

Project plan

Life style and allergy - importance of environmental and life style factors on pregnancy, infancy and childhood - Aladdin

Overall aim

The overall aim of the project is to assess the role of environmental and life style factors for the development of allergy in children. A novel approach is to focus primarily on factors offering protection in relation to allergy. Reduced exposure to such factors may contribute to explain the rise in allergy in many Western countries in recent decades. We are thus interested in investigating factors regulating the immune system in an allergy protective direction and how internal regulating mechanisms could be utilised for preventive or therapeutic purposes.

Specific aims are:

- Can a causal relation between allergic disease in children and life style factors during pregnancy, neonatal period and infancy be found?
- Does the intrauterine environment differ between mothers with and without allergy, as well as those with anthroposophical and traditional life style?
- Is the expression of Toll-Like-Receptors in cell populations from the specific and un-specific immune system associated to life style factors and allergic disease?
- In which way does the humoral and cellular immune response on allergens differ between an atopic and a non-atopic individual?
- Can a causal association between commensal microbial flora in the gut, production of antimicrobial peptides on the skin and induction of allergy be shown?
- Does the level of psychological stress in family, assessed by saliva cortisol, influence the risk in the child to develop allergy?

Research survey

IgE-mediated allergy

The prevalence of IgE-mediated allergic diseases has increased markedly during the past decades, especially in children, although recent reports indicate that the occurrence has stabilised (1). Allergic IgE-mediated diseases – atopic eczema, food allergy, allergic rhino-conjunctivitis and asthma – affect about 30% of children and youngsters. The reason for the rapid increase in IgE-mediated allergy is uncertain and several hypotheses exist (2). Today allergic diseases are a major public health problem, which, apart from personal suffering, bring extensive costs to medical care as well as consequences for employment and social life. Therefore it is urgent to identify, not only risk factors, but also protective factors, for preventive purposes.

The atopic constitution is characterised by the ability of an individual to produce IgE-immunoglobulin (IgE) following exposure to common environmental substances, i.e. allergens. Cell-bound IgE-antibodies cross-link the allergen resulting in cellular degranulation of substances, e.g. histamine and prostaglandins, causing allergic symptoms. Allergen-reactive T-helper (Th) cells of Th2-type play an important role in inducting and sustaining the allergic reaction (3). It is still uncertain what drives the immunological response towards Th2-activity. The initial contact between allergen and the native and specific immune response, however, seems to be of crucial importance. It is indicated that the localisation of antigen-presenting cells (APC) and their functional capacity could dictate whether a cellular (Th1) or an IgE-mediated (Th2) immune response (memory) is established, or if T-regulating (Treg) cells will develop inducing tolerance against allergen (4).

Environmental and life style factors

We have in previous epidemiological studies found factors in the farming environment and in the anthroposophical life style protective for the risk of developing allergy (5, 6, 7). A protecting effect

is thought to be caused, among others, by components from microbes in the environment (2, 8). The intestinal microbiota constitutes a prerequisite for oral tolerance by inducing immune responses in the human body, and may thus be of importance for the development of atopy (2). Milk allergic infants with atopic dermatitis showed milder symptoms when their formula was fortified with lactobacilli (2). In animals some antibiotics seem to, by influencing microbiota, induce an allergic immune response (9). We have previously found that life style seems to influence the microbiota in infants (10).

Toll-Like Receptors (TLRs) and LPS-receptor CD14 seem to be of importance for microbial signalling to immune-competent cells, and for the regulation of the Th1/Th2-balance, allergic reactions and IgE-production (11). House dust could contain microbial compounds, such as lipopolysaccharides (LPS), which thus may stimulate innate immunity and suppress atopic sensitisation (8). Children in farm families, who run less risk of developing allergy, had significantly higher levels of TLR-2 mRNA in blood cells compared to non-farm children (12).

