IN SITU CYTOKINE ANALYSIS IN HUMAN CONTACT DERMATITIS

IMPLICATIONS FOR A DIFFERENTIATION MARKER AND AN *EX VIVO* HUMAN CONTACT DERMATITIS MODEL

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IN SITU CYTOKINE ANALYSE IN CONTACT DERMATITIS IN DE MENS

IMPLICATIES VOOR EEN DIFFERENTIATIE MARKER EN EEN *EX VIXO* CONTACT DERMATITIS MODEL VOOR DE MENS

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Ter nagedachtenis aan mijn moeder
Voor mijn vader, Ineke, Tom en Imke
Voor Erik

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1.1 GENERAL INTRODUCTION

Our skin is under constant environmental stress resulting from personal care (soap, cosmetics, clothes), home (plants, wood, vegetables), leisure (glues, photodevelopers, paints) and work. Although the skin is an efficient barrier and body protector a cutaneous disorder may be induced by many substances or conditions. The causes of skin injury can be classified in one of the following categories: chemical, mechanical, physical and biological. Within these categories chemical injuries constitute an important part.

Toxic reactions to the skin from environmentally encountered substances may include ulcerations, pigmentary abnormalities, folliculitis with the development of acne-type lesions and neoplasms, but the skin disorder that most frequently occurs is contact dermatitis. Contact dermatitis is a common non-contagious inflammatory skin reaction elicited by an external factor. The group of contact dermatitis reactions includes allergic contact dermatitis, irritant contact dermatitis (acute/cumulative), phototoxic contact dermatitis and photoallergic contact dermatitis, and the immediate type of contact dermatitis (e.g. urticaria). This thesis will be limited to allergic and irritant contact dermatitis.

Contact dermatitis accounts for 60-95 per cent of all occupational skin diseases, whereas occupational skin diseases account for 9-34 per cent of all notified occupational diseases. The incidence of notified occupational skin diseases in three european countries and the US is 0.5 to 0.7 cases per 1000 workers a year. The frequency of sick leave due to skin diseases among workers is approximately 7 cases per 1000 workers a year. Approximately 3 per cent of the cases do not recover within one year and consequently receive disability pension. The prevalence of skin diseases in the workers population, however, is much higher than would be expected from the data on sick leave, disability pension or notified occupational diseases. This is probably caused by the fact that only a small fraction of workers reports sick due to skin diseases. The prevalence of eczema on hands and forearms in occupations in the construction industry and the metal industry was higher than 10 per cent Therefore the identification of risk factors and the prevention of occupational skin diseases especially contact dermatitis deserves more attention.

Allergic contact dermatitis (ACD) is a very common mechanism, representing a mode of defense through which invasive substances can be rejected. ACD is a specific inflammatory reaction due to chemical agents (plants, medicaments, cosmetics, metals, fabrics). In ACD epidermal and dermal oedema, vesiculation and erythema are seen. Site and configuration of skin lesions are clues to the causal agent; patch tests may

confirm the diagnosis identifying the substance responsible for ACD. Avoidance of the causal agent usually decreases clinically manifest dermatitis. The contact allergens involved in ACD are usually chemicals of low molecular weight. In general, ACD can be elicited by extremely low doses.

In allergic contact dermatitis there is an afferent (induction or sensitization phase) and an efferent (elicitation or challenge phase) limb. The afferent limb includes the events following allergen presentation to the immunologically naive system and is complete when the subject is sensitized and capable of giving a positive challenge reaction. The efferent limb is activated during the elicitation (challenge) phase. The main events which occur in the afferent phase after skin penetration of the small antigens are (1) the binding of contact allergens to skin constituents notably Major Histocompatibility Complex (MHC) Class II molecules present on the Langerhans cell as antigen presenting cell or binding of the contact allergen to a skin protein which in turn is taken up by Langerhans cells, is processed and is presented by the Langerhans cell in the contect of MHC Class II molecules, (2) migration of the antigen-carrying Langerhans cells as veiled cells via the afferent lymphatics to the regional lymph nodes where they settle in the paracortical areas, (3) recognition of the antigen presented on MHC-class II molecules by the T-cell receptor and (4) proliferation and subsequent dissemination of specific (memory) T-cells throughout the body.

After sensitization has been completed a subsequent contact with the appropriate allergen may initiate the elicitation reaction. Upon this renewed skin contact the allergen is locally presented to allergen specific T-cells, which in turn are triggered to produce cytokines. The release of these T-cell derived cytokines together with the cytokines produced by the epidermal cells after skin application cause an increase in the number of CD4 positive T-cells, cytotoxic T-cells and macrophages that amplify the local mediator release and lead to an eczematous reaction after 48-72 hours.

Irritant contact dermatitis (ICD) in contrast to ACD, is a non specific toxic inflammation of the skin. ICD may occur after short term exposure to large quantities of (highly) irritative agents or may result from repeated or long term exposure to less irritative agents. The latter form of ICD caused by repeated or long term contact (without recovery) in most individuals looks like ACD and is described in this thesis.

In ICD epidermal and dermal oedema, vesiculation and erythema are seen. The eczematous reaction caused by an irritant is restricted to the exposed skin in contrast to the reaction caused by an allergen. Under normal conditions adequate hygienic measures are effective to prevent ICD. In contrast to ICD protection against ACD by hygienic measures is difficult, since skin contact with even very low quantities of an allergen may give rise to such an effect once sensibilization has taken place. Thus, in ACD every contact has to be avoided.

In irritant contact dermatitis there is an induction of damage to the epidermal cell layers resulting in cytokine release by these epidermal cells, non-specific migration of Langerhans cells, infiltration of inflammatory CD4 positive T-cells, cytotoxic T-cells and macrophages from the circulation into the dermal compartment. In contrast to ACD, ICD is not dependent on pre-existing sensitization and therefore ICD is described as a non-specific inflammatory reaction.

Exposure to several chemical substances may result in allergic as well as irritant contact dermatitis. The correct assessment and diagnosis of allergic and irritant contact dermatitis may pose considerable problems in dermatologic practice even when using epicutaneous patch testing. Until now both types of contact dermatitis, despite of their induction by different mechanisms, are difficult to differentiate from the macroscopic appearance, electron-microscopy, and light microscopic histology. To establish the type of contact dermatitis and the possible measures of protection against contact with the chemical a differentiation marker between ACD and ICD is required.

As already stated, ACD depends primarily on the activation of specifically sensitized T-cells. Upon local allergen triggering these T-cells initiate an inflammatory cascade leading to overt skin disease. In contrast to ACD, ICD is caused by a non-specific inflammatory reaction. Since different mechanisms are involved in ACD and ICD the search for a differentiation marker continues. In our study, we have focussed on epidermal cells and T-cells with their mediators produced.

Epidermal cells such as keratinocytes and Langerhans cells are the first cells in the skin which are encountered by a chemical substance. After exposure to a chemical, these cells immediately produce common inflammatory cytokines (a.o. interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α)) which enable them to cause a local inflammatory reaction in the skin. In ACD the Langerhans cells have a role in the activation and expansion of antigen-specific T-cells. In ICD and ACD non-specific inflammatory T-cells are present in the cellular infiltrates in the dermis.

Upon activation, T-cells produce cytokines. These cytokines may lead to the enhancement of the inflammatory reaction. Cytokines are glycosylated polypeptides, produced by a variety of cell types. Originally, these polypeptides were called lymphokines, monokines or interleukins based on their source or their role in the communication between leucocytes. Detailed investigations revealed that most of these polypeptides were produced by more than one cell type and exerted their regulatory role on a variety of other cell types. In general, cytokine producing cells are paracrine cells, meaning that their regulatory activity is directed to target cells which are localized in close conjunction.

Activated T-cells will express CD40 ligand (gp39) even before they start producing cytokines. CD40 is expressed on B-cells and on human professional antigen presenting cells such as Langerhans cells and other dendritic cells. The role of CD40 ligand-CD40 interaction is established in different humoral (a.o.hyper-IgM syndrome in man) and cellular immune responses (a.o. acute graft versus host response in mice). This suggests that both the cell derived cytokines as well as CD40 ligand and CD40 could be involved in contact dermatitis. Therefore, detailed knowledge of the cytokine profiles as well as knowledge of the functional role for CD40 ligand-CD40 interaction in contact dermatitis is required to establish the possibility to use these antigens or surface markers to differentiate between allergic and irritant contact dermatitis.

Patients presented with the clinical picture of contact dermatitis are currently skin tested (epicutaneous patch test) to verify whether the dermatitis is of allergic origin. Present skin test parameters are based on erythema, induration and vesiculation of the reaction. It is sometimes difficult to differentiate between allergic and irritant reactions at the time of patch test reading. Another disadvantage of patch testing is the risk of sensitization during the test. To solve this problem a suitable test to establish the *ex vivo* individual sensitivity for a specific contact allergen could be helpful. A routine test to establish the individual predisposition to be sensitized in the future is also not available yet. Workers take the risk of sensitization during their work. Therefore, the development of a suitable test to establish the individual predisposition could be of great value in occupational health practice.

The diagnostic use of blood samples and/or human skin in *in vitro* tests have not obtained a routine status to establish the *ex vivo* individual sensitivity or predict the predisposition to be sensitized in the future for a specific contact allergen. The search for an adequate *ex vivo* test has started. Immune-deficient mice are widely used for human skin graft experiments. They accept grafts from many human skin diseases such as psoriasis, cutaneous lupus, pemphigus and vitiligo. They can be reconstituted with human PBL's (full house mouse). To develop an adequate human-skin-grafted-immune-deficient-mice model for allergic contact dermatitis many experiments should be designed. In our study, we have focussed on the prerequisites of such a model; the persistence of human antigen presenting cells for the uptake of allergen and migration, the persistance of human antigen specific T-cells and non-specific T-cells in the graft or circulation during several weeks, and signals for homing or recruitment of human T-cells to the human skin graft after challenge.

1.2 AIM OF THE STUDY AND INTRODUCTION TO THE CHAPTERS

The aim of the studies presented in this thesis was to find a differentiation marker between allergic and irritant contact dermatitis, and to develop an *ex vivo* model to establish the individual sensitivity, or predisposition to be sensitized in the future, for a specific given allergen.

Special emphasis was put on the development of antibody and probe based *in situ* detection methods to analyse cytokine protein and mRNA profiles in skin tissue respectively. Therefore chapter 2 presents the *in situ* approach. Basic information is given. The variety of immunohistochemical methods and *in situ* hybridization methods that have been used for demonstration of cytokines and cytokine-mRNAs in different human tissues are described (chapter 2.1). The specificity controls for primary antibody and regular procedure controls in cytokine detection are listed and described. The advantages and disadvantages of each immunohistochemical and *in situ* hybridization method are evaluated. Furthermore, the selection of the method of choice for cytokine protein as well as cytokine-mRNA *in situ* detection is discussed.

The usefulness of the two step indirect immunohistochemical approach with unlabelled primary antibody to detect cytokine producing cells is demonstrated in the human tonsil (chapter 2.2). The localization of cytokine and antibody producing cells in human palatine tonsil is described. The implications of these findings with regard to T-B cell interactions taking place in the extrafollicular area of human palatine tonsils is discussed.

Based on the selections as proposed in chapter 2 the two step indirect immunohistochemical detection method with unlabelled primary antibody is also used for the *in situ* cytokine analysis (IL-1α, TNF-α, IFN-γ, IL-2) in human contact dermatitis in chapter 3.1 and 3.2. In chapter 3.3. different antibody based detection methods dependent on the cytokine (IL-1ß, IL-4 or IL-10) were applied to visualize the presence of cytokine producing cells. To find a differentiation marker between allergic and irritant contact dermatitis the local cytokine profiles in skin biopsies from allergic and irritant patch test reactions are determined by immunohistochemistry and *in situ* hybridization. The frequencies of IL-1α, IL-1ß, IL-2, IL-4, IL-10, TNF-α and IFN-γ producing cells are investigated.

Specifically activated T-cells are present in allergic contact dermatitis. Our *in vivo* mouse studies revealed that activated T-cells express CD40 ligand in thymus dependent responses. In addition, it is known that cultured human Langerhans cells express CD40 antigen. In chapter 4, we determined the kinetics of appearance of

CD40 ligand, CD40 positive cells and cytokine producing cells in a delayed type hypersensitivity (DTH) response in murine skin using immunohistochemistry. The detection of CD40 ligand and CD40 positive cells in human allergic contact dermatitis is described. Until now CD40 ligand-CD40 interaction was suggested to be important in mainly humoral immune responses. In this chapter, the functional role of CD40 ligand-CD40 interaction in ear swelling (cellular immune response) is investigated and compared to the role in specific local antibody production (humoral immune response) after skin exposure to a contact allergen in mice. The implications of these new findings with regard to CD40 ligand as possible marker to differentiate between allergic and irritant contact dermatitis in man is discussed.

The epicutaneous patch test provides a sensitive methodology for diagnosing hypersensitivity in man. A suitable *in vivo* or *in vitro* test to establish the *ex vivo* individual sensitivity or predisposition to be sensitized in the future for a specific contact allergen is not available. We started the search for a human-skin-onto-immune-deficient-mice model that retains the possibility to develop an allergic contact dermatitis reaction. The main events in the induction or sensibilization phase are the association of the allergen with MHC class II molecules present on antigen presenting cells "Langerhans cells" and migration of these allergen-carrying Langerhans cells to the draining lymph node. In chapter 5 it is determined whether the functional activity in a contact sensitization response in a human-skin-graft-onto-nude-mice model is affected at the level of antigen uptake by human Langerhans cells and human Langerhans cell migration (chapter 5.1). Furthermore, it is investigated whether human antigen presenting cells expressing MHC class II molecules are still present in the human skin graft after transplantation. The migration capability of these human antigen presenting cells is studied after RITC application on the human skin graft. The frequencies of RITC-carrying and human MHC class II positive cells are determined in the draining lymph nodes before and after RITC application on the skin graft using *in situ* immunohistochemical staining methods.

The persistance of antigen specific T-cells and non-specific T-cells in the graft or circulation during several weeks, and signals for homing or recruitment of human T-cells to the human skin graft after challenge are additional prerequisites in a human-skin-onto-immune-deficient-mice model for allergic contact dermatitis. In the efferent phase, Langerhans cells present the antigen to T-cells in the skin in order to initiate an appropriate antigen-specific immune response with cellular infiltration in the skin. In case of a human-skin-onto-immune-deficient-mice model it is investigated whether T-cells present in the skin or circulation have the capability for homing and recruitment to the human skin graft after challenge. Therefore, in chapter 5.2 the

possibility to imitate the efferent phase in a human-skin-onto-mice model is described. Biopsies are obtained from allergic affected skin sites and grafted onto the immune-deficient mice. It is investigated whether T-cells and other characteristic human antigens are still present in the human skin graft 2 weeks after transplantation. The expression of human CD3, MHC class I and II molecules and adhesion molecules in the human skin graft on immune-deficient mice two weeks after transplantation and specific allergen application is analysed (chapter 5.2).

In chapter 6 the main points emerging from the experimental studies are discussed.

Chapter 2

The *in situ* approach

2.1

Detection of human cytokines *in situ* using antibody and probe based methods
Journal of Immunological Methods 185: 149-175, 1995.

2.2

Immunohistochemical detection of co-localizing cytokine and antibody producing cells in the extrafollicular area of human palatine tonsils
Clinical Experimental Immunology 93: 223-228, 1993.

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Review article

Detection of human cytokines in situ using antibody and probe based methods

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1. Introduction

Cytokines are extremely potent glycosylated proteins, acting as biological chemical messengers. Cytokines are known under a variety of other names, such as lymphokines, monokines, interleukins (IL), growth factors, interferons (IFN) and tumor necrosis factor (TNF), based on their source or their role in the communication between cells. Cytokines are produced by a variety of cell types of both hematopoietic and non-hematopoietic origin, and are produced when and where they are needed. Cytokines form a partly degenerated system in that they can deliver different specific messages to one cell, or bring the same message to different cells. The way this message is interpreted depends on the cytokine, its receptor, which cell it acts on, and what other messages that particular cell is receiving at the same time. Cytokines may regulate production of their own receptor, secondary messengers, proliferation, differentiation, and effector aspects of target cells (Balkwill, 1993; Romagnani, 1994; Callard and Gearing, 1994). Furthermore, they are important in neoplastic transformation, destruction, repair, remodelling of tissue and defence mechanisms. IL, TNF, platelet-derived growth factor, and transforming growth factors are known to mediate processes in many infectious and inflammatory diseases involving organ systems such as the pulmonary, gastrointestinal, renal, and musculo-skeletal systems. Furthermore, cytokines are important in foreign-body granulomatous reactions, such as silicosis and asbestosis (Jiranek et al., 1993).

Both in vitro and in vivo approaches are used to increase our knowledge of the role of cytokines in human tissue. In vitro research uses tools to determinate which individual factors and cells

Abbreviations: AP, alkaline phosphatase; HRP, horseradish peroxidase; APAAP, phosphatase-anti-alkaline phosphatase; PAP, peroxidase-anti-peroxidase; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Ig, immunoglobulin; TNP trinitrophenyl; c, complementary; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolylphosphate; AEC, 3-amino-9-ethylcarbazole; DAB, 3,3'-diaminobenzidine.

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may be required for cell activation, proliferation, and differentiation. In vitro research intrinsically disregards the complex organization of human organs and tissues. It reflects the full potential of cells and factors under defined, and sometimes artificial circumstances, whereas in vivo research reflects what cells and factors are actually doing at a given moment. Experiments with modulating agents in man, e.g. cytokines, neutralizing antibodies or agonists and antagonists are typical examples of in vivo cytokine research. In vivo research can also be based on in situ techniques, such as immunohistochemistry and in situ hybridization (ISH), which allow the in vivo study of the localization, activity, and interactions of various cells and cytokines in relation to their direct unchanged original microenvironment. This can be achieved with all types of tissue at any given moment, provided biopsies can be taken, without inducing artefacts by manipulations such as cell separation and culture. These and other advantages are listed in Table 1. Disadvantages include the fact that quantitative results are rather laborious to procure. In addition, the interpretation of cell-cell interactions and identification of tissue compartments require specific training.

Regulation of immune responses by cytokines is mainly a local and strongly compartmentalized process, and therefore in most studies immunohistochemical and/or ISH methods are desirable to investigate the localization of cytokine producing cells at the single cell level. The choice of a specific detection method is based on the individual needs of the laboratory, such as the type of specimen and molecular structure being investigated, the degree of signal to 'noise' ratio, the processing time, safety, and costs. Here we discuss a few typical examples of methods for the detection of human cytokines in tissue sections using antibody as well as the probe based methods, currently used. Although we will limit ourselves to the discussion of immune system regula-

Table 1
Advantages of cytokine and cytokine-mRNA detection in tissue sections [a]

Detection level	
1.	Detection of rare cytokine producing cells (1 in 10^6–10^7)
2.	Detection of cytokine producing cells with low cytokine production levels or blocked secretion
3.	Real time analysis (sample frozen within seconds)
4.	No cell or tissue manipulation such as cell preparation and (pre-)culture (induction of 'background' synthesis)
Detection and localization	
1.	Determination of anatomical localization in tissue compartments
2.	Determination of clone size
3.	Potential for quantification of cytokine producing cells
Detection and combinations of methods	
1.	Double simultaneous immunohistochemical detection of multiple antigen specificities in a single sample (with antigen, antibody or other cells)
2.	Combination immunohistochemistry with ISH
3.	Combination of cytokine detection with enzyme histochemistry (for endogenous enzymes e.g. acid phosphatase for macrophages)
4.	Sequential application and analysis of cross-reactive (re-)agents is possible
Detection and source	
1.	Small sample size (biopsies) possible
2.	Mounted stained slides provide permanent record of results
3.	Use of archive material, retrospective studies, possible
4.	Use of frozen tissue is convenient for timing in experimental design

[a] As compared to cytokine detection in body fluids, isolated cells, and in vitro assays.

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tory cytokines IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, and TNF-α, the methods described are or may also be applicable to other cytokines.

2. Antibody based cytokine detection

2.1. Immunohistochemistry

Immunohistochemical methods permit localization of defined structures through a classical antibody-antigen reaction. The antibody-antigen reaction can be visualized in various ways using reporter molecules such as fluorochromes or enzymes (with their specific substrates and chromogens). In general, immunohistochemical staining involves fixation, incubation of antibodies and/or other reagents, and visualization of the reporter molecules.

When using immunohistochemical methods tissues and/or tissue sections are fixed, and sometimes pre-treated in a microwave oven or treated with proteolytic enzymes to facilitate penetration of antibodies or other reagents necessary for detection and staining. Recently, Andersson and Andersson (1994) have reported that incubation using saponin improves tissue accessibility and this is improving the success of cytokine staining. One should always be aware that epitopes present on the antigen can be changed or damaged using tissue preparation methods. The fixation of tissue sections is a crucial point, because the antibody needs to penetrate the tissue and cell membrane to detect intracellular cytokines. The fixation method should not result in formation of neo-determinants on the cytokine (protein). In this regard acetone fixation is the optimal fixation method for immunohistochemical purposes (Laman et al., 1991), and therefore is frequently used. Acetone induces a very mild fixation based on coagulation since the proteins in the acetone fixed tissue become insoluble after precipitation. There is no cross-linking, no neodeterminants are formed and a low diffusion barrier is provided since the cell membrane becomes permeable by lipid extraction. The morphology of the tissue is less well preserved (but still well within acceptable limits) in acetone fixed cryostat sections as compared to sections from formalin fixed paraffin-embedded tissue.

In the case of formalin fixation is induced by the addition and conjugation which results in cross-linking and a high diffusion barrier. No coagulation and no lipid extraction take place. To enhance the detection of antigens in paraffin sections pre-treatment in a microwave oven or treatment with proteolytic enzymes is frequently used.

For the detection of cytokine producing cells liquid nitrogen frozen tissue and acetone fixed cryostat sections are predominantly used because of the aforementioned advantages of this method.

2.2. Antibody based methodology

Monoclonal and polyclonal antibodies specific for human cytokines

Mouse, rat, rabbit, goat and/or sheep can be immunized with the cytokine protein in the presence of adjuvant. However, species homology in cytokine sequences can give problems i.e. unresponsiveness to the complete cytokine protein. An option is to elicit antibodies with synthetic peptides selected after analysis of tentative antigenic sites in the amino-acid sequence. With this method antibodies can be developed avoiding species homology problems (for review see Boersma et al., 1993). The handbooks 'Current Protocols in Immunology' (Coligan et al., 1994) and the 'Antibodies: A Laboratory Manual' (Harlow and Lane, 1988) provide details on the development of antibodies in general.

A variety of monoclonal and polyclonal antibodies against human cytokines are frequently used for immunohistochemical purposes (Table 2). In general, monoclonal antibodies are preferable for cytokine staining, since they tend to give less background than polyclonal antisera. Cytokine specificity is established using immunological assays, including a neutralization assay, immunoblotting, and immunoprecipitation. However, only rarely are antibodies evaluated for use and specificity in an immunohistochemical assay. In order to find an antibody suitable for staining on fixed tissues numerous different antibodies have to be screened.

Table 2
Anti-human cytokine antibodies used for immunohistochemistry

Cytokine	Antibody	Species	Source	References
IL-1α	Vhp18	Mouse	IRIS Department of Immunopharmacology, Siena, Italy	Ruco et al., 1989,1990; Hoefakker et al., 1992,1993,1995
	LP-710	Rabbit	Genzyme, Cambridge, MA, USA	Yoshioka et al., 1993; Hansen et al., 1991; Ruco et al., 1990; Heufelder and Bahn, 1993
IL-1β	Vmp20	Mouse	IRIS Department of Immunopharmacology, Siena, Italy	Ruco et al., 1990; Hoefakker et al., 1992,1993,1995
	mAb [a]	Mouse	Upjohn., Kalamazoo, MI, USA	Koch et al., 1992; Chensue et al., 1992
	02-1025	Mouse (IgM)	Cistron Biotechnology, Pine Brook, NJ, USA	Prens et al., 1990; Cooper et al., 1990; Wood et al., 1992a; Anttila et al., 1990
	LP-712	Rabbit	Genzyme	Yoshioka et al., 1993; Hansen et al., 1991; Noronha et al., 1993
	02-1100	Rabbit	Cistron	Asano et al., 1991; Anttila et al., 1990
IL-2	DMS-1	Mouse	Genzyme	Hoefakker et al., 1992,1993,1995; Sato et al., 1993
IL-4	3H8 and 4D9 [b]	Mouse	Ciba-Geigy, Basel, Switzerland	Bradding et al., 1993,1994
	1842-01	Mouse	Genzyme	Hoefakker et al., 1992,1993,1995
IL-5	mAb7 and mAb8[b]	Mouse	Glaxo, Greenford, Middlesex, UK	Bradding et al., 1993,1994
IL-6	104-B11, 83-E9 and 32112 [b]	Mouse	Glaxo	Bradding et al., 1993,1994
	LP-716	Rabbit	Genzyme	Yoshioka et al., 1993; Tyor et al., 1992
IL-10	MCA926	Mouse	Serotec, Oxford, England	Hoefakker et al., 1992,1993,1995
IFN-γ	MD-2	Mouse	Biosource, Int., Camarillo, CA, USA	Hoefakker et al., 1992,1993,1995
	pAb	Rabbit	Genzyme	Tyor et al., 1992; Heufelder and Bahn, 1993
TNF-α	61E71	Mouse	Dr. W. Buurman, Maastricht	Hoefakker et al., 1992,1993,1995
	MTN-1	Mouse	Hayashibara Biochemical Laboratories, Okayama, Japan	Yoshioka et al., 1993; Misaki et al., 1992
	mAb	Mouse	Genentech, South San Francisco, CA, USA	Hofman and Hinton, 1992; McCall et al., 1989
	CD 0006	Mouse	Celltech, Berkshire, UK	Noronha et al., 1992,1993
	B154.2, 6, 7, 9 and 10	Mouse	Wistar Institute, Philadelphia, PA, USA	Ruco et al., 1989; Murphy et al., 1992; Hoffmann et al., 1993
	TNF-E	Mouse	Boehringer Institute, Vienna, Austria	Arnoldi et al., 1990; Kretschmer et al., 1990
	IP-300 [c]	Rabbit	Genzyme	Heufelder and Bahn, 1993; Sasaguri et al., 1992; Tillie-Leblond et al., 1994; Tsuchida et al., 1992

All the antibodies are used on cryostat sections from liquid nitrogen frozen tissue.
[a] Only tested on paraffin sections.
[b] Only tested on sections from GMA embedded tissue.
[c] Tested on both cryostat as well as paraffin sections.

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When cells in tissue sections are stained with anti-cytokine antibodies, the specificity of the antibody (Table 3) and staining controls (Table 4) which establish the efficiency of the staining procedure under the conditions of the assay must always be tested.

Specificity controls for primary antibody in cytokine detection

In performing in situ analysis of cytokines on tissue sections one of the main problems encountered is the specificity of the primary antibodies, i.e. the cross-reaction of the antibody with non-cytokine epitopes in the fixed tissue, producing a false positive result. The essential immunohistochemical specificity controls for cytokine detection, in order of importance, are listed in Table 3. To evaluate an anti-cytokine antibody in an immunohistochemical assay the antibody has, first of all, to be titrated. Titration of each individual antibody is mandatory since the use of antibody which is too dilute as well as antibody which is too concentrated will result in weak or negative staining (compare with prozone effect in ELISA). Furthermore, the avidity of the binding between the primary and the secondary antibody, dependent on the concentration of the secondary antibody used and the frequency of immobilized primary antibody in the tissue, proves to be important in signal amplification. Using the optimal concentration of the secondary antibody two times more isotypic determinants as well as hapten

Table 3
Specificity controls for primary antibody (Ab) in cytokine detection in direct and indirect immunohistochemical staining methods

Method	Result	Demonstrates	Does not exclude
1. Dose response curve of the primary antibody	[Ab] dependent staining	Antibody and dilution dependent staining (optimum concentration ($\pm 20\ \mu g/ml$)	Cross-reactivity [d]
2. Competition with unlabelled primary antibody with the same antigen specificity but different epitope [a,b]	Reduced staining [Ab] dependent staining	Detection of same antigen, confirms antigen specificity of Abs	Tissue antigen cross-reactivity [d] by both Abs
3. Double staining with labelled primary antibodies with the same antigen specificity but different epitope (i.e. one primary antibody revealed with enzyme X and the other with enzyme Y on one section)	Double stained cells	Detection of same antigen, confirms antigen specificity of Abs	Tissue antigen cross-reactivity [d] by both Abs
4. Competition of the primary antibody with antigen (section) [b]	Reduced staining [Ab] dependent staining	Antigen recognition after tissue preparation	Tissue antigen cross-reactivity [d]
5. Preincubation of the primary antibody with antigen (tube) [b]	Reduced staining [Ab] dependent staining	Antigen recognition	Tissue antigen cross-reactivity [d]
6. Comparing the staining of the antibody specific for the cytokine with preimmune serum or normal pooled serum of the same species [c]	no staining	Preimmune serum contains no specific or cross-reactive antibodies	Tissue antigen cross-reactivity [d]
7. Double staining for phenotype	Double stained cells	Cell phenotype dependent staining	Tissue antigen cross-reactivity [d]
8. To study the antigen expression with the primary antibody in tissue during immune responses (kinetics) [b]	Staining	Time dependent staining Indication of specificity	Tissue antigen cross-reactivity [d]
9. To study the corresponding cytokine mRNA expression	Staining	Indication of specificity	Tissue antigen cross-reactivity [d]

[a] Not feasible with polyclonal antibodies.
[b] Dose-response curves are used.
[d] Controls applicable in only the methods based on unlabelled primary antibody.
[d] Tissue antigen cross-reactivity means cross-reactivity of simultaneous emerging cross-reactive epitopes.

molecules bind to the same secondary antibody based on the presence of two antigen binding sites per antibody (this is comparable to the hook effect in ELISA). The relation between the staining intensity and the optimum antibody concentration (5–20 μg/ml) found in the dose response staining curve gives an indication of whether it is possible to use the antibody in immunohistochemical assays.

Control of cross-reactivity of the primary antibody with non-cytokine epitopes present in the tissue is essential. For example, in recent experiments using frozen spleen sections of trinitrophenyl-Ficoll immunized mice we found positive staining with a monoclonal antibody against mouse TNF-α. However, this staining could be blocked by adding trinitrophenyl-glycine and by a monoclonal antibody against trinitrophenyl. From these results we concluded that the monoclonal antibody against TNF-α also recognized the completely unrelated molecule trinitrophenyl. Only by competition with antigen or a second monoclonal antibody (not recognizing trinitrophenyl) directed against a different antigenic determinant of TNF-α were we able to show that the staining observed with the first antibody was non-specific.

Competition of the antibody (labelled) used for cytokine detection on the tissue section with a second monoclonal antibody (unlabelled) with the same specificity provides the best specificity control. Double staining with two monoclonal antibodies with the same antigen specificity, recognizing an epitope close to the epitope recognized by the first antibody, could also be used as a specificity control. However, two monoclonal antibodies with the same antigen specificity and both suitable for use in immunohistochemistry are rarely available.

The antigen specificity of an antibody can also be tested by competition of the antibody with cytokine present in the tissue section and highly purified cytokine antigen added to the section after tissue preparation. Secondly, pre-incubation of the primary antibody with antigen (in the test tube) before tissue application to the tissue is another option for testing antigen specificity.

Pre-or non-immune pooled antiserum can only be used as a control for non-specific binding but this does not give any information on the specificity of the primary antibody. Upon staining with the affinity absorbed antibody or pre-or non-immune pooled antiserum it is still possible that cells can stain for a non-specific protein in the tissue due to antigen cross-reactivity.

When antigen cross-reactivity is expected, double staining of cytokine producing cells with an

Table 4
Regular procedure controls for immunohistochemical cytokine detection

Control	Method	Demonstrates
Tissue	Positive tissue in which detection is ensured (e.g. tonsil for cytokines)	Tissue specific staining
	Negative tissue in which antigen is not present	Tissue specific staining
Primary antibody	Absence of primary antibody	Non-specific staining due to secondary reagents (back-ground)
	Isotype matched relevant antibody (antigen present in tissue section)	Validity of protocol
	Isotype matched irrelevant antibody (antigen not present in tissue section)	Any non-specific staining due to reagents
Secondary reagents used after primary antibody but before histochemical revelation [a]	Absence of secondary reagents before histochemical revelation	Non-specific staining due to secondary reagents
Histochemistry	Substrates only	Non-specific staining. Endogenous enzyme activity
	Subsequent different histochemical revelations	Incompatibility of techniques

[a] Controls applicable in only the indirect methods.

antibody against a known cell marker could give an indication of the antigen specificity of the cytokine staining. In addition, the in vivo kinetics of cytokine producing cells may help to differentiate between specific and non-specific cytokine staining due to evident differences in the number of cytokine producing cells at different time points (Van den Eertwegh et al., 1991). To confirm antibody based results with respect to one particular cytokine, probe based detection of corresponding mRNA can be simultaneously performed (Hoefakker et al., 1995).

Although much emphasis is placed on controls pertaining to the staining as a whole, we feel that more stress should be placed on the actual immunohistochemical properties of specific primary antibodies.

Regular procedure controls

Day to day procedure controls for immunohistochemical cytokine detection are listed in Table 4. To establish the correctness of the staining procedure and particularly to ascertain the absence of non-specific reactions of the chemicals and reagents used for cytokine detection an irrelevant isotype matched antibody (of the same immunoglobulin (Ig) class) for an antigen not present in the tissue under investigation has to be used as a negative primary antibody control. In order to demonstrate positive results in a particular tissue, the sections are incubated with a relevant antibody for a non-cytokine antigen present in the tissue under investigation (isotype matched). Tissues that do not contain the specific cytokine and tissues that contain the cytokine should be used as negative and positive tissue controls respectively. These tissue controls should reveal the correct distribution of reactivity and non-reactivity respectively (Boenisch et al., 1989).

Omission of the primary anti-cytokine antibody can only be used to exclude non-specific background staining due to secondary reagents. To decrease cross-reactions of secondary reagents with endogenous immunoglobulins only antisera absorbed with human immunoglobulins should be used. In addition, application of the substrates and chromogens only is necessary for histochemical revelation of non-specific staining due to endogenous enzyme activity.

Immunohistochemical detection of a specific cytokine in human tissue in general provides positive cytoplasmic staining with or without evident typical Golgi staining for cytokine producing cells (dependent on the cytokine), but not target cells, which have absorbed or endocytosed the cytokine. The possibility that cytoplasmic staining represents uptake of the specific cytokine rather than production, can be ruled out using the method of Andersson et al. (1986). These authors observed that incubation of mononuclear cells with high concentrations of IFN-γ did not lead to any detectable IFN-γ staining. Similarly, no staining should be observed in tissue supplied with cytokines or tissue sections after incubation with different concentrations of cytokines.

