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Making Minds Matter

CELLULAR ASPECTS OF CUTANEOUS INFLAMMATION

Clinical and *in vitro* studies of allergic contact dermatitis and allergic drug eruptions

CELLULAIRE ASPECTEN VAN ONTSTEKING IN DE HUID

Klinisch en *in vitro* onderzoek van
allergisch contacteczeem en allergische geneesmiddelen-erupties

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CONTENTS

CHAPTER 1

CUTANEOUS INFLAMMATION

1.1 Introduction

This thesis is about the application of immunological insights and techniques to improve diagnosis, treatment and follow-up of inflammatory skin diseases, like allergic contact dermatitis (ACD) and allergic drug eruptions (ADE). The cells and mediators involved in cutaneous inflammation, allergic skin reactions in particular, will be discussed below. In chapter 1.2 and 1.3, clinical, epidemiological and immunological aspects of ACD and ADE, respectively, will be discussed. Finally, the various immunological techniques used in this thesis will be summarized in chapter 1.4.

Cutaneous inflammation
Healthy, uninflamed skin, with the horny layer as the first barrier, protects the body from loss of cells, fluids and electrolytes and from penetration of harmful substances like chemicals and infectious agents. In inflammatory skin conditions, such as psoriasis, contact allergy and wound healing, this barrier is disrupted or indirectly disturbed by an inflammatory infiltrate in the epidermis. Usually, inflammation is an effective response. It is the basic reaction of living tissue to several types of injuries leading to its complete or incomplete healing. The classical signs of inflammation, irrespective of the tissue(s) involved, are rubor, calor, dolor, tumor and functio laesa, i.e. redness, heat, pain, swelling and disturbed function, respectively. However, inflammatory skin disease may become so severe and widespread that it intervenes with physiological functions of the skin, like protection against external agents, prevention of water and heat loss, and, in a broader sense, psychosocial functions of the affected individual.

In inflammatory skin diseases, allergic skin reactions included, various resident, recruited and/or recirculating cells and their mediators participate. Together they constitute the Skin Immune System (SIS) [1, 2]. The SIS includes cells of the epidermis, the dermis, the blood vessels, the lymphatics, and their mediators. The cutaneous nervous system interacts with several of these components of the SIS. The various constituents mentioned will now be looked at more closely in the following sections.

1.1.1 Cells involved in cutaneous inflammation

The cellular component of the SIS consists of keratinocytes (KC), Langerhans cells (LC), melanocytes, lymphocytes, cells from the monocyte/macrophage lineage, granulocytes, mast cells (MC) and endothelial cells (EN).

Keratinocytes
KC make up 95 percent of the epidermis and produce keratins which give the skin its strength. The innermost or basal KC actively proliferate. They differentiate and give rise to, subsequently, the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The stratum corneum is the outermost layer which consists of cornified and flattened KC and forms the prin-

ciple barrier to exogenous agents. Unlike lymphocytes, monocytes and LC, KC are sessile. They grow, differentiate and constitutively produce some cytokines, even in the absence of stimuli [3]. When stimulated, however, they produce and release a wide array of biologically active substances, such as proteolytic enzymes (e.g. acid phosphatase and proteases), growth factors, proinflammatory cytokines (e.g. interleukin(IL)-1, IL-6 and tumor necrosis factor(TNF)-α), chemokines and inhibitory substances like IL-10, prostaglandin(PG)-E_2, hydroxy-eicosatetraenoic acids (HETE), transforming growth factor(TGF)-β and α-melanocyte stimulating hormone (MSH) [1, 4-8]. On their cell membranes KC express cytokine receptors and human leukocyte antigen (HLA) class I. When exposed to interferon(IFN)-γ, KC express HLA class II, adhesion molecules (e.g. intercellular adhesion molecule (ICAM)) and B7-3, but not B7-1 or B7-2 [4, 8-10]. There is increasing evidence that such 'activated' KC can act as non-professional antigen (Ag) presenting cells (APC) and activate T cells in a direct or indirect manner via adhesion molecules (e.g. ICAM-1), cytokines (e.g. IL-1 and IL-10) and costimulatory molecules (e.g. B7-3) [11, 12]. In contrast, T cell anergy following interaction between T cells and KC has also been noted [10].

Langerhans cells

Epidermal LC are distinct tissue dendritic cells (DC). They develop from bone marrow-derived precursor cells, that are related to monocytes and circulate in the peripheral blood [13-15]. In human skin, LC represent the key APC that control the T cell response to Ag [12, 16]. They possess the characteristic Birbeck granules and express a distinct pattern of phenotypic markers, such as CD1a (a specific LC marker), b and c, CD15s (ligand for E-selectin (CD62e)), CD45, the adhesion molecules CD18 (β-chain of lymphocyte function-associated antigen(LFA)-1) and CD58 (LFA-3), high and low (CD23) affinity Fc receptors for IgE and for IgG (CD32), HLA class I and class II molecules and the cutaneous lymphocyte-associated antigen (CLA) [15]. The HLA class I and II molecules are part of the human major histocompatibility complex (MHC). This is a highly polymorphic cluster of genes that code for MHC molecules on the cell surface of APC, B cells and activated T cells (HLA class II) or all nucleated cells (HLA class I). The HLA molecules represent 'self' during presentation of (foreign) Ag to T cells (see below).

LC produce a variety of cytokines, including IL-1 and IL-6. As such, LC are optimally equipped for their task as professional APC of the skin. They take up offending Ag that has passed the stratum corneum and process it into small peptides which associate intracellularly with HLA class II. Subsequently, the LC migrate via afferent lymphatics from the skin to the paracortical area of the draining lymph node [17, 18] and differentiate into B7 expressing DC with potent costimulatory activity. The peptide-HLA complex is expressed on the cell surface, ready for recognition by the T cell receptor (TcR).

Lymphocytes

B lymphocytes are sporadically observed in either healthy or inflamed human skin. They can produce antibodies (Ab) but normally do not infiltrate the skin. T cells, on

the other hand, are present in healthy skin. Naive T cells may live for many years without dividing. They can be induced to differentiate into at least three functionally different effector T cells: T helper type 1 (Th_1), T helper type 2 (Th_2) and $CD8^+$ cytotoxic T cells. What causes differentiation into Th_1 or Th_2 cells is not fully understood. The decision may be directed by the type and density of Ag-HLA complexes, the costimulatory properties of the APC and by paracrine or hormonal effects of cytokines produced by other cells involved in the immune reaction [19]. Naive T cells leave the blood and enter the lymph nodes via interaction of L-selectin and mucin-like vascular addressins (e.g. CD34 and GlyCAM-1) in high endothelial venules. In the T cell areas, they can bind transiently to professional APC. This binding is mediated by LFA-1 and CD2 on the T cell and adhesion molecules of the immunoglobulin superfamily (e.g. ICAM-1, -2 and -3 and LFA-3) on the APC. During this period of contact the HLA class II on the APC is sampled for a specific peptide. In many cases a specific peptide is not found and the T cell separates from the APC. If the Ag is recognized in the absence of adequate costimulation, anergic T cells are induced which are unable to produce IL-2. This anergy ensures T cell tolerance to self tissue Ag. After recognition, intracellular signaling through the T cell receptor (TcR) leads to higher affinity of LFA-1 for its ligands. If the appropriate costimulatory signal to CD28 on the T cell is provided simultaneously through B7 on the same APC, the naive T cell is activated. Interactions between CD40L on the activated T cell and CD40 on the APC may sustain, enhance or prolong the presentation of B7-1 or B7-2 on the APC and thus prevent the induction of anergy and ineffective or abortive T-cell stimulation [20]. Synthesis of the T cell growth factor IL-2, and its high affinity receptor (IL-2R), is induced via increased transcription and stabilization of IL-2 mRNA. The T cell re-enters the cell cycle, starts to proliferate and its progeny differentiates into effector T cells. Upon renewed presentation and recognition of the Ag, the effector T cells will produce cytokines and initiate an inflammatory response without the need for costimulation. Due to loss of L-selectin and expresssion of very late activating antigen(VLA)-4, they no longer recirculate and as a result of increased levels of LFA-1 and CD2, they display more effective adhesion to target cells. Also, their TcR signal transduction is more efficient due to altered conformation by CD45R0 and the increased expression of CTLA-4, another receptor for B7. All effector T cells mediate their function through expression of membrane-bound (e.g. CD40 ligand, Fas ligand) or secreted (cytokines, cytotoxins) molecules. In inflammatory skin diseases, such as psoriasis, contact dermatitis, drug eruptions, (late phase) atopic dermatitis, bullous diseases, cutaneous T cell lymphoma and lichen planus, T cells are abundantly present in the skin. At such sites of cutaneous inflammation, almost all T cells bear the skin homing receptor CLA which is the major T cell ligand for the vascular adhesion molecule E-selectin on dermal postcapillary venules [21, 22]. Other lymphocytes (specific for unrelated Ag), granulocytes and macrophages are also recruited, and augment the inflammatory reaction. The prominent role of T cells in allergic contact dermatitis will be discussed in chapter 1.2.

Monocytes
Monocytes are bone marrow-derived cells which circulate in the peripheral blood until they exit and differentiate into tissue macrophages and DC [13, 14]. During their differentiation, the number and properties of their abundant membrane receptors, secretory products and phagocytic capacity change. The main functions of macrophages are ingestion and degradation or storage of foreign particles (including micro-organisms) and tissue debris. Many of their specialized degrading enzymes are stored in the lysosomes and released during inflammation. After processing, antigenic peptides are presented in the MHC class I or II groove to T cells. In addition, arachidonic acid derivatives and cytokines like IL-1, TNF and IFN-α/β are synthesized and secreted. When chronically activated, as in mycobacterial infections, they may become epitheloid cells or get incorporated into giant cells.

Granulocytes
Neutrophil granulocytes are the earliest (within a few hours) bone marrow-derived cells found at sites of inflammation. Their main function is ingestion, digestion and removal of microbes and tissue debris. The cytoplasm of the neutrophil contains numerous granules filled with degradative enzymes and other bioactive substances. Following ingestion, bactericidal H_2O_2 and O_2^- are formed from oxygen in the respiratory burst. Additionally, neutrophils can synthesize inflammatory mediators, such as platelet activating factor (PAF), 5-HETE, thromboxanes and leukotrienes (LT). They express membrane receptors for a.o. Fcγ (the constant part of IgG), complement and cytokines.

Eosinophil granulocytes are also phagocytic but only weakly bactericidal. Their numbers are increased in normal wound healing, allergic conditions associated with high IgE, parasitic infestations and some neoplasias. Their differentiation and activation is mediated by a.o. IL-5 and they are responsive to chemotactic factors, PAF, $LTB_{4,}$, PGD_2, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Their membranes contain receptors for IgE, complement, cytokines and glucocorticoids. In addition, eosinophils produce a multitude of inflammatory mediators that contribute to the micromilieu for local and infiltrating cells, such as cytokines, eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN), several degradative enzymes, LT and PAF.

Basophil granulocytes contribute to wound healing, participate in delayed hypersensitivity reactions and are increased in atopic individuals. They express receptors for IgE (FcεRI) and can be activated by specific Ag, anti-IgE, complement and eosinophil MBP.

Mast cells
MC participate in wound healing, acute inflammatory skin diseases (a.o. allergy) and chronic conditions like cutaneous systemic mastocytosis. They express a.o. FcεRI and receptors for neuropeptides and cytokines [23, 24] and contain more preformed inflammatory mediators than basophils. Activated MC synthesize and

release TNF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, PG, LT and GM-CSF [25]. MC are a heterogenous group of cells. On the basis of their neutral serine protease content, human MC can be divided into two phenotypes: (1) the 'connective-tissue-type' MC, which is tryptase and chymase positive (MC_{TC}), is present in human dermis and contains IL-4 and little IL-5 or IL-6, and (2) its counterpart, the tryptase positive, chymase negative 'mucosal-type' MC which contains IL-4, IL-5 and IL-6 and generally is not present in skin.

Endothelial cells
EN, especially of the postcapillary venules, play a critical role in controlling adhesion and subsequent extravasation of leukocytes. In a multistep process, circulating leukocytes decelerate, become activated, adhere to, and finally pass the EN layer [1]. This process is mediated by a number of adhesion molecules on EN, such as E-selectin, P-selectin, ICAM-1, ICAM-2, and vascular cell adhesion molecule(VCAM)-1, and soluble adhesion molecules like sE-selectin and sICAM-1, that interact with counter adhesion molecules like CLA, LFA-1 and VLA-4 on granulocytes, monocytes and T cells. The density of the adhesion molecules is upregulated by inflammatory mediators such as IL-1, TNF-α and IFN-γ.

1.1.2 Cytokines involved in cutaneous inflammation

Cytokines are small, soluble polypeptide molecules that are produced by virtually every cell type in response to exogenous stimuli. They interact with specific receptors to mediate intercellular or intracellular communication. Being active in picomolar concentrations, cytokines regulate the amplitude and duration of inflammation by acting locally on nearby cells (paracrine actions), on the same cells that secreted them (autocrine) and occasionally via the circulation on distant target cells (endocrine). Cytokines may display both pleiotropy (one cytokine induces several different biological effects) and redundancy (different cytokines induce similar biological effects). The biological response to cytokines reflects a balance between their production, their half-life (decay), the expression of cytokine receptors on the target cells, and the influence of natural inhibitors. Epidermal cells (EC) are capable of producing a wide array of mutually interacting cytokines.

In Table I, epidermal cytokines which are important in cutaneous inflammation [1, 5, 6, 26-29] are grouped into proinflammatory, chemotactic and immunomodulatory cytokines and discussed below.

Proinflammatory cytokines
The so-called proinflammatory cytokines include IL-1, IL-6 and TNF-α. IL-1 was originally denoted by different names such as lymphocyte activating factor (LAF) and EC-derived thymocyte activating factor (ETAF). In human skin it is produced by macrophages, KC, EN and fibroblasts (FB). Different isoforms of IL-1, termed IL-1α, IL-1β, IL-1γ and IL-1ra have been identified. While IL-1α is biologically active, IL-1β is not until it is proteolytically processed by IL-1β converting enzyme (ICE). In contrast to monocytes and macrophages, which mainly produce IL-1β,

Table I. Three classes of cytokines important in cutaneous inflammation.

Cytokine	Stimulus	Producer	Target	Effect
I. Proinflammatory				
IL-1	LPS, PMA, UV, injury, bacterial products, heat shock proteins, cytokines	Mφ, KC, EN, FB	KC LC T B NK Mφ FB EN	prol, IL-1, IL-6, IL-8, ICAM diff act, IL-2(R), IL-4, IFN-γ act act act, IL-1, IL-6, TNF-α prol, IL-6, ICAM-1 ICAM-1
IL-6	LPS, PMA, UV, injury, cytokines, T_{act}	KC, FB, Mφ	KC T B NK Mφ	prol act, prol act, prol, diff, Ig-prod act act
TNF-α	LPS, TPA, UV, uroshiol, T_{act}	Mφ, KC, T	KC LC FB EN	cytostatic, ICAM-1 diff prol ICAM-1, ELAM-1, VCAM-1
II. Chemotactic				
IL-8	IL-1, TNF-α	KC, FB, EN	T, Neutro	chem
Gro-α	IL-1, TNF-α	KC, FB, EN	T, Neutro	chem
IP-10	IFN-γ	KC, EN	T, Mo	chem

Table I. Three classes of cytokines important in cutaneous inflammation (continued).

Cytokine	Stimulus	Producer	Target	Effect
III. Immunomodulatory				
IL-2	CD28-B7 interaction	Th_1, CTL	KC T B NK	? act, prol, IL-2R prol prol
IL-4	T_{act}	Th_2, MC_{TC}	KC T B Mφ	IL-6, prol prol, surv prol, act, IgE, MHC act, MHC
IFN-γ	T_{act}	Th_1, CTL	KC LC T B NK Mφ	ICAM-1, HLA-DR, IP-10 MHC prol, diff diff, IgG_{2a} act act, MHC

act, activation; B, B cell; chem, chemotaxis; diff, differentiation; ELAM, endothelial leukocyte adhesion molecule; EN, endothelial cell; FB, fibroblast; ICAM, intercellular adhesion molecule; IP-10, IFN-γ-inducable protein 10; IFN, interferon; Ig, immunoglobulin; IL, interleukin; KC, keratinocyte; LC, Langerhans cell; LPS, lipopolysaccharide; MC_{TC}, tryptase and chymase positive mast cell; Mφ, macrophage; MHC, major histocompatibility complex; Mo, monocyte; NK, natural killer cell; Neutro, neutrophil; PMA, phorbol myristate acetate; prod, production; prol, proliferation; R, receptor; surv, survival; T, T cell; T_{act}, T cell activation; Th, T helper; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; UV, ultraviolet; VCAM, vascular cell adhesion molecule.

KC predominantly produce IL-1α which they release after injury. They are capable of producing IL-1β, but do not use classical ICE to process it into the biologically active form. Released pro-IL-1β may be processed by infiltrating inflammatory cells. Also, some evidence for activation of human epidermal IL-1β by proteinases other than ICE has recently been reported [30]. The multiple functions of IL-1 (see Table I) support the notion of its central role in inflammation and immunity [5, 27, 31].

IL-6, another multifunctional proinflammatory cytokine, is also released by monocytes, FB, EN and KC (see Table I). After stimulation, KC produce several biologically active IL-6 isoforms. IL-6 participates in both physiological and pathological cutaneous processes as part of a cytokine network which consists of IL-1, TNF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), IFN-γ, TGF-α and TGF-β. In addition, locally produced IL-6 can diffuse into the peripheral circulation, and mediate systemic features of the acute phase response [5].

TNF comprises two forms with similar biological activity: TNF-α, originally named cachectin, and TNF-β or lymphotoxin. TNF acts on 2 receptors, i.e. a 55 kDa TNFRI and a 75 kDa TNFRII. TNF-α is produced by macrophages, activated T cells, MC and KC. Together with IL-1, it may indirectly (via induction of B7-1/B7-2 on APC) and directly enhance the clonal expansion of Ag-stimulated T cells [32]. Environmental stimuli, like UV-B radiation and contact allergens, may induce TNF-α production by KC and consequently increased ICAM-1 and VCAM-1 expression on KC and EN [5, 7].

Chemokines

The family of chemotactic cytokines, or chemokines, are partly responsible for the disease-characteristic tissue infiltrate composition and T cell accumulation seen in chronic skin diseases. Members of the C-X-C type subfamily (α chemokines), like IL-8, Gro-α and IP-10, are selectively chemotactic for T cells and neutrophils but not for eosinophils, basophils or monocytes. IL-8 and Gro-α are produced in the skin by KC, FB and EN after stimulation with IL-1α, IL-1β and TNF-α and regulate recruitment of T cells and neutrophils to the skin. Like IL-1α, IL-8 is probably preformed and released in diseased skin. IP-10 is expressed in KC after stimulation with IFN-γ. Members of the C-C type subfamily (β chemokines) attract monocytes, certain T cell subsets, eosinophils and basophils, but not neutrophils. They include monocyte-chemotactic and activating factor (MCP-1/MCAF), the factor Regulated And Normal T cell Expressed and Secreted (RANTES) and macrophage inflammatory protein(MIP)-1α and β. Little is known about the role of C-C chemokines in the skin, but RANTES may play an important role in delayed type hypersensitivity (DTH) reactions [1, 6].

Immunomodulatory cytokines

Typical immunomodulatory cytokines include IL-2, IL-4 and IFN-γ. IL-2 plays a key role in T cell activation and T and B cell proliferation. Both IL-2 and its receptor (IL-2R) are synthesized by activated T cells after Ag presentation. Ligands binding to the surface receptor CD2 can also induce IL-2 production.

IL-4 is a Th_2 cytokine, but it is also produced by MC. It enhances T cell proliferation and induces class II MHC expression and the IgG to IgE isotype switch of B cells. IL-4 may directly induce proliferation of KC, independent of IL-6 [33], suppresses Th_1-mediated immune responses and is an important down-regulator of skin inflammation [34].

IFN-γ is produced by Th_1 cells. It induces activation of macrophages. IFN-γ also induces or upregulates class II MHC expression on macrophages, LC and KC, and the production of IP-10 and expression of ICAM-1 by KC.

Besides the classical proinflammatory and immunomodulatory cytokines mentioned in Table I and described above, in recent years the importance of IL-10 and IL-12 as additional immunomodulatory cytokines has become clear. Both cytokines may be produced by a.o. activated KC and play a critical role in the polarization of immune reactions towards Th_2 or Th_1 responses [1, 19].

Other mediators of inflammation
In addition to the cytokines and chemokines mentioned, the humoral component of the SIS comprises several other mediators of inflammation, such as antimicrobial peptides, complement, immunoglobulins, eicosanoids and neuropeptides. The enzymes acid phosphatase and cathepsins are released by epidermal cells and contribute further to inflammation. Neuropeptides like calcitonin gene-related protein (CGRP), substance P (SP), neurokinin A (NKA) and somatostatin (SOM) are thought to mediate the complex interplay between the cutaneous nervous system and several components of the SIS. For instance, intradermal injection of SP is known to induce wheal and flare reactions [35] and CGRP has been found to inhibit the induction of contact hypersensitivity in mice [36].

Other inflammatory mediators that derive from the cells of damaged tissue, infiltrating leukocytes or plasma, such as histamine, PG, LT, PAF, and acute phase proteins are beyond the scope of this thesis and will not be discussed here.

1.1.3 Allergic skin reactions

Allergic skin reactions are inflammatory skin conditions initially caused by a specific immune response to an Ag. Normally, the adaptive immune response against infectious agents is beneficial. However, adaptive immune responses may also be directed against harmless Ag that are not associated with infectious agents, causing serious disease like allergy, autoimmunity or tissue rejection. Coombs and Gell have introduced a classification in which the immune responses are divided into four main reaction types depending on the Ag and mechanism involved [37]. Many skin reactions are good examples of these immune reactions at work: for example, type I reactions in urticaria and angioedema, type II reactions in pemphigus and pemphigoid, type III reactions in the Arthus reaction and vasculitis, and type IV reactions in allergic contact dermatitis and granulomatous reactions in tuberculosis, leprosy, leishmaniasis or due to zirconium and beryllium. Although some re-

sponses cannot be readily classified into a single reaction type, the classification is very useful for didactic and mechanistic purposes. The allergic reaction types I-IV will now be looked at more closely.

Type I or immediate allergic reactions are IgE-mediated responses. They can be detected by intracutaneous skin tests and *in vitro* assays like the radioallergosorbent test (RAST) and the basophil degranulation test. The reaction is triggered by an allergen that crosslinks two IgE Ab bound to FcεR1 on the same MC, leading to its activation and consequently the release of preformed histamine. Later on, LT, IL-3, IL-4, IL-5 and TNF-α are also produced, leading to an influx of monocytes, T cells and eosinophils. These cells dominate the so-called late-phase reaction. The clinical signs and symptoms of a type I reaction are dependent on the dose of the allergen and its route of administration. If given systemically, or even locally in the case of a potent allergen and a highly sensitized individual, connective tissue MC throughout the body may become activated. As a result, histamine and other mediators are systemically released causing systemic anaphylaxis. Insect venoms and drugs may cause this type of reaction. Local administration of small amounts of allergen in the skin, as done in intracutaneous skin tests, induces activation of local MC resulting in a wheal and flare reaction. Inhalation of allergen may induce activation of local mucosal MC causing allergic rhinitis or allergic asthma. Finally, ingestion of allergen may cause activation of local mucosal MC and deep skin connective tissue MC leading to vomiting, diarrhea and urticaria.

Type II allergic reactions are mediated by IgG or IgM Ab. These Ab bind to cell-surface or matrix-associated Ag (for example a drug), leading to activation of complement and FcR^+ accessory cells, and lysis of the Ag-modified cell. Erythrocytes and thrombocytes are particularly vulnerable to this type of response. Associated clinical syndromes are hemolytic anemia and thrombocytopenia, but also pemphigus and pemphigoid.

Type III allergic reactions are caused by deposition of Ag-Ab immune complexes in many tissues. The Ag to Ab ratio controls the size and precipitation of the complexes. The dose and route of Ag administration determine the pathogenic potential. Systemically delivered drugs may induce serum-sickness-like reactions. Local injection into the skin may result in an Arthus reaction. Vasculitis allergica and extrinsic allergic alveolitis, or hypersensitivity pneumonitis due to certain inhaled Ag, are examples of type III reactions.

Type IV or DTH reactions are mediated by specifically sensitized T cells. This reaction type is described in greater detail in chapter 1.2 and in chapter 2 on allergic contact dermatitis.

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1.2 Allergic contact dermatitis

1.2.1 Clinical and epidemiological aspects

Introduction

Contact dermatitis (CD) is an inflammatory skin disease that may occur after repeated contact with a broad array of chemical compounds. It is a world-wide problem and a major cause of occupational disease, interfering with work, household and recreational activities [1-5].

Based on etiology, two different types of CD can be distinguished, i.e. irritant and allergic CD. Morphologically, they are very similar. The main difference is that allergic CD (ACD) is mediated by specifically sensitized T cells, whereas irritant CD (ICD) is not. ACD will be the focus of this chapter. It is a classic type IV reaction and clinically characterized by erythema, induration, papules, vesicles and itching. Additionally, fissuring and lichenification may develop in chronic cases. The hands are most commonly affected and the symptoms usually remain restricted to the area of contact. However, secondary lesions may develop elsewhere. Histologically, ACD is characterized by a predominantly mononuclear infiltrate and spongiosis of the epidermis.

The incapacitating ability of ACD and the constantly increasing number of potential contact allergens are important motives for intense research into its immunological background and for searching new strategies to counteract or prevent this unwanted immune response.

Prevalence

Incidence figures for CD among the general population are grossly lacking. The majority of the available studies are cross-sectional and among patient populations of dermatology clinics and thus provide only estimates of the prevalence. The populations studied often differ in many respects. Differences in the study period, the distribution of age and gender, the localization of the eczema, occupational and environmental exposure and the type of allergen make comparison of those studies extremely difficult [1]. Reliable comparison is complicated even more by the use of different diagnostic criteria for contact dermatitis, which vary from self-administered questionnaires or routine examination to full medical and exposure history, including clinical examination and patch testing. With these limitations in mind, estimates of the prevalence of contact dermatitis in the general population range between 1% and 10% [1, 5], with a preponderance of ICD. However, in many dermatology clinics the frequency of ACD is often higher than that of ICD. This may be explained by the more severe and persisting symptoms of ACD and consequently a tendency to seek medical care more often.

The likelihood of ACD is tightly correlated with the presence or absence of certain risk factors [1, 2]. Several of such possible risk factors for ACD are shown in Table I, grouped into patient, environment, and allergen-related factors.

Table I. Possible risk factors for allergic contact dermatitis.

Patient (susceptibility)	Environment (exposure)	Allergen (sensitizing potential)
- age	- sensitizers / irritants	- chemical reactivity
- gender	- concentration; frequency	- vehicle / alloy
- genetic predisposition / atopy?	- site of exposure	- cross-reactivity
- pre-existent skin injury (e.g. ear-piercing, dryness)	- low humidity	
	- high temperature	
	- occlusion	
	- sweating	

In most studies, the prevalence of ACD among women is approximately twice as high as among men [1, 6, 7]. The highest prevalence of ACD is seen among young women, between 20 and 30 years of age [1, 2]. In men, no clear age trend is seen.

As ear piercing and wearing of inexpensive jewelry have become very popular, the prevalence of nickel sensitivity is increasing, especially among schoolgirls [6, 8, 9]. Sweat and especially plasma were found to be very effective in releasing nickel from jewelry [10]. Among Norwegian school children between 7 and 12 years of age, 17% had proven contact allergy to one or more metals. Among these allergies, nickel was the most common allergen (86%). Girls were affected twice as frequent as boys (10% and 4.5%, respectively). Also, nickel sensitivity in girls with pierced ears was 2 times more frequent than in those without, and increased with the number of holes in the earlobes. Positive metal tests among atopic girls were 2 times more frequent than among non-atopics [6]. The poor barrier function of atopic skin may explain the higher incidence of ACD in atopic individuals. However, the role of atopy in the development of ACD is still controversial as lower sensitization rates among atopics have also been reported [1]. Possible explanations for the latter observation could be (1) a change in the immunological reactivity favoring a T helper (Th) type 2 reaction over a Th type 1 reaction, or (2) a behavioral change leading to effective avoidance of nickel exposure.

High occupational exposure occurs in wet work, like household duties, hairdressing / cosmetics, the cleaning industry, food industry and health services, but also in dry work, like the rubber, chemical, metal and construction industry. Frequent and prolonged exposure to water, soaps, perfumes, dyes, bleaches or other aggressive chemicals and metal equipment facilitate the development of both irritant and allergic contact dermatitis. Among female cleaners [4] and hairdressers [11] CD is a major problem. Sensitization to the allergens in the hairdressers' series, as recommended by the International Contact Dermatitis Research Group (ICDRG) and its European counterpart the EECDRG, was seen in up to 51% of European hairdressers [11].

Many risk factors are tightly interrelated. For example, certain types of 'risky' work are almost exclusively performed by young women. Gender-related differences may thus be based on differences in occupational exposure instead of susceptibility. Additionally, negative risk factors may also exist. For example, early oral exposure to nickel from dental braces was found to reduce sensitization rates upon subsequent ear piercing in a retrospective study among patients attending patch test clinics [12].