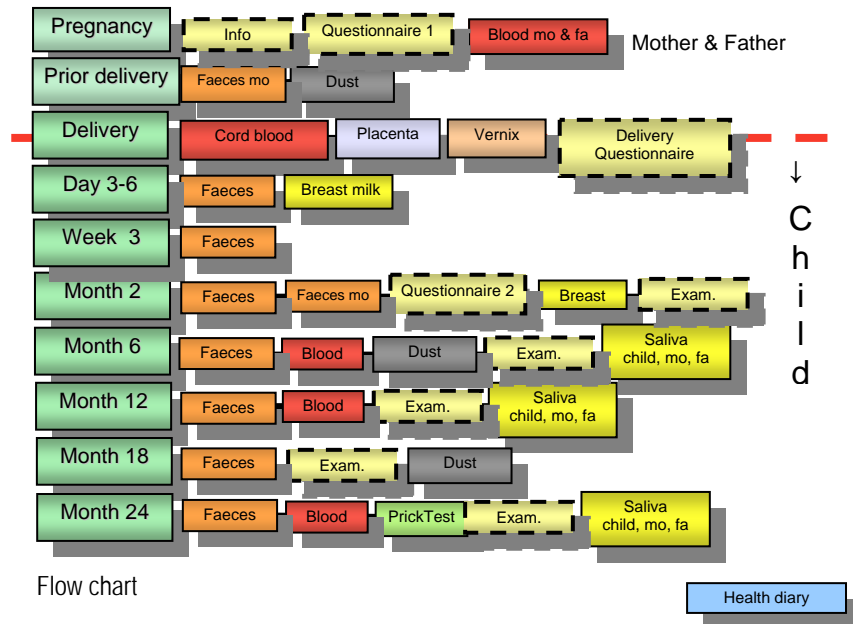
An association is supposed between the propensity in the individual to develop atopic disease and the level of microbial stimulus during foetal period and/or first year of life (13). The immunological interplay between mother and foetus takes place through the placenta. It has been supposed that the cellular part of the maternal immune response is down-regulated in order to prevent rejection of the foetus. It has also been supposed that newborns could be characterised by a dominating cytokine profile of Th2-type (14). Normally, most of them rapidly deviate towards an immune response of Th1-type, counteracting atopic phenotype. In the infant prone to allergy this transition, for unclear reasons, is thought not to occur (14). We have found that umbilical cord cells from offspring with maternal and paternal allergic heredity are polarised towards a Th2- balance to a higher extent with larger IL-4/IFN- γ relation and a smaller amount of IL-12-producing cells (15). Accordingly, at two years of age, only children with few IL-12 producing cells were IgE-sensitised. In parallel, more children with few IFN- γ producing cells at birth had atopic dermatitis during their first two years of life (16).

Project plan

We have found that children with an anthroposophic life style have a reduced risk of allergic disease (4) and we have confirmed this within a European research network, in a large study in five European countries (PARSIFAL) (6, 7). These cross-sectional studies strengthen the hypothesis on importance of interaction between ambient microbes and our immune system during childhood regarding allergy. However, prospective studies are needed to focus on a causal relationship. The anthroposophic life style is characterised by a high frequency of home deliveries, and a diet - even for small children - of mostly local food produced according to biodynamic principles, including vegetables preserved by spontaneous fermentation containing live lactobacilli. Furthermore, anthroposophically oriented doctors restrict the use of antibiotics, antipyretics, and vaccinations.

Participants

Three age groups of families enrolled in the anthroposophic maternity welfare centres at the Vidar clinic (MWC) in Järna, south of Stockholm, will be asked to participate in the project (n=150). 180 controls will be recruited in the same way from the conventional MWC in Järna. In addition, results from a present investigation at Sachs' Children's Hospital, comprising 280 five-year-old children, and with a very similar protocol, however not regarding anthroposophical life style, will constitute reference data. At present, 175 families are recruited and 130 children have been born, of which 110 have passed the 2-month, 75 the 6-month and 20 children the 1-year examination. The last of the 330 children are estimated to be born in spring 2007, and thus be 2 years of age in 2009. This is followed by extensive analyses and reports, and final report is estimated to 2010. Dropouts are few, at present five families.



Questionnaire: Information on environment and life style of families involved will be collected through questionnaires prepared on the basis of experiences from previous studies (5, 6, 7). Questions will be asked regarding feeding practises for children, family diet, use of antibiotics, atopy-associated symptoms, atopic heredity and environmental factors like tobacco smoking and furred pets in immediate surroundings.

Infection diary: Parents will be asked to conduct an infection diary on the child, in which passed infections and use of antibiotics is continuously registered. Survey and validation of diary data will be made during clinical controls at 6, 12 and 24 months of age.

