.9.6.1) completely prevents the successful transfer of IDDM by diabetic spleen cells.

(b) Treatment with anti-CD8 antibodies prevents transfer of IDDM

Administration of anti-CD8 antibodies (the depleting monoclonal, YTS 169.4) within two weeks after diabetic spleen cell transfer prevented the onset of IDDM in recipient mice (Fig. 1). If anti-CD8 treatment was delayed until two weeks or later, the protective effect of this antibody was lost.

Detailed histological analysis of the pancreas of treated animals revealed that depletion of CD8+ cells within two weeks of transfer of spleen cells from a diabetic animal prevented intra-islet infiltration by CD4+ T cells and macrophages (Fig. 1). It
Table 1
Treatment with YTS 177.9.6.1 (non-depleting anti-CD4) prevents the transfer of diabetes

<table>
<thead>
<tr>
<th>Day 12</th>
<th>Day 29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>Untreated</td>
</tr>
<tr>
<td>7.3</td>
<td>9.9</td>
</tr>
<tr>
<td>6.1</td>
<td>5.2</td>
</tr>
<tr>
<td>8.1</td>
<td>7.0</td>
</tr>
<tr>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>7.2</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Values are blood glucose in mEq/l, estimated using a Glucometer which registers a maximum of 22.2 mEq/l. Values are for individual mice.

Fig. 1
Sublethally irradiated male NOD mice were given 2 x 10^7 spleen cells from diabetic donors and treated with anti-CD8 before week 2 (day 1 or day 8) or after week 2 (day 15 or day 21). Untreated animals were given diabetic spleen cells but no anti-CD8 and the control males were age-matched stock animals. Pancreases were examined 5 weeks after transfer. ■, CD4+; □, CD8+; □, Mac-1.

(c) NOD CD4+ T cell lines only infiltrate the islets in the presence of CD8+ cells

CD4+ NOD T cell lines have been generated which are capable of causing a transient hyperglycaemia. Because recipients of such T cell lines did not develop sustained disease, the effect of CD8+ T cells on T cell line mediated β-cell destruction was assessed.

The results of this experiment are presented in Table 2. It can be seen from this Table that diabetic spleen cells depleted of CD4+ T cells (using YTS 191.1) could not infiltrate the islet or transfer overt disease. This is in line with data from Wicker and co-workers [2]. However, if CD4+ splenic T cells from a CD8+ depleted diabetic donor were also transferred, the recipient developed hyperglycaemia (Table 2b) and addition of the CD4+ T cell line also restored the ability of such CD8+ T cells to mediate an intra-islet infiltration (Table 2a).

Our previous studies have shown that intra-islet infiltration by T cells and macrophages induces an increase in class I MHC antigen expression in all endocrine cells in the islet and in exocrine cells adjacent to the area of infiltration. Islets in the pancreas of animals receiving a combination of CD8+ T cells with either CD4+ T cells from a diabetic spleen or CD4+ cells from a T cell line, manifest increased class I MHC antigen expression whereas islets in the pancreas of animals receiving only CD8+ T cell enriched splenocytes did not show increased MHC class I expression (Table 2a).

In addition to T cells, the intra-islet infiltration observed in the prediabetic pancreas is also composed of mature inflammatory macrophages. Table 2(a) demonstrates that the recruitment of macrophages into the islet requires both CD4+ T cells and CD8+ T cells. The CD4+ T cell line can mediate such macrophage recruitment in the presence of CD8+ T cells.

Discussion
The development of IDDM in the NOD mouse has been shown to require CD4+ T cells, CD8+ T cells and additionally, macrophages. Intra-islet infiltration by such cells results in pancreatic β-cell destruction but the exact mechanism by which such specific destruction is effected remains to be clarified.

The hyperexpression of class I MHC antigens on the pre-diabetic pancreatic endocrine and exocrine makes it tempting to assume that the β-cell is selectively destroyed by a CD8+ (class I restricted) T cell. However, studies demonstrating the exquisite sensitivity of β-cells to macrophage-derived products [14], as well as the presence of inflamma-
CD8+ T cells require CD4+ T cells to mediate intra-islet infiltration and diabetes

N.D., not determined. *Spleen cells enriched for CD4+ and CD8+ T cells were taken from diabetic donors depleted in vivo with monoclonal antibodies as described in the Methods section. 10^7 cells of each subpopulation and/or T line cells, were injected intravenously into irradiated recipients which were sacrificed at 2 weeks for immunohistological studies or monitored for diabetes until week 4.