Extracellular and membrane bound cytokine staining are generally not observed, possibly due to the relatively low extracellular concentration of cytokines and membrane bound specific cytokine receptors. The inability of the primary antibody in the immunohistochemical detection methods to detect receptor-bound 'membrane' cytokine may be due to the masking of epitopes resulting from steric hindrance by the receptor.

Since there are many different immunohistochemical detection steps in which non-specific staining or background staining could be generated, the staining protocol has to be evaluated again when any step is changed.

2.3. Antibody based methods to visualize the presence of cytokine producing cells

The simplest cytokine-protein detection method is the direct method based on anti-cytokine antibodies labelled with enzymes or fluorochromes. In more complex techniques, including amplification steps, the cytokine producing cells are demonstrated by staining based on different indirect detection methods. As shown in Fig. 1 several indirect detection methods have been developed using isotypic determinants or species specificity or haptens present on the anti-cytokine primary antibody or on the secondary antibody. During the last incubation step,

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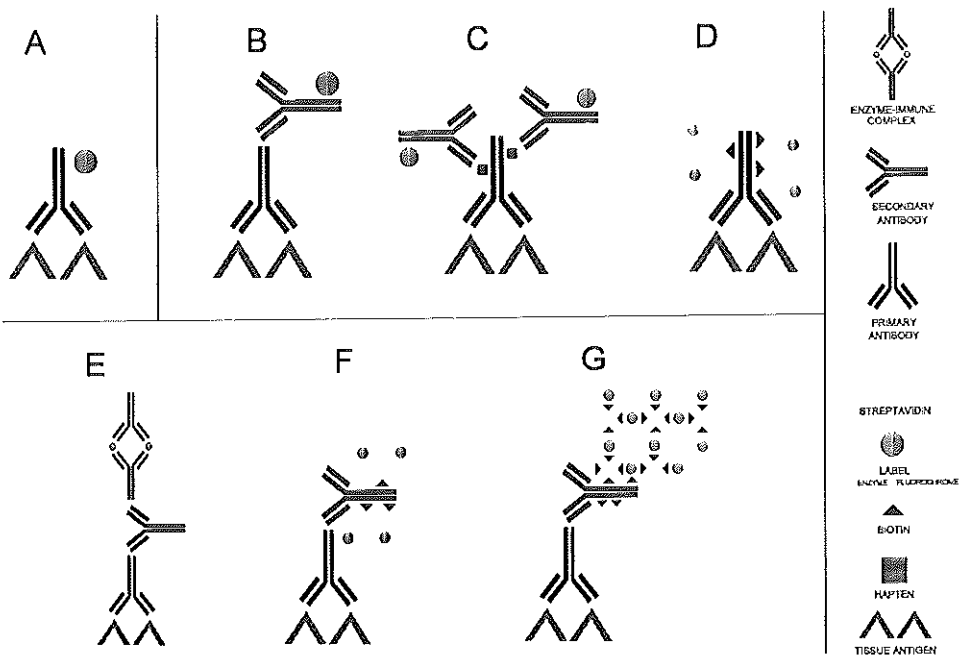

Fig. 1. Immunohistochemical staining methods to demonstrate cytokine producing cells. *A*: direct method based on an enzyme-or fluorochrome-labelled antibody. *B*: indirect method based on an unlabelled primary antibody and an isotype or species specific enzyme-or fluorochrome-labelled secondary antibody. *C*: indirect method based on a hapten-labelled primary antibody and a hapten specific enzyme-or fluorochrome-labelled secondary antibody. *D*: indirect method based on a biotinylated primary antibody and streptavidin-conjugated to enzyme or fluorochrome. *E*: indirect method visualized with soluble enzyme-anti-enzyme immune complex. *F*: indirect method based on three steps included anti-cytokine antibody, isotype or species specific biotinylated secondary antibody, and streptavidin conjugated to enzyme or fluorochrome. *G*: indirect method based on three steps included anti-cytokine antibody, isotype or species specific biotinylated secondary antibody, and a complex consisted of several streptavidin molecules conjugated to biotinylated enzyme molecules. *B–D*: two-step procedures. *E–G*: three-step procedures.

enzyme or fluorochrome labelled reagents are used. Both in the direct as well as in the indirect detection methods the enzymes are visualized histochemically and the fluorochromes are detected by fluorescent microscopy. The advantages of using enzymes over fluorochromes were reviewed by Claassen et al. (1986). The main advantage is the possibility of studying the histology of the tissue without autofluorescence and fading problems. This section describes different cytokine staining methods, and subsequently the advantages and disadvantages of each method.

Table 5 lists the in situ immunohistochemical methods used for the detection of human cytokines, the indirect three step method based on the streptavidin-biotin interaction is the most commonly used cytokine staining method followed by the indirect three step soluble enzyme immune complex method. Furthermore, the methods used include the indirect two step method with an unlabelled primary antibody and an enzyme-or fluorochrome-labelled secondary antibody, and the indirect method using biotinylated primary antibodies and enzyme-or fluo-

Table 5
Cytokine indirect immunohistochemical staining methods

Cytokine	Indirect method	Studies
IL-1α/β	Unlabelled primary antibody	Tyor et al., 1992; Hoefakker et al., 1992,1993; Emilie et al., 1992c; Prens et al., 1990; Didierjean et al., 1989; Simon et al., 1993; Hoffmann et al., 1993; Cooper et al., 1990; Yoshioka et al., 1993; Fielding et al., 1993
	Biotinylated primary antibody	Zheng et al., 1991; Chu et al., 1992
	Soluble enzyme immune complex	Tipping and Hancock, 1993; Sasaguri et al., 1992; Ruco et al., 1989,1990; Kristensen et al., 1992; Anttila et al., 1990; Noronha et al., 1993; Arnoldi et al., 1990
	Streptavidin-biotin 3 steps	Graeber et al., 1993; Oxholm et al., 1988,1991; Ruco et al., 1989,1990; Hansen et al., 1991; Heufelder and Bahn, 1993; Didierjean et al., 1989; Sato et al., 1993; Tyor et al., 1992; Anttila et al., 1990; Wood et al., 1992a; Simon et al., 1993; Koch et al., 1992; Chensue et al., 1992; Asano et al., 1991; Umehara et al., 1994; Jiranek et al., 1993; Olson et al., 1993; Andersson and Andersson, 1994; Andersson et al., 1994
IL-2	Unlabelled primary antibody	Hoefakker et al., 1992,1993; Howell et al., 1991
	Streptavidin-biotin 3 steps	Sato et al., 1993; Ruan et al., 1992; Andersson and Andersson, 1994
IL-4	Streptavidin-biotin 3 steps	Bradding et al., 1993,1994; Andersson and Andersson, 1994
IL-5	Soluble enzyme immune complex	Dubucquoi et al., 1994
	Streptavidin-biotin 3 steps	Bradding et al., 1993,1994; Andersson and Andersson, 1994
IL-6	Unlabelled primary antibody	Neuner et al., 1991; Fukatsu et al., 1991; Tyor et al., 1992; Prens et al., 1990; Strauss et al., 1992; Yoshioka et al., 1993; Gogesev et al., 1993; Raasveld et al., 1993
	Biotinylated primary antibody	Chu et al., 1992; Zheng et al., 1991
	Soluble enzyme immune complex	Leger-Ravet et al., 1991
	Streptavidin-biotin 3 steps	Oxholm et al., 1991; Miles et al., 1990; Emilie et al., 1992b; Grossman et al., 1989; Romero and Pincus, 1992; Umehara et al., 1994; Graeber et al., 1993; Tyor et al., 1992; Velkeniers et al., 1994; Bradding et al., 1993,1994; Castells-Rodellas et al., 1992; Ruan et al., 1992; Koch et al., 1993; Andersson and Andersson, 1994
IL-8	Unlabelled primary antibody	Nickoloff et al., 1991; Yoshioka et al., 1993
	Streptavidin-biotin 3 steps	Sticherling et al., 1992; Smith et al., 1994a; Schmouder et al., 1992; Bradding et al., 1993; Koch et al., 1993; Nickoloff et al., 1991; Anttila et al., 1992; Graeber et al., 1993; Chimoya et al., 1992; Andersson and Andersson, 1994
IL-10	Unlabelled primary antibody	Emilie et al., 1992c
	Streptavidin-biotin 3 steps	Smith et al., 1994b
IFN-γ	Unlabelled primary antibody	Tyor et al., 1992; Hoefakker et al., 1992,1993; Brett et al., 1992
	Biotinylated primary antibody	Zheng et al., 1992
	Soluble enzyme immune complex	Arnoldi et al., 1990; Noronha et al., 1992
	Streptavidin-biotin 3 steps	Heufelder and Bahn, 1993; Tyor et al., 1992; Umehara et al., 1994; Sato et al., 1993; Asano et al., 1991; Ruan et al., 1992; Andersson and Andersson, 1994
TNF-α	Unlabelled primary antibody	Tyor et al., 1992; Hoefakker et al., 1992,1993; Nickoloff et al., 1991; Gonzalez-Amaro et al., 1994; Hoffmann et al., 1993; Tsuchida et al., 1992; Yoshioka et al., 1993; Gogesev et al., 1993
	Biotinylated primary antibody	Chu et al., 1992; Gonzalez-Amaro et al., 1994; Zheng et al., 1992
	Soluble enzyme immune complex	Arnoldi et al., 1990; Noronha et al., 1992,1993; Ruco et al., 1989; Kretschmer et al., 1990; Tipping and Hancock, 1993; Sasaguri et al., 1992; Gonzalez-Amaro et al., 1994
	Streptavidin-biotin 3 steps	Tyor et al., 1992; Oxholm et al., 1988,1991; Murphy et al., 1992; Tillie-Leblond et al., 1994; Graeber et al., 1993; Hofman and Hinton, 1992; Heufelder and Bahn, 1993; Bradding et al., 1994; Nickoloff et al., 1991, Ruco et al., 1989; Asano et al., 1991; McCall et al., 1989; Tan et al., 1993; Gonzalez-Amaro et al., 1994; Misaki et al., 1992; Umehara et al., 1994; Tabibzadeh, 1991; Takeyama et al., 1991; Sato et al., 1993; Olson et al., 1993; Hunt et al., 1992; Reis et al., 1993; Andersson and Andersson, 1994

rochrome-labelled streptavidin. The success of in situ detection depends on numerous factors, including the cellular cytokine concentration, the preservation of the cytokine protein, the properties of the primary antibody, the type of detection method, and the signal to 'noise' ratio of the method.

Direct in situ detection method

Direct detection requires enzyme labelled anti-cytokine antibodies (A in Fig. 1). Enzyme-labelled anti-cytokine antibodies to detect cytokine producing cells in mouse tissue sections were used by Van den Eertwegh et al. (1991). For example horseradish peroxidase(HRP)-or alkaline phosphatase(AP)-labelled antibodies against murine IFN-γ revealed positive cells in spleen tissue sections from mice immunized with TNP-Ficoll. In contrast, only with a few direct enzyme-labelled monoclonal antibodies could cytokine specific staining be observed in human tissue sections (tonsil, skin). AP-labelled monoclonal antibody against IL-1α, but not AP-labelled monoclonal antibodies against IFN-γ, IL-2 or IL-4 permitted us to demonstrate cytokine producing cells in a direct detection method in contact allergic skin sections. Coupling AP to the primary anti-cytokine antibodies was performed as described by Claassen and Adler (1988). Keratinocytes are a source of abundant IL-1α production and therefore could give positive staining using a direct AP-labelled monoclonal antibody (E and F in Fig. 2). In human tissues from which biopsies are taken during a resting stage or after a weak immune response a relatively poor expression of cytokines is expected compared to the cytokine production in the tissues of immunized mice. Since the detection level is dependent on the intracellular concentration of the cytokine produced and signal amplification is absent using the direct method, the detection of cytokine producing cells in human tissue using a direct method could be a practical problem.

Basically, the advantages of the direct method are simplicity, relatively low costs and a short procedure time (one step). Furthermore, enzyme-labelled antibodies can be used successfully for double staining if a high intracellular concentration of the cytokine is present. The disadvantage is that there is no signal amplification which results in low to intermediate signal to 'noise' ratios. Direct labelled antibodies are not commercially available but a number of simple coupling procedures to label the specific antibodies have recently been described by Claassen and Jeurissen (1995a). The immmunodecoration method was performed as described in detail elsewhere (Claassen and Jeurissen, 1995a,b; Jeurissen et al., 1995).

Indirect in situ detection methods

Two-step indirect immunohistochemical detection methods. In this method to detect cytokines in situ, an unlabelled or hapten-labelled anti-cytokine primary antibody is allowed to bind to the cytokine antigen (B–D in Fig. 1). Subsequently, enzyme-labelled or fluorochrome-labelled secondary (usually polyclonal) antibody specific for the isotypic or species determinants (B in Fig. 1) or for the hapten present on the anti-cytokine primary antibody are applied. In general for the hapten biotin either enzyme-or fluorochrome-labelled streptavidin is applied as the secondary reagent (C in Fig. 1). Compared to the use of avidin, streptavidin gives rise to lower background staining. Therefore streptavidin is discussed below.

Digoxigenin and FITC have also been used as haptens conjugated to the anti-cytokine antibody (Kessler, 1991; Van der Loos et al., 1989). Detection is achieved with a commercially available enzyme-or fluorochrome-labelled secondary antibody specific for the hapten (D in Fig. 1). The detection of cytokines with this indirect staining method based on two steps can be performed with both monoclonal as well as polyclonal primary antibodies. The coupling of enzymes to the second step antibodies and the immunohistochemical procedure can be performed as described in detail elsewhere (Claassen and Jeurissen, 1995a,b; Jeurissen et al., 1995).

We have previously examined cytokine production in skin biopsies from patients with contact allergies (Hoefakker et al., 1992,1995) and in tonsil (Hoefakker et al., 1993) in situ. The two-

step indirect detection method was performed using an anti-IFN-γ primary monoclonal antibody and HRP-labelled polyclonal secondary antibody as shown in Fig. 2C and D. Furthermore, biotinylated as well as digoxigenin-labelled primary antibodies can be successfully used to detect human cytokines in tonsil and in skin sections (G and H in Fig. 2).

Basically, the advantages of this method are simplicity, use of ready made commercially available reagents, relatively low costs and a short processing time (2 steps), signal amplification and low to intermediate backgrounds, and subsequently an intermediate to high signal to 'noise' ratio. Compared to the direct detection method the indirect methods are designed to attach more enzyme molecules at the site of the antigen. An advantage of hapten-labelled antibodies is the fact that they can be used in double staining using different detector molecules. A minor practical point is that biotinylated or digoxigenin-labelled antibodies specific for human cytokines are not yet commercially available, but coupling is relatively easy.

The use of streptavidin conjugated to enzyme or fluorochrome is the most frequently used two step method because it offers several important advantages. Compared to the large enzyme-or fluorochrome-labelled secondary antibodies the small streptavidin molecules conjugated to enzyme or fluorochrome are likely to penetrate rapidly into the tissue if similar enzymes or fluorochromes are compared. Moreover, the affinity of streptavidin for biotin (dissociation constant 10^{-15} M^{-1}) is higher than the affinity of antigen (hapten) for antibody (dissociation constant 10^{-8}–10^{-11} M^{-1}). The presence of four active binding sites for biotin on streptavidin and the relatively high labelling index of biotinylated antibodies as compared to the number of isotypic determinants per antibody are also important considerations.

Indirect three step immunohistochemical detection using soluble enzyme immune complexes. This method utilizes as reporter a preformed soluble enzyme-anti-enzyme immune complex (E in Fig. 1). This type of immune complex consists of an enzyme (the antigen), and an antibody directed against the enzyme. To obtain soluble enzyme-anti-enzyme complexes, the enzyme is added in excess and precipitates are removed by low speed centrifugation. The staining sequence is: (i) unlabelled anti-cytokine primary antibody, (ii) unlabelled secondary antibody, (iii) soluble enzyme-anti-enzyme complex, and substrate with chromogen solution. The anti-cytokine primary antibody and the antibody of the soluble enzyme-anti-enzyme immune complex should be made in the same species so that the secondary antibody can link the two together. The alkaline phosphatase-anti-alkaline phosphatase complex (APAAP) method and the peroxidase-anti-peroxidase complex (PAP) method are used to detect cytokines in situ (Mason and Sammons, 1978). Both polyclonal as well as monoclonal antibodies can be used as primary antibodies. Enzyme activity can be revealed as described by Claassen et al. (1986).

The advantages of this method are simplicity, use of ready made commercially available reagents, high signal amplification and low to intermediate background, and subsequently an intermediate to high signal to 'noise' ratio. The disadvantages are relatively high costs and a long procedure time (3 steps). Furthermore, the molecular size of the complex used in the PAP method is large and hence might give problems in tissue penetration or diffusion (Hsu et al., 1981).

Indirect three step immunohistochemical detection using the streptavidin-biotin interaction. The indirect method based on three steps including streptavidin-biotin interactions is widely used for immunohistochemical detection of proteins in tissue. Two almost identical three step methods are used based on the interaction between streptavidin and biotin. The sequence of reagents and incubations are: (i) anti-cytokine primary antibody, (ii) biotinylated secondary antibody, (iii) enzyme-or fluorochrome-labelled streptavidin (F in Fig. 1) or a complex consisting of several biotinylated enzyme-labelled streptavidin molecules (G in Fig. 1), and substrate and chromogen. The indirect method based on three steps including streptavidin-biotin interactions can be

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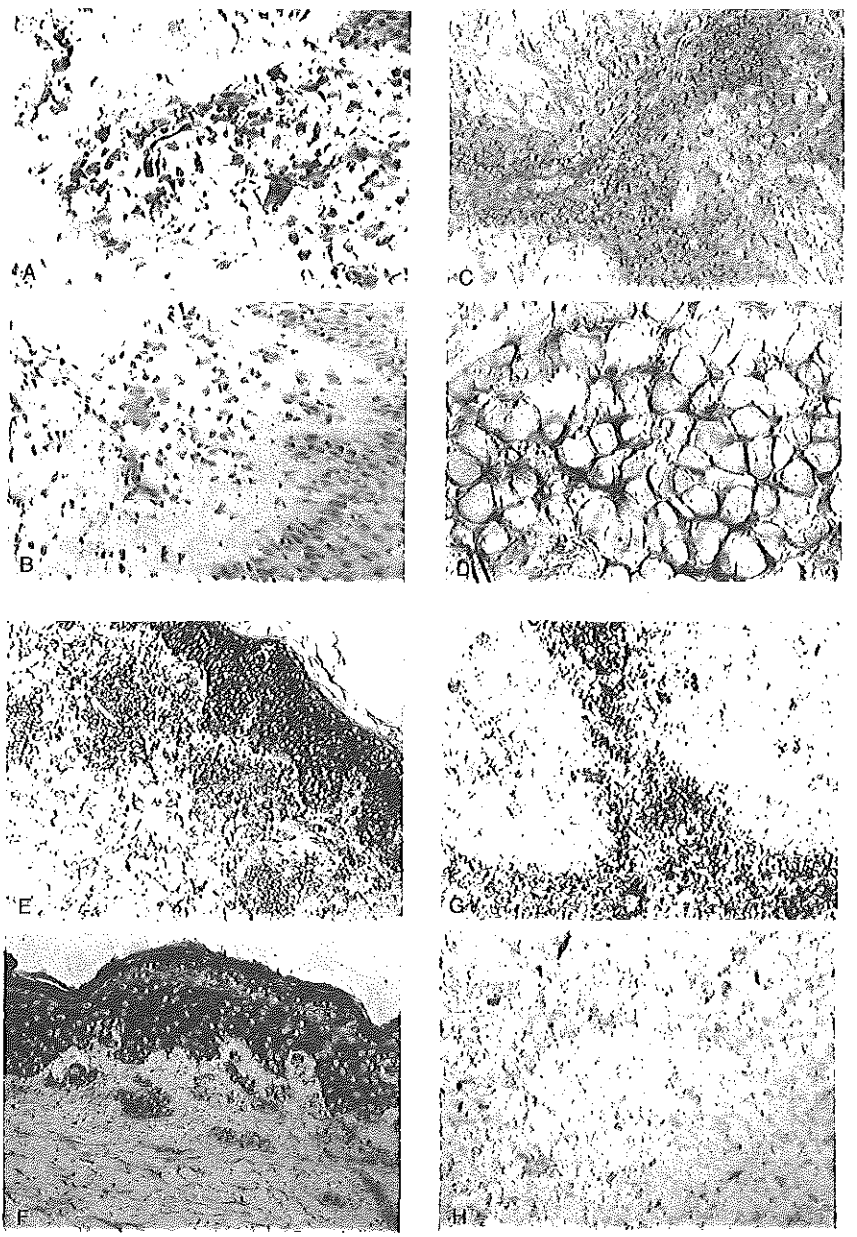

performed with monoclonal and polyclonal antibodies as primary anti-cytokine antibodies. Biotinylated isotypic or species specific secondary antibodies are required as linking antibody between the primary antibody and enzyme-or fluorochrome-labelled streptavidin or the complexes consisting of streptavidin molecules and biotinylated enzyme. These biotinylated antibodies are often commercially available or can be easily conjugated to biotin as described by Guesdon et al. (1972). Enzyme activity can be revealed as described by Claassen et al. (1986).

The indirect detection method based on three steps including anti-IL-4 antibody, biotinylated horse anti-mouse, and streptavidin conjugated to AP has been used successfully for the demonstration of IL-4 expression in the skin of patients with atopic allergy (A and B in Fig. 2).

The advantages of this method are simplicity, the use of ready made commercially available reagents, high signal amplification and low to intermediate backgrounds, and subsequently high signal to 'noise' ratios. This indirect detection method was found to be very efficient (Giorno, 1984) and is the most frequently used method (Table 5). The multiple biotin molecules present on the secondary immunoglobulin or third step enzyme molecules increase the eventual number of enzyme molecules. The molecular size of the complex which consists of several streptavidin molecules conjugated to biotinylated HRP is small compared to the complex used in the PAP method and hence might better facilitate tissue penetration or diffusion (Hsu et al., 1981).

The method using *enzyme-labelled streptavidin* in the third incubation step was shown to be approximately four-to eight-fold more sensitive (signal to 'noise' ratio) than *a complex consisting of biotinylated enzyme-labelled streptavidin* in the third incubation step possibly because the complex cannot readily interact with the biotin-labelled secondary antibody due to steric hindrance (Giorno, 1984).

A disadvantage of both these three step indirect methods is the long processing time of the three incubation steps to generate results and the relatively high costs.

3. Probe based cytokine detection

3.1. *In situ hybridization (ISH)*

This method utilizes specific genetic probes which are complementary for mRNA sequences. Furthermore, it permits identification of the pre-

Fig. 2. Immunohistochemical visualization of cytokine producing cells in human tissues using direct and indirect staining methods in sequence. *A + B*: IL-4 producing cells characterized by a blue cytoplasm were detected in the dermal infiltrates in the skin of a patient with atopic allergy using the three-step indirect method based on unlabelled mouse monoclonal anti-IL-4 antibody (1842-01, Genzyme), biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA), and streptavidin conjugated to AP (Life Technologies, Gaithersburg, MD, USA) (*A*). The histochemical revelation was performed with naphthol AS MX and fast blue as substrate and chromogen (Claassen et al., 1986). The anti-cytokine antibody was omitted in the negative control (*B*). *C + D*: IFN-γ producing cells characterized by a red cytoplasm were detected in the dermal infiltrates in the affected skin of a patient with contact allergy using the indirect method based on unlabelled mouse monoclonal anti-IFN-γ antibody (MD-2 from P. van der Meide, BPRC, Rijswijk, Netherlands) and a HRP-labelled rabbit anti-mouse secondary antibody (Dakopatts, Copenhagen, Denmark). The histochemical revelation was performed with AEC (Hoefakker et al., 1992,1993,1995). Low magnification (*C*) and high magnification (*D*). *E + F*: IL-1α producing cells characterized by a blue cytoplasm were detected in the epidermis and infiltrates in the dermis in the skin of a patient with contact allergy (*E*) and predominantly in the epidermis in normal skin (*F*) using a direct method with AP-labelled monoclonal mouse anti-IL-1α antibody (Vhp18, P. Ghiara, IRIS Department of Immunopharmacology, Siena, Italy). The AP-labelling was performed according to Claassen and Adler (1988). The histochemical revelation was performed with naphthol AS MX and fast blue as substrate and chromogen (Claassen et al., 1986). *G + H*: IFN-γ producing cells characterized by a dark blue (purple) cytoplasm were detected in the extrafollicular areas in tonsil using an indirect method based on digoxigenin-labelled monoclonal mouse anti-IFN-γ antibody (MD-2 from P. van der Meide, BPRC, Rijswijk, Netherlands) and a sheep AP-labelled secondary antibody (Boehringer Mannheim, Germany) (*G*). The DIG-labelling was performed using a DIG antibody labelling kit (Boehringer Mannheim, Germany). The histochemical revelation was performed with NBT/BCIP (Hoefakker et al., 1995). A control skin section treated by omission of primary antibody demonstrated no positive cells (*H*). *E* and *F*, ×100; *A*, *B*, *C*, *G*, *H*, ×200; *D*, ×400. (All the results presented here were obtained after titration of the anti-cytokine antibodies and mostly used at optimal concentrations between 5–20 μg/ml.)

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cise morphological tissue distribution of mRNA. There are many different protocols for ISH. In general, a protocol involves slide preparation, fixation of the tissue sections, pretreatment of material on the slide, prehybridization, hybridization, post-hybridization washings, and visualization by immunocytochemistry or autoradiography.

Cryostat sections on slides coated with poly-L-lysine are fixed in buffered 4% paraformaldehyde. Paraformaldehyde fixation is most frequently used in ISH. This fixation method prevents diffusion of mRNA within the tissue section or out of the tissue section. As described for immunohistochemical tissue preparation the tissue sections used for ISH are sometimes pretreated with proteolytic enzymes to facilitate penetration of the probe and other reagents used in the hybridization.

3.2. Probe based methodology

Basically, labelled complimentary DNA (cDNA) and RNA (cRNA) probes can be em-

Table 6
Cytokine mRNA ISH methods

Cytokine mRNA	Probe	Label	Studies
IL-1α/β	cDNA[a]	^{35}S	Whiteside et al., 1993
		^{32}P	Wood et al., 1992a; Aime et al., 1993
		S	Zheng et al., 1991
		Biotin	Ohta et al., 1991
	OLIGO[a]	^{35}S	Matsuki et al., 1992; Yoshioka et al., 1993
		DIG	Woodroofe et al., 1992
	cRNA	^{35}S	Tovey et al., 1991; Leger-Ravet et al., 1991; Naylor et al., 1993; Foss et al., 1994; Noronha et al., 1993; Fromont et al., 1993; Emilie et al., 1990,1991,1992a; Firestein et al., 1990; Rutenfranz et al., 1992; Jiranek et al., 1993; Devergne et al., 1992; Cappello et al., 1992
IL-2	cDNA [a]	^{35}S	Boehm et al., 1989; Vitolo et al., 1992; Whiteside et al., 1993; Borish et al., 1993
	OLIGO[a]	^{32}P	Warren et al., 1991
		DIG	Woodroofe et al., 1992
		Biotin	Howell et al., 1991
	cRNA	^{35}S	Peuchmaur et al., 1990,1991; Bentley et al., 1993; Leger-Ravet et al., 1991; Emilie et al., 1990,1991; Devergne et al., 1992; Tovey et al., 1993
IL-4	cDNA[a]	^{35}S	Borish et al., 1993
		DIG	Bromley et al., 1994
	OLIGO[a]	DIG	Woodroofe et al., 1992; Ying et al., 1994
	cRNA	^{35}S	Masuyama et al., 1994; Merz et al., 1991; Bentley et al., 1993; Varney et al., 1993; Tsicopoulos et al., 1992; Durham et al., 1992; Kay et al., 1991
IL-5	OLIGO[a]	DIG	Martinez et al., 1993
	cRNA[a]	^{35}S	Masuyama et al., 1994; Bentley et al., 1993; Merz et al., 1991; Dubucquoi et al., 1994; Varney et al., 1993; Tsicopoulos et al., 1992; Durham et al., 1992; Kay et al., 1991
		^{32}P	Hamid et al., 1991
IL-6	cDNA[a]	^{35}S	Whiteside et al., 1993; Velkeniers et al., 1994; Bosseloir et al., 1989; Hoyland et al., 1994
		^{32}P	Wood et al., 1992b
		S	Zheng et al., 1991
		Biotin	Ohta et al., 1991
	OLIGO[a]	^{35}S	Matsuki et al., 1992; Yoshioka et al., 1993
		Biotin	Seino et al., 1993
		DIG	Woodroofe et al., 1992
	cRNA	^{35}S	Gillitzer et al., 1991; Naylor et al., 1993; Foss et al., 1994; Emilie et al., 1990,1992a,b; Tovey et al., 1991,1993; Fromont et al., 1993; Devergne et al., 1992; Merz et al., 1991; Foss et al., 1993; VandenBroecke et al., 1991; Villiger et al., 1991; Neuner et al., 1991; Jücker et al., 1991; Rutenfranz et al., 1992; Firestein et al., 1990; Grossman et al., 1989
IL-8	OLIGO [a]	^{35}S	Matsuki et al., 1992
	cRNA [a]	^{35}S	Mazzucchelli et al., 1994; Foss et al., 1994; Gillitzer et al., 1991

Table 6 (continued)

Cytokine mRNA	Probe	Label	Studies
IL-10	cRNA	^{35}S	Emilie et al., 1992c
IFN-γ	cDNA[a]	^{35}S	Vitolo et al., 1992; Whiteside et al., 1993; Borish et al., 1993
		^{32}P	Murphy et al., 1992
		S	Zheng et al., 1992
	OLIGO[a]	DIG	Woodroofe et al., 1992
	cRNA	^{35}S	Varney et al., 1993; Tsicopoulos et al., 1992; Durham et al., 1992; Kay et al., 1991; Fromont et al., 1993; Emilie et al., 1990,1991; Devergne et al., 1992; Pirmez et al., 1990; Cooper et al., 1989; Bentley et al., 1993; Rutenfranz et al., 1992; Barnes et al., 1990; VandenBroecke et al., 1991; Tovey et al., 1991; Peuchmaur et al., 1991; Firestein et al., 1990
		^{32}P	Klein et al., 1993
TNF-α	cDNA[a]	^{35}S	Vitolo et al., 1992; Whiteside et al., 1993
		^{32}P	Murphy et al., 1992
		S	Zheng et al., 1992
		Biotin	Takeyama et al., 1991; Tarlow et al., 1993; Hunt et al., 1992
	OLIGO[a]	^{35}S	Matsuki et al., 1992; Yoshioka et al., 1993
		Biotin	Kretschmer et al., 1990
		DIG	Woodroofe et al., 1992
	cRNA	^{35}S	Foss et al., 1993,1994; Naylor et al., 1990,1993; Firestein et al., 1990; VandenBroecke et al., 1991; Devergne et al., 1992; Tovey et al., 1991; Majewski et al., 1991; Ying et al., 1991; Gillitzer et al., 1991; Noronha et al., 1993; Tan et al., 1993; Barnes et al., 1990; Reka and Kotler, 1993; Cappello et al., 1992
		DIG	Gonzalez-Amaro et al., 1994

S: probe labelled by sodium bisulfide; DIG: probe labelled with digoxigenin.
[a] cDNA: non-synthetic cDNA probe; OLIGO: synthetic cDNA probe.

ployed to localize cytokine mRNA. Nick-translated or randomly primed non-synthetic double stranded cDNA probes (produced in large quantities using DNA cloning or PCR), synthetic oligonucleotide cDNA probes, and single stranded antisense cRNA probes prepared in a transcription vector are used.

As with all immunohistological methods, if a hybridization signal is found control experiments have to be performed to establish the specificity of the ISH reaction and to show the correctness of the procedure. In general, to check the specificity of the probe a northern blot on total RNA should be performed. The probe which hybridizes to cytokine mRNA is antisense in sequence. A sense probe is identical in sequence to the mRNA target and hence will not bind to the target. If hybridizations with a sense probe produce a signal comparable to hybridizations with an antisense probe, the labelling is clearly non-specific. The procedure controls for cytokine mRNA hybridization are similar to the controls used for cytokine immunohistochemistry (Table 4). To establish tissue specific staining a positive and a negative tissue control are used as described for immunohistochemistry (see sections 2.3.2.1 and 2.3.2.2). For the probe control, a 'no probe', a positive probe, and a sense probe or non-homologous probe should generally be included to establish non-specific staining due to the visualization methods and non-sequence dependent staining. In addition, to demonstrate that the target nucleic acid is RNA, tissue sections can be incubated with RNase before application of the probe. mRNA within the section will be destroyed and any remaining signals are non-specific. Non-specific staining due to endogenous enzyme activity in the case of immunohistochemical revelation could be ruled out after determination of staining using substrates only.