The number of possible contact allergens is constantly increasing with some substances disappearing but more newly developed substances appearing. The sensitization rate of an allergen in a population is determined mainly by the combination of its inherent sensitizing potential and its frequency of occurrence or intensity of exposure. In Table II the European standard series of commonly encountered contact allergens is summarized along with some estimated sensitization rates based on European studies among suspected ACD patients [13].

Nickel, chromium and cobalt are the highest ranking sensitizers in many population-based and patient-based studies in Europe as well as in the United States [1]. Sensitivity to nickel was found in 86% of confirmed metal allergies in Norwegian school children [6]. Even supposedly inert metals, such as gold, gold salts [14, 15] and platinum, have been recognized as contact sensitizers. In chapter 2.1 the contact allergen nickel is discussed in greater detail.

Cosmetics are also a frequent cause of CD, in females as well as in males, and amount to 2-4% of dermatologic consultations [16]. The permanent wave ingredient glyceryl-monothioglycolate and hair dye ingredient p-phenylenediamine have been reported as the leading sensitizers among European hairdressers in 19% and 15%, respectively [11].

Acrylates are increasingly used in the medical, dental, industrial and domestic environment (a.o. nail varnish). Occupational contact sensitivity to acrylates has been reported in up to 13%, especially among dental laboratory technicians [3, 17, 18].

Latex is increasingly used in health care, a.o. in disposable latex protective gloves, catheters, IV tubes, and injection caps. The preventive measures related to the HIV epidemic have lead to a rise in prevalence of latex allergy up to 10 percent, which is comparable to the prevalence among the highly exposed group of operating theater personnel [3, 19-26]. Spina bifida patients are also at risk due to long-term exposure to latex containing gloves, catheters and tubes [19, 27]. Clinical manifestations range from mild delayed eczematous skin reactions to severe, even lethal, systemic reactions [19]. Most of the reactions are, however, caused by irritation or type I allergy.

Table II. European standard series and sensitization rates among suspected ACD patients.

Contact allergen	Percentage of ACD
Metals	
Nickel sulfate	13-19
Cobalt chloride	4.6-9.0
Potassium dichromate	1.4-8.1
Cosmetics	
Fragrance mix	6.4-9.4
Balsam of Peru	4.0-6.7
Isothiazolinone	0.9-8.4
Formaldehyde	1.4-5.2
p-phenylenediamine dihydrochloride	0.3-4.9
Quaternium-15	0.3-2.2
Pharmaceuticals	
Neomycin sulfate	1.3-6.9
Colophony	1.7-4.7
Wool alcohols	1.2-3.9
Benzocaine	0.4-3.3
Ethylenediamine dihydrochloride	0.6-2.7
Parabens	0.5-2.6
Quinolone mix	0.7-2.2
Rubber chemicals	
Carba mix	0.8-4.7
Thiuram mix	2.0-2.6
Mercapto mix	0.6-1.2
Black rubber mix	0.3-1.3
Miscellaneous	
p-tert-butylphenolformaldehyde resin	0.3-1.4
Epoxy resin	0.6-1.1
Primin	0.3-1.4

Modified from Wilkinson and Rycroft [13].
ACD, allergic contact dermatitis.

Diagnosis

Every suspected contact allergy should be evaluated thoroughly, starting with a detailed history and physical examination in which the previously described risk factors should be considered. However, the assessment of a causal relation between exposure to an allergen and its supposed contact allergic reaction is not a light task, especially if exposure to multiple allergens occurs. Still, precise details on the frequency and type of exposure, the type of allergen involved, the risk factors present, and the localization of the eczema are of great practical importance because they may influence prognosis, as measured by persistence of symptoms, tendency for medical consultation, sick leave and permanent disability [1, 2, 13].

Indications on the role of specific allergens in the etiology of ACD can be obtained from patch testing. For optimal comparison between different clinics, standard patch test (PT) series should be performed according to guidelines of the ICDRG [13]. As an example, the European standard series of contact allergens is shown in Table II. A positive PT for an allergen is not sufficient for the clinical diagnosis of ACD. Sensitization to one or more allergens is not exceptional in the general population and is not necessarily followed by clinically overt ACD. Therefore, the relevance of a positive PT should always be judged by the exposure history [1]. The combination of a positive exposure history and a positive PT for the same allergen is highly significant for the clinical diagnosis and relevance of ACD.

Additional evidence of sensitization to an allergen can be obtained from *in vitro* lymphocyte proliferation assays (LPA) [28, 29]. In chapter 2.2, the skin PT and the LPA are described as parameters for monitoring a hyposensitization procedure in nickel ACD patients.

Predictive testing, to assess the risk of future ACD in an individual case, is not (yet) possible.

Treatment

Satisfactory treatment of CD is often difficult. Despite improvements in diagnosis and treatment of CD, the outcome does not appear to have changed much over the past 30 years, especially in occupational CD [2].

Rigorous reduction of allergen exposure should be the primary goal in prevention of occupational dermatitis [11] since even occasional contact with the allergen in the home or working environment is sufficient to maintain the condition [1]. Examples of successfully reduced exposure are the addition of ferrous sulfate to cement which significantly reduced skin problems related to chromium exposure from cement, and the legal measures in Denmark to prevent nickel exposure from a.o. jewelry. Since damaged skin is more susceptible to developing CD, protection of the skin with gloves and creams may also be helpful. However, the wearing of gloves is not popular and may induce its own skin problems.

Emollients, topical or even systemic corticosteroids and phototherapy may also be beneficial. For some contact allergens, e.g. nickel, restriction of dietary intake may also be useful. In chapter 2.1 the presence of nickel in daily used objects and in food is discussed. In chapter 2.2 a possible hyposensitization procedure for nickel ACD is described [29].

Rigorous avoidance of exposure normally leads to resolution of the clinical signs of acute allergic or irritant CD. However, for chronic CD the outcome is less favorable. In a prospective study 1-16 years after the initial presentation, 70% of irritant hand dermatitis patients still had active disease [30]. Reported percentages for remission or significant improvement of ACD are 30% after 6 years [31] and 27% after 10 years [32].

Exposure and gender are the main predictors associated with outcome. Although more frequently affected, women may have a better outcome [2]. However, as mentioned earlier many risk factors are tightly interrelated, which may mask their individual effects.

1.2.2 Immunological aspects

In chapter 1.1, the cells and mediators involved in cutaneous inflammation and allergic skin reactions in general were discussed. In the following section, the immunopathophysiology of ACD will be discussed in greater detail.

Allergic contact dermatitis is a classical type IV or delayed type hypersensitivity (DTH) reaction. This unwanted immune response is mediated by T cells previously sensitized to one or more contact allergens. It nicely illustrates the tension that exists between protective immunity against non-self and reactivity to modified self due to reactive chemicals that may convert normally tolerated proteins into highly immunogenic substances [33].

Substances capable of inducing ACD are generally low molecular weight chemically reactive compounds, called haptens. They can penetrate the skin and bind to skin- or non-skin-related proteins to form a complete antigen (Ag), capable of inducing an immune response. Metal haptens such as nickel may also bind directly to, and modify, particular self peptides in the MHC groove [15]. Nickel as a contact allergen is discussed in chapter 2.1.

The sequence of events leading to ACD following first and second exposures to a reactive hapten can be organized into two phases, i.e. the induction or acquisition phase and the elicitation or expression phase [33]. Table III summarizes these events and some of their mediators.

In the afferent limb Ag is taken up, processed and presented in the groove of HLA class II on the surface of Ag presenting cells (APC). After migration to the regional lymph nodes, the HLA-peptide complex on the APC can be recognized by the TcR of specific T cells, leading to their activation. However, for optimal T cell activation and IL-2 production a second signal, the so-called costimulation, is needed. Multiple accessory signals may be involved in costimulation, including IL-1 and interaction of LFA-1, CD28 (and other B7 counter receptors) and CD40L on the T cell and ICAM-1, B7-1 (CD80), B7-2 (CD86) and CD40 on the APC [34]. Lack of costimulation can result in prolonged unresponsiveness of the T cell (anergy) or its death.

Table III. Events leading to ACD and some of their mediators.

Event	Mediators involved
I. Induction phase	
- Penetration of hapten and formation of complete Ag	
- Uptake and processing of Ag by LC	IL-1β, IL-12, PGE_2
- Migration of LC to regional lymph nodes	chemokines
- Activation of specific T cells via	
- Apposition of LC and T cell	ICAM-1/LFA-1, CD3/LFA-2
- Presentation of Ag-fragment	HLA class II/TcR
- Costimulation	IL-1, B7/CD28, CD40/CD40L
- Activation and clonal expansion of selected Th_1	IL-2
- Recirculation and migration of memory T cells	CLA
II. Elicitation phase	
- Penetration of hapten and formation of complete Ag	
- Recruitment and activation of specific and nonspecific $CD4^+$ and $CD8^+$ T cells and Mφ via	CLA
- expression of chemokines by LC, KC and EN	IFN-γ, IL-1, TNF-α
- expression of adhesion molecules and HLA class II by LC, KC and EN	IFN-γ, TNF-α
- Modulation of ACD by cutaneous innervation	neuropeptides
- Down-regulation of ACD by regulatory cytokines	IL-4, IL-10

ACD, allergic contact dermatitis; Ag, antigen; CLA, cutaneous lymphocyte-associated antigen; EN, endothelial cell; HLA, human leukocyte antigen; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; KC, keratinocyte; LC, Langerhans cell; LFA, lymphocyte-associated antigen; Mφ, macrophage; PGE, prostaglandin E; TcR, T cell receptor; Th, T helper; TNF, tumor necrosis factor.

In the efferent limb the previously sensitized Ag-specific $CD4^+$ T helper type 1 and $CD8^+$ cytotoxic T cells migrate to the skin where they may get activated upon renewed Ag presentation. Cutaneous lymphocyte-associated antigen (CLA) may play a prominent role in this homing behavior [35]. Release of cytokines and chemokines like IFN-γ, TNF-β, IL-3, GM-CSF, MIP and MCP/MCAF leads to recruitment and activation of other T cells, specific for unrelated Ag, and macrophages which amplify the ACD response.

It has been shown that the skin is essential in the development of ACD. Therefore, many of its intrinsic cell types, cytokines and other cellular constituents have been studied extensively for their roles in the induction, elicitation and down-modulation of ACD. Most of these cells and mediators have already been discussed in chapter 1.1. In addition, application of allergen to the epidermis was found to cause an up-regulation of HLA class II on epidermal LC [36, 37], an allergen-specific cytokine pattern consisting of early epidermal LC-produced IL-1β and KC-produced IL-1α

and IP-10, and non-specifically induced cytokines like KC-produced TNF-α and T cell derived IFN-γ [38, 39]. Administration of the Th_1-promoting cytokine IL-12 during hapten sensitization was found to induce effector $CD4^+$ T cells that produced IFN-γ, but not IL-4 in response to T cell receptor-mediated stimulation [40]. On the other hand, KC-derived IL-10, which is specifically induced by contact allergens, inhibits the APC function of LC via inhibition of costimulatory signals and thereby enables counter-regulation of ACD and even tolerance induction [39, 41]. In addition, KC can also activate T cells as non-professional APC, thereby inducing T cell anergy due to the absence of proper costimulatory signals [42, 43].

In chapter 2, allergic contact dermatitis due to nickel will be described in greater detail.

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1.3 Allergic drug eruptions

1.3.1 Clinical and epidemiological aspects

Introduction

Allergic drug reactions are a small but important part of the broad range of adverse drug reactions (ADR). An accepted definition of ADR is: any undesired and unintended response that occurs at certain doses of a drug given for therapeutic, diagnostic or prophylactic benefit of the patient [1]. An ADR can be considered allergic if it is mediated by an antigen(Ag)-dependent immunological mechanism, i.e. via antibodies (Ab) or T cells. Although 'hypersensitivity' is widely used as a synonym, we prefer the term 'allergy' as it indicates an underlying immunological mechanism, whereas 'hypersensitivity' does not. Table I shows drug allergy in the context of a classification of all ADR based on the predictability and dose-dependency of the reaction.

Table I. Classification of adverse drug reactions.

Predictable (dose-dependent / in normal patients)	Unpredictable (dose-independent / in susceptible patients)
- overdose / toxicity	- intolerance
- side effects	- idiosyncrasy
- secondary / indirect effects	- **allergy**
- drug interactions	- pseudoallergy

Modified from DeSwarte [1].

The two main categories of ADR are predictable reactions, which are usually dose-dependent and occur in otherwise healthy patients, and unpredictable reactions, which are often dose-independent and limited to a small group of susceptible patients. Among the predictable reactions, side effects are the most common accounting for up to 80% of ADR.

Unpredictable ADR comprise intolerance, idiosyncrasy, allergy and pseudoallergy. The term intolerance is used for quantitatively increased, pharmacologically characteristic effects of a drug, often at unusually small doses. Idiosyncrasy is reserved for qualitatively abnormal responses to a drug, unrelated to its pharmacological actions and not the result of an immune response. Pseudoallergy is used for qualitatively abnormal reactions to a drug that may resemble an allergic ADR in any aspect. Especially type I allergic reactions (Coombs and Gell) are often mimicked but the main difference from true allergy is that interaction of specific Ab or sensitized T cells with the drug is not involved. Likewise, a sensitization period is absent and skin tests are not helpful. Finally, drug allergy, which comprises about 5 to 10% of ADR, will now be looked at more closely.

Allergic ADR may or may not follow the well-known allergic reaction types originally described by Coombs and Gell [2], which are reviewed in section 1.3.2. Actually, drug allergy may mimic many diseases that range from mild to even fatal conditions. The majority of allergic ADR, however, involve the skin [1] and are consequently also termed allergic drug eruptions. They may be accompanied by fever, hematological, hepatic, renal, musculoskeletal, cardiorespiratory, autoimmune or other systemic manifestations. The variety of dermatological appearances includes exanthematous and eczematous eruptions, urticaria and angioedema, (photo)allergic contact dermatitis, fixed drug eruptions, exfoliative dermatitis, Stevens-Johnson syndrome and toxic epidermal necrolysis (TEN) [1, 3-5].

Prevalence

Data on drug eruptions are voluntarily reported and recorded in Europe [6]. Similar reporting and registration systems are maintained elsewhere, e.g. by the Food and Drug Administration in the United States, and are vital for detection, notification, and epidemiological purposes [7-9]. Nonetheless, systematic studies on the incidence and prevalence of allergic ADR in a general population are grossly lacking [3]. Currently available studies are often difficult to compare as they differ with respect to the drugs and populations studied, and the criteria used for the diagnosis of drug allergy. Additionally, these studies are likely to be biased due to the voluntary reporting system used.

Reported frequencies of ADR (allergic and nonallergic) range from 0.3% to 30% per drug treatment [10]. Allergic ADR are estimated to account for 5-10% of all observed ADR [5, 10]. For most common drugs, however, the risk of an allergic reaction is between 1% and 3% [1]. Reported frequencies of hospitalization due to ADR vary significantly from 0.2% to 29%. The frequency of drug-attributed deaths is estimated at 0.1-0.01% of inpatients [1].

The likelihood of an allergic ADR is tightly correlated with the presence or absence of certain risk factors [1, 4, 5, 10]. A number of risk factors that have been identified for drug allergy are shown in Table II and grouped into patient, treatment, and drug-related factors.

Age is an example of a patient-related risk factor: the incidence of drug allergy in children and the aged is lower than that in adults. A familial predisposition to drug allergy has also been noted but the genetic factors involved have not yet been elucidated [1, 5]. Whether atopy represents a risk factor for allergic ADR is still controversial [1, 3, 11]. Previous allergic, and possibly even nonallergic ADR, are also a risk for the development of new ADR.

Treatment-related factors include dose and duration of treatment and concomitant use of multiple drugs [10]. High dose and long-term drug therapy increase exposure and therefore the risk of allergic ADR.

Table II. Risk factors for allergic adverse drug reactions.

Patient (susceptibility)	Treatment (exposure)	Drug (or metabolite)
- age	- current drug exposure (dose; frequency; duration)	- molecular size
- gender	- previous exposure	- chemical reactivity
- genetic predisposition / atopy?	- route of administration	- cross-reactivity
- previous (non)allergic ADR	- polypharmacy	
- concurrent disease		

The route of drug administration is another risk-determining factor. For example, topical application of some drugs (e.g. antibiotics and antihistamines) may induce allergic contact dermatitis, and oral therapy is considerably less likely to induce severe reactions, such as anaphylaxis, than parenteral administration [5].

Chemical reactivity is a crucial drug-related risk factor, since it determines the likelihood that a drug will bind to a carrier molecule, turning it into a multivalent complete allergen [4]. Certain drugs induce allergic ADR more frequently than others. Commonly used allergenic drug groups are antibiotics (including penicillins, cephalosporins and sulfonamides), anti-epileptic drugs (AED), anesthetics (including muscle relaxants and thiopental), hormones (a.o. insulin), immunoglobulins and vaccines [4, 5]. In an interview-based Canadian study among patients scheduled for surgery, antibiotics (50%), opioids (27%), non-steroidal anti-inflammatory drugs (NSAID) (10%) and sedatives (5%) were found to account for more than 90% of reported drug allergies [10].

The large influence of risk factors and diagnostic criteria on the reported frequency of drug allergy is illustrated by the following data on penicillin allergy. The incidence of penicillin 'allergy' as reported by patients was found seven times higher (7.8%) than the actual figure (1.1%) as judged from a detailed history in a British study among hospital patients [12]. Among patients on long-term antibiotic treatment for cystic fibrosis, allergy to beta-lactam antibiotics, as defined by drug-induced fever and rash, was seen in 29% [13]. In another study, penicillin allergy, as determined by skin tests and radioallergosorbent test (RAST), was found in 19.4% of patients with a history of a cutaneous reaction following penicillin administration [14]. Among patients with a previous history of penicillin allergy, positive skin tests with major determinant benzylpenicilloyl polylysine and a minor-determinant mixture were seen in 7.1%, as opposed to 1.7% of subjects negative by history. Acute allergic reactions upon subsequent penicillin administration to individuals with negative skin tests were seen in 2.9% of subjects positive by history as opposed to 0.5% of patients negative by history [11].

The incidence of anaphylactic shock, the most severe type of allergic ADR, is estimated at 1 in 1,000-10,000 treatments. Its main symptom is a drop in blood pressure immediately or within 1 hour after administration of the drug. Penicillin is

its main elicitor, but preparations used for hyposensitization and blood or plasma-derived components may also provoke this type of reaction. Fatal cases of severe generalized anaphylaxis due to penicillin are estimated at 1 in 50.000-100.000. Over half of these occur in patients with no previous history of penicillin allergy [1, 3, 5]. Nonimmediate reactions, as evidenced by a positive delayed response to direct challenge with penicillin intramuscularly and orally, were seen in 7.6% of patients with a history of a cutaneous reaction following penicillin administration. Immunological mechanisms were demonstrated by a positive delayed skin test in 65% of these patients [14].

AED also frequently cause ADR, including allergy. They will be discussed in depth in chapter 3.

Pseudoallergic reactions may mimick true drug allergy in any aspect, sometimes posing serious differential diagnostic difficulties. In the next sections, pseudoallergic reactions to NSAID and iodinated contrast media are discussed.

Pseudoallergic ADR to mild analgesics or NSAID were seen in less than 1% in a Swiss survey of over 19,000 exposed patients [7]. Pseudoallergic bronchoconstrictive reactions and urticaria to aspirin and other NSAID occur especially in asthmatics [4, 5]. The frequency of such aspirin 'intolerance' increases with age and is slightly higher in females [3]. The underlying mechanism is not entirely clear but direct non-specific release of histamine from basophils or mast cells, and inhibition of cyclooxygenase have been suggested as possible mechanisms [1, 4].

Iodinated contrast media may also induce pseudoallergic or anaphylactoid reactions. These reactions are usually mild and self-limited, but serious reactions do occur in about 0.1% of radiocontrast procedures [4]. Nonionic contrast material has been shown to induce fewer and less severe adverse reactions. As such, they are a suitable alternative for patients with previous serious or life-threatening reactions to the hyperosmolar iodinated contrast materials [15, 16].

Diagnosis

Drug allergy is often suspected but rarely properly analyzed, proven and documented. The patients statement of being allergic is often accepted without further investigation [10, 12]. Also, the assessment of causality between a drug and its supposed allergic reaction is not a light task, especially if multiple drugs are prescribed. Discrimination between allergic and other ADR, however, is essential since it influences future drug administration. Incorrect labeling of a patient as allergic may lead to substitution of beneficial medication for other possibly less effective more dangerous and/or more expensive drugs [10]. Therefore, suspected allergic ADR should be evaluated thoroughly, starting with a detailed history and physical examination in which the previously described risk factors should be taken into consideration.

In Table III the variety of drug induced cutaneous manifestations are classified with respect to the likeliness of an underlying allergic mechanism [1].

Table III. Cutaneous manifestations of drug allergy.

I. Likely allergic
- exanthema
- urticaria / angioedema
- contact dermatitis* and photosensitivity
- fixed drug eruption
- erythema multiforme
- exfoliative dermatitis
- vasculitis
- toxic epidermal necrolysis (TEN)
- erythema nodosum

II. Possibly allergic
- eczematous eruptions
- vesiculobullous reactions
- lichen planus
- pityriasis rosea

III. Not allergic
- acneiform eruptions
- pigmentary changes
- alopecia
- tumors

*allergic contact dermatitis is discussed in chapter 1.2.

Additional clinical and laboratory criteria suggestive of drug allergy are summarized in Table IV. These clinical criteria are often insufficient for a clear distinction between allergic and non-allergic ADR. However, such distinction is highly relevant as it influences future drug administration. Therefore, additional tests are often needed. Predictive tests, to assess the risk of future drug allergy in an individual case, are not (yet) available, despite many attempts [1, 17]. Several diagnostic tests have been suggested. However, the lack of a 'golden standard' for the diagnosis of allergic ADR seriously hampers the proper validation of such tests. Rechallenge is the most definite test but it may be hazardous and a life-threatening anaphylactic reaction can be the result. It should therefore be restricted to cases in which no alternatives exist and may only be executed after informed consent, in an intensive care setting, and by experienced physicians [5].

Skin tests, like intradermal and patch tests (PT), and *in vitro* tests, like the RAST, the enzyme-linked immunosorbent assay (ELISA), the basophil degranulation test, and the lymphocyte proliferation assay (LPA), have all been practiced. However, the suitability of these tests is highly dependent on the drug involved. In addition, many tests are time critical and may be disturbed by concurrent symptomatic drug therapy, like antihistamines and corticosteroids. Proper positive and negative controls should therefore always be included.

Table IV. Clinical and laboratory criteria suggestive of drug allergy.

- occurrence in minority of (susceptible) patients
- sensitization period > 1 week (no prior exposure)
- manifestations unrelated to pharmacological actions of the drug
- manifestations matching known allergic reaction types (e.g. anaphylaxis, urticaria, serum sickness-like reactions)
- blood / tissue eosinophilia
- demonstration of drug-specific antibodies or sensitized T cells
- disappearance within several days after discontinuation
- reproduction by minute doses of the same or immunochemically related drugs

Modified from DeSwarte [1].

A suitable diagnostic test for allergic ADR should be quick, inexpensive, able to demonstrate either specific Ab or sensitized T cells (which may be the only difference between a true allergic and a pseudoallergic ADR), and above all safe. *In vitro* tests are likely to be the safest alternatives to rechallenge. Skin tests seem quite safe as generally mild self-limited adverse reactions to skin tests with major and minor determinants of penicillin were seen in only 1.2% of patients with a positive history. The safety could be increased even more by doing preliminary scratch tests before intradermal tests [11].

Demonstration of drug-specific Ab, as was often the only analysis in the past, may be suggestive for sensitization but is not sufficient for the clinical diagnosis allergic ADR. Actually, many people have penicillin-specific IgG and IgM without developing clinically overt allergy to the drug [1, 12]. However, the concomitant presence of suggestive clinical and immunological parameters are highly significant for the diagnosis.

In chapter 3, the skin PT and the LPA are investigated for their suitability to detect allergy to the AED carbamazepine and its metabolites. A flow diagram for diagnosing anti-epileptic drug allergy is also proposed.

Treatment

For didactic purposes, treatment is discussed here after diagnosis. However, in suspected allergic ADR the offending drug should be discontinued as soon as possible, usually before extensive diagnostic exploration can be done. In most cases prompt withdrawal of the causative drug will halt the allergic response. A non-cross-reactive substitute drug may be started if needed. Symptomatic treatment with corticosteroids or antihistamines can be of help. Systemic adrenaline and/or antihistamines may be needed in more severe cases.

Table V. Classification of drug allergic reaction types according to Coombs and Gell [2].

Reaction type	Mechanism	Manifestations	Examples of drugs involved
I. Immediate	drug cross-links specific IgE on mast cells and basophils resulting in degranulation	anaphylaxis, urticaria / angioedema bronchoconstriction	penicillin, antisera, hyposensitization preparations
II. Antibody-dependent cytotoxic	antibody (IgG/IgM) binds to drug or drug-altered cell membrane resulting in complement-mediated cytotoxicity	hemolytic anemia, thrombocytopenia, leukocytopenia	penicillin, cephalosporins, quinine, α-methyldopa
III. Immune complex	drug-antibody (IgG) complexes are deposited in blood vessel walls and basement membranes leading to complement-mediated tissue damage	serum sickness (urticaria / rash, fever, arthritis, lymphadenitis), vasculitis, nephritis, hemolytic anemia, thrombocytopenia, drug-induced LE	penicillin, carbamazepine
IV. Cell-mediated, DTH	tissue damage is mediated directly by cytotoxic T cells, or indirectly via release of inflammatory cytokines by activated Th_1 cells	allergic contact dermatitis, allergic photodermatitis, hepatocellular injury	penicillin, antihistamines (also topically applied), carbamazepine

DTH, delayed type hypersensitivity; Ig, immunoglobulin; LE, lupus erythematosus; Th, T helper.

For some drugs (e.g. penicillin and insulin) rush desensitization, as used for bee or wasp allergy, has been successful. However, the procedure is not without risk and should only be undertaken if no alternative is available [4, 12]. The mechanism of action of desensitization is unclear but generation of so-called blocking Ab and gradual and subclinical mast cell degranulation and thus depletion have been suggested [5]. The restrictions described earlier for rechallenge are also valid for desensitization.

1.3.2 Immunological aspects

In chapter 1.1, the cells and mediators involved in cutaneous inflammation and allergic skin reactions in general were discussed. In the following section, the immunopathophysiology of allergic ADR will be discussed in greater detail.

Allergic ADR depend on a drug-specific immune response. However, most drugs are low molecular substances and consequently they cannot induce such an immune response on their own. The drug itself, or one of its (mostly reactive) metabolites first needs to bind covalently to carrier molecules, like proteins (e.g. albumin), glycoproteins and sometimes polysaccharides or cell membranes (e.g. erythrocytes and platelets). This need for binding to certain carrier molecules may explain why allergic reactions to some drugs remain restricted to particular tissues or organs, and why detection of allergy to some drugs may fail since such a carrier molecule is not present in *in vitro* test conditions [1].

The actual response of the immune system is dependent on the relative concentrations of multivalent drug-carrier complexes and univalent free drug or metabolite molecules. The multivalent hapten-carrier complexes, or complete Ag, are then processed (or not [18]) and presented by APC to T cells. If the Ag is recognized, proliferation and formation of effector and memory T cells or production of specific Ab by B cells follows. As of now the patient is sensitized and renewed contact with the allergen may lead to an allergic reaction. The underlying immune responses may follow one or more of the allergic reaction types I-IV as originally described by Coombs and Gell [2]. These 'prototype' allergic reactions were discussed in detail in chapter 1.1. In Table V, they are briefly summarized for drug allergy along with their mechanisms, clinical manifestations, and some examples of drugs involved.

In chapter 3, symptoms, diagnosis and treatment of allergic reactions to carbamazepine (CBZ) and other AED, are described in detail.

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1.4 Introduction to the experimental work

In this chapter the various immunological techniques used in this thesis will be put into perspective. Their purpose, procedure, possibilities and limitations will be discussed. However, only a broad outline of each procedure will be given here. For details the reader is referred to the methods section of each chapter. First, the methods used in each chapter will be briefly mentioned. After that, each method will be discussed in greater detail.

Most of the immunological techniques described below focus on T cell function. In chapter 2.2 the *patch test (PT)*, the *lymphocyte proliferation assay (LPA)* and *flow cytometric analysis* are used for monitoring immunological parameters during hyposensitization treatment of nickel allergic contact dermatitis (ACD) patients. The details of the hyposensitization procedure are given in chapter 2.2 and will be discussed in chapter 4. In chapter 2.3 *immunohistochemistry (IHC)* and *computer-assisted image analysis (IA)* are used for detection of cytokines and other markers of inflammation in inflammatory skin sections. In the chapters 3.2, 3.3 and 3.4 the *PT* and the *LPA* are validated and used to support the diagnosis of allergic adverse drug reactions (ADR) to the anti-epileptic drugs carbamazepine and its metabolites, and to bromocriptine.

