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PhD thesis

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Allergic Contact Dermatitis and Autoimmune Diseases

- Epidemiological and Experimental studies

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&

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Study part 1

I. Engkilde K, Menné T, Johansen JD. Inverse relationship between allergic contact dermatitis and type 1 diabetes mellitus: a retrospective clinic-based study. *Diabetologia 2006*;49(4):644-7.

II. Engkilde K, Menne T, Johansen JD. Inflammatory bowel disease in relation to contact allergy: a patient-based study. *Scand J Gastroenterol 2007*;42(5):572-6.

Study part 2

III. Engkilde K, Buschard K, Hansen AK, Menné T, Johansen JD. Prevention of Type 1 Diabetes by Exposure to a Contact Allergen Inducing a Sub-Clinical Dermatitis. Manuscript submitted to *Diabetes* 2009.

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1 Summary

1.1 Summary (English)

Allergic contact dermatitis is a frequent disease, and up to twenty percent of the adult population is sensitized to one or more contact allergens. There does not appear to be genetic predispositions, which could explain this high quantity of sensitized individuals, and allergic contact dermatitis is therefore in essence an environmental acquired disease, where development of disease is due to exposure patterns. Allergic contact dermatitis is a cellular mediated disease, which involves both the innate and the adaptive immune system, and is the prototype of a delayed-type hypersensitivity reaction. Allergic contact dermatitis is diagnosed by patch testing, an internationally standardized in vivo test. Due to the involvement of various different immune cells, it is possible that allergic contact dermatitis could influence the occurrence of other diseases involving the immune system.

The aim of this PhD study was to investigate associations between allergic contact dermatitis and autoimmune disease.

The PhD work was done as two separate projects: an epidemiological study (Part 1) and an experimental murine study (Part 2).

For Part 1, the register from the Dermatological Department at Gentofte Hospital, which contains results of patch tests, was linked with the Danish National Patient Register, which contains discharge diagnoses. The relationship between a positive patch test and type 1 diabetes or inflammatory bowel disease (IBD) was studied. A significant inverse association to type 1 diabetes (OR: 0.63) and IBD (OR: 0.71) was found. In the IBD study, Crohns disease appeared to be the driving factor for the association. Type 1 diabetes and Crohns disease are two autoimmune diseases where genetic predispositions play a large role, and it is therefore interesting that an environmental disease is associated inversely with these diseases.

For Part 2, the Non-Obese Diabetic (NOD) mouse was used to investigate whether an experimental allergic contact dermatitis influenced the development of diabetes. NOD mice were first treated according to the Local Lymph Node Assay (LLNA) with the potent allergens p-phenylenediamine (PPD) and 2,4-dinitrochlorobenzene (DNCB) to establish that they could respond to allergens. Subsequently, an incidence study was initiated to evaluate the effect of allergic contact dermatitis on diabetes development, and it was found that repeated treatment with PPD reduced the diabetes development (P= 0.048), while this was not the case for DNCB. For further study of the effect of allergens on the innate immune system, C57BL/6 mice were treated with PPD and DNCB, and it was establish that both allergens stimulated Natural Killer T cells (NK-T) in the liver, while seemingly only PPD resulted in an increase of NK-T cells in the draining lymph node.

In conclusion, allergic contact dermatitis appeared to be inversely correlated with autoimmune diseases, such as type 1 diabetes and Crohns disease. This could be due to environmental and/or genetic factors. Support for the role of an environmental factor was found in animal experiments, and additionally we found that the allergens increased the quantity of NK-T cells in the liver.

1.2 Resumé (Danish)

Allergisk kontakteksem er en hyppig sygdom, og op mod tyve procent af den voksne befolkning er allergiske over for et eller flere kontaktallergener. Der synes ikke at være nogen genetiske prædisponeringer, som kan forklare dette høje antal, og allergisk kontakteksem anses derfor for at være en overvejende miljøbetinget sygdom. Allergisk kontakteksem er en cellulært medieret sygdom, som involverer både det medfødte og det tilpassede immunsystem, og er prototypen på en forsinket hypersensitivitets (DTH) reaktion. Allergisk kontakteksem diagnosticeres ved epikutantest, som er en international standardiseret in vivo test. Involvering af mange forskellige celler i den kontaktallergiske reaktion gør, at det er muligt, at kontaktallergi kan influere udviklingen af andre sygdomme.

Formålet med ph.d.-studiet var at undersøge sammenhængen mellem allergisk kontakteksem og autoimmune sygdomme.

Ph.d.-studiet blev udført som to selvstændige projekter. Et epidemiologisk studie (Studie 1) og et eksperimentelt dyreforsøgsstudie (Studie 2).

I studie 1 blev et register fra Dermato-allergologisk afdeling på Gentofte Hospital, som indeholder information om resultatet af epikutantests, koblet med Landspatientregistret. Sammenhængen mellem en positiv epikutantest og type 1 diabetes eller inflammatorisk tarmsygdom blev undersøgt. En signifikant invers association til type 1 diabetes (OR: 0,63) og inflammatorisk tarmsygdom (OR: 0,71) blev fundet. Crohns sygdom synes at være årsagen til den inverse sammenhæng.

I studie 2 blev Non-Obese Diabetic (NOD) mus anvendt til at undersøge om eksperimentelt induceret allergisk kontakteksem kan påvirke udviklingen af diabetes. NOD musene er en musestamme, der spontant udvikler type 1 diabetes. NOD mus blev først behandlet svarende til protokollen for Local Lymph Node Assay med to potente allergener, p-phenylenediamin (PPD) og 2,4-dinitrochlorobenzen (DNCB), for at fastslå, at musene kunne reagere allergisk. Herefter blev et incidensstudie med NOD mus igangsat for at undersøge betydning af allergisk kontakteksem i relation til diabetes udviklingen. Det blev fundet, at gentagne behandlinger med PPD reducerede udviklingen af diabetes (p=0,048), mens dette ikke var tilfældet for behandling med DNCB.
For yderligere at undersøge effekten af kontaktallergener på immunsystemet blev C57BL/6 mus behandlet med henholdsvis PPD og DNCB, og det blev fundet at begge allergener øgede antallet af NK-T celler i leveren, mens kun PPD resulterede i en øgning af antallet af NK-T cellerne i de drænende lymfeknuder.

Vi fandt således, at allergisk kontakteksem synes at være inverst korreleret til autoimmune sygdomme som type 1 diabetes og Crohns sygdom. Denne sammenhæng kan skyldes ukendte miljømæssige og/eller genetiske faktorer.

Vi har i dyreforsøg fundet støtte for en miljømæssig komponent, og vi har yderlige fundet, at kontaktallergenerne øgede antallet af NK-T celler i leveren

2 Background

Allergic contact dermatitis is a reaction in the skin to contact allergens in the environment. It can be a distressing disease for those concerned and may cause sick leave, occupational impairment and increased national health service expenses. The disease was most likely recognized as an entity even in ancient times. One of the first recorded reports is by Pliny the Younger, who in the first century A.D. noticed that individuals experienced severe itching when felling pine trees (2). However, scientific inquiry in the disease was limited until the development of the epicutanous patch test, which made it possible to accurately identifying the contact allergen causing the disease.
The patch test technique, which is now the gold standard for testing, was presented by Josef Jadassohn in 1895 at the "Fünfter Congress der Deutschen Dermatologischen Gesellschaft" (2).

2.1 Allergic Contact Dermatitis

Contact allergy is caused by skin contact with haptens and may evolve to allergic contact dermatitis (ACD) if exposure exceeds the individual's threshold for reaction (3). Allergic contact dermatitis (ACD) is a cell-mediated inflammatory skin condition and is generally considered to be an example of a type IV hypersensitivity reaction according to Gell and Coombs classification (4).
The disease primarily involves exposed skin areas, most frequently on the hands, face and feet. The clinical definition of the disease is based on the history of the patient, clinical examination, patch testing, and a detailed exposure assessment (5).
The degree of contact allergy can be graded according to the patch test outcome, which demonstrates whether the individual is sensitized. A positive reaction is graded from + to +++, where + is defined by homogenous erythema, infiltration and possibly papules to +++, which is intense erythema, infiltration and coalescing vesicles (6).
In the acute stage, ACD lesions are characterized by papules, vesicles and weeping, while the lesions in the chronic stage may appear dry, thickened and scaly.

2.1.1 Sensitizing Molecules

The sensitizing substances are small molecules (haptens) with a molecular weight of less than 500 Dalton (7). Due to their size and electrophilic nature they can penetrate the stratum corneum and interact with proteins, making the hapten-protein molecule immunogenic (8). Some allergens may even interact directly with the human histocompatibility leukocyte antigen (HLA) in a peptide-independent manner (9). There are several thousand chemicals described that can cause ACD; however, the number of allergens that actually cause general problems in the population is much smaller. The present European baseline series contains 28 items (10), but as seven of these are mixes, a number of additional allergens are applied. Common contact allergens include metals, biocides/preservatives, fragrance chemicals and dyes.

2.1.2 Immunological Mechanisms

Knowledge of the pathophysiology of ACD is derived chiefly from animal models in which the skin inflammation induced by allergen painting of the skin is referred to as contact hypersensitivity (CHS) (11). ACD consists of two distinct phases: the induction (sensitization) and the effector (elicitation or challenge) phase (12). The induction phase includes the events following the first contact with the allergen and is complete when an individual is sensitized and capable of giving an ACD response. The effector phase begins upon challenge with the same allergen and results in the clinical manifestation of ACD (3).

The induction phase may take more than one exposure, as ACD to moderate or weak allergens almost never occurs after the first contact but can require years of permanent skin exposure to develop (12). When the dose of the contact allergen is sufficient it provides a danger signal, leading to a local production of proinflammatory cytokines that induce Langerhans cell (LC) mobilization to the draining lymph nodes (13). In the lymphoid tissue the HLA-allergen complexes are presented to naïve T-cells. If a naïve T-cell recognizes the complex and receives co-stimulatory signals, memory T-cells may be generated (3). The individual is then able to generate an ACD reaction.

The elicitation phase begins upon contact with the allergen in question, after which allergen specific T-cells are directed to the skin at the site of exposure, resulting in clinical manifestation of ACD.

2.1.3 Epidemiology of contact allergy

Most studies on epidemiology of contact allergy have been performed in Western Europe and North America, and from these studies a weighted prevalence of 19.5% has been calculated (14). The prevalence in Denmark is close to this value, as the point prevalence in the general population was found to be 18.6% in the age group 15–41 years (15). As there are such high numbers of contact allergy in the general population, many people are at risk of developing ACD.

There have been some studies on contact allergy in infants and children, as summarized in Thyssen et al. 2007 (14). These studies have generally reported a relatively high prevalence of allergy at young age, e.g., Röckl et al 1966 (16) found 40.6% to be sensitized, and the nature of these reactions has been questioned. (17).

In adolescents, however, it appears as if the prevalence is close to that in adulthood. In 2002 Mortz et al. reported the prevalence in adolescents aged 12–16 years to be 15.2% (18).

2.1.4 Genetics in Allergic Contact Dermatitis

In the older literature, there are studies that showed that there might be a genetic predisposition to ACD. For example, Fleming et al. (19) found that relatives of patients with nickel ACD had an increased risk of developing the condition, and it therefore appeared that there might be a family predisposition. Others have shown that patients with multiple allergies are more readily sensitized experimentally (20). More recent studies have tried to elucidate which genetic polymorphisms might predispose an individual to ACD. Most of these studies looked at nickel-allergic or p-phenylenediamine- (PPD) allergic patients. The human leukocyte antigens HLA-B7, -B21, -B12, -Bw22, -B35, -B40, -DR4, and -DRw6 have been shown to be increased in nickel-allergic patients (21-23). However, these studies were small and none found the same HLA antigens. Others have looked at cytokine polymorphisms. It has been found that a single nucleotide polymorphism (guanine to adenine) located at nucleotide-308 upstream of the transcription start site of tumor necrosis factor-alpha (TNF-α) can be a susceptibility factor for contact allergy (24;25). In addition, polymorphisms at nucleotide-295 in IL-16 have been shown to be associated with contact allergy (26) and also acetylation polymorphism in N-acetyltransferase 2 (NAT2) appears to be a susceptibility marker for PPD sensitization (27;28). Furthermore a loss-of-function mutations in the filaggrin has been found to be associated with sensitization to nickel (29). These filaggrin mutations can give an impaired skin barrier and can thereby allow greater penetration of chemical haptens; however, the study did not find associations with other contact allergens, but this could be due to power reductions, as nickel is the foremost common contact allergen. A thorough review of genetics involved in nickel allergy can be found in Schram et al (30), and of multiple allergies in Carlsen et al (31).

Despite these studies, the general view is that ACD is controlled by genetics to only a small extent and is mainly due to dose and exposure patterns, a view supported by a twin study by Bryld et al. (32). Likewise, the gender difference is also assumed to be due to a larger exposure to fragrances, cosmetics and the like; female constitutes about 2/3 of patients with ACD, (33;34). The gender difference is still a matter of discussion (35), but it might be due to hormones as it has reported that the response differs during the menstrual cycle (36;37). Furthermore, it has been suggested that 17β-estradiol augments the oedema in a mouse model of ACD by enhancing the expression of inflammatory cytokines (38).

2.1.5 New findings:

As the ACD reaction is easily studied in mice models (CHS), there are many investigative studies on the immune system using this immune reaction, and through these studies much new insight has been gained into the immunological reactions leading to ACD.

Doubt exists as to whether Langerhans cells (LC) really are the dendritic cells that present allergens to T-cells in the lymph nodes. Kaplan et al. and others have done several animal studies in which the LC were ablated. Their results point toward a regulatory role of LC, while a novel population of dermal

dendritic cells appears to be the cells that capture, process and present antigen (39). Fukunaga et al. (40) give support to the lack of a role for LC in generation of the hypersensitivity response.
Other studies have revealed other cell types that may be involved in allergic contact dermatitis, for example: mast cells (41), platelets (42), Natural Killer cells (43;44), Natural Killer T-cells (45-47), and IL-17-producing CD8 and CD4 T cells (48;49).
For the Natural Killer T-cell (NKT), it is the invariant CD1d restricted NKT cells that have been studied in gene-knockout studies (45), in tolerance studies (47) or in immunization studies (46).
An involvement of IL17 producing T-cells and Natural Killer T-cells may indicate that ACD can influence other diseases. These cell types have recently received a great deal of attention due to their involvement in several diseases; furthermore, there has been speculation about targeting them in vaccine development (50;51).

2.1.6 Therapeutic Use of Contact Allergens

The allergic contact dermatitis reaction has been used in topical therapy of alopecia areata and viral warts (52). A practice that according to Buckley et al (52) goes back to the 1960s. Furthermore, the immunological response towards the contact allergen 2,4-dinitrochlorobenzene (DNCB) has shown to be correlated with the prognosis for cutaneous T cell lymphoma (53); and dacarbazine mixed with DNCB is effective in patients with regionally metastasized melanoma (54). There is even a unorthodox study from 1997 showing DNCB therapy to be an inexpensive treatment approach for HIV disease (55).

2.2 Diabetes Mellitus

Diabetes mellitus (DM) has been known for several millenaries. It comprises a group of metabolic disorders, in which hyperglycaemia is the phenotype. DM is classified on the basis of the pathogenic process that leads to hyperglycaemia (56). The two main types are designated type 1 and type 2. Other forms relate to gestational or medical disorders.