Parents: Before delivery, skin prick tests for air-borne allergens will be performed, and blood collected for analysis of IgE-antibodies against 11 common allergens (Phadiatop; Phadia AB, Uppsala) in order to confirm/exclude atopic airway allergy, and for immunological characteristics of maternal blood (see below).

Children: I) *Clinical evaluation* by a physician will be repeatedly performed until at least 24 months of age. II) *In vivo* – skin prick test will be completed at 24 months of age against cow’s milk, hen’s egg, cat, and, following agreement with parents, additional allergens. III) *In vitro* – blood is collected at birth (15-20 mL umbilical cord blood) and venous blood samples at 6, 12 and 24 months of age following previous topical anaesthesia (EMLA-ointment).

Serological analyses

IgE – the blood samples will be analysed for IgE-antibodies against cow’s milk, hen’s egg, birch and cat (Phadia AB, Uppsala).

Viral serology: Epidemiological and experimental studies have confirmed, albeit not consistently, a possible allergy-preventive effect of several infectious antigens (2). Early attendance in day nursery is found to reduce atopy in children from small families, but an opposite association has also been described (2). In our similar on-going prospective investigation of 280 children, however not consider anthroposophic life style, we have found that children having had serologically verified Epstein-Barr Virus (EBV) infection before their third year of life were less often sensitised (IgE) to food and airborne allergens (17). This was most pronounced in those having had Cytomegalovirus infection in parallel (17) and not related to a lower production of Th-2 type cytokines (Nilsson, et al., in manuscript). In collaboration with Prof. Annika Linde at SMI, Karolinska Institutet, signs of

passed viral infections (respiratory syncytial virus (RSV), influenza, para-influenza, metapneumovirus, adenovirus, rhinovirus, enterovirus, cytomegalovirus, herpes simplex, chicken pox, Epstein Barr-virus, and human herpes virus 6 and 7) will be examined in cord blood (maternally transferred antibodies) and in sera collected at 6, 12 and 24 months of age.

Cellular tests (mother and child): In order to study if also factors related to life style and not only to heredity (15), influence the immunological polarisation of umbilical cord cells, cytokine analyses will be performed on cells isolated from blood samples from mother and child at delivery, and from the child at 24 months of age. Production of IL-4, IL-10, IL-12, IL-13 and IFN- γ following *in vitro* activation with ovalbumin, birch, cat, LPS, peptidoglycan and the control PHA will be assessed. Cytokine production by certain cells will be determined by ELISA, ELISpot (18), real-time PCR and flow cytometry with CBA (cytometric bead array) technique allowing for quantitative analysis of several cytokines in parallel and in small sample volumes. Phenotypic characterisation of isolated cells will be performed by immunocytochemistry and by flow cytometry. The ability by antigen presenting cells (APC) to present antigen/allergen to autolog peripher blood mononuclear (PBMC)/T-cells, including Treg, will be measured by thymidine incorporation-/proliferation test.

Placenta: The immunological interplay between mother and foetus takes place through the placenta. Trophoblasts are important for inherited native immunity in the placenta during pregnancy (19). IgE+ macrophages are located behind the trophoblast barrier in the foetal part of the placenta, independently of whether the mother is atopic or not (20), and TLR-2 and TLR-4 are highly expressed by the placental trophoblasts in full term pregnant healthy women (20). In addition we have found that LPS-receptor CD14 in neonates correlated to maternal atopy, however, contradictory to other studies, not to atopy in the child (20).

Biopsy specimens from placenta will be analysed by immuno-histochemistry regarding occurrence of IgE, TLR, APC, and other cells/cell receptors of importance for native and specific immune defence (20). For functional studies, explants will be cultured *in vitro* in presence of certain microbial stimuli and allergen. Both frozen and fresh tissue will be used for real-time PCR analysis of mRNA expression of genes under study (20). In order to investigate allergen transportation cross placenta-barrier, *in vitro* models for placenta passage are set up (21), and passage of IgE/IgG-immune complex will be studied by confocal microscopy following isolating and culturing trophoblasts into a monolayer (22), in which transport of FITC-labelled immune complex will be studied. The results will be correlated to IgE-levels, life style and allergic disease in mother and child.