Patch test
Purpose. PT are used as an aid in detecting the antigen (Ag) or drug that caused ACD or allergic ADR.
Procedure. PT chambers with the suspected contact allergens are applied to the skin of the back. After 48 hours the chambers are removed. At 48 and 72 hours, the skin reaction to each allergen is read and scored according to international standard criteria. The resulting score can be - (negative), 1+, 2+, 3+, and occasionally 4+.
Possibilities. A large number of possible contact allergens can be tested at once for their relevance. The severity of the allergy is reflected in the height of the PT score. The method is safe, quick and cheap.
Limitations. PT are not suitable for diagnosing type I, II and III allergic reactions. Reproducible and reliable application and reading of PT requires proper training. Irritant reactions, induced by some substances, can mimic allergic reactions. Negative as well as positive control subjects and Ag should be included for internal standardization. Sunbathing can make proper PT analysis less reliable due to the immunosuppressive action of ultraviolet radiation. Sporadically, the local allergic skin reaction develops into a more generalized allergic reaction or a reactivation of previously affected areas.

Lymphocyte proliferation assay
Purpose. The LPA is used to demonstrate lymphocyte reactivity to allergens in patients with suspected allergy.
Procedure. Peripheral blood mononuclear cells (PBMC, i.e. lymphocytes and monocytes) are isolated from venous blood of the patient, cultured and exposed to suspected Ag. If the Ag is recognized, T lymphocytes start proliferating. After several

days of culture the proliferation rate of exposed lymphocytes is measured and compared to that of unexposed lymphocytes.
Possibilities. The difference in proliferation rates between exposed and unexposed lymphocytes is a measure of the severity of the allergy or the degree of sensitization. As the lymphocyte exposure to Ag is done entirely *in vitro*, i.e. outside the body, the risk of a generalized allergic reaction to the test is completely absent.
Limitations. Negative as well as positive control subjects and Ag should be included for internal standardization. The number of Ag that can be tested at once is limited due to the relatively large number of PBMC that is needed per Ag. Ag need to be water soluble at the desired concentrations. Seemingly false positive results can be found as the LPA can detect lymphocyte reactivity to an Ag before clinically overt allergy manifests itself. Finally, the LPA requires extensive laboratory facilities and expertise, is labor intensive and costly.

Flow cytometric analysis
Purpose. Flow cytometric analysis is used to rapidly extract objective and reproducible qualitative and quantitative information about the presence of particular cell types, structures or products from a large number of immunofluorescently stained cells in suspension. In this thesis it is used to type PBMC as an immunological parameter during hyposensitization of nickel ACD patients.
Procedure. Cell samples are processed into suspensions and exposed to monoclonal and/or polyclonal antibodies (Ab) that were raised specifically against the Ag to be demonstrated, and directly or indirectly coupled to an immunofluorescent or otherwise detectable marker substance. After calibration, the attributes of interest (e.g. forward and side scatter, stain, cell number, etc.) are measured and the resulting data are stored for later processing.
Possibilities. The presence of certain Ag in or on a large number of cells in suspension, its frequency and its quantity can be rapidly measured with a high degree of objectivity and accuracy.
Limitations. Because samples are processed into suspensions, information about the localization of the Ag is lost. The possibility of non-specific reactivity with other cells requires a large number of method controls (i.e. positive and negative control Ab and cell samples) and reduces the applicability of many Ab. Some Ag cannot (yet) be detected as they structurally change, disappear or cannot be reached by Ab in the staining procedure. High quality flow cytometric analysis requires expensive software and computer equipment.

Immunohistochemistry
Purpose. IHC is used to demonstrate particular cell types and structures or products that may be present in certain types of tissue. In this thesis it is used to detect cytokines and other markers of inflammation in inflammatory skin of ACD and psoriasis patients.
Procedure. Skin samples are taken by biopsy, prepared into small tissue sections and mounted onto microscope glass slides. The tissue sections are then exposed to monoclonal and/or polyclonal Ab that were raised specifically against the Ag to be demonstrated, and directly or indirectly coupled to a visually or otherwise detectable marker substance.

Possibilities. The presence and localization of certain Ag in the tissue, and an indication of its quantity, can be determined by microscopic analysis.
Limitations. The possibility of non-specific reactivity with other tissue components requires a large number of method controls (i.e. positive and negative control Ab and tissue samples) and reduces the applicability of many Ab. Since the method is highly sensitive to small known or unknown deviations from the protocol, all samples that need to be compared should be processed in one run. Some Ag cannot (yet) be detected as they structurally change, disappear or cannot be reached by Ab in the IHC procedure. Finally, because the quality of the resulting slides decreases with time, microscopical analysis and scoring should be done within a relatively short period.

Computer-assisted image analysis
Purpose. IA strives to extract objective and reproducible qualitative and/or quantitative information from images that is not immediately obvious from visual inspection alone.
Procedure. The images that need analysis are digitized and stored electronically. After calibration and pre-analysis image optimization, the attributes of interest (e.g. distances, areas, shapes, color, intensity, etc.) are measured and the resulting data are stored for later processing.
Possibilities. Digital storage guarantees absolute conservation of image quality. If IA is performed well, a high degree of objectivity and accuracy can be reached.
Limitations. The handling of high quality digital images (high resolution and true color) requires expensive image analysis software and computer equipment with large memory and storage capacities. Because some of the methods and algorithms are still under development, reliable IA requires caution and a sound understanding of information theory and computer technology.

In both ACD and allergic ADR, the skin is the target organ of immunological reactivity to an allergen. This thesis describes the application of immunological insights and techniques to improve the diagnosis, treatment and follow-up of ACD and allergic ADR. In the following chapters, the techniques described above are used for the following aims: (1) to monitor clinical and immunological parameters during hyposensitization treatment of nickel ACD patients (chapter 2.2), (2) to improve quantification of immunostained skin sections in inflammatory skin diseases like ACD and psoriasis (chapter 2.3), and (3) to assess immunological reactivity to the drugs carbamazepine, oxcarbazepine, phenytoin and bromocriptine in patients with a possibly allergic ADR to one of these drugs (chapters 3.2, 3.3 and 3.4).

CHAPTER 2

STUDIES ON ALLERGIC CONTACT DERMATITIS

CHAPTER 2.1

NICKEL AS A CONTACT ALLERGEN

In this short chapter our focus will be mainly on nickel as a contact sensitizer. This metal deserves special attention for a number of reasons discussed below.

Nickel, the ubiquitous allergen

Nickel is a widespread element. It is one of the main components of magma in the earth's core. Many daily used objects, such as coins, keys, scissors, wrist watches, jewelry and blue jeans buttons contain nickel. Even so-called hypoallergenic jewelry contains considerable amounts of nickel. Sweat and plasma were found to be very effective in releasing available nickel from such jewelry, especially at low pH and after prolonged contact [1]. Nickel is also present in many sorts of food. Naturally rich in nickel content are nuts (walnut, peanut, hazelnut, almond), bitter chocolate, oats, buckwheat, soy products and dried legumes. Finally, nickel is an essential component of many enzymes in bacteria, plants, invertebrates and probably also higher animals [2].

The primary routes of nickel exposure and uptake are ingestion in the general population, inhalation in the occupational setting, and, to a lesser extent, release from surgical implants and dental prostheses. On average, daily dietary intake is 150-250 μg leading to a normal nickel serum level of 0.5-5 μg/L. Elimination is via urinary and biliary excretion with a half-time of ~28 hours [3, 4]. Although uptake of nickel via the stratum corneum, sweat ducts and hair follicles of the skin is important in the induction and elicitation of nickel allergic contact dermatitis (ACD), its contribution to overall nickel uptake is fairly small [4]. However, the skin can act as a reservoir for nickel with a mean retention time of many months [5]. Although nickel concentrations in plasma and urine of nickel allergic and non-allergic individuals are not different, in occupationally exposed individuals they may be ~10 times higher [6].

As stated in chapter 1.2, the sensitization rate of an allergen in a population is determined mainly by the combined effects of the intensity of exposure and its sensitizing potential [7]. Therefore, substances to which the human skin is frequently exposed bear a higher risk of inducing ACD. For nickel, the combination of a mild sensitizing potential and widespread exposure in the general population results in the high frequency of sensitization of 10-20%. Moreover, since (ear) piercing and wearing of inexpensive jewelry has become very popular, the prevalence of nickel sensitivity has also increased [1, 8, 9]. In many population-based and patient-based studies in Europe as well as the United States, nickel, chromium and cobalt were the highest ranking sensitizers [7]. Although a strong clinical association between nickel and cobalt allergy was found [10], immunological cross-reactivity could not be demonstrated.

Nickel directly affects the immune system

Metals, such as nickel, chromium, cobalt, gold, platinum, mercury and beryllium, are directly immunogenic and capable of inducing a wide range of pathologic and

non-pathologic conditions including ACD, asthma, autoimmune disease and under special conditions also immunological tolerance. Their immunogenicity depends on the ability to form complexes with proteins, polysaccharides and even nucleic acids. The various actions of metals on the immune system and non-specific defense mechanisms include mostly inhibitory but also stimulatory effects on phagocytosis, proliferation, cytokine production, synthesis of antibodies, and cell mediated immune reactions. Target cells are keratinocytes, macrophages, monocytes, dendritic cells, B and T lymphocytes, natural killer cells and endothelial cells [11]. At high (>1-10 mg Ni/m^3 air) concentrations, inhalation of certain industrially used nickel compounds may also cause acute toxicity, and lung and nasal cancer [12, 13]. However, of greatest public concern is nickel ACD.

Among the substances capable of inducing ACD, the metals nickel, chromium and cobalt are the most common. Nickel can induce ACD via direct actions on many immunocompetent cells, including Langerhans cells, keratinocytes and endothelial cells. A direct effect on keratinocyte viability and IL-1 production has been reported [5]. Nickel can also directly induce endothelial expression of adhesion molecules (ICAM-1, VCAM-1 and ELAM-1) and production of IL-6 via activation of the transcription factor NFκB [14, 15]. Finally, nickel can directly modify the peptide-HLA class II complex that is recognized by specific T cells [16].

In the next two chapters, hyposensitization and immunohistochemical analysis of cytokines in nickel ACD will be discussed.

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CHAPTER 2.2

HYPOSENSITIZATION IN NICKEL ALLERGIC CONTACT DERMATITIS: CLINICAL AND IMMUNOLOGIC MONITORING*

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ABSTRACT

Background: In allergic contact dermatitis (ACD) previously sensitized T cells cause skin damage. If an ubiquitous allergen like nickel is involved, no effective treatment is available. Down-regulation of this allergic response has been described after antigen presentation in the absence of adequate costimulatory signals. UV exposure can enhance such hyposensitization.
Objective: The aim of this study was to establish the capability of a hyposensitization procedure to induce antigen-specific tolerance.
Methods: Twenty-one patients with nickel ACD were randomly assigned to either a hyposensitized or control group. A schedule, consisting of UVB treatment and subcutaneous nickel sulfate administration (hyposensitization) or UVB only (control), was applied. During the ensuing two years, several clinical and immunologic features were monitored.
Results: During UVB treatment we observed a significant clinical improvement in both groups that persisted in the hyposensitized group. Except for increased slope variances of specific lymphocyte proliferation in time, no clear changes were seen in the immunologic findings.
Conclusion: Despite significant clinical improvement induced by UVB, hyposensitization did not induce significant changes in the immunologic findings in patients with nickel ACD.

INTRODUCTION

The type of allergic contact dermatitis (ACD) most frequently diagnosed involves nickel allergy. Treatment of chronic ACD has been largely symptomatic because consistent avoidance of skin contact with and dietary intake of nickel are difficult to achieve. Allergen-specific T lymphocytes are crucial in the pathogenesis of ACD. Nickel-specific T lymphocyte clones have been isolated from peripheral blood of nickel-allergic patients [1-4]. When activated, these T lymphocytes produce IL-2 and high levels of IFN-γ [1, 4] and cause inflammatory skin injury. Prevention of skin injury may be obtained through inhibition of specific T cell activation (e.g. through hyposensitization).
A state of specific tolerance or anergy has been described after antigen presentation in the absence of costimulatory signals [5-7]. In both mice [8-21] and human beings [5, 22], UVB induces specific hyporesponsiveness when administered simultaneously with allergen. UV exposure (even suberythemagenic doses) has significant down-modulatory effects on T cell-mediated responses to contact allergens. In a previous study, we demonstrated a clear effect of UVB on patch test responses to nickel, possibly through depletion of Langerhans cells (LC) and induction of $CD1a^-DR^+$ antigen presenting cells (APC) [22].
Until now, hyposensitization treatment has been restricted to immediate hypersensitivity reactions such as to insect venoms and pollen. In view of the clear effects of UVB on LC and ACD, we designed a hyposensitization schedule that bypasses the highly sensitizing potential of epidermal LC by combining UVB treatment with subcutaneous injection of nickel sulfate solutions.

MATERIAL AND METHODS

Patch tests

Before the start of the study a European standard patch test series of contact allergens (van der Bend, Brielle, The Netherlands), including nickel sulfate, potassium dichromate and cobalt chloride, was performed according to guidelines of the International Contact Dermatitis Research Group. After 48 hours the patch chambers were removed. At 48 hours and 72 hours the skin reaction was scored as described earlier [23]. At the start of the study, patch tests with nickel, chromium and cobalt were repeated to obtain baseline values. Because of the risk of further sensitization or boostering, patch tests were not repeated.

Patients

Twenty-one patients allergic to nickel as judged by history and clinical signs of contact allergy (predominantly on the hands and face) indicating nickel as a likely cause, and confirmed by a patch test response of 2+ or greater to nickel sulfate were enrolled in and completed the study. All subjects were nonpregnant women.
Each patient was randomly assigned to either the hyposensitized group, scheduled for UVB plus nickel sulfate administration, or to the control group, which was to receive only UVB treatment. The protocol and informed consent documents were approved by the committee on medical ethics of our hospital. The hyposensitized group consisted of 12 women, 28 ± 5 years of age. Nine patients, 38 ± 16 years of age, formed the control group.

UVB treatment

A Waldmann UV1000 standing UV cabinet was used for UV treatment. All patients (hyposensitized and control groups) received UVB pretreatment during the first 3 to 6 months. On the basis of skin type, exposure started at 5 to 10 mJ/cm^2 three times a week and gradually increased, allowing only mild erythema. Nickel sulfate injections were started in the hyposensitized group as soon as a cumulative dose of 1.0 J/cm^2 was reached. At this point, UV exposure was continued once a week until a cumulative dose of approximately 1.25 mJ/cm^2 was reached.

Hyposensitization procedure

Sterile, pyrogen-free, solutions of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} mol/L nickel sulfate were prepared. The lowest concentration equals the nickel concentration normally measured in body fluids [24-27]. In a manner similar to hyposensitization schedules used in type I allergy, weekly subcutaneous injections were started with 0.1 ml of the lowest nickel sulfate concentration in the arm. After each injection the patients were observed for at least 20 minutes. If possible, the next doses were subsequently 0.2, 0.4, 0.7 and 1.0 ml of the same concentration, and finally 0.1 ml of the next concentration. Doses were increased until either minimal local symptoms occurred or the highest concentration was reached. At this point the maximal dose achieved was continued and the interval was gradually prolonged up to 1 month. The total duration of the nickel sulfate hyposensitization was 2 years.

Clinical evaluation

The clinical follow-up period was 24 months. At three defined time points - at the start of the study (month 0), at maximal UVB exposure (month 6), and at the end (month 18) of the hyposensitization treatment - clinical evaluation was done. The affected area of involved skin, severity (itching, papules, vesicles and fissure formation), frequency of symptoms, therapeutic need (use of corticosteroids and its potency), and a subjective quality of life assessment were scored on a standard evaluation form. With respect to clinical scores, the control group consisted of eight patients because we excluded one patient because of lack of clinical follow-up information.

Lymphocyte proliferation assays

Lymphocyte proliferation assays (LPA) were performed as described earlier [23]. Lymphocyte proliferation was expressed as counts per minute (cpm). Stimulation indices (SI) (i.e. relative proliferation) were calculated by dividing specific by background proliferation. In our laboratory an SI greater than 3 was considered to be indicative of prior lymphocyte sensitization to nickel.

Fluorescence-activated cell sorter immunophenotyping

Peripheral blood samples were immunophenotyped with the monoclonal antibodies listed in Table I. Except for lymphocyte function-associated antigen (LFA)-1/2 and BBA4, all monoclonal antibodies were directly conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Measurement and analysis was done with a FACScan flow cytometer with Simulset, FACScan, Consort 30 and Lysis research software (Becton Dickinson, San Jose, CA). Both absolute cell numbers per microliter and percentages were calculated.

Statistical analysis

Professional STATA 3.0 Statistics/Data Analysis (Computing Resource Center, Santa Monica, CA) and EGRET (version 0.26.6, 1991, Serc and Cytel, Seattle) were used for data analysis and statistical calculations.

The change of the disease activity scores during treatment was analyzed, within each group, with the Wilcoxon signed-rank test. An exact trend test was used to compare the hyposensitized and control groups with regard to the distribution of the scores at the end of the study.

The distributions of SI and cell counts were transformed to normal by taking natural log values. This enables use of parametric statistical methods. The resulting distributions were checked for normality in normal plots.

Linear regression was used to analyze time trends of lymphocyte proliferation and activated T cell numbers during treatment [28]. The resulting slope values of both groups were compared in a *t* test modified for unequal variances (Welch test). Equality of variances was tested in the variance ratio test (*F* test).

Table I. Monoclonal antibodies used in this study.

Antibody	CD code (antigen recognized)	Source
Leu-4 (FITC/PE)	CD3	BD
Leu-3 (FITC/PE)	CD4	BD
Leu-1	CD5	BD
Leu-2 (PE)	CD8	BD
LFA1/2	CD11a	CLB
My4	CD14	CC
Leu-11c (PE)	CD16	BD
B1 (FITC)	CD19	CC
B4 (FITC)	CD20	CC
2A3 (PE)	CD25	BD
HLe-1	CD45	BD
BBA4	CD54	BB
Leu-19 (PE)	CD56	BD
L243 (PE)	HLA-DR	BD

BB, British Biotechnology, Oxon, UK; BD, Becton Dickinson, Sunnyvale, CA; CLB, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; CC, Coulter Clone, Hialeah, FL; FITC, fluorescein isothiocyanate; LFA, lymphocyte function-associated antigen; PE, phycoerythrin.

RESULTS

Baseline values of patch tests and LPA

The distributions of baseline values for both patch tests and LPA in the hyposensitized and control groups were compared. Patch test scores and specific lymphocyte proliferation were comparable in both groups. In both the hyposensitized and the control groups, a patient previously (before study) reactive to nickel showed a negative baseline value of the patch test at the start of treatment. However, LPA results showed that lymphocyte reactivity to nickel still existed.

UVB dose

Individual UVB doses ranged between 5 and 50 mJ/cm^2. In both groups the cumulative dose of 1.0 J/cm^2 was reached within the first 4 months.

Nickel sulfate administration

Most patients tolerated a 100- to 1000-fold increase of the nickel dose within 3 months. In two patients the maximum dose of 1.0 ml of 10^{-3} mol/L nickel sulfate was reached. Adverse effects remained limited to transient local induration that developed at the site of injection within 8 to 24 hours. These lesions were histologically characterized by a perivascular accumulation of mononuclear cells. To avoid this reaction, doses were temporarily lowered. As a result, in some patients doses varied, occasionally by 10-fold, because of variable nickel tolerability.

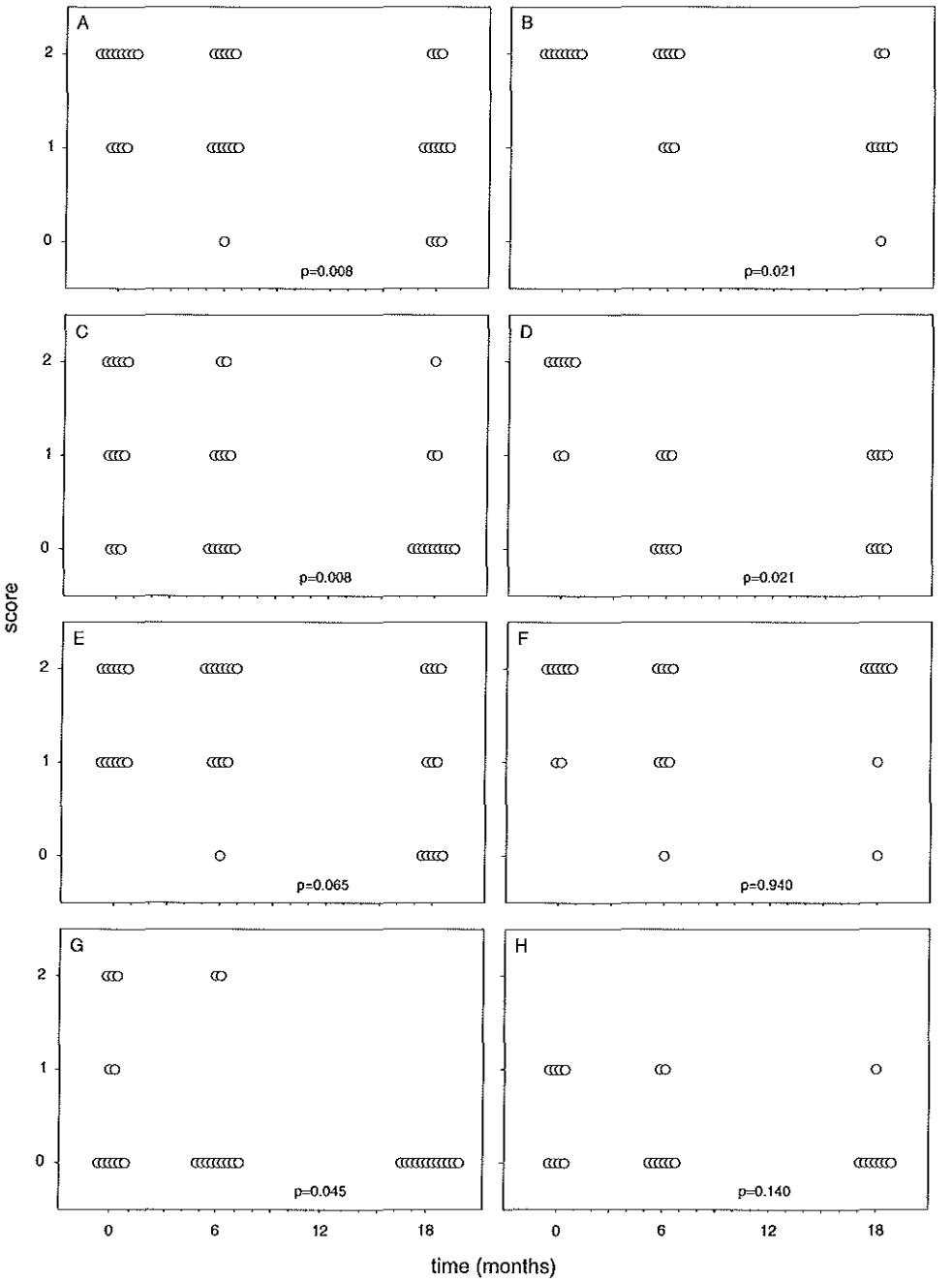

Figure 1. Time course of disease activity scores in hyposensitized (left panels) and control (right panels) groups with regard to affected area (A and B), severity (C and D), frequency (E and F) and therapy (G and H).

Clinical evaluation

Figure 1 shows dot plots of the four disease activity scores in both groups at three time points: at the start of the study (month 0), during maximal UVB exposure (month 6), and at the end of the study (month 18). Overall, scores within both groups dropped during UVB treatment. This trend continued even after UVB exposure was stopped. In the hyposensitized group this led to p values of 0.0076, 0.0076, 0.065 and 0.045 for the affected area, severity, frequency and therapy scores respectively. In the control group these p values were larger, namely 0.021, 0.021, 0.94 and 0.14, respectively. The change of the disease activity scores was most evident for the affected area and severity scores. However, between the hyposensitized and control groups no statistically significant score differences were found ($p = 0.84$, 0.33, 0.26 and 0.40, respectively).

Lymphocyte proliferation

Specific lymphocyte reactivity against nickel was monitored during the hyposensitization therapy. Within each patient, some variation of SI was found during the 2-year observation period. Neither SI nor net counts per minute values showed significant correlation with clinical scores.

In figure 2, A, mean SI values and 95% confidence intervals of all patients within each group are plotted against duration of treatment. Mean proliferation indices over time varied between 10 and 30. No significant difference was seen between the groups. In an attempt to characterize further the time course of lymphocyte reactivity, slopes of individual time plots were calculated by linear regression analysis. The resulting slope values indicate ascending or descending trends of lymphocyte reactivity with duration of treatment. However, neither positive nor negative slope values corresponded with clinical improvement. Figure 2, B, shows approximately equivalent mean slope values but increased slope variance in the hyposensitized group as compared with control subjects. This difference of variances was highly significant ($p<0.001$).

Immunophenotyping of peripheral blood mononuclear cells

Numbers of B cells, T cells (including the subpopulations) and natural killer cells were within normal limits, comparable in both groups and fairly constant over time (Table II).

Table II. Distribution of lymphocyte phenotypes.

Cell type	Nickel allergic		Nonallergic controls
	Hyposensitized	Control	
Lymphocytes	2.0 (1.4-4.1)	1.3 (1.0-2.5)	1.7 (1.1-2.6)
B cells	0.2 (0.08-0.4)	0.1 (0.05-0.2)	0.1 (0.1-0.2)
T cells	1.4 (1.1-3.2)	1.1 (0.7-2.2)	1.2 (0.8-2.2)
$CD4^+$ cells	1.1 (0.6-2.5)	0.7 (0.5-1.4)	0.7 (0.4-1.5)
$CD8^+$ cells	0.5 (0.3-1.0)	0.3 (0.1-0.7)	0.4 (0.2-0.7)
Natural killer cells	0.2 (0.08-0.3)	0.1 (0.1-0.2)	0.2 (0.06-0.6)

Values are median absolute cell counts per milliliter; 95% confidence intervals shown in parentheses.

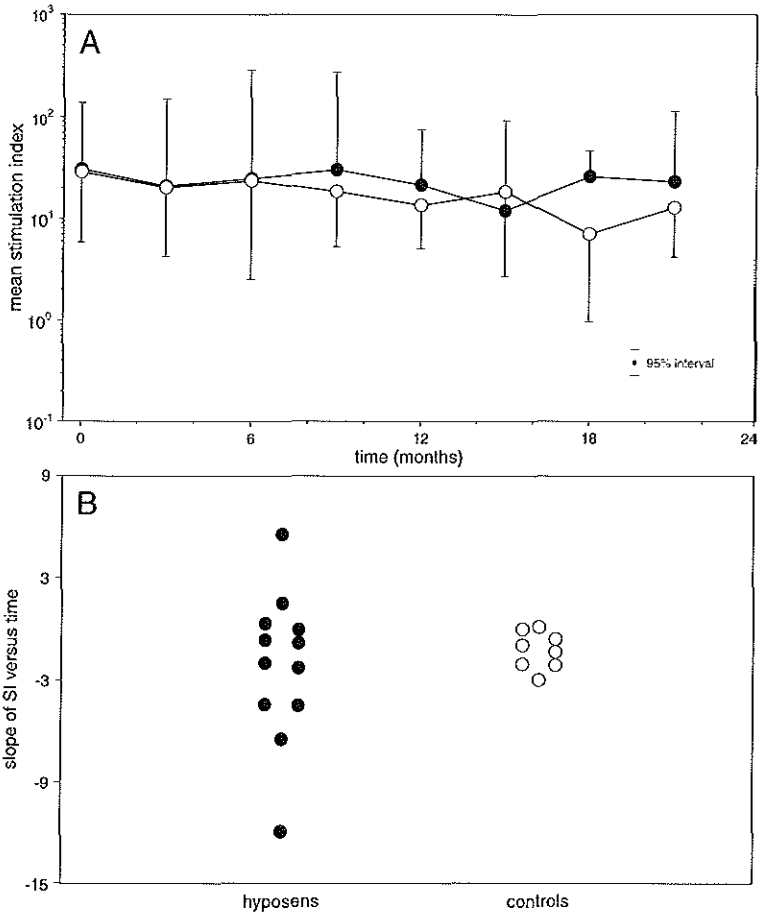

Figure 2. Level of nickel reactivity in time as measured by nickel-specific lymphocyte proliferation: (A) mean SI values over time in all patients within each group. Bars represent 95% confidence intervals. (B) slopes of individual time plots as calculated by linear regression analysis. ● hyposensitized group ○ control group.

In an attempt to characterize further the nickel reactive lymphocytes, we did flowcytometric analysis of peripheral blood mononuclear cells for activated T cells with CD3/HLA-DR and CD3/CD25 double-staining. Actual double-positive cell numbers per microliter were calculated and plotted against time. Values ranged from 10 to 1000 cells/μl in both groups. Figure 3 shows the time course of mean $CD3^+$/IL-2 $receptor^+(CD25^+)$ and $CD3^+/HLA\text{-}DR^+$ cell counts and 95% confidence intervals of all patients within each group. On average, $CD25^+$ counts were 1.7 times higher than $HLA\text{-}DR^+$ counts. As previously described [29], co-expression of IL-2 receptor and HLA-DR on T cells is limited. Apparently these markers represent at least partially different types or phases of activation. The average number of activated T cells was estimated at approximately 100 to 150 cells/μl (i.e. 5% to 20% of circulating T cells). These values are within normal limits.