In 1979 and 1980, both the U.S. National Diabetes Data Group (57) and the World Health Organization (58) tackled the task of classification and criteria of diabetes. The terms "juvenile-onset diabetes" and "maturity-onset diabetes" were replaced by insulin-dependent diabetes mellitus (IDDM) or type 1 diabetes and non-insulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes. As the terms insulin-dependent and non-insulin-dependent DM were considered to be confusing, resulting in classifying the patients based on treatment rather than etiology, the terms were eliminated while the terms type 1 and type 2 diabetes were retained (59;60).

2.2.1 Type 1 DM

Type 1 DM is a chronic disease and is the result of an autoimmune cellular-mediated destruction of the beta cells in the islets of Langerhans, leading to a lifelong insulin dependency. Islet cell antibodies (ICAs), glutamic acid decarboxylase antibody (GADA), insulin autoantibodies, or autoantibodies to tyrosine phosphatases are present in the majority of people with type 1 DM (61). These antibodies are markers of the destruction of the pancreas, which is T-cell mediated (62).

Type 1 DM commonly occurs in childhood and adolescence but can occur at any age even in the 8th and 9th decades of life (63); 50% of cases have their onset after the age of 20 years (64).

The main genetic predispositions to type 1 DM is found in the genes encoding HLA and insulin (65). The HLA region accounts for about half of the familial clustering of type 1 DM through a large variety of protective and predisposing haplotypes (62).

The concordance rate for monozygotic twins has been reported to be 23%–43% (probandwise); however, as the concordance rate for dizygotic twins is much smaller, it emphasizes the importance of genetics (66;67). A longer follow-up of discordant twins might, however, result in a higher concordance (68). As 85% of new cases occur sporadically in individuals without a first-degree relative with the disease (65), this, combined with the rise in the incidence of type 1 DM in recent decades (69), points to hitherto unknown environmental factors. Both vitamin D and virus infections have been suggested to be the environmental factors, as studies have shown seasonal variation where the incidence is at its maximum in the winter period (70).

Type 1 DM patients require life-long treatment with insulin and have a high risk of medical complications; accordingly, preventive or curative therapies are needed (71).

Research in type 1 DM, especially in the pre-diabetic stage, is mainly done in two spontaneously animal models of type 1 DM, the non-obese diabetic (NOD)

mouse and the bio-breeding (BB) rat (72). In female NOD mice, diabetes onset occurs typically at 12 to 14 weeks of age, at which time a large number of β-cells have been destroyed. From as early as 3 to 4 weeks of age mononuclear infiltrates can be seen in the pancreas, and when they reach 25–30 week of age, around 80% of female NOD mice have diabetes (73;74). Factors that help promote a type 1 cytokine over a type 2 cytokine response or disturb this balance, can directly affect diabetes susceptibility in NOD mice (73).
Type 1 DM is considered a Th1 disease and has therefore been inversely associated with type I allergies. However, since most β-cells are already destroyed when type 1 DM presents, the Th1 driven pancreatic inflammation diminishes with length of disease and therefore the inverse association is probably found only in pre-diabetics and newly diagnosed patients (75). Asthma, however, is apparently still inversely associated with type 1 DM and autoimmune diseases in general (76;77).
Recently, there has been focus on the NKT cell and the possibility of manipulating the progression of diabetes in the NOD mouse by augmenting the NKT cell activity as this has been shown to suppress the development of autoimmune disease (74).

2.2.2 Type 2 DM

Type 2 DM is a group of chronic disorders characterized by a variable degree of insulin resistance. Type 2 DM results from the interaction between genetic and lifestyle factors (78). It accounts for about 90% of all diabetes cases and is estimated to be increasing rapidly, especially in the developing countries (79). The increase is the result of profound changes in patterns of environmental exposures, such as diet, exercise and other lifestyle changes that predispose to obesity (80). Obesity and physical inactivity are therefore considered to be strong risk factors for Type 2 DM (81). Nevertheless, new studies indicate that type 2 DM and related intermediate traits are influenced by genetic variations that modify the individual response (80;82).
Type 2 DM develops primary from two mechanisms where glycaemic control is lost. The early stage is characterized by insulin resistance, in which uptake of glucose by tissue cells is decreased in response to insulin, resulting in hyperglycemia after eating and defects in pancreatic beta-cell function (83). In the early stage, the beta-cells will try to compensate for the insulin resistance by secreting more insulin (84), but as the insulin response to food intake worsens, the beta-cell function deteriorates, which results in a more lasting hyperglycemia (85).
Risk factors for type 2 DM seem to differ among ethnic populations; nonetheless, across ethnic groups, family history confers an increased risk of type 2 DM (82;86). Several genome-wide association studies have recently been done, and these have found a number of single-nucleotide polymorphisms associated with type 2 DM (87-91). These genetic variants represent common variants that are shared by a large proportion of the population and may, therefore, have only modest effect (82).
Type 2 DM typically develops with increasing age; it does, however, occur in young people too, particularly in obese adolescents (92).

2.3 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is an idiopathic and chronic intestinal inflammation. The two main clinical entities of IBD are Crohn's disease (CD) and Ulcerative Colitis (UC). Both are chronic relapsing inflammatory conditions of the gastrointestinal tract.

The generally accepted notion is that the mucosa of patients with CD is dominated by CD4+ T-cells with a Th1 phenotype, while the mucosa of patients with UC is dominated by an atypical Th2 phenotype (93-95). Susceptibility to IBD is influenced by genetic predispositions, especially in CD where the concordance rate for monozygotic twins is relatively high, while the concordance rate for UC is low (96-98). Nevertheless, since the concordance rate is well below 100%, non-genetic factors, primarily environmental or luminal, may be required to trigger IBD in a genetically susceptible host. Once a diagnosis of IBD is made, distinguishing between UC and CD is impossible in 10–15% of the cases (99)

In the Western world, there was an increase in incidence in both CD and UC during the 1960s and 1970s. The incidence tends to stabilize in areas with high incidence such as Northern Europe and America, while it seems to increase in areas with low incidence (100).

Genes predisposing for IBD have been difficult to discover due to complex genetics, including factors such as lack of simple Mendelian inheritance patterns, involvement of several genes, and the influence of environmental factors and intestinal microflora on disease development (101). For CD, however, an association between CD and variants in the nucleotide-binding oligomerization domain 2 (NOD2) gene—also known as caspase recruitment domain 15 (CARD15)—has been found (102;103). In addition, no association with UC was found, which further shows these are different diseases and that separate genes predispose (102;103). Conversely, the IL23R gene has been associated with both CD and UC, which indicates that IL17 plays a role in both diseases (104).

3 Aims of The Study

The aim of the thesis was to investigate associations between allergic contact dermatitis and autoimmune disease.

Study I To investigate a possible association between a positive patch test and diabetes mellitus in patients with contact dermatitis.

Study II To investigate a possible association between a positive patch test and inflammatory bowel disease in patients with contact dermatitis.

Study III To study the effect of either contact allergy or allergic contact dermatitis on diabetes development in the Non-Obese Diabetic mouse.

Study IV To study the effect on NKT cells in liver and draining lymph nodes after challenge with allergen.

4 Materials and Methods

The PhD work was done as two separate projects: an epidemiological study (Part 1) and an experimental murine study (Part 2).

4.1 Part 1: The Epidemiological Study

4.1.1 Database Containing Results of Patch Tests

The Department of Dermato-Allergology at Gentofte Hospital holds records on all patch tests performed at the department from 1979 until the present time. The test series used were equivalent to the European baseline series recommended at the time. The series contains the most important contact allergens in the environment. The epicutanous patch testing was done according to international standards—on the upper back using Trolab (Hermal, Reinbek, Germany) and Finn chambers (Epitest, Tuusula, Finland). The occlusion time was 48 hours and the patches were read on Day 2, 3, and 5 or 7 according to international criteria from the International Contact Dermatitis Research Group (ICDRG) (6;105). Accordingly, a positive allergic reaction was defined as at least homogenous erythema and infiltration on the test site.
The period selected for the epidemiological studies was from 1 January 1985 to 31 December 2003 (18 years). This selection yielded 13,315 individual patients (4818 males and 8497 females). If an individual had a positive reading on any of the three reading days, this individual was considered to have allergic contact dermatitis. A binary variable was constructed with the information on whether an individual had ever had a positive patch test. If an individual had been patch tested more than once during this period, the age for this individual was calculated as the age at the first patch test with a positive reaction. If the individual had never reacted positively to a patch test, the age at the first patch test was used. After age calculation, the patients where stratified into five age groups: 0–15, 16–30, 31–50, 51–70 and more than 70 years of age.

Table 1. The applied allergens from the European Baseline Series

Allergen	Concentration	Time in effect
Potassium dichromate	0.5% pet	1/1-85—31/12-03
Neomycin sulphate	20% pet	1/1-85—31/12-03
Thiuram mix	1.0% pet	1/1-85—31/12-03
p-Phenylenediamine dihydrochloride	0.5% pet	1/1-85—31/12-88
----p-Phenylenediamine (free base)	1.0% pet	1/1-89—31/12-03
Cobalt chloride	1.0% pet	1/1-85—31/12-03
Benzocaine	5.0% pet	1/1-85—31/12-03
Formaldehyde	1.0% aq	1/1-85—31/12-03
Colophony	60% pet	1/1-85—31/12-85
----Colophony	20% pet	1/1-86—31/12-03
Quinoline mix	6.0% pet	1/1-85—1/1-95
----Clioquinol	5.0% pet	1/1-95—31/12-03
Myroxylon pereirae (Balsam of peru)	25% pet	1/1-85—31/12-03
N-Isopropyl-N-phenyl-4-phenylenediamine	0.1% pet	1/4-93—31/12-03
----Black rubber mix	0.6% pet	1/1-85—1/4-93
Wool alcohols (lanolin alcohol)	30% pet	1/1-85—31/12-03
Mercapto mix	1.0% pet	15/2-95—31/12-03

----Mercapto mix	2.0% pet	1/1-85—14/2-95
Epoxy resin	1.0% pet	1/1-85—31/12-03
Paraben mix	15% pet	1/1-85—31/12-94
---- Paraben mix	16% pet	1/1-95—31/12-03
Para-tertiary-butylphenol-formaldehyde resin (PTBP resin)	1.0% pet	1/1-85—31/12-03
Fragrance mix 1	8.0% pet	1/1-85—31/12-03
Sesquiterpene Lactone Mix (ChemoTechnique)	0.1% pet	27/5-87—31/12-03
Quaternium-15	1.0% pet	1/1-85—31/12-03
Nickel sulphate	5.0% pet	1/1-85—31/12-03
Primin	0.01% pet	1/1-85—31/12-03
Ethylenediamine dihydrochloride	1.0% pet	1/1-85—2/9-92
Methylchloroisothiazolinone/Methylisothiazolinone (MCI/MI)	0.01% aq	1/1-85—31/12-03
Carba mix	3.0% pet	1/1-85—31/12-88
Mercaptobenzothiazol	2.0% pet	1/1-85—31/12-03

4.1.2 The Danish National Patient Registry

At birth or on immigration, all residents in Denmark receive a unique and personal identifier number, a CPR-number, which is used for identification in databases. This enables linkage of individual data over time and between databases.

Using the unique identifier (CPR), patients from the database were linked with the Danish National Patient Registry (DNPR). The DNPR contains information on all patient contact with clinical hospital departments in Denmark and includes variables such as CPR number, date of admission, date of discharge and diagnoses. The diagnoses in the DNPR are coded according to the International Classification of Diseases, 8th and 10th revision (ICD8 and ICD10). The DNPR contains information on all hospital admissions, discharges diagnoses and operations since 1977, outpatient and emergency patients were included from 1 January 1995. From 1977 to 1993, the DNPR used the ICD8, and since 1994, the ICD10 has been used.

Andersen et al. 1999 reviewed the DNPR (106).

After the linkage, patients with the ICD8 code 249 or the ICD10 code E10 were categorized as having type 1 DM, while patients coded with the ICD8 code 250 or the ICD10 code E11 were categorized as having type 2 DM—providing that they did not also have a code for type 1 DM. The standard WHO version of the ICD8 contains only a single code for diabetes (250); however, in Denmark the code 249 was introduced in January 1987 to account for type 1 DM.

Patients with the ICD8 code 56301 or the ICD10 code DK50 were categorized as having CD, while patients with the ICD8 code 56319 or the ICD10 code DK51 were categorized as having UC. Patients with either an ICD code for CD or UC were categorized as having IBD.

After linkage of the patch test database with the DNPR, the resulting data file was analyzed using logistic regression with the patch test outcome as the dependent and the diagnoses from the DNPR as independent variables. Further, the regressions were controlled for sex and age and odds ratios with 95% confidence intervals were estimated. Data analysis was done using SPSS version 12 and 13 (SPSS Inc., Chicago, IL, USA).

4.2 Part 2: The Murine Study

The Danish Animal Experimental Council (Journal number 2006/561-1116) approved the following experiments.
The murine study consisted of two studies: a diabetes incidence study, which used female Non-Obese Diabetic (NOD) mice that spontaneously develop diabetes equivalent to type 1 DM in humans; and a smaller study that focused on the activation of Natural Killer T-cells (NKT) study using female C57BL/6 mice.
Two different, potent contact allergens were used in the murine study to investigate both an experimental allergen and an allergen with relevance to consumer products and thus wide exposure of significant parts of the general population. The chosen allergens were 2,4-dinitrochlorobenzene (DNCB) and p-phenylenediamine (PPD) respectively.
An evaluation of the sensitizing potential of the chosen allergens was done by performing local lymph node assays (LLNA) in NOD mice, which have not previously been used for sensitization studies. The C57BL/6 mouse strain appears to have a dose sensitizing potential comparable to that of the CBA mouse, which is the mouse strain used the most in LLNA analysis (107).

4.2.1 Local Lymph node Assay (prestudy in NOD mice)

The LLNA protocol was performed as previously described (108). Briefly, 28 NOD/BomTac mice (Taconic, Ry, Denmark) aged 11 weeks were exposed to one of two known potent allergens, either p-phenylenediamine (PPD) or 2,4-dinitrochlorobenzene (DNCB) (Sigma-Aldrich, Brøndby, Denmark) or with vehicle. The vehicle was acetone and olive oil in the ratio four to one (AOO). The mice were treated with 25µL on the dorsal side of each ear. The concentration of the three PPD groups was 1.0, 0.25, and 0.05%(w/v) and for the three DNCB groups it was 0.25, 0.05, and 0.01%(w/v). The mice were treated once daily for three days followed by two days' rest. On Day 6, the mice were injected with tritiated thymidine (TRA310, GE Healthcare). Approximate five hours after injection, the mice were euthanized and their ear-draining lymph nodes were removed, and incorporation of tritiated thymidine into DNA was measured.

4.2.2 Diabetes study

To study the incidence rate of diabetes in contact-allergen exposed mice, we took receipt of 104 female, 3-week-old NOD/BomTac mice (Taconic, Ry, Denmark.
The mice were allocated to seven groups. These groups were further allocated to exposure with PPD, DNCB, vehicle (AOO) or water (one group). All groups received three topical exposures a week after being delivered, equivalent to the first three days of the LLNA.
Four groups were further exposed repeatedly every two weeks until they were 32 weeks of age. These four groups consisted of one group from each of the PPD, DNCB and AOO exposure groups and the group from the water exposure group. The first exposure, on the fourth week of life, was epicutaneous

application of 25µL of a concentration of 0.1%(w/v) of the allergens (PPD or DNCB) and pure solvent for the other exposure groups (AOO and water). For the repeated exposures (four groups) the amount remained at 25µL, whereas the concentration of allergen was reduced to 0.01%(w/v). The vehicle (AOO) and water repeated exposure groups received only AOO or water, respectively. The dilutions were freshly made immediately before application. The outline of the seven groups is presented in Table 2.
The experiment ended when the mice were 32 weeks old, at which time the mice in the repeatedly exposed groups had received 14 applications of the repeated dose. At 12 weeks of age, weekly measurements of tail blood glucose commenced. If the glucose level was equal to or above 14 mmol/L on two consecutive days, the mice were considered diabetic and were euthanized. At the end of the study, the mice still alive in the PPD repeatedly exposed group were provoked with 0.1%(w/v) PPD and euthanized two days later. A histological examination was made to assess cellular infiltration of the ears (Figure 6). All samples were fixed in formalin and stained in hematoxylin and eosin. The mice that were still alive at the end of the study were euthanized, and their ears were examined for cellular infiltration.
The cumulative diabetes incidence was evaluated by the Kaplan-Meier estimation, and the significance was estimated by the log-rank test.