3.3. Probe based methods to visualize cytokine-mRNA producing cells

The detection of cellular cytokine mRNA has been demonstrated with different methods based

on the choice of probe and label. Table 6 lists the methods used for the detection of cytokine mRNA. Non-synthetic nick-translated or randomly primed double stranded cDNA probes, synthetic oligonucleotide cDNA probes, and antisense cRNA probes are used, each with their own

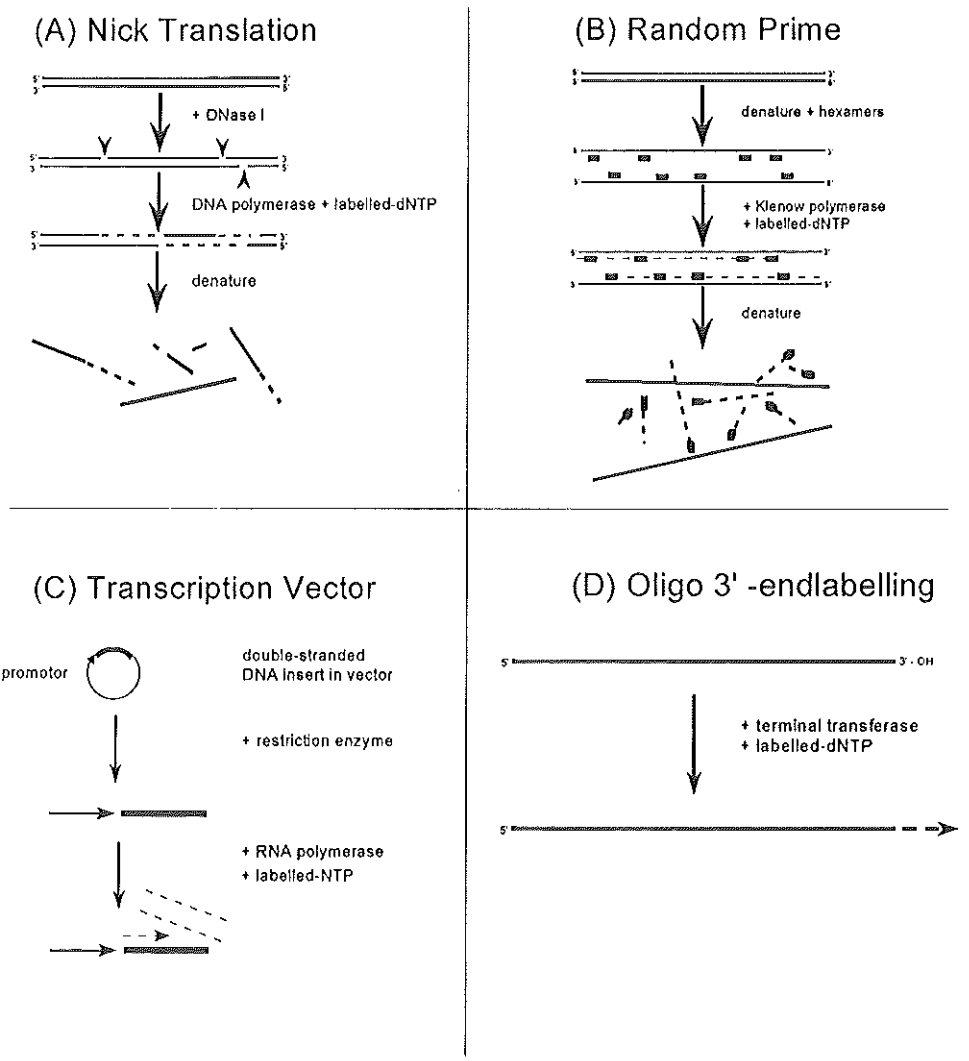

Fig. 3. Procedures for labelling cytokine-mRNA probes for ISH. Predominantly nick translation (*A*) and random prime (*B*) procedures are used to obtain labelled cDNA probes. Labelled cRNA probes are obtained using a transcription vector (*C*). Oligonucleotides are labelled using 3' end labelling (*D*).

methods to visualize the hybridization sequence. The success of ISH depends on numerous factors, including the number of copies of mRNA, the preservation of mRNA, the type of probe, single probe or multiple probe detection, hybridization and stringency conditions, methods of probe labelling (radioactive or nonradioactive), and the techniques of signal detection. This section describes the different detection methods and subsequently the advantages and disadvantages of each method.

Primary detection with cDNA probes

Non-synthetic cDNA probes. Clonal propagation of defined segments of DNA coding for human cytokine mRNA, which became possible with DNA cloning, involve the insertion of a fragment of DNA into a genetic element that replicates (a plasmid or virus). Nick-translation and random prime as double stranded DNA (< 1000 bp) labelling methods are used to obtain labelled double-stranded cDNA probes (Fig. 3). In both methods a heterogeneous population of probe strands (< 500 bp), many of which have overlapping complementary regions, are produced. This results in multiple probe detection and subsequently signal amplification in the hybridization experiment.

The nick-translation method is based on the use of the enzyme *E. coli* DNA polymerase I. Starting at a nick (gap) in the DNA, this enzyme removes nucleotides from the 5′ side of the nick using 5′ exonuclease activity. At the same time the enzyme adds radio-labelled or otherwise labelled nucleotides to the 3′ side of the nick using its DNA polymerase activity. After denaturation labelled probes are obtained. Nick-translation can be performed with the specific insert only, or with the specific DNA (insert) in a plasmid vector.

cDNA probes can also be labelled by random prime. This method employs the 3′ DNA polymerase activity of the Klenow fragment of *E. coli* polymerase I enzyme. After first melting the DNA, random sequence hexamers are added that serve as a primer to the polymerase reaction with labelled nucleotide-triphosphates. After denaturation labelled probes are obtained. As described for the probes obtained by nick-translation a heterogeneous population of probe strands, many of which have overlapping complementary regions, are produced. Sambrook et al. (1989) provides details about both the nick-translation and the random prime labelling methods.

Two other labelling methods are based on the chemical addition of hapten molecules to the probe. Using sodium bisulfite (Zheng et al., 1991,1992) or biotin after photochemical conjugation (Ohta et al., 1991) cDNA can be labelled with sulfonated cytidine and biotin respectively. Although both these labelling methods are very simple to perform they are not frequently used.

To detect human cytokine mRNA mainly randomly primed labelled non-synthetic cDNA probes are applied labelled with predominantly ^{35}S, ^{32}P, and biotin. ^{35}S and ^{32}P are labels which are detected by autoradiography. Specific hybridization is recognized as clear dense deposits of silver grains in the photographic emulsion overlaying the tissue sections. ^{32}P-labelled probes need a relatively short exposure time resulting in a lower resolution as compared to detection using ^{35}S-labelled probes (DeLellis, 1994). For the detection of the biotinylated cDNA probes, immunohistochemical revelation is necessary based on visualization using enzyme-labelled streptavidin. ISH using sulfonated probes has been applied to study cytokine mRNA profiles in autoimmune thyroiditis, non-toxic goitre and normal thyroid tissue using the three-step indirect immunohistochemical detection method visualized with APAAP (Zheng et al., 1991,1992).

Synthetic cDNA probes. With the development of a new generation of DNA synthesizers which allow cheap oligonucleotide production, increased attention has turned to the use of short single stranded oligodeoxynucleotide probes (20–50 bp) in single probe detection and multiple probe detection (double, triple detection). The application depends on the specific labelling of the oligonucleotide, which permits a later identification or recovery of the probe. Traditionally, the labelling is performed by the enzymatic coupling of radioactively labelled deoxynucleotides to the 3′ end (Schmitz et al., 1991; DeLellis, 1994)

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(Fig. 3). As shown in Table 6 ^{35}S, ^{32}P, biotin, and digoxigenin are frequently used as labels.

Matsuki et al. (1992) demonstrated cytokine mRNA producing cells in chronically inflamed gingival tissue with a cocktail of ^{35}S-labelled oligonucleotide probes, whereas Warren et al. (1991) demonstrated IL-2 mRNA in rheumatoid arthritis tissue using a ^{32}P-labelled oligonucleotide probe cocktail. IFN-γ mRNA producing cells can be visualized using a digoxigenin-labelled oligonucleotide probe cocktail and a digoxigenin specific AP-labelled antibody, and histochemical revelation with NBT/BCIP (Hoefakker et al., 1995).

Evidently, these synthetic oligonucleotide probes have several advantages over non-synthetic cDNA probes; (i) they can be used to achieve high levels of specificity under conditions of high stringency, (ii) it is possible to construct different probes for a particular sequence with a high reproducibility and high specific activity, (iii) the use of oligonucleotide probes does not require detailed expertise with basic molecular technologies because they do not need to be cloned and labelling can be carried out in a single step by either 3′ or 5′ labelling, and (iv) oligonucleotide probes are commercially produced relatively inexpensively (Höfler, 1987).

Primary detection with cRNA probes

cRNA probes are prepared in transcription vectors (plasmids) that contain promotors located upstream from multiple cloning sites (Fig. 3). The DNA of interest is inserted into the cloning site and can be transcribed in the presence of labelled nucleotides to provide sense or antisense probes (< 2500 bp) after linearization. A sense probe will have the same 5′ to 3′ orientation as the transcribed mRNA. It will therefore not hybridize to mRNA but will hybridize to one of the DNA strands, provided that the DNA has been denatured or melted before the reaction. The length of the probe is defined by the distance between the restriction site used for linearization and the start of transcription. This guarantees that the probes all have the same length.

Until now, as shown in Table 6, predominantly ^{35}S, ^{32}P, and digoxigenin-labelled cRNA probes have been used to detect cytokine mRNA in situ. As compared to non-synthetic cDNA probes, cRNA probes can be labelled to a higher specific activity and have greater thermal stability. Moreover the efficient reduction of background problems using RNase to digest unhybridized single strand probes is another significant advantage of cRNA probes. Antisense cRNA probes have a constant probe size and do not contain vector sequences. This leads to highly reproducible signals and low non-specific hybridization. As a result, interexperimental comparisons are possible. In general, compared to non-synthetic cDNA probes, a relatively high signal to 'noise' ratio and high reproducibility of reactions is observed using cRNA probes. Furthermore, using cRNA probes competitive hybridization to the complementary strand (which causes background problems as it occurs with cDNA probes) is excluded (Höfler, 1987).

4. Simultaneous detection

4.1. Double staining: simultaneous immunohistochemical detection of two or more antigens

Phenotypes (subpopulations) of the cytokine producing cells may be studied in serial sections. However, the phenotype of the individual cytokine producing cell can be exactly identified within one section. Demonstration of double stained cells within one section indicates that a single defined cell (particular phenotype) produces the cytokine. Such double immunofluorescence staining methods were used by Chu et al. (1992) and Cooper et al. (1990). For example Chu et al. (1992) used a method based on the demonstration of species specific determinants and hapten on the different primary antibodies used, whereas Cooper et al. (1990) used a method based on the demonstration of species specific determinants and isotypic determinants on the two different primary antibodies. In addition, combinations of two three-step indirect methods have been applied by Ruco et al. (1990) and Wood et al. (1992a). For detection of the first antigen Ruco et al. (1990) used a method based

on (i) unlabelled antibody, (ii) biotinylated secondary antibody, and (iii) HRP-labelled streptavidin and for the detection of the second antigen a method based on the three-step indirect APAAP method. Wood et al. (1992a) used two similar three-step indirect methods based on (i) unlabelled anti-IL-1 antibody, (ii) biotinylated secondary antibody, and (iii) a complex consisting of biotinylated AP-labelled streptavidin for the first antigen and HRP-labelled streptavidin for the second antigen.

In contrast to the combinations of methods described above, the combination of the indirect method based on three steps including (i) unlabelled primary antibody, (ii) a biotinylated secondary antibody, and (iii) enzyme-or fluorochrome-labelled streptavidin to detect cytokine producing cells, together with the indirect method based on two steps including an unlabelled anti-cytokine antibody and an enzyme or fluorochrome-labelled secondary antibody to identify the cell phenotype is the most frequently used double staining method. Using this method two different enzymes (McCall et al., 1989), two different fluorochomes (Nickoloff et al., 1991), or one enzyme and one fluorochrome can be used for visualization (Oxholm et al., 1991).

Undesired reactions may occur when using secondary antibodies in the indirect detection which also react with other primary antibodies. Although the cross-reactivity could be detected using specificity controls (Table 3), these unwanted antibody interactions may be avoided by using two different direct enzyme-or fluorochrome-labelled primary antibodies. Direct enzyme-labelled monoclonal antibodies permit the detection of two different cytokines simultaneously in mouse tissue from immunized mice (Van den Eertwegh et al., 1993). However, in unstimulated human tissue or human tissue taken after a weak immune response or during a resting stage, the low optimal cytokine signal certainly needs to be amplified and will probably not be detected with direct enzyme-or fluorochrome-labelled primary antibodies. Hapten-labelled primary antibodies visualized by enzyme-or fluorochrome-labelled secondary reagents are suitable alternatives when applied in a double staining technique (Van der Loos et al., 1989) (Kessler, 1991). Preliminary results have recently been obtained with simultaneous use of digoxigenin and biotin as haptens on primary antibodies (Hoefakker et al., unpublished observations).

4.2. Simultaneous detection using a combination of antibody and probe based methods

A combination of immunohistochemical staining methods with ISH methods can be used in the identification of cytokine-mRNA containing cells. The conditions required for ISH differ from those required for immunohistochemistry and often damage protein antigens. Nevertheless, a few reports have described the successful use of a combination of an ISH method to detect mRNA with an immunohistochemical staining method to demonstrate the phenotypic cell surface determinant in a single tissue section. For example, Wood et al. (1992a) identified the CD14-positive macrophage as the predominant cell type producing IL-1β mRNA in rheumatoid arthritis synovium. Similarly colocalization of CD30 and cytokine gene transcripts has been demonstrated in Reed-Sternberg cells in Hodgkin's disease (Xerri et al., 1992; Foss et al., 1993) with a double labelling procedure which consisted of an immunohistochemical staining prior to ISH with a radioactive-labelled probe. Interestingly, in a recent report double stained cells were demonstrated in a nasal biopsy after allergen challenge using non-radioactive ISH with a digoxigenin-labelled IL-4 probe and subsequent visualization of the probe using an enzyme-labelled anti-digoxigenin antibody with an unlabelled anti-CD3 monoclonal antibody followed by staining using the indirect three-step APAAP method (Ying et al., 1994).

5. Conclusions

A variety of immunohistochemical methods and a variety of ISH methods that have been used for the demonstration of cytokines and cytokine-mRNAs in different human tissues, is available.

When the different methods for the immunohistochemical detection of cytokines are compared the success of a specific method in the detection of cytokine producing cells seems to be determined predominantly by the specificity of the anti-cytokine antibody (Table 3), the results of the regular procedure controls (Table 4), and the signal to 'noise' ratio of the method to visualize the presence of nanograms or picograms of intracellular cytokine. It is important to note that without a specific anti-cytokine antibody successful staining experiments cannot be obtained. It should be emphasized again that not all controls are specificity controls (actually most are staining/background controls) and that one should repeatedly ask the specificity question with every change in the protocol. The differences in signal to 'noise' ratios between the direct method and the indirect detection methods are predominantly caused by the signal amplification steps used in the indirect methods. The number of incubation steps, the affinity between hapten-labelled or unlabelled primary antibody and the secondary antibody, the labelling index of the haptenated antibodies as well as the avidity of the binding between the primary and secondary antibody under the assay condition are also important in signal amplification.

Immunohistochemical methods based on streptavidin-biotin binding clearly have distinct advantages over other methods. The main advantage is the high signal to 'noise' ratio as is obtained in the indirect two-step as well as the indirect three-step method. This is predominantly based on the high affinity between biotin and streptavidin and the efficient labelling of antibody with biotin. Since reliable biotinylated primary anti-cytokine antibodies are not available commercially, successful staining using biotinylated primary antibodies in the two step indirect staining method has been demonstrated in only a few studies. In addition, biotinylated primary antibodies visualized with enzyme-or fluorochrome-labelled streptavidin together with other hapten-labelled primary antibodies visualized with specific enzyme-or fluorochrome-labelled secondary antibody provide the opportunity to combine different primary antibodies in elegant double staining detection methods which avoid unwanted cross-reactivities.

ISH protocols using ^{35}S-labelled cRNA probes have predominantly been used to detect cytokine mRNA in many studies. This method permits the target to be hybridized with high specificity and with extremely low backgrounds. However, the choice of probe and label to detect cytokine mRNA has shifted from cRNA and ^{35}S to synthetic oligonucleotide probes and non-radioactive labels. This shift is based predominantly on the high specific signal obtained, easy production and labelling without the need for radioactive facilities, and rapid processing.

Finally, regardless of the different immunohistochemical or ISH detection methods which are used, these methods are generally applicable. However, the experimental character of various methods (variability of conditions e.g. tissue preparation, type of tissue) and the interpretation of staining patterns in specific tissue compartments require specific training and expertise. Nevertheless, we have found that in most cases at least one of the aforementioned methods gives good results. Selection of the method of choice will depend on the reagents available and individual preference (time, costs, lab routine and facilities). In general, the indirect immunohistochemical staining methods based on streptavidin-biotin interactions and non-radioactive synthetic cDNA probes have advantages over the other methods.

In this review we have given some typical examples of currently used cytokine stainings in human tissues (for mouse see Van den Eertwegh et al., 1992). Although this work cannot be all inclusive we hope it will provide a stepping stone for those who wish to enter the field of in situ detection of cytokines in tissue sections. A step by step guide for those who have no previous experience in tissue sampling, processing, fixation, incubation and substrate/chromogen revelation is given in detail elsewhere (Claassen and Jeurissen, 1995a,b; Jeurissen et al., 1995). In any case, the ultimate advantage of an in situ approach lies in the ability to study the localization and activity of cytokines in relation to their direct unchanged original microenvironment (including the demonstration of cell-cell interactions), in all

types of tissue at any given moment, without inducing artefacts by manipulations such as cell separation and (pre)-culture.

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Immunohistochemical detection of co-localizing cytokine and antibody producing cells in the extrafollicular area of human palatine tonsils

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SUMMARY

In vitro experiments have documented the role of cytokines in the regulation of the human humoral immune response. Which cytokines are operative *in vivo* and in which lymphoid compartment interactions between cytokine-producing T cells and antibody-forming B cells occur is still unclear. For that reason we studied human tonsils using immunohistochemical techniques. In tissue sections from tonsils in a resting stage after recurrent tonsillitis we observed cells producing IL-1α and tumour necrosis factor-alpha (TNF-α) which were exclusively localized in the mantle zone of the follicle and in the extrafollicular area. Furthermore, a high frequency of interferon-gamma (IFN-γ)-producing cells was detected in the extrafollicular area, but not inside the follicles. Occasional IL-2- and IL-4-producing cells were found in the extrafollicular area. Immunohistochemical detection of antibody isotypes revealed that B cells, IgM-membrane-positive, were localized inside the follicles and mantle zones, whereas IgD-membrane-positive cells were mainly found in the mantle zones of secondary follicles. In contrast, plasma cells producing IgG1–4 and IgA1–2 were found in the extrafollicular area. No IgD and IgE antibody-forming cells were detected in tonsils, whereas IgM antibody-forming cells were detected in the extrafollicular area. The co-localization of cytokine-producing cells and antibody-forming cells in human tonsil suggests that T–B cell interactions, required for B cell differentiation and isotype switching, take place in the extrafollicular area.

Keywords cytokine-producing cells antibody-forming cells palatine tonsils isotype switching *in situ* human histochemistry

INTRODUCTION

One major function of cytokines produced by T cells in lymphoid tissues is to provide signals necessary for activation, proliferation and differentiation of B cells that have been exposed to antigen. It is still largely unknown which cytokines are important in the human humoral immune response *in vivo*, and in which anatomical compartment(s) the T–B cell interactions are localized. Therefore, we immunohistochemically studied the distribution of cytokine-producing cells (CPC) and antibody-forming cells (AFC) in human palatine tonsils.

Palatine tonsils are located at the entrance of both the respiratory and digestive tubes, and are exposed to a variety of exogenous antigens in food and air. The extrafollicular area contains interdigitating cells (IDC), macrophages, T cells, AFC and high endothelial venules (HEV) (Fig. 1). These HEV take care of the recirculation of T cells and B cells from blood (reviewed by Kuper *et al.* [1]). Follicles, found inside various lymphoid organs such as spleen, lymph nodes, tonsils, Peyer's patches and the appendix, are globular structures, and can be subdivided into primary and secondary follicles. The former consist of clusters of resting B cells, follicular dendritic cells (FDC), and are mainly found in non-stimulated tonsils, the latter consist of a germinal centre with a mantle zone of predominantly memory B cells, a few T cells, FDC and tingible body macrophages (TBM) [2–4]. Germinal centres are foci of intense B cell proliferative activity which develop in response to antigenic restimulation. The primary function of germinal centres appears to be the establishment and maintenance of B cell memory (reviewed by Liu *et al.* [5]). The FDC in the follicles trap antigen and have a function in antigen presentation to B cells, B cell proliferation, selection and affinity maturation [6].

In contrast, the histological localization of T–B cell interactions, B cell differentiation and isotype switching remains unknown. Many investigators have suggested that in general the follicles in lymphoid tissue appear the main sites where T cell-dependent antibody responses and affinity maturation take

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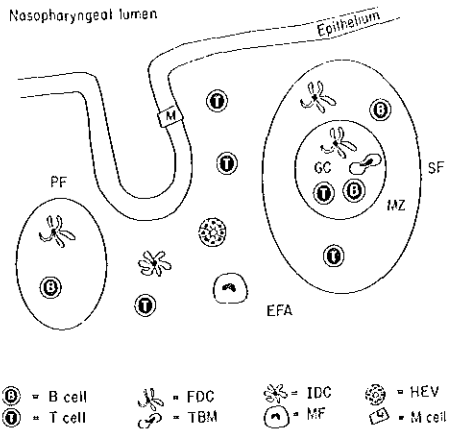

Fig. 1. Schematic representation of compartments and cellular composition of palatine tonsils. C, Crypt; EFA, extrafollicular area; GC, germinal centre; M cell, microfold-cell; MF, macrophage; MZ, mantle zone; PF, primary follicle; SF, secondary follicle. (The drawing is modified after Kuper *et al.* [1].)

place (reviewed by Liu *et al.* [5]). There is ample *in vitro* evidence for the role of cytokines in B cell differentiation and isotype switching in man [7–9]. Our studies in mouse lymphoid tissues revealed evidence for a co-localization of cytokine production, together with specific antibody formation in the extrafollicular area [10,11]. However, the co-localization of CPC and AFC in human lymphoid tissue has not been investigated yet.

IL-1α and tumour necrosis factor-alpha (TNF-α) are mainly released by antigen-presenting cells (APC), such as macrophages and IDC [12–14], whereas IL-2, IL-4 and interferon-gamma (IFN-γ) are mainly produced by activated T cells [15]. These three T cell-derived cytokines are suggested to play an important role in B cell growth and differentiation [16–19]. From a review by Vitetta *et al.* [20] on the *in vitro* effects of cytokines on immune cells we concluded that IL-1α and TNF-α production may be expected at sites of antigen presentation, whereas IL-2, IL-4 and IFN-γ are anticipated to be produced at sites of T–B cell interactions.

To determine in which lymphoid compartment the T–B cell interactions required for antibody production take place in human lymphoid tissue, the present study details the localization of CPC (IL-1α, TNF-α, IL-2, IL-4, IFN-γ) together with AFC (IgG1–4, IgA1–2, IgD, IgE, IgM) in palatine tonsil after recurrent tonsillitis.

MATERIALS AND METHODS

Human palatine tonsils

Tonsils were obtained under general anaesthesia from seven young children (4–6 years old) with recurrent tonsillitis. At the time of tonsillectomy the tonsils were clinically in a resting stage. The fresh surgical specimens were snap-frozen in liquid nitrogen and stored at −70°C.

Antibodies

Vmp18, a mouse MoAb specific for human IL-1α, was kindly provided by Dr D. Boraschi [13]. Mouse MoAb 61E71, recognizing human TNF-α, was a generous gift from Dr W. Buurman [21]. DMS-1, a mouse MoAb to recombinant human IL-2, and BL-4-P, a rabbit anti-human-IL-4 polyclonal antibody were purchased from Sanbio (Uden, The Netherlands). The mouse MoAb MD-2 to human IFN-γ was kindly provided by Dr P. van der Meide [22]. The MoAbs against the different immunoglobulin classes respectively IgG1–4, IgA1–2, IgD, IgE and IgM were generated in our laboratory and tested for their specificity [23–27]. The MoAbs were all from the IgG1 isotype. A mouse IgG1 MoAb specific for HIV-1 gp120 (IIIB-V3-21 isolate) was used as an isotype-matched control antibody [28]. As negative control polyclonal antibody, a rabbit polyclonal antiserum specific for KLH-MBS-SP66 (SP66 is a peptide from the androgen receptor) was used [29]. The secondary antibodies, rabbit anti-mouse (total immunoglobulin) and goat anti-rabbit (total immunoglobulin), both horseradish peroxidase (HRP)-conjugated, were purchased from Dakopatts (Copenhagen, Denmark).

Immunohistochemical staining

Cryostat sections (8 μm) were fixed for 10 min in fresh acetone, containing 0·02% H_2O_2. Slides were incubated overnight at 4°C with the panel of antibodies. All reagents were diluted in PBS containing 0·1% bovine serum albumin (BSA) and titrated to get optimal results. After incubation the slides were washed with PBS and incubated for another 60 min at room temperature with secondary conjugates in appropriate dilutions in PBS containing 1% BSA and 1% normal human serum. Sections were washed with PBS and histochemical revelation of HRP was performed with 3-amino-9-ethylcarbazole (AEC) as previously described [30]. Slides were counterstained with haematoxylin for 15 s and mounted in glycerol/gelatin. Two different sections from each tonsil were subjected to histological evaluation for CPC and AFC.

Inhibition experiments

To verify the specificity of the staining, cytokine inhibition experiments were performed on palatine tonsil sections as well as on human allergic skin tissue sections. The skin biopsy was obtained after 72 h of application with epoxy resin (1%) and used for IL-2-positive control and inhibition experiments.

Table 1. Distribution of cytokine-producing cells within various human tonsillar tissue compartments

Cytokines produced	Germinal centre	Mantle zone	Extrafollicular area
IL-1α	−	+*	++
TNF-α	−	+*	+
IFN-γ	−	−	++
IL-2	−	−	±
IL-4	−	−	±

* Almost the whole mantle zone stained positive.

−, No cytokine-producing cells (CPC) found; ±, occasional CPC (< 10 per section); +, high frequency of CPC (< 1000 per section); ++, very high frequency of CPC (> 1000 per section) (descriptions used in text).

Solutions with different concentrations of the antibodies 61E71, DMS-1, BL-4-P and MD-2 were prepared and mixed with respectively the affinity-purified recombinant cytokines TNF-α (Cetus Corporation, Emeryville, CA), IL-2 (Boehringer, Mannheim, Germany), IL-4 (HIL-4-C, Sanbio) and IFN-γ (HG-IFN, Sanbio) in two different concentrations. These mixtures were incubated for 2 h at room temperature and cleared of aggregates and complexes by centrifugation (12 000 *g*, 2 min, 20°C). The tissue sections were incubated directly with these mixtures and the normal immunohistochemical staining method was subsequently applied.

RESULTS

Histology

In the haematoxylin-stained tonsil sections, primary and secondary follicles were observed. The frequency of secondary follicles compared with the primary follicles was dependent on

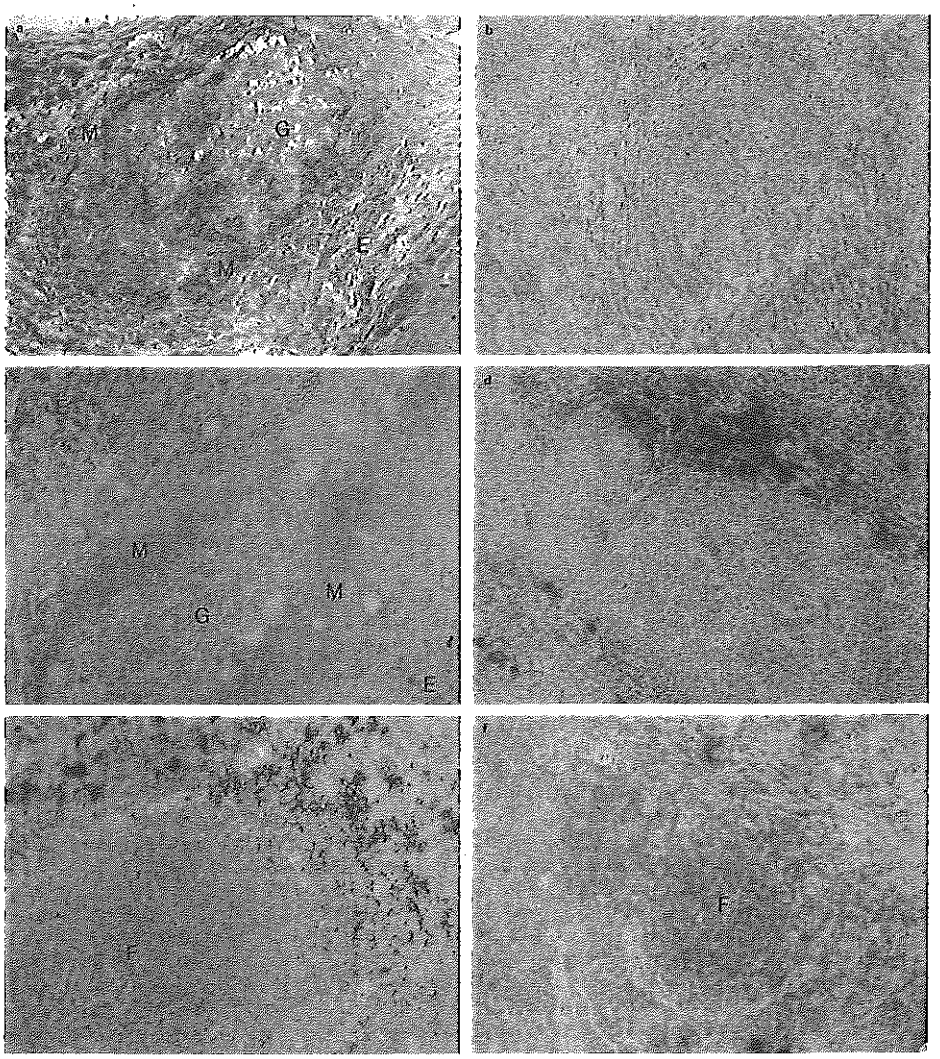

Fig. 2. Immunoperoxidase staining for cytokine-producing cells and antibody-forming cells (AFC) in tonsillar tissue compartments. (a) A tonsil section stained using MoAb against IL-1α demonstrates positive cells in mantle zone and extrafollicular area. (b) Absence of IL-2 staining in the extrafollicular area. (c) Positive IFN-γ-producing cells present in extrafollicular area. (d) IgG1-AFC in the extrafollicular area. (e) IgM membrane staining inside follicle and mantle zone, IgM-AFC in extrafollicular area. (f) IgD membrane staining inside primary follicle. F, Follicle; G, germinal centre; M, mantle zone; E, extrafollicular area. (× 200.)

Table 2. (Sub)class distribution of antibody-forming cells (AFC) within various compartments in human tonsils

Immunoglobulin subclass	Germinal centre	Mantle zone	Extrafollicular area
IgG1	–	–	++
IgG2	–	–	+
IgG3	–	–	++
IgG4	–	–	+
IgA1	–	–	++
IgA2	–	–	+
IgD	–	+†	+
IgE	–	–	–
IgM	+*	±*	±

* IgM-positive immune complexes or IgM membrane-positive cells were found.

† IgD membrane-positive cells.

–, No AFC found; ±, occasional AFC (<100 per section); +, high frequency of AFC (100–500 per section); ++, very high frequency of AFC (>500 per section) (descriptions used in text).

the general reactive state of the tonsil. All seven tonsils showed far more secondary follicles than primary follicles.

Specificity of the staining

Inhibition experiments on palatine tonsil and allergic skin sections verified the specificity of the TNF-α, IL-2, IL-4 and IFN-γ staining we performed. Preincubation of 61E71 (25 μg/ml) with TNF-α (50 μg/ml), DMS-1 (35 μg/ml) with IL-2 (8 μg/ml), BL-4P (12 μg/ml) with IL-4 (1 μg/ml) and MD-2 (35 μg/ml) with IFN-γ (50 μg/ml) inhibited the cytokine-specific staining. No staining was seen in sections treated by omission of the primary antibody or after staining with the control MoAb and polyclonal antibody.

Immunohistochemical detection of CPC

In this study the presence of IL-1α-, TNF-α-, IL-2-, IL-4- and IFN-γ-producing cells was investigated in palatine tonsil sections. Theoretically, cytoplasmic cytokine production, membrane-bound cytokine and uptake of cytokines by target cells can be detected using immunohistochemistry. However, we found no evidence for membrane-bound cytokines and uptake of cytokines by target cells. The expected low concentration of cytokines bound to membrane-localized cytokine receptors compared with high cytoplasmic concentration of cytokines may prohibit simultaneous detection by immunohistochemistry. The possibility that cytoplasmic staining represents uptake of cytokines rather than production has already been ruled out previously by Andersson *et al.* [31]. They observed that incubation of mononuclear cells with high concentrations of IFN-γ did not lead to any detectable IFN-γ staining. From these and our experiments we concluded that the cytoplasmic staining found using cytokine-specific MoAbs reflects actual cytokine production.

The distribution patterns of IL-1α- and TNF-α-producing cells were similar, and consisted of a high frequency of cells with cytoplasmic staining in the mantle zone of secondary follicles (Table 1). IL-1α-producing cells (Fig. 2a) and TNF-α-producing cells were also detected in the extrafollicular area, especially in the T cell zones. However, in this latter compartment the frequency of IL-1α-producing cells was very high compared with the high frequency of TNF-α-producing cells. Only occasional cells positive for IL-2 (Fig. 2b) and IL-4 were detected exclusively in the extrafollicular area (Table 1). In contrast, a high frequency of IFN-γ-producing cells was found restricted to the extrafollicular area (Fig. 2c) (Table 1). The IL-2-, IL-4- and IFN-γ-producing cells were generally detected in the T cell zones.

Detection of AFC producing different immunoglobulin subclasses

Tonsils were examined for IgG-AFC, IgA-AFC, IgD-AFC, IgE-AFC and IgM-AFC using MoAbs known to recognize these different immunoglobulin subclasses (Table 2). IgG-AFC and IgA-AFC were found in high or very high frequencies. In addition, we detected IgM-AFC. IgD-AFC and IgE-AFC were not found. The proportion of IgG1-AFC and IgG3-AFC was almost similar, and exceeded the number of IgG2-AFC and IgG4-AFC. More IgA1-AFC than IgA2-AFC were found.

The IgG1–4-AFC as well as the IgA1–2-AFC were mainly localized in the extrafollicular areas, especially in the T cell zones and occasionally in association with the cript epithelium (Fig. 2d). IgM membrane staining was seen inside the B cell follicles and mantle zones. IgM-AFC were detected in the extrafollicular area, mainly in the T cell zones (Fig. 2e). IgD membrane staining was found in the mantle zones of secondary follicles and inside primary follicles (Fig. 2f).

DISCUSSION

In this study we demonstrated immunohistochemically that cytokine-producing T cells as well as antibody-forming B cells were localized in the extrafollicular compartment in human tonsillar tissue. These findings suggest that in resting tonsils T–B cell interaction might be restricted to this tonsillar compartment.

Our *in situ* cytokine detection revealed that IL-1α- and TNF-α-producing cells are localized in the same compartments: the mantle zone and the extrafollicular area, especially the T cell zones. IFN-γ-producing cells were not found in the mantle zone, but were only localized in the T cell zones in the extrafollicular area. In contrast, occasional IL-2- and IL-4-producing cells were detected in the T cell zones in the extrafollicular area. These findings suggest that mainly T cells were producing IL-2, IL-4 and IFN-γ. Ruco *et al.* [14] demonstrated by double staining that IL-1α-producing macrophages, IL-1α-producing IDC and TNF-α-producing macrophages were localized in T cell-dependent areas, whereas FDC inside the germinal centres were consistently negative for these two cytokines. Our *in situ* findings are supported by evidence from Bowen *et al* [32], who showed *in vitro* that the Leu-7 (CD57)$^+$ T cells isolated from human tonsillar follicles did not produce TNF-α, IL-2, IL-4 and IFN-γ in significant amounts. Furthermore, *in situ* IL-4 mRNA production was not observed inside the germinal centres of tonsils from young children with recurrent infections, but was only detected in the extrafollicular area [33].