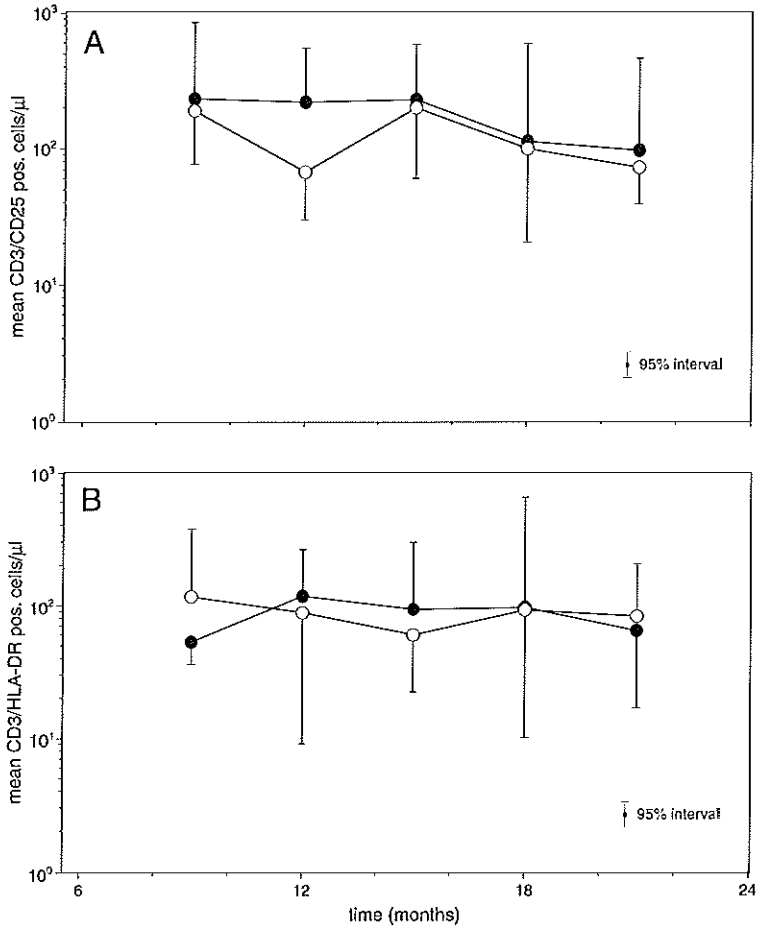

Figure 3. Mean $CD3^+$/IL-2 $receptor^+$ (A) and $CD3^+$/$HLA\text{-}DR^+$ (B) cell numbers over time in all patients within each group. Bars represent 95% confidence intervals. ● hyposensitized group ○ control group.

LFA-1α and intercellular adhesion molecule-I expression of both peripheral blood lymphocytes and monocytes was also measured. Lymphocytes showed a bimodal LFA-1α expression, that is, normal ($LFA\text{-}1\alpha^+$) and high ($LFA\text{-}1\alpha^{++}$), mainly because of the expression of this molecule on T cells. During the study, these two levels of expression appeared to be inversely correlated. These time trends, however, did not match clinical disease activity.

DISCUSSION

Hyposensitization with contact allergens has previously been shown to be effective [30-34]. The present study describes monitoring of clinical and immunologic features during such nickel hyposensitization.

Considerable clinical improvement was observed during UVB pretreatment. In both groups this trend persisted even after UVB withdrawal. No statistically significant difference between the groups was seen.
Lymphocyte reactivity to nickel appeared to fluctuate with time. Height of SI showed no correlation with clinical scores. Discordance between clinical and *in vitro* findings has previously been described [35-40].
Overall, no significant change in the *in vitro* lymphocyte reactivity to nickel was seen during the monitored period. However, time-trend analysis of each patient revealed a significantly larger slope variability within the hyposensitized group. Its meaning is not entirely clear. Possibly the effect of hyposensitization varies in different persons [21, 41].
Activated T lymphocytes in peripheral blood, measured as $CD3^+/HLA\text{-}DR^+$ and $CD3^+/CD25^+$ cells, remained within normal limits during treatment. It is conceivable that the number of nickel-specific memory T lymphocytes is too low to allow detection of changes in the peripheral circulation.
As in type I hyposensitization, the mechanisms by which clinical improvement might be explained, remain to be elucidated. Evidence is emerging that besides LC and T lymphocytes, keratinocytes are also actively involved in immunologic reactions in the skin. Insight into the process of induction of central or peripheral tolerance, clonal anergy or active suppression is only beginning to appear [42-45]. Analysis of epidermal cells and lymphocytes for the presence and susceptibility of costimulatory factors, such as adhesion molecules [46-48] and cytokines [7, 49, 50], and the influence of immunomodulators, such as UV radiation on these, may provide more insight into this process.

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CHAPTER 2.3

COMPUTER-ASSISTED AREA MEASUREMENT TO IMPROVE THE IMMUNOHISTOCHEMICAL ANALYSIS OF INFLAMMATORY SKIN DISEASE*

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ABSTRACT

Accurate and reproducible quantification of immunostaining is difficult. A number of different procedures have been described, producing results that cannot be easily compared. Computer-assisted image analysis (IA) may help to overcome some of these difficulties. However, reliable IA of immunostained slides requires sound knowledge of information theory and computer technology, besides familiarity with histology and immunohistochemistry (IHC). Moreover, while IA has been used successfully to quantify immunostained cytological preparations and sections of a number of other tissues, application of the technique to skin sections has rarely been described.
We developed and validated a semiautomatic procedure (macro) for area measurement by IA. The accuracy and reproducibility of this area measurement procedure were high, i.e. less than 0.5% and 1%, respectively. When compared to traditional grid-based area measurement, the mean values of both methods were similar but the standard deviation of IA was somewhat smaller. As an example, the IA area measurement procedure was applied to immunostained inflammatory skin sections. The computer-assisted IA allowed us to quantify immunostaining as cell counts per square millimeter of epidermal, dermal or infiltrate section area.

INTRODUCTION

Quantification of immunostaining is difficult. Firstly, reliable and reproducible immunohistochemistry (IHC) depends heavily on a variety of critical factors, such as time between biopsy and embedding, embedding medium, variation of section thickness, fixation, species, isotype and subtype of the primary and secondary antibody (Ab) and proper Ab and tissue controls [1]. Secondly, quantification of IHC is done by widely different methods. The number of positively stained cells, for example, may be expressed per field [2], per unit of basement membrane [3], or as a fraction of the largest positively stained cluster observed [4]. This obvious lack of standardized procedures may explain the poor reproducibility of many methods, the inter- and intraobserver variance and the difficult interpretation of the results obtained from different methods.

Computer-assisted image analysis (IA) may seem a logical step to overcome some of these difficulties. However, reliable IA of immunostained slides requires sound knowledge of information theory and computer technology, besides of course familiarity with histology and IHC. Although IA has been applied to immunostained (single cell) cytological preparations and sections of tissues like bone marrow, lymph nodes, brain, nerve tissue, breast, endometrium and cervix [5], application of the technique to immunostained skin sections has rarely been described.

We describe a standardized, semiautomatic IA procedure to measure epidermal, dermal and infiltrate areas in inflammatory skin sections. As an example, the technique is applied to inflammatory skin sections that were immunostained for cytokines

and other markers of inflammation. This method enabled us to quantify the number of stained cells per square millimeter of epidermal, dermal or infiltrate section area.

PATIENTS AND METHODS

Patients and controls

Six ACD patients (Hospital Walcheren, Vlissingen, The Netherlands), 6 psoriatic patients (University Hospital Dijkzigt, Rotterdam, The Netherlands) and nine healthy controls, scheduled for cosmetic breast or abdominal reduction surgery (Sint Franciscus Hospital, Rotterdam, The Netherlands), were enrolled in our study after informed consent.

The presence and activity of ACD was judged by history, clinical signs and a patch test (PT) response of 2+ or greater. PT were performed according to ICDRG guidelines with nickel sulfate 5% in petrolatum in commercially available PT chambers (van der Bend, Brielle, The Netherlands). After 48 hours the PT chambers were removed. At 48 hours and 72 hours the skin reactions were scored as previously described [6]. Three millimeter punch biopsies were taken from positive PT, lesional psoriatic and healthy control skin.

Tissue processing

The skin biopsies were snap frozen in Tissue-Tek® O.C.T. compound (Miles Laboratories, Elkhart, IN, USA) and stored at -80 °C until further processing. Six micrometer sections were prepared using a Jung Frigocut 2800E cryostat (Leica, Rijswijk, The Netherlands), mounted on 3' aminopropyltriethoxysilane(APES)-coated (A3648, Sigma Chemical Company, St. Louis, MO, USA) glass slides, and air-dried. Previous experiments had shown APES coating to give the lowest background staining. For optimal comparison, each slide contained normal, ACD and lesional psoriatic skin sections in duplo. Sections of tonsil, thymus, spleen, cutaneous T cell lymphoma (mycosis fungoides), bronchial tissue, and cytospin preparations of stimulated and unstimulated peripheral blood mononuclear cells were included as additional tissue controls.

Image analysis

The IA hardware consisted of a three chip charge-coupled device (3CCD) color videocamera (DXC-930, Sony, Tokyo, Japan) mounted to a Zeiss Axioplan light microscope (Zeiss, Oberkochen, Germany) at 10x magnification and connected to a frame grabber (Kontron Electronics, München, Germany) in a 80486 66 MHz personal computer. Automatic gain control was intentionally turned off for optimal comparability. Illumination was provided by a halogen light source (Philips 7023, Philips, Eindhoven, The Netherlands), connected to a stabilized adjustable power supply (12V / 100W). Since the emission of light sources and the noise and sensitivity of CCD cameras are known to vary with temperature, and therefore may interfere with reliable measurement [7-9], all hardware was allowed a 1 hour warming period to minimize these unwanted thermal effects.

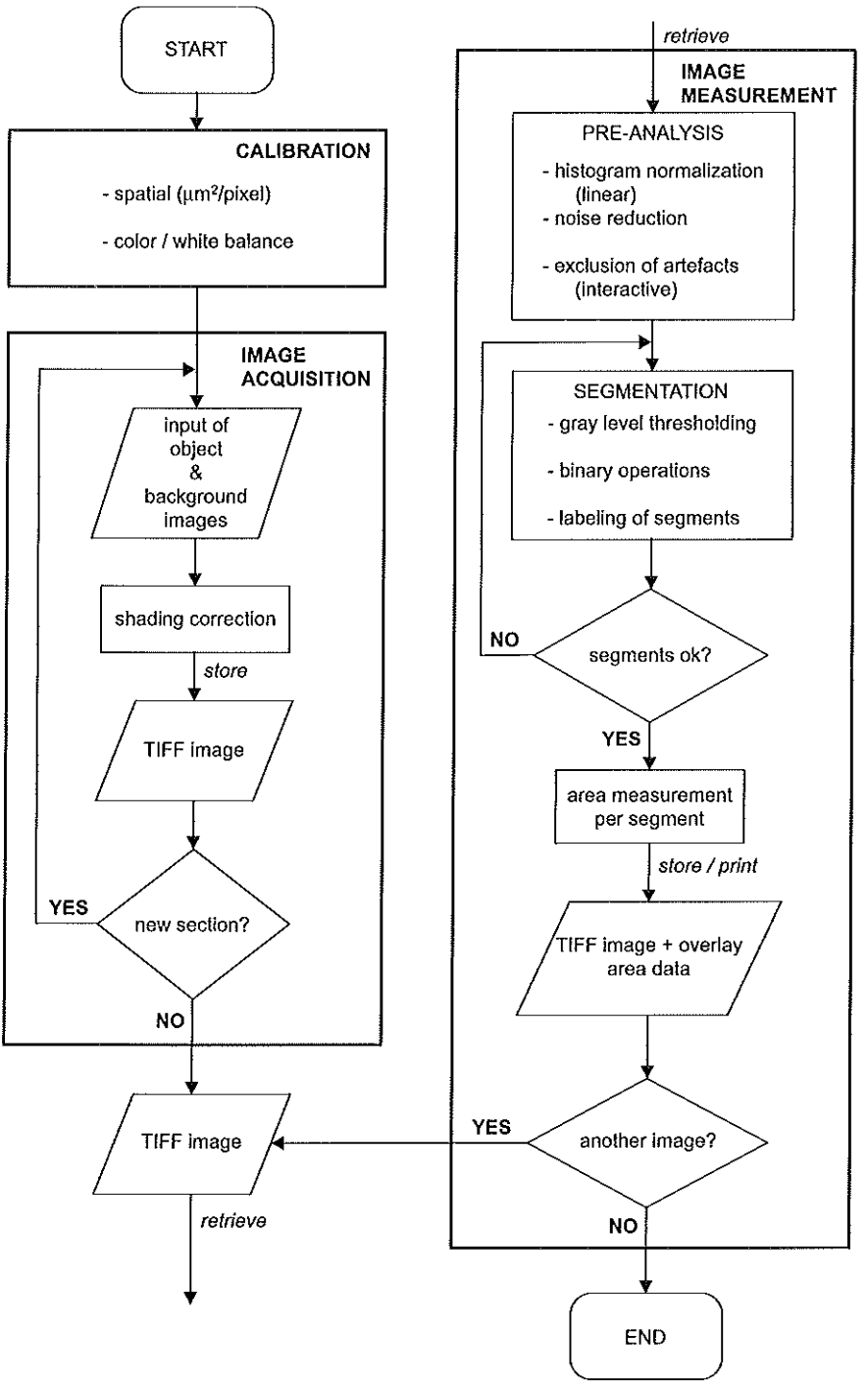

Figure 1. Flow diagram of the macro for semiautomatic measurement of epidermal, dermal and infiltrate section areas.

The IA software consisted of the KS-400 v1.2 software package (Kontron Electronics) running under Microsoft Windows. This macro-based IA software allows automatic execution of a user-defined sequence (macro) of image processing commands. Figure 1 is a flow diagram of the macro we developed for semiautomatic measurement of epidermal, dermal and infiltrate section areas. The procedure is divided into three main parts: (1) calibration of the microscope and the CCD camera, (2) image acquisition and (3) image measurement. For optimal comparability, calibration and acquisition were done in a single session.

Calibration
Color calibration of the red, green and blue (RGB) channels of the CCD camera was done via white balance of a blank image. In addition, spatial calibration was done to allow conversion of pixels into mm^2. However, because pixels are square instead of round, the distance between two pixels that touch diagonally is greater ($\sqrt{2}$ at most) than their distance orthogonally. Thus, to enable accurate image measurement, the resulting error should be corrected by the IA software [8]. We checked this correction by measuring a known 0.55x0.55 mm^2 grid at 0, 15, 45 and 60 degrees angles of rotation, respectively.

Image acquisition
The acquisition of all images was done in a single session. For each object image, a site-matched background (blank) image was captured. The captured area amounted to >50% of the total skin section area. Each frame was corrected for nonuniform illumination (shading correction) by subtraction of the background image from the object image. The resulting image was stored on optical disk in an uncompressed 24 bits tagged image file format (TIFF) with a spatial resolution of 756x512 pixels and a range of 256 values for each of the R, G, and B channels.

Image measurement
First, the contrast of each image was enhanced by linear normalization of the gray value histogram. Noise was then suppressed by the application of an edge preserving 3x3 median filter [8]. Histologic artifacts (e.g. tissue folds and bubbles) were delineated and excluded from further analysis. Next, the hematoxylin counterstained epidermal and infiltrate section areas were selected by interactively setting segmentation thresholds in the gray value histogram of the image. The number of small scattered segments was reduced by application of binary morphologic operations, such as opening and closing. The resulting segments were labeled and their outer limits were visually checked by superimposing them on their corresponding original image. The surface area of each segment was then automatically calculated and stored. Finally, a hard copy was made for reference during cell counting (see quantification of immunostaining). A similar procedure was followed to measure the total area of the tissue section. This method appeared highly reproducible due to the characteristic gray value histogram of the image (Figure 2), with peaks corresponding to the darker infiltrate and epidermis and the lighter dermis, respectively.

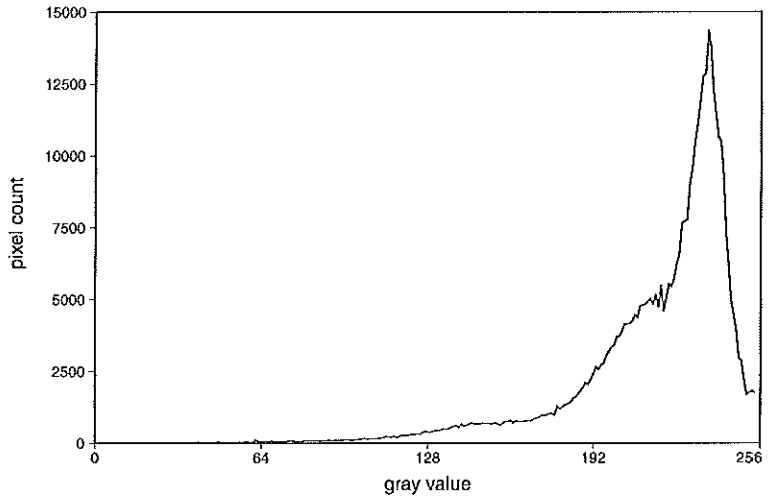

Figure 2. Characteristic gray value histogram of inflammatory skin, showing three peaks corresponding to the infiltrate (128-160), epidermis (192-224) and dermis (224-256), respectively.

The reproducibility of IA area measurement was assessed by measuring a single normal skin section 10 times. Individual measurements were spaced at least 1 hour apart and every three successive measurements the microscope and CCD camera were turned off, postponing further measurements to the next day. Additionally, area measurement by IA was compared with the more traditional grid-based method in 20 serial ACD skin sections (see results).

Immunohistochemistry

Antibodies and controls

We used a number of Ab specific for cytokines and other markers of inflammation. The Ab used in this study and their sources are listed in Table I. The specificity of the primary and secondary Ab was checked by using a protein concentration-matched non-relevant (third-party) monoclonal Ab, a pre-immune mouse IgG_1 and phosphate-buffered saline (PBS).

Immunosupersensitive alkaline-phosphatase staining

All incubations and washes were done in a Sequenza immunostaining center (Shandon Scientific Ltd, Cheshire, UK). The tissue sections were fixed in acetone (10 minutes at room temperature), washed once in PBS and washed twice in PBS with 1% bovine serum albumin (BSA, Sigma Chemical Company). Subsequently, the sections were preincubated with 10% normal rabbit serum for 10 minutes. After that, they were incubated with the primary monoclonal Ab for 1 hour at room temperature, washed in PBS-BSA 1% and incubated for 30 minutes with a 1:50 dilution of biotinylated rabbit anti-mouse immunoglobulin (Biogenex, San Ramon,

CA, USA) supplemented with 10% normal human serum. After washing twice in PBS, the sections were incubated for 30 minutes with a 1:50 dilution of streptavidin-alkaline phosphatase (Biogenex, San Ramon, CA, USA). Naphtol-AS-MX-phosphate (0.30 mg/ml, Sigma Chemical Company) and new fuchsine (160 mg/ml in 2 mol/L HCl, Chroma-Gesellschaft, Köngen, Germany) were used as a substrate for alkaline phosphatase (AP). Levamisol (0.25 mg/ml, Sigma Chemical Company) was added to block endogenous AP activity. Finally, sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol-gelatin (Merck).

Table I. Antibodies used in this study.

Antibody / antigen	Clone / code	Species / isotype	Conc (µg/ml)	Source
CD1a	OKT6	$MIgG_1$	10	ATC
CD3	Leu-4	$MIgG_1$	0.125	BD
IL-1α	1C12.1	$MIgG_1$	5.0	ONC
IL-4[a]	BMS129	$MIgG_1$	5.0	BM
IL-8[b]	52E8	$MIgG_1$	1:5	-
IFN-γ[c]	MD-2	$MIgG_1$	20	-
chymase	MAB1254	$MIgG_1$	0.10	CH
control 1	9040	$MIgG_1$	20	BD
control 2	9050	$MIgG_{2a}$	20	BD
secondary Ab	ZA000-4M	RαMIgG-biotin	1:50	BG

[a] Kindly provided by Bender MedSystems, Vienna, Austria. [b] Supernatant, kindly provided by Dr. M. Sticherling, University of Kiel, Kiel, Germany. [c] Kindly provided by Dr. P. van der Meyden, TNO Primate Centre, Rijswijk, The Netherlands.
Ab, antibody; CD, cluster of differentiation; Conc, protein concentration; IL, interleukin; IFN, interferon; MIg, mouse immunoglobulin; RαM, rabbit anti-mouse. ATC, American Type Culture Collection, Rockville, MA, USA; BD, Becton Dickinson, San Jose, CA, USA; BG, Biogenex, San Ramon, CA, USA; BM, Bender MedSystems, Vienna, Austria; CH, Chemicon, Temecula, CA, USA; ONC, Oncogene Science Inc., Uniondale, NY, USA.

Quantification of immunostaining

Quantification of the staining was done in duplicate by two independent observers (MS, JH) on direct light microscopic images at magnifications of 10x10 or 10x25 (Leitz Dialux, Leica, Rijswijk, The Netherlands). Using the hardcopy of the IA measured areas as a reference, positive cells within the epidermis, infiltrate and dermis were counted and subsequently expressed as the number of cells per mm^2 of the corresponding section area as measured by IA. $CD3^+$ cells were calculated slightly different: the percentage of positively stained infiltrate cells was determined. Cytokines causing diffuse staining were scored semi-quantitatively as shown in Table II.

Table II. Semiquantitative scores of staining intensity and stained area fraction.

Score	Staining intensity	Stained area fraction (%)
0	negative	< 20
1	weak	< 40
2	moderate	< 60
3	strong	< 80
4	very strong	< 100

Statistical analysis

Professional STATA™ 4.0 Statistics / Data Analysis (Stata Corporation, College Station, TX, USA) was used for data analysis and statistical calculations. For variables with a normal distribution, parametric statistical methods were used, otherwise non-parametric methods were applied. Coefficients of variation were calculated as the standard deviation (SD) divided by the mean times 100 percent [10]. Differences between the ACD, psoriatic and normal control groups were tested for significance using the non-parametric two-sample Wilcoxon Rank-Sum Test [11].

RESULTS

PT scores of the ACD patients

All ACD patients were patch tested with nickel sulfate to induce a local elicitation reaction from which the ACD skin biopsies were taken. The PT scores, which are a measure of disease activity, are shown in Table III. All but one patient had a PT score of 2+ or greater.

Table III. Patch test scores of the ACD patients.

Patient	PT score
1	+++
2	++
3	++
4	+
5	+++
6	++

Validation of image analysis for area measurement

Figure 3 is a visual representation of the IA procedure for measurement of section areas as depicted in Figure 1. The original image (A) of an ACD skin section was converted into a binary image (B) by setting a threshold in the gray value histogram. After that, the number of small scattered segments in the binary image was

reduced by applying binary morphologic operations as illustrated in image (C). Finally, the outer limits of the epidermal and infiltrate segments were superimposed on the original image to enable visual verification of the procedure (D).

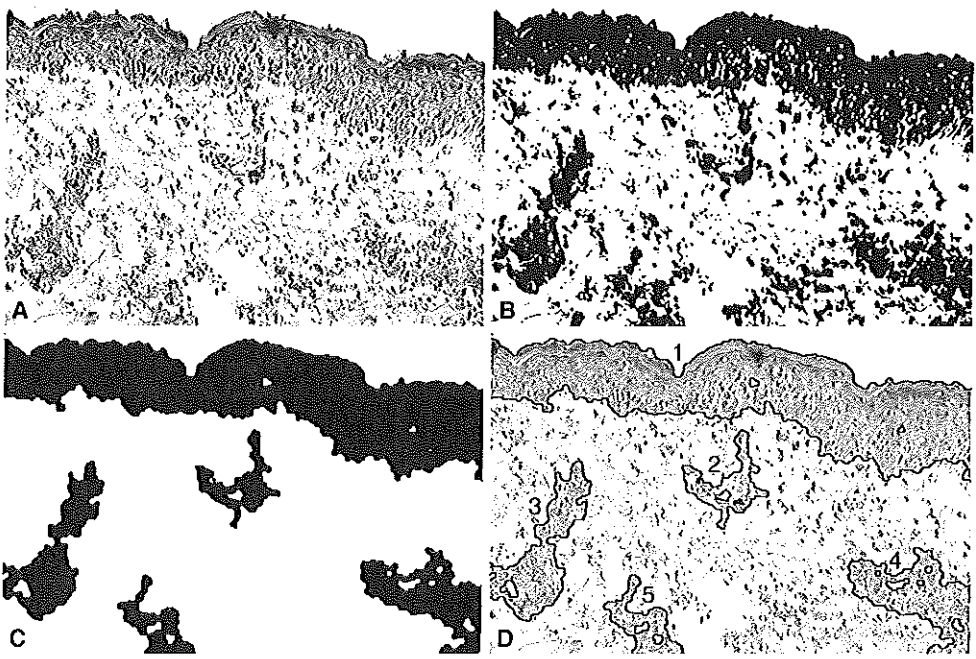

Figure 3. Visual representation of the image analysis procedure for measurement of section areas (see Figure 1), showing (A) original object image of a contactallergic skin section, (B) binary image after thresholding of the gray value histogram, (C) binary image after binary morphologic operations, and (D) the epidermal and infiltrate segments as deduced from the binary image, labeled and superimposed on the original object image. (10x)

Accuracy and reproducibility

The accuracy of IA area measurement and its sensitivity to orientation was checked by measuring a grid of known dimensions at various angles of rotation. The resulting deviations from the actual value were small, i.e. between -0.44 and +0.23 percent. At 10x magnification of the microscope objective, a standard image sized 756x512 pixels corresponded to 1.202 mm^2 section area.

The reproducibility of our IA area measurement procedure was calculated from repeated measurement of a single normal skin section. The resulting coefficients of variation were small, i.e. below 1.0 percent (data not shown).

Variation of serial skin section areas

To assess the section-to-section variation, serial ACD, psoriatic and normal skin

sections were measured by IA. In Figure 4, the resulting epidermal, dermal, infiltrate and total section areas are shown for 20 serial ACD skin sections. For epidermal, dermal and total section areas, the coefficients of variation (SD as a percentage of the mean) were 7.4-8.6 percent. However, for infiltrate section areas the coefficient of variation was much larger, i.e. 17 percent. This is probably due to the irregular dimensions and scatter of infiltrate cell clusters as compared to the rather constant epidermal and dermal section areas.

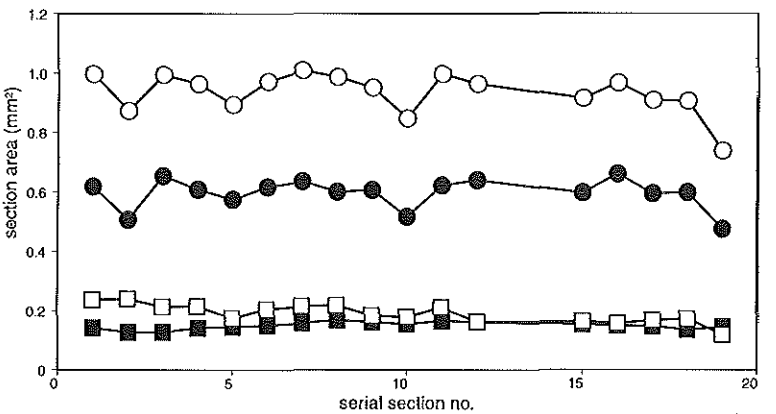

Figure 4. Variation of epidermal (closed squares), infiltrate (open squares), dermal (closed circles) and total (open circles) section areas as measured by image analysis in 20 serial contactallergic skin sections (sections no. 13, 14 and 20 were excluded as they contained large artifacts).

Comparison of area measurements by IA and grid

Area measurement by IA was compared with the traditional grid-based method in 20 serial ACD and psoriatic skin sections. Table IV shows the distributions of epidermal and infiltrate section areas for both methods and of their absolute and relative differences. As psoriatic epidermis is much thicker than ACD and normal epidermis, the total section area of psoriatic skin was almost identical to the epidermal section area. Therefore, for psoriatic skin only the epidermal section area is listed. The mean epidermal and mean infiltrate section areas of both ACD and psoriatic skin were quite similar for the two methods. However, the SD of ACD epidermal section areas as measured by the grid-based method was somewhat larger. The SD of psoriatic epidermal and ACD infiltrate section areas were much larger and no differences between the IA and grid-based methods were noted here. The agreement between both methods was determined by analysis of the distribution of the differences [11]. The mean difference, which is an estimate of the average bias of one method relative to the other, ranged between -4 and +11% (relative difference). The SD of the differences, which is an estimate of the individual agreement between both methods, was 6 and 11% (relative difference) for psoriasis and ACD, respectively.

Table IV. Comparison of IA and grid-based measurement of epidermal and infiltrate section areas in 20 serial ACD and psoriatic skin sections.

	IA	Grid-based	Δ (absolute)	Δ (relative)
ACD				
Epidermis	0.15 ± 0.01	0.13 ± 0.02	0.02 ± 0.02	0.11 ± 0.11
Infiltrate	0.19 ± 0.04	0.17 ± 0.04	0.02 ± 0.02	0.10 ± 0.10
Psoriasis				
Epidermis	0.37 ± 0.08	0.39 ± 0.08	-0.02 ± 0.03	-0.04 ± 0.06

Values are mean areas in mm^2 ± SD. The relative difference (Δ) is calculated as the difference divided by the mean of both methods. ACD, allergic contact dermatitis; IA, image analysis.