Table 2. Design and dose-groups in the murine diabetes study

Exposure substance	PPD		DNCB		AOO		Water
Groups	Induction only	Repeated exposure	Induction only	Repeated exposure	Induction only	Repeated exposure	Repeated exposure
Conc.	0.1%(w/v)	0.01%(w/v)	0.1%(w/v)	0.01%(w/v)			

4.2.3 NKT study

We took receipt of 20 female, 10-week-old C57BL/6JBomTac mice (Taconic, Ry, Denmark). One week after arrival, 10 mice were sensitized with 0.5% (w/v) DNCB in AOO and 10 mice with PPD 0.5% (w/v). Two weeks after sensitization the mice were separated into ten groups of two mice—accordingly there were five groups in each interval. In eight groups, the respective allergen in a concentration of 0.5% (w/v) was re-applied. At the following points after re-application (18, 24, 48, and 72 hours) a group of mice was anaesthetized, fixated and vena porta was ligated. After ligation, vena cava was cut and the mice bled. Subsequently the liver was perfused with Phosphate-buffered saline (PBS) containing heparin until pale; followed by removal of the liver along with the ear-draining lymph nodes (A-LN). The groups that did not receive a re-application were euthanized at the 48-hour point.
The livers and A-LNs were strained (70 µm; BD Biosciences, Brøndby, Denmark) to gain a single cell suspensions, and the liver lymphocytes were isolated using Lympholyte-M (CEDARLANE Laboratories, Burlington, Canada). Subsequently, the cell suspensions were stained with APC-conjugated CD1d tetramers (ProImmune, Oxford, UK) loaded with α-galactosylceramide (Alexis Biochemicals/Axxora, San Diego, USA) according to the manufacture's guidelines. The following cellular receptors were then stained FITC conjugated anti-CD3, PE-Cy5.5 conjugated anti-TCR-β, PE-Cy7 conjugated anti-NK1.1 (eBioscience, San Diego, USA) and APC-Cy7 conjugated CD19 (BioLegend, San Diego, USA). Afterwards the cells were analyzed on a FACSCanto II (BD Biosciences, Brøndby, Denmark), and the CD19 positive cells were gated out, and TCR-β positive cells were selected. Subsequently, cells in the quadrant containing CD3 and CD1d positive cells were designated as CD1d restricted NKT cells. Figure 1 shows the single cell suspension of the liver for the PPD exposed mice euthanized 72h after challenge. Paired samples t-test was used to evaluate the difference in NKT cell stimulation in the A-LNs for the two allergens.

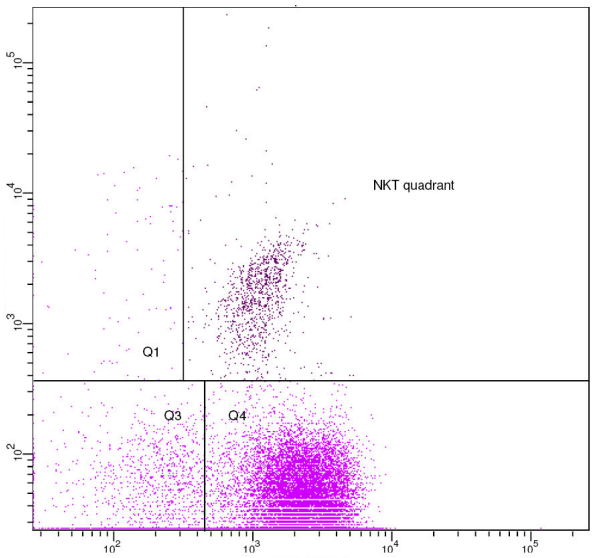

Figure 1. Picture of the gate containing the NKT cells
Cell suspension from the liver of PPD exposed mice euthanized 72 hours after challenge

5 Results

5.1 Part 1: The Epidemiological Study

The selected year span (1985–2003) from the database containing patch test results resulted in 14,214 patch tests with the European Baseline Series in a total of 13,315 individuals. Some patients received more than one patch test in the time span; however, most received only one patch test (94%) as can be seen in Figure 3. A patient was defined as reactive or positive if he or she reacted with a positive reaction to a patch test. Females constituted about two thirds (63.8%) of the tested patients, and 42% of the females tested had a positive reaction. Figure 2 shows the distribution of sex and the result of the patch tests. The age span of the patch-tested patients was from 6 to 99 years of age, with a median age of 47 years. The age group with the highest proportion of patients was 31–50 years of age (35%). Figure 4 shows the age group distribution.

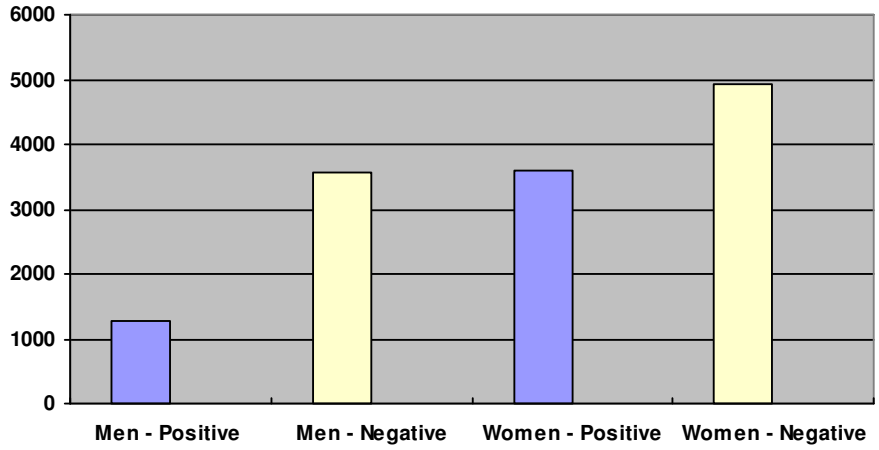

Figure 2. Distribution of sex and patch test result
The figure shows the distribution within the 13,315 patients from the database

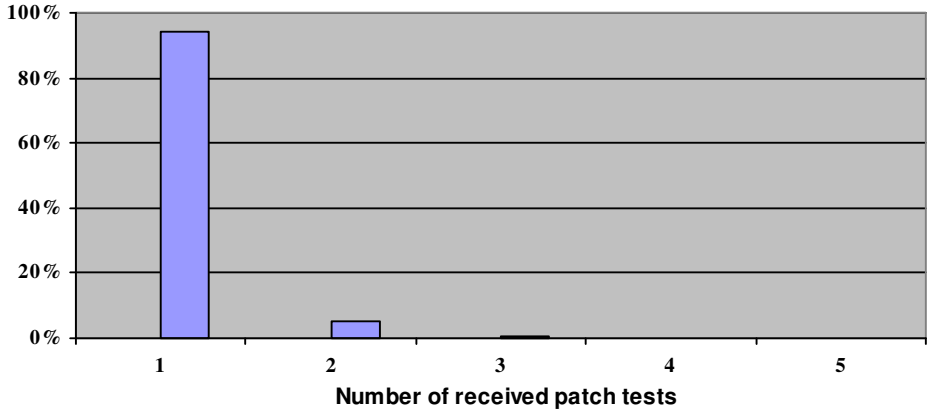

Figure 3. Number of times patients registered in the database had been patch tested
Shows the percentage of times a patient was tested in the selected period: 12,517 were tested once only; 712 were tested twice; 73 were tested three times; 11 were tested four times; and 2 were tested five times.

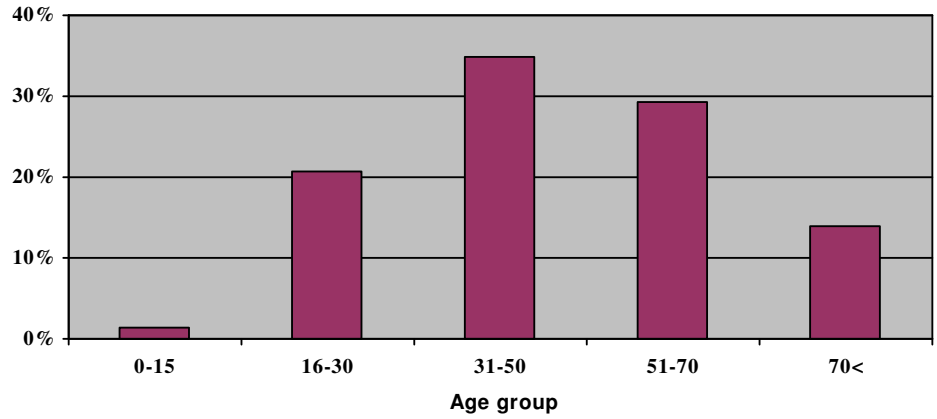

Figure 4. The distribution of the 13,315 patients within age groups

5.1.1 Register study of Diabetes Mellitus

After linkage with the DNPR, 632 patients with a DM diagnoses were identified, of these, 229 had an ICD code for type 1 DM (ICD8 249 or ICD10 E10), and 403 had an ICD code for type 2 DM without also having an ICD code for type 1 DM. Their patch test result distribution can be seen in Table 3. An inverse association was found between type 1 DM and ACD.
The odds ratio for a person with type 1 DM to have ACD was 0.62 (with CI 95% 0.46–0.83), when adjusting for sex and age the odds ratio was 0.63 (with CI 95% 0.47–0.86). No association was found between type 2 DM and ACD. The odds ratio for a person with type 2 diabetes to have ACD was 0.90 (with CI 95% 0.73–1.11), and when adjusting for sex and age group 0.98 (with CI 95% 0.79-1.21). Table 4 shows the result of the logistic regression.

Table 3. Patch test result for patients with a diagnosis of either type 1 DM or type 2 DM in the patient register

	Result of the patch test				Total
	Negative		Positive		
	Sex				
	Male	Female	Male	Female	
Type 1 DM	84	85	28	32	229
Type 2 DM	158	108	63	74	403
No DM diagnosis	3311	4721	1174	3477	12683
Total	3553	4914	1265	3583	13315

Table 4. Result of logistic regression with diabetes as outcome and ACD as explanatory variable

	Adjusted for	P-value	Odds ratio	CI 95% odds ratio
Type 1 DM		0.001	0.62	0.46–0.83
Type 1 DM	Age and sex	0.003	0.63	0.47–0.86
Type 2 DM		0.31	0.90	0.73–1.11
Type 2 DM	Age and sex	0.82	0.98	0.79–1.21

5.1.2 Register study of Inflammatory Bowel Disease

Of the 13,315 patch-tested patients, 225 had at least one ICD8 or ICD10 code for IBD (ICD8 56301 or 56319; ICD10 DE50 or DE51); of these, 34 had the diagnosis of both UC and CD. Of the remaining 191 patients, 73 had a CD diagnosis only and 118 had a UC diagnosis. Table 5 shows the number of patients with IBD and their patch test result.
The odds ratio for a person with an IBD diagnosis to have ACD was 0.71 (with CI 95% 0.53–0.94). Adjusting for sex and age barely influenced the odds ratio, the odds ratio being 0.71 (CI 95% 0.53–0.95). The IBD diagnosis was further split into three patient groups: patients with a CD diagnosis only, patients with a UC diagnosis only, and patients with both a CD and a UC diagnosis. Only the group with a CD diagnosis had a significant relation to ACD. A patient with a CD diagnosis had an odds ratio of 0.42 for having ACD (CI 95% 0.23–0.76). Table 6 shows the result of the logistic regression.

Table 5. Patch test result for patients with an IBD diagnosis in the patient register

		Result of the patch test				Total
		Negative		Positive		
		Sex				
		Male	Female	Male	Female	
Both a CD and a UC diagnosis	CD & UC	8	15	3	8	34
CD diagnosis only	CD	26	33	0	14	73
UC diagnosis only	UC	32	46	14	26	118
No diagnosis for IBD	No IBD	3487	4820	1248	3535	13090

Table 6. Result of logistic regression with IBD as outcome and ACD as explanatory variable

	Adjusted for:	P-value	Odds ratio	CI 95%
IBD		0.019	0.71	0.53–0.94
IBD	Age and sex	0.02	0.71	0.53–0.95
Both a UC and a CD diagnosis	Age and sex	0.64	0.83	0.40–1.73
UC	Age and sex	0.53	0.88	0.60–1.30
CD	Age and sex	0.004	0.42	0.23–0.76

5.2 Part 2: The Murine Study

In the LLNA the NOD mice gave a response similar to the standard strain (CBA), evaluated by the EC3 values, which is the standard measure for potency of an allergen tested in LLNA. The EC3 values in our experiment were 0.169 and 0.037 for PPD and DNCB, respectively, which is in accordance with results from CBA mice (109;110). Based on the EC3 values, we decided to use a concentration of 0.1% (w/v) for sensitization and 0.01% (w/v) for repeated treatment in the diabetes incidence study.

In the diabetes study, the NOD mice exposed to PPD repeatedly displayed a cumulative diabetes incidence of 47%, in contrast to 93% for the water treated group (P=0.004). The group repeatedly exposed to DNCB had an incidence of 92%, while the group that was exposed to PPD only in the fourth week of life—a sensitizing regime—had a cumulative diabetes incidence of 93% Figure 5. The remaining three groups developed diabetes at a similar rate with a cumulative incidence from 80–86% (data not shown).The P-value for all seven groups was 0.0048. Table 7 shows the diabetes progression in each exposure on a weekly base. Dermatitis was not noticeable by visual inspection; however, sensitization in the group repeatedly treated with PPD was confirmed by a typical lymphocytic infiltration in the ears following PPD provocation; a more moderate infiltrate was also noticeable in the group repeatedly treated with DNCB Figure 6.

The NKT study showed that in the liver, both allergens influenced the quantity of NKT cells in an analogous manner, data shown in Figure 8, which is in contrast to the ear-draining lymph nodes (A-LN) where PPD appeared to influence the quantity of NKT-cells more strongly than DNCB (P (one-tailed) = 0.043), data shown in Figure 7.