In our study the observed low frequencies of IL-2- and IL-4-producing cells may be explained by the kinetics of cytokine production. In *in vitro* experiments by Secrist *et al.* [34], phytohaemagglutinin (PHA) activation of tonsillar mono-

nuclear cells, which stimulates mainly T cells, induced expression of IL-2 and IL-4 mRNA after 8 h of stimulation. No IL-2 mRNA or IL-4 mRNA could be detected after 24 h and 40 h of stimulation.

Since the tonsils were obtained in a clinically resting stage, it is expected that only a small number of CPC were present. In contrast, a high frequency of IFN-γ-producing cells were observed in the extrafollicular area. This is in agreement with *in vitro* experiments showing a high production of IFN-γ in freshly isolated tonsillar cell suspensions [35] and tonsillar lymphocytes stimulated with PHA or Sendai virus [36,37]. Moreover, allogeneic stimulation of BALB/c ($H\text{-}2^d$) mouse splenic T cells with CBA/Ca ($H\text{-}2^k$) mouse dendritic cells or FcR^+ macrophages generated elevated levels of IFN-γ detectable throughout the 7-day culture period. In contrast, only negligible amounts of IL-2 and IL-4 were detected in this study [38]. These data suggest that the differences in kinetics of distinct CPC are the most likely explanation for the frequencies of IL-2-, IL-4- and IFN-γ-producing cells found. Moreover, another explanation for the persistence of IFN-γ- compared with IL-4-producing cells could be the putative role of this cytokine as a down-regulator of IL-4 production, which was also suggested by Fernandez-Botran *et al.* [39]. In their study, they demonstrated that IFN-γ down-regulated the production of IL-4 by murine T-helper type-2 cells.

Our results concerning the localization of AFC and the isotype distribution of AFC within the tonsillar tissue support the study described by Brandtzaeg *et al.* [40]. They demonstrated the presence of IgG-AFC and IgA-AFC in mainly the extrafollicular area, and a low frequency of these AFC inside the germinal centres and mantle zones of human tonsils from patients with facial traumas or tumours. In their study, IgM-AFC and IgD-AFC were hardly found in the different tonsillar compartments. The absence of IgE-AFC in the present study is also in agreement with observations from Brandtzaeg *et al.* [40], who found neither IgE-AFC *in situ* nor significant serum IgE concentrations.

IL-2-, IL-4- and IFN-γ-producing cells were localized in the same compartment as the IgG-AFC and IgA-AFC. This co-localization of CPC and AFC is similar to the distribution of analogous cell types in mouse spleen. There we detected IL-2-, IL-4- and IFN-γ-producing cells together with antigen-specific AFC in the extrafollicular compartments [10,11].

The high frequency of IFN-γ-producing cells in the present experiments is suggestive for stimulation of antibody production by IFN-γ in a humoral immune response. *In vitro* experiments by Jelinek *et al.* [17] also demonstrated the stimulatory effect of IFN-γ on the growth and differentiation of immunoglobulin-secreting tonsillar B cells after stimulation with *Staphylococcus aureus*.

The detection of occasional IL-4-producing cells and the absence of IgE-AFC in tonsillar tissue might be ascribed to the high production of IFN-γ, which down-regulates IL-4-induced IgE production, as earlier described by Del Prete *et al.* [18]. They demonstrated *in vitro* in human T cell clones that IFN-γ antagonizes largely the effects of IL-4 on B cells, including IgE production.

The suggestion of interactions between CPC and AFC occurring in the extrafollicular area, as discussed above, suggests that T–B cell interactions might be restricted to this compartment. It appears that the sequence of events leading to an antibody response as described by Van den Eertwegh *et al.* [11] may have its human counterpart in tonsillitis. Antigens will be taken up and processed by macrophages or IDC in the extrafollicular area. Upon contact, antigen-specific T cells are activated and proliferate. B cells inside the germinal centre that bind soluble or processed antigen migrate into the mantle zone and extrafollicular area. Subsequently, these B cells encounter the specific T cells in the extrafollicular area, and T–B cell interactions with cytokine and antibody production, including isotype switching, are likely to occur.

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Chapter 3

In situ cytokine profiles in contact dermatitis

3.1

Enhanced IL-1α, IL-2, IFN-γ, and TNF-α production in allergic contact dermatitis in human skin: an *in situ* immunohistochemical study
In: New advances on cytokines. S. Romagnani, T.R. Mosmann and A.K. Abbas (Eds.), Serona Symposia Publications, Raven Press, Vol. 92, 334-340, 1992.

3.2

In vivo cytokine profiles in allergic and irritant contact dermatitis (IL-1α, IL-2, IFN-γ and TNF-α)
Contact Dermatitis (in press).

3.3

In situ expression of IL-1ß, IL-4 and IL-10 in human allergic and irritant contact dermatitis: implications for differentiation *(submitted).*

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ENHANCED IL-1α, IL-2, IFN-γ AND TNF-α PRODUCTION IN ALLERGIC CONTACT DERMATITIS IN HUMAN SKIN: AN *IN SITU* IMMUNOHISTOCHEMICAL STUDY

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INTRODUCTION

Allergic contact dermatitis (ACD) is a cell-mediated inflammatory response of delayed onset (26-72h) to the epicutaneous application of highly reactive simple chemical compounds (haptens). Cells of the dermis as well as the epidermis play an active role in the local inflammatory events in ACD.

The presence of various cytokines in the epidermis as well as in the dermis has frequently been reported (4,24,20,17,19). Keratinocytes in the epidermis in normal and allergic affected skin and both Langerhans cells and macrophages penetrating the dermis in allergic affected skin, are capable of producing a variety of cytokines including the commonly produced inflammatory cytokines IL-1α and TNF-α.

In vivo modulation experiments in both animal (12,22,8) and man (9) demonstrated that injection of IL-1α produced leukocyte-rich inflammatory reactions. Anti-TNF-α antibody treatment was an effective inhibitor in ACD based on an *in vivo* study in sensitized mice (21).

During the course of an ACD reaction in the skin, leukocytes especially T-lymphocytes are recruited from the blood into the inflammatory site (13). There is ample evidence that predominantly $CD4^+$ T-lymphocytes play a central role in ACD. Transfer of ACD in the mouse can be mediated by $CD4^+$ T-helper-1 (TH1) lymphocytes (6). Within panels of T-lymphocyte clones randomly prepared from biopsies of experimentally nickel-induced skin lesions in nickel-allergic individuals, it was found that nickel-specific $CD4^+$ T-lymphocytes occur in relatively higher frequencies in these lesions when compared to non-allergic control individuals (15).

Interferon-γ (IFN-γ) and interleukin-2 (IL-2) are cytokines produced by activated T-lymphocytes. Based on *in vitro* experiments (25,16) and *in vivo* modulation studies in both animal (10,28,14) and man (3) they may be expected to appear particularly in ACD.

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Considering the fact that *in vitro* manipulations like cell separations and culture may introduce artefacts and at least essentially change the microenvironment of the cells, it is somewhat premature to predict the role of cytokines in a disease process based on *in vitro* experiments. *In vivo* modulation studies provide an alternative approach to study the role of cytokines in a disease process. In contrast to *in vitro* experiments, *in vivo* modulation experiments allow to study the effect of cytokine or anti-cytokine treatment in an unchanged context of immune cells *in vivo*.

Detection of cytokines by *in situ* immunohistochemical techniques enables to study the cytokine expression of cells in their unchanged original microenvironment, without cytokine or anti-cytokine treatment. Feasibility of the *in situ* immunohistochemical detection of cytokines at single cell levels by cytokine specific monoclonal antibodies has been demonstrated in different human skin diseases (2,11,26).

To further extent the understanding of the role of cytokines in the regulation of allergic responses, we investigated the potentials of immunohistochemical techniques in the *in vivo* analysis of a cytokine-mediated contact allergic response. Furthermore, the study was aimed to immunohistochemically compare the cytokine secretion pattern in skin biopsies from epoxy resin and formaldehyde allergic individuals, control "normal" skin biopsies from these persons and normal skin from control persons.

MATERIAL AND METHODS

Participants

Six volunteers (male adults) took part in the study after giving informed consent. Persons known to be allergic for epoxy resin (n=2) and persons known to be allergic for formaldehyde (n=2) participated in the allergic patch-test. Non-allergic individuals (n=2) were the control persons.

The allergic patch-test was performed with 1% formaldehyde in aquadest and 1% epoxy resin in petrolatum. Finn chambers (10 mm) (Epitest, LTD. Oy, Payala, Finland) were used for occlusion and fixed on the back skin of the participants for 48 hours. As controls, simultaneous patch-tests with petrolatum and aquadest were performed at an area sufficiently distant from the allergic patch-tests.

Biopsy specimens

Three five-millimeter punch biopsies were taken from each person which participated in the allergic patch test. One biopsy was a control at t=0, one was a control from clinically unaffected skin at t=72 hours and one was from clinically affected skin at t=72 hours. One biopsy was taken from the non-allergic individuals. Biopsy specimen were snapfrozen and stored at -70°C.

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Monoclonal antibodies

Vmp18, a specific mouse monoclonal antibody (MAb) to the synthetic peptide 199-208 of mouse anti IL-1α, corresponds to 196-205 in the human sequence and was kindly provided by Dr. O. Boraschi (23). DMS-1, a mouse MAb to recombinant humaan IL-2, was purchased from Sanbio (Uden, the Netherlands). The mouse MAb MD-2, which neutralizes human IFN-γ, was kindly provided by Dr. P. van der Meide (27). Mouse MAb 61E71, recognizing human TNF-α, was a generous gift from Dr. W. Buurman (5).

These MAb's were all from the isotype IgG1. As control for anti-isotype and concentration-match, a mouse MAb against HIV-1 gp120 (IIIB isolate) was used and made available by Dr. J. Laman (18).

Immunohistochemical staining

Cryostate sections (8 μm) were fixed for 10 min. in fresh acetone, containing 0.02% H_2O_2. Slides were incubated overnight at 4°C with Vmp18, MD-2, DMS-1, 61E71 and 1033. All reagents were diluted in PBS containing 0.1% BSA and titrated to get optimal results. After incubation the slides were washed with PBS and incubated for another 60 min. at room temperature with second-step Rabbit-anti-Mouse Ig(total) conjugated to horseradish-peroxydase (RaM-HRP) (Dakopatts, Denmark) diluted optimally in PBS containing 1% BSA and 1% normal human serum. Sections were washed with PBS and histochemical revelation of HRP was performed with 3-amino-9-ethylcarbazole (AEC) as the substrate. Slides were counterstained with hematoxylin for 15 sec. and mounted in glycerol/gelatin (7).

RESULTS AND DISCUSSION

We investigated cytokine expression by *in situ* immunohistochemistry on cryostat sections of clinically manifest contact allergic skin after application of epoxy resin and formaldehyde, on "normal" skin from allergic persons at t=0 and t=72 hours and on normal skin from control persons.

No staining was seen in control slides from skin sections treated by omission, concentration- and isotype-matched substitution of the primary antibody (FIG. 1.a, 1.c). After incubation with Vmp18 and histochemical revelation, IL-1α positive keratinocytes, characterized by a cytoplasmic staining, were detected in the epidermis of "normal" skin from allergic persons at t=0 and t=72 hours as well as in the normal skin from control persons. In general, significantly enhanced IL-1α production was found in epidermis and in infiltrates in the dermis in both epoxy resin and formaldehyde (FIG. 1.b) allergic affected skin.

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Analysis of IL-2, IFN-γ and TNF-α producing cells revealed that in the cell clusters in "normal" skin from allergic persons and normal skin from control persons no significant increase in cytokine producing cells was observed. In allergic affected skin an enhanced frequency of IL-2, IFN-γ and TNF-α producing cells was detected in the dense infiltrates. In addition, in allergic affected skin we could demonstrate IFN-γ positive cells in infiltrates in the dermis (FIG. 1.d), which were not seen in control stained slides from allergic affected skin (FIG. 1.c).

The staining pattern and localization of IL-1α producing cells in epidermis in control skin in this study was similar to the patterns as described by Antilla et al. (1). So far IL-1α has not been detected immunohistochemically in allergic affected skin. Dowd et al. (9) mimicked a contact allergic respons. They reported that intradermal injection of 10-100 U recombinant IL-1α in human volunteers produced erythematous reactions lasting more than 24 hours and containing mixed leukocyte infiltrates, but the presence of IL-1α was not demonstrated immunohistochemically at the local level. *In situ* detection of IL-1α producing cells in both epidermis as well as dermis in allergic affected skin in higher frequencies than in "normal" skin from allergic persons and normal skin from control persons strongly supports the notion that this cytokine may be an important mediator in contact hypersensitivity reactions.

In contact allergy the frequencies of IL-2, TNF-α and IFN-γ positive cells were increased compared to normal or "normal" sensitized skin. These results indicate that IL-2, TNF-α and IFN-γ are important mediators in contact allergic reactions as already suggested by the results of *in vitro* and *in vivo* modulation studies (25,16,10,28,14,3,21).

In conclusion, this study demonstrates the potentials of the *in situ* immunohistochemical approach to the *in vivo* analysis of a cytokine-mediated contact allergic response in man. With respect to the possible role of cytokines in allergic contact dermatitis these findings indicate that IL-1α, IL-2, IFN-γ and TNF-α may be used as differential markers for "normal" and allergic effected skin. Furthermore, in combination with other techniques, this method will facilitate the analysis of the role of these cytokines and the *in vivo* kinetics during an allergic contact reaction.

In addition, studies with more participants and a quantitative scoring-methodology are presently under investigation.

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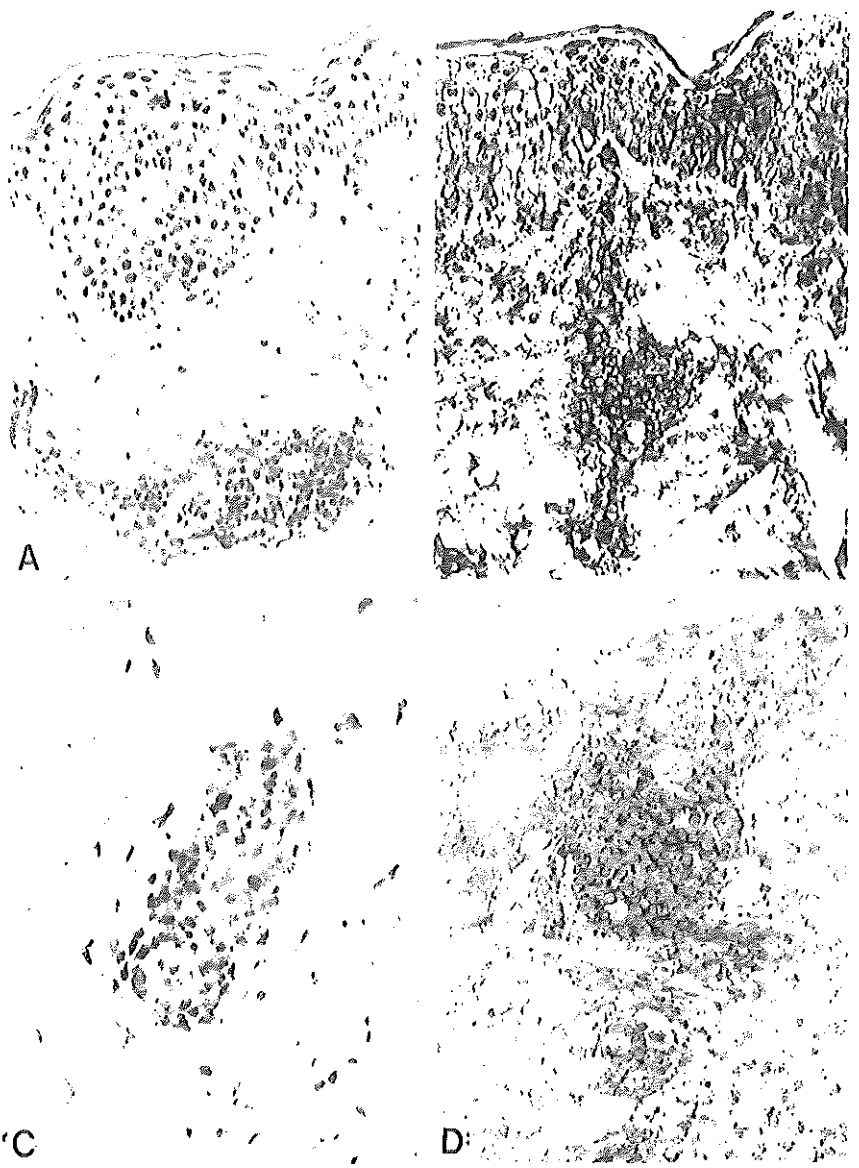

FIG. 1: Tissue sections from an allergic skin lesion counterstained with hematoxylin; a) control stained section without primary antibody, b) IL-1α positive cells detected in the epidermis and in infiltrates in the dermis, c) control stained section without primary antibody, and d) IFN-γ positive cells detected in infiltrates in the dermis.

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IN VIVO CYTOKINE PROFILES IN ALLERGIC AND IRRITANT CONTACT DERMATITIS

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ABSTRACT

Local cytokine profiles in skin biopsies from allergic and irritant patch test reactions were determined by *in vivo* immunohistochemistry to differentiate between these two clinically identical afflictions especially at the time of final reading in diagnostic dermatology. Biopsies were taken from established allergic persons after specific allergic patch tests for epoxy resin (1%) and formaldehyde (1%) and from non-allergic individuals with irritant patch tests for sodium lauryl sulphate (10%) and formaldehyde (8%). At 72 hours after application of the agents, significant enhanced frequencies of dermal infiltrating cells producing IL-1α, TNF-α, IL-2, and IFN-γ per 100 infiltrating cells in the dermis were observed in allergic as well as in irritant patch test reactions as compared to normal skin. Significant higher frequencies of IL-1α producing cells were observed in biopsies from epoxy resin (1%) allergic and sodium lauryl sulphate (10%) irritant affected skin as compared to formaldehyde (1%) allergic affected skin. In addition, significant higher frequencies of TNF-α producing cells were observed in epoxy resin allergic affected skin as compared to formaldehyde (1%) allergic and formaldehyde (8%) irritant affected skin. The allergic and irritant patch test reactions showed similar levels of expression of the Th1 cytokines IL-2 and IFN-γ in the dermis, confirmed by probe based detection of IL-2 mRNA and IFN-γ mRNA.

In conclusion, the described similarity shows that allergens as well as irritants can induce the same profile of IL-1α, TNF-α, IL-2, and IFN-γ production resulting in the almost impossibility to discriminate between allergic and irritant contact dermatitis at the time of patch test reading.

INTRODUCTION

Exposure to several chemical substances may result in allergic as well as irritant contact dermatitis (1). The correct assessment of allergic and irritant contact dermatitis may pose considerable problems in dermatologic practice. Especially difficulties may arise when differentiating allergic and irritant patch test reactions at the time of their final reading, in general 72 hours after application. Until now both types of contact dermatitis, despite of their induction by different mechanisms, could not be differentiated from the macroscopic appearance, electron-microscopy, and light microscopic histology predominantly based on the detection of adhesion molecules and cell phenotypes (2,3). Since epidermal cells and inflammatory cells have the capacity to produce different cytokines (4-10), the *in vivo* determination of cytokine production profiles in the two types of contact dermatitis may provide a differentiation marker.

In vitro experiments showed that keratinocytes in normal and allergic affected skin, and both Langerhans cells and macrophages penetrating the dermis in allergic affected skin, were capable of producing a variety of cytokines, including the cytokines IL-1 and TNF-α (4,5). *In vivo* modulation experiments in man (6) demonstrated that injection of recombinant IL-1α produced leukocyte-rich inflammatory reactions in the skin. Furthermore, the involvement of TNF-α in allergic as well as in irritant contact dermatitis was demonstrated by the observation that anti-TNF-α antibody treatment was able to inhibit both types of contact dermatitis reactions in mice (7).

Based on *in vivo* modulation studies, IL-2 and IFN-γ are considered to be major factors in skin inflammation in both animal and man. A recruitment of lymphocytes into the skin of rats was found after intradermal injection of IFN-γ (8) whereas intradermal IFN-γ injection in man induced a moderate perivascular dermal infiltrate (9). Systemic administration of IL-2 induced an enhancement of a delayed hypersensitivity reaction to a contact allergen (10). Mosmann et al. (11) distinguished two main types of T-cells in the mouse: Th1 cells produce IL-2 and IFN-γ, whereas Th2 cells express IL-4 and IL-5. As a result of these findings in the mouse, different human inflammatory diseases have been associated with cells preferentially expressing a Th1 or Th2 profile (12). In allergic contact dermatitis Kapsenberg et al. (13) demonstrated Th1 cytokine production profiles of nickel-specific $CD4^+$ T-cell clones from nickel contact allergic individuals.

Studies into the role of the Th1 cytokines IL-2 and IFN-γ in irritant reactions are scarce. Hunziker et al. (14) observed a 2-3-fold increase of IL-2 in human skin lymph derived from sodium lauryl sulphate induced contact dermatitis 72 hours after the first application of the irritant. Moreover, Enk and Katz (15) demonstrated in mice, by sensitive reverse transcriptase-polymerase chain reaction technique, an upregulation of

IFN-γ mRNA after both allergen and irritant painting during the first 4 hours of contact dermatitis.

In the present study, we compared the expression of "IL-1α and TNF-α" and the expression of the Th1 cytokines "IL-2 and IFN-γ" in patch test reactions elicited with the industrial agents epoxy resin (1%) and formaldehyde (1%) for the allergic reactions and with sodium lauryl sulphate (10%) and formaldehyde (8%) for the irritant reactions.

PATIENTS AND METHODS

Participants and skin testing methods

Volunteers (n=23) took part in the study after giving informed consent. Persons proven to be allergic for epoxy resin (n=6) and persons proven to be allergic for formaldehyde (n=6) participated in the allergic patch test. The allergic patch test was performed on the back with 1% formaldehyde in aquadest and 1% epoxy resin in petrolatum; these concentrations are routinely used in diagnostic procedures. For the irritant application non-allergic individuals were treated on the back with 10% sodium lauryl sulphate in petrolatum (n=5) and 8% formaldehyde in aquadest (n=6). These concentrations were established to cause an intensity of inflammation equal to the allergic reaction in a pilot dose-response study. This means that, determined by clinical inspection, the same degree of intense erythema and moderate infiltration was seen in the allergic and irritant patch test reactions. According to the established international criteria (16) all reactions were classified as moderate allergic (++) or irritant (++) at the final reading (72 hours). Finn chambers (10 mm) (Epitest-Ltd. Oy, Payala, Finland) were used for occlusion and fixed on the back skin of the participants for 48 hours. Two 4mm punch skin biopsies were obtained; one from allergen or irritant challenged skin at 72 hours after application and one from normal control skin at an area on the back sufficiently distant (>10 cm) from the allergic or irritant patch test. To ensure the non-allergic individuals treated with 8% formaldehyde were not allergic, the reaction to 1% formaldehyde was also determined by clinical inspection without taking a biopsy. Biopsy specimens were immediately frozen in liquid nitrogen and stored at -70°C. The time choices of 48 hours of application and 72 hours to evaluate the results and to take biopsies are generally established in patch test procedures (16).

Chemicals

3-amino-9-ethylcarbazole (AEC; A-5754), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; B-8503), levamisole, salmon sperm DNA (D-1626), dextran sulphate (D-8906),

bovine serum albumin (BSA;
A-9647), poly-L-lysine (P-1274), nitro blue tetrazolium salt (NBT) and polyvinyl pyrrolidone (PVP) were obtained from Sigma (St. Louis, MO, U.S.A.). Diethyl pyrocarbonate (DEPC) was obtained from Serva Feinbiochemica (Heidelberg, Germany). DNA tailing kit (1028 707) and sheep-anti-digoxigenin-alkaline-phosphatase(AP) Fab Fragments (1093 274) were purchased from Boehringer (Mannheim, Germany). Ficoll (17- 0400-01) was obtained from Pharmacia (Uppsala, Sweden). Formamide (D-6100) was purchased from Merck (Darmstadt, Germany). Ion exchange resin (AG-501-X8) was obtained from Bio-Rad (Richmond, CA, U.S.A.).

Antibodies and immunohistochemistry

Vmp18, a mouse monoclonal antibody (MAb) specific for human IL-1α was kindly provided by Dr. D. Boraschi, Laboratorio di Immunofarmacologia, Centro Ricerche Sclavo, Siena, Italy (17). Mouse MAb 61E71, recognizing human TNF-α, was a generous gift from Dr. W. Buurman, Department of Surgery, Biomedical Center, University of Limburg, Maastricht, The Netherlands (18). DMS-1, a mouse MAb to recombinant human IL-2 was purchased from Sanbio (Uden, The Netherlands). The mouse MAb MD-2 to human IFN-γ was donated by Dr. P. Van der Meide from our institute (19). The MAbs were all of the IgG1-isotype. 61E71, DMS-1 and MD-2 were tested for their specificity in a previous study (20). A mouse IgG1 MAb specific for HIV-1 gp120 (IIIB-V3-21 isolate) was used as an isotype matched negative reagent control (21). The secondary antibody, rabbit-anti-mouse (total-Ig) horseradish-peroxidase (HRP) conjugated, was purchased from Dakopatts (Copenhagen, Denmark).

Cryostat sections (8 μm) were fixed for 10 min in fresh acetone, containing 0.02% H_2O_2. Slides were incubated overnight at 4°C with the panel of antibodies. All reagents were diluted in PBS containing 0.1% BSA and titrated to get optimal results. After incubation the slides were washed with PBS and incubated for another 60 min at room temperature with rabbit-anti-mouse-HRP diluted optimally in PBS containing 1% BSA and 1% normal human serum. Sections were washed with PBS and histochemical revelation of HRP was performed with AEC as the substrate according to Claassen and Adler (22). Slides were counterstained with hematoxylin for 15 s and mounted in glycerol/gelatin.

Probes and mRNA-probe hybridisation

The synthetic digoxigenin labelled oligonucleotide probe cocktail complementary to human IL-2 mRNA (BPR13) was purchased from British Biotechnology Limited, Oxford, U.K. The probe cocktail against IFN-γ mRNA was kindly provided by Dr. M.N. Woodroofe (23). The negative control probe also used by Matsuki et al. (24), a

synthetic oligonucleotide (GGCGACG-CGCCGTATTTATAATTCATTATG), was prepared on a DNA synthesizer (Cyclone plus DNA synthesizer, BioGen/Biosearch Division of Millipore, Etten-Leur, The Netherlands), and labelled with digoxigenin using terminal deoxynucleotidyl transferase as previously described by Schmitz et al. (25).

The hybridisation technique was adapted from Woodroofe et al. (26). Cryostat sections (8 μm) were cut onto poly-L-lysine-coated slides. Sections were fixed for 10 min in 4% paraformaldehyde in 0.1 M PBS and 0.02% DEPC (pH 7.4). Washing consisted of three sequential 10 min baths: 1) 1M PBS (pH 7.4); 2) 1M PBS (pH 7.4); 3) 2x standard saline citrate (SSC) (0.3 M NaCl, 0.03 M Na-citrate, pH 7). Slides were prehybridized for 2 h at 37°C by using a hybridisation mix consisting of 50% deionized formamide (by ion exchange resin), 10% dextran sulphate, 30% 20xSSC, 5x Denhart's (0.1% Ficoll, 0.1% BSA, 0.1% PVP) and 100 μg/ml denatured salm sperm DNA. For hybridisation a probe mixture was added consisting of the probe (2 μg/ml) in hybridisation mix under parafilm. Hybridisation proceeded for 17 h at 37°C. Slides were washed in six sequential 5 min baths: 1) 30% deionized formamide in 4xSSC, 2) 30% deionized formamide in 4xSSC, 3) 30% deionized formamide in 2xSSC, 4) 30% deionized formamide in 2xSSC, 5) 30% deionized formamide in 0.2xSSC at 37°C, and 6) 30% deionized formamide in 0.2xSSC at 37°C. Sections were prepared for immunological detection by washing for 5 min in buffer 1 (0.1 M Tris-HCl,0.15 M NaCl, pH 7.5). Slides were incubated for 30 min with 2% normal sheep serum (NSS) in buffer 1. AP-conjugated sheep-anti-digoxigenin was applied at 1:500 in 2% NSS in buffer 1 for 3 h at RT. Unbound conjugate was removed by washing in two changes of buffer 1 followed by one wash in buffer 2 (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). For the visualization of AP-activity, sections were incubated for several hours with 240 μg/ml levamisole, 0.34 mg/ml NBT and 0.18 mg/ml BCIP in buffer 2. The staining reaction was stopped with buffer 3 (0.01M Tris-HCl, 1mM EDTA, pH 8.0). Slides were mounted in glycerol/gelatin.

Quantification and statistical analysis

Frequencies of cells with evident cytokine production and the total number of cells in the dermis were determined by using an eyepiece graticule. For each biopsy, one section was stained and at least 2 fields were analysed. Frequencies were expressed as the mean ±SD per 100 cells in the dermis of the group.

Results were analysed by the two-sample Student's t-test for comparison of two empirical means in a normally distributed population ($P<0.05$).

RESULTS

Cytokine production profiles in allergic reactions to epoxy resin (1%) and formaldehyde (1%) and in irritant reactions to formaldehyde (8%) and sodium lauryl sulphate (10%) were compared.

In the present study, as was found in our previous study (20), no staining was seen in control slides from any specimen treated by omission (Fig. 1a) or substitution of the primary antibodies specific for the human cytokines by a mouse IgG1 MAb specific for HIV-1 gp120 (Fig. 1g).

In normal skin biopsied before application IL-1α-producing-cells(PC) were detected in the epidermis. For TNF-α we found weak staining inside the epidermis, especially in the high epidermal layers. Staining with the MAbs specific for IL-2 and IFN-γ (Fig. 1d) revealed that no or a few little rounded red cells were observed producing these cytokines in normal skin.

To evaluate the effect of skin treatment with two different allergens (Fig. 2) and irritants (Fig. 3) to normal skin the numbers of cells inside the dermis producing a specific cytokine (per 100 cells) were determined. At 72 hours after application with the two allergens as well as the two irritants significant enhanced frequencies of IL-1α-PC (Fig. 1b), TNF-α-PC (Fig. 1c), IFN-γ-PC (Fig. 1e,f), and IL-2-PC (Fig. 1h,i) were observed in mononuclear cell infiltrates in the dermis as compared to normal skin. For IL-2 and IFN-γ no significant differences were found between the frequencies of cytokine-PC in biopsies from allergic affected skin after exposure to epoxy resin (1%) as compared to formaldehyde (1%) (Fig. 2), and in biopsies from irritant affected skin after exposure to formaldehyde (8%) as compared to sodium lauryl sulphate (10%) (Fig. 3). In the biopsies from epoxy resin (1%) exposed skin the frequencies of IL-1α-PC and TNF-α-PC were significantly higher than the frequencies of IL-1α-PC and TNF-α-PC after exposure to formaldehyde (1%) (Fig. 2). For the frequencies of IL-1α-PC and TNF-α-PC no significant differences were found dependent on the irritant applied (Fig. 3).

We then compared the cytokine production between allergic and irritant contact dermatitis. With respect to IL-2 and IFN-γ a remarkable resemblance was observed in biopsies from allergic affected skin due to formaldehyde (1%) and epoxy resin (1%) as compared to the biopsies from irritant affected skin due to formaldehyde (8%) and sodium lauryl sulphate (10%). Significant higher frequencies of IL-1α-PC were observed in sodium lauryl sulphate (10%) irritant as compared to formaldehyde (1%) allergic affected skin, whereas significant higher frequencies of TNF-α-PC were observed in epoxy resin (1%) allergic affected skin as compared to formaldehyde (8%) irritant affected skin.

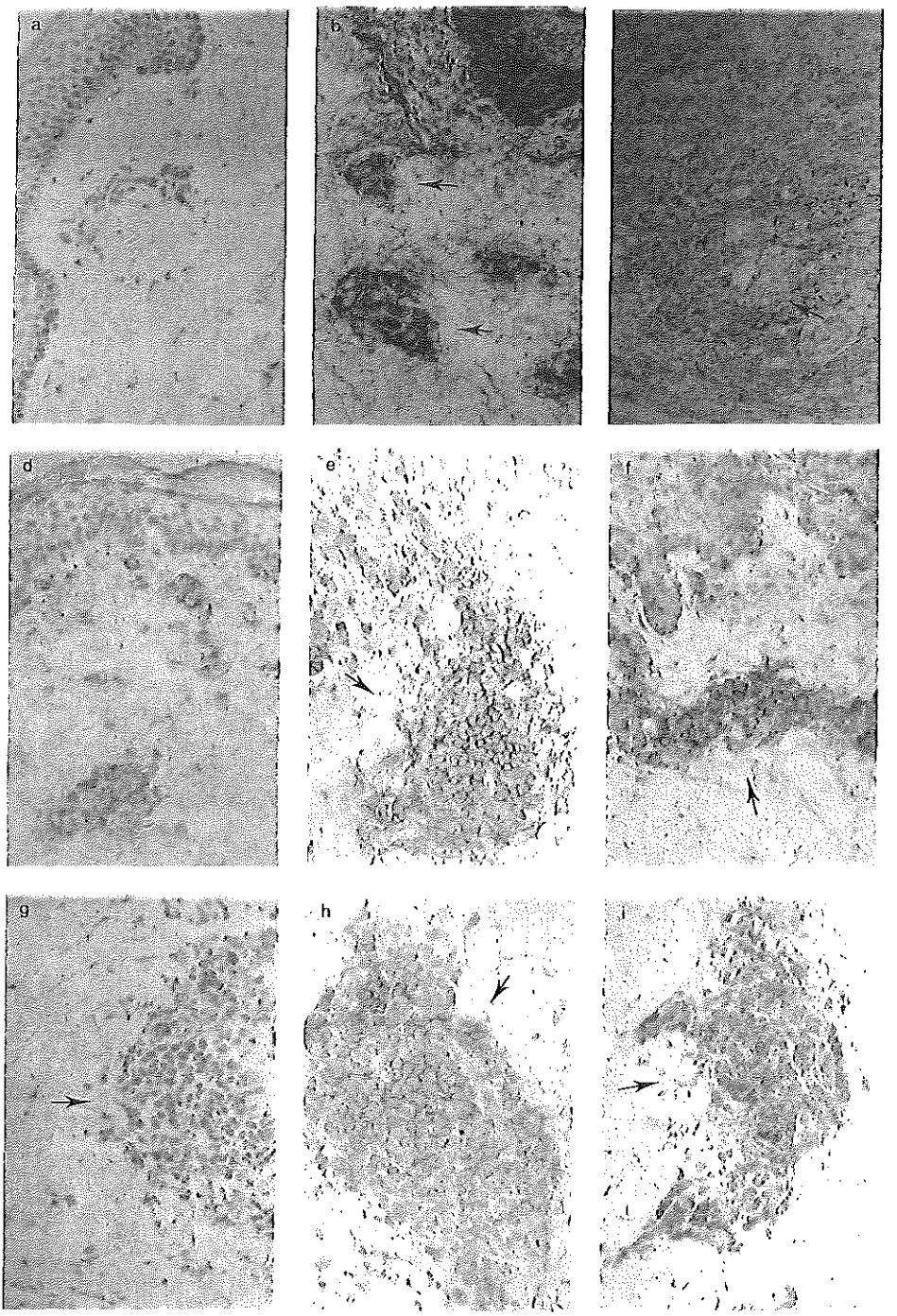
In the epidermis, investigation of the localisation of IL-1α-PC showed that the

patterns of IL-1α-PC were highly similar at 72 hours after application of allergen or irritant.