Application of IA to IHC-stained inflammatory skin sections

Application of IA area measurement to immunostained inflammatory skin sections is illustrated in Table V for CD1a, CD3 and chymase, three commonly used inflammatory markers specific for Langerhans cells, T cells and mast cells, respectively.

Table V. Inflammatory markers in ACD, psoriatic and normal skin.

Localization	CD1a	CD3	Chymase
ACD			
Epidermis	110 (53-380)	19 (0-230)[c]	0 (0-0)
Infiltrate	230 (35-510)[a]	24 (6.1-34)[d]	210 (100-270)[g]
Dermis	14 (2.3-210)	ND	24 (20-37)
Psoriasis			
Epidermis	56 (13-77)	86 (21-120)[e]	4.3 (0-20)[h]
Infiltrate	360 (130-680)[b]	28 (22-34)[f]	130 (0-280)[i]
Dermis	32 (1.8-99)	150 (110-220)	36 (0-43)
Normal			
Epidermis	71 (27-210)	0 (0-3.8)	0 (0-3.5)
Dermis	14 (5.5-38)	6.7 (5.0-14)	25 (11-36)

[a] p=0.0043; [b] p=0027; [c] p=0.024; [d] p=0.028; [e] p=0.0019; [f] p=0.0034; [g] p=0.0027; [h] p=0.024; [i] p=0.046. p values were calculated in comparison with normal skin. Values are median cell counts per mm^2. Minimum and maximum values are shown in parentheses. $CD3^+$ infiltrate cells are expressed as the percentage of positively stained infiltrate area. ACD, allergic contact dermatitis; CD, cluster of differentiation; ND, no data available.

The numbers of $CD1a^+$ (Langerhans) cells in ACD and psoriatic infiltrates were significantly larger than in normal skin. $CD3^+$ (T) cell numbers were significantly increased in the epidermis and infiltrates of both ACD and psoriatic skin. $Chymase^+$

mast cells were significantly increased in the infiltrates of both inflammatory skin diseases.

Additionally, the Tables VI and VII show the results of visual densitometry of immunostaining of markers specific for the proinflammatory cytokine interleukin(IL)-1α, the chemokine IL-8 and the regulatory cytokines IL-4 and interferon(IFN)-γ. These diffusely staining markers could not be quantified in a reliable and reproducible way by IA.

Table VI. Inflammatory cytokines in the epidermis of ACD, psoriatic and normal skin.

Localization	IL-1α	IL-8
ACD		
SB area fraction	4 (4-4)	0 (0-0)
SB intensity	1 (1-2)	0 (0-0)
SPG area fraction	4 (0-4)	2.5 (2-4)
SPG intensity	1 (1-2)	3 (1-3)
SC area fraction	4 (1-4)	0 (0-2)
Psoriasis		
SB area fraction	4 (4-4)	0 (0-0)
SB intensity	1 (1-1)[a]	0 (0-0)
SPG area fraction	3.5 (0-4)	3 (0-4)
SPG intensity	1 (0-1)	1.5 (0-3)
SC area fraction	ND	ND
Normal		
SB area fraction	4 (4-4)	0 (0-0)
SB intensity	3 (1-4)	0 (0-0)
SPG area fraction	4 (4-4)	3 (1-4)
SPG intensity	2 (1-2)	2 (1-4)
SC area fraction	4 (4-4)	1 (0-2)

[a] p=0.032. p values were calculated in comparison with normal skin. Values are median scores. Minimum and maximum values are shown in parentheses. ACD, allergic contact dermatitis; IL, interleukin; ND, no data available; SB, stratum basale; SC, stratum corneum; SPG, stratum spinosum and granulosum.

The intensity of IL-1α staining of the suprabasal keratinocytes was significantly decreased in psoriatic skin. This seemingly paradoxical finding can be explained by dilution of the staining due to the increased epidermal thickness and the high percentage of IL-1α$^+$ cells in psoriatic epidermis. A similar trend was seen in ACD. IL-8 staining was localized in the stratum spinosum and granulosum but not in the basal or suprabasal layer as has been reported by some authors [12, 13]. For IL-8, no significant differences between the inflammatory and normal control skin were seen.

Table VII. Regulatory cytokines in the epidermis and infiltrates of ACD, psoriatic and normal skin.

Localization		IL-4	IFN-γ
ACD			
Epidermis	- area fraction	0 (0-4)	0 (0-1)
	- intensity	0.5 (0-1)	1 (0-2)
Infiltrate	- intensity	2 (1-4)	2 (1-3)[a]
Psoriasis			
Epidermis	- area fraction	4 (0-4)	ND
	- intensity	1 (0-1)	ND
Infiltrate	- intensity	2 (1-2)	ND
Normal			
Epidermis	- area fraction	3 (0-4)	0 (0-1)
	- intensity	1 (0-2)	0 (0-1)
Infiltrate	- intensity	3 (0-3)	1 (0-3)

[a] p=0.046. p values were calculated in comparison with normal skin. Values are median scores. Minimum and maximum values are shown in parentheses. ACD, allergic contact dermatitis; IFN, interferon; IL, interleukin; ND, no data available.

The intensity of IFN-γ staining of infiltrate cells was significantly increased in ACD compared with normal skin. No significant differences were seen for IL-4.

DISCUSSION

The continuously dropping cost of computer hardware and the evolution of software towards easier, more intuitive graphical user interfaces has brought computer-assisted IA within the reach of almost every IHC researcher. However, reliable IA of immunostained slides requires sound knowledge of information theory and computer technology, besides of course familiarity with histology and IHC. We investigated the suitability of IA area measurement for application to immunostained inflammatory skin sections. The accuracy and reproducibility of IA area measurement was high, i.e. deviations of less than 0.5% and 1.0%, respectively. When compared to traditional grid-based area measurement, the mean values of the areas as measured by both methods were similar but the standard deviation was somewhat smaller for IA (Table IV). However, as the large section-to-section variation (Figure 4) may have troubled these results, the distribution of the differences between both methods was also analyzed. The mean difference was at most 11% of the measured area (Table IV). As an example, the IA area measurement procedure was applied to immunostained inflammatory skin sections (Table V). The procedure could also easily be modified to give a measure of spongiosis (a valuable parameter in inflammatory skin disease) by calculating the binary closed minus unclosed epidermal section area.

IA was used by a number of authors to acquire more consistent data, a.o. for cell counting and densitometric analysis of cytokine production at the single cell level [14-17]. When compared to the classical methods, like the use of grids and fields, IA of immunostained tissue enables accurate, reproducible and objective image measurement with minimal human interaction, less field selection or sampling bias, lower risk of errors due to manual copying of data and reasonable speed of execution [5, 7, 18, 19]. Additional advantages of digitized IHC images include degradation-free storing and copying (as opposed to deterioration of the original sections on glass slides), the opportunity to correct for slight image defects and the ability to do structural and statistical analysis of the image and three dimensional (3D) reconstruction of serial sections. An excellent review on quantitative IA was written by Oberholzer et al. [20]. More extended information can be found in textbooks on IA such as the ones by Russ [8] and Castleman [9].

Quantification of immunostaining by computer-assisted IA has been reported by a number of authors [7, 19, 21] and is said to have several advantages over more traditional methods: it is more objective, less laborious and faster than visual estimation and, like confocal laser microscopy, it preserves morphologic information when compared to biochemical assays and flow cytometry. However, in practice the use of computer-assisted densitometry for quantification of immunostaining of cytokines requires rigorous standardization of IHC (e.g. thickness of sections, mounting substance, permeabilization, type of fixation, species and isotype of antibody), microscopy (e.g. light level and focus of the microscope) and of IA (e.g. choice of segmentation thresholds in RGB or HSL color space). Also, IA cannot eradicate the difficulties associated with quantification of cytokines that cause diffuse staining. Jagoe et al. have criticized uncontrolled thresholding, particularly in singlemodal image histograms, showing significant intra- and interobserver variation in the range of gray values labeled as 'positively stained' [22]. The importance of a well-defined and objective quantification-protocol, explained in detail in the methods section, cannot be overestimated. In our hands and with available hard- and software, direct quantification of immunostaining intensity of cytokines in inflammatory skin sections proved unreliable.

In conclusion, IA is a useful and exciting addition to IHC. Although quantification of immunostaining *intensity* by IA remains difficult and requires thorough standardization of both IHC and IA, the IA procedure we describe enables measurement of inflammatory skin section areas, that is more accurate, reproducible and objective than conventional area measurement methods.

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The first and second author have equally contributed to this manuscript.

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CHAPTER 3

STUDIES ON ALLERGIC DRUG ERUPTIONS

CHAPTER 3.1

ADVERSE DRUG REACTIONS, WITH EMPHASIS ON ALLERGY TO ANTI-EPILEPTIC DRUGS*

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Introduction

Anti-epileptic drugs (AED) are for more than one reason an important group of drugs with respect to adverse drug reactions. Long-term use, multi-drug therapy, structural similarity (cross-reactivity; mimicry), and a relative lack of alternatives, make the occurrence of adverse reactions to AED a difficult condition. Moreover, the choice of an AED may be largely determined by the incidence of the adverse effects as the effectiveness of most AED is comparable [1].

Roughly three to 30 percent of skin eruptions due to AED (depending on the source) are reported to have an allergic origin [2-8]. In practice, many severe reactions, which are frequently accompanied by fever, impaired liver and renal function, appear to be immunologically mediated [9-14]. For some life-threatening skin reactions (e.g. Stevens-Johnson syndrome and toxic epidermal necrolysis), AED are among the most important perpetrators [15, 16]. Therefore, research into the mechanisms underlying these conditions is of great practical importance.

The AED carbamazepine, phenytoin, barbiturates and valproate are frequently used and responsible for the majority of adverse drug reactions [1, 17]. Recently, however, new AED like vigabatrin, lamotrigin, oxcarbazepine, felbamate and gabapentin have appeared. These drugs are claimed to match good effectiveness to rare adverse effects [18-20]. Since these drugs have been available for only a few years, further experience with these drugs in a larger population is needed to prove this claim to be true or false.

Clinical manifestations of adverse reactions to anti-epileptic drugs

Skin rashes are the most pronounced adverse reactions to AED, besides fever, lymphadenopathy, hematological abnormalities and impaired liver function. The diversity of dermatological appearances is summarized in Table I [6, 21].

Table I. Skin eruptions due to anti-epileptic drugs.

- maculopapular exanthema
- urticaria
- eczematous reactions
- pruritus
- erythema exsudativum multiforme / Stevens-Johnson syndrome
- exfoliative dermatitis
- drug induced lupus erythematosus
- alopecia
- lichenoid eruptions

In general, the risk of adverse drug reactions, both allergic and non-allergic, increases with age due to physiological changes of pharmacodynamics and -kinetics [15, 22], and as a result of age-related risk factors, like concurrent disease and exposure [23-26].

A number of specific AED and their accompanying skin rashes will be discussed in some detail below.

Carbamazepine (CBZ)
The long-standing experience with CBZ has provided a rich literature about the skin rashes it may induce. Skin rashes have been reported in 3 to 20 percent of patients using CBZ [3, 4, 7, 8]. The variability of the data is partly due to differences in study design, and the definition of the rashes included. Erythematous, morbilliform and urticarial eruptions as well as purpura are the most frequently reported. Toxic epidermal necrolysis (TEN) and exfoliative dermatitis have also been described. Eczema, photoreactivity, drug-induced lupus erythematosus (LE), erythema exsudativum multiforme and pustular reactions are less frequently encountered [21, 27, 28]. Hematological abnormalities like thrombocytopenia and leukopenia, and liver function disorders are also commonly reported [7, 29, 30]. The severe anti-convulsant hypersensitivity syndrome (AHS), described below in greater detail, can also be induced by CBZ [31]. Cross-reactivity between CBZ and the more recently introduced oxcarbazepine has been described, especially in patients with severe skin eruptions [18, 19, 32].

Phenytoin
A transient maculopapular exanthema is commonly seen during the first three weeks after the onset of phenytoin therapy. Pseudolymphomatous reactions have also been described. During long-term use gingival hyperplasia and even acromegalic features may develop due to excessive proliferation of fibroblasts. Erythema exsudativum multiforme, toxic epidermal necrolysis, drug-induced LE and fixed drug eruptions are also commonly seen [16, 27, 28]. Phenytoin can also induce AHS.

Valproate
Transient exanthemas are occasionally reported during initial valproate treatment. Induction of drug-induced LE and haematological abnormalities, like thrombocytopenia and macrocytosis, have also been described. Usually these symptoms disappear after dose reduction or cessation of the drug [13, 28, 33].

Barbiturates
Fixed drug eruptions, especially of the glans penis, are commonly reported during barbiturate treatment. Allergic reactions, like immunologically mediated urticaria and serum sickness, are seen less frequently. However, fatal cases of exfoliative dermatitis and erythema exsudativum multiforme, as well as toxic epidermal necrolysis, purpura and photoreactivity have also been reported. Barbiturates can also induce AHS. Phenobarbital and benzodiazepines have, unlike the above-mentioned AED, not been reported to induce LE with characteristic clinical and laboratory abnormalities[13, 27, 28].

Clinical manifestations of the anti-convulsant hypersensitivity syndrome (AHS)
Especially aromatic AED, like phenytoin, CBZ and the barbiturates can induce

AHS [34]. The syndrome usually starts with fever after a mean period of three weeks of drug intake. Next, lymphadenopathy and skin rashes occur, that may vary from mild exanthemas to life threatening skin conditions like toxic epidermal necrolysis. Subsequently, damage to internal organs, like hepatitis (50%), nephritis (10%) and hematological abnormalities (a.o. atypical lymphocytosis and eosinophilia) develop. Pseudolymphoma may also be part of the syndrome [12]. Usually, cessation of the causative drug does not lead to prompt disappearance of the disorder and prednisone therapy is required.

Pathogenesis of adverse reactions to anti-epileptic drugs

The pathogenesis of adverse reactions to AED may be based on immunological or non-immunological responses to the drug itself or one or more of its (reactive) metabolites.

Individual (genetic) diversity in the metabolism of AED may cause accumulation of toxic metabolites [35, 36]. For example, cyclic AED, like phenytoin, phenobarbital and CBZ, are metabolized by cytochrome P-450 in the liver. In this process epoxides are formed which can be reactive or induce an immune response [10]. Generally, these epoxides are broken down by epoxide hydrolases. Deficiency of these enzymes can lead to adverse effects due to accumulation, especially in the case of phenytoin. The underlying mechanism is probably idiosyncrasy, a qualitatively abnormal, unexpected response to a drug, differing from its pharmacologic actions [23].

Concurrent treatment with phenytoin, cimethidin and glucocorticoids, as sometimes used for epilepsy due to intracranial processes, may cause thrombocytopenia. This can be ascribed to accumulation of toxic metabolites as a result of downregulation of the epoxide-hydrolases by glucocorticoids [37].

As CBZ is known to induce its own detoxification enzymes, adverse effects due to toxic metabolites can usually be prevented by gradual introduction of the drug. However, reactive metabolites, formed from CBZ by the myeloperoxidase(MPO)-system of activated leukocytes, have been reported as a possible cause of agranulocytosis and lupus erythematosus [38]. In the metabolic pathway of oxcarbazepine (OCBZ, a CBZ-derived AED) no epoxides are thought to be formed. This may explain why adverse effects due to OCBZ are less frequently seen. However, the blood level of concomitant drugs may rise if OCBZ substitutes CBZ, as the former does not induce detoxification enzymes.

Immune mediated reactions

AED can influence both antigen(Ag)-dependent and Ag-independent immune reactivity. They may induce allergic reactions, auto-immunity, pseudolymphomatous reactions, and non-specific changes of the humoral and cellular immune response [12-14, 39].

Drug allergy can be defined as an immunologically mediated reaction in which the drug acts as an allergen. For proper induction of an allergic drug reaction, most

AED need to bind to carrier molecules (glycoproteins) first. Subsequently, the hapten-carrier complex or 'complete Ag' is presented to T cells by Ag presenting cells. If the Ag is recognized, proliferation and formation of memory T cells follows. As of now the patient is sensitized and renewed contact with the allergen may lead to an allergic reaction according to one or more immunological reaction types originally described by Coombs and Gell. These reaction types were described in greater detail in the chapters 1.1 and 1.3. In addition, drug-induced adherence between leukocytes and keratinocytes may be an important initial step in the pathogenesis of immunological cytotoxicity [40, 41].

Diagnosis of adverse reactions to anti-epileptic drugs
Routine laboratory tests cannot (yet) reliably predict the likelihood of drug allergy in a single patient [42]. Nevertheless, the distinction between allergy and non-immunological reactions is of great importance for future drug use.
Clinically, serious reactions can often be recognized on the basis of simultaneous occurrence of skin eruptions, fever and involvement of internal organs [43].

Although epicutaneous patch tests are sometimes considered to be of little use for detection of allergy to systemically used drugs, they are definitely useful for detection of allergy to CBZ (and possibly other AED) [8, 34, 44, 45]. Additionally, the lymphocyte proliferation assay (LPA), if correctly performed, is an important technique to confirm immunological reactivity [34, 45]. To avoid false-negative test results, it is recommended to perform the LPA at least 2 months after the period of clinical disease [34, 45].

In patients with a genetic deficiency of detoxification enzymes, accumulation of reactive metabolites can be detected by a cytotoxicity test [10]. In this test, lymphocytes of a patient are exposed *in vitro* to toxic epoxides preformed by cytochrome P-450. If epoxide hydrolases are deficient in these lymphocytes, cell death can occur as a result of accumulation of toxic epoxides. The enzyme deficiency can then be quantified as a percentage of cytotoxicity. Routine use of this test is not recommended since the prevalence of genetic enzyme deficiency is low and the costs of the cytotoxicity test are high [36].

Therapy
In most cases of adverse reactions to AED, prompt withdrawal (if possible) of the causative drug will halt the response. The AHS may, however, remain well after stopping the causative drug.

If drug allergy is likely, an alternative non-cross-reactive AED should be sought. The newly developed AED, representing additional therapeutic possibilities with possibly fewer adverse reactions, may lighten this task. The risk of 'status epilepticus' is not high if adequate alternative drug therapy is instituted [6].

Especially allergic drug reactions may require additional treatment. Antihistamines may be helpful to control pruritis. Effective treatment of drug-induced serum sick-

ness may require the use of corticosteroids and antihistamines. Corticosteroids are also needed for severe or progressive drug eruptions. Severe reactions like anaphylaxis require the combined use of corticosteroids, antihistamines and sympaticomimetics.

Desensitization has been successful for allergy to bee and wasp venom and some drugs (e.g. penicillin and insulin). The mechanism of desensitization is unclear but generation of 'blocking' IgG antibodies and gradual and subclinical mast cell degranulation have been suggested [26]. The procedure is not without risk and should only be tried if no alternative is available [24, 46]. Reports of successful clinical hyposensitization with CBZ have been sporadic [47-49]. This may be due to differences in the allergic reaction types involved in allergy to CBZ as opposed to allergy to bee or wasp venom.

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CHAPTER 3.2

ALLERGY TO CARBAMAZEPINE: PARALLEL *IN VIVO* AND *IN VITRO* DETECTION*

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ABSTRACT

Purpose: Five to 20% of patients discontinue antiepileptic drug (AED) therapy because of adverse reactions. Careful reintroduction, however, may be considered if true drug allergy can be ruled out. Definitive assessment of such immunologically mediated reactions requires demonstration of either specific antibodies or sensitized lymphocytes.
Methods: We investigated whether skin patch tests (PT) and *in vitro* lymphocyte proliferation assays (LPA) were suitable for detection of allergy to carbamazepine (CBZ) and the possibly cross-reactive oxcarbazepine (OCBZ). Data of 65 patients displaying a wide range of possibly allergic side effects to CBZ were available for analysis. CBZ users without any side effects and healthy volunteers served as controls. Both PT and LPA were done with CBZ, OCBZ and three metabolites [CBZ-10,11-epoxide (CBZ-E), 10-monohydroxy-CBZ (MHD), and 10,11-dihydroxy-CBZ (DIOL)].
Results: Positive PT with CBZ were seen in 20% and with OCBZ in 14% of the patients. Positive LPA results with CBZ and OCBZ, respectively, were found in 40 and 19%. Both tests were positive in 14 and 7% of the patients. Cross-reactivity to OCBZ was seen in ~ 40% of CBZ-reactive patients in both PT and LPA.
Conclusion: These data illustrate the additional value of LPA in the detection of CBZ allergy while showing that a major part of side effects to CBZ and OCBZ is not immunologically mediated, according to PT and LPA.

INTRODUCTION

Discontinuation of antiepileptic drug (AED) intake because of possibly allergic side effects is necessary in 5-20% of patients [1-5]. In some cases these reactions are even life threatening [6-9]. Especially severe adverse reactions are thought to be immunologically mediated [10-15].
Careful analysis of adverse drug reactions is needed to determine their nature as this will influence future drug administration [16]. Cautious readministration may even be considered if true (immunologically mediated) drug allergy can be ruled out. Rechallenge may confirm the allergic nature of adverse effects, but it bears the risk of severe reactions [17]. Therefore, safe and reliable methods are urgently needed to make a clear distinction between drug allergy and non-allergic responses. Moreover, recognition of cross-reactivity may help in selecting an alternative drug. Skin patch tests (PT) and lymphocyte proliferation assays (LPA) have previously been advocated to establish the immunologic basis of adverse reactions [4, 8, 18-26]. Most studies have been performed by using a limited number of metabolites and a small number of patients usually with severe symptoms. For thorough evaluation of the test it is essential to test a large number of individuals comprising a wide spectrum of clinical signs and symptoms of possibly allergic nature. We report the results of parallel skin PT and LPA for the detection of carbamazepine (CBZ) allergy and oxcarbazepine (OCBZ) cross-reactivity. All currently available metabolites were tested using a large number of patients receiving CBZ therapy with a wide range of side effects. We have studied allergy to CBZ as it is widely used and well documented to cause allergic cutaneous eruptions in 3-20% of its users [1-4, 21, 27, 28].

METHODS

Patients

Data from 65 patients with epilepsy displaying a wide range of side effects to CBZ were collected for analysis. Skin rashes consisted of mild maculopapular, urticarial, erythematous, exfoliative and eczematous skin reactions. Systemic manifestations included fever, hematologic abnormalities (leukocytopenia and thrombocytopenia), lymphadenopathy and impaired liver function. Fifteen CBZ users without adverse effects and six healthy individuals served as controls.

Clinical evaluation

A standardized checklist was used for documentation of age and gender of the patient, clinical signs and symptoms of the adverse reaction and its treatment, duration of prior CBZ intake, current medication, and previous allergies or atopy of the patient and his or her family.

Patch tests

Patch tests (PT) were performed according to international standard procedures [29]. Pure CBZ, its main metabolite CBZ-10,11-epoxide (CBZ-E, GP49.023), oxcarbazepine (OCBZ, GP47.680), its active metabolite 10-monohydroxy-CBZ (MHD, GP47.779) and the common metabolite 10,11-dihydroxy-CBZ (DIOL, CGP10.000) as well as ground CBZ (Tegretol®) and OCBZ (Trileptal®) tablets (Ciba Geigy, Basel, Switzerland) were used. Figure 1 depicts these metabolites in the metabolic pathway of CBZ and OCBZ [30]. Of all substances 5 milligrams was retained in PT chambers (van der Bend, Brielle, The Netherlands) with 50 µl ethanol 70% and applied to the left side of the back. After 2 days the chambers were removed and the skin reaction was scored at 48 and 72 hours.

Lymphocyte proliferation assays

Sodium-heparinized venous blood samples were drawn before application of the PT. Peripheral blood mononuclear cells (PBMC) were isolated from these samples by using Ficoll-Paque (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density-gradient centrifugation. After washing, cells were suspended in complete RPMI 1640 medium (supplemented with 20 mM Hepes, L-glutamine, antibiotics and 15% heat-inactivated pooled human serum), and seeded at 2x10^5 cells/well in 96 wells round-bottom culture plates (Falcon, San Jose, CA, USA). Stock solutions of CBZ, OCBZ and the three metabolites were prepared at concentrations of 5-15 mg/ml in pure ethanol (pro analysi 99.8%, Merck, Darmstadt, Germany). Previous testing showed that final concentrations of 5, 10 and 15 µg/ml in culture medium were optimal. The levels are grossly comparable to usual serum levels. Tetanus toxoid (3 Lf/ml, RIVM, Bilthoven, The Netherlands) was used as a positive control. After 6, 7 and 8 days, respectively, 0.5 µCi ^{3}H-TdR (Amersham International, Amersham, UK) was added to each well. After 8 hours, cells were harvested by using an automatic cell harvester (Skatron, Lier, Norway). Tritiated thymidine incorporation was measured with a BetaPlate liquid scintillation counter (LKB Wallac, Turku, Finland). All cultures were done in quadruplicate. Lymphocyte proliferation was expressed as counts per mi-

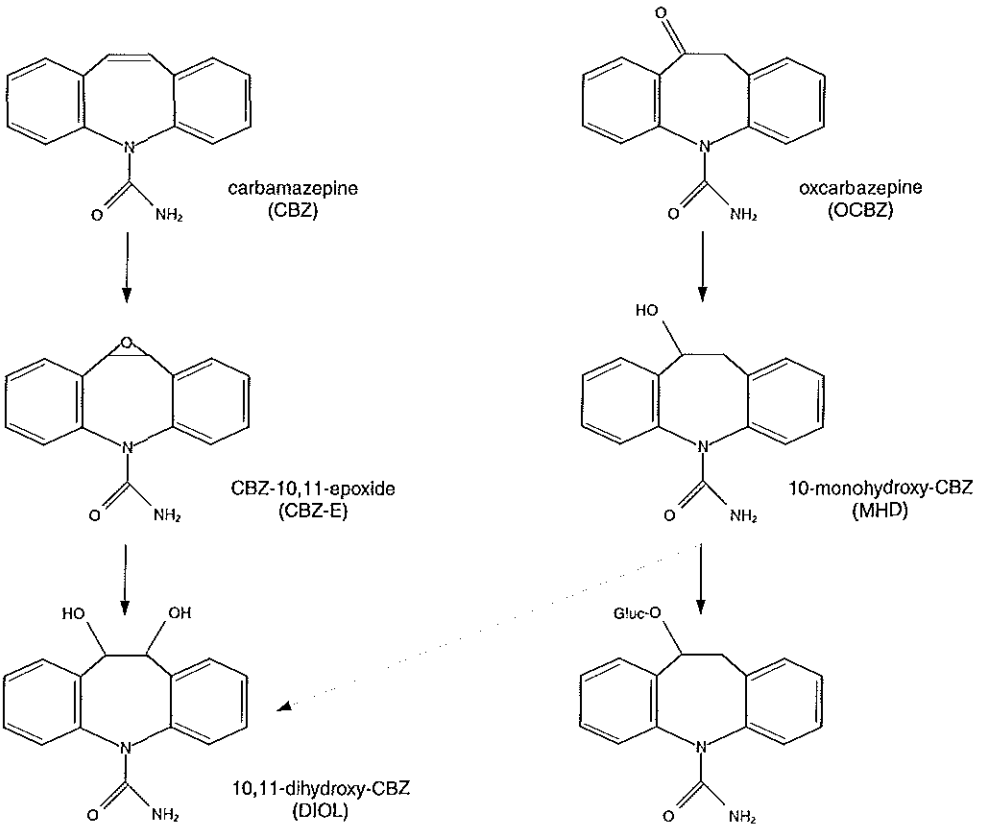

Figure 1. Metabolism of carbamazepine and oxcarbazepine.

nute (cpm). Stimulation indices (SI; i.e. relative proliferation) were calculated by dividing maximum specific proliferation by background proliferation. SI >3 were considered indicative of prior lymphocyte sensitization. Values between 3 and 10 were considered low positive, those >10, high positive.

Statistical analysis

Professional STATA 3.0 Statistics/Data Analysis (Computing Resource Center, Santa Monica, CA, USA) was used for data analysis and statistical calculations. Means and standard deviations were calculated from quadruplicate cultures. Differences between the patient and control groups were tested for significance using the Wilcoxon Rank-Sum Test for numerical data and χ^2 and Fisher's exact tests for categorical data. Spearman's rank correlation coefficients were calculated as a measure of association between parameters [31, 32]

RESULTS

Clinical evaluation

The clinical features of the patients and controls are summarized in Table I. The median duration of prior CBZ intake was 1 month. Skin rashes including mild maculopapular, urticarial, erythematous, exfoliative and eczematous skin reactions constituted a major part of the reactions [overall 60 (92%) of 65 cases]. Systemic manifestations such as fever, hematologic abnormalities (leukocytopenia and thrombocytopenia), lymphadenopathy or impaired liver function were documented in 14 (22%) of 65 cases. These rather high frequencies reflect bias due to selective referral.

Table I. Clinical features of patients and controls tested for CBZ allergy.

	Patients with side effects	Controls
No. of patients	65	21
M/F-ratio	1:2.0	1:1.9
Age (yr)[a]	31 (4-82)	28 (19-68)
Drug intake prior to side effects (mo)[a]	1 (0.1-240)	-
Skin reactions	92%	0%
Systemic manifestations[b]	22%	0%

[a]median (range); [b]fever, hematologic abnormalities, lymphadenopathy or impaired liver function; CBZ, carbamazepine.