Skin samples: Antimicrobial peptides, also occurring in amnion fluid, vernix and human milk, are involved in local defence against microbes on skin and mucous surfaces, by activating antigen-presenting dendritic cells via Toll-Like Receptors. It is hypothesised that individuals with atopic disorders have a defective interplay between skin barrier, normal skin flora, and production of antimicrobial polypeptides. At delivery vernix from the neonate will be collected for analysis regarding antimicrobial peptides, in collaboration with Ass. Prof. Birgitta Agerberth, BMM, KI (23). The results will be correlated to occurrence of allergen-specific IgE in serum and to allergic disease.

Breast milk: Human breast milk is a complex liquid with diverse components such as, immune competent cells, antimicrobial peptides and soluble proteins that provide specific immunity and affect the maturation of the infant's immune system. Breast milk has been attributed to immuno-stimulatory or immuno-regulatory functions and is believed to protect infants from allergy development. However, some studies have raised the question whether breast-feeding in some instances can have the reverse effect, at least in subgroups of the population. Exosomes are MHC class I and II bearing nano-vesicles (30-100 nm) secreted by a diverse range of cell types and carry

immunorelevant structures, which play important roles in immune responses (24). They can be isolated in physiological fluids such as bronchoalveolar lavage (25). We have preliminary data that exosomes also can be detected in colostrum and mature milk (Gabrielsson 2006, personal message). Their role regarding allergy is still unknown. We hypothesize that exosomes in human breast milk might have a regulatory role on the infant's immune system and development of allergy.

50 mL human milk 3-6 days following delivery and at 2 months of age are collected and stored frozen until analysis for presence of cytokines, antimicrobial peptides, and exosomes, which might influence the development of the child's immune response.

Faeces: We also found that the life style factors related to the anthroposophic way of life influenced the composition of the gut flora in infants, which may contribute to the lower prevalence of atopic disease previously observed in children in anthroposophic families (10, 26).

Faecal samples will be collected at birth, 3 w, 2, 6, 12, 18 and 24 months of age. Maternal faeces will be sampled prior to and 2 months following delivery. Material will directly be frozen and later stored at -70° C for future characterisation by bacterial enumeration, bacterial typing, and by genetic technologies (TRFLP and conventional DNA-analysis) in collaboration with Janet Jansson Swedish University of Agricultural sciences (SLU), Uppsala. (26)

Psychosocial situation/stress: Psycho-neuro-endocrine-immunological research supports that psychological factors influence our immune system, also regarding allergy, e.g., the level of IgE in cord blood (27). Saliva cortisol is considered to estimate the level of stress in an individual in a valid and easy way (28).

During pregnancy, both parents will separately answer a questionnaire regarding his or her psychosocial situation (KASAM) (29). During delivery, the mother answers ten validated questions on delivery fear (30). In collaboration with Prof. Töres Theorell and Ass. Prof. Frank Lindblad, Institute for psychosocial medicine (IPM), Karolinska Institutet, saliva cortisol is assessed at 3 occasions within 24 hours in the child at 6, 12 and 24 months of age and its parents. A cotton roll kept in mouth for apx 30 secs performs the method. We have preliminary found interesting variation in saliva cortisol between, but also within, the first 50 families, in this study.

Dust samples: Analyses of dust collected from the homes of the participants regarding cat allergen and endotoxins will be performed prior to delivery ("0-sample"), and at the age of 6, and 18 months, by well-known sampling protocols. Presence of cat allergen (*Fel d 1*) in dust will be analysed in collaboration with Prof. Marianne v Hage, Dep. Clin. Imm., Karolinska Inst. and Univ. Hospital, Solna (31). Endotoxin will be analysed, in collaboration with Ass. Prof. Lennart Larsson, Inst f MMDI, Lunds University, using two complementary methods in parallel: a chromogen, *Limulus*-based method, and a mass spectrometry-based method (32).

Power analysis and statistical processing: If 1/3 of the children in the control group develop allergic disease, the testing power would be 85 % to, with statistical significance (alpha=5%), prove a relative risk at 0.5 by children grown up in an anthroposophical environment to develop atopic disease. Detailed epidemiological study design, and management of bias and effect modifying of certain exposures, will be developed with the assistance of competence at the Institute of Environmental Medicine at Karolinska Institutet, where a co-worker, Göran Pershagen, is affiliated. Statistical processing will also be coordinated from there.