Table 7. Result of the diabetes incidence study in NOD mice

	PPD repeated		PPD induction		DNCB repeated		DNCB induction		AOO repeated		AOO induction		Water repeated	
Age of mice	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
12 weeks	14	1	15	0	15	0	15	0	15	0	15	0	14	0
13 weeks	14	1	15	0	15	0	15	0	15	0	15	0	14	0
14 weeks	14	1	15	0	14	1	14	1	15	0	15	0	14	0
15 weeks	14	1	14	1	13	2	12	3	15	0	14	1	14	0
16 weeks	14	1	14	1	11	4	11	4	12	3	12	3	11	3
17 weeks	13	2	12	3	10	5	10	5	12	3	11	4	11	3
18 weeks	13	2	10	5	10	5	10	5	12	3	10	5	9	5
19 weeks	13	2	7	8	9	6	10	5	12	3	7	8	8	6
20 weeks	13	2	6	9	8	7	10	5	9	6	7	8	8	6
21 weeks	11	4	4	11	7	8	8	7	8	7	7	8	6	8
22 weeks	11	4	3	12	6	9	6	9	7	8	7	8	5	9
23 weeks	10	5	2	13	6	9	5	10	7	8	5	10	3	11
24 weeks	10	5	2	13	6	9	4	11	7	8	3	12	3	11
25 weeks	10	5	2	13	6	9	4	11	7	8	3	12	3	11
26 weeks	10	5	2	13	3	12	4	11	7	8	2	13	2	12
27 weeks	10	5	2	13	3	12	4	11	6	9	2	13	1	13
28 weeks	9	6	2	13	3	12	4	11	4	11	2	13	1	13
29 weeks	9	6	2	13	3	12	3	12	4	11	2	13	1	13
30 weeks	9	6	2	13	3	12	3	12	4	11	2	13	1	13
31 weeks	8	7	1	14	3	12	3	12	3	12	2	13	1	13
32 weeks	8	7	1	14	1	14	3	12	3	12	2	13	1	13

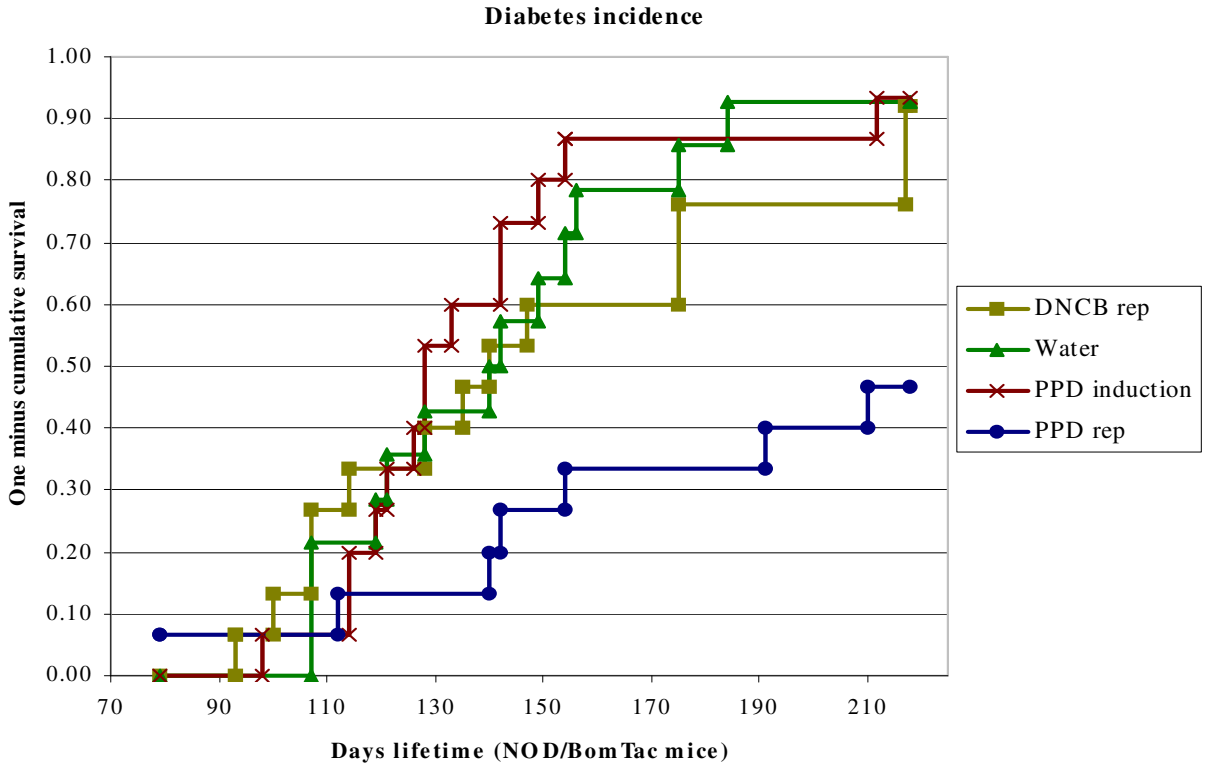

Figure 5. Repeated application of PPD protects NOD mice against development of type 1 diabetes.
The figure shows a Kaplan-Meier curve of the diabetes incidence for four groups of mice. Mice repeatedly exposed to DNCB, PPD, and Water, and a group of mice that were only sensitized. The group of NOD mice that was exposed to PPD repeatedly, displayed a cumulative diabetes incidence of 47%, in contrast to 93% of the water treated group (P=0.004). The log-rank test for all the shown curves gives a significance of P=0.008.

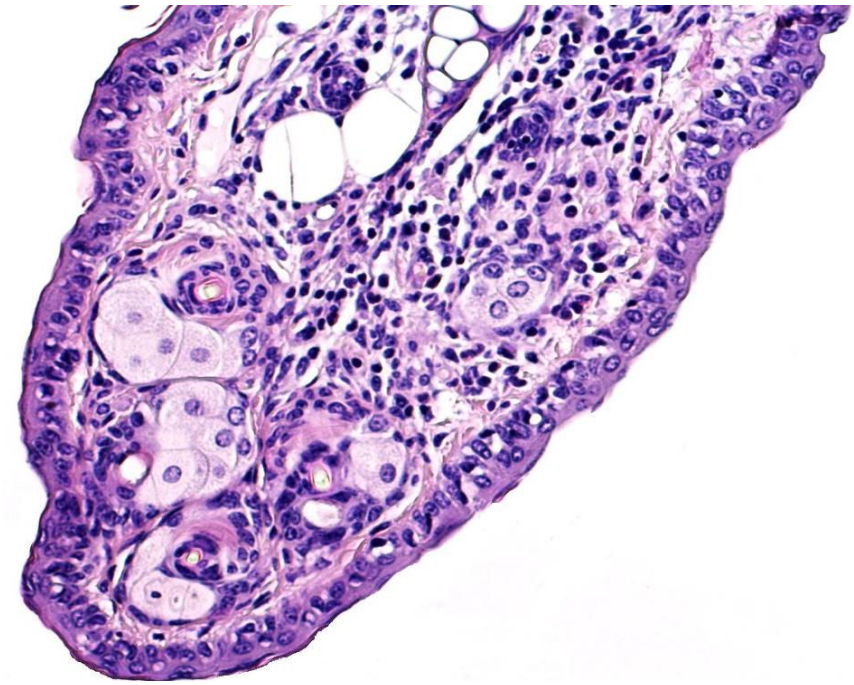

Figure 6. Mononuclear infiltrations in the ear after provocation with PPD at the end of the study.
The figure shows a hematoxylin and eosin stained ear from the group of mice repeatedly exposed to PPD. Ears from the group repeatedly exposed to DNCB show a similar, though weaker, infiltrate (not shown).

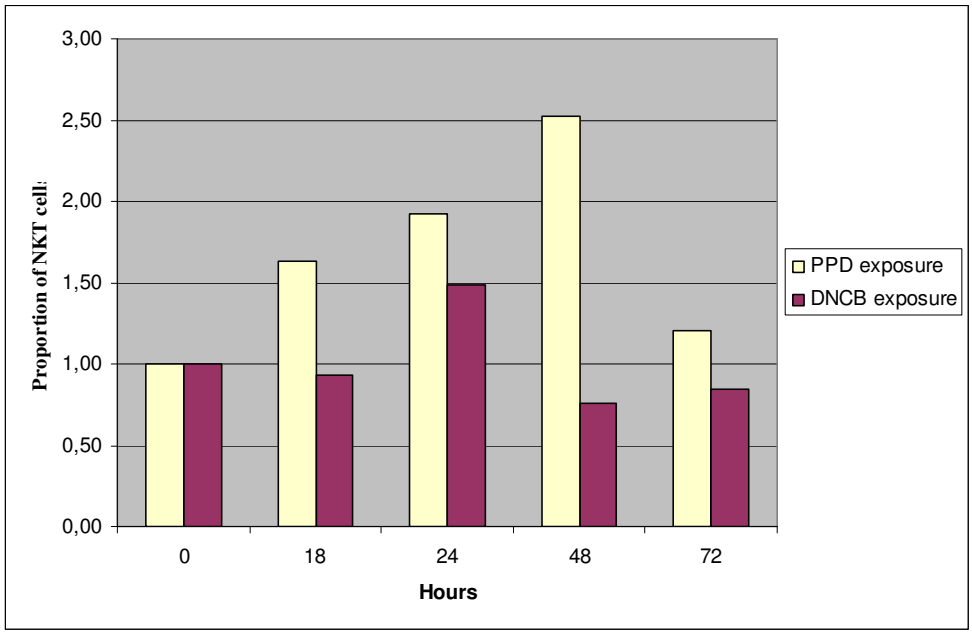

Figure 7. PPD is a strong peripheral activator of NKT cells.
The figure shows the proportion of NKT cells in the ear-draining lymph nodes for the mice exposed to either PPD or DNCB. The proportions were found by dividing by the number of NKT from the control groups (0 hours). As can be seen from the figure, the proportion of NKT cells appears to rise in the beginning, but at 48–72 hours there is a sharp drop, which is probably related to a down regulation of receptors. Apparently, the PPD allergen is a strong peripheral stimulator of NKT cells (P (one-tailed) = 0.043).
The NKT cells were stained for TCR-β, CD3 and CD1D loaded tetramers (α-GalCer), CD19 positive cells were gated out.

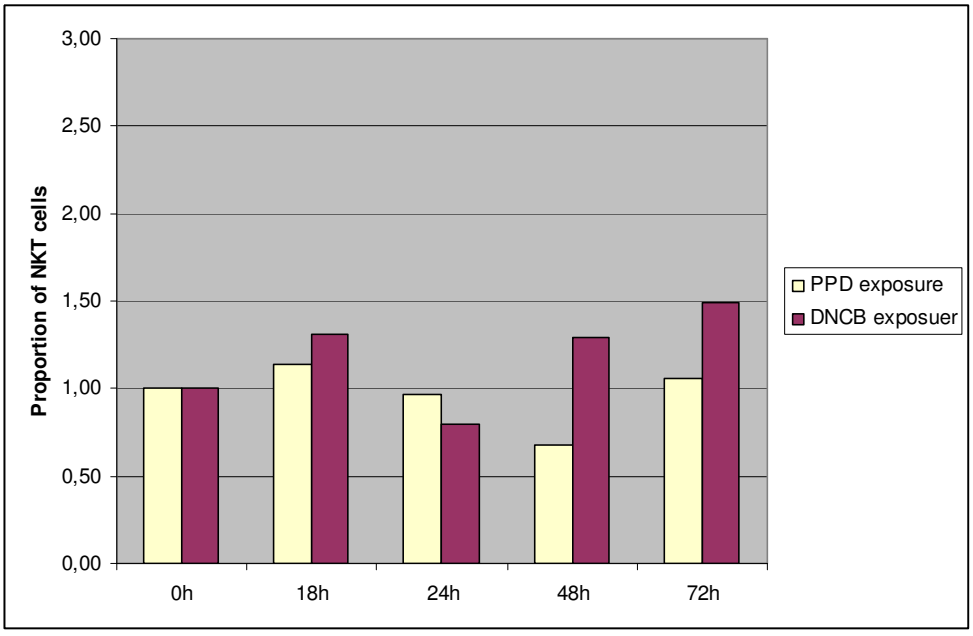

Figure 8. Topical exposure to contact allergens stimulates NKT cells in the liver.
The figure shows the proportion of NKT cells in the liver for the mice exposed to PPD or DNCB. The proportions were found by dividing by the number of NKT cells from the control groups (0 hours). Both allergens increased the number of NKT cells in the liver in a comparable manner. As can be seen in the figure, the proportion of NKT cells peaks at 18 hours, followed by a decrease, and yet another expansion. The observation of a rise followed by a steep drop may be due to down-regulation of receptors, which has previously been reported (1).
The NKT cells were stained for TCR-β, CD3 and CD1D loaded tetramers (α-GalCer), CD19 positive cells were gated out.

6 Discussion

6.1 Part 1: Epidemiological studies

6.1.1 Register study of Diabetes Mellitus

We found an inverse relation between type 1 DM and ACD (Table 4). We also investigated the relationship between type 2 DM and ACD but found no association (Table 4).

We may have underestimated the number of allergic patients, as we studied patients patch tested with only the European Baseline Series, and a previous study performed in the same geographical region as our study found that 20% of the patch tested patients were allergic to allergens not present in the European Baseline Series (111). The old part of the database holds no information about patch tests with substances other than those in the European Baseline Series; therefore, we cannot investigate this bias. However, we do not believe that patients wrongfully classified as having a negative patch test were unequally distributed.

Information about exposures would have been desirable. This information is not included in the database, and information not collected in a standardized manner is unreliable.

We cannot discount false positives being a possible bias. Nevertheless, the bias is minimized as a small number of specially trained personnel performed all patch tests.

The patients with ACD were entered in the database because of referral to a hospital clinic due to their dermatitis. Patch testing classified the patients in groups with and without ACD (a positive patch test). Patients with type 1 DM had a decreased risk of ACD, independent of age and sex. There were more female than male patients in the database; and the number of positive patch tests was higher for females, which is in accordance with the literature (34).

We identified cases of type 1 and type 2 DM in the DNPR using ICD coded diagnosis. The quality of the DNPR registry with reference to type 1 DM diagnosis has been evaluated for 1987–1993 by Nielsen et al(112). In their study they found the specificity of the diagnoses to be 96.3% and the completeness to be 91% (112). A problem with the DNPR, which is also addressed by Nielsen et al., is that the registry does not contain outpatient contacts before 1 January 1995, which means that patients with a milder course of the disease could have escaped registration in the DNPR until 1995. Nevertheless, in Denmark, patients with type 1 DM are usually treated at hospital departments (113) and would therefore appear in the DNPR.

The ICD8 code 249, which distinguishes type 1 from type 2 DM, was not introduced in the DNPR until 1987. This may have resulted in too few individuals being classified as having type 1 DM; however, the prevalence of type 1 DM in our dataset is relatively high, which indicates that we accounted for nearly all persons with type 1 diabetes. The prevalence in our data is even higher than in most studies on childhood onset type 1 DM; however, these studies often focused on younger individuals. Supporting our high number of type 1 diabetes patients is the article by Molbak et al., who found a high incidence of type 1 diabetes in Denmark, even in the age group above thirty

years and, further, estimated the lifetime risk of developing type 1 DM to be as high as 1.5% (114). Hviid et al. have previously concluded that the completeness and validity of the data on type 1 DM acquired in a similar way, was unlikely to be a cause of concern (115). If all diabetic patients are considered as a whole, (i.e. also type 2 DM) 80–90% per cent of those in medical treatment can be traced by the DNPR. A number of patients, however, are assumed to be undiagnosed and are therefore not in the DNPR (116). The undiagnosed patients are mainly men (116).

An advantage of the present data set is a median age of 47 years: it is anticipated that most of those who have either the genetic predisposition or environmental exposures leading to type 1 DM or ACD will have developed these diseases by the age of 47 years.