Patch tests

The concordances between patch tests with pure CBZ and OCBZ on the one hand and ground Tegretol and Trileptal tablets on the other, were 95 and 92%, respectively, the tablets giving slightly more positive tests. In Table II, the results of PT with CBZ and OCBZ among patients with side effects and controls are summarized. The difference between both groups was not statistically significant (CBZ: p=0.4; OCBZ: p=0.19). Positive PT with CBZ and OCBZ were found in 12 (20%) of 61 and 8 (14%) of 59, respectively. Cross-reactivity to OCBZ was found in five (45%) of 11 of CBZ-reactive cases. When metabolites of CBZ and OCBZ were also taken into account, the frequency increased to 55 percent. No side effects of the patch tests were noticed.

Lymphocyte proliferation

In Figure 2, results of the LPA among suspected patients and controls are summarized. Stimulation indices varied between 0.1 and 100. Highly significant differences were seen with CBZ, CBZ-E and OCBZ (p=0.0038, p=0.0020 and p=0.0010, respectively). Reactivity to CBZ-E was comparable to its precursor CBZ. OCBZ displayed an inhibitory effect on the proliferation of PBMC *in vitro*, as it frequently gave rise to SI <1. Stimulation with OCBZ was also significantly less than with MHD, its active metabolite. *In vitro* lymphocyte reactivity to CBZ and OCBZ was found in 26 (40%) of 65 and 12 (19%) of 64, respectively. Among the controls, one patient using CBZ

showed low reactivity to CBZ. For MHD an SI cut-off level >3 may be desirable. *In vitro* cross-reactivity to OCBZ was seen in 10 (40%) of 25 of CBZ-reactive cases. This value increased to 73% if metabolites of both drugs were also taken into account.

Table II. Distribution of skin PT reactions to CBZ and OCBZ among patients with side effects to CBZ and controls.

Drug	PT-score[a]	Patients with side effects	Controls
CBZ[b]	-	49	10
	+	3	0
	++	8	1
	+++	1	0
OCBZ[c]	-	51	11
	+	5	0
	++	2	0
	+++	1	0

[a]patch test score according to international guidelines; [b]p=0.40; [c] p=0.19; CBZ, carbamazepine; OCBZ, oxcarbazepine; PT, patch test; LPA, lymphocyte proliferation assay.

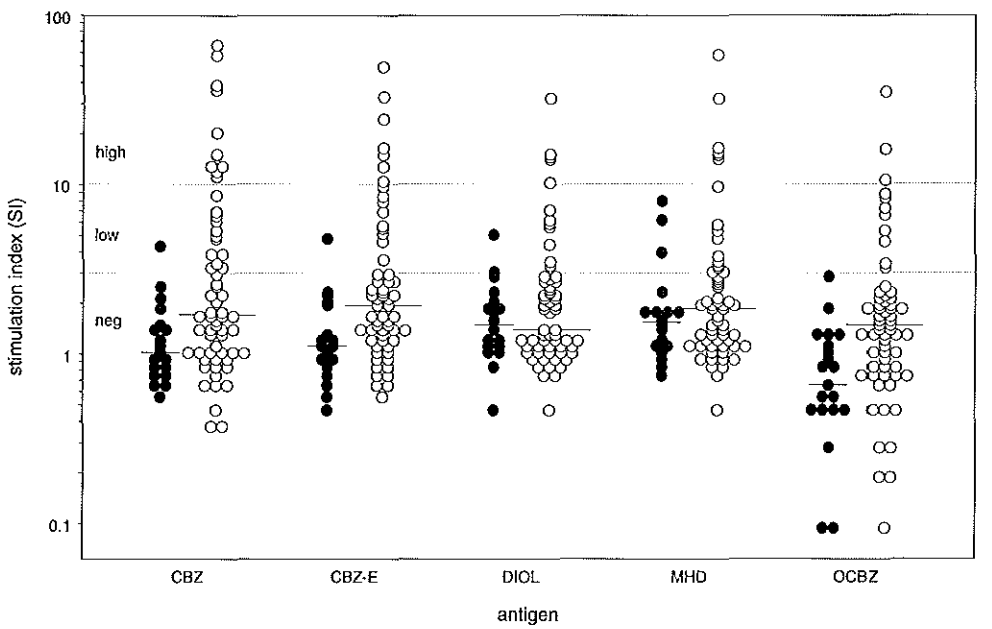

Figure 2. Distributions of stimulation indices (SI) with carbamazepine (CBZ, p=0.0038), its metabolite CBZ-10,11-epoxide (CBZ-E, p=0.0020), the common metabolite 10,11-dihydroxy-CBZ (DIOL, p=0.56), the OCBZ metabolite 10-monohydroxy-CBZ (MHD, p=0.26) and oxcarbazepine (OCBZ, p=0.0010) among patients with side effects (open circles) and controls (closed circles). Medians are represented by horizontal lines.

Table III shows PT and LPA results for CBZ and OCBZ. Simultaneous positivity in both tests was seen in eight (14%) of 59 with CBZ and four (7%) of 56 with OCBZ. The correlation between the results of PT and LPA, although statistically significant, was rather low (r=0.39, p=0.0022) for both CBZ and OCBZ.

Table III. Patch test and LPA for CBZ and OCBZ.

	Patch test		Totals
	-	+	
CBZ LPA			
SI ≤ 3	33	3	36
SI > 3	15	8	23
Total	48	11	59
OCBZ LPA			
SI ≤ 3	41	3	44
SI > 3	8	4	12
Total	49	7	56

LPA, lymphocyte proliferation assay; CBZ, carbamazepine; OCBZ, oxcarbazepine; SI, stimulation index.

In Figure 3 the distribution of the sensitization period (i.e. time between start of CBZ use and the occurrence of side effects) and its relation with CBZ-specific lymphocyte proliferation is depicted. Almost all reactions associated with a positive LPA were seen within the first 3 months of drug intake. Of the reactions appearing after 3 months, only one resulted in low reactivity in the LPA.

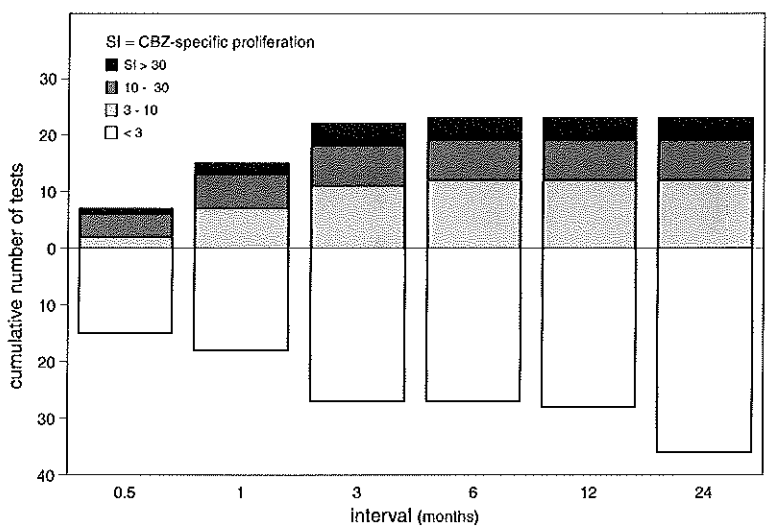

Figure 3. Distribution of sensitization period and its relation with carbamazepine-specific lymphocyte proliferation (white: negative LPA; light grey: low positive; dark grey: high positive; black: very high positive).

Figure 4 shows the distribution of the test interval (i.e. time between the occurrence of side effects and performance of LPA) and its relation with CBZ-specific lymphocyte proliferation. CBZ-specific lymphocytes could be detected between one month and as long as 10 years after the initial sensitization. Very high *in vitro* reactivity to CBZ (SI≥30) was not seen until 3 months after clinically active drug reactions.

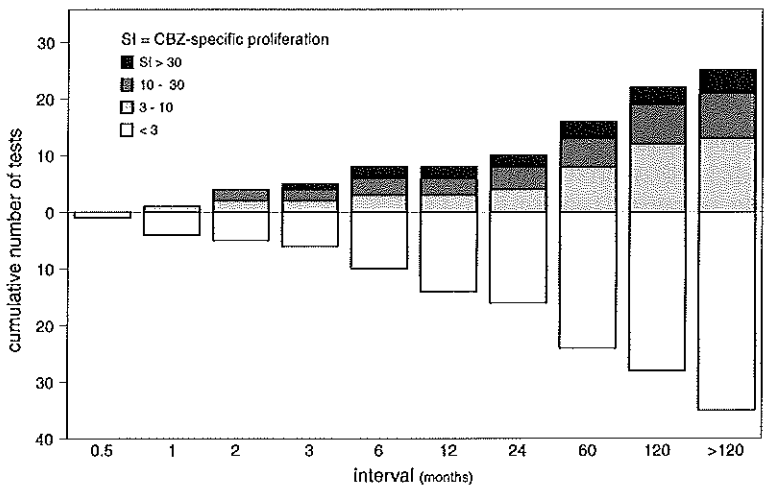

Figure 4. Distribution of test interval and its relation with CBZ-specific lymphocyte proliferation (white: negative LPA; light grey: low positive; dark grey: high positive; black: very high positive).

DISCUSSION

In clinical practice, frequently the dilemma exists whether to stop drug intake if allergy to that drug is suspected. As the severity of allergic responses tends to increase after repeated boostering, it is vital to stop drug intake as early as possible until the nature of the adverse reaction is unraveled. If true drug allergy can be ruled out, reintroduction may be considered. The choice of an alternative drug may be helped by *in vitro* detection of cross-reactivity. A safe and reliable method is therefore needed to detect allergic responses and cross-reactivity. With AED, long-term use, combination therapy, structural similarity (cross-reactivity), and a relative lack of alternatives, stress the need for such diagnostic tests. One of the principal *in vitro* assays to assess cell-mediated immune reactivity is the antigen-specific LPA. Our study concerned the parallel use of skin PT and LPA with all available metabolites for the detection of CBZ allergy in a large group of patients with a wide range of side effects during CBZ therapy.

We found 20% positive PT to CBZ, whereas *in vitro* lymphocyte proliferation was positive in 40%. Among the CBZ-using controls one low-positive SI was seen. Experience with the LPA in nickel allergy has shown that this may imply early subclinical sensitization.

The stable 10,11-epoxide of CBZ (not an arene oxide) has been thought to be responsible for many adverse reactions including immunologically mediated reactions. However, we did not see either a larger number of positive tests or a higher reactivity with CBZ-E as compared to CBZ. This confirms previous *in vivo* and *in vitro* observations [11, 33]. The role of other short-lived epoxides of CBZ, which are known to be formed *in vivo* [34], remains unclear because these metabolites have not been available for testing.

OCBZ has previously been described as equally effective as CBZ, but with a better tolerability and a favorable interaction profile, possibly due to its different metabolism [30, 35-43]. *In vitro*, however, OCBZ was found to inhibit lymphocyte proliferation. The rapid *in vivo* metabolism of OCBZ into MHD may explain this discrepancy.

In vivo and *in vitro* cross-reactivity of OCBZ in CBZ-reactive patients has been reported in 15-25% [21, 40]. With regard to chemical cross-reactivity between aromatic AED, frequencies as low as 0 and as high as 80% [11, 12] have been described. We found ~ 40% cross-reactivity in both PT and LPA.

The finding of a sensitization period with a maximum of 3 months is in line with previous studies [2, 3, 11, 16] and stresses the diagnostic importance of this temporal relation. The distribution of the test interval showed a 'refractory period' of ~ 1 month, during which false-negative test results may occur. The interval appears to be longer after strong sensitization. Such a refractory period has been noted before and was ascribed to 'impaired lymphocyte responsiveness' [20]. However, we propose that selective recruitment of antigen-specific lymphocytes into target organs, transiently leading to low specific T cell numbers in the peripheral blood, is the cause of this phenomenon. Remarkably, with drug-allergic hepatic injury, quite the opposite was recently described, resulting in an advice to test within 1-3 weeks after onset of the event and retest for negative result after 3-6 months [44].

Finally, a flowchart for diagnosing AED allergy is proposed in Figure 5. Test characteristics of both PT, LPA and cytotoxicity assays have been taken into account. The chart starts with a suggestive history or physical examination or both providing a reasonable working diagnosis. The importance of clinical information as a starting point for further investigation needs to be stressed. Clinical criteria helpful in distinguishing allergic from other adverse reactions have been reviewed elsewhere [9, 16, 45]. Patch testing, if possible with both the pure drug as well as the used formulation, is then suggested as a quick and cheap first step. LPA can then be done if a patient with a suspect history has a negative PT. As cytotoxic reactions are probably not detected in our tests, an assay that detects deficiency of detoxification enzymes will be needed to recognize these reactions [11,12]. The combination of serious adverse effects with both negative PT and LPA results may call for such an *in vitro* cytotoxicity assay.

After this approach several diagnostic categories are possible: non-allergic reaction or allergic reaction with or without a deficiency of detoxification enzymes. Therapeutic options include (a) choice of an alternative non-cross-reactive anti-epileptic drug, (b) repetition of the diagnostic sequence if false-negative results are suspected, (c) careful reintroduction of the drug involved, and (d) in some sporadic cases, if no alternative is available, desensitization [46-49].

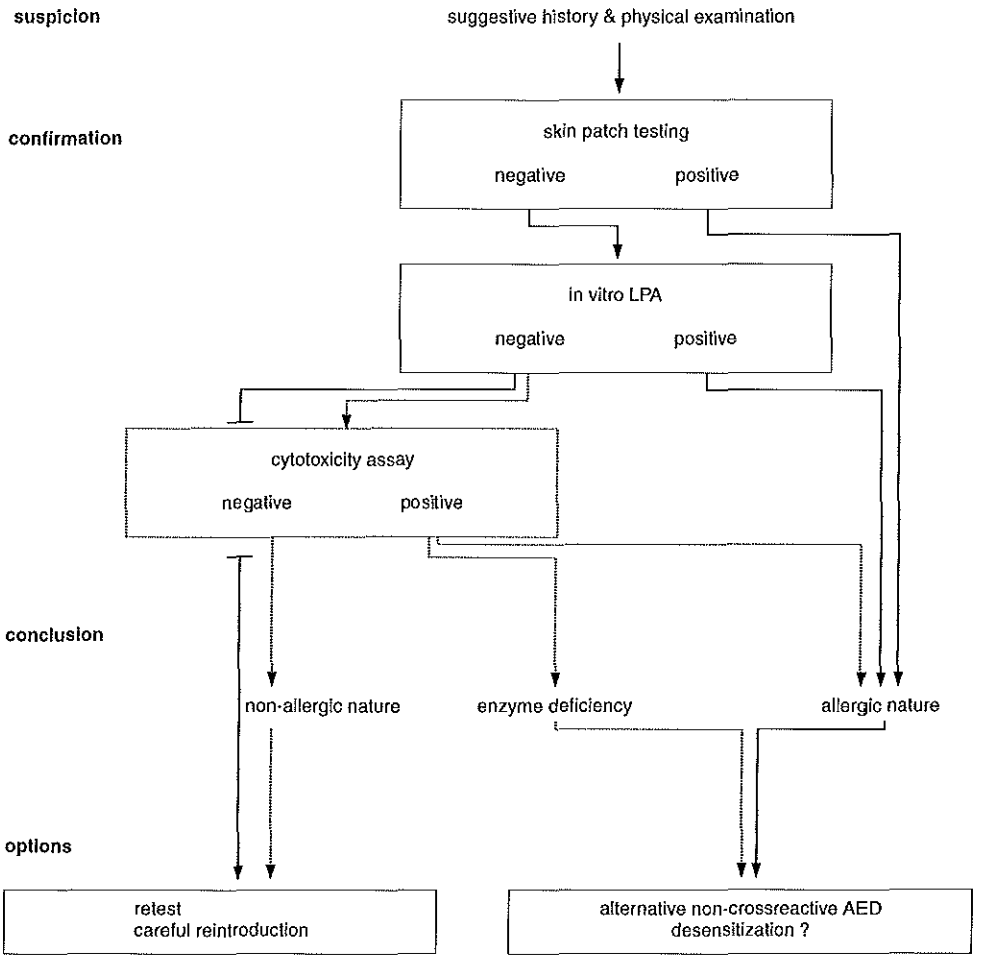

Figure 5. Suggested flowchart for diagnosing antiepileptic drug allergy.

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CHAPTER 3.3

EXFOLIATIVE DERMATITIS DUE TO IMMUNOLOGICALLY CONFIRMED CARBAMAZEPINE HYPERSENSITIVITY*

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Ped Dermatol 1996;13:316-320

ABSTRACT

A 6-year-old Caucasian girl experienced a generalized erythematous skin rash during carbamazepine therapy. In the next four days the eruption worsened into erythroderma with fever and generalized lymphadenopathy. Routine laboratory studies revealed increased serum levels of liver enzymes and eosinophilia. Immunologic reactivity to the anticonvulsant carbamazepine was confirmed both *in vivo* and *in vitro* by patch tests and lymphocyte proliferation assays, respectively.

INTRODUCTION

The anticonvulsant hypersensitivity syndrome (AHS) is generally characterized by fever, skin rash, lymphadenopathy and hepatitis that develop between three weeks to three months after initiation of antiepileptic drug therapy, especially aromatic agents. Cutaneous manifestations are variable and include erythema, a maculopapular eruption, erythema multiforme and even exfoliative dermatitis. Immunologic mechanisms are thought to play a major role in the pathogenesis of this syndrome, but are rarely verified by appropriate testing. We combined clinical and immunologic analyses to confirm immunologic reactivity to the anticonvulsant carbamazepine in our patient. Although the term 'hypersensitivity' is widely used, we prefer the word 'allergic' as it indicates an underlying immunologic mechanism whereas 'hypersensitivity' does not.

CASE REPORT

A 6-year-old Caucasian girl was admitted for a generalized skin eruption with fever, abdominal pain, vomiting and diarrhea of two weeks duration. As scarlet fever was suspected, oral penicillin V therapy was given, but without effect. Next, oral cefuroxim was given for four days. Nevertheless, the rash evolved into an erythroderma and the patient had a fever of 39 °C. She suffered several epileptic insults and refused to drink. Carbamazepine 400 mg/day had been started for partial epilepsy four weeks earlier.

Clinical examination revealed a pulse rate of 112/minute, blood pressure 110/70 mm Hg, proper hydration and consciousness, occipital lymphadenopathy, palpable liver and spleen, and generalized blue-red erythema of the skin and conjunctivae with edema of the hands, feet and periorbital areas (Figure 1). The conjunctivae were red but the mucous membranes were not involved.

Laboratory data

Relevant study results included hemoglobin 7.7 mmol/L (normal 6.8-8.1 mmol/L); white blood cell count 35.8×10^9/L (normal $4.0\text{-}10 \times 10^9$/L) with eosinophilia 1.35×10^9/L (normal $0.050\text{-}0.250 \times 10^9$/L); aspartate aminotransferase 81 U/L (normal <30 U/L); alanine aminotransferase 59 U/L (normal <30 U/L); alkaline phosphatase 122 U/L (normal 80-225 U/L); γ-glutamyl transferase 113 U/L (normal <30 U/L); lactate dehydrogenase 1359 U/L (normal 160-360 U/L); creatinine 51 mmol/L

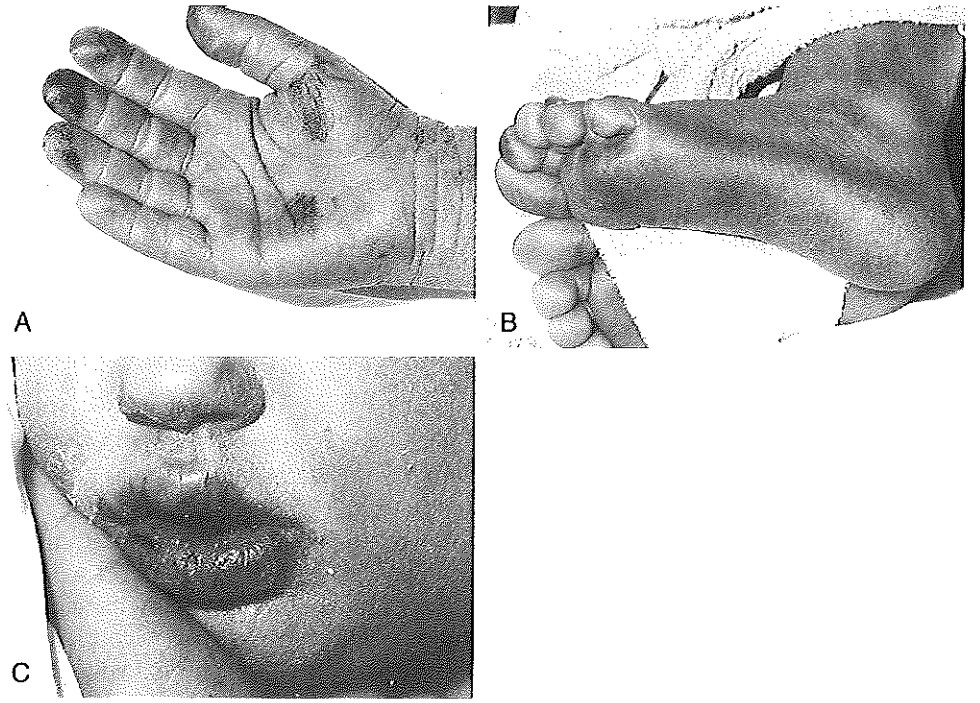

Figure 1. Erythema, edema and purpura of the hands (A), the feet (B) and exfoliative dermatitis with perioral crusts (C).

(normal 25-50 mmol/L) ; C_3 0.73 g/L (normal 0.83-1.59 g/L); C_4 0.08 g/L (normal 0.15-0.50 g/L). Other tests were normal. Viral and bacterial cultures and serology were all negative.

Diagnosis and treatment

Kawasaki disease was ruled out, as only three out of six classic criteria were present. Fever, edema and erythema, followed by scaling of the extremities, and hyperemic conjunctivae were present; redness of the lips and oropharynx, polymorphous exanthema with target lesions, and cervical lymphadopathy were not.

Carbamazepine was suspected as a possible cause, so it was stopped and replaced with oxcarbazepine. Oxcarbazepine is widely used outside the United States. Its efficiency and chemical structure are similar to those of carbamazepine, and cross-reactivity has been reported in 15-25% of patients [1-3]. The girl's erythema and scaling disappeared, her temperature dropped, and laboratory parameters improved. However, after 10 days the rash reappeared with similar clinical and laboratory characteristics and she experienced a remarkable loss of hair. Oxcarbazepine was stopped and phenytoin was started, and within three weeks the rash disappeared. After two months, patch tests (PT) and the *in vitro* lymphocyte proliferation assay (LPA) were done to confirm an immunologic basis for the skin eruption. Patch tests were performed according to international standard procedures [4] with pulverized tablets of carbamazepine, oxcarbazepine (Tegretol and Trileptal, Ciba Geigy, Basel, Switzer-

land) and phenytoin (Diphantoine; Katwijk, Katwijk, The Netherlands). Five milligrams of each substance was retained in PT chambers (van der Bend, Brielle, The Netherlands) with 50 µl ethanol 70% and applied to the left side of the back. Previous testing of 80 subjects showed this concentration to be nonirritating (Troost, unpublished observations). After two days the chambers were removed and the skin reaction was scored at 48 and 72 hours. The PT was positive with all three antiepileptic drugs (Figure 2).

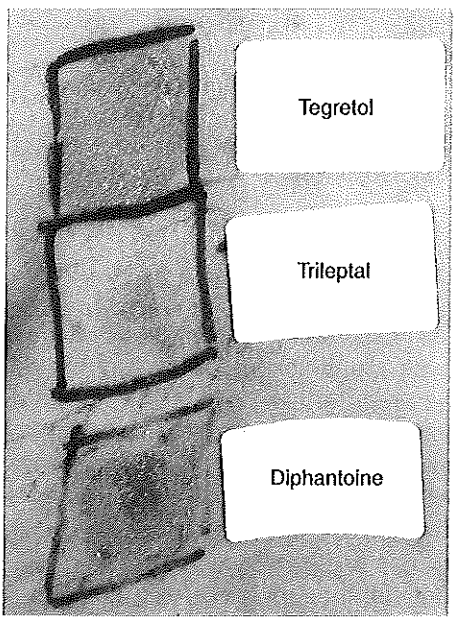

Figure 2. Positive patch test results with carbamazepine, oxcarbazepine and phenytoin.

In vitro LPA was performed with carbamazepine (CBZ), its main metabolite CBZ-10,11-epoxide (CBZ-E, GP49.023), oxcarbazepine (OCBZ, GP47.680), its metabolite 10-monohydroxy-CBZ (MHD, GP47.779) and the common metabolite 10,11-dihydroxy-CBZ (DIOL, CGP10.000)[5] as well as phenytoin (PHEN). Previous testing in allergic and nonallergic CBZ-users showed that 5 to 15 µg/ml were optimal *in vitro* concentrations for CBZ and metabolites, and 2.5 to 50 µg/ml for PHEN (Troost, unpublished observations). These concentrations are grossly comparable with the usual serum levels of these drugs. Tetanus toxoid (3 Lf/ml, RIVM, Bilthoven, The Netherlands) was used as a positive control.
Stimulation indices (SI, i.e. relative proliferation) were calculated by dividing maximum specific proliferation by background proliferation. An SI above 3 was considered indicative of prior lymphocyte sensitization. Lymphocyte reactivity to CBZ and its main metabolite CBZ-10,11-epoxide was found, but not to OCBZ or PHEN. The results of PT and *in vitro* LPA are summarized in Table I.
AHS with exfoliative dermatitis was considered the most likely diagnosis. Phenytoin was then instituted and was well tolerated.

Table I. Clinical response to, and results of, patch tests and lymphocyte proliferation assays with several anti-epileptic drugs.

Drug	Clinical response	Test results PT	LPA (SI*)
Carbamazepine	++	++	14.2
CBZ-epoxide	ND	ND	5.8
Dihydroxy-CBZ	ND	ND	1.3
Monohydroxy-CBZ	ND	ND	2.1
Oxcarbazepine	++	++	2.3
Phenytoin	-	+++	2.3

*An SI above 3 is considered positive. PT, patch test; LPA, lymphocyte proliferation assay; ND, not done.

DISCUSSION

Carbamazepine is an aromatic anti-epileptic drug that is widely administered and well documented to cause allergic skin eruptions in 3% to 20% of users [1, 6-11]. Exfoliative dermatitis was reported in 2.5% to 10% of dermatological reactions to CBZ [10, 12, 13]. Less frequently the rash is part of the AHS. The syndrome is characterized by fever, rash, lymphadenopathy, and hepatitis, usually occurring within three weeks to three months after the start of drug therapy. Other possible features are hematologic abnormalities, nephritis, edema, especially of the face, conjunctivitis, pharyngitis and allergic pneumonitis.

Severe adverse reactions are thought to be immunologically mediated [12, 14-18]. Careful analysis of these reactions is therefore necessary to determine their nature, as this influences future drug administration. Cautious readministration may be considered if true (immunologically mediated) drug allergy can be ruled out. Clinical criteria helpful in distinguishing allergic from other adverse reactions are reviewed elsewhere [19-21]. The temporal relationship between start of drug intake and appearance of allergic symptoms is often the most vital information in determining the causative agent. Although challenge tests may confirm the allergic nature of adverse effects, they bear the risk of severe reactions [22]. Skin PT and LPA can be performed safely to establish the immunologic basis of the effects [1, 11, 23-32]. In addition, detection of cross-reactivity may help in choosing an alternative drug.

Our patient was probably sensitized twice: first to CBZ and after another sensitization period of 10 days to OCBZ. Although PHEN cross-reactivity has been described, this drug was well tolerated by our patient. Patch tests confirmed the reactivity to both CBZ and OCBZ, but showed a positive skin reaction to PHEN as well. *In vitro*, however, no lymphocyte reactivity to PHEN was detected. Therefore, the patch test may have been a false positive result due to excited skin syndrome. This could not be confirmed, however, as the parents of the child refused further testing. The patient was diagnosed as having AHS due to CBZ.

In vitro LPA confirmed reactivity to CBZ and CBZ-10,11-epoxide. This stable epoxide is thought to be responsible for many adverse reactions, including the immunologically mediated reactions.

Previously, OCBZ was described as equally effective as CBZ and better tolerated, and having a favorable interaction profile, possibly due to its different metabolism [3, 5, 33-40]. *In vivo* and *in vitro* cross-reactivity of OCBZ was reported in 15% to 25% in CBZ reactive patients [1, 3]. Even higher frequencies are seen after serious skin eruptions [12]. Our patient showed reactivity to both CBZ and OCBZ, clinically as well as with PT; however, *in vitro* lymphocyte reactivity to OCBZ was absent. The rapid *in vivo* metabolism of OCBZ and the significant inhibition of *in vitro* lymphocyte proliferation caused by OCBZ in many other patients may explain this discrepancy. A combined clinical and laboratory study to assess further the performance of PT and LPA in detecting CBZ allergy and OCB cross-reactivity in a large group of subjects taking CBZ is currently in review for publication elsewhere.