Ethical considerations: How "free" or "forced" do parents feel to participate in a project aimed to investigate causes to a disease, which has had a dramatic increase in children? What does blood sampling and skin prick test mean mentally to a child, not suffering from any disease? To our experience, previous similar investigations have shown that parents feel great satisfaction by contributing to a pressing issue. Besides, by participating they got their child examined and tested with regard to allergy or other diseases. The children seldom or never seem to feel any discomfort

during sampling, which always is performed by a practised nurse. We believe that the advantages of these studies outweigh the disadvantages by, hopefully, generating new knowledge on life style factors, which could lower the risk to become allergic. Furthermore, the families could benefit by getting the opportunity to obtain general knowledge on allergy and allergy prevention. In accordance with “biobankslagen” we will obtain permission for storing samples in case further supplementary analysis is required. In that case, a renewed application for ethical approval will be submitted.

Relevance for community and public health

Allergic disease constitutes huge problems to the individual and the community resulting in considerable expenses to medical care, morbidity and occupational rearrangements. The project is expected to supply new knowledge on the development of our immune system in relation to life style- and environmental factors, and could bring about new preventive and therapeutic measures. The anthroposophical population has been shown to offer a unique possibility to study the consequence of various life style exposures. Increased knowledge about the mechanisms behind undesired immunologic reactions is necessary to in a radical way enable prevention or early treatment of allergy, and thus improve living conditions for those affected today and tomorrow.

Gender perspective All families included in the study will be followed according to an identical standardised protocol. Allergic disease dominates in early childhood in boys, but in teen years in girls. In addition, heredity seems to affect differentially in boys and girls. Thus, statistical analyses will include not only adjustments but also stratification for gender. The research network consists of 7 male and 17 females.

Equipment and methodology needed to carry out the project are available partly at the research laboratory at Södersjukhuset including the latest version for IgE-serology, but also through well established routines for caretaking and analysis of samples at Clin. Allergy Research Unit, Dept. of Medicine, Karolinska Univ. Hospital, Solna. At Stockholm’s University we have relevant equipment, including a 3-channelFACS (fluorescence-activated cell sorter) and quantitative real time PCR. For the saliva analyses we have radio-immunologic equipment. In addition, other members of the staff have complete and relevant equipment for each analysis in question.

Research group

Sachsska Barnsjh/SÖS: Gunnar Lilja, Ass.prof.; Johan Alm, MD, PhD; Caroline Nilsson, MD, PhD-stud; Fredrik Stenius, MD, PhD-stud; Marie–Louise Klingsäter, MD, prospective PhD-stud; Natalia Ballardini, MD; Margareta Eriksson, research nurse; Monica Nordlund, BMA.

Vidarkliniken, Järna: Jackie Swartz, MD; Diane Sjögren, midwife.

Kirstens familjehälsa, Järna: Kirsten Nisted, midwife.

Clin. Allergy Research Unit. f.,Dept. of Medicine, Karolinska Univ. Hosp, Solna: Annika Scheynius, Prof.; Susanne Gabrielsson, PhD; Catharina Johansson, PhD; Erika Rindsjö, PhD-stud; Agneta Beinhoff, BMA; Anna Andersson, lab. assistant.

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Inst. f. Immunologi, Stockholms Univ.: Marita Troye-Blomberg, Prof., Yvonne Sundström, PhD-stud.

Inst. f. Psychosocial. medicine (IPM): Töres Teorell Prof. and Frank Lindblad, Ass. Prof.

Delivery ward, Södertälje sjukhus: Maria Ställberg, midwife.

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Dr Christine Edwards (Senior Lecturer in Human Nutrition, Infabio Project coordinator) Glasgow Univ., Glasgow
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National collaborators

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Marianne van Hage, Prof., MD, Department of Medicine, Karolinska Institutet and Univ. Hospital, Solna, analyses of allergen in dust samples.
Lennart Larsson, Ass. Prof., Dept. MMDI, University Lund, analyses of endotoxins in dust samples.
Annika Linde, Prof., Swedish Institute for Infectious Disease Control (SMI), Stockholm, virological analyses.
Ylva Linde, Ass. Prof., MD, Dermatology Clinic, South Stockholm General Hospital, clinical dermatology.
Nikos Papadogiannakis, Ass. Prof., MD, Dept. of Pathology, Karolinska Univ. Hospital, Huddinge, placenta pathology

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