(a) Staining for macrophages and class I

<table>
<thead>
<tr>
<th></th>
<th>Islets positive for F4/80 (%)</th>
<th>Islets positive for class I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peri-</td>
<td>Intra-</td>
</tr>
<tr>
<td>CD8+ T cells (10^7)*</td>
<td>9.1</td>
<td>5.0</td>
</tr>
<tr>
<td>CD8+ T cells + CD4+ T cells</td>
<td>N.D.</td>
<td>90.0</td>
</tr>
<tr>
<td>CD8+ T cells + T cell line</td>
<td>1.0</td>
<td>71.0</td>
</tr>
</tbody>
</table>

(b) Incidence of diabetes

<table>
<thead>
<tr>
<th>Cells administered</th>
<th>Blood glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ T cells alone</td>
<td>Normoglycaemic at week 4</td>
</tr>
<tr>
<td>T line cells alone or with CD8+ T cells</td>
<td>Early, slight and transient hyperglycaemia</td>
</tr>
<tr>
<td>CD4+ T cells alone</td>
<td>Normoglycaemic at week 4</td>
</tr>
<tr>
<td>CD4+ T cells mixed with CD8+ T cells</td>
<td>Overt diabetes at week 4</td>
</tr>
</tbody>
</table>

...tory macrophages within the islet [15] suggest an alternative mechanism. Our recent studies demonstrating that blockade of the adhesion molecule (CD11/CD18) prevents recruitment of T cells to the islet and the subsequent transfer of IDDM by diabetic spleen cells, emphasizes the pivotal role of the macrophage in β-cell destruction.

To try to dissect out the relative contributions of different lymphoid subpopulations in β-cell destruction we have selectively depleted diabetic spleen cell populations. The studies presented in this paper underline the need for three cell types in the genesis of IDDM: CD4+ T cells, CD8+ T cells and macrophages. Our studies suggest that CD8+ T cells play a role in facilitating the influx of CD4+ T cells and macrophages to an intra-islet location. Since CD8+ T cells on their own are unable to mediate β-cell destruction, this suggests that CD8+ T cells may act by secreting cytokines which affect vascular permeability, migration and recruitment of effector cells to the site of inflammation. Therefore, if, as we have shown in recent studies [Nature (London) in the press], arrest of macrophage migration inhibits T cell recruitment to the islets and if depletion of CD8+ T cells or CD4+ T cells prevents not only T cell infiltration but also the influx of macrophages, then it is apparent that a complex cellular interdependence exists in the genesis of IDDM, all the components of which, are required to be present at the target site.

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The molecular basis of halothane-induced hepatitis

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Halothane hepatitis

The anaesthetic agent halothane (CF₃CHClBr) causes two distinct types of liver damage. About 20% of halothane-exposed patients exhibit mild hepatotoxicity, characterized by transient elevations in liver enzymes. In contrast, a much smaller fraction of patients exposed to halothane (between 1 in 3500 and 1 in 35 000) develop severe hepatotoxicity, commonly termed 'halothane hepatitis', which frequently may progress to liver failure and death [1]. Typically, patients with halothane hepatitis tend to be obese females in late middle age, although a minority of patients are not obese, some are male and the syndrome has been described in children [2]. In common with many other adverse drug reactions which are of clinical importance, halothane hepatitis is idiosyncratic and dose-independent. The underlying pathogenic mechanisms have yet to be resolved unequivocally, although various clinical and laboratory findings indicate that immune processes are involved [1]. A series of recent investigations have outlined a likely mechanism, which involves an immune response to a group of novel, halothane metabolite-modified liver protein antigens.

Immune response to halothane-induced liver antigens

Initially, Vergani et al. [3] found that leucocytes from patients with halothane hepatitis were sensitized to undefined components present in homogenates of livers from halothane-anaesthetized rabbits, but not in homogenates from ether-anaesthetized rabbits, as assessed using a crude in vitro leucocyte migration test. Subsequently, indirect immunofluorescence and induced cellular cytotoxicity studies demonstrated that the patients' sera contained antibodies to antigens present on hepatocytes isolated from livers of halothane-anaesthetized rabbits, but not on hepatocytes from untreated or ether-anaesthetized rabbits [4]. These studies showed that the halothane-induced antigens were expressed on the surface membrane of isolated rabbit hepatocytes and that rabbit hepatocytes bearing the novel antigens were susceptible to antibody-directed cytotoxic killing by normal human lymphocytes in vitro. In addition, antibodies to the antigens were detected only in sera from patients with halothane hepatitis and not in a wide range of control sera, including sera from patients who received multiple halothane anaesthetics but did not develop liver damage, and sera from patients who sustained hepatitis attributable to recent viral infection and who had undergone recent anaesthesia with halothane. More recent studies, performed using other methods for antibody detection (c.e.i.s.a. and immunoblotting) [5, 6], have confirmed that antibodies to halothane-induced liver antigens are restricted to patients with halothane hepatitis. Thus sensitization to halothane-induced antigens is not simply a secondary consequence of halothane exposure, or of halothane exposure plus liver damage.

Abbreviation used: TFA, trifluoroacetyl.