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CHAPTER 3.4

SUGGESTIVE EVIDENCE FOR BROMOCRIPTINE-INDUCED PLEURISY*

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ABSTRACT

Pleurisy of initially unknown origin was found in a patient who was treated with bromocriptine for Parkinson's disease for 6 years. At presentation, bilateral pleural thickening existed that caused severe restriction of pulmonary function. There were an elevated erythrocyte sedimentation rate, polyclonal hypergammaglobulinaemia, increased levels of acute phase proteins and anaemia. After withdrawal of the bromocriptine the patient's complaints as well as the laboratory parameters markedly improved. Further loss of pulmonary function did not occur. However, the pleural thickening did not resolve, not even upon subsequent corticosteroid treatment, probably due to fibrosis. Together, these findings strongly suggest a causative role of bromocriptine. The results of the laboratory studies suggested an immunopathogenetic mechanism, but *in vitro* lymphocyte-proliferation studies and skin patch tests with bromocriptine were negative. Bromocriptine should be considered as a cause of pleurisy. The drug must be stopped immediately upon the occurrence of pleural thickening in order to prevent impairment of pulmonary function. In addition, periodic laboratory and X-ray studies in patients on long-term bromocriptine treatment should be considered.

INTRODUCTION

Pleurisy can be a manifestation of a large number of different clinical entities, such as infectious, malignant or autoimmune diseases or toxic conditions. Here we report on a case of bilateral pleurisy associated with the use of bromocriptine for Parkinson's disease.

CASE REPORT

A 66-year-old man suffering from mild Parkinson's disease for 15 years was admitted in September 1993 because of progressive thoracic discomfort, dry cough and dyspnoea that had existed for 7 months. An involuntary weight loss of 5 kg had occurred in this period. Neither the patient nor his family had a history of pulmonary, cardiac or other systemic diseases. He had been a heavy smoker but stopped smoking 20 years ago. He denied occupational exposure to asbestos. His medication consisted of bromocriptine (10-15 mg daily since 1987), levodopa-carbidopa and baclofen.

Physical examination disclosed no abnormalities other than bilaterally slightly dimmed percussion of the latero-basal thorax with fine inspiratory crackles, but was otherwise unremarkable. The temperature was 37.3°C. Laboratory studies showed a normochromic, normocytic anaemia, an elevated erythrocyte sedimentation rate and CRP concentration (Fig. 1) as well as increased levels of the acute-phase proteins haptoglobin (3.4 g/l, normal range 0.4-2.5 g/l) and ferritin (473 mg/l, normal range 30-240 mg/l). Analysis of the serum protein spectrum revealed increased levels of α_2-globulins and a polyclonal hypergammaglobulinaemia.

IgG, IgA and IgM concentrations were 25.4 g/l (normal range 8.0 - 18.0 g/l), 4.84 g/l (normal range 0.9 - 4.5 g/l) and 1.26 g/l (normal range 0.6 - 2.8 g/l), respectively. The white-blood-cell count was just below the upper normal limit (9.8 x 10^9/l, 72% neutrophils, 20% lymphocytes, 8% monocytes). Autoantibodies (ANA, anti-ds-DNA, rheumatoid factors) were not detectable. Arterial blood gas analysis was normal.

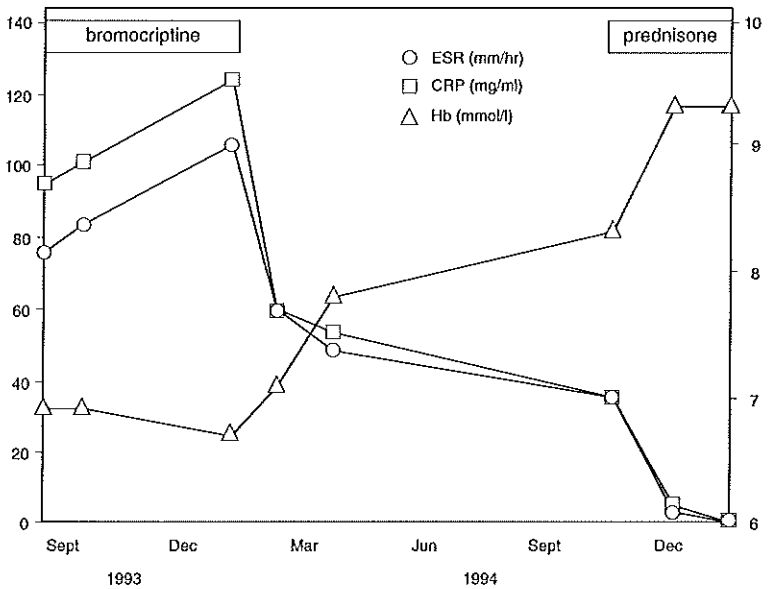

Figure 1. Laboratory parameters (ESR, the concentration of C-reactive protein and haemoglobin) during bromocriptine treatment (15 mg daily) after withdrawal of this drug and during prednisone treatment (20 mg daily).

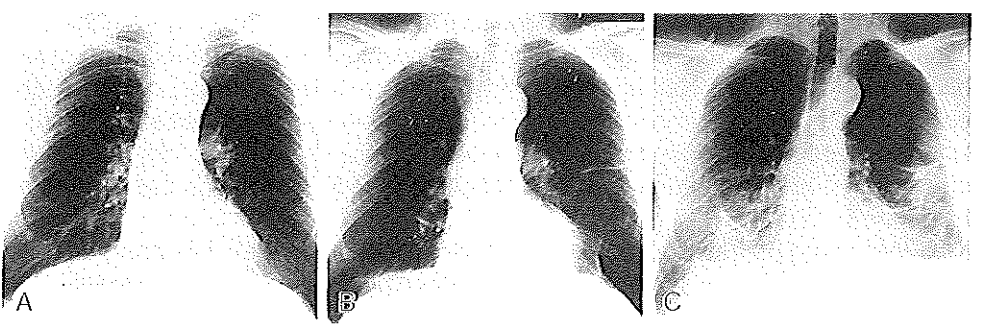

Figure 2. Chest X-ray films 3 years before (A), during 4 (B) and 6 (C) years of treatment with bromocriptine for Parkinson's disease.

The chest X-ray showed bilateral laterobasal pleural thickening with partial compression atelectasis of the left lung (Fig. 2c). A previous chest X-ray made in 1984 because of transient coughing was normal (Fig. 2a). The chest X-ray made in 1991, also because of transient coughing, showed only blunting of the costo-phrenic sinuses, a pleural streak in the midfield of the left lung and small pleural adhesions above the right diaphragm and beside the apex of the heart (Fig. 2b). Further investigations or follow-up chest X-ray studies were not carried out at that time. The findings on the present X-ray were confirmed by CT-scan. Neither parenchymal abnormalities of the lungs nor enlarged thoracic or abdominal lymphomas nor retroperitoneal fibrosis were present. Pleural fluid could not be obtained by puncture and was not demonstrated by subsequent echography. Pulmonary function tests showed a restrictive disorder (TLC 66%, FRC 61% of the predicted value) without airway obstruction.

Further aetiological studies including bronchoscopy with transbronchial biopsy, cultures of the sputum and PPD-skin test were all unremarkable. In the meantime the complaints of the patient worsened as did the results of the laboratory studies: the ESR and CRP concentration rose further, and the haemoglobin concentration further decreased (Fig. 1). Because of several reports that suggested a relation between pleuropulmonary disease and the use of bromocriptine [1-9] we conducted *in vivo* and *in vitro* immunological studies to confirm bromocriptine hypersensitivity as the aetiological factor in our patient. Skin patch tests and *in vitro* lymphocyte-proliferation studies using the patient's peripheral blood mononuclear cells were carried out with different concentrations of bromocriptine mesylate (final concentrations ranging from 0.2 to 125 ng/ml; therapeutic plasma concentration in Parkinson's disease patients ranging from 0.4 to 1.3 ng/ml). Both tests were negative with positive response to control antigens.

Notwithstanding these results, we discontinued the bromocriptine medication by tapering it off in 2 weeks time. Four weeks after the bromocriptine had been completely stopped, the patient reported a substantial improvement of the thoracic discomfort, disappearance of the dry cough, reduction of dyspnoea and a weight gain of 3 kg. The laboratory abnormalities also improved (Fig. 1). The IgG concentration in the serum decreased slightly to 21.8 g/l. Although the complaints and laboratory abnormalities further declined in the next months, they remained at a lower level and the X-rays of the chest showed no improvement. Therefore, prednisone 20 mg daily was instituted. Within 6 weeks, the remaining thoracic discomfort and dyspnoea completely resolved. Laboratory parameters normalized (Fig. 1), including the hypergammaglobulinaemia (IgG and IgA concentrations declined to 14.8 and 2.84 g/l, respectively). However, neither the chest X-ray and the CT-scan nor the pulmonary function tests (TLC 68%, FRC 61% of the predicted value) showed any improvement. The corticosteroid therapy is currently being tapered off without any sign of reactivation of the inflammatory process.

DISCUSSION

Long-term bromocriptine therapy has been used world-wide over the past two decades in large numbers of patients. Indications for bromocriptine include Parkinson's disease, prolactinoma and acromegaly. More recently, it has been used in inflammatory diseases such as arthritis psoriatica [10] and iridocyclitis [11], and in immunosuppressive regimens after transplantation [12,13], because bromocriptine interferes with the intracellular signalling of several cytokines [14]. However, to our knowledge, to date, only 30 cases of bromocriptine-induced pleurisy have been described in the English language medical literature [1-9,15]. This suggests that pleurisy is a rare complication of the use of bromocriptine. Alternatively, it is also possible that bromocriptine is often not recognized as a causative agent in patients with pleurisy. Upon introduction of bromocriptine, initial studies on the safety of this drug described pleurisy in 6 out of 123 patients treated [1]. The daily doses in that study were substantially higher than those used nowadays and ranged from 30 to 100 mg. This might explain the relatively high frequency of pleurisy in those patients. However, more recently pleurisy was also found in 2 out of 62 patients with Parkinson's disease taking less than 30 mg daily in a prospective 5-year follow-up study on the use of bromocriptine [15]. Similar to our patient, all patients described until now were men over 50 years of age treated for Parkinson's disease. In all cases complaints and abnormalities in laboratory and X-ray investigations developed after 1-5 years of treatment with bromocriptine in daily doses of at least 15 mg. Retrospectively, it might be argued that the mild abnormalities present on the chest X-ray film of our patient in 1991 were also caused by bromocriptine.

In addition, pleural effusion with non-specific lymphocytic pleocytosis was frequently described [1-9,15]. Our patient, however, had no detectable amounts of fluid in the pleural cavity. Presumably, he had a more advanced stage of pleurisy with organisation and subsequent fibrosis of the pleural exudate that had been present earlier in the disease. The poor response to withdrawal of bromocriptine and the subsequent institution of prednisone therapy, as indicated by the X-ray studies and pulmonary function tests, support this hypothesis.

The striking relation between the withdrawal of bromocriptine in this patient and those described previously [1-9] and the improvement of the clinical and laboratory findings provides strong circumstantial evidence for a causative role of bromocriptine in the development of pleurisy. However, the pathogenesis is unclear. Idiosyncrasy cannot be ruled out, but the increased levels of acute-phase proteins and the polyclonal hypergammaglobulinaemia suggest that immunological mechanisms might be operating. To our knowledge we are the first to address this possibility by immunological studies. Definite proof is lacking as the skin tests and lymphocyte stimulation tests were negative. This might be due to the fact that bromocriptine does not provoke an immune reaction directly, but merely acts like a hapten, which together with pleural determinants forms the antigenic stimulus for immunologically mediated injury. In addition to pleural fibrosis, bromocriptine has also been implicated in the development of retroperitoneal fibrosis with similar abnormalities in laboratory studies, suggesting a common pathogenetic mechanism [4,15-18]. Fibrotic inflammatory disorders of the pleura and retroperitoneal fibrosis have also been

described during the long-term use of the alkylated ergot derivative, methysergide, which has a molecular structure related to that of bromocriptine [19-21]. Direct proof that methysergide plays a causative role in these disorders is also lacking, but the abnormalities remitted after withdrawal of the drug, as we described for bromocriptine. In conclusion, bromocriptine should be considered as a causative agent when pleurisy occurs, even if the daily dose is low. In such cases the drug should be stopped and corticosteroid treatment is possibly beneficial in order to prevent formation of irreversible pleural thickening with impairment of pulmonary function. In addition, periodic laboratory and X-ray studies in patients on long-term bromocriptine therapy are recommended.

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CHAPTER 4

GENERAL DISCUSSION

This thesis describes the application of immunological insights and techniques to improve the diagnosis, treatment and follow-up of inflammatory skin diseases, in particular allergic contact dermatitis (ACD) and allergic drug eruptions. In this last chapter, the most important findings and conclusions of the previous chapters will be discussed in a broader sense and in the light of recent developments in research on allergic and inflammatory skin disease. The figure on the cover is explained and some concluding remarks are made about the immune system of the skin, manipulation of the immune system (hyposensitization and tolerance induction) and diagnostic tests for drug allergy. Suggestions for further research are also given.

In both ACD and allergic drug eruptions, the skin is the target organ of immunological reactivity to an allergen. The figure on the cover is a schematic representation of human skin exposed to antigens (Ag) or allergens from inside the body, like most drugs (the tablet in the figure), and from outside the body, such as contact allergens (the metal ring in the figure). A migrating T cell, specific for the Ag in question or an unrelated Ag, is depicted strategically at the border between epidermis and dermis.

4.1 The immune system of the skin

The knowledge about the cells, mediators and mechanisms that are involved in allergic skin reactions has increased vastly in the last two decades and it will continue to increase in the next. Therefore, it seems inevitable for investigators that concepts, that were up-to-date at the start of their study, will be partly outdated when the study finishes. This thesis is no exception to that. Nevertheless, chapter 1 attempts to review the current insights into the cells and cytokines that are involved in cutaneous inflammation. Although this thesis is for the most part about 'cellular aspects' of lymphocytes and keratinocytes, various other resident, recruited and/or recirculating skin cells, that make up the Skin Immune System (SIS) [1], deserve further study. Especially Langerhans cells (LC) are promising targets for immunomodulation. Cytokine production profiles are another topic of intense research and studies on apoptosis may also provide clues for future immunotherapy.

In the chapters 1 and 2, ACD was described as a typical T helper type 1 response. This is, however, an oversimplification. Although many infectious, autoimmune and allergic diseases have been described as typical T helper type 1 or type 2 responses [2], the original paradigm cannot entirely account for the true complexity of the *in vivo* situation [3]. The role that T cells, cytokines and other effector cells play in a certain disease may not always fall easily into the discrete T helper type 1 or 2 pattern. Indeed, based on cytokine production patterns, other $CD4^+$ T helper effector phenotypes (a.o. Th0A, Th0B and Th3) and also $CD8^+$ cytotoxic T cell

subsets (Tc1 and Tc2) have been described [4]. In various diseases, both T helper type 1 and 2 responses are present. Many responses are also mediated by cytokines produced by cells other than T helper type 1 or 2 (e.g. keratinocyte-produced IL-1 and IL-6, mast cell and eosinophil-produced IL-4, NK cell-produced IFN-γ and macrophage-produced IL-12). In addition, the cell types, cytokines and their effects in one stage of a disease may be completely different in another. For example, atopic dermatitis is initially a T helper type 2 reaction but after 24-48 hours the T helper type 1 cytokine profile dominates. Thus, analogous to the classifications based on the immune reaction types I-IV [5] or on CD4/CD8-ratios, oversimplification may be useful for didactic and mechanistic purposes but in most *in vivo* conditions the processes are more complex.

4.2 Manipulation of the immune system

Hyposensitization and tolerance induction

In chapter 2.1, nickel was shown to be ubiquitous. Its presence in many objects for daily use and in food makes effective avoidance virtually impossible. That is the main reason for our attempts to hyposensitize nickel ACD patients. Some investigators believe that an allergy that has already developed is irreversible [6] as opposed to primary induction of Ag-specific tolerance, which prevents the subsequent development of an allergy to that Ag. However, the tendency of some allergies to fade away over a period of many years proves that down regulation is possible. Also, for type I allergy, the efficacy of hyposensitization has already been proven. Therefore, we believe the question is not *whether* it is possible, but *how* it should be done.

According to generally accepted concepts, tolerance is acquired by clonal deletion (after ubiquitous Ag presentation), clonal inactivation (after Ag presentation without appropriate co-stimulatory signals) and possibly also through active suppression (suppressor T cells). Chapter 2.2. is the first published report of a clinical hyposensitization procedure using ultraviolet B (UVB) and subcutaneous nickel sulfate administration to induce Ag-specific tolerance in patients with nickel ACD. It is based on publications by Kripke [7, 8] and derived from analogous hyposensitization procedures used in type I allergy, such as to pollen. UVB is now known to act on various cells of the SIS, including epidermal LC, keratinocytes and T lymphocytes, and on chromophores, such as DNA and trans-urocanic acid [9-11]. The net results of UVB on the SIS, that have been reported, are local and systemic immunosuppression, possibly mediated via IL-10 and TNF-α. When UVB exposure is followed by application of a contact allergen, a subsequent allergen-specific ACD reaction is suppressed [8]. In our hyposensitization study, low dose UVB and subcutaneous nickel sulfate injections were administered to nickel ACD patients. The nickel sulfate doses were raised from 10^{-6} to 10^{-3} mol/L and its frequency was once a week until the highest concentration was reached and once a month in the subsequent follow-up period. The hyposensitization treatment was monitored using clinical disease activity scores and immunological parameters,

such as *in vitro* reactivity to nickel in the lymphocyte proliferation assay (LPA) and flow cytometric analysis of the expression of the adhesion molecules LFA-1α and ICAM-1 on peripheral blood mononuclear cells (PBMC), and of activated $CD3^+$/$HLA\text{-}DR^+$ and $CD3^+$/IL-2 $receptor^+$ T cells. Although the clinical parameters studied improved significantly, no changes were seen in the immunological parameters evaluated. Possible explanations for these findings include (1) the changes induced were too small to detect, (2) the size of the group was too small to detect any changes, (3) the immunological follow-up parameters were inappropriate, and (4) the hyposensitization procedure is not yet optimal. Suggestions for further research include repetition of the procedure in a larger group, the use of epidermal LC as antigen presenting cells in the *in vitro* LPA, the follow-up of other immunological parameters, such as detection of cytokine production profiles after allergen-specific T cell stimulation and expression of costimulatory factors, and other hyposensitization procedures, for example via the oral route [12]. Oral exposure to nickel from dental braces was found to reduce sensitization upon subsequent ear piercing in a large retrospective study [6]. Combination of CsA and B7-1/B7-2-blocking agents has also been reported as an effective method to induce T cell anergy and thus complete immunosuppression [13]. In addition, gamma delta T cells have recently been reported to play a possible role in downregulation of ACD and high-dose Ag tolerance [14, 15].

In chapter 2.3, an attempt was made to study *in vivo* cytokine production after Ag-specific stimulation (patch test) through immunohistochemistry of ACD skin sections. However, reliable and reproducible quantification of cytokine staining proved extremely difficult. Although image analysis (IA) was demonstrated to be a valid and useful method to improve quantification of immunostained inflammatory skin biopsies, it could not circumvent the difficulties associated with quantification of cytokines that cause diffuse staining. A limited study is currently done to see whether IA density measurement of positively stained section areas corresponds with the results of traditional visual densitometric scoring systems. The IA procedure used in this study is analogous to well-described methods used in flow cytometric analysis. First, the 'red range' is selected via thresholding of Hue in HSL color space. After that, the resulting image is gray transformed and exclusion limits are set on the histogram of the negative control section (stained with an isotype-matched control Ab). These limits are then applied to the corresponding immunostained sections, effectively leading to subtraction of the negative control. The final image is visually checked to contain only the positively stained section areas. The sizes of these section areas are measured and compared to visual estimations. The results of this study will clarify whether IA can also be used to estimate staining intensity, besides section area measurement.

4.3 Diagnostic tests for allergy

In chapter 3.1, clinical manifestations and diagnostic aspects of allergic adverse drug reactions (ADR) caused by anti-epileptic drugs (AED) were reviewed. It was stated that in the case of AED, the search for the drug that caused the ADR and a

suitable alternative drug is complicated by the long-term use, multi-drug therapy, structural similarity (cross-reactivity; mimicry) and the relative lack of alternatives. Also, the relevance and importance of the distinction between allergy and non-immunological reactions for future drug choice was stressed. The epicutaneous PT and the *in vitro* LPA were suggested to confirm immunological reactivity to AED [16, 17]. In chapter 3.2, the suitability of skin PT and *in vitro* LPA for detection of allergy to CBZ and the possibly cross-reactive oxcarbazepine (OCBZ) was investigated in patients on CBZ, displaying a wide range of possibly allergic side effects. True allergy for CBZ and OCBZ was found in up to 40% and 20% of the patients with suspected reactions, respectively. The PT and the LPA did not always denote the same patients as being allergic. Therefore, these tests should be considered additive instead of mutually exclusive, as depicted in the flowchart for diagnosing allergy to AED at the end of chapter 3.2. In the chapters 3.3 and 3.4 we described how the PT and the LPA were used to support the diagnosis of drug allergy in two case reports on exfoliative dermatitis due to CBZ and on bromocriptine-induced pleurisy, respectively. In the first case, immunological reactivity was confirmed, in the second it was not.

As the LPA is done completely *in vitro*, it is very safe. However, its main drawbacks are that extensive controls and large cell numbers are needed for every Ag tested, and that it takes about one week to get interpretable results. In addition, its readout concerns the overall proliferation of PBMC, not of specific T cells. A more appropriate readout might be the cytokine production profile after Ag-specific stimulation. Also, the LPA could be modified to incorporate antibody production and cytotoxicity aspects. Cross-reactivity between CBZ and phenytoin is another issue that needs further study [17, 18]. Its main problem, however, is that reliable detection of immunological cross-reactivity between these drugs requires CBZ allergic subjects without prior phenytoin intake or phenytoin allergic patients without prior exposure to CBZ. In practice, this proves to be a serious problem as prior medication is not always properly known and recorded.

In conclusion: (1) hyposensitization of nickel ACD patients via low dose UVB exposure and subcutaneous nickel sulfate injections induced a significant improvement of clinical ACD parameters, but needs further refinement and verification by appropriate immunological parameters, (2) quantification of immunostained inflammatory skin sections was improved by computer-assisted IA area measurement, and (3) suspected drug allergy to the anti-epileptic drugs CBZ, OCBZ and phenytoin could be confirmed by parallel *in vivo* and *in vitro* detection of immunological reactivity to the drug via PT and LPA, respectively.

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SUMMARY

This thesis is about the application of immunological insights and techniques to improve the diagnosis, treatment and follow-up of inflammatory skin diseases, in particular allergic contact dermatitis and allergic drug eruptions.

In **chapter 1.1** the cells and cytokines that are involved in cutaneous inflammation are discussed. Firstly, the various resident, recruited and / or recirculating skin cells, that make up the so-called Skin Immune System, are summarized. The Skin Immune System includes keratinocytes, Langerhans cells, melanocytes, lymphocytes, cells from the monocyte / macrophage lineage, granulocytes, mast cells and endothelial cells. Secondly, cytokines (small, soluble polypeptide molecules that are produced by virtually every cell type in response to exogenous stimuli) are discussed. Cytokines interact with specific receptors on the cell membrane or inside cells to mediate intercellular or intracellular communication. A number of cytokines important in the induction and regulation of cutaneous inflammation are summarized, along with their stimuli, producer cells, target cells and effects. Finally, the classification of immune responses according to Gell and Coombs is summarized for a better understanding of the immune reactions at work in the various inflammatory skin diseases.

Contact dermatitis is a world-wide disorder and a major cause of occupational disease. Estimates of its prevalence in the general population range between 1% and 10%. Two different types can be distinguished, i.e. allergic and irritant contact dermatitis. In **chapter 1.2**, clinical, epidemiological and immunological aspects of allergic contact dermatitis are discussed. Allergic contact dermatitis is seen most frequently among young women, between 20 and 30 years of age. It is an inflammatory skin disease that results from repeated contact with contact allergens or sensitizers. Allergic contact dermatitis is very persistent as even occasional contact with allergens is sufficient to maintain the condition. The occurrance of allergic contact dermatitis is tightly correlated with the presence or absence of certain risk factors. Precise details on the frequency and type of exposure, the type of allergen involved, the risk factors present and the localization of the eczema are of great practical importance for estimating the prognosis. However, the search for the causal contact allergen(s) in individual allergic contact dermatitis patients is not a light task, especially if allergy to multiple allergens exists. Patch tests and the *in vitro* lymphocyte proliferation assay may be of help here. The combination of a positive exposure history and a positive patch test for the same allergen is highly significant for the clinical diagnosis of allergic contact dermatitis. To date, avoidance of allergen exposure seems to be the only truly effective measure. Immunologically, allergic contact dermatitis can be characterized as a classical type IV or delayed type hypersensitivity reaction. This reaction is mediated by T cells previously sensitized to one or more contact allergens. Substances capable of inducing allergic contact dermatitis are generally low molecular weight chemically reactive compounds, called haptens. They penetrate the skin and bind to skin proteins to form a complete antigen, capable of inducing an immune response. Nickel, chro-

mium and cobalt are among the most frequent contact allergens. The sequence of events leading to allergic contact dermatitis, after initial and subsequent repeated exposure to a reactive hapten, is described as two phases, (1) the induction or acquisition phase and (2) the elicitation or expression phase. In the afferent limb, antigen is taken up, processed and presented in the groove of HLA class II on the surface of antigen presenting cells. After migration to the regional lymph nodes, the HLA-peptide complex on the antigen presenting cell can be recognized by the T cell receptor of specific T cells, leading to their activation. In the efferent limb the previously sensitized antigen-specific $CD4^+$ T helper type 1 cells and $CD8^+$ cytotoxic T cells migrate to the skin where they may get activated upon renewed antigen presentation. Release of cytokines and chemokines like interferon-γ, tumor necrosis factor-β, interleukin-3, granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein and monocyte-chemotactic and activating factor leads to recruitment and activation of other T cells, specific for unrelated antigens, and macrophages which amplify the inflammatory response.

Chapter 1.3 discusses clinical, epidemiological and immunological aspects of allergic drug reactions, a small but important part of the broad range of adverse drug reactions. Reported frequencies of adverse drug reactions (allergic and nonallergic) range from 0.3% to 30% per drug treatment. In general, the risk of an allergic adverse drug reaction is between 1% and 3%. An adverse drug reaction can be considered allergic if it is mediated by antigen-specific immunological mechanisms, i.e. via antibodies or lymphocytes. Drug allergy may mimic many diseases, ranging from mild to even fatal conditions. The majority of allergic adverse drug reactions, however, involve the skin and are consequently also termed allergic drug eruptions. The likelihood of allergic adverse drug reactions is tightly correlated with the presence or absence of certain risk factors.

Although drug allergy is often suspected, it is rarely properly analyzed. Discrimination between allergic and other adverse drug reactions is essential since it influences future drug administration. Incorrect labeling of a patient as allergic may lead to substitution of beneficial medication for other, possibly less effective, more dangerous and/or more expensive drugs. If an allergic adverse drug reaction is suspected, the offending drug should be discontinued as soon as possible, usually before extensive diagnostic exploration can be done. Several diagnostic tests have been suggested. However, the lack of a 'golden standard' for the diagnosis of allergic adverse drug reactions seriously hampers the proper validation of such tests. *In vitro* tests, such as the lymphocyte proliferation assay, are likely to be the safest alternatives to rechallenge.

Allergic adverse drug reactions may or may not follow the allergic reaction types as described by Coombs and Gell. Chemical reactivity, a drug-related risk factor, is crucial since it determines the likelihood that a drug will bind to a carrier molecule. As most drugs have a low molecular weight, they cannot induce an immune response on their own. Frequently, the drug itself or one of its (mostly reactive) metabolites first needs to bind covalently to carrier molecules, like proteins, glycoproteins and sometimes polysaccharides or cell membranes, turning it into a mul-

tivalent complete allergen. Certain drugs induce allergic adverse drug reactions more frequently than others. Commonly used allergenic drugs are antibiotics (including β-lactam penicillins, cephalosporins and sulfonamides), anti-epileptic drugs, anesthetics (including muscle relaxants and thiopental), hormones (a.o. insulin), immunoglobulins and vaccines.

The various immunological techniques used in this thesis are put into perspective in **chapter 1.4**. Their purpose, technical procedure, possibilities and limitations are discussed. The patch test, the lymphocyte proliferation assay and flow cytometric analysis were used for monitoring immunological parameters during hyposensitization treatment of nickel allergic contact dermatitis patients. The patch test and the lymphocyte proliferation assay were also used and validated to support the diagnosis of allergic adverse drug reactions to the anti-epileptic drug carbamazepine and its metabolites, and to bromocriptine. Immunohistochemistry and computer-assisted image analysis were used for detection of cytokines and other markers of inflammation in inflammatory skin sections.