IL-1α-PC were observed in all epidermal cell layers. In addition, the pattern was similar as was observed in normal skin. The epidermal TNF-α staining in allergic as well as in irritant affected skin was found to be more intense as compared to staining of TNF-α in normal skin and predominantly observed in the lower epidermal cell layers including the basal cell layer next to the dermal infiltrates.

To confirm part of these immunohistochemical findings, probe based *in vivo* mRNA-hybridisation for IL-2 and IFN-γ was performed. Mononuclear cells with distinct cytoplasmic expression of IL-2 mRNA and IFN-γ mRNA (Fig. 4b) were observed in the infiltrates in the dermis in both allergic and irritant affected skin. No staining was observed in sections from affected skin treated by omission of the specific IL-2 mRNA or IFN-γ mRNA probe (Fig. 4a) or in sections treated by the negative control probe.

>>*Figure 1. Antibody based in vivo immunoperoxidase staining for cytokine-producing cells in cryostat sections of skin biopsies from allergic and irritant patch test reactions. (a) Absence of staining in a section from normal skin treated by omission of primary antibody (x 100). (b) MAb to IL-1α show IL-1α-PC with red cytoplasm in the epidermis and in the infiltrates in the dermis in irritant affected skin due to formaldehyde (8%) (x 200). (c) TNF-α producing cells present in the infiltrates in epoxy resin (1%) affected skin (x 200). (d) IFN-γ staining of a normal skin section (x 100). (e) Presence of little rounded red IFN-γ-PC in allergic affected skin due to epoxy resin (1%) (x 200). (f) Presence of little rounded red IFN-γ-PC in irritant affected skin due to formaldehyde (8%) (x 200). (g) Absence of staining of infiltrating mononuclear cells in a section from epoxy resin (1%) affected skin treated by substitution of primary antibody specific for human cytokines by a mouse IgG1 MAb specific for HIV-1 gp120 (x 400). (h) Presence of little rounded red IL-2-PC in allergic affected skin due to epoxy resin (1%) (x 400). (i) Presence of little rounded red IL-2-PC in irritant affected skin due to formaldehyde (8%) (x 400). Sections were counterstained with hematoxilin. Arrows indicate mononuclear cell infiltrates.*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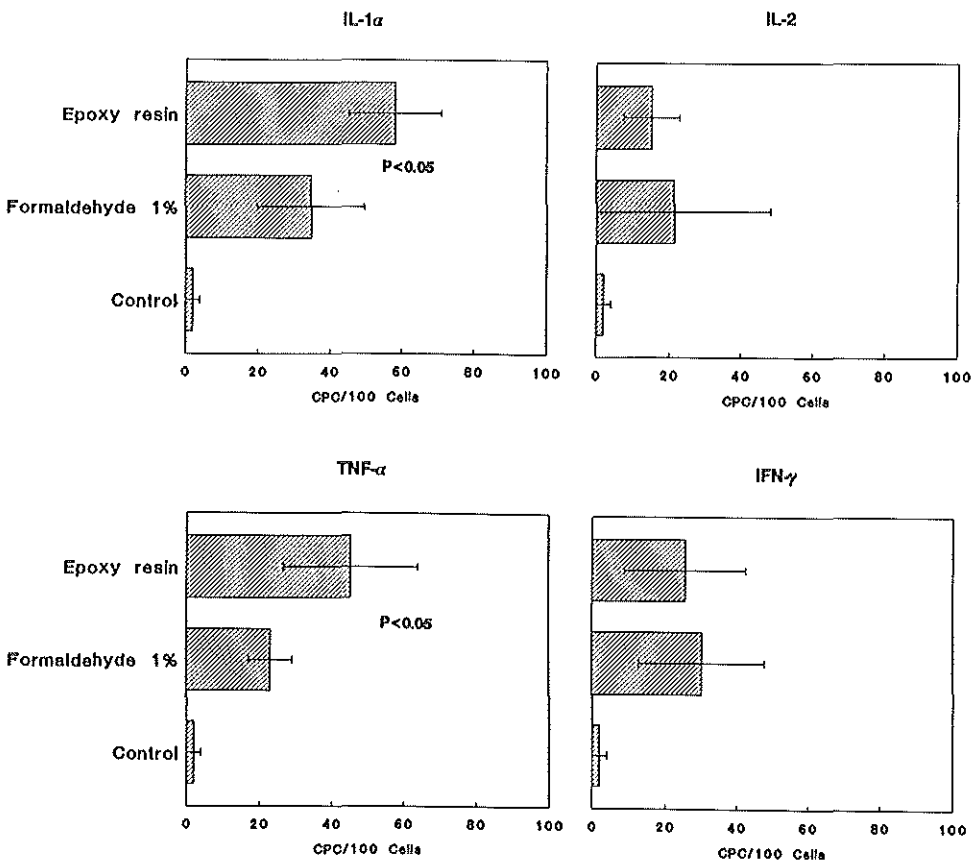

Figure 2. Immunohistochemical revelation of cytokine profiles in skin biopsies from allergic individuals with established allergic patch test reactions and normal skin using MAbs for IL-1α, TNF-α, IL-2 and IFN-γ. Comparisons were made between epoxy resin (1%) and formaldehyde (1%) allergic affected skin and normal skin. After immunohistochemical revelation the numbers of cytokine producing cells (CPC) were quantified as described in ***patients and methods****. Bars represent means of frequencies with standard deviation per 100 cells in the dermis. A significant enhancement of IL-1α and TNF-α expression was observed in epoxy resin (1%) affected skin as compared to formaldehyde (1%) affected skin (P<0.05).*

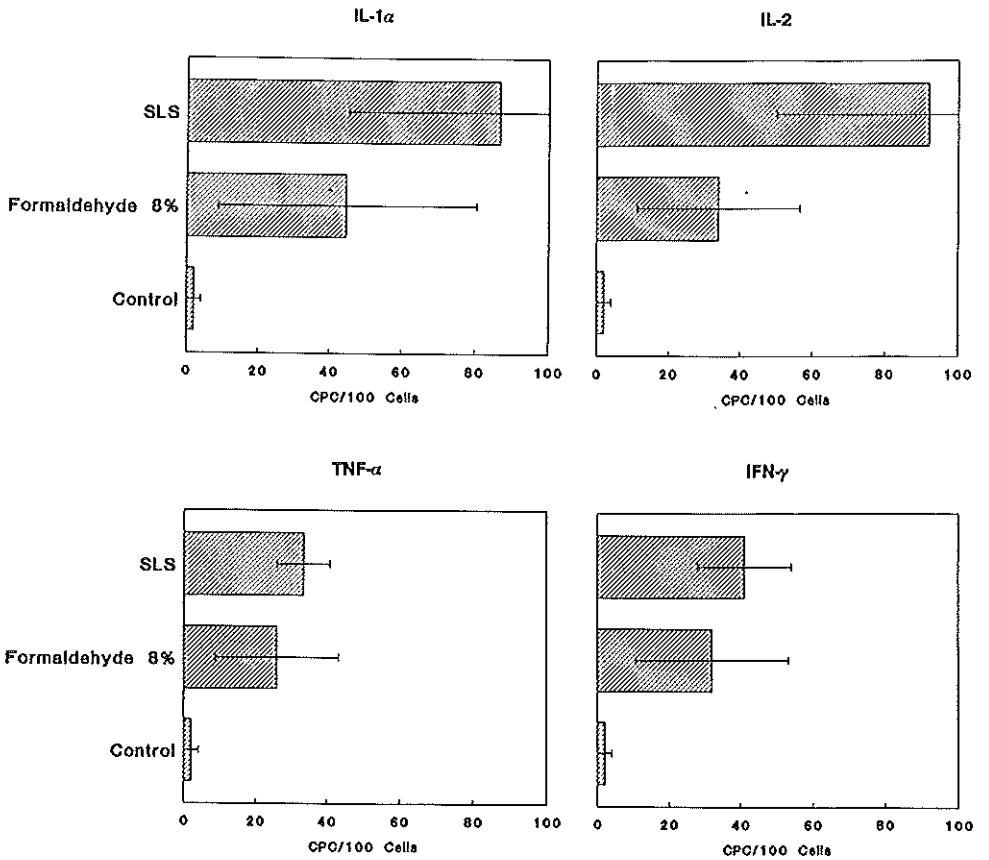

Figure 3. Immunohistochemical revelation of cytokine profiles in skin biopsies from individuals with established irritant patch test reactions and normal skin using MAbs for IL-1α, TNF-α, IL-2 and IFN-γ. Comparisons were made between sodium lauryl sulphate (SLS) (10%) and formaldehyde (8%) irritant affected skin and normal skin. After immuno-histochemical revelation the numbers of cytokine producing cells (CPC) were quantified as described in ***patients and methods****. Bars represent means of frequencies with standard deviation per 100 cells in the dermis.*

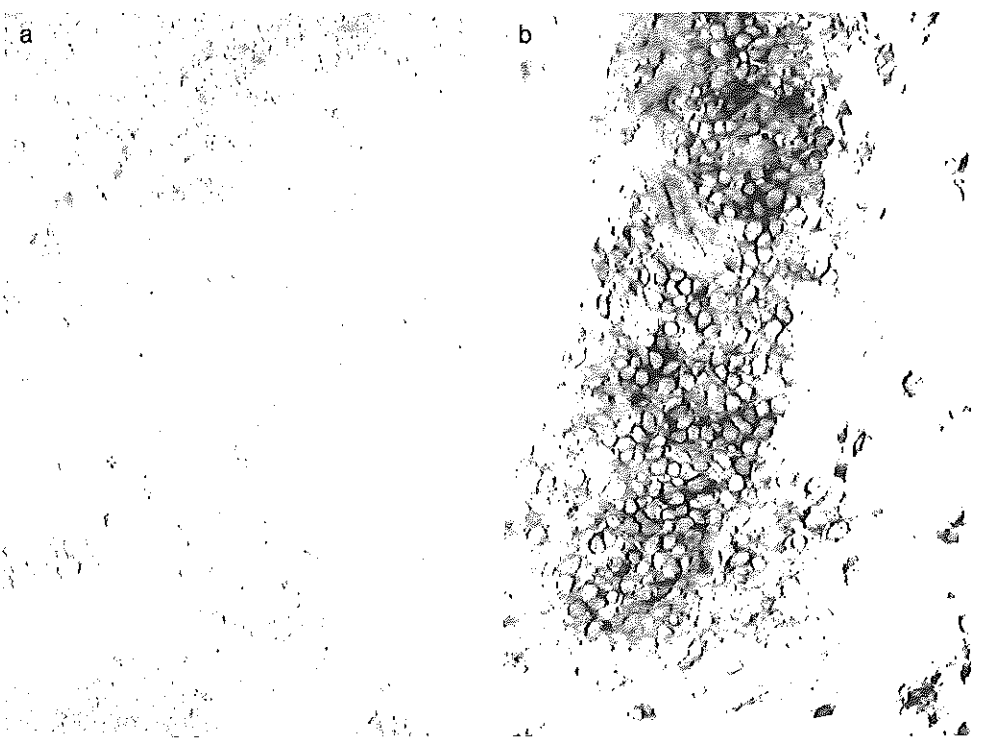

Figure 4. Probe based in vivo mRNA hybridisation. (a) Absence of staining in a section from allergic affected skin due to formaldehyde (1%) treated by omission of the probe to IFN-γ mRNA (x 200). (b) IFN-γ mRNA-PC with a dark blue cytoplasm localized inside the infiltrate in the dermis in allergic affected skin due to formaldehyde (1%) (x 400).

DISCUSSION

This study has demonstrated that based on the *in vivo* detection of the Th1 cytokines "IL-2 and IFN-γ" and the common inflammatory cytokines "IL-1 and TNF-α" definitive discrimination between allergic and irritant contact dermatitis could not be established at the time of patch test reading (72h).

The production of IL-1α and TNF-α was upregulated during both allergic and irritant contact dermatitis. Interestingly, significant differences in frequencies of IL-1α-PC and TNF-α-PC between the four different treated groups were observed. These findings suggested a relatively large non-specific inflammatory component in epoxy resin (1%) allergic as compared to formaldehyde (1%) allergic affected skin. We identified an intense infiltration of mononuclear cells in epoxy resin (1%) allergic as compared to formaldehyde (1%) allergic affected skin. Thus the contribution of a non-specific component in allergic inflammation may be dependent on the allergen applied. The upregulation of IL-1α and TNF-α has not been detected before with *in situ* methodology, although Dowd et al. (6) demonstrated by *in vivo* modulation experiments in man that injection of IL-1α caused leukocyte-rich inflammatory reactions. Furthermore, the involvement of TNF-α in allergic as well as in irritant contact dermatitis was confirmed by Piquet et al. (7) who showed that anti-TNF-α antibody treatment could be an effective inhibitor of both types of contact dermatitis in mice.

The *in vivo* demonstration revealed a significant increase in the frequencies of IL-2-PC and IFN-γ-PC in both allergen and irritant affected skin as compared to the frequencies in normal skin. The presence of IL-2 and IFN-γ in the infiltrates in the dermis in both allergic and irritant reactions was confirmed by the presence of respectively the corresponding IL-2 mRNA and IFN-γ mRNA also localized in the dermal infiltrates in the same biopsies. These findings indicate an involvement of Th1 cytokines in the process of generation of antigen and non-antigen specific T-cell responses.

We chose the 72 hour time point to evaluate the effect of skin exposure to different allergens or irritants, because differentiation of the reaction type (allergic versus irritant) based on routine clinical observation especially at later points in time is sometimes difficult. Furthermore, pilot studies pointed out that the amount of infiltrating inflammatory cells producing cytokines does not reach a maximum before this time point.

Using *in vivo* techniques reproducibility is determined by several factors in particular inter-patient and assay to assay or day to day variation. As with all studies using human volunteers and contact allergens or irritants, reproducibility depends on

standardisation of dose and form of application on the skin. The results obtained in this study as shown by the standard deviations were within acceptable limits. For each assay and day well defined standardized reagents with minimal or no batch to batch variation were used. As observed in the sections analysed, the results were completely reproducible concerning qualification.

To ensure that cells producing cytokines detected in the biopsies from allergic and irritant contact dermatitis patch test reactions (at 72 hours) are generated by challenge of the skin with an allergen or irritant, normal control skin biopsies were also obtained at 72 hours. IL-1α-PC and TNF-α-PC were detected in the epidermis in normal control skin. This is in agreement with previous studies. Antilla et al. (26) detected IL-1α-PC in all epidermal cell layers in normal human skin *in vivo*. Furthermore, an *in vitro* study with cultured human keratinocytes, described by Kupper et al. (27,28) supported our *in vivo* findings. Cultured keratinocytes appear to produce IL-1 spontaneously. TNF-α mRNA-PC were detected in normal mouse ears by Piquet et al. (7) while TNF-α-PC were detected in the stratum granulosum and stratum spinosum in normal human skin by Oxholm et al. (29).

The observed cytokine profiles in allergic and irritant contact dermatitis, as described here, strongly suggest that a similar stage of inflammation, with non-naive T-cells, at 72 hours after application of an allergen or irritant might be induced in the two reaction types. This is supported by an extensive immunophenotypic study performed by Brasch et al. (3). In allergic contact dermatitis epidermal cells will produce and secrete pro-inflammatory cytokines such as IL-1α and TNF-α while allergens will be taken up by Langerhans cells or other dendritic cells. In the case of antigen recognition antigen-specific T-cells are activated and produce IL-2, IFN-γ, and TNF-α. Furthermore, antigen-non-specific T-cells are activated by cytokines produced by antigen-specific T-cells or epidermal cells. In the case of irritant contact dermatitis epidermal cells will produce and secrete IL-1α and TNF-α. The cytokine release by the epidermal cells induces an antigen-non-specific T-cell activation and subsequently IL-2, IFN-γ, and TNF-α production. Thus, in both reaction types infiltrating inflammatory mononuclear cells might be responsible for the IL-1α, TNF-α, IL-2 and IFN-γ production inside the infiltrates in the dermis. Until now, concerning differences in cytokine production between allergic and irritant contact dermatitis, enhanced Langerhans cell-derived IL-1ß mRNA was detected as early as 15 min after skin painting with allergens and not with irritants in mice (15).

In conclusion, the described similarity in cytokine profiles in both allergic and irritant contact dermatitis suggest that common inflammatory stages are reached. Furthermore, the enhanced cytokine production fails to distinguish between the two reaction types after 72 hours of application. Further investigation into the kinetics of

cytokine production "including IL-1ß", double/triple staining of two or three different cytokines (Th1/Th2), other low molecular weight response mediators or activation markers might be useful to discriminate between allergic and irritant contact dermatitis.

Acknowledgments

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IN SITU EXPRESSION OF IL-1ß, IL-4 AND IL-10 IN HUMAN ALLERGIC AND IRRITANT CONTACT DERMATITIS: IMPLICATIONS FOR DIFFERENTIATION

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ABSTRACT

In vitro and *in vivo* modulation experiments indicate the existence of a balance between Th1(IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine producing cells in the modulation of human allergic contact dermatitis. Allergic and irritant contact dermatitis, despite their induction by different mechanisms, could not be differentiated based on the expression of the Th1 like cytokines IL-2 and IFN-γ at 72 h after application of the allergen or irritant. In mice IL-10 mRNA and IL-1ß mRNA are produced by epidermal cells after skin application of contact sensitizers but not after skin application of irritants. To assess whether the cytokine profile based on the differential expression of IL-4, IL-10 and IL-1ß could be used as marker, we studied the expression of these cytokines in cryostat sections of skin biopsies. The biopsies were obtained from allergen and irritant contact dermatitis affected human skin sites at 72 h after application of the allergen or irritant. In the epidermal part of the biopsies enhanced expression was observed of IL-10 and IL-1ß both in allergic and irritant affected skin as compared to normal skin. No epidermal IL-4 producing cells were found. In the dermal part enhanced expression was observed for IL-4, IL-10 and IL-1ß in both allergic as well as irritant contact dermatitis. Minor differences were found between the IL-4, IL-10 and IL-1ß data in the allergic and irritant affected groups of patients.

Cytokine based immune modulation is the result of a critical balance between the actions of various cytokines. As a consequence, an obvious difference was established between the ratios of IL-4 : IL-10 : IL-1ß in allergic and irritant contact dermatitis.

INTRODUCTION

Exposure to several chemical substances may result in allergic as well as irritant contact dermatitis dependent on local concentration and the nature of the chemical substance (1). However, the correct assessment of allergic and irritant contact dermatitis may pose considerable problems in dermatologic practice. Even differentiation of the reaction type based on routine clinical observation, electron-microscopy and light microscopical histology is sometimes difficult (2,3).

In previous studies we attempted to distinguish between allergic contact dermatitis and irritant contact dermatitis on the basis of local cytokine profiles. Allergic contact dermatitis is based on a specific immune response, whereas irritant contact dermatitis is based on non-specific cell activation. We therefore chose to differentiate dermatitis on the basis of the expression of specific cytokines, i.e. IL-2 and IFN-γ, as well as the expresion of common inflammatory cytokines, i.e. IL-1α and TNF-α. We demonstrated that the common inflammatory cytokines were produced in both allergic as well as irritant reaction types at 72 h after reagent application (4). In addition, the Th1-related cytokines IL-2 and IFN-γ which were expected to be predominantly present in specific immune responses were observed in both the allergic as well as in the irritant reaction types at the time point investigated. As a consequence, we still felt the need for differentiation markers in contact dermatitis.

IL-4 and IL-10 were shown to be produced by Th2 type cells and play a role in down-regulation of the function of Th1 cells in skin (5-7). IL-1ß and IL-10 can be produced by epidermal cells (8-10) and modulate inflammatory responses. Langerhans' cell-derived IL-1ß mRNA signal strength is increased within 15 min and persists at peak levels at least for 24 h after allergen but not after irritant painting on the mouse skin (8). In addition, the signal strength of keratinocyte-derived IL-10 mRNA is enhanced by hapten application to mouse skin (9). Recombinant IL-10 when injected into the skin before antigenic challenge, prevented the elicitation of contact hypersensitivity in previously sensitized mice (10). These and other studies into the role of IL-4, IL-10 and IL-1ß in contact dermatitis so far suggest that both the down-regulating Th2 cytokines IL-4 and IL-10 as well as the Langerhans' cell-derived IL-1ß might be differentially involved in allergic dermatitis as compared to irritant contact dermatitis. In the present study we have investigated the expression of the cytokines IL-1ß, IL-4 and IL-10 in allergic and irritant affected human skin. The biopsies were taken from human volunteers 72 h after allergen or irritant application. This time point was chosen since routine dermato-pathology is generally presented at that time and differentiation of the reaction type is sometimes difficult. Furthermore, at that time infiltrating cells producing cytokines are present and especially cells with down-regulating capacity may be expected.

MATERIALS AND METHODS

Participants, skin testing and biopsies

Informed consent was given by eight volunteers of which four had a history of specific allergy (ethylene-diamine (1), cobalt chloride (1) and nickel sulphate (2)). The allergic patch test was performed on the skin of the back of the volunteers with the known allergen in concentrations used in standardized clinical diagnostic procedures. Skin of four non-allergic individuals were treated on the back with 10% sodium lauryl sulphate which was used as an irritant. Finn chambers (10 mm) (Epitest-Ltd. Oy, Tuusula, Finland) were used for occlusion and fixed on the back skin of the participants for 48 h. According to the established international criteria (11) all reactions could be classified either as moderate allergic or irritant at the final reading (72 h). No erythema, redness or moderate infiltration was seen in vehicle controls. Two 4mm punch skin biopsies from each volunteer were obtained under the local effect of an anaesthetic: one from normal skin and one from the allergen or irritant reaction at 72 h after application. Biopsy specimens were immediately frozen in liquid nitrogen and stored at -70°C.

Chemicals, antibodies and immunohistochemistry

Immunohistochemistry was performed essentially as described by Hoefakker et al. (12). 3-amino-9-ethylcarbazole (AEC; A-5754), Fast blue BB Base (F-125), naphthol AS MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), levamisole and bovine serum albumin (BSA; A-9647) were obtained from Sigma (St. Louis, MO, U.S.A.).

The mouse monoclonal antibody Vhp20 specific for human IL-1ß was kindly provided by Dr. P. Ghiara, IRIS Department of Immunopharmacology, Siena, Italy (13). The mouse monoclonal antibody (1842-01) to human IL-4 was purchased from Genzyme, Cambridge, MA, USA. The mouse monoclonal antibody MCA 926 to human IL-10 was purchased from Serotec, Oxford, England.

Vhp20 was used as biotin-labelled antibody conjugated as previously described (14) and streptavidin-alkaline-phosphatase (AP) (Life Technologies, Gaithersburg MD, USA) was used for visualization. The mouse monoclonal antibody 1842-01 was used in an indirect three-step method with horse-anti-mouse-biotin-labelled secondary antibody (Vector Laboratories, Burlingame CA, USA) and streptavidin-horseradish peroxidase (HRP) (Life Technologies, Gaithersburg MD, USA) for visualization. For the detection of IL-10 an indirect two-step method was used with rabbit-anti-mouse HRP-labelled antibody (Dakopatts, Copenhagen, Denmark) to visualize IL-10 producing cells. The specificity of Vhp20 was established in a previous study (13). In this study the specificity of 1842-01 and MCA926 was established by preabsorption of

the antibodies with the recombinant cytokines IL-4 and IL-10, respectively. Solutions with different concentrations of the antibodies 1842-01 and MCA926 were prepared and mixed with respectively the affinity-purified recombinant cytokines IL-4 (Sandoz, Base, Switzerland) and IL-10 (DNAX, Research Institute, Palo Alto, CA, USA) in two different concentrations. These mixtures were incubated for 2 h at room temperature and cleared of aggregates and complexes by centrigugation (12000 g, 2 min, 20°C). The tissues sections from affected skin were incubated directly with these mixtures and the immunohistochemical staining method was subsequently applied. Regular procedure controles were included on each slide (12).

For immunohistochemistry cryostat sections (8 μm) were fixed for 10 min in fresh acetone, containing 0.02% H_2O_2. Slides were incubated overnight at 4°C with the panel of antibodies. All reagents were diluted in PBS containing 0.1% BSA and titrated to get optimal results. After incubation the slides were washed with PBS and incubated for another 60 min at room temperature with the secondary steps diluted optimally in PBS containing 1% BSA and 1% normal human serum. For the IL-4 detection sections were washed with PBS and incubated with the third step reagent for 60 min. Slides were washed with PBS and histochemical revelation of HRP and AP was done according to Claassen & Adler (15). Slides were counterstained with hematoxylin for 15 s and mounted in glycerol/gelatin.

Quantification and statistical analysis

Frequencies of cytokine producing cells and the total number of cells in the dermis were determined by using an eyepiece graticule. For each biopsy, one section was stained with each of the specific antibodies and at least 2 fields were analysed. For the sections from both allergic as well as irritant affected skin at least 100 cells per field were counted and analysed for cytokine producing cells. Frequencies of cytokine producing cells were calculated as the mean per 100 cells ± SD in the dermis in one biopsy. Results of the allergic group of patients and the irritant group of patients were analyzed by the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population. The mean frequencies of IL-4 producing cells observed in the allergic group of patients were compared to the mean frequencies of IL-4 producing cells observed in the irritant group of patients. Similar comparisons were made for the mean frequencies of IL-10 and IL-1ß producing cells. The ratios of the total mean frequencies of IL-4, IL-10 and IL-1ß producing cells in the allergic and irritant group of patients were determined.

RESULTS

In normal skin erythema and infiltration of mononuclear cells was not observed. In contrast in both allergic as well as irritant affected skin sites intense erythema and moderate infiltration with accumulation of mononuclear cells in the dermis was found.

To verify the specificity of the IL-4 and IL-10 staining, inhibition experiments were performed. Preincubation of 1842-01 with recombinant IL-4 and MCA926 with recombinant IL-10 inhibited the cytokine-specific staining. No staining was seen in control sections treated by omission of the primary antibody or after staining with a isotype matched irrelevant antibody (negative reagent; antigen not present in the tissue).

In the epidermis IL-10 and IL-1ß producing cells were localized in all the cell-layers in both normal as well as in the allergic and irritant affected skin biopsies. However, in the allergic and irritant reactions, the intensity of IL-1ß cytoplasmic staining was increased as compared to staining in normal skin. The lower epidermal cell layers close to the dermal infiltrates in biopsies from allergic and irritant affected skin showed varying degrees of epidermal spongiosis with large keratinocytes. Predominantly these keratinocytes had an intense red colour. In contrast to the presence of IL-10 and IL-1ß producing cells, no IL-4 producing cells were found in the epidermis. Fig. 1 illustrates representative cytokine staining results.

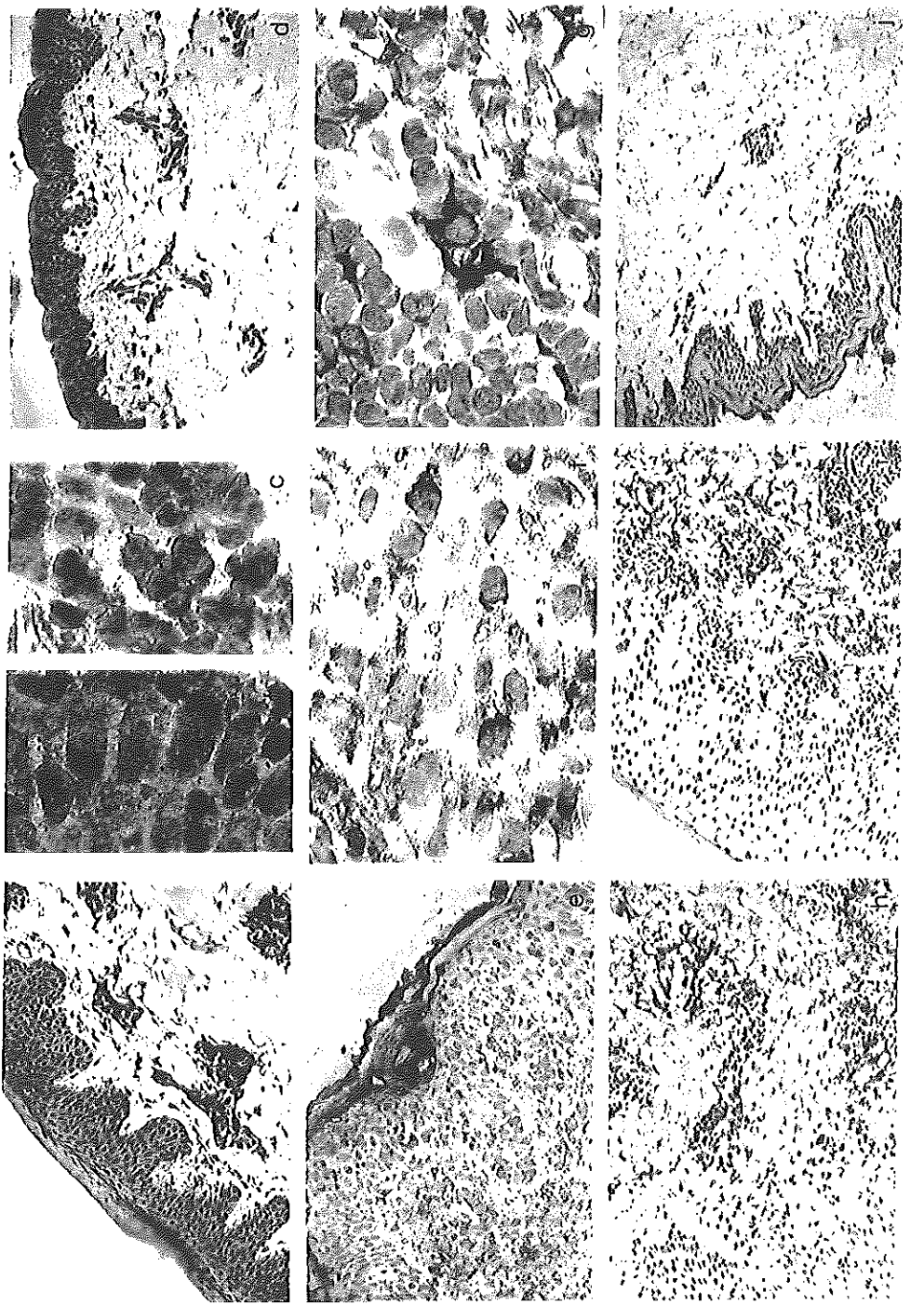
In the dermis in normal skin the total number of cytokine producing cells (IL-4, IL-10, IL-1ß) was lower than 8% of nucleated cells. In the dermal part of allergic or irritant affected skin the IL-4, IL-10 and IL-1ß producing cells were found in the perivascular spaces. The IL-4 and IL-10 producing cells were found scattered in groups of small mononuclear cells in the infiltrates. IL-1ß producing cells showed cytoplasmic staining of cells with a dendritic appearance.

>>Figure 1. Antibody based in vivo staining for cytokine positive cells in allergic and irritant patch test reactions:

(a) IL-10 positive cells with red cytoplasm in the epidermis and in the infiltrates in the dermis in irritant affected skin. (b) IL-10 positive keratinocytes with red cytoplasm in irritant affected skin. (c) IL-10 positive mononuclear cells with red cytoplasm in a dermal infiltrate in irritant affected skin. (d) IL-10 positive cells with red cytoplasm in the epidermis in normal skin.

(e) IL-1ß positive cells with blue cytoplasm in the epidermis and in the dermis in allergic affected skin. (f) IL-1ß positive keratinocytes with blue cytoplasm in allergic affected skin. (g) IL-1ß positive cells with a dendritic appearance in a dermal infiltrate in allergic affected skin.

(h) Presence of red IL-4 positive cells in the dermis in allergic affected skin and (i) in irritant affected skin. (j) Absence of IL-4 positive cells in the dermis in a section from normal skin. Sections were counterstained with hematoxilin. (a,d,j) x100, (e,h,i) x200 and (b,c,f,g) x1000.


a
b
c
d

In allergic as well as irritant reactions IL-4 producing cells represented a minor component of the nucleated cells in the dermis, ranging from 5 to 20%. The IL-10 producing cells made up approximately 20 to 30% of the nucleated cells in the dermis. IL-1ß producing cells were found in the dermis ranging from 10 to 30% of all nucleated cells in the dermis.

Minor differences were found between the frequencies of IL-4 producing cells in biopsies from allergic affected skin as compared to the frequencies of cytokine producing cells in biopsies from irritant affected skin (Table 1). This is also found for the cytokines IL-10 and IL-1ß (Table 1).

Cytokine based immune modulation is the result of a critical balance between the actions of various cytokines. The results of our experiment revealed differences between the ratios of total mean frequencies of IL-4, IL-10 and IL-1ß producing cells in the allergic and irritant group of patients. The ratio of IL-4 : IL-10 : IL-1ß is 1 : 3 : 2.5 in the allergic group of patients and 1 : 2 : 1 in the irritant group of patients. This means that predominantly in the allergic group of patients and not in the irritant group of patients more IL-1ß producing cells were observed as compared to IL-4 producing cells. The mean frequencies of IL-1ß producing cells per IL-4 producing cell in the allergic group of patients (2.7 ± 1.2) was significantly different ($p < 0.05$) as compared to the irritant group of patients (1.2 ± 0.4) (Table 1, Fig. 2).