As the prevalence of type 2 DM increases with age, some of the patients will develop type 2 DM when they get older. It is therefore probable that we have underestimated the number of patients with type 2 DM. Furthermore, patients with type 2 DM are generally treated in private practice and are therefore not accounted for in the DNPR until 1995. The prevalence of type 2 DM in our dataset is 3%, while the prevalence in Denmark for patients with recognized type 2 DM is about 5–6% for individuals of 60 years of age (117) while the prevalence in our dataset is 3%. It is unclear how an underestimation of type 2 DM would influence an association between type 2 DM and ACD; however, as smoking is a risk factor for both diseases (118;119), and social class appears to be a risk factor too (120-122), a positive correlation could be expected. Findings from an animal study suggested the ACD response is diminished in obese individuals (123). This could have resulted in a false negative patch test. The lack of association between type 2 DM and ACD could therefore be due to an underestimation of type 2 DM.

As the investigation was based on hospital material, any sampling bias would have led to a positive rather than a negative association. ACD is not associated with an increased death rate, which could have interfered with the data. Any medical treatment of the conditions is unlikely to introduce bias. Most of the ACD patients had been treated intermittently with topical steroids and only a minority with systemic immunosuppressive agents. This type of treatment is unlikely to have increased or decreased the risk for type 1 or type 2 DM.

Several studies have evaluated the potential relationship between atopic dermatitis and type 1 diabetes; the outcome is not uniform, but most point to a negative association (76). Atopic dermatitis is defined as a clinical syndrome, and is not based on a specific immunological event in contrast to ACD, which might explain this variation.

ACD has not previously been investigated with respect to autoimmune diseases, probably because ACD has not been regarded as a Th2 dominated illness (124), in contrast to other hypersensitivity reactions such as asthma. Some studies do, however, show that in patients or mice with long-lasting dermatitis, as in the present patient material, a type 2 cytokine response is suspected (125-127). Others have found the response to be a mixed cytokine response (128;129), and its been suggested that the immunological response may be different for different allergens (130).

An immunological background for the observed inverse association could be due to stimulation of NKT. Animal studies have shown that contact allergens

can increase the level of NKT cells in the liver (131), while stimulation of the same cell type can protect NOD mice against diabetes (132-134). In addition, monozygotic twins discordant for type 1 DM appear to have different gene expressions of NKT cells (135). NKT cells have experimentally been shown to be necessary for elicitation of ACD in mice (45;131).
In conclusion, an inverse relationship between type 1 DM and ACD was found. This is intriguing as type 1 DM is an autoimmune disease with a strong genetic component, while ACD is a type IV hypersensitivity reaction where genetics appear to play a minor role. Nonetheless, the association could be due to shared genetic or common environmental determinates.
This could stimulate research in the immunological interaction between environmental skin exposure and autoimmune diseases.

6.1.2 Register study of inflammatory bowel disease

An inverse association was found between ACD and IBD (Table 6). When the IBD was further split into the aforementioned three groups the association appeared mainly to be due to an inverse association between ACD and CD. The odds ratio for CD adjusted for age and sex was 0.42 (CI 95% 0.23–0.76) (Table 6).
There are the same potential biases with the patch test database, as previously mentioned in chapter 6.1.1.
We identified cases with inflammatory bowel disease (IBD) by record linking patch-tested patients from the Department of Dermato-Allergology with the DNPR using diagnosis for Crohn's disease (CD) and ulcerative colitis (UC). Patients with either disease were considered to have IBD. The codes used were the ICD8 codes 56301 and 56319 and the ICD10 codes DK50 and DK50. These codes cover the diagnosis in the 8^{th} and 10^{th} revision for CD and UC, respectively. As CD and UC are two different clinical entities of IBD, they were further split into three groups: one consisting of patients with CD diagnosis only, one with UC diagnosis only, and one consisting of patients with a diagnosis of both diseases. This was done as it appears that the immunological response in these two clinical entities differs; however, in a minor portion of patients with IBD, a distinction between CD and UC, cannot be made (99). The completeness and validity of these two diagnoses have previously been estimated to be 94% and 97%, respectively, for the CD diagnosis and 94% and 97%, respectively, for the UC diagnosis (136). The estimation was made in the County of Northern Jutland where the regional hospital system was validated using the pathology system as a reference standard (136). As the DNPR is based on the regional hospital systems, its completeness and validity is considered to be the same. As the present study included mainly patients from the County of Copenhagen, the validity and completeness of the DNPR in this area is at least at the same level as in the County of Northern Jutland, as patients with IBD have been registered in Copenhagen over a long period (137;138).
In general, ACD is more frequent among females (34), which explains the sex difference in the database. This difference is reflected among the patients with a CD or UC diagnosis (Table 5). In Denmark CD is more frequent among females, whereas this is not the case for UC (138;139).

Studies have found the estimated prevalence of UC and CD as respectively 160 and 54 per 100,000 inhabitants (137;138). However, more recently a study covering the period from 1981–1992 found an increasing incidence of CD (140), which would result in a higher prevalence.
The study was based on discharge diagnoses, which may vary in quality between hospitals and over time; however, this potential bias would be similar for CD and UC, while only the odds ratio for CD is significant.
As the investigation was based on a hospital material, any sampling bias would have led to a positive rather than a negative association. ACD is not associated with an increased death rate, which could have interfered with the data. Most of the ACD patients had been treated intermittently with topical steroids and only a minority with systemic immunosuppressive agents. This treatment is unlikely to have influenced the risk of IBD.
A possible confounding factor in our study could be smoking, as smoking increases the risk of CD but has a protective effect in UC (141). Studies have found that smoking might increase the risk of ACD (118), but this common risk factor for CD and ACD would have resulted in a positive rather than a negative association, as found in this study. It might, however, have reduced a positive association between UC and UC.
Another possible confounder is medical treatment of IBD with immunosuppressants, e.g. azathioprin or prednisolon; however, if this treatment had an effect on risk of ACD, it would most likely be equal for both UC and CD. Medical therapy with anti-tumour necrosis factor α (TNF-α) is presumably a confounder, since migration of Langerhans cells is inhibited by anti-TNF-α treatment (142); mice deficient in the TNF-α gene have an impaired contact hypersensitivity reaction (143). Treatment of CD with anti-TNF-α might therefore interfere with the patch test result. However, Infliximab was not introduced in Denmark until late 1999 (144) and is therefore unlikely to be a problem in this study. Furthermore, patients with a down-regulated elicitation response would most likely not have been patch tested and therefore would not be included in the database.
IBD has previously been studied in relation to allergy; the results were diverging (145-148) and only a few and relatively small studies focused on delayed hypersensitivity (149;150). Triantafillidis et. al. found that patients with CD had a reduced delayed hypersensitivity response to microbial antigens in the multi-test (149), whereas D'Arienzo et. al. found no statistical differences between UC and controls in regard to contact allergy (150).
Animal models have been developed to study mucosal inflammation and to try to immunologically differentiate between CD and UC (95). Some of these models use contact allergens in which intrarectal administration gives inflammation. From these it appears that trinitrobenzenesulfonic acid (TNBS)-initiated colitis has a transmural inflammation as seen in CD (151), while in oxazolone-initiated colitis the histology resembles UC (152). Studies of these murine models and human studies suggest that CD is both a Th1- and a Th17-driven inflammation, while in UC the inflammation is driven by IL4 and IL13 (151). The cytokine IL23 appears to play a role in both diseases, as gene polymorphisms in the gene encoding IL23R are associated with both CD and UC (153). IL23 causes the differentiation of naïve helper T cells into T cellsthat produce IL17, IL17F, IL6, and TNF-α (Th17) (104). There are a number of

studies indicating that IL17 participates in the ACD response and that T-cells producing IL17 can be found in skin biopsies (48;49;154). An association between IBD and ACD due to IL17 would, however, probably not have shown an inverse association.
Involvement of NKT cells is also likely, as stimulation of these cells by α-galactosylceramide and an analogue named OCH (155) can improve colitis in the dextran sulfate sodium (DSS)-induced colitis mouse model (156;157). The number of circulating NKT cells is reduced in patients with CD or UC (158), but the frequency and function of NKT cells from different organs might be unrelated (159). NKT cells appear to be pivotal in the ACD response (45), and apparently contact allergens quickly and potently activate NKT cells in the liver (131). Therefore, it is probable that the number of NKT cells is low in IBD and high in ACD, but this does not explain the difference in the association with CD and UC.
In conclusion, we found and inverse association between IBD and ACD, which appears to be due to an inverse association between CD and ACD. The association might be related to shared genetic factors or common environmental determinates.

6.2 Part 2: The Murine Study

As a follow-up on the epidemiological studies, we did an animal experiment. In the animal studies, we found that repeated treatment with contact allergen PPD significantly reduced the cumulative incidence of diabetes in female NOD mice.

We chose to study diabetes progression in the NOD mouse, an animal model for type 1 DM, as there was seemingly no definitive animal model for CD. We decided upon using DNCB and PPD as contact allergens in the animal studies. Both allergens are potent, which insured that the mice would be sensitized, and they represent an experimental and an environmental allergen. We started by addressing whether the NOD mouse had an abnormal response to the chosen allergens. We did this by conducting the LLNA protocol in this strain of mice. We found that mice aged 11 weeks responded in a manner similar to that of the mouse strain usually used. At this age, most of the islet of Langerhans are infiltrated by T-cells or destroyed, and diabetes therefore precipitates from around 12 weeks of age (74). However, the inflammation in the pancreas does not appear to influence the immunology of the skin and skin-draining lymph nodes.

Accordingly, we started a diabetes incidence study in NOD mice, in which NOD mice were sensitized in the fourth week of life. At this early time, leucocytes have already begun to enter the pancreas; however, the mice are still in a benign state (74). After sensitization, some mice were followed for diabetes progression only, while others were repeatedly exposed to allergens at two-week intervals. The mice were followed for diabetes development until 32 weeks of age, at which time most had developed diabetes. In all groups, except the mice repeatedly treated with PPD, the cumulative incidence was more than 80%. Repeated applications of PPD therefore appear to reduce the diabetes incidence in NOD mice (Figure 5). The mice repeatedly exposed to either of the two allergens developed a sub-clinical dermatitis. The dermatitis was not noticeable from visual inspection and could be confirmed only by histology. A difference in dermatitis generation between the two allergens does not therefore explain the difference in diabetes development. As NKT cells influence ACD both in developing the dermatitis (45;46;160) and in the oral tolerance induction (47), we set up a study to investigate the effect of elicitation with the two allergens on NKT cells in the ear-draining lymph node and in the liver. In this study we found that both allergens modulated the quantity of NKT cells in the liver (Figure 8), which is in accordance with previous published data (46). PPD, however, gave a more pronounced NKT cell response in the ear-draining lymph nodes (A-LNs) (Figure 7).

The peripheral stimulation of NKT cells by contact allergens is in agreement with a recent human study; however, in the study by Gober et al. PPD was not the allergen causing the most pronounced quantity of NKT cells (160).

The mechanism for the protective effect of ACD on diabetes is unknown, but activation of NKT cells could be an explanation, since up-regulation of NKT cells has been shown to protect NOD mice against diabetes (74;132-134). The difference between PPD and DNCB in the peripheral activation of NKT cells might then explain the selective protective effect of PPD.

Repeated application of a contact allergen may possibly give a Th2 cytokine milieu (127;161-163), which might also contribute as a mechanism for the protective effect of repeated application of PPD. The lack of effect of DNCB on diabetes development may then be due to difference in potency between the two allergens. DNCB may, however, also be a special contact allergen, in that it rarely gives systemic eczema when used for treatment of alopecia areata. In conclusion, we found that repeated exposure to PPD giving a sub-clinical dermatitis significantly reduced the cumulative diabetes incidence in female NOD mice. The protection appeared to be allergen specific, which could be due to potency differences or differences in cellular stimulation.

7 General Discussion

In the epidemiological studies, we found an inverse association between two autoimmune diseases and ACD, as defined by a positive patch test in dermatitis patients. If we look at type 1 DM and Crohn's disease (CD), patients with these diseases appear to develop ACD at a lower rate than patients without these diseases. Type 1 DM and CD do not appear to be associated, and mutually adjusting for the diseases does not change their odds ratio. The diseases do not appear to have a common environmental or genetic predisposition (164) that could explain why they are both inversely correlated with ACD. Both are driven by type 1 cytokines, though Th17 appears to play some a role in CD, but this does not appear to be the case for type 1 DM (165;166). We did a preliminary study of the association between patients with rheumatoid arthritis (RA) and ACD, and in this study we also found an inverse association (167). There may, however, be problems with the validity of RA diagnosis (168), and the disease is associated with advanced age (169), so some patients registered in our database may later develop RA. Adjusting for RA in a logistic regression with type 1 DM and CD does not affect the odds ratios of type 1 DM and CD (Table 8). RA is as CD related to type 1 cytokines and Th17 and as for both CD and type 1 DM positive family history is a risk factor (169;170). Therefore, it appears that there is an(164) association between autoimmune diseases and a (somewhat) type 1 cytokine environment. The reason for this association is unclear as the studies in autoimmune diseases are not strongly correlated (164). Based on information in the database and registers, we cannot determine what came first: the autoimmune diseases or ACD. To study the interplay between an autoimmune disease and ACD, we initiated the animal studies. In these, we could control precisely the time of exposure to the contact allergens and could identify the time at which the NOD mice developed clinical type 1 DM. These studies showed that contact allergen exposure could protect against diabetes development; however, the exposure had to be repeated often, as sensitization was not enough to protect against diabetes development. Further, the protection appeared to be allergen specific, as repeated exposure to DNCB did not protect against diabetes. These observations could indicate that the protection observed in both murine and epidemiological studies is due to stimulation of the immune system by challenge with allergen. This stimulation could activate the innate immune system, such as the NKT cells, which could hinder diseases progression. Another possibility is that the repeated exposure diverges the immune system towards a type 2 cytokine response, which has been reported for murine studies (127;163;171;172), and type 2 cytokine also appears to play a role in chronic ACD in humans (173). Additionally, NKT cells have been reported to release IL4 after exposure to allergen (46).

Since the protection is allergen specific, it could be connected with different signal transduction pathways, which has previously been reported (174). It may also be connected with dose levels, as DNCB is a more potent allergen than PPD, but it was used in the same amount and concentration in our study. As PPD appears to be the protective allergen of the two used, genetics may also play a role, as N-acetyltransferase 2 polymorphisms coding for rapid

acetylation are more frequent in patients suffering from allergy towards PPD (28).

Table 8. Result of logistic regression with autoimmune diseases
Result of logistic regression with ACD as the response variable and type 1 DM, Crohn's disease (CD) and rheumatoid arthritis (RA) as the independent variable, adjusted for sex and age (unpublished results).

	P-value	Odds ratio	CI 95%
Type 1 DM	0.003	0.64	0.47-0.90
CD	0.004	0.42	0.23-0.76
RA	0.037	0.78	0.62-0.99

8 Conclusion

The aim of the thesis was to investigate associations between allergic contact dermatitis and autoimmune disease.
From this the following conclusions can be drawn:

- Patients with type 1 diabetes mellitus investigated at the Dermato-Allergology department have a lower odds ratio of having a positive patch test.
- Patients with inflammatory bowel disease investigated at the Dermato-Allergology department have a lower odds ratio of having a positive patch test. The association appears to be driven by Crohn's disease.
- Allergic contact dermatitis caused by topical application of PPD protects Non-Obese Diabetic mice from developing diabetes. Repeated application of DNCB, or merely induction of allergy, did not appear to have an effect.
- NKT cells are stimulated in the liver by both DNCB and PPD, whereas only PPD appears to stimulate NKT cells in the draining lymph nodes.

From the above studies we can conclude that autoimmune diseases are apparently inversely associated with ACD as defined by a positive patch test reaction in dermatitis patients. From the epidemiological studies we cannot say whether autoimmune diseases protect against ACD or vice versus; nevertheless, the murine studies demonstrate that ACD may protect against diabetes. Additionally prediabetic NOD mice had a normal response towards the studied contact allergens.