Chapter 2.1 focuses on nickel as a contact sensitizer. The combination of a mild sensitizing potential and widespread exposure of nickel in the general population results in the high frequency of sensitization of 10-20%. Many objects that are frequently used in daily life contain nickel, e.g. coins, keys, scissors, wrist watches, jewelry (a.o. earrings) and blue jeans buttons. Being a trace element, nickel is also present in many sorts of food, such as nuts (walnut, peanut, hazelnut, almond), bitter chocolate, oats, buckwheat, soy products and dried legumes. The primary routes of nickel exposure and uptake are ingestion in the general population, inhalation in the occupational setting, and, to a lesser extent, release from surgical implants and dental prostheses. Although uptake of nickel via the stratum corneum, sweat ducts and hair follicles of the skin is important in the induction and elicitation of nickel allergic contact dermatitis, its contribution to overall nickel uptake of the body is fairly small. However, the skin can act as a reservoir for nickel with a mean retention time of many months. Metals, such as nickel, chromium, cobalt, gold, platinum, mercury and beryllium, are directly immunogenic and capable of inducing a wide range of pathological conditions including allergic contact dermatitis, asthma and autoimmune disease, but can, under special conditions, also induce immunological tolerance. Nickel induces allergic contact dermatitis via effects on many immunocompetent cells, including Langerhans cells, keratinocytes and endothelial cells.

Chapter 2.2 is the first published report of a clinical hyposensitization procedure using ultraviolet B and subcutaneous nickel sulfate administration to induce antigen-specific tolerance in patients with nickel allergic contact dermatitis. The incentive for this study was the finding that the development of allergy could be down-regulated if the antigen was presented in the absence of adequate costimulatory signals, as can be induced by ultraviolet B exposure. Twenty-one patients with nickel allergic contact dermatitis were randomly assigned to either a hyposensitized or control group. A schedule, consisting of ultraviolet B treatment and

subcutaneous nickel sulfate administration (hyposensitization) or ultraviolet B only (control), was applied. During the ensuing two years, several clinical and immunological features were monitored. During ultraviolet B treatment we observed significant clinical improvement in both groups that persisted in the hyposensitized group. However, no clear changes were seen in the immunological parameters evaluated.

Allergic contact dermatitis is studied widely by immunostaining of skin biopsies. Accurate and reproducible quantification of immunostaining is difficult. A number of dissimilar procedures have been reported, producing results that cannot be easily compared. In **chapter 2.3**, image analysis is demonstrated as a valid and useful method to improve quantification of immunostained inflammatory skin biopsies. A semiautomatic procedure (macro) for area measurement by image analysis was developed and validated. The technique allowed quantification of immunostaining as cell counts per square millimeter of epidermal, dermal or infiltrate section area. The accuracy and reproducibility of the image analysis area measurement procedure was high, i.e. the deviations were less than 0.5% and 1%, respectively. When compared to traditional grid-based area measurement, the mean values of both methods were similar.

Chapter 3.1 discusses clinical manifestations and diagnostic aspects of allergic adverse drug reactions caused by anti-epileptic drugs. Skin rashes are the most pronounced adverse drug reactions to anti-epileptic drugs, besides fever, lymphadenopathy, hematological abnormalities and impaired liver function. In the case of anti-epileptic drugs, the search for the drug that caused the adverse reaction and a suitable alternative drug is complicated by the long-term use, multi-drug therapy, structural similarity (cross-reactivity; mimicry) and the relative lack of alternatives. Carbamazepine, phenytoin, barbiturates and sodium valproate are responsible for the majority of adverse drug reactions to anti-epileptic drugs. Although routine laboratory tests cannot reliably predict the likelihood of drug allergy in a single patient, epicutaneous patch tests and the *in vitro* lymphocyte proliferation assay can be used to confirm immunological reactivity to anti-epileptic drugs. Such distinction between allergy and non-immunological reactions is of great importance for future drug use.

Five to 20% of patients discontinue anti-epileptic drug therapy because of adverse reactions. However, careful reintroduction may be considered if true drug allergy can be ruled out. Definitive assessment of such immunologically mediated reactions requires demonstration of either specific antibodies or sensitized lymphocytes. In **chapter 3.2**, the suitability of skin patch tests and *in vitro* lymphocyte proliferation assays for detection of allergy to carbamazepine and the possibly cross-reactive oxcarbazepine was investigated. Sixty-five patients on carbamazepine, displaying a wide range of possibly allergic side effects, carbamazepine users without any side effects and healthy volunteers were tested. Both patch tests and lymphocyte proliferation assays were done with carbamazepine, oxcarbazepine and three metabolites (carbamazepine-10,11-epoxide, 10-mono-

hydroxy-carbamazepine and 10,11-dihydroxy-carbamazepine). Positive patch tests with carbamazepine were seen in 20% and with oxcarbazepine in 14% of the patients. Positive lymphocyte proliferation assay results with carbamazepine and oxcarbazepine were found in 40 and 19% of the patients, respectively. Both tests were positive in 14 and 7% of the patients, respectively. Cross-reactivity to oxcarbazepine was seen in approximately 40% of carbamazepine-reactive patients in both patch tests and lymphocyte proliferation assays.

In **chapter 3.3**, a case of exfoliative dermatitis due to carbamazepine allergy is described. The patient is a 6-year-old Caucasian girl who experienced a generalized erythematous skin rash during carbamazepine therapy. The eruption worsened into erythroderma with fever and generalized lymphadenopathy in the next four days. Routine laboratory studies revealed increased serum levels of liver enzymes and eosinophilia. Immunological reactivity to carbamazepine was confirmed both *in vivo* and *in vitro* by patch tests and lymphocyte proliferation assays, respectively.

Chapter 3.4 describes a possible case of bromocriptine-induced pleurisy. The pleurisy of initially unknown origin was discovered in a patient who was treated with bromocriptine for Parkinson's disease for 6 years. At presentation, bilateral pleural thickening existed that caused severe restriction of pulmonary function. Laboratory tests revealed an elevated erythrocyte sedimentation rate, polyclonal hypergammaglobulinaemia, increased levels of acute phase proteins and anaemia. After withdrawal of the bromocriptine the patient's complaints as well as the laboratory parameters markedly improved. However, the pleural thickening did not resolve. Although these findings strongly suggested a causative role of bromocriptine, the *in vitro* lymphocyte proliferation assay and the skin patch test with bromocriptine were negative.

Finally in **chapter 4**, the figure on the cover is explained and some concluding remarks are made about the immune system of the skin, modulation of the immune system via hyposensitization and tolerance induction, and diagnostic tests for drug allergy. In this chapter, the experimental and clinical studies described in this thesis are discussed in the light of recent developments in research on allergic and inflammatory skin disease.

SAMENVATTING

Dit proefschrift beschrijft de toepassing van immunologische inzichten en technieken ter verbetering van de diagnose, behandeling en follow-up van inflammatoire huidziekten, in het bijzonder allergisch contacteczeem en allergische geneesmiddelenerupties.

In **hoofdstuk 1.1** worden de cellen en cytokinen, die betrokken zijn bij cutane ontsteking, besproken. Eerst worden de diverse residente, gerekruteerde en recirculerende cellen in de huid, die gezamenlijk het zogenaamde Skin Immune System vormen, besproken. Het Skin Immune System omvat keratinocyten, Langerhans cellen, melanocyten, lymfocyten, cellen van de monocyt/macrofaaglijn, granulocyten, mestcellen en endotheelcellen. Daarna worden cytokinen (kleine oplosbare polypeptiden die door vrijwel elk celtype worden geproduceerd als reactie op exogene prikkels) besproken. Cytokinen binden aan specifieke receptoren op de celmembraan of binnenin de cel en verzorgen zo de inter- en intracellulaire communicatie. Een aantal cytokinen die een belangrijke rol spelen bij het ontstaan en de regulatie van cutane ontsteking, worden samengevat tezamen met hun stimuli, cellulaire oorsprong, doelwitcellen en effecten. Tenslotte wordt de indeling van immuunreacties volgens Gell en Coombs besproken om de immuunreacties, die bij de diverse inflammatoire huidziekten optreden, beter te kunnen begrijpen.

Contacteczeem is een wereldwijd voorkomende aandoening en een belangrijke veroorzaker van beroepsziekten. Schattingen van de prevalentie in de algemene populatie liggen tussen 1% en 10%. Er kunnen twee verschillende vormen worden onderscheiden, nl. allergisch en orthoergisch (irritant) contacteczeem. In **hoofdstuk 1.2** worden klinische, epidemiologische en immunologische aspecten van allergisch contacteczeem besproken. Allergisch contacteczeem komt het meest voor bij jonge vrouwen in de leeftijdsgroep van 20 tot 30 jaar. Het is een inflammatoire huidziekte die het gevolg is van herhaald contact met contactallergenen of sensibilisatoren. Allergisch contacteczeem is een zeer hardnekkige aandoening daar zelfs incidenteel contact met het allergeen voldoende is om de aandoening te onderhouden. Het voorkomen van allergisch contacteczeem is nauw gecorreleerd met de aan- of afwezigheid van bepaalde risicofactoren. Nauwkeurige informatie over de frequentie en het type van blootstelling, het type allergeen, de aanwezigheid van risicofactoren en de lokalisatie van het eczeem zijn van groot praktisch belang om de prognose te kunnen beoordelen. De zoektocht naar het oorzakelijke contactallergeen in individuele allergisch contacteczeem patiënten is echter geen sinecure, zeker bij polyvalente allergie. Patch tests en *in vitro* lymfocyten proliferatie assays kunnen hierbij van nut zijn. Het gezamenlijk voorkomen van een anamnese, die positief is voor blootstelling aan een allergeen, en een positieve patch test voor hetzelfde allergeen is van groot belang voor de klinische diagnose van allergisch contacteczeem. Tot nu toe lijkt vermijding van expositie aan het allergeen de enige effectieve maatregel te zijn. Immunologisch kan allergisch contacteczeem worden gekarakteriseerd als een klassieke type IV of delayed type hypersensitivity reactie. Dit reactietype wordt veroorzaakt door T cellen die

gesensibiliseerd zijn tegen één of meer contactallergenen. De stoffen die in staat zijn om allergisch contacteczeem te induceren, zijn meestal chemisch reactieve componenten met een laag molecuulgewicht, ook wel haptenen genoemd. Zij dringen de huid binnen, binden aan eiwitten en vormen een compleet antigeen dat in staat is een immuunrespons te induceren. Nikkel, chroom en kobalt behoren tot de meest voorkomende contactallergenen. Het ontstaan van allergisch contacteczeem, na de eerste en daarna herhaalde blootstelling aan een reactief hapteen, wordt veelal beschreven in twee fasen, en wel (1) de inductie- of acquisitiefase en (2) de elicitatie- of expressiefase. In de afferente route wordt antigeen opgenomen, geprocest en gepresenteerd in de groeve van HLA klasse II moleculen op het oppervlak van antigeen presenterende cellen. Na migratie naar de regionale lymfklieren kan het HLA-peptide complex op de antigeen presenterende cellen worden herkend door de T cel receptor van specifieke T cellen, hetgeen leidt tot hun activering. In de efferente route migreren de gesensibiliseerde antigeen-specifieke $CD4^+$ T helper type 1 cellen en $CD8^+$ cytotoxische T cellen naar de huid, waar zij bij hernieuwde presentatie van het antigeen geactiveerd kunnen worden. Afgifte van cytokinen en chemokinen, zoals interferon-γ, tumor necrosis factor-β, interleukine-3, granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein en monocyte-chemotactic and activating factor, leidt tot rekrutering en activering van andere T cellen, die specifiek zijn voor andere antigenen, en macrofagen waardoor de ontstekingsreactie wordt versterkt.

Hoofdstuk 1.3 bespreekt klinische, epidemiologische en immunologische aspecten van allergische geneesmiddelenreacties, die een klein maar belangrijk deel van het brede spectrum van bijwerkingen van geneesmiddelen vormen. De gerapporteerde frequentie van geneesmiddelenreacties (allergisch en niet-allergisch) ligt tussen 0.3% en 30% per behandeling met een geneesmiddel. In het algemeen is het risico op een allergische geneesmiddelenreactie 1% tot 3%. Een geneesmiddelenreactie kan als allergisch worden beschouwd als deze wordt gemedieerd door antigeen-specifieke immunologische mechanismen, zoals antistoffen en lymfocyten. Geneesmiddelenallergie kan veel ziekten, in ernst variërend van mild tot zelfs fataal, nabootsen. De meerderheid van de allergische geneesmiddelenreacties betreffen echter de huid en worden daarom ook wel allergische geneesmiddelenerupties genoemd. De kans op een allergische geneesmiddelenreactie is nauw gecorreleerd met de aan- of afwezigheid van bepaalde risicofactoren.

Hoewel geneesmiddelenallergie vaak wordt vermoed, wordt deze zelden goed geanalyseerd. Onderscheid tussen allergische en andere geneesmiddelenreacties is van groot belang aangezien het toekomstige medicatiebeleid erdoor wordt bepaald. Het foutief als allergisch bestempelen van een patiënt kan er namelijk toe leiden dat nuttige medicatie wordt vervangen door andere, mogelijk minder effectieve en / of duurdere geneesmiddelen. Indien een allergische geneesmiddelenreactie wordt vermoed, moet het betreffende geneesmiddel zo snel mogelijk worden gestaakt, vrijwel altijd vóórdat uitgebreide diagnostiek kan worden verricht. Enkele diagnostische tests zijn geopperd. Echter, het gebrek aan een 'gouden standaard' voor de diagnose allergische geneesmiddelenreactie belemmert een

goede validering van zulke tests. *In vitro* tests, zoals de lymfocyten proliferatie assay, zijn de veiligste alternatieven voor rechallenge.

Allergische geneesmiddelenreacties kunnen al of niet volgens de allergische reactietypen zoals door Gell en Coombs beschreven, verlopen. De chemische reactiviteit van een stof is een cruciale geneesmiddel-gerelateerde risicofactor omdat deze de kans, dat een geneesmiddel aan een carrier bindt, bepaalt. Aangezien de meeste geneesmiddelen een laag molecuulgewicht hebben, kunnen zij zelfstandig geen immuunrespons induceren. Vaak moet het geneesmiddel zelf, of een van zijn (veelal reactieve) metabolieten, eerst covalent binden aan carriermoleculen, zoals eiwitten, glycoproteïnen en soms polysacchariden of celmembranen, waardoor een multivalent compleet allergeen ontstaat. Bepaalde geneesmiddelen induceren vaker een allergische geneesmiddelenreactie dan andere. Veel gebruikte allergene geneesmiddelen zijn antibiotica (inclusief de β-lactam penicillinen en sulfonamiden), anti-epileptica, anaesthetica (inclusief de spierverslappers en thiopental), hormonen (o.a. insuline), immunoglobulinen en vaccins.

De verschillende in dit proefschrift gebruikte immunologische technieken worden in perspectief geplaatst in **hoofdstuk 1.4**. Hun doel, technische procedure, mogelijkheden en beperkingen worden besproken. De patch test, lymfocyten proliferatie assay en flowcytometrische analyse werden gebruikt voor het vervolgen van immunologische parameters tijdens hyposensibilisatie van nikkel allergisch contacteczeem patiënten. De patch test en lymfocyten proliferatie assay werden tevens gebruikt en gevalideerd ter ondersteuning van de diagnose allergische geneesmiddelenreactie door het anti-epilepticum carbamazepine en zijn metabolieten, en door bromocriptine. Immunohistochemie en computer-assisted image analysis werden gebruikt voor de detectie van cytokinen en andere ontstekingsmarkers in huidcoupes van patiënten met inflammatoire huidziekten.

Hoofdstuk 2.1 richt zich op nikkel als contactallergeen. De combinatie van een mild sensibiliserend vermogen en de omvangrijke blootstelling aan nikkel in de algemene bevolking resulteren in de hoge sensibilisatiefrequentie van 10-20%. Veel voorwerpen voor dagelijks gebruik, zoals geld, sleutels, scharen, horloges, sieraden (bijv. oorbellen) en metalen knopen aan spijkergoed, bevatten nikkel. Als sporenelement is nikkel ook aanwezig in veel voedingsmiddelen, zoals noten (walnoot, pinda, hazelnoot, amandel), pure chocolade, haver, boekweit, sojaprodukten en gedroogde groenten. De belangrijkste wegen van blootstelling en opname van nikkel zijn via de voeding in de algemene populatie, via inhalatie in bepaalde beroepsgroepen en in mindere mate vanuit chirurgische implantaten en tandheelkundige prothesen. Hoewel de opname van nikkel via het stratum corneum, de zweetklieren en de haarfollikels van de huid belangrijk is voor de inductie en elicitatie van nikkel allergisch contacteczeem, is de bijdrage daarvan aan de totale nikkelopname van het lichaam vrij gering. De huid kan echter wel als een reservoir voor nikkel fungeren met een gemiddelde retentietijd van vele maanden. Metalen als nikkel, chroom, kobalt, goud, platina, kwik en beryllium zijn direct immunogeen

en kunnen een scala van pathologische aandoeningen veroorzaken, waaronder allergisch contacteczeem, astma en autoimmuunziekten, maar kunnen, onder speciale omstandigheden, ook tolerantie induceren. Nikkel induceert allergisch contacteczeem via effecten op diverse immunocompetente cellen, onder andere Langerhans cellen, keratinocyten en endotheelcellen.

Hoofdstuk 2.2 is de eerste gepubliceerde beschrijving van een klinische hyposensibilisatieprocedure, waarbij ultraviolet B licht en subcutane toediening van nikkelsulfaat is gebruikt om antigeen-specifieke tolerantie in patiënten met nikkel allergisch contacteczeem te induceren. De aanzet tot deze studie werd gevormd door de bevinding dat het ontstaan van een allergie kon worden verminderd door het betreffende antigeen aan te bieden in afwezigheid van adequate costimulatie, zoals door ultraviolet B kan worden geïnduceerd. Eenentwintig patiënten met nikkel allergisch contacteczeem werden random toegewezen aan de hyposensibilisatie- of de controlegroep. Een behandelingsschema werd toegepast bestaande uit ultraviolet B-expositie en subcutane toediening van nikkelsulfaat (hyposensibilisatiegroep) of uitsluitend ultraviolet B-expositie (controlegroep). Gedurende de daaropvolgende twee jaar werden diverse klinische en immunologische parameters vervolgd. Tijdens de ultraviolet B-behandeling werd significante klinische verbetering in beide groepen gezien, welke bleef voortbestaan in de hyposensibilisatiegroep. Er werden evenwel geen duidelijke veranderingen in de immunologische parameters gezien.

Allergisch contacteczeem wordt vaak bestudeerd via immunohistochemie op huidbiopten. Precieze en reproduceerbare kwantificering van immunohistochemische aankleuring is moeilijk. Een aantal verschillende procedures zijn beschreven, waarvan de resultaten onderling moeilijk vergelijkbaar zijn. In **hoofdstuk 2.3** wordt image analysis gedemonstreerd als een valide en bruikbare methode ter verbetering van de kwantificering van immunohistochemisch gekleurde coupes van ontstekingshuid. Een semi-automatische image analysis procedure (macro) voor meting van oppervlak werd ontwikkeld en gevalideerd. Deze techniek maakte het mogelijk om immunohistochemische aankleuring te kwantificeren als het aantal positieve cellen per vierkante millimeter epidermaal, dermaal of infiltraatoppervlak. De precisie en reproduceerbaarheid van deze image analysis procedure voor meting van oppervlakken was hoog, d.w.z. de afwijkingen waren kleiner dan respectievelijk 0.5% en 1%. Bij vergelijking met traditionele oppervlaktemeting met rasters bleken de gemiddelde waarden van beide methoden equivalent.

Hoofdstuk 3.1 bespreekt klinische manifestaties en diagnostische aspecten van allergische geneesmiddelenreacties veroorzaakt door anti-epileptica. Huiderupties zijn de meest uitgesproken verschijnselen, naast koorts, lymfadenopathie, hematologische afwijkingen en gestoorde leverfunctie. Bij anti-epileptica wordt de zoektocht naar het veroorzakend medicament en een geschikt alternatief nog bemoeilijkt door een vaak langdurig gebruik, combinatietherapie, structurele verwantschap (kruisreacties, mimicry) en een relatief tekort aan alternatieven.

Carbamazepine, fenytoïne, barbituraten en natriumvalproaat zijn verantwoordelijk voor de meerderheid van de allergische geneesmiddelenreacties door anti-epileptica. Hoewel routine laboratoriumtesten de waarschijnlijkheid van geneesmiddelenallergie in een individuele patiënt niet betrouwbaar kunnen voorspellen, zijn de patch test en de *in vitro* lymfocyten proliferatie assay wel bruikbaar ter bevestiging van immunologische reactiviteit tegen anti-epileptica. Een dergelijk onderscheid tussen allergie en niet-immunologische reacties is van groot belang voor het toekomstig medicatiebeleid.

Vijf tot 20% van de patiënten staken de behandeling met anti-epileptica vanwege bijwerkingen. Voorzichtige herintroductie kan echter worden overwogen indien een echte geneesmiddelenallergie kan worden uitgesloten. Voor definitieve bepaling van zulke immunologisch gemedieerde reacties is het aantonen van specifieke antistoffen of gesensibiliseerde lymfocyten vereist. In **hoofdstuk 3.2** wordt de geschiktheid van de patch test en de *in vitro* lymfocyten proliferatie assay onderzocht voor de detectie van allergie door carbamazepine en het mogelijk kruisreagerende oxcarbazepine. Zesenvijftig carbamazepine-gebruikende patiënten met een breed scala van mogelijk allergische bijwerkingen werden getest, evenals carbamazepine-gebruikers zonder bijwerkingen en gezonde vrijwilligers. Zowel de patch test als de lymfocyten proliferatie assay werden gedaan met carbamazepine, oxcarbazepine en drie metabolieten (carbamazepine-10,11-epoxide, 10-monohydroxy-carbamazepine en 10,11-dihydroxy-carbamazepine). Een positieve patch test met carbamazepine werd gezien bij 20% en met oxcarbazepine bij 14% van de patiënten. Positieve lymfocyten proliferatie assay resultaten met carbamazepine en oxcarbazepine werden gevonden bij respectievelijk 40% en 19% van de patiënten. Beide testen waren positief bij respectievelijk 14% en 7% van de patiënten. Kruisreactiviteit tegen oxcarbazepine werd gezien bij ongeveer 40% van de carbamazepine-reactieve patiënten, zowel in de patch test als de lymfocyten proliferatie assay.

In **hoofdstuk 3.3** wordt een casus van exfoliatieve dermatitis door carbamazepine-allergie beschreven. De patiënt is een 6-jarig Kaukasisch meisje dat een gegeneraliseerde erythemateuze huideruptie doormaakte tijdens gebruik van carbamazepine. In de daaropvolgende 4 dagen verergerde de huideruptie tot erythrodermie met koorts en gegeneraliseerde lymfadenopathie. Routine laboratoriumonderzoek leverde verhoogde serumconcentraties van leverenzymen en eosinofilie op. Immunologische reactiviteit tegen carbamazepine werd *in vivo* en *in vitro* bevestigd door respectievelijk de patch test en de lymfocyten proliferatie assay.

Hoofdstuk 3.4 beschrijft een casus van een mogelijk door bromocriptine veroorzaakte pleuritis. De pleuritis, door aanvankelijk onbekende oorzaak, werd gevonden bij een patiënt die reeds 6 jaar werd behandeld met bromocriptine wegens de ziekte van Parkinson. Bij presentatie was bilateraal pleuraverdikking aanwezig, die ernstige beperking van de longfunctie veroorzaakte. Bij laboratoriumonderzoek werden een verhoogde bezinkingssnelheid van de erythrocyten, een polyklonale hypergammaglobulinemie, verhoogde serumconcentraties van de acute fase eiwitten en anemie gevonden. Na het staken van bromocriptine werd duidelijke verbe-

tering van zowel klinische als laboratoriumparameters gezien. De pleuraverdikking verdween echter niet. Hoewel deze bevindingen sterk wijzen op een oorzakelijke rol van bromocriptine, waren zowel de *in vitro* lymfocyten proliferatie assay als de patch test met bromocriptine negatief.

Tenslotte wordt in **hoofdstuk 4** de figuur op de omslag verklaard en worden enkele afsluitende opmerkingen gemaakt over het immuunsysteem van de huid, de modulatie van het immuunsysteem door hyposensibilisatie en de inductie van tolerantie, en diagnostische tests voor geneesmiddelenallergie. In dit hoofdstuk worden de in dit proefschrift beschreven experimentele en klinische studies besproken in het licht van recente ontwikkelingen in het onderzoek van allergische en inflammatoire huidziekten.

ABBREVIATIONS

3D	:	three dimensional
Ab	:	antibody (-bodies)
ACD	:	allergic contact dermatitis
ADE	:	allergic drug eruption(s)
ADR	:	adverse drug reaction(s)
AED	:	anti-epileptic drug(s)
Ag	:	antigen(s)
AHS	:	anticonvulsant hypersensitivity syndrome
ANA	:	antinuclear antibody(ies)
AP	:	alkaline phosphatase
APAAP	:	alkaline-phosphatase-anti-alkaline-phosphatase
APC	:	antigen presenting cell(s)
APES	:	3' aminopropyltriethoxy-silane
BSA	:	bovine serum albumin
C	:	complement
CBZ	:	carbamazepine
CBZ-E	:	CBZ-10,11-epoxide
CCD	:	charge-coupled device
CD1	:	cluster of differentiation-1
CD	:	contact dermatitis
CGRP	:	calcitonin gene-related peptide
CLA	:	cutaneous lymphocyte-associated antigen
cpm	:	counts per minute
CRP	:	c-reactive protein
CS	:	corticosteroid(s)
CT	:	computer tomography
DC	:	dendritic cell(s)
DIOL	:	10,11-dihydroxy-CBZ
DNA	:	deoxyribonucleic acid
DTH	:	delayed type hypersensitivity
EN	:	endothelial cell(s)
EC	:	epidermal cell(s)
ECP	:	eosinophil cationic protein
EDN	:	eosinophil-derived neurotoxin
EGF	:	epidermal growth factor
ELISA	:	enzyme-linked immunosorbent assay(s)
ETAF	:	epidermal cell-derived thymocyte activating factor
ESR	:	erythrocyte sedimentation rate
FB	:	fibroblast(s)
FACS	:	fluorescence-activated cell sorter
FITC	:	fluorescein isothiocyanate
FRC	:	free residual capacity
GM-CSF	:	granulocyte-macrophage colony-stimulating factor
h	:	hour(s)
Hb	:	hemoglobin
^{3}H-TdR	:	tritiated thymidine
HE	:	haematoxylin eosin
HETE	:	hydroxy-eicosa-tetraenoic acid
HLA	:	human leukocyte antigen
HLS	:	hue, lightness, saturation
HS	:	human serum
Hu	:	human
IA	:	image analysis
ICAM	:	intercellular adhesion molecule
ICD	:	irritant contact dermatitis
ICDRG	:	international contact dermatitis research group
ICE	:	interleukin-1β converting enzyme
IFN	:	interferon
Ig	:	immunoglobulin(s)
IHC	:	immunohistochemistry
IL	:	interleukin
IL-1ra	:	interleukin-1 receptor antagonist
IP-10	:	IFN-γ-inducible protein 10
KC	:	keratinocyte(s)
LAF	:	lymphocyte activating factor
LC	:	Langerhans cell(s)
LE	:	lupus erythematosus
LFA	:	leukocyte function-associated antigen

LPA	:	lymphocyte proliferation assay(s)
LPS	:	lipopolysaccharide
LT	:	leukotriene(s)
MBP	:	major basic protein
MCAF	:	monocyte-chemotactic and activating factor
MCP	:	monocyte-chemotactic protein
MC	:	mast cell(s)
MC_{TC}	:	tryptase and chymase positive mast cell(s)
MHC	:	major histocompatibility complex
MHD	:	10-monohydroxy-CBZ
MIg	:	mouse immunoglobulin
MIP	:	macrophage inflammatory protein
MPO	:	myeloperoxidase
moAb	:	monoclonal antibody
NK	:	natural killer
NKA	:	neurokinin A
NF	:	nuclear factor(s)
NSAID	:	non-steroidal anti-inflammatory drug(s)
OCBZ	:	oxcarbazepine
OD	:	optical density
p	:	probability
PAF	:	platelet activating factor
PBMC	:	peripheral blood mononuclear cell(s)
PBS	:	phosphate-buffered saline
PDGF	:	platelet-derived growth factor
PE	:	phycoerythrin
PG	:	prostaglandin(s)
PHEN	:	phenytoin
PMA	:	phorbol myristate acetate
PPD	:	purified protein derivative
PT	:	patch test(s)
R	:	receptor(s)
RαM	:	rabbit anti-mouse
RANTES	:	regulated and normal T cell expressed and secreted
RAST	:	radioallergosorbent test(s)
RGB	:	red, green and blue
RNA	:	ribonucleic acid
RPMI	:	Roswell Park Memorial Institute
SALT	:	skin-associated lymphoid tissue
SD	:	standard deviation(s)
SEM	:	standard error(s) of the mean
SI	:	stimulation index(-ices)
SIS	:	skin immune system
SLE	:	systemic lupus erythematosus
SOM	:	somatostatin
SP	:	substance P
TcR	:	T cell receptor(s)
TEN	:	toxic epidermal necrolysis
TGF	:	transforming growth factor
Th	:	T helper
TIFF	:	tagged image file format
TLC	:	total lung capacity
TNF	:	tumor necrosis factor
TPA	:	12-O-tetradecanoyl-phorbol-13-acetate
UV	:	ultraviolet
VCAM	:	vascular cell adhesion molecule
VLA	:	very late activating antigen
WBC	:	white blood cell(s)

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