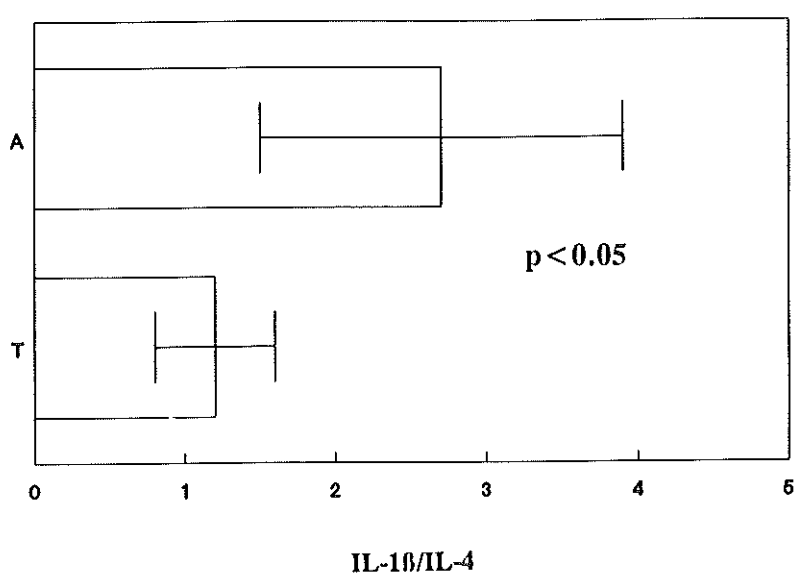


Figure 2. IL-1β producing cells per IL-4 producing cell in the dermis in allergic (A) and irritant contact dermatitis (T). The data presented are the ±SD from four patients.

Table 1. Frequencies of cytokine producing cells in dermal infiltrates in allergic and irritant patch-test reactions[a]

	Allergic contact dermatitis				
	Patients				
Cytokine	1	2	3	4	mean total
IL-4	8.4±0.2[b]	9.4±0.3	5.5±3.6	12.1±9.1	8.9±.4.5
IL-10	30±8.5	25±2.9	25±2.1	26±6.4	26.2±4.8
IL-1ß	32±5.3	17.9±4.0	19.6±3.0	17.7±1.8	21.8±7
IL1ß/ IL-4	3.8[c]	1.9	3.6	1.5	2.7±1.2

	Irritant contact dermatitis				
	Patients				
Cytokine	5	6	7	8	mean total
IL-4	9.4±0.9	8.0±5.7	19.1±2.6	16.9±3.1	13.4±5.8
IL-10	33±11.3	27.4±8.4	24.1±2.8	31.3±14	28.9±8.5
IL-1ß	9.5±0.7	9.4±0.9	13±2.6	28.4±16.5	15.1±10.5
IL1ß/ IL-4	1	1.2	0.7	1.7	1.2±0.4

[a]Allergic affected skin due to ethylene-diamine (1), allergic affected skin due to nickel sulphate (2), allergic affected skin due to cobalt chloride (3),allergic affected skin due to nickel sulphate (4). Irritant affected skin due to sodium lauryl sulphate (5-8).

[b]Cytokine producing cells were stained with the appropriate monoclonal antibodies and visualization methods (material and methods). After immunohistochemical revelation the numbers of cytokine producing cells were quantified as described in material and methods. Frequencies of IL-4, IL-10 and IL-1ß producing cells per 100 infiltrating cells ± SD in the dermis in the different biopsies.

[c]Individual ratios between IL-1ß and IL-4.

DISCUSSION

This study demonstrates that the frequencies of IL-4, IL-10 and IL-1ß producing cells as such in allergic and irritant reactions did not allow to differentiate between these two reaction types. However, the ratios of total mean frequencies of IL-4, IL-10 and IL-1ß producing cells show differences between allergic and irritant contact dermatitis. Interestingly, a significant difference was observed using the ratio between IL-4 and IL-1ß i.e. the mean frequencies of IL-1ß producing cells per IL-4 producing cell. This emphasizes that investigation based on a combination of cytokines in a "cytokine-profile" may be essential for differentiation between allergic and irritant contact dermatitis.

In the presently evaluated specimen of biopsies no differences between allergic and irritant contact dermatitis were observed in the human situation for IL-10 producing cells. This is in contrast with findings in the mouse system. Enk & Katz (9) found upregulation of IL-10mRNA *in vivo* after skin exposure to a contact allergen but not to an irritant. This is confirmed by the enhancement of IL-10 as well as IL-10mRNA production after stimulation with a contact allergen using a murine keratinocyte-cell line (9). The differences observed between the results in this study and the data presented by Enk & Katz (9) may be explained by the differences of species (human versus mice), time points (72h versus 24h) and techniques used in the two studies. In the mouse studies the primary immune response in the skin was investigated, whereas in this study the secondary immune response was investigated. Furthermore, in the mouse studies immunoprecipitation to detect IL-10 and PCR to detect IL-10mRNA was used, whereas we used immunohistochemical staining techniques.

In the present study the mean frequencies of IL-1ß producing cells localized in the dermis were higher after challenging with allergens than observed after irritant application. The higher frequency of IL-1ß producing cells in allergic contact dermatitis suggests that activated Langerhans' cells present in the dermis are producing IL-1ß. This hypothesis is confirmed by experiments described by Enk & Katz (8). However, they described that only epidermal Langerhans cells are responsible for IL-1ß production after painting mouse skin with allergens and not with irritants. In our study we observed IL-1ß positive keratinocytes in both allergic and irritant affected skin. These epidermal findings are also in contrast with the mouse studies in which no specific induction of IL-1ß mRNA in keratinocytes by skin exposure to an allergen or irritant was demonstrated (8). The discrepancies between our study in man and the mouse study could be explained by the use of a monoclonal antibody specific for MHC-class II-Ia in the cell depletion study performed by Enk & Katz (8). Not only Langerhans' cells present in the epidermis as stated by Enk & Katz (8) but also Langerhans' cells present in the dermis and keratinocytes may have been

depleted due to the expression of MHC-class II-Ia molecules on the surface on both cell-types (16).

The findings with respect to epidermal IL-1ß expression in normal skin are in agreement with previous human studies. Anttila et al. (17) detected IL-1ß producing cells in all epidermal cell layers in normal human skin *in vivo*. The *in vivo* expression of IL-10 in normal human skin is demonstrated for the first time in this study. Moreover, the *in vivo* enhanced expression of IL-1ß and IL-10 in the epidermal cell layers in allergic as well as irritant affected human skin was not described before.

In the dermal compartment in both allergic as well as irritant affected skin IL-4, IL-10 and IL-1ß producing cells were detected. It has been shown in *in vitro* experiments with nickel specific human T-cell clones that IL-4 is produced in low amounts in allergic contact dermatitis. Furthermore, modulation experiments (18) demonstrated that demonstration of IL-10 in human skin inhibits the elicitation phase of allergic contact hypersensitivity due to the suppression of IFN-γ mRNA upregulation in ongoing immune responses. The simultaneous expression of IL-4 and IL-10 in this study suggests an important role for both IL-4 as well as for IL-10 as immunoregulators by inhibition of IL-2 and IFN-γ production. Although we have investigated the staining patterns and the frequencies of IL-4, IL-10 and IL-1ß producing cells in allergic and irritant contact dermatitis only at one time-point (72h) our results indicate that both allergic and irritant responses determined *in vivo* are not predominantly Th1 or Th2 responses. It appears that there is a similar balance between Th1 and Th2 cytokine producing cells in allergic and irritant contact dermatitis. This is in agreement with experiments described by Thomson et al. (19) in which both IL-4-mRNA as well as IFN-γ-mRNA was measured after 24h culture with anti-CD3 antibody in oxazolone primed lymph node cells after 3 days after skin application with oxazolone in mice.

Differences between the total mean frequencies of IL-4, IL-10 and IL-1ß producing cells in allergic and irritant contact dermatitis were limited. However, differences between the ratios of total mean frequencies of IL-4 : IL-10 : IL-1ß producing cells in the allergic as compared to the irritant group of patients were found, which might allow to distinguish these dermatitis. Using the ratios between IL-4 and IL-1ß an obvious difference was found between the two reaction types. This indicates that 72 h after application of an allergen or irritant the balance between IL-4 and IL-1ß production is dependent on the type of contact dermatitis reaction. In any case differentiation between allergic and irritant contact dermatitis based on a combination of different cytokines "cytokine profile" might be possible in the future. The combination of IL-4 with IL-1ß is an example.

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Chapter 4

Functional role for CD40 ligand-CD40 interaction in antigen specific antibody production, but not in Delayed-Type Hypersensitivity, after skin exposure to contact allergen
(submitted).

FUNCTIONAL ROLE FOR CD40 LIGAND-CD40 INTERACTION IN ANTIGEN SPECIFIC ANTIBODY PRODUCTION, BUT NOT IN DELAYED-TYPE HYPERSENSITIVITY AFTER SKIN EXPOSURE TO CONTACT ALLERGEN

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ABSTRACT

A central role for interaction between CD40 expression on B-cells and CD40 ligand (gp39) expressed on activated CD4 positive T-cells has been shown for thymus dependent antibody responses. Recently it was established that cultured Langerhans cells express CD40. Furthermore, it is known that antigen specific activated T-cells are present in the skin during a contact hypersensitivity reaction (categorized as a delayed-type hypersensitivity response, DTH). This suggests that CD40-bearing Langerhans cells could be involved in both the DTH and the humoral response induced by contact allergens. Therefore, in this study we investigated the role of CD40 ligand-CD40 interaction in the humoral response and the DTH response after skin exposure to the contact allergen dinitrochlorobenzene (DNCB).

We analysed the expression of CD40 and CD40 ligand in contact hypersensitivity in human skin as well as in mice. In mice, we evaluated the humoral response by looking at the effect of *in vivo* anti-CD40 ligand antibody treatment on the frequencies of antigen specific antibody forming cells and germinal centre formation in the draining lymph node. The DTH response was evaluated by measuring the increase in ear thickness (cellular response) after *in vivo* anti-CD40 ligand antibody treatment. The data show that the frequency of antigen specific antibody forming cells and the percentage of secondary follicles (germinal centres) are markedly decreased after injection of anti-CD40 ligand antibody. The administration of anti-CD40 ligand antibody did not influence the DTH response.

The results demonstrate that a cognate CD40 ligand-CD40 interaction is essential in the regulation of the humoral response after skin exposure to a contact allergen.

However, our data do not support a role for CD40 ligand-CD40 interaction in contact hypersensitivity. Therefore, this model shows that anti-CD40 ligand antibody treatment given systemically or topically might be used for therapy in skin diseases which are dependent on antibody formation such as pemphigus and subacute cutaneous lupus erythematosus.

INTRODUCTION

CD40 ligand-CD40 interactions are involved in different types of cellular communication. They are involved in immune response induction, memory formation and development of autoimmunity [1]. In this study we investigated the role of CD40 ligand-CD40 interaction in a local humoral immune response and contact hypersensitivity induced by skin exposure to a contact allergen.
CD40 surface antigen is expressed on B-cells [2, 3] and human professional antigen presenting cells such as cultured Langerhans cells (veiled cells) and other dendritic cells [4-12]. The human and murine forms of the CD40 ligand (gp39) were recently cloned and demonstrated to be type II integral membrane proteins expressed primarily on activated $CD4^+$ T-cells [13, 14]. It is the cognate CD40 ligand-CD40 interaction that plays a crucial role in humoral immune responses: proliferation, immunoglobulin production, isotype switching and memory formation [1]. Patients suffering from hyper-IgM syndrome are unable to produce antibody isotypes other than IgM. It has been shown that this results from mutations in the gene encoding CD40 ligand leading to expression of defective CD40 ligand [15-18]. In mice, *in vivo* administration of antibody to CD40 ligand blocked the occurrence of chronic graft versus host disease [19], experimental allergic encephalomyelitis (K. Gerritse, paper submitted for publication), collagen induced arthritis [20] and thymus dependent humoral immunity after intravenous immunization with TNP-KLH [21].

In many dermatological diseases the involvement of antibodies is established [22]. In pemphigus and subacute cutaneous lupus erythematosus (SCLE) autoantibodies appear to mediate the skin disease. Pemphigus may be considered an intraepidermal blistering disease group characterized by autoantibodies reactive with antigens located in the intercellular spaces or on the surface of epidermal cells. SCLE is characterized by large annular erythema gyratum or erythamosquamous psoriasiform lesions, disseminated on both sides of upper trunk, face, and arms and by marked photosensitivity. The involvement of antibodies in pemphigus and SCLE opens up the possibility that blocking of CD40 ligand-CD40 interaction may inhibit local antibody production resulting in suppression of the clinical symptoms.

Analogous to humoral immune responses induced by autoantigens as in pemphigus and SCLE, and intravenously administered antigens [21], some contact allergens may also induce antigen specific antibody forming cells in the draining lymph node [23]. Therefore, mice exposed to contact allergens can be used as models for antibody mediated skin diseases caused by antigen coming from or through the skin. Besides a local humoral immune response contact hypersensitivity may also be induced after skin exposure to a contact allergen. Contact hypersensitivity is categorized as a DTH response. The response can be subdivided in an afferent (induction or sensitization phase) and an efferent (elicitation or challenge phase) limb. The afferent limb includes the events following allergen presentation to the immunologically naive system and is complete when the subject is sensitized and capable of giving a positive challenge reaction. The main events which occur in the afferent phase after skin penetration of the small antigens are (1) the binding of contact allergens to skin constituents notably major histocompatibility complex (MHC) class II molecules present on the Langerhans cell as antigen presenting cell or binding of the contact allergen to a skin protein which in turn is taken up by Langerhans cells, processed and presented in the context of MHC-Class II molecules, (2) migration of the antigen-carrying Langerhans veiled cells via the afferent lymphatics to the regional lymph nodes where they settle in the paracortical areas, (3) recognition of the antigen presented on MHC-class II molecules by the T-cell receptor and (4) proliferation and subsequent dissemination of specific (memory) T-cells throughout the body. In the efferent phase, which follows a second epicutaneous exposure, activation and recruitment of the recirculating antigen specific immune effector T-cells will initiate a localized inflammatory response at the challenge site. Since specifically activated T-cells and Langerhans cells are able to express CD40 ligand and CD40 respectively, we investigated whether CD40 ligand-CD40 interaction would be functional in contact hypersensitivity in mice.

MATERIALS AND METHODS

Experimental design

To study the role of CD40 ligand-CD40 interaction the expression of CD40 and CD40 ligand was investigated in normal skin and contact hypersensitivity in man by using immunohistochemical staining experiments. Furthermore, the kinetics of CD40 and CD40 ligand positive cells during a contact hypersensitivity response in mice was analysed by using immunohistochemical staining. To demonstrate the microscopical development of skin inflammation, we analysed the involvement of mononuclear cells (MNC), CD4 positive cells, and IL-2, IL-4 and IFN-γ cytokine producing cells by using

immunohistochemistry. BALB/c mice were sensitized to 1% DNCB in an aceton/olive oil vehicle (4:1 v/v) on day 0 to the shaved abdominal skin (200 μl) and 25 μl on each hind footpad. Five days later, the mice were challenged by applying 25 μl of 1% DNCB to each ear. Mice were anesthetized and killed at various time intervals after challenge: at day 0, 1, 2, 3, 4, 5, 7, 9 and 12.

For the *in vivo* modulation experiments the optimal condition to assess the effect of anti-CD40 ligand antibody treatment on the humoral and cellular (DTH) immune response were established. Auricular lymph nodes were removed at various time intervals after challenge (at day 0, 4, 5, 6, 7 and 10), and immediatly frozen in liquid nitrogen and stored at -70°C. Mouse DNP-specific IgM and IgG antibody forming cells (AFC) were determined in the draining auricular lymph nodes by immunohistochemistry using a TNP-enzyme conjugate (TNP-DNP crossreaction). To study the kinetics of IgM and IgG production after challenge (at day 0, 4, 5, 6, 7 and 10) total mouse serum IgM, IgG and serum DNP-specific IgM and IgG antibody titers were measured by ELISA [21, 24]. Total serum and DNP-specific IgG antibody titers were related to that of total serum and DNP-specific IgG antibody titers after TNP-KLH immunization day 14. Total serum and DNP-specific IgM antibody titers were related to that of total serum and DNP-specific IgM antibody titers after TNP-Ficoll immunization day 5. The ear swelling responses were evaluated 0-5 days after challenge. The *in vivo* modulation experiments using the optimal conditions were performed as outlined in 2.7 *in vivo* modulation.

Skin testing and biopsies

Volunteers took part in the study after giving informed consent. The epicutaneous patch test was performed on the back with test substances in concentrations routinely used in diagnostic procedures. Finn chambers (10 mm) (Epitest-Ltd. Oy, Payala, Finland) were used for occlusion and fixed on the skin of the back of participants for 48 hours. Two 4mm punch skin biopsies were obtained; one from normal skin and one from the contact hypersensitivity reaction site at 72 hours after application. Biopsy specimens were immediately frozen in liquid nitrogen and stored at -70°C. According to the established international criteria [25] the reactions were classified as moderately allergic (++) at the final reading (72 hours).

Animals

BALB/c mice were purchased from Charles River Wiga, Sulzfeld, Germany and were used at 9 to 12 weeks of age.

Chemicals

DNCB, alkaline phosphatase (AP; P-6774, type VII-T, 1020 U/mg protein), fast blue BB Base (F-0125), levamisole, naphthol AS MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), horseradish peroxidase (HRP), and 3-amino-9-ethylcarbazole (A-5754) were obtained from Sigma (St. Louis, MO).

Reagents

Ascites from the cell line MR1, a hamster mAb directed to mouse CD40 ligand [26], was purified by means of a protein-A column. Control polyclonal hamster antibody was purchased from Serva (Feinbiochemica, Heidelberg, Germany). For immunohistochemistry we used the murine mAb 5D12F10 (PanGenetics, Heemskerk, The Netherlands) directed to human CD40. The rat mAb MCA-1143 directed to mouse CD40 was purchased from Instruchemie (Hilversum, The Netherlands). A soluble fusion protein composed of the extracellular domain of human CD40 genetically fused with the Fc domain of human IgG_1 (CD40-Ig) to detect human CD40 ligand positive cells was a gift of Dr. R. J. Noelle [26]. The murine mAb DB-1, S4B6 and 11B11 were used to detect murine IFN-γ, IL-2 and IL-4 respectively [27]. CD40-Ig, the mAb MR-1, DB-1, S4B6 and 11B11 were used after biotin conjugation as previously described [28]. The rat mAb L3T4 (CD4)(clone GK-1.5)[29] was used as T-cell marker. The mouse mAb against the endothelial leukocyte adhesion molecule-1 (ELAM-1) (13P) was a kind gift from Dr. J. Y. Bonnefoy (Glaxo Institute for molecular Biology, Geneva, Switzerland). For the detection of human and mouse CD40 we used a three step detection method to amplify the signal [30]. In that case the secondary conjugates biotinylated horse anti-mouse from Vector Laboratories (Burlingame, CA) and biotinylated goat anti-rat (Becton Dickinson) were used. Rabbit-HRP anti-rat Ig, rabbit-HRP anti-mouse Ig (Dakopatts, Copenhagen, Denmark) and streptavidin-HRP or AP (Life Technologies, Gaithersburg, MD) were used as second step or third step reagents. TNP-HRP was prepared according to the previously described methods [31, 32].

Evaluation of ear swelling

Ear thickness was measured before challenge and at 1, 2, 3, 4, and 5 days post-challenge, using a micrometer (Helios, Heinrich Böker). The results are expressed as the mean percentage increase in ear thickness relative to pre-challenge values at 1, 2, 3, 4 and 5 days after challenge.

***In vivo* modulation**

To study the effect of anti-CD40 ligand antibody treatment we used two different approaches. First we investigated the effect of anti-CD40 ligand antibody treatment

before sensibilization and second, we investigated the effect of anti-CD40 ligand antibody treatment after sensibilization (before challenge). Mice were divided into four groups of five mice per group. The first group received anti-CD40 ligand antibodies, the second control hamster antibodies, the third group PBS and the fourth group represented the specificity control. For the first approach, mice received an i.p. injection of 250 μg of purified anti-CD40 ligand mAb or 250 μg purified hamster Ig or PBS at day -1, 1, and 4. For the second approach, mice received an i.p. injection of 250 μg of purified anti-CD40 ligand mAb (MR1) or 250 μg purified hamster Ig or PBS at day 5, 4 hours prior to challenge. On day 0 mice were sensitized and at day 5 the sensitized mice were challenged (as described under experimental design). All injections were administered in a total volume of 200 μl pyrogen-free, azide-free PBS. Unsensitized mice, exposed only to aceton/olive oil at the time of sensitization, and later challenged with 1% DNCB, were considered as specificity controls.

To study the effect of treatment on the humoral immune response the auricular lymph nodes were removed at day 5 after challenge. Mouse DNP-specific IgM and IgG AFC were determined in the draining auricular lymph nodes by immunohistochemistry. Furthermore, the frequencies of primary and secondary follicles were determined after hematoxilin staining. To study the effect of treatment on the DTH response the ear thickness was measured before challenge and 4 days after challenge.

Immunohistochemistry

Cryostat sections (-20°C, 8 μm) of human skin (2 sections at different heights), mouse skin (two sections from either the left or the right ear at different heights) and mouse auricular lymph nodes (five sections from either the left or righ lymph node at different heights), were picked up on glass slides and kept overnight under high humidity at room temperature. Slides were air dried and stored in air-tight boxes at RT until use. Cryostat sections were fixed for 10 min in aceton (p.a.). Slides were incubated horizontally overnight at 4°C with reagents (5D12F10, MCA-1143, L3T4, 13P or TNP-HRP and the biotinylated reagents CD40-Ig, MR1, DB-1, S4B6, 11B11). All reagents were diluted in PBS containing 0.1% BSA and titrated to obtain optimal results. Subsequently slides were washed with PBS (three times for 5 min) and incubated for 60 min at room temperature with secondary reagents diluted optimally in PBS containing 1% BSA and 1% normal human (human tissue) or mouse (mouse tissue) serum. After incubation with the secondary biotinylated antibodies streptavidin-HRP/AP was used. Simultaneous staining for human CD40 ligand with the endothelial marker ELAM-1 was performed. Skin sections were first incubated with anti-ELAM-1 and biotinylated CD40-Ig, followed by streptavidin-AP and rabbit-HRP anti-mouse Ig. The histochemical revelation of AP and HRP was performed according to Claassen and Adler [33]. For double staining the immunohistochemical revelation

of AP was performed prior to HRP. Sections were rinsed with PBS, counterstained with hematoxylin and mounted in glycerin-gelatine [30]. Control staining sections with an isotype-matched control antibody and with omission of the primary antibody were included in each staining experiment. In these controls no staining was seen.

Quantification and statistical evaluation

Frequencies of CD40 ligand, CD40, CD4 positive cells, cytokine producing cells and MNC were determined in two sections from either the left and right ear from three mice at one time-point after challenge by using an eyepiece graticule. At least three fields, which together represent approximately the whole area in which infiltration was seen, were analysed in one ear section. Frequencies were expressed as the mean ±SEM per mm^2 dermis. For the analysis of the humoral response cryostat sections were made of the entire left and right auricular lymph nodes from five mice per group. The frequencies of DNP-AFC in the auricular lymph node were expressed in numbers of cells per mm^2 based on at least 5 sections at different heights. The frequencies of primary and secondary follicles were expressed in numbers of follicles per mm^2 based on at least 5 sections at different heights. The results obtained were analysed by the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population.

RESULTS

CD40 expression in normal human and mouse skin

We investigated whether CD40 was expressed on dendritic cells or resting Langerhans cells in normal human and mouse skin by using immunohistochemical staining methods. CD40 positive cells characterized by red membrane staining were found in human tonsil and mouse spleen. However, CD40 positive cells were not observed in normal human and mouse skin (Fig. 1).

CD40 and CD40 ligand positive cells in contact hypersensitivity in man and mice

To study the expression and kinetics of CD40 positive cells we investigated the expression of CD40 and CD40 ligand *in situ* during a DTH response. In the contact hypersensitivity reaction in man obtained 72 hours after skin exposure to the contact allergen, there was an increase in numbers of MNC as well as CD4 positive cells as compared to normal human skin. As shown in Fig. 1 CD40 ligand positive cells were found in clusters around the blood vessels (ELAM-1 positive), and CD40 positive cells were found in the dermis in contact hypersensitivity in man 72 hours after skin exposure to the contact allergen. In the dermis in mouse skin relatively high

frequencies of MNC (883/mm²) and CD4 positive cells (110/mm²) were reached at day 4 after challenge corresponding to an ear thickness increase of 60-70% (data not shown). The maximum frequencies of CD40 ligand (Fig. 1), CD40 positive cells and IL-2, IL-4 (Fig. 1) and IFN-γ cytokine producing cells per mm² dermis were observed at the same time as the maximum frequencies of MNC, CD4 positive cells (Fig. 1) and ear swelling response. The kinetics of appearance of CD40 ligand, CD40, cytokine producing cells, CD4 positive and MNC after challenge are shown in Table 1. Interestingly, in the epidermis some CD40 positive cells with a dendritic morphology were detected 2-3 days after challenge (Fig. 1).

Table 1. Kinetics of appearance of CD40 ligand, CD40 positive cells and cytokine producing cells in the dermis after exposure to the skin with the contact allergen DNCB [a)]

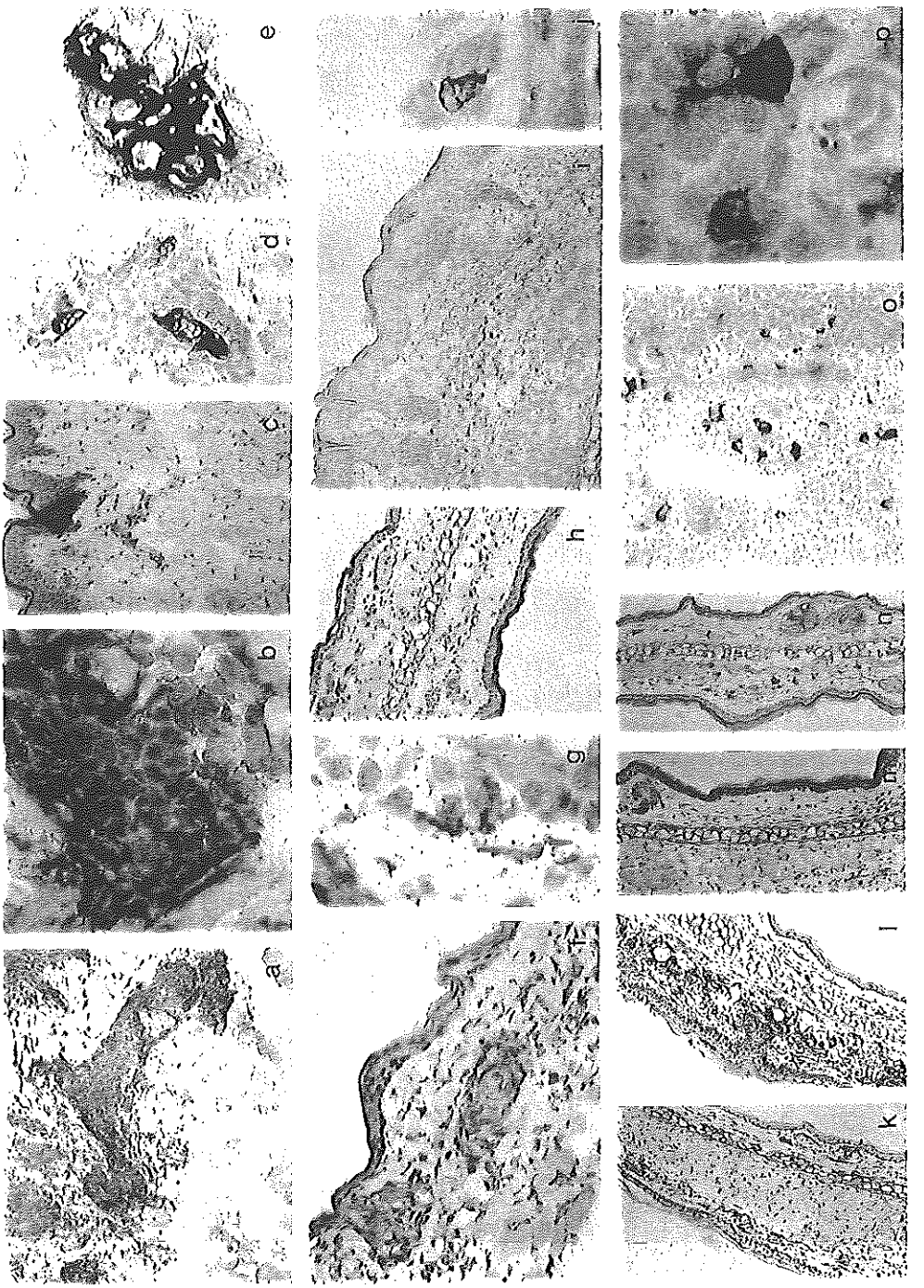
	Days after challenge								
Marker	0	1	2	3	4	5	7	9	12
CD40 ligand	0	0	0	19±12	59±19	8±3	0	0	0
CD40	0	0	2±2	18±14	22±13	6±4	0	0	0
IL-2	0	0	9±5	20±8	27±14	32±18	0	0	0
IL-4	0	0	57±15	16±6	48±16	10±6	0	0	0
IFN-γ	0	0	327±74	215±33	315±93	217±80	267±69	128±61	0
CD4	0	1±1	7±4	34±7	110±24	26±10	40±19	20±19	0
MNC	188±20	417±97	503±83	528±117	883±106	448±77	437±72	140±15	148±12

a) Groups of three mice were sensitized with 1% DNCB on day 0 and challenged with 1% DNCB on day 5. Different groups of mice were sacrificed at the indicated days after challenge. Two cryostat sections were prepared from the right and left ears and immunohistochemical demonstration of CD40 ligand, CD40, IL-2, IL-4, IFN-γ and CD4 positive cells was performed. Frequencies of cells with evident expression and the total frequencies of mononuclear cells infiltrating the dermis were determined in at least three fields per section from each ear by using an eyepiece graticule. Frequencies were expressed as the mean ± SEM per mm² in the dermal part of the skin of each group.

***In vivo* anti-CD40 ligand antibody administration inhibits the specific antibody (humoral) response to DNCB**

We observed CD40 and CD40 ligand positive cells in contact hypersensitivity in mice after skin exposure to DNCB. In order to investigate whether the CD40 ligand-CD40 interactions were essential for the antibody response caused by skin exposure to DNCB the optimal conditions with regard to time after challenge to assess the effect of anti-CD40 ligand antibody treatment were established. Mice were sensitized and challenged with 1% DNCB and the kinetics of DNP-AFC were studied. As shown in Fig. 2, challenge with DNCB resulted in an increase of the number DNP-AFC in lymph node sections of mice 5 days after application. As could be expected after skin exposure the serum IgG and IgM antibody titers were low and the DNP-specific IgM and IgG antibody titers were extremely low as compared to serum antibody titers after TNP-KLH or TNP-Ficoll immunization (data not shown). Hardly any difference in total and DNP-specific antibody production between two time-points after challenge was observed.

*>>**Figure 1.** Immunohistochemical visualization of positive cells in normal skin and contact hypersensitivity in human or mice. Cryostat sections of human and murine skin tissue were incubated with specific immuno-conjugates, followed by immunohistochemical revelation. Conjugates and substrates used are indicated between the square brackets. a) 3 days after allergen application on human skin; red stained cells are CD40 positive cells localized in the infiltrates in the dermis [5D12F10 + biotinylated horse-anti-mouse + streptavidin-HRP; AEC]. b) Higher magnification of (a). c) normal human skin incubated as in (a) showing no positive cells. d) 3 days after allergen application on human skin; blue stained cells are CD40 ligand positive cells localized in the infiltrates around or within the endotelium [biotinylated-CD40Ig + streptavidin-AP; Fast blue]; red stained cells are ELAM-1 positive endothelial cells [biotinylated-13P + streptavidin HRP; AEC]. e) Higher magnification of (d). f) 2 days after DNCB challenge on murine skin; red stained cells are CD40 positive cells localized in the epidermis [MCA-1143 + biotinylated-goat-anti-rat + streptavidin-HRP; AEC]. g) Higher magnification of (f). h) normal murine skin incubated as in (f) showing no positive cells. i) 4 days after DNCB challenge on murine skin; red stained cells are CD40 ligand positive cells localized in the infiltrates in the dermis [biotinylated MR-1 + streptavidin-HRP; AEC]. j) Higher magnification of (i). k) normal murine skin without positive cells incubated as (l). l) 4 days after DNCB challenge on murine skin; red stained cells are CD4 positive cells localized in the infiltrates in the dermis [L3T4 + Rabbit-HRP anti-mouse; AEC]. m) normal murine skin without positive cells incubated as (n). n) 4 days after DNCB challenge on murine skin; red stained cells are IL-4 positive cells localized in the dermis [biotinylated-11B11 + streptavidin-HRP; AEC]. o) 5 days after DNCB challenge on murine skin; red stained cells are DNP-AFC localized along medullary cords in the auricular lymph node [TNP-HRP; AEC]. p) Higher magnification of (o). (a,c,h,i,k,l,m,n) x100, (d,f) x200, (b,o) x400 and (e,g,j,p) x1000.*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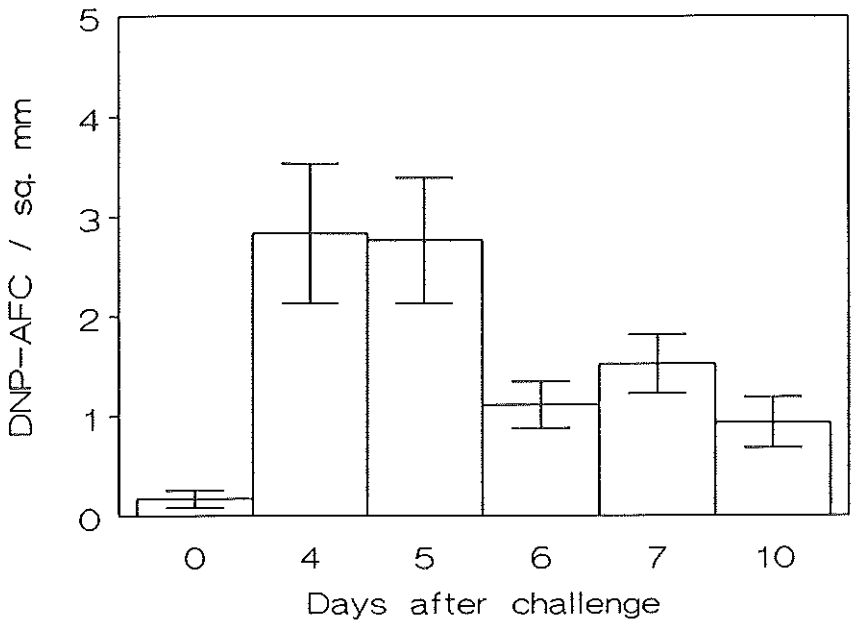

Figure 2. Kinetics of appearance of DNP-AFC in draining lymph node after application of DNCB on the mouse skin. BALB/c mice were sensitized (at day 0) and challenged (at day 5) with 1% DNCB and sacrificed at the indicated time-points after challenge. Auricular lymph nodes were removed and immunohistochemical visualization and quantification using image analysis was performed as described in the materials and methods section. Values represent the mean ± SEM of number of positive cells per mm².