9 Perspectives and future studies

The studies in this thesis show that two autoimmune diseases, type 1 DM and CD, are inversely correlated with ACD in a selection of patients referred to a dermatological department for elucidation of their eczema. Both autoimmune diseases appear to be somewhat driven by a type 1 cytokine environment, and, as shown in the murine study, a sub-clinical ACD reduces the diabetes incidence. Accordingly, it could be considered that an ACD might to some extent protect an individual against type 1 DM and CD. It is therefore probable that knowledge of the immune stimulation created by contact allergens on organs other than the skin could lead to new treatments.
Further studies should be conducted:

- To reveal whether the correlations are due to genetics. Genome-wide association studies (GWAS) that could identify genetic factors that influence ACD would be rewarding. As the murine study shows an allergen specific effect, a GWAS should be done on ACD due to different allergens.

- To reveal whether PPD is a specific allergen or whether the protection against diabetes was due to a specific dose or concentration, supplementary diabetes incidence studies should be initiated using other allergens and other doses. Along with this, the cytokine and genetic response should be measured.

- To reveal whether the protection against diabetes is possible only if allergen exposure is implemented at a young age, a study should be done using NOD mice at an older age.

- To reveal the signal transduction to NKT cells, a study should be done to investigate whether contact allergens stimulate NKT cells directly or whether the NKT cells are activated by an unknown danger signal

- To reveal whether some of the NKT cells have a memory function, naïve mice should be injected with carboxyfluorescein succinimidyl ester (CFSE) labeled NKT cells from sensitized mice. It would thereby be possible to see if these labeled cells were guided to the periphery and whether they proliferate.

- To reveal whether the inverse correlation between autoimmune diseases and ACD is a common occurrence, it would also be revealing to do further association studies, such as a possible association between multiple sclerosis and ACD.

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ARTICLE

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Inverse relationship between allergic contact dermatitis and type 1 diabetes mellitus: a retrospective clinic-based study

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Abstract *Aims/hypothesis:* Contact allergy (CA) is a disease induced and maintained by environmental factors, which mainly has a Th2 pattern in its chronic form. Environmental factors play a major role in CA, while genetic factors are of minor importance. Type 1 diabetes is an autoimmune disease of the islets of Langerhans, which has a Th1 cytokine pattern and in which modulators of risk are both genetic and environmental. To investigate whether environmental exposure to chemicals leading to CA could influence the risk of type 1 diabetes, we conducted a retrospective clinic-based study of patients subjected to diagnostic patch testing of CA. *Methods:* We undertook a retrospective clinic-based study of 13,315 patients who were patch-tested between 1985 and 2003, and linked it with the Danish National Patient Registry containing diabetic mellitus discharge diagnoses from 1987 to 2003. The 13,315 patch-tested patients gave rise to 4,848 CA patients. Using logistic regression, we calculated odds ratios for persons with CA of having type 1 diabetes. *Results:* Type 1 diabetes was diagnosed in 229 of the patch-tested patients. CA patients had a reduced risk of having type 1 diabetes, with an odds ratio 0.62 (95% CI 0.46–0.86). After adjusting for sex and age, the odds ratio was 0.63 (95% CI 0.47–0.86). *Conclusions/interpretation:* An inverse relationship between CA and type 1 diabetes was found. Thus there may be a protective effect of having CA in relation to the risk of type 1 diabetes, or vice versa type 1 diabetes may lead to tolerance rather than hypersensitivity. Alternatively, these two diseases may share common genetic factors, although at present these are unknown.

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Abbreviations CA: contact allergy · DM: diabetes mellitus · ICD: International Classification of Diseases · NKT cells: natural killer T cells

Introduction

Contact allergy (CA) is a delayed type IV reaction involving T lymphocytes. It is caused by skin contact with substances of small molecular weight in the environment. Once a patient is sensitised to an allergen, re-exposure will result in allergic contact dermatitis, which is an inflammatory skin disease characterised by erythema and infiltration at the site of contact. CA is common in the general population, where 15 to 20% are sensitised to substances in the environment [1]. CA is rare among infants but increases rapidly in adolescents, with 15% of schoolchildren aged 12 to 16 years having a CA to one or more common environmental allergens, close to the level established in adults [2]. Individual predispositions to CA exist; however, the total number of sensitised individuals in a population has mainly to do with environmental factors [3], while genetic factors only play a minor role [4–6].

T cells and Langerhans cells are of importance in the induction and elicitation of the allergic response. Moreover, it was recently established that the invariant natural killer T cell (NKT) plays a pivotal role in CA [7, 8].

Type 1 diabetes mellitus is also a chronic disease, and is the result of an autoimmune Th1 cell-mediated destruction of the beta cells. The rise in the incidence of type 1 diabetes mellitus in recent decades points to hitherto unknown environmental factors [9].

Studies have found NKT cell numbers to be reduced in type 1 diabetes mellitus [10, 11], while activation of these cells by α-galactosylceramide prevents the onset of type 1 diabetes mellitus [12, 13]. The relationship between NKT cells and CA seems to be different from the relationship between NKT cells and type 1 diabetes mellitus, since NKT

cells seem to be necessary for elicitation of CA [8], while NKT cells suppress the development of type 1 diabetes mellitus.

The aim of the present study was to investigate the association between CA and type 1 diabetes mellitus, and thus whether environmental exposure to chemicals leading to CA might influence the risk of type 1 diabetes mellitus.

Subjects, materials and methods

From 1985 to 2003, a total of 13,315 patients (4,818 males and 8,497 females), were patch-tested at the Department of Dermatology, Gentofte Hospital, University of Copenhagen, Denmark. The patients were referred to the department because of chronic dermatitis. The outcome of the patch test together with information regarding sex and date of birth were recorded in the allergy database. All patients were patch-tested with the European Standard Series recommended at the time of testing. The European Standard Series contains the most important contact allergens in the environment for continental Europe. Patch testing was performed, according to international standards, on the upper back using Trolab allergens (Hermal, Reinbek, Germany) and Finn chambers for occlusion (Epitest, Tuusula, Finland). Occlusion time was 48 h and the patches were read on days 2, 3 and 7 according to international criteria from the International Contact Dermatitis Research Group [14, 15]. A positive allergic reaction was defined as at least erythema and infiltration in the test area. Of the 13,315 people tested, 4,848 had been tested at least once with a positive response to one of the test allergens.

At birth, or in the case of immigration, all Danish citizens are given a unique and personal identifier number, a CPR number, which is used for identification in databases. This enables linking of individuals' data over time and between databases.

Using the CPR number, the allergy database was linked with the Danish National Patient Registry, which contains the codes of diagnoses from the International Classification of Diseases 8th and 10th revisions (ICD8 and ICD10). The Danish National Patient Registry contains information on all hospital admissions, discharge diagnoses and operations performed since 1977, and furthermore contains outpatient contacts from 1 January 1995. Patients with the ICD8 code 249 or the ICD10 code E10 were categorised as having type 1 diabetes mellitus. From 1977 to 1993 the Danish National Patient Registry used the ICD8, and since 1994 the ICD10 has been used. The standard WHO version of the ICD8 contains only one diabetic code (250); however, the Danish National Patient Registry introduced the code 249 in January 1987 to account for type 1 diabetes mellitus. We can therefore identify type 1 diabetes mellitus patients treated from 1987 to 1994 in hospital, and from 1995 to 2003 we can also identify type 1 diabetes mellitus patients treated as outpatients.

Age was calculated as the age at the first positive patch test; if there was no positive patch test, the age at the first patch test was used. The patients were stratified into five age groups: 0–15, 16–30, 31–50, 51–70 and >70 years of age.

The resulting data file was analysed using logistic regression with the patch test outcome as the dependent variable and type 1 diabetes mellitus as the independent variable, and controlled for sex and age. Odds ratios with 95% CIs were estimated using logistic regression. All data analysis was done using SPSS version 12 (SPSS, Chicago, IL, USA).

Results

The 13,315 patients were patch-tested between 1985 and 2003; of these, 229 had an ICD code for type 1 diabetes mellitus (ICD8 249 or ICD10 E10). There were more females than males in the registry, and the number of females with a positive patch test was also higher than males (42.2 vs 26.3%). The age (defined as the age at the first positive patch test or if there where no positive patch test, the age at the first patch test) spanned from 6 to 99 years with a median of 47 years. Some of the patient categories can be found in Table 1.

An inverse association was found between CA and type 1 diabetes mellitus. The odds ratio for a person with CA of having type 1 diabetes mellitus was 0.62 (95% CI 0.46–0.83); when adjusted for sex and age, the odds ratio was 0.63 (95% CI 0.47–0.86). The result of the logistic regression can also be found in Table 2.

Discussion

An inverse relationship between CA, a type IV immunological reaction, and type 1 diabetes mellitus, an autoimmune disease, was found by record linking two databases: one of allergy and one of patient discharge diagnoses. Such a study has never been performed before and was only

Table 1 Overview of patient contact allergy categories in the allergy database

	Negative patch test (n=8,467)						Positive patch test (n=4,848)					
	Age group (years)						Age group (years)					
	0–15	16–30	31–50	51–70	>70	Total	0–15	16–30	31–50	51–70	>70	Total
Type 1 DM	0	12	39	80	38	169	0	4	17	28	11	60
No type 1 DM	166	1,866	2,766	2,359	1,141	8,298	36	866	1,808	1,421	657	4,788

DM, Diabetes mellitus

Table 2 Results from the logistic regressions with CA as the response variable and type 1 diabetes mellitus as the independent variable

	p	Odds ratio	95% CI of odds ratio
Type 1 DM	0.001	0.62	0.46–0.83
Type 1 DM adjusted for sex and age	0.003	0.63	0.47–0.86

There is an inverse relationship between CA and type 1 DM
DM, Diabetes mellitus

possible due to the collection of large patient populations and the unique identifier system in Denmark, which makes a linkage possible between such registers. The inverse relationship may lead to new theories and insight into disease mechanisms.

We identified cases of type 1 diabetes mellitus from discharge diagnoses from the Danish National Patient Registry for the period 1987 to 2003. The Danish National Patient Registry does not contain outpatient contacts until 1 January 1995, which means that patients with a milder course of type 1 diabetes mellitus may have escaped registration in the Registry until 1995. However, the period from 1987 to 1993 has previously been evaluated with respect to type 1 diabetes mellitus by Nielsen et al. [16], who found the specificity of the diagnoses to be 96.3% and the completeness to be 91%. In the period from 1987 to 1993 the ICD8 was used and we expect that the introduction of ICD10 in 1994 has not decreased the specificity and completeness of the registry. We therefore anticipate that most type 1 diabetes mellitus patients are accounted for. Additionally the prevalence of type 1 diabetes mellitus in our dataset is relatively high, which further indicated that we did account for most type 1 diabetes mellitus cases. The prevalence in our data is even higher than in most studies on childhood-onset type 1 diabetes mellitus; these studies, however, often focused on younger individuals.

Supporting our high number of type 1 diabetes mellitus patients is the article by Mølbak et al. 1994 [17], who found a high incidence of type 1 diabetes mellitus in Denmark, even in the age group above 30 years.

The patients with contact dermatitis entered the database because of referral to a university clinic, generally because of chronic dermatitis. Patch testing classified the patients in groups with and without CA (a positive patch test). Those with CA had decreased risk of type 1 diabetes mellitus independently of age and sex. A sampling bias because the investigation was based on hospital material would have led to a positive and not a negative association.

Even though population-based studies are often preferable to patient-based studies by virtue of unbiased estimation of prevalence and risk, when evaluating risk factors for CA they have limited power [18]. CA is not associated with an increased death rate, which could have interfered with the data. Any medical treatment of the two conditions is unlikely to introduce bias. Most of the CA patients had been treated intermittently with topical steroids and only a minority with systemic immunosuppressants. This type of treatment is unlikely to have increased or decreased the risk of type 1 diabetes mellitus. None of the patients in the allergy database had been referred because of dermatitis reaction at the injection site, which otherwise could have induced a selection bias. The high median age of 47 years of the analysed patients means that most of those who during their lifetime will develop CA and/or type 1 diabetes mellitus have already done so at the time of allergy testing (patch testing).

There are more females than males in the allergy database and there are also more females than males with a positive patch test, which is in accord with the literature [3].

Several studies have evaluated the potential relationship between atopic dermatitis and type 1 diabetes mellitus. The outcome is not uniform, but most studies point to a negative association [19–22]. Those studies, however, deal with children and recorded atopic illnesses prior to the development of diabetes mellitus. One study found the association only to be significant for the older age group (10–14 years) [22] and one study only found an inverse association if atopic dermatitis was diagnosed before onset of type 1 diabetes mellitus [23]. Atopic dermatitis is defined as a clinical syndrome, and is not based on a specific immunological event, in contrast to CA, which might explain this variation.

CA has not previously been investigated with respect to autoimmune diseases, probably because CA has not been regarded as a Th2-dominated illness [24]. However, recent studies have found that in patients with long-lasting dermatitis, as in the present patient material, a Th2 response is anticipated [25, 26]. In this study, we found an inverse relationship between CA and type 1 diabetes mellitus. This finding could lead to the hypothesis that CA is an environmentally acquired factor modulating the risk of developing type 1 diabetes mellitus. Alternatively the inverse relationship could be explained by genetic factor or environmental determinates; however, at present genetic factors are not considered to play a major role in CA and there are no known shared environmental factors.

CD1d-restricted NKT cells have shown interesting abilities in regulating the immune system in both a suppressing and an enhancing direction [27]. Therefore, the NKT cell has been studied in the pathogenesis of a number of autoimmune disease, including type 1 diabetes mellitus. The nonobese diabetic mouse has been found to be numerically and functionally deficient in NKT cells [10]. Furthermore a study of identical twins discordant for type 1 diabetes mellitus also showed differences in gene expression of NKT cells [11]. The NKT cells respond to α-galactosylceramide, a glycolipid, in the context of CD1d. Treatment of nonobese diabetic mice with α-galactosylceramide has been found to protect against diabetes [12, 13, 28]; a review of NKT cells in relation to type 1 diabetes mellitus is found in [29].

In an animal model of CA, Campos et al. [7] found an increased level of NKT cells in the liver after induction of

CA. Furthermore, recent studies have shown that NKT cells are important in the elicitation of CA [7, 8].

We have also investigated the relationship between CA and type 2 diabetes mellitus, but found no association (data not shown). This can be explained by the fact that type 2 diabetes mellitus is not a Th1-mediated autoimmune disease. However, patients with type 2 diabetes mellitus are not followed in the same way through the hospital system as patients with type 1 diabetes mellitus, since most are treated by a general practitioner, which probably leads to an underassessment of type 2 diabetes mellitus patients.

In conclusion, we found an inverse relationship between CA and type 1 diabetes mellitus. This association might be related to shared genetic factors or common environmental determinates; however, genetics is generally thought to be a minor factor in the development of CA. Another possibility is that development of CA influences the immune system, so that the risk of developing type 1 diabetes mellitus is reduced, or vice versa.

CA has not previously been found to have an association with autoimmune diseases, which is why this study may stimulate research into the immunological interaction between environmental skin exposure and autoimmune diseases.

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ORIGINAL ARTICLE

Inflammatory bowel disease in relation to contact allergy: A patient-based study

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Abstract

Objective. Inflammatory bowel disease (IBD) has previously been investigated with relation to allergic conditions; however, diverging results were found and there are only a few small studies focusing on delayed hypersensitivity. The aim of this study was to investigate whether there was an association between contact allergy (CA), which is a type IV hypersensitivity reaction of the skin, and IBD. ***Material and methods.*** A database consisting of a cohort of 13,315 patients, patch tested between 1985 and 2003, was linked with the Danish National Patient Registry using a unique personal identifier number. The patients were patch tested at a dermatology department with a long history of research in CA. By record linking with the Danish National Patient Registry, patients were identified who had either an International Classification of Disease (ICD) code for Crohn's disease (CD) or an ICD code for ulcerative colitis (UC) diagnosis. Using logistic regression, with the result of the patch test as the dependent variable, we calculated the odds ratios for IBD, CD and UC, adjusted for gender and age. ***Results.*** An inverse association between CA and IBD was found, odds ratio adjusted for age and gender 0.71 (CI 95% 0.53–0.94), which is mainly the result of an inverse association between CA and CD, odds ratio adjusted for age and gender 0.42 (CI 95% 0.23–0.76). ***Conclusions.*** The association found between CA and IBD might be related to shared genetic factors or common environmental determinates. It may also be that having either disease result in skewness of the immune system might lead to an inverse disease association.

Key Words: *Allergic contact dermatitis, contact allergy, Crohn disease, patch tests, ulcerative colitis*

Introduction

Contact allergy (CA) is a disease caused by skin contact with substances in the environment of small molecular weight. CA is a delayed-type hypersensitivity reaction, following a T-cell-mediated response. The T-cell response consists of both a Th-1 and a Th-2 response [1], but it may verge more toward a Th-2 response in chronic eczema following CA [2,3], which may be directed toward reducing a harmful Th-1 response, since $CD4^+$ T cells can have a down-regulatory effect [4].

CA develops in two phases, which are defined operationally as the induction (sensitization) and elicitation phase. In the induction phase, skin contact with a contact allergen of a sufficient dose causes activation and expansion of contact allergen-specific T lymphocytes. The elicitation phase begins upon contact with the allergen in question and results in clinical manifestation of allergic contact dermatitis (ACD). ACD is characterized by erythema and infiltration at the skin site of contact.

The main cells of importance in CA are, besides the T cells, the Langerhans cells and dermal dendritic cells, and the natural killer T (NKT) cell may also play a pivotal role [5].

CA is rare among infants but common in adolescents: 15% of children aged 12–16 years have CA to one or more allergens [6], which is similar to the level in the adult population [7]. Risk factors for CA are mostly related to environmental exposure, while genetic factors seemingly play only a minor role [8–11]; accordingly, CA is considered a disease mainly driven by the environment.

Inflammatory bowel disease (IBD) is an idiopathic and chronic intestinal inflammation. The two main clinical entities of IBD are Crohn's disease (CD) and

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ulcerative colitis (UC). Both are chronic relapsing inflammatory conditions of the gastrointestinal tract.

The generally accepted notion is that the mucosa of patients with CD is dominated by CD4+ T cells with a Th-1 phenotype, while the mucosa of patients with UC is dominated by an atypical Th-2 phenotype [12–14].

Susceptibility to IBD is influenced by genetic predisposition, especially in CD where the concordance rate for monozygotic twins is relatively high, whereas the concordance rate for UC is low [15–17]. Nevertheless, since the concordance rate is well below 100%, non-genetic factors, primarily environmental or luminal, may be required to trigger IBD in a genetically susceptible host.

To investigate the relation between IBD and CA, we studied a large cohort of patients tested for CA in a university department of dermatology.

Material and methods

From 1985 to 2003, 13,315 patients (4818 M, 8497 F) were patch tested at the Department of Dermatology, Gentofte Hospital, University of Copenhagen, Denmark. Patch testing is the international standard test that is used to diagnose CA. Patients were typically referred to the department because of relapsing or chronic dermatitis. The outcome of the patch test, gender and date of birth were recorded in the allergy database. All patients were patch tested with the European Standard Series recommended at the time of testing; this series contains the most important contact allergens in the environment for the European continent. Patch testing was performed, according to international standards, on the upper back using Trolab allergens (Hermal, Reinbek, Germany) and Finn chambers for occlusion (Epitest, Tuusula, Finland). Occlusion time was 48 h and the patches were read on days 2, 3 and 7 according to the international criteria from the International Contact Dermatitis Research Group (ICDRG) [18,19]. A positive allergic reaction was defined as at least homogeneous erythema and infiltration in the test area. The database has information on the test result for each of the days, but in the present study a binary variable was constructed; a positive test on any of the days to any of the allergens of the European Standard Series at any test time rendered the patient positive. Of the 13,315 people tested, 4848 had a positive response, at least once, to one of the tested allergens.

At birth, or on immigration, all Danish residents are given a unique and personal identification number (a CPR number) which is used for identification in databases. This enables linkage of individual data over time and between databases.

Using the unique identifier (CPR), the allergy database was linked with the Danish National Patient Registry, which contains the codes of diagnoses from the International Classification of Diseases, 8th and 10th revision (ICD8 and ICD10). The Danish National Patient Registry contains information on all hospital admissions, discharge diagnoses and operations performed since 1977, and outpatient contacts from 1 January 1995. Patients with the ICD8 code 56301 or the ICD10 code DK50 were categorized as having CD, while patients with the ICD8 code 56319 or the ICD10 code DK51 were categorized as having UC. Patients with either an ICD code for CD or UC were categorized as having IBD. From 1977 to 1993 the Danish National Patient Registry used the ICD8, and since 1994 the ICD10 has been used [20].

Age was calculated as age at the first positive patch test; if there was no positive patch test, the age at the first patch test was used. The patients were stratified into five age groups: 0–15, 16–30, 31–50, 51–70 and >70 years.

The resulting data file was analysed using logistic regression, with the patch test outcome as the dependent variable and IBD or CD or UC as the independent variable, and controlled for gender and age. Odds ratios with 95% confidence intervals (CIs) were estimated using logistic regression. All data analyses were done using SPSS version 12 (SPSS Inc., Chicago, Ill., USA).

Results

Of the 13,315 patients, 225 had at least one ICD8 or ICD10 code for IBD (ICD8 56301 or 56319; ICD10 DE50 or DE51); of these, 34 had diagnoses of both UC and CD, and of the remaining 191 patients, 73 had a CD diagnosis and 118 had a UC diagnosis (Table I).

The age span was 6–99 years (median 47 years): for patients with a UC diagnosis only, the age span was 15–91 years (median 49 years); for the CD group, 9–86 years (median 43 years); and for those with an ICD code for both CD and UC, the span was 13–73 years (median 42.5 years).

There were more females than males in the registry, and also more females with a positive patch test than males (42.2% versus 26.3%).

When adjusting for gender and age group, patients with an IBD diagnosis had an odds ratio of 0.71 of having CA (CI 95% 0.53–0.95).

The IBD diagnosis was further spilt into three groups: patients with a CD diagnosis only, patients with a UC diagnosis only and patients with both a CD and a UC diagnosis. Only the group with a CD diagnosis had a significant association with CA

Table I. Overview of the number of patients in the CD and UC categories.

Number of patients in the various groups		Negative patch test		Positive patch test		Total
		Male	Female	Male	Female	
Both a CD and a UC diagnosis	CD & UC	8	15	3	8	34
CD diagnosis only	CD	26	33	0	14	73
UC diagnosis only	UC	32	46	14	26	118
No diagnosis for IBD	No IBD	3487	4820	1248	3535	13090

Abbreviations: CD = Crohn's disease; UC = ulcerative colitis; IBD = inflammatory bowel disease.

($p = 0.004$). The odds ratio for a patient with a CD diagnosis only of having a positive patch test was 0.42 (CI 95% 0.23–0.76).

Discussion

We found an inverse association between CA and IBD, which was mainly due to an inverse association between CA and CD. The odds ratio for CD adjusted for age and gender was 0.42 (CI 95% 0.23–0.76) (Table II). This investigation is unique: as it is based on a large cohort of patients followed over 19 years at a university department of dermatology, and the record linking used is possible only in Denmark, where a unique personal identifier system has long been used in all registers.

The study concerned patients referred to the dermatology department for patch testing. CA is generally more common among females [8], and this explains the gender difference in the database. This difference is reflected among the patients with a CD or UC diagnosis (Table I). In Denmark, CD is more common among females, but this is not the case for UC [21,22].

In two studies the estimated prevalence of UC and CD was found to be, respectively, 160 and 54 per 100,000 inhabitants [21,23]. However, more recently, a study covering the period from 1981 to 1992 found an increasing incidence of CD [24], which would result in a higher prevalence. Furthermore, outpatients were first included in the Danish National Patient Registry in 1995.

The study was based on discharge diagnoses, which may vary in quality between hospitals and over time; however, this potential bias would be similar for CD and UC, while only the odds ratio for CD is significant. The Danish National Patient Registry has been investigated previously in the county of Northern Jutland, and here the completeness of the Danish National Patient Registry using the pathology system as a reference standard was 94% for both CD and UC, and the validity was 97% and 90%, respectively [25]. The present study largely included patients from the county of Copenhagen, which is an area where patients with IBD have been registered over a long period [21,23], and the validity and completeness of the Danish National Patient Registry in this area is therefore at least on the same level as those of Northern Jutland.

A sampling bias caused by the investigation being based on hospital material would have led to a positive and not a negative association. Although population-based studies are often preferable to patient-based studies by virtue of unbiased estimation of prevalence and risk, when evaluating risk factors for CA they have limited power [26]. CA is not associated with an increased death rate, which could have interfered with the data. Most of the CA patients had been treated intermittently with topical steroids, and only a minority with systemic immunosuppressive drugs. This treatment is unlikely to have influenced the risk of IBD.

A possible confounding factor in our study could be smoking, as smoking increases the risk of CD, but has a protective effect in UC [27]. Studies have found that smoking might increase the risk of CA [28], but this common risk factor for CA and CD would have resulted in a positive rather than a negative association as found in this study. It might, however, have reduced a positive association between CA and UC.

Table II. Results from the logistic regression with CA as the response variable and inflammatory bowel disease, CD or UC as the independent variables.

Association between contact allergy and IBD	Adjusted for	*p*-value	Odds ratio	CI 95%
IBD		0.019	0.71	0.53–0.94
IBD	Age and gender	0.02	0.71	0.53–0.95
Both a CD and a UC diagnosis	Age and gender	0.64	0.83	0.40–1.73
CD	Age and gender	0.004	0.42	0.23–0.76
UC	Age and gender	0.53	0.88	0.60–1.30

Abbreviations: CA = contact allergy; CD = Crohn's disease; UC = ulcerative colitis; IBD = inflammatory bowel disease.

Another possible confounder is medical treatment of IBD with immunosuppressants, e.g. azathioprine or prednisolone; however, if this treatment had an effect on the risk of CA, it would most likely be the same for both UC and CD. Medical therapy with anti-tumour necrosis factor alpha (TNF-α) is presumably a confounder, since migration of Langerhans cells is inhibited by anti-TNF-α treatment [29]; mice deficient in the TNF-α gene have an impaired contact hypersensitivity reaction [30]. Treatment of CD with anti-TNF-α might therefore interfere with the patch test result. However, infliximab was not introduced in Denmark until late 1999 [31], and is therefore unlikely to be a problem in this study. Furthermore, patients with a down-regulated elicitation response would most likely not have been patch tested, and therefore would not be included in the database.

IBD has previously been studied in relation to allergy; the results were divergent [32–35], and only a few and relatively small studies focused on delayed hypersensitivity [36,37]. Triantafillidis et al. [36] found that patients with CD had a reduced delayed hypersensitivity response to microbial antigens in the multi-test [36], while D'Arienzo et al. found no statistical differences between CA in a small cohort of patients with UC and controls [37].

In our study we found an inverse association between CA and IBD, which was mainly caused by the inverse association between CA and CD. CA is generally considered to be a Th-1 disease; however, studies have suggested that in chronic CA dermatitis, the cytokine milieu is more of a Th-2 profile [2]. The patients in our study mostly had chronic or relapsing contact dermatitis and could therefore have had a Th-2 cytokine profile, which could explain the inverse association to CD.

A possible relation between CA and IBD could be the natural killer T (NKT) cells. In 2004, Fuss et al. found non-classical CD1d restricted NKT cells involved in oxazolone-induced colitis, an animal model for UC [38]. In the dextran sulphate sodium (DSS)-induced colitis mouse model, it has been shown that administration of both α-galactosylceramide and OCH improved the colitis [39,40], which indicates that activation of the NKT cell improves resolution from this induced colitis model. The number of circulating NKT cells is reduced in patients with CD or UC [41], but the frequency and function of NKT cells from different organs might be unrelated [42] and the number in blood may therefore not be relevant. Nieuwenhuis et al. found that in oxazolone-induced contact hypersensitivity, CD1d-restricted NKT cells play a pivotal role [5]. Campos et al. found that the level of NKT cells in the liver increased after induction of CA [43]. Seemingly, the level of NKT cells is inversed in CA and IBD.

Using the same cohort, we previously investigated the relation between CA and type 1 diabetes and found and an inverse association [44]. This and the previous study both point to an inverse association between CA and a Th-1-driven disease.

In conclusion we found an inverse relation between CA and IBD in the context of CD. This association might be related to shared genetic factors or common environmental determinates. However, genetic factors are generally thought to be a minor determinant in developing CA. Another possibility is that development of CA influences the immune system, thus reducing the risk of developing CD.

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Prevention of Type 1 Diabetes By Exposure to a Contact Allergen Inducing a Sub-Clinical Dermatitis

Repeated exposure to allergen prevents diabetes

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ABSTRACT

Context: Type 1 diabetes is an autoimmune disease resulting from the destruction of the insulin-producing β-cells by autoreactive T cells. Allergic contact dermatitis is primarily an environmental disease, which develops following epicutanous contact with reactive low-molecular chemicals. We have previously shown an inverse association between these two diseases in an epidemiological study, if this finding is confirmed experimentally, it could bring forth new treatment possibilities for diabetic patients.

Objective: The objective of the study was to determine whether contact allergens could influence the development of diabetes in Non-Obese Diabetic mice, and if Natural Killer-T (NKT) cells could play a role.

Method: Non-Obese Diabetic mice were exposed to two potent allergens, p-phenylenediamine and 2,4-dinitrochlorobenzene respectively, to investigate the diabetes development. The mice were followed for a maximum of 32 weeks.

The stimulation of NKT cells by the two allergens were furthermore studied in C57BL/6 mice.

Results: It was found that repeated application of p-phenylenediamine halted the development of diabetes compared to application of water (P= 0.004). Moreover it was shown that both allergens increased the quantity of NKT cells in the liver, while only p-phenylenediamine increased the quantity of NKT cells in the draining lymph node.

Conclusion: These experimental data support our earlier epidemiological study, which showed that allergic contact dermatitis is inversely associated with diabetes. This may be due to stimulation of the innate immune system in the context of NKT cells. This knowledge could pave the way for new preventive interventions and treatment modalities for type 1 diabetes.

INTRODUCTION

Type 1 diabetes is an autoimmune disease in which the β-cells in the pancreas are destroyed. The incidence of this disease is increasing (1), and it is therefore of interest to investigate environmental factors which could modulate the development of disease. Non-Obese Diabetic (NOD) mice are used to study type 1 diabetes in the animal setting, as female NOD mice spontaneously develop a disease equivalent to type 1 diabetes.