To study the effect of anti-CD40 ligand antibody treatment, quantification of DNP-AFC per lymph node (mm²) was performed at 5 days after challenge in the anti-CD40 ligand antibody treated group and compared to the control hamster antibody and PBS treated groups. Both anti-CD40 ligand antibody treatment before sensibilization in the first experiment (data not shown) or after sensibilization in the second experiment (Fig. 3) completely prevented the development of DNP-AFC after skin exposure to DNCB. Anti-CD40 ligand antibody treatment is also reflected in germinal centre formation. Quantification of primary and secondary follicles per lymph node (mm²) was performed at 5 days after challenge in the anti-CD40 ligand antibody treated group and compared to the control hamster antibody and PBS treated groups (Fig. 4). CD40 ligand antibody treatment before sensibilization suppressed the germinal centre formation after skin exposure to DNCB. This means that CD40 ligand antibody

treatment given before sensibilization blocks the transformation of primary follicles to secondary follicles. In the CD40 ligand antibody treated group 14% secondary follicles over total numbers of primary and secondary follicles per lymph node (mm^2) were observed. In the control antibody and PBS treated groups of mice 61% and 58% secondary follicles over total numbers of follicles were observed respectively. The total numbers of primary and secondary follicles per lymph node (mm^2) were in the same range in the three different groups of mice. In conclusion, anti-CD40 ligand antibody treatment had an effect on the humoral immune response after DNCB sensibilization and challenge by skin exposure indicating that the anti-CD40 ligand antibody treatment is effective.

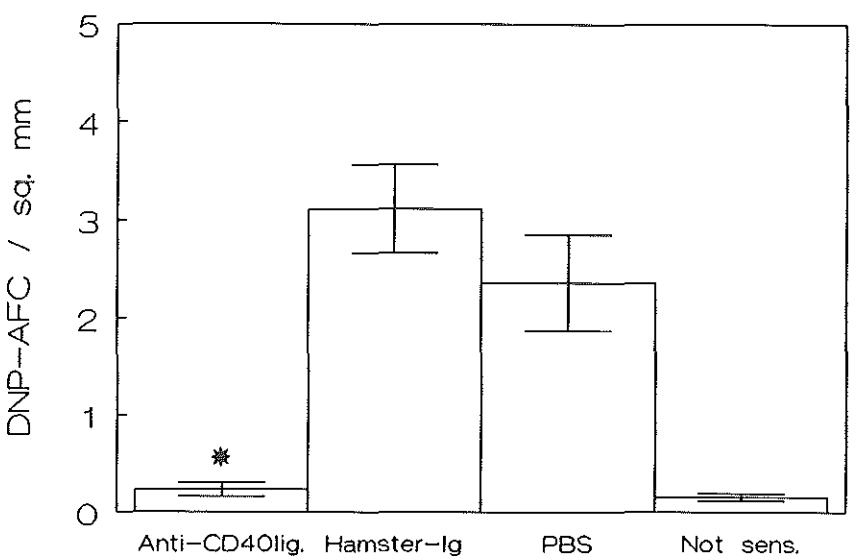

Figure 3. *DNP-AFC response suppression in the auricular lymph node after treatment with anti-CD40 ligand mAb. BALB/c mice were sensitized with 1% DNCB on day 0, and injected with 250 µg anti-CD40 ligand mAb, control hamster antibodies or PBS on day 5, 4 hours prior to challenge with 1% DNCB. Mice, not sensitized on day 0 but challenged on day 5 with 1% DNCB, were included as specificity controls. Mice were killed 10 days after sensibilization and auricular lymph nodes were removed. Immunohistochemical visualization and quantification using image analysis was performed as described in the materials and methods section. Values represent the mean ± SEM of number of positive cells per mm^2. A significant decrease of DNP-AFC per mm^2 was found after anti-CD40 ligand antibody treatment as compared to control hamster antibody treatment ($p < 0.001$).*

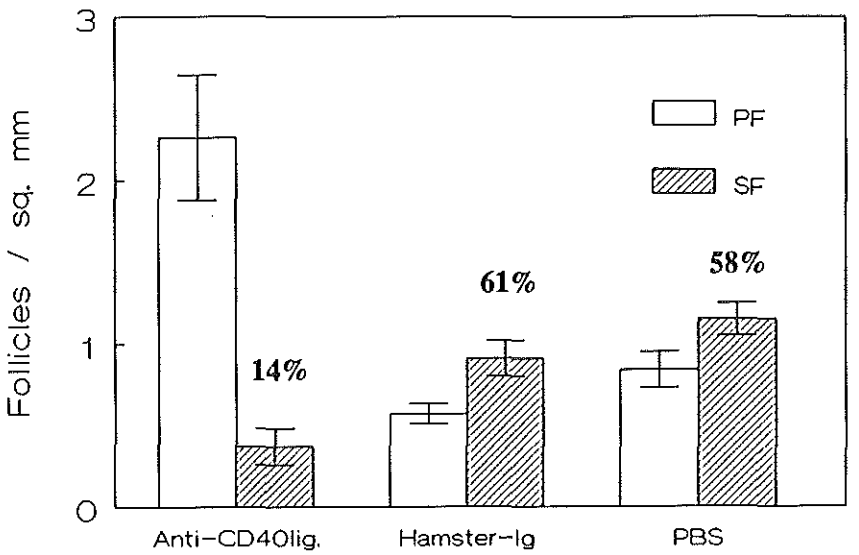

Figure 4. Impaired germinal centre formation in the auricular lymph node after treatment with anti-CD40 ligand mAb before sensibilization. BALB/c mice were injected with 250 µg anti-CD40 ligand mAb, control hamster antibodies or PBS on days -1, 1, and 4. Mice were sensitized with 1% DNCB on day 0 and challenged with 1% DNCB on day 5. Mice were killed 10 days after sensibilization and auricular lymph nodes were removed. Hematoxilin staining and quantification using image analysis was performed as described in the materials and methods section. Values represent the mean ± SEM of number of follicles per mm². 14% secondary follicles over total follicles per mm² was found after anti-CD40 ligand antibody treatment as compared to 61% and 58% secondary follicles over total follicles after control hamster antibody and PBS treatment respectively. PF, primary follicles; SF, secondary follicles.

In vivo anti-CD40 ligand antibody treatment does not have an effect on DTH

As CD40 and CD40 ligand are present on Langerhans (veiled) cells and activated T-cells respectively, we asked whether a DTH response could be influenced by anti-CD40 ligand antibody treatment. In order to investigate whether the CD40 ligand-CD40 interactions were essential for the DTH response caused by skin exposure to DNCB the optimal condition to establish the effect of anti-CD40 ligand antibody

treatment was established. Evaluation of ear swelling revealed that the highest specific increase in ear thickness was seen at day 4 after DNCB challenge (Fig. 5). The moderate increase in the control group can be explained by the irritant effect of aceton/olive oil. Subsequently, groups of mice were treated with anti-CD40 ligand antibody or control hamster antibody or PBS either before sensibilization in the first experiment or after sensibilization (before challenge) in the second experiment. The mean increase in ear thickness was calculated at 4 days after challenge in the treated groups. The ear swelling responses after anti-CD40 ligand antibody treatment in both experiments (Fig. 6a and b) were not affected. The control group represents the mean increase in ear thickness responses after challenge without sensibilization, confirming that the responses in the other groups were DNCB specific.

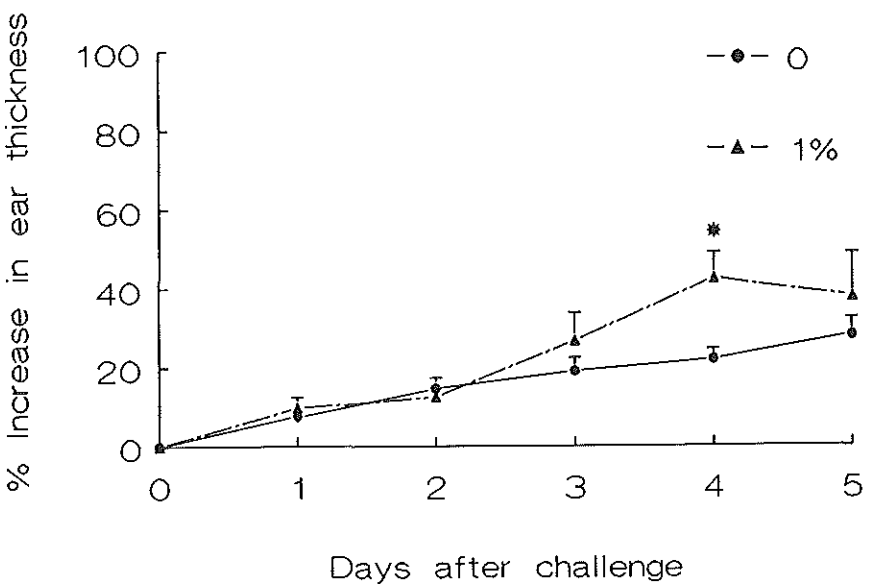

Figure 5. Kinetics in ear swelling during a contact hypersensitivity reaction. BALB/c mice were sensibilized with 1% DNCB on day 0 and challenged with 1% DNCB on day 5. Increase in ear thickness in challenged mice (broken line) and in unsensitized mice (solid line) was determined 0 to 5 days after challenge. Data represent the mean ± SEM of percentage increase in ear thickness of three mice (6 ears) at each time point. A significant difference was found between ear swelling in challenged mice as compared to ear swelling in non-sensitized mice ($p < 0.02$).

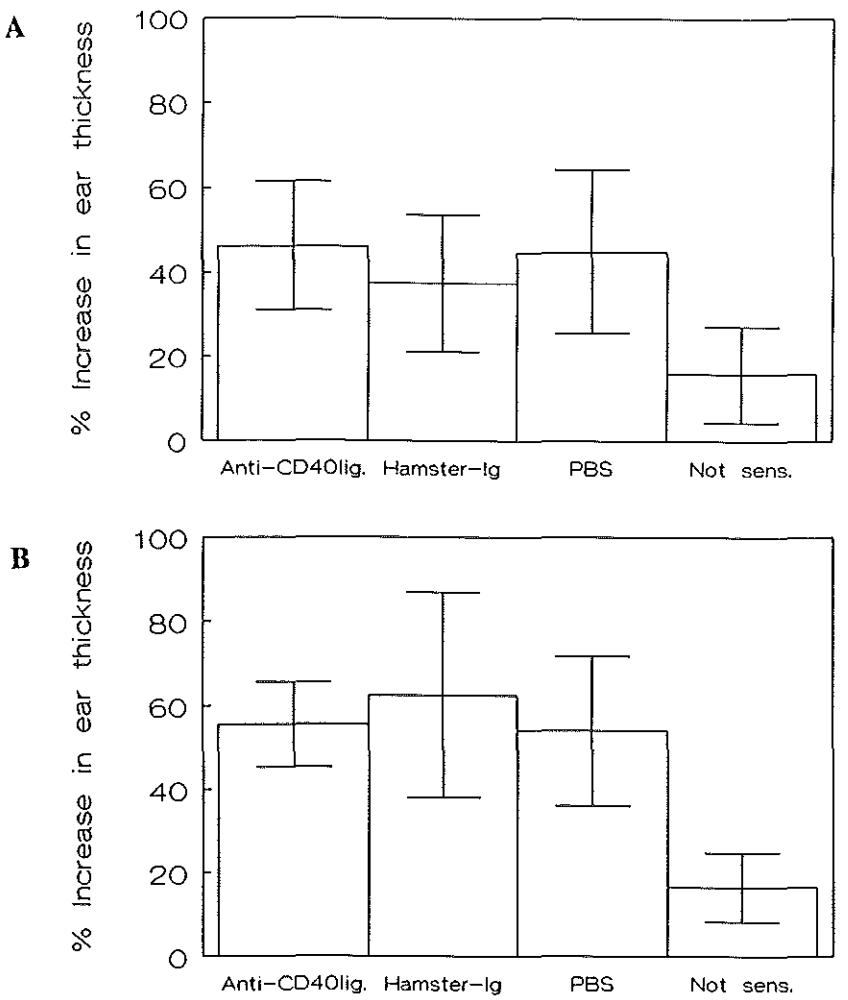

Figure 6. No influence on ear swelling by treatment with anti-CD40 ligand antibodies either before (a) or after sensibilization (b). (a) BALB/c mice were injected with 250 µg anti-CD40 ligand mAb, control hamster antibodies or PBS on days -1, 1, and 4. Mice were sensitized with 1% DNCB on day 0 and challenged with 1% DNCB on day 5. (b) BALB/c mice were sensitized with 1% DNCB on day 0, and injected with 250 µg anti-CD40 ligand mAb, control hamster antibodies or PBS on day 5, 4 hours prior to challenge with 1% DNCB. (a+b) Mice, not sensitized on day 0 but challenged on day 5 with 1% DNCB, were included as specificity controls in experiment a and b. Ear swelling was measured and evaluated as described in the materials and methods section. Values represent the means ± SD of percentages increase in ear thickness.

DISCUSSION

The present study demonstrates that cells involved in contact hypersensitivity in human and mice do express CD40 and CD40 ligand. In normal resting human and mouse skin dendritic cells expressing CD40 were not observed. *In vivo* blocking experiments in mice showed that CD40 ligand-CD40 interactions were essential for the specific DNP antibody (humoral) response. In contrast, no influence on ear swelling (DTH) was found, indicating that CD40 ligand-CD40 interactions are not required for DTH in this system.

The presence of CD40 positive cells in contact hypersensitivity in man and in mice has not been described before. However, data are available that following culture, Langerhans cells express CD40 at high levels. Dendritic cells can be generated *in vitro* by culturing $CD34^+$ hematopoietic progenitor cells in the presence of GMCSF/IL-3 and TNF-α [11]. These cells, which resemble Langerhans cells as they express CD1a and display Birbeck granules, express CD40 at high levels and may thus represent cells at a stage of differentiation between Langerhans cells and interdigitating dendritic cells. In fact, high levels of CD40 on interdigitating dendritic cells in the T-cell-rich areas of secondary lymphoid organs were described [34, 35]. Furthermore, dendritic cells isolated from peripheral blood express CD40 [36]. These observations together with the upregulation of CD40 in contact hypersensitivity suggest that CD40 expression will be enhanced after antigen application or immunization. Probably, the CD40 molecule present on cells derived from skin/mucosal Langerhans cells, which only weakly express CD40 [37] and therefore difficult to detect in normal tissue using immunohistochemistry, become functional during presentation of antigen in the secondary lymph node. During the DTH reaction we could detect some CD40 ligand positive cells around blood venules in mouse skin, indicating that CD40 ligand expression is less important in the skin during a DTH reaction. This was confirmed with the observation of some CD40 ligand positive cells in contact hypersensitivity in the human skin biopsies. During migration of T-cells from the draining lymph node the amount of CD40 ligand molecules may be reduced. Van den Eertwegh et al. [21] showed abundant expression of CD40 ligand in the spleen and the draining lymph node after TNP-KLH immunization, indicating that CD40 ligand has an important role in these compartments.

Significant numbers of DNP-AFC were found in the draining lymph node, whereas serum DNP-specific IgM and IgG could not be detected after skin exposure to a contact allergen. This indicates that the humoral immune response was primarily localized in the auricular lymph node. Anti-CD40 ligand antibody treatment resulted

in lower frequencies of DNP-AFC in the lymph node as compared to control treatment and has an effect on the formation of DNP-AFC after treatment before sensibilization and after sensibilization. This finding suggests that the humoral immune response caused by skin exposure to a contact allergen and antigen presentation in the draining lymph node could be inhibited by blocking CD40 ligand-CD40 interaction. This is in concordance with the situation for systemically administered antigens [21]. Recently, Durie et al. [1] discussed the co-stimulatory role of the CD40 ligand-CD40 interaction between B and T-cells and suggested that molecules which optimalize signaling via T-cell receptor (antigen-specific MHC-restricted interaction) regulate CD40 ligand expression. This results in the expression of the co-stimulatory molecules B7 on the B-cell, B-cell activation and subsequently antibody production. Therefore, it is clear that the effect of anti-CD40 ligand antibody treatment on co-stimulation between B and T-cells found in this study caused inhibition of antibody production. The decrease in the percentage of secondary follicles over total primary and secondary follicles after anti-CD40 ligand antibody treatment before sensibilization demonstrates the important role of CD40 ligand-CD40 interaction for germinal centre formation in the draining lymph node as was described by Foy et al. [38] for the spleen.

Anti-CD40 ligand antibody treatment before sensibilization did not affect ear swelling. This suggests that CD40 ligand-CD40 interaction between Langerhans cells and T-cells in the draining lymph node is not essential for the presentation of antigen to T-cells and subsequent sensibilization. The observation that we were not able to block the DTH response with anti-CD40 ligand antibody treatment after sensibilization suggests that CD40 ligand-CD40 interaction and the presence of CD40 ligand-molecules on activated T-cells are not important in that phase of the antigen specific cellular response. The reason for the lack of effect on the development of a DTH response in the skin could be the induction of the co-stimulatory molecules B7 on CD40-bearing antigen presenting cells irrespective of the expression of CD40 ligand. Probably, B7-CD28 interactions occur before the up-regulation of CD40 on Langerhans cells and CD40 ligand on T-cells [39]. Another reason for the absence of an effect of anti-CD40 ligand treatment on ear swelling could be the possibility that specific CD40 ligand positive cells in the skin escaped from antibody treatment given i.p or the amount of antibody given is not high enough. However, Scheynius et al. [40] did find a reduction of contact hypersensitivity reactions in mice treated with mAb to leukocyte function-associated molecule-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) after i.p. injection of the mAb in similar concentrations. Furthermore, the inhibition of the humoral immune response in the same mice strongly argues against this possibility. Alternatively, or in addition, the absence of an effect of anti-CD40 ligand treatment on ear swelling could be due to the presence of a major

fraction of antigen non-specific CD40 ligand negative T-cells or MNC in the inflammatory site. The presence of a high frequency of IFN-γ producing cells and a relatively low frequency of CD4 and CD40 ligand positive cells observed in this study indicates that another cell population is involved.

In many mouse disease models clinical disease symptoms could be reduced with anti-CD40 ligand antibody treatment given systemically [19, 20]. This study shows the effects of anti-CD40 ligand antibody treatment on a humoral immune response and not on a cellular immune response caused by skin exposure to a contact allergen. The present data reveal the possibility to treat patients suffering from antibody-mediated skin diseases. In addition to treatment given systemically, it will be of interest in the future to determine the effects of topical application of e.g. liposomes containing anti-CD40 ligand antibody on antibody production in the draining lymph node and localized clinical symptoms.

Acknowledgments

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Chapter 5

Ex vivo contact dermatitis model

5.1

Migration of human antigen presenting cells in a human-skin-graft-onto-nude-mice model after contact sensitization
Immunology 86: in press, 1995.

5.2

The fate of allergic affected skin after transplantation onto immune deficient mice *(submitted).*

Migration of human antigen-presenting cells in a human skin graft onto nude mice model after contact sensitization

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SUMMARY

Fluorescent contact chemical allergens provoke sensitization after application on both syngeneic and allogeneic skin grafts in mice. We attempted to determine whether the functional activity in a contact sensitization response of human skin graft was affected at the level of antigen uptake and migration. After xenogeneic skin transplantation, we examined the effect of topical exposure of the graft to rhodamine B isothiocyanate (RITC). This paper describes the migration of RITC-carrying cells and human major histocompatibility complex (MHC) class II DR (HLA-DR)$^+$ cells, from the graft to mouse draining lymph nodes. As demonstrated by immunohistochemistry, grafting resulted in a time-dependent decrease of human HLA-DR$^+$ and CD1a$^+$ cells, and an increase of mouse MHC class II (Ia)$^+$ cells within the graft. Application of RITC on a 3-week-old human skin graft showed optimal migration capability compared to 6- or 9-week-old grafts. In addition, the time-dependent increase of frequencies of RITC$^+$ and HLA-DR$^+$ cells in the draining lymph nodes, and the time-dependent decrease of HLA-DR$^+$ cells in the 3-week-old human skin graft, were concurrent. Supporting these data, human cytokines interleukin-1 (IL-1α), IL-1β and tumour necrosis factor-α (TNF-α), analysed *in situ*, revealed that cytokine production by keratinocytes, a property associated with dendritic cell migration, was preserved in the human skin graft. Thus, like dendritic cells in contact sensitization in allografted skin, dendritic cells from human xenografted skin onto nude mice are capable of migration to mouse draining lymph nodes after allergen application. Induction of contact hypersensitivity is possible in a human skin graft onto nude mice model, although the use of this *ex vivo* model to analyze contact sensitivity is probably limited to 3 weeks after transplantation.

INTRODUCTION

In mice, both cutaneous dermal as well as epidermal antigen-presenting cells (APC) play an important role in the induction phase of contact sensitization. Langerhans' cells from the epidermis are considered to be the most important cells in contact sensitization.[1] Recently, it has been demonstrated that Langerhans' cells are not indispensable.[2] Contact hypersensitivity could be induced in tape-stripped skin devoid of Langerhans' cells, whereas surgical excision of hapten-painted skin within 1 hr of hapten-application prevented the development of contact hypersensitivity in mice. It was concluded that either Langerhans' cells or dendritic dermal cells are sufficient for antigen presentation in the induction of contact hypersensitivity. This conclusion was supported by demonstrating that Ia$^+$ cells haptenated *in vitro* could elicit contact hypersensitivity after intradermal injection *in vivo*.[3] Except for the lack of Birbeck granules and expression of lower levels of CD1a, the mouse epidermal and dermal dendritic cells are equivalent to epidermis-derived dendritic cells in humans.[4] In addition, there is evidence that human HLA-DR$^+$ dendritic cells in the perivascular space of the dermis express potent APC activity.[5] To provide evidence for the role of cutaneous APC in murine contact sensitivity a number of investigators has shown that, following topical exposure of mice to sensitizing fluorescent chemicals, cells carrying the antigen rapidly accumulate in lymph nodes draining the site of application.[6–8] These cells, with dendritic appearance, were able to support lymphocyte stimulation *in vitro* and rendered recipient mice sensitive after transfer. Using a combination of allogeneic skin transplantation with fluorescein isothiocyanate (FITC) contact sensitivity, Kripke *et al.*[9] demonstrated that APC isolated from lymph

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Abbreviations: AP, alkaline phosphatase; APC, antigen-presenting cell; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; Hu, human; RITC, rhodamine B isothiocyanate.

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nodes draining the site of FITC sensitization from C3H mouse skin-grafted BALB/c nude mice rendered C3H recipients sensitive to FITC. This suggests that, after epicutaneous sensitization of allografted skin, APC could leave the skin graft and migrate to the draining lymph nodes in nude mice.

Unlike the functional description of normal allografted skin onto nude mice in contact sensitivity, the functional description of xenografted skin, human skin onto nude mice, in contact sensitization has not been established. Until now, the behaviour of host and donor cells in human split-thickness skin grafts onto nude mice has been studied using immunohistochemical methods. In such a model the epidermis of the human graft is not replaced by mouse keratinocytes[10] or mouse Langerhans' cells.[11] Revascularization of the grafts with mouse microcirculatory bed and mouse endothelial cells, together with migration of mouse fibroblasts into the graft dermis within a few days after transplantation, has been observed.[10] The presence of murine (host) Langerhans' cells infiltrating the human-grafted skin onto nude mice was observed from 5 months after grafting.[12]

Recent studies of human contact dermatitis have led to the conclusion that the proinflammatory cytokines (interleukin-1α) (IL-1α), IL-1β and tumour necrosis factor-α (TNF-α) are major factors in primary as well as in recurrent immune responses.[13–17] During the primary immune response these initiating cytokines seem to support the capability of APC localized in human skin to migrate after stimulation, whereas during a recurrent immune response IL-1α, IL-1β and TNF-α induce the production of more and different cytokines that have chemotactic and leucocyte-activating properties as well as direct effects on the expression of cell adhesion molecules on endothelial cells.[18,19] Xenogeneic skin grafting, transplantation of, for example, human skin on nude or SCID mice, will allow individual *ex vivo* analysis of sensitivity to a given allergen or immunotoxin without risking sensitization by exposure of the individual (skin donor). In order to establish and validate the human skin graft onto nude mice model, a functional description of human skin grafted onto nude mice is presented in this paper. The human skin grafts were painted with the contact allergen rhodamine B isothiocyanate (RITC). As murine Langerhans' cells are capable of infiltrating the graft after transplantation, we evaluated the human skin grafts at various time-points after transplantation. Furthermore, we determined whether and when (time after transplantation) human APC inside the skin graft were able to carry and transfer the antigen to draining lymph nodes after painting the graft.

MATERIALS AND METHODS

Animals

BALB/c-nu/nu mice, 9–12 weeks old, were purchased from Harlan Olac Ltd, (Blackthorn, UK) and were kept under specific pathogen-free conditions. The mice were housed in Macrolon, type II cages (ITL, the Netherlands) with filter tops and were provided with sterile bedding, autoclaved food pellets and acidified-chlorinated water *ad libitum*.

Skin grafting

Human skin was obtained from female individuals (±30 years old) undergoing mammary reductions. The mammary skin was prepared by gently scraping away all subcutaneous fascia. Varying amounts of the dermis were trimmed so that the thickness of the human grafts approximated 0·5 mm. The mice were anaesthetized with sodium hexabarbital (Evipan; Bayer AG, Leverkusen, Germany). A piece of skin 1 cm^2, localized in the mouse flank, was removed to the level of the panniculus carnosus. After human skin was cut down to the size of 1 cm^2, and could be fitted into the prepared graft site, it was secured by stitching (Perma-Hand Seide, 5–0; Ethicon, Germany). Thereafter, the graft was dressed with Op-Site (T.J. Smith and Nephew Ltd, UK) and Microfoam (3M; Leiden, the Netherlands), which were firmly applied around the entire thorax. Only one graft was applied on each animal. Mice with grafts were housed individually. At 3 weeks after transplantation the human skin graft stabilized, and subsequently at 3, 6 and 9 weeks after transplantation the bandage (frequently changed) was removed before application of the allergen or solvent.

Graft exposure

At various times after grafting, 100 μl of a 2% (W/V) solution of RITC (Sigma, St Louis, MO), dissolved in DMSO: aceton: dibutylphthalate (10:45:45), was painted onto the grafted human skin as established for mouse skin.[20] A ring of petroleum jelly was applied bordering the graft to prevent spreading of the hapten solution beyond the grafted human skin. At 3 days after exposure of the 3-, 6- and 9-week-old human skin grafts, and subsequently at 1, 2 and 3 days after exposure of the 3-week-old human skin grafts, the draining axillary, brachial and inguinal lymph nodes were removed, immediately frozen in liquid nitrogen and stored at −70°.

Antibodies

The monoclonal antibody (mAb) L234, directed to human HLA-DR, was a kind gift of Dr A. Morgan (Celltech Ltd, Slough, UK). Cells of the mouse hybridoma CRL 8020 (OKT6), which produce mAb against CD1a, were obtained from ATCC (Rockville, MD). The rat mAb M5/114, which recognizes Ia from the mouse, was a gift from Dr E. van Wilsem (Department of Cellbiology, Faculty of Medicine, VU, Amsterdam, the Netherlands). Vmp18 and Vhp20, both mouse mAb specific for, respectively, human IL-1α and IL-1β, were kindly provided by Dr D. Boraschi (Laboratorio di Immunofarmacologia, Centro Ricerche Sclavo, Siena, Italy).[21] Mouse mAb 61E71, recognizing human TNF-α, was a generous gift from Dr W. Buurman (Department of Surgery, Biomedical Center, University of Limburg, Maastricht, the Netherlands).[22]

L234, OKT6, Vmp18, Vhp20 and 61E71 were used as biotin-labelled antibodies conjugated as previously described.[23] The secondary antibody rabbit anti-rat–horseradish conjugate (RaRa–HRP), used for mouse Ia detection, was purchased from Dakopatts (Copenhagen, Denmark). The avidin–alkaline–phosphatase (AP) and avidin–FITC conjugate used for biotin detection were obtained from Sigma.

Immunofluorescense staining and immunohistochemistry

Cells carrying RITC were counted, in cryostat sections (8 μm) from each draining lymph node, using a fluorescence microscope, Vanox AH2-SLH Olympus (BP545 excitation filter, 476–456 nm excitation wavelengths). After counting the $RITC^+$ cells, the lymph node sections and the skin graft sections were fixed for 10 min in fresh acetone (containing 0·02% H_2O_2 for blocking of the endogenous peroxidase). Slides

were incubated overnight at 4° in the dark with L234 and OKT6. All reagents were diluted in phosphate-buffered saline (PBS) containing 0·1% bovine serum albumin (BSA) and titrated to get optimal results. After incubation for another 60 min at room temperature with avidin–FITC, diluted optimally in PBS containing 1% BSA and 1% normal human serum, the sections were washed with PBS. The slides were mounted in glycerol/saline (9:1 v/v) and sealed with nail varnish. Cells expressing FITC (BP495 excitation filter, 460–490 nm excitation wavelengths) were counted in cryostat sections (8 μm) from each draining lymph node.

For the detection of Ia^+ cells in the graft, mAb M5/114 and the secondary antibody RaRa–HRP were used. To detect HLA-DR^+, CD1a^+, IL-1α^+, IL-1β^+ and TNF-α^+ cells in the graft, avidin–AP was used. The histochemical revelation of HRP and AP was performed according to Claassen & Adler.[24] The slides were counterstained with haematoxylin for 15 seconds and mounted in glycerol/gelatin. Control staining sections with an isotype-matched control antibody and with omission of the primary antibody were included in each staining experiment.[25] In these controls no staining was seen.

Quantification and statistical analysis

In the draining lymph nodes the RITC$^+$, HLA-DR^+ and CD1a^+ cells were enumerated in at least 50 sections per draining lymph node (approximately half of a node). The relative frequencies of RITC$^+$ cells in the brachial and inguinal draining lymph nodes 3 days after RITC application on a 3-week-old human skin graft and a 3-week-old BALB/c-nu/nu skin graft were calculated based on the maximum number of RITC$^+$ cells found in the axillary lymph nodes (100%). Differences between means of frequencies of positive cells from 50 lymph node sections were calculated using the Student's *t*-test.

In the human skin graft in the dermis, HLA-DR^+, CD1a^+ and Ia^+ cells were counted in two fields (0·5 mm^2/field) in at least two sections at three different sites of each human skin graft. In the epidermis the HLA-DR^+, CD1a^+ and Ia^+ cells were counted per mm in at least two sections at three different sites of each human skin graft. Because of the heterogeneous distribution of human Langerhans' cells expressing CD1a, human dendritic cells expressing HLA-DR and mouse dendritic cells expressing Ia cells in the human skin graft, we used the varying intervals of positive cell numbers as a measure for the behaviour of these cells in the graft.

RESULTS

General histological observation

As shown in Table 1, the frequencies of HLA-DR^+ and CD1a^+ human cells localized in the epidermis of the graft decreased rapidly during the first 3 weeks after transplantation compared to the frequencies of HLA-DR^+ and CD1a^+ cells found before grafting (Fig. 1a–d). In the dermis, by 3 weeks after engraftment a relatively low number of HLA-DR^+ as well as CD1a^+ cells was found, compared to the frequencies observed before transplantation and markedly decreased frequencies 6 and 9 weeks after transplantation. From week 3 after transplantation the number of Ia^+ cells dramatically increased in the dermis and less so in the epidermis of the human graft (Fig. 1e).

Although the quantification of epidermal human cells expressing CD1a or HLA-DR or epidermal mouse cells expressing Ia^+ could be simplified by using epidermal sheets (transversal sections) from the human skin graft, we used cryostat cross-sections, the advantage of this approach being the possibility to study the expression of several antigens in serial sections. Furthermore, epidermal sheets represent just one plane in the skin and therefore the migrating Langerhans' cells leaving the epidermal part or dendritic cells localized in the dermal compartment, will be missed by analysis of these sheets.

Time after transplantation in relation to the migration of dendritic cells to draining lymph nodes after painting

Averaged frequencies of RITC-carrying cells and HLA-DR^+ cells from 50 lymph node sections are shown in Fig. 2. The frequencies of RITC-fluorescent cells, as well as of human HLA-DR^+ cells, decreased rapidly 6 and 9 weeks after transplantation. RITC-carrying cells (Fig. 1f) and HLA-DR^+ cells (Fig. 1g) were predominantly localized in the deep cortex of the lymph node. A negligible number of HLA-DR^+ cells (< 10) was found in the draining lymph nodes before RITC application and after solvent application. No HLA-DR^+ cells were found in the lymph nodes from ungrafted BALB/c-nu/nu mice. No auto-fluorescent cells expressing red or green fluorescence were detected in draining lymph nodes obtained from a BALB/c-nu/nu mouse after grafting of human skin either before or after application of solvent.

The frequencies of HLA-DR^+ cells and RITC-carrying cells were determined by using the immunofluorescense detection of RITC-carrying cells prior to fixation of the

Table 1. Surface antigen expression in human skin grafts

Time after transplantation	Human HLA-DR		Human CD1a		Mouse Ia	
	Epidermis	Dermis	Epidermis	Dermis	Epidermis	Dermis
0 (control)	3–10	21–60	3–10	1–2	0	0
3 weeks	1–2	3–20	1–2	0	0	1–25
6 weeks	1–2	0	1–2	0	3–20	26–50
9 weeks	1–2	0	1–2	0	3–20	51–125

Number of positive cells per mm epidermis and per mm^2 dermis is indicated.