Allergic contact dermatitis (ACD) is a skin disease, driven by skin exposures to contact allergens (2), which are often present in consumer products. These allergens are small electrophilic chemicals (haptens) which due to their size and lipophilic nature are able to penetrate the skin and interact with proteins making the hapten-protein molecule immunogenic(3). The standard method for estimating the relative potency of contact allergens is the Local Lymph Node Assay (LLNA) (4). Recent studies using potent contact allergens have shown that $CD8^+$ T cells are the effector cells of ACD in mice, whereas CD4+ T cells behave as down-regulatory cells (5). Studies using a repeated application protocol, have however shown a shift from a Th1 cytokine milieu to a Th2 milieu (6-9).

In an epidemiological study, we have previously shown an inverse association between type 1 diabetes and ACD (10), and we therefore wished to examine, whether exposure to a contact allergen could prevent the development of diabetes in the spontaneously diabetic NOD mouse.

It has been establish that stimulation of Natural Killer-T (NKT) cells can protect NOD mice against diabetes (11). Since some allergens have been shown to stimulate NKT cells (12;13), we additionally explored whether the used allergens actually did influence NKT cells.

METHODS

Local Lymph Node Assay (LLNA) - dose-finding study

The LLNA protocol was performed as previously described (14). Briefly 28 NOD/BomTac (Taconic) mice at the age of eleven weeks were exposed to one of two known potent allergens, either p-phenylenediamine (PPD), or 2,4-dinitrochlorobenzene (DNCB) (Sigma-Aldrich) or with the vehicle. The vehicle was acetone and olive oil 4:1 (AOO). For the PPD groups the concentrations were 1.0, 0.25, and 0.05% (w/v) and for the DNCB groups the concentrations were 0.25, 0.05, and 0.01% (w/v).

Diabetes study
To study the incidence rate of diabetes in allergen exposed mice, 114 female NOD/BomTac mice (Taconic) were received at the age of three weeks. The mice were allocated to seven groups, and all groups received either a sensitizing or a control regime. In the sensitizing regime the mice were exposed three times in the fourth week of life (equivalent to the first three days of the LLNA). Four groups were additionally exposed repeatedly every other week to either vehicle, water, or one of the allergens until a maximum of 32 weeks of life. The exposures were topically application of 25µL substance on the dorsal side of each ear. For sensitization the concentration for the allergens were 0.1 %(w/v), and for the repeated exposure regime a concentration of 0.01 %(w/v) was used. The dilutions were freshly made just prior to application.
The experiment ended when the mice were 32 weeks old, at which time the mice in the repeatedly exposed groups had received 14 applications of the repeated dose. At 12 weeks of age, weekly measurements of tail blood glucose were started. If the glucose level was equal to or above 14 mmol/L on two consecutive days, the mice were considered to be diabetic and were euthanized. At the end of the study the mice still alive in the PPD repeatedly exposed group were provoked with 0.1 %(w/v) PPD, and euthanized two days later. A histological examination was made to assess cellular infiltration of the ears (Fig. 2). All samples were fixed in formalin and stained in hematoxylin and eosin. The rest of the mice still alive at the end of the study, were euthanized and their ears were also examined for cellular infiltration.
The cumulative diabetes incidence was evaluated by the Kaplan-Meier estimation, and the significance was estimated by the log-rank test.
NKT study
Twenty female C57BL/6JBomTac (Taconic) mice were received, at the age of ten weeks. One week after arrival ten mice were sensitized with 0.5 % (w/v) DNCB in AOO and ten mice with PPD 0.5 % (w/v). Two weeks after sensitization the mice were separated into ten groups of two mice – accordingly there were five groups in each interval. In eight groups the respective allergen in a concentration of 0.5 % (w/v) was reapplied. At the following time points after reapplication (18, 24, 48, and 72 hours) a group of mice were anaesthetized, fixated and vena portae was ligated. Vena cava was cut and the mice bled. The liver was perfused with PBS containing heparin until pale; afterwards the liver was removed along with the ear-draining lymph nodes (A-LN). The groups which did not receive a reapplication were euthanized at 48hour.
The livers and A-LNs were strained (70 µm; BD Biosciences, Brøndby, Denmark), to gain a single cell suspensions, and the liver lymphocytes were isolated using Lympholyte-M (CEDARLANE Laboratories, Burlington, Canada). Subsequently the cell suspensions were stained with APC-conjugated CD1d tetramers (ProImmune, Oxford Science Park, UK) loaded with α-galactocylceramide (Alexis Biochemicals/Axxora, San Diego, USA) according to the manufacture guidelines. Thereupon the following cellular receptors were stained FITC conjugated anti-CD3, PE-Cy5.5 conjugated anti-TCR-β, PE-Cy7 conjugated anti-NK1.1 (eBioscience, San Diego, USA) and APC-Cy7 conjugated CD19 (BioLegend, San Diego, USA). Afterwards the cells were analyzed on a FACSCanto II (BD Biosciences, Brøndby, Denmark), and the CD19 positive cells were gated out, while the CD3 and TCR-β positive cells were selected and the amount of CD1d positive cells within this gate was found.
Paired samples t-test was used to evaluate the difference in NKT cell stimulation in the A-LN's.

RESULTS

In the LLNA the NOD mice gave a response similar to the standard strain (CBA), evaluated by the EC3 values, which is the standard measure for potency of an allergen tested in LLNA. The EC3 values in our experiment was 0.169 and 0.037 for PPD and DNCB, respectively, which is in accordance with results from CBA mice (15;16). On the basis of the EC3 values we decided to use a concentration of 0.1 for sensitization and 0.01 for repeated treatment, in the diabetes study.
In the diabetes study, the NOD mice exposed to PPD repeatedly, displayed a cumulative diabetes incidence of 47%, in contrast to 93% of the water treated group (P=0.004). The group repeatedly

exposed to DNCB had an incidence of 92%, and the group which only received the PPD sensitizing regime 93% (Fig.1). The three other groups developed diabetes by a rate similarly with a cumulative incidence from 80-86% (data not shown).
Dermatitis was not noticeable by visual inspection, however sensitization in the group repeatedly exposed to PPD, was confirmed by a typical lymphocytic infiltration in the ears following PPD provocation, and a more moderate infiltrate was also noticeable in the group repeatedly exposed to DNCB (Fig. 2).
The NKT study showed that in the liver both allergens influenced the amount of NKT cells in an analogous manner, data for PPD shown in Fig. 4., which is in contrast to the ear-draining lymph nodes (A-LN) where PPD seems to influence the amount of NKT-cells more strongly than DNCB (P (one-tailed) = 0.043), data shown in Fig. 3.

DISCUSSION

We showed that repeated application of PPD reduces the diabetes incidence in NOD mice. The repeated application of allergen gave a sub-clinical dermatitis. This could not be explained by an abnormal response in NOD mice to the used allergens, as the NOD mice reacted similar to the standard mouse strain used for LLNA. Moreover we found that both allergens modulated the number of NKT cells in the liver (Fig. 4), which is in accordance with previous published data [13].
PPD gave a more pronounced NKT cell response in the ear-draining lymph nodes (Fig. 3). This peripheral stimulation of NKT cells by contact allergens is in agreement with a recent human study (17). The mechanism for the protective effect of ACD on diabetes is unknown, but activation of NKT cells could be an explanation, since up-regulation of NKT cells have been shown to protect NOD mice against diabetes (11;18-20). The difference between PPD and DNCB in the peripheral activation of NKT cells might then explain the selective protective effect of PPD.
Repeated application of a contact allergen may possibly give a Th2 cytokine milieu (6-9), which as well might contribute as a mechanism for the protective effect of repeated application of PPD. The lack of effect of DNCB on diabetes development may then be due to the differences in potency.
In conclusion, we show that controlled repeated epicutanous exposures to a contact allergen prevent development of diabetes in the NOD mouse. The mechanism may be a peripheral up-regulation of NKT cells and or Th2 cells. This observation may lead to a new understanding of the integrated immune function and might lead to a new strategy for prevention of type 1 diabetes.

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LEGENDS TO FIGURES

Figure 1 Repeated application of PPD protects NOD mice against development of type 1 diabetes.

The figure shows a Kaplan-Meier curve of the diabetes incidence for four groups of mice. DNCB-rep, PPD-rep and Water, refers to the groups of mice that were repeatedly exposed to DNCB, PPD or water every other week. PPD-sensi refers to the group of mice which were only exposed to PPD in the fourth week of life. The NOD mice exposed to PPD repeatedly, displayed a cumulative diabetes incidence of 47%, in contrast to 93% of the water treated group (P=0.004).
The log-rank test for all the shown curves gives a significance of P=0.008.

Figure 2 Mononuclear infiltrations in the ear after provocation with PPD at the end of the study.

The figure shows a hematoxylin and eosin stained ear from the group of mice repeatedly exposed to PPD. Ears from the group repeatedly exposed to DNCB shows a similar, though weaker infiltrate (not shown).

Figure 3 PPD is a strong peripheral activator of NKT cells.

The figure shows the proportion of NKT cells in the ear-draining lymph nodes, for the mice exposed to either PPD or DNCB. The proportions were found, by dividing with the number of NKT from the control groups (0 hours). As can be seen from the figure the proportion of NKT cells appears to rise in the beginning, but at 48-72 hours there is a sharp drop which probably have to do with a down regulation of receptors. Apparently the PPD allergen is a strong peripheral stimulator of NKT cells (P (one-tailed) = 0.043).
The NKT cells were stained for TCR-β, CD3 and CD1D loaded tetramers (α-GalCer), CD19 positive cells were gated out.

Figure 4 Topical exposure to contact allergens stimulates NKT cells in the liver.

The figure shows the proportion of NKT cells in the liver, for the mice exposed to PPD or DNCB. The proportions were found by dividing with the number of NKT cells from the control groups (0 hours). Both allergens increased the number of NKT cells in the liver in a comparable manner. As can be seen in the figure, the proportion of NKT cells peaks at 18 hours, followed by a decrease, and yet another expansion. The observation of a rise followed by a steep drop may be due to down-regulation of receptors which have previously been reported [(21)].
The NKT cells were stained for TCR-β, CD3 and CD1D loaded tetramers (α-GalCer), CD19 positive cells were gated out.

Figure 1

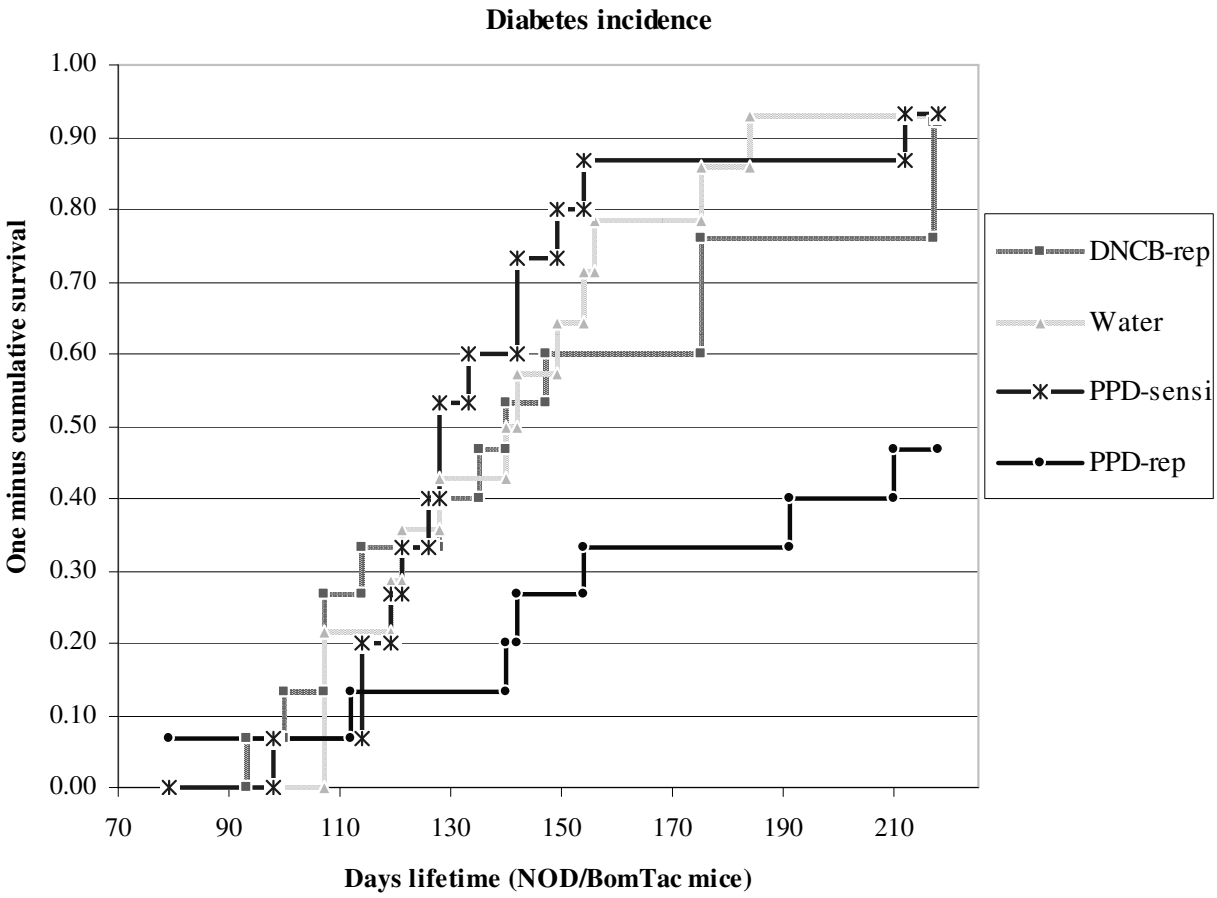

Figure 2

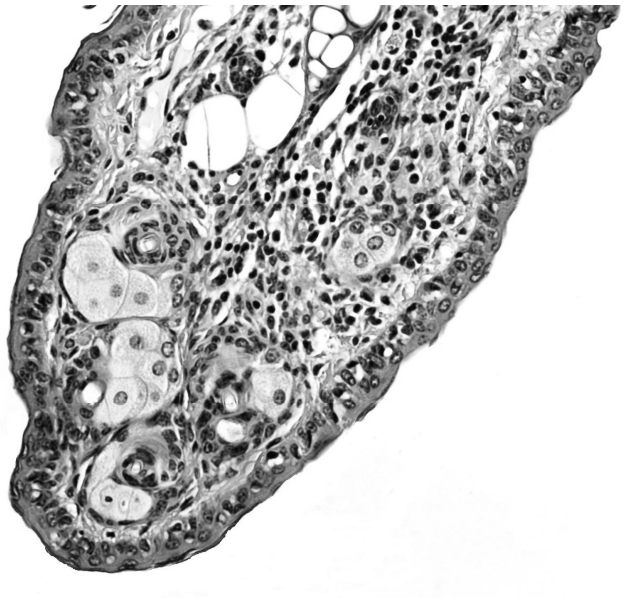

Figure 3

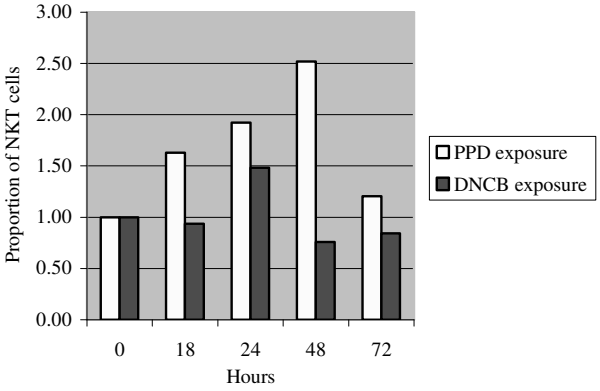

Figure 4