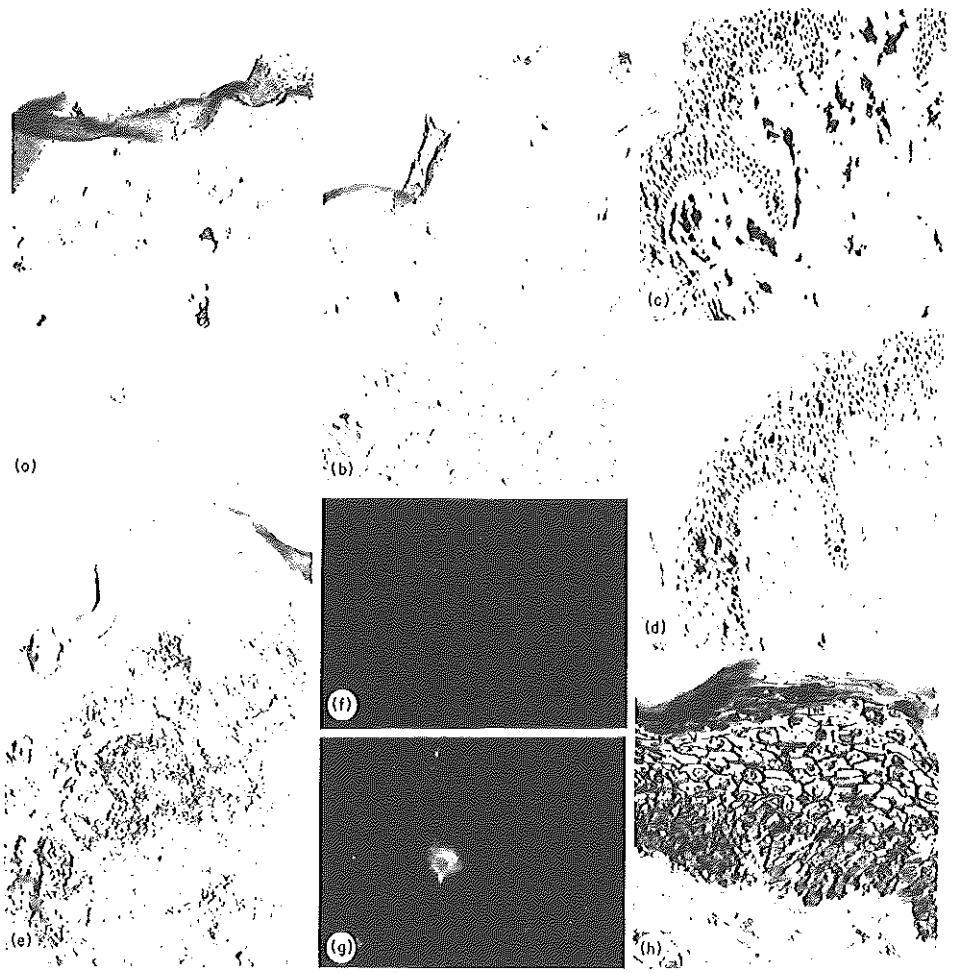

Figure 1. Immunohistochemical and immunofluorescent characterization of the graft and the draining lymph nodes in a human skin graft onto nude mice model after allergen application on the skin graft. (a) HLA-DR expression in a 3-week-old human skin graft. (b) CD1a expression in a 3-week-old human skin graft. (c) HLA-DR expression in normal human skin before transplantation (×100). (d) CD1a expression in normal skin before transplantation. Note the decrease in HLA-DR and CD1a expression in normal human skin compared to a 3-week-old human skin graft. (e) Ia^+ cells are distributed all over the dermis, with a few towards the epidermis, in a 9-week-old human skin graft. (f) Strong fluorescent RITC carrying cells in a draining axillary lymph node after allergen application. (g) A dendritic cell in the draining axillary lymph node after allergen application shows an intense staining with the mAb against HLA-DR at fluorescence microscopy. (h) IL-1α-producing cells in the epidermis in a 3-week-old human skin graft. Magnifications (a–e), ×200; (f–h), ×400.

section, and immunohistochemical staining of $HLA\text{-}DR^+$ cells. Because fixation dramatically decreased the red fluorescense, double fluorescense staining was not able to give reliable information.

Influx of fluorescent cells in the lymph nodes after painting the human skin graft 3 weeks after transplantation

The relative frequencies are shown in Fig. 3. The majority of RITC-fluorescent cells that migrated from the human skin graft were found in the axillary and brachial lymph nodes, whereas lower frequencies were detected in the inguinal lymph nodes localized at the same flank as the skin graft. In addition, in both control syngeneic transplantation experiments and xenogeneic transplantation experiments low relative frequencies of $RITC^+$ cells were found in the inguinal lymph nodes at the same flank as the skin graft. A comparatively high frequency of $RITC^+$ cells (722) was detected in the draining axillary lymph node

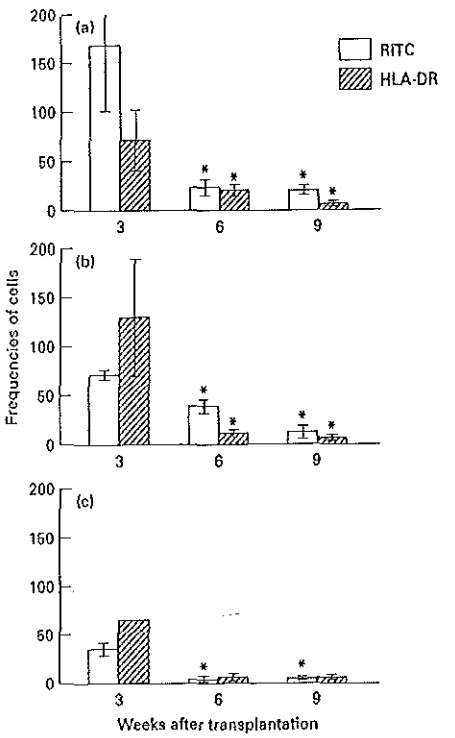

Figure 2. Changes in means of frequencies of RITC$^+$ and HLA-DR$^+$ cells in the draining lymph nodes at 6 and 9 weeks compared to 3 weeks after transplantation. Mice were painted with 2% RITC and 3 days after application the relative migration capacity of RITC$^+$ and HLA-DR$^+$ cells was established in the draining axillary (a), brachial (b) and inguinal (c) lymph nodes. Bars represent the mean (± SEM) frequency. *Statistical difference of $P < 0{\cdot}1$ for RITC$^+$ and HLA-DR$^+$ cells between 6- and 9-week-old grafts compared to 3-week-old grafts.

from a nude mouse grafted with BALB/c-nu/nu skin, compared to the frequency of RITC$^+$ cells (168) detected in the draining axillary lymph node from a nude mouse grafted with human skin.

Influx of human dendritic cells in the lymph nodes after painting the human skin graft 3 weeks after transplantation

The frequencies of HLA-DR$^+$ cells increased in the draining axillary and brachial lymph nodes during the first 3 days after painting (Fig. 4). The frequency of HLA-DR$^+$ cells (< 10) in the draining lymph nodes after RITC painting was negligible before and after solvent application.

In addition, the frequencies of CD1a$^+$ cells were determined in draining lymph nodes from human skin graft 3 weeks after grafting and 3 days after RITC painting. Evidently less CD1a$^+$ cells (< 20%) were counted compared to the frequencies of HLA-DR$^+$ cells in the draining lymph nodes (seven versus 71 in the axillary, 24 versus 129 in the brachial, and 2 versus 65 in the inguinal lymph node).

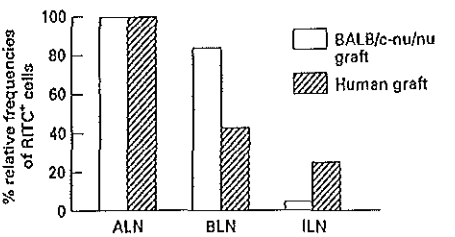

Figure 3. Effect of syngeneic and xenogeneic skin transplantation on the relative frequencies of RITC$^+$ cells in the draining axillary (ALN), brachial (BLN) and inguinal (ILN) lymph nodes at 3 weeks after transplantation and 3 days after application. Bars represent the relative frequency of one mouse.

Localization of human dendritic cells in 3-week-old human skin graft after RITC application

No differences in frequencies of HLA-DR$^+$ cells were observed inside the epidermis and dermis 1 and 2 days after RITC application, compared to the frequencies of HLA-DR$^+$ cells observed before RITC application (Table 2). At day 3 after painting, the frequency of HLA-DR$^+$ cells decreased compared to the frequencies of HLA-DR$^+$ cells detected in the dermis before or 1 and 2 days after painting. Changes in the already low frequencies of dendritic cells in the epidermis were not observed.

Exact quantification of fluorescent cells within the human skin graft after RITC application was impossible, because RITC as a hapten binds to proteins in the skin, which at the dose used causes high levels of background fluorescence not bound to cells.

Cytokine production by epidermal cells

To establish the capability of the keratinocytes in the human skin graft to produce human(hu)IL-1α, huIL-1β and huTNF-α (proinflammatory cytokines that have an important role in migration of dendritic cells to draining lymph nodes), the expression of these cytokines was examined at various times after RITC painting of the human skin graft and compared to

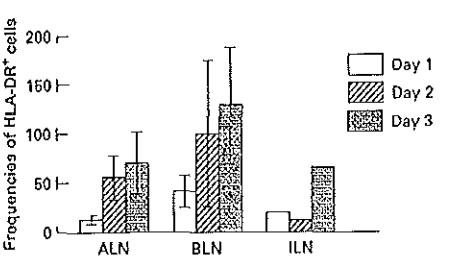

Figure 4. Changes in numbers of HLA-DR$^+$ cells in the draining ALN, BLN and ILN lymph nodes examined 1, 2 and 3 days after painting the human skin graft with 2% RITC 3 weeks after transplantation. Bars represent the mean (± SEM) frequency at one time-point after RITC application.

Table 2. Human HLA-DR expression in 3-week-old human skin grafts after RITC painting

	Human MHC II	
Time after painting	Epidermis	Dermis
0 (control)	1–2	3–20
1 day	1–2	3–20
2 days	1–2	3–20
3 days	1–2	1–2

Number of positive cells per mm epidermis and per mm^2 dermis is indicated.

previous data.[13] After qualitative determination, we found that the expression of huIL-1α (Fig. 1h), huIL-1β and huTNF-α was similar at days 1, 2 and 3 after skin painting, compared to the expression before painting. Human IL-1α- and huIL-1β-producing cells were detected in the different epidermal cell layers, whereas huTNF-α-producing cells were mainly observed in the high epidermal cell layers. The epidermis of the human skin graft could be predominantly considered as 'human' 3, 6 and 9 weeks after grafting, based on the normal histology observed in the graft. The human epidermal part was thick compared to the mouse epidermal part. A multi-layered thick stratified epithelium could be clearly recognized.

DISCUSSION

In this report we describe the functional activity of human dendritic cells from a human skin graft after xenogeneic transplantation. These cells were present in high frequencies in the human skin graft at 3 weeks after transplantation. Their frequency in the dermis decreased 3 days after allergen application, and subsequently, this resulted in a time-dependent increase of human dendritic cells in the mouse draining axillary and brachial lymph nodes (in which no human cells were present before RITC application). The preservation of human cytokine production in the human skin graft supports the migration data. These results definitively establish that the human skin graft onto nude mice model can be employed to analyse contact sensitivity.

We observed a relatively high frequency of human dendritic cells expressing HLA-DR in the human skin graft 3 weeks after transplantation, compared to the frequency of these cells determined 6 and 9 weeks after transplantation. Therefore, these results strongly suggest that at least the capability to develop contact sensitivity, based on the presence of sufficient human dendritic cells in the human skin graft, is not preserved longer than a minimum of 3 weeks and a maximum of 6 weeks after transplantation.

After grafting of split-thickness normal human skin onto nude mice, mouse Langerhans' cells were not observed during 2 months after grafting.[12] In addition, after human skin transplantation on nude mice the human dermis was reconstructed with mouse-specific elements such as endothelial cells and fibroblasts.[10] In the present study, the human skin graft was progressively invaded by mouse dendritic cells expressing Ia.

To test the capability of human dendritic cells from the graft to migrate to draining lymph nodes after painting, we performed experiments to establish the optimal time-point after transplantation and subsequent application of the allergen RITC. This was required to conclude that there is migration of human dendritic cells. The present study focused on dendritic cells expressing HLA-DR, because these dendritic cells take up the allergen RITC[7,8] and migrate to a draining lymph node. Three weeks after transplantation, and 2–3 days after application of the allergen, numerous human dendritic cells ($HLA\text{-}DR^+$) were found in the axillary and brachial lymph nodes draining the skin graft. The inguinal lymph nodes localized at the same flank as the human skin graft did not play an important draining role. These data provide evidence for insufficient antigen-presenting cells within the skin graft later than 3 weeks after transplantation.

The moment of appearance of the human $HLA\text{-}DR^+$ dendritic cells within the draining lymph nodes coincided with the time-course of the numbers of $HLA\text{-}DR^+$ cells within the human skin graft (3 week old) during the first 3 days after application. The changes in the human skin graft were consistent with a previous report in which the behaviour of mouse Langerhans' cells was examined in epidermal sheets from allografts or isografts.[26] The simultaneous changes in the graft and the draining lymph nodes provide evidence that, in our model, similar to that described for contact sensitization in previous studies,[9,26] a pathway might exist between the graft and the draining lymph nodes.

The kinetics of dendritic cell accumulation in the lymph node from mouse skin have been described by Hill *et al.*[27] In those studies, the frequencies of fluorescent cells increased rapidly and reached a maximum at days 1–3. The present study showed a gradual increase of $HLA\text{-}DR^+$ cells in the draining lymph node between 0 and 3 days after RITC application on the human skin graft. Moreover, we found a high frequency of RITC-carrying cells after syngeneic transplantation, compared to a low frequency of those cells after xenogeneic transplantation in the draining lymph nodes 3 days after RITC application on a 3-week-old skin graft. Therefore, it is concluded that syngeneic transplantation might differ markedly from xenogeneic transplantation from the histological point of view. However, it is also possible that in syngeneic skin grafts host mouse Langerhans' cells migrate into the epidermis of the graft or are generated in the epidermis during the first 3 weeks, and subsequently take up the allergen.[11]

Although RITC-carrying cells were present in high frequencies in the draining lymph nodes after xenogeneic skin grafting, only a small proportion of these $RITC^+$ cells were $HLA\text{-}DR^+$ and $CD1a^+$ cells (frequency $RITC^+ > HLA\text{-}DR^+ > CD1a^+$). The difference between RITC and HLA-DR expression could be the result of a failure to restrict the allergen to the grafted human skin. Lateral spread of the topically applied RITC could have enabled the participation of additional cells from the mouse skin outside the region grafted with human skin.

The differences between HLA-DR and CD1a expression demonstrated that a proportion of the total number of $HLA\text{-}DR^+$ cells found in the draining lymph nodes was Langerhans' cells. This suggests that other dendritic cells in the human skin graft could be important in the contact sensitization reaction. Experiments done by Meunier *et al.*[5] in humans and by Tse &

Cooper[3] in mice have demonstrated that both cutaneous dermal HLA-DR$^+$ cells in humans as well as in mice possess antigen-presenting capacity and play a role in contact sensitization. The existence of dermal dendritic cells with low levels of CD1a could support the small proportion of CD1a$^+$ cells detected in the draining lymph nodes.[4] In addition, it is possible that an increase of HLA-DR expression on individual APC during migration to the draining lymph nodes might be partially responsible for the differences between the frequencies of CD1a$^+$ and HLA-DR$^+$ cells observed in the draining lymph nodes after exposure. Cumberbatch *et al.*[28] presented data from mice that indicate that by the time Langerhans' cells reach the regional lymph nodes, there has been a significant increase in Ia antigen expression, consistent with phenotypic maturation, and the acquisition of antigen-presenting cell function.

The observed expression of huIL-1α, huIL-1β and huTNF-α by epidermal cells within the 3-week-old human skin graft, was similar to that detected in normal human skin.[13] This indicates that there is no defect in proinflammatory cytokine production in the grafted human skin in order to develop an immune response. In agreement with the findings in mice,[14–17] it is strongly suggested that the observed migration of Langerhans' cells and other dendritic cells from the human skin graft to draining lymph nodes after allergen application is related to the produced epidermal cytokines.

In conclusion, the model system described here makes it possible to establish migration of human dendritic cells during a contact sensitization response at least 3 weeks after transplantation and 2–3 days after RITC application. However, it should be kept in mind that this model could be of limited use for induction of a primary contact hypersensitivity reaction (sensitization and challenge) that needs at least several weeks to develop. Additional studies with SCID mice with human skin grafts from normal, sensitized or allergic individuals, reconstituted with human peripheral blood lymphocytes (full house mouse), are in progress to establish *ex vivo* individual sensitivity to a given allergen.

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THE FATE OF HUMAN ALLERGIC AFFECTED SKIN AFTER TRANSPLANTATION ONTO IMMUNE-DEFICIENT MICE: AN IMMUNOHISTOCHEMICAL STUDY

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BRIEF COMMUNICATION

Allergic contact dermatitis still represents a serious problem in occupational health. The epicutaneous patch test provides a sensitive methodology to establish the diagnosis of allergic contact dermatitis in man. A disadvantage of the epicutaneous patch test however is the risk of unwanted sensitization during the test depending on the allergen used. A suitable *in vivo* or *in vitro* model to establish the individual sensitivity or predisposition to be sensitized for a specific allergen is not available. The purpose of this study was to establish an ex vivo human-skin-onto-mice model that retains the possibility to develop the challenge phase of an allergic contact dermatitis reaction. In such a model the prerequisites are: the persistence of human antigen presenting cells for the uptake of allergen and migration, the persistance of human antigen specific T-cells and non-specific T-cells in the graft or circulation during several weeks, and signals for homing or recruitment of human T-cells to the human skin graft after challenge.

Immune-deficient mice are widely used for xenograft experiments (1). They accept human skin grafts from normal skin as well as from skin diseases such as psoriasis, cutaneous lupus erythematosus, pemphigus and vitiligo (2). These human skin grafts on immune-deficient mice remained viable (3) and thus provided a powerful tool for understanding the etiology and mechanism of these pathological processes in the skin and the effect of topical application of pharmacologically active compounds (4,5). However, this skin transplantation model was not used so far to establish the sensitivity or predisposition of an individual to develop allergic contact dermatitis.

In general after allergen exposure, activated Langerhans cells migrate to the

draining lymph nodes (sensitization phase) or to the dermis (challenge phase) to present the antigen to naive or specific T-cells respectively. In case of a human-skin-onto-immune-deficient-mice model it can be questioned whether it is possible to initiate an antigen-specific immune response after exposure of the human skin graft. Recently we established that the antigen presentation function remained intact for a limited period of time after transplantation of normal human skin onto nude mice i.e the capability to take up allergen by human antigen presenting cells and migration of these cells from normal human skin grafts to mouse draining lymph nodes (6). In the present study we investigated the preservation of the challenge function in this transplantation model. For this purpose we followed the preservation of human characteristics and the ingrowth of murine cells in the human skin graft obtained from allergic affected skin. Therefore, the human skin grafts were analysed for expression of human MHC-class I and II (HLA-DR) antigens and mouse MHC-class II (Ia) antigen. In addition we studied the feasibility of elicitation of an effective challenge phase in a skin graft from human allergic affected skin after allergen application on the graft. The persistence of human T-cells in this model and the presence of specific markers for homing or recruitment from the circulation into the human skin graft was examined (adhesion molecules ICAM-1 and ELAM-1). Human skin grafts were obtained from allergic affected skin in order to ensure the presence of human T-cells (±1% specific) in the skin graft to persist in the graft or in circulation after transplantation.

Eighteen biopsies, two from each patient (one 5 mm and one 3mm in diameter) with allergic affected skin, were all classified as moderate allergic (++) at the final reading (72 hours). The 5 mm biopsies were used for transplantation, the 3mm biopsies were used as immunohistochemical staining controles.

Antigens were dissolved in aquadest or petrolatum at concentrations routinely used in epicutaneous patch test. The allergens were applied on the human skin grafts using subsequently a piece of filter from the finn chambers (Epitest-Ltd. Oy, Payala, Finland), op-site and microfoam for occlusion (Fig. 1). Three days after allergen exposure the grafts were removed, snapfrozen and stored at -70°C

For transplantation, the biopsies were prepared by gently scraping away all subcutaneous fascia. Varying amounts of the dermis were trimmed so that the thickness of the human graft approximated 0.5 mm. The mice were anaesthetized with sodium hexabarbital (Evipan; Bayer AG, Leverkusen, Germany). A piece of skin of 25 mm^2 localized in one flank was removed to the level of panniculus carnosus. After the graft fitted into the prepared graft site, the grafts were transplanted onto Balb/c-nude mice (n=4, T-cell deficient) (Harlan Olac Limited Blackthorn, Bicester, Oxon, England) or BNX mice (n=5, BNX-nu/nu bg/bg xid mice: T-cell deficient, recessive B-cell defect (xid) and NK deficient (bg)) (TNO, Rijswijk, The Netherlands). The graft was dressed

with op-site (T.J. Smith and Nephew Limited, England) and microfoam (3M, Leiden, The Netherlands), which was firmly applied around the entire thorax. Mice with grafts were housed individually and the bandage was removed 2 weeks after transplantation, just before application of the specific allergen. Two human skin grafts transplanted onto Balb/c mice and three human skin grafts transplanted onto BNX mice were rechallenged epicutaneously with the same allergen as was applied for the *in vivo* patch test which was used to generate the allergic skin biopsies. The allergic affected skin biopsies used in this study were classified as moderately allergic. The concentration of the different allergens used was established to give reproducible moderately allergic reactions in *in vivo* patch tests. The allergen treated group of mice was compared to the non-treated group of mice independently on the type of allergen.
Two human skin grafts transplanted onto Balb/c mice and two human skin grafts transplanted onto BNX mice were not rechallenged and were used as treatment controles (Table 1).

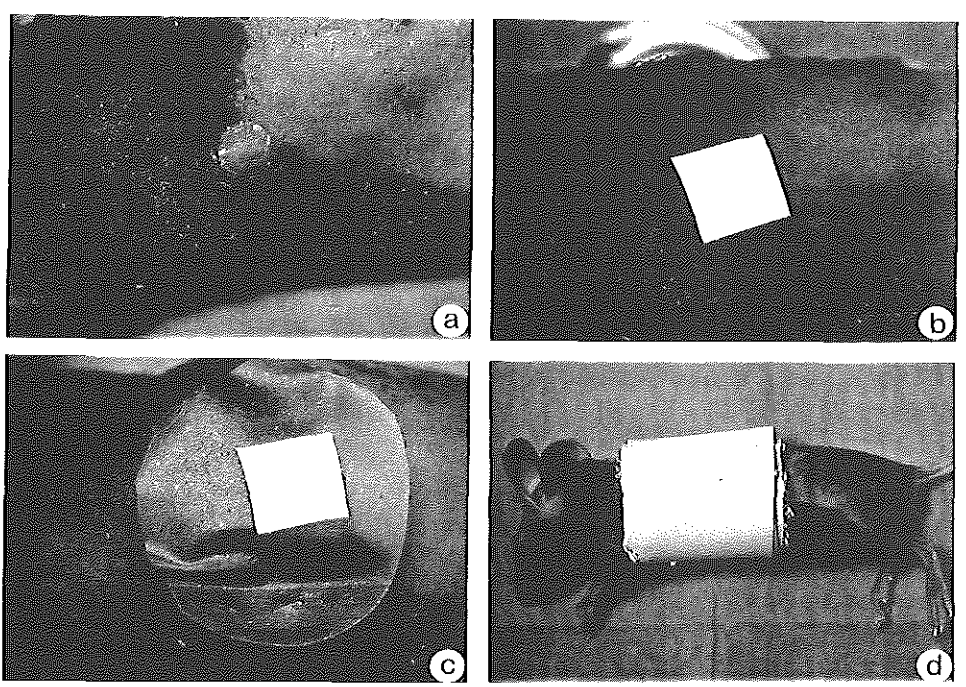

Figure 1. *The allergen application procedure of the human allergic skin grafts on the mouse. a, Appearance of a human allergic skin graft (5mm) on a BNX mouse 2 weeks after transplantation. b, Application method of the allergen using a piece of filter from the finn chamber. c, The op-site applied on the filter. d, The dressing of microfoam around the animal's thorax.*

Table 1. Patients, specific allergen and mice

Patient Number	Allergen	Transplantation onto Balb/c-nude or BNX mice	Allergen treated
1	p-aminoazobenzene	Balb/c-nude	yes
2	mercury chloride	Balb/c-nude	yes
3	cobalt chloride	Balb/c-nude	no
4	nickel sulphate	Balb/c-nude	no
5	nickel sulphate	BNX	yes
6	cobalt chloride	BNX	yes
7	ethylenediamine	BNX	yes
8	ethylenediamine	BNX	no
9	nickel sulphate	BNX	no

Tissue sections made from the human skin grafts were stained with biotinylated monoclonal antibodies (MAb) including 1) the rat MAb M5/114 which recognizes MHC-class II (Ia^+ cells) from the mouse (Dr. E. van Wilsem, Department of Cellbiology, Medical Faculty Free University, Amsterdam), 2) the mouse MAb L234 directed to human MHC-class II ($HLA\text{-}DR^+$ cells) (Dr. A. Morgan, Celltech LTD, Slough, U.K.), 3) the mouse MAb directed to human MHC-class I made by the mouse hybridoma W6/32 (ATCC, Rockville, Maryland, USA), 4) the mouse MAb 19C1 specific for ICAM-1 (Dr. J. Y. Bonnefoy, Glaxo Institute for Molecular Biology S.A. Geneva, Switzerland) and 5) the mouse MAb 13P specific for ELAM-1 (E-selectin) (Dr. J. Y. Bonnefoy). As the 6th antibody, rabbit polyclonal antibody coupled to an inert polymer backbone (DAKO EPOS) and horseradish peroxidase (HRP) was used to mark human CD3 positive T-cells (Dakopatts, Copenhagen, Denmark). Details on reagents and staining were essentially similar as described previously (6).

Graft acceptance was achieved in the four Balb/c and five BNX mice without evidence of either technical failure or immunologic rejection (Table 1). To the grafts on Balb/c nude and BNX mice specific allergens were applied. At 72 hours after application of the specific allergen the grafts didnot show a macroscopically allergic reaction such as erythema. Biopsies were taken including the graft surrounding mouse skin and underlying mouse tissue.

The staining results are summarized in Table 2. The mouse portion of the biopsy adjacent to the human graft showed positivity with the anti-mouse MHC-class II antibody. In all grafts the presence of mouse cells was established. The human skin grafts on Balb/c or BNX mice exhibited a normal human MHC-class I expression in the epidermal cell layers irrespective of the treatment received. In the dermal part of the graft significantly more human MHC-class I positive cells were found in the grafts on BNX mice as compared to the grafted Balb/c-nude mice in both the untreated as well as the treated grafts. In the murine epidermal part of the sections no human MHC-class I positive cells were detected. Fig. 2 illustrates representative sections from: grafted human skin on BNX mice treated with allergen (a) and allergic affected human skin before transplantation (b) both stained for human MHC-class I. In the dermis of 2 out of 3 treated human skin grafts onto BNX mice MHC-class II positive cells were detected. In the untreated human skin grafts on BNX mice no MHC-class II positive cells were found in the dermis (Table 2). The staining of sections with the anti-human MHC-class II antibody is shown in Fig. 2c-e. Fig. 2c shows grafted human skin on BNX mice, graft not treated with allergen, Fig. 2d. shows grafted human skin on BNX mice, graft treated with allergen and Fig. 2e represents allergic affected human skin before transplantation. The difference in MHC-class II expression between the treated and untreated grafts is an indication for activation during the challenge phase. Human MHC-class II positive cells in the epidermal part were found in similar frequencies in the skin grafts on Balb/c and BNX mice as previously determined (6). No differences in frequencies of epidermal MHC-class II positive cells were found between the treated and the untreated human skin grafts. The observation of increasing MHC-class II expression after allergen application on human grafted skin is in agreement with previous reports in which an upregulation of MHC-class II after allergen application *in vitro* and *in vivo* was described in mouse skin (7).

>>***Figure 2.*** *Immunolocalization of human tissue antigens using immunoperoxidase staining in grafted human skin on BNX mice. MHC-class I positive cells in human skin graft treated with allergen (a) and in a biopsy from allergic affected human skin before transplantation (b). Immunolocalization of human MHC-class II positive cells in grafted human skin untreated (c), treated with allergen (d) and in a biopsy from allergic affected human skin (e). Absence of human CD3 positive cells in grafted human skin untreated (f) and treated with allergen (g). Presence of human CD3 positive cells in infiltrates in the dermis in a biopsy from allergic affected human skin (h). Immunolocalization of ICAM-1 positive cells in grafted human skin treated with allergen (i) and in a biopsy from allergic affected human skin (j). Magnifications x 200.*

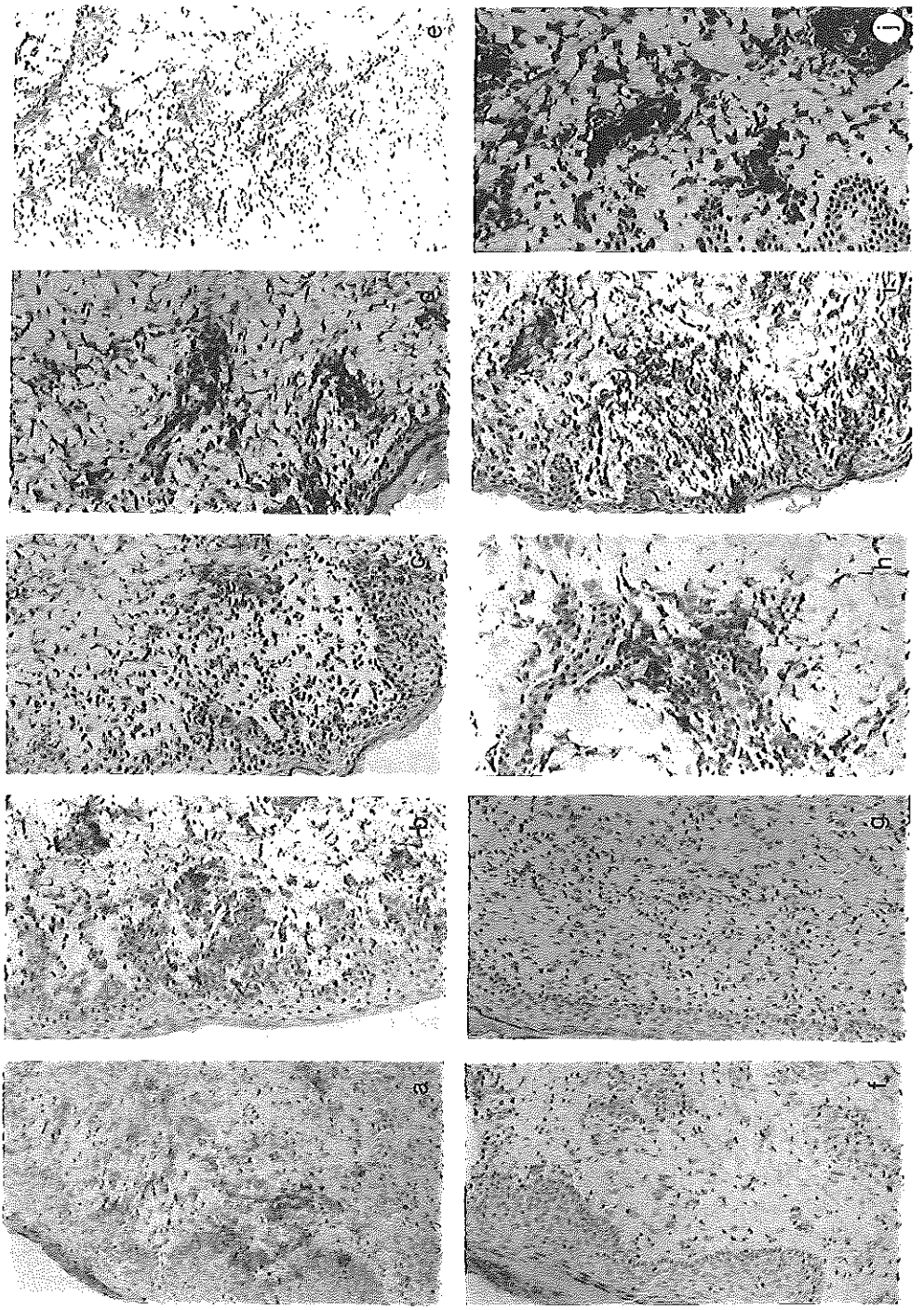
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Table 2. Summary of presence of cells expressing a specific cell-marker in allergen or non allergen treated human skin grafts on Balb/c-nude or BNX mice.*

	Balb/c-nude mice (n=4)				BNX mice (n=5)			
Cell-marker	not treated (n=2)		treated (n=2)		not treated (n=2)		treated (n=3)	
Epidermis/Dermis	E	D	E	D	E	D	E	D
Murine MHC-II (Ia)	0/2	2/2	0/2	2/2	0/2	2/2	0/3	3/3
Human MHC-I	2/2	0/2	2/2	0/2	2/2	2/2	3/3	3/3
Human MHC-II (HLA-DR)	2/2	0/2	2/2	0/2	2/2	0/2	3/3	2/3
Human CD3	0/2	0/2	0/2	0/2	0/2	0/2	0/3	0/3
ICAM-1	0/2	0/2	0/2	0/2	0/2	1/2	0/3	3/3
ELAM-1	0/2	0/2	0/2	0/2	0/2	0/2	0/3	1/3

Two sections at different heights for each antibody were scored on the presence of obvious positive cells (>5 per mm^2 dermis; >2 per mm epidermis). The sections of the human skin grafts onto Balb/c-nude mice were compared with sections from the human skin grafts onto BNX-mice. Moreover, sections of human skin grafts prior to allergen exposure were compared with sections of human skin grafts after 3 days of allergen exposure on a 2 week old skin graft.

* x/n; number of grafts with a positive graft score (=x) per total number of grafts (=n).

To investigate the capability of T-cells to home or to be recruited in the human skin graft, we analysed the frequencies of CD3 positive T-cells in the human skin grafts obtained from allergic affected skin and in allergic affected skin before transplantation. No human CD3 positive T-cells were detected in the different grafts. Fig. 2f and Fig. 2g are representative anti-CD3 stained sections of grafted human skin on BNX mice untreated (Fig. 2f) and treated with allergen (Fig. 2g). Fig. 2h is a representative section of allergic affected human skin before transplantation stained for CD3. So, by using this CD3-staining method we demonstrated that no human CD3 positive T-cells were present in the skin before or after challenge of a human skin graft onto immune-deficient mice. These results suggest that the T-cells disappear from the skin and persist in circulation in a very low frequency. Moreover, these data indicate that the circulated T-cells are not able to home to the human skin graft after allergen application.

To further address the requirement of homing and recruitment we investigated the presence of adhesion molecules ICAM-1 and ELAM-1. Homing of human T-cells has been proven to be dependent on the expression of adhesion molecules (8,9). ICAM-1 and ELAM-1 positive cells were not found in the human skin grafts on Balb/c-nude mice. In contrast, ICAM-1 and ELAM-1 positive cells were observed in treated human skin grafts on BNX mice. ICAM-1 and no ELAM-1 positive cells were found in untreated human skin grafts on BNX mice. In all three allergen treated human skin grafts on BNX mice few ICAM-1 positive cells were found in the dermal compartment and not in the epidermal compartment (Fig. 2i). This is in contrast to the high frequencies of ICAM-1 positive cells observed in allergic affected human skin before transplantation (Fig. 2j). In one out of three allergen treated human skin grafts on BNX mice relatively few positive ELAM-1 positive endothelial cells were found in skin grafts onto BNX mice as compared to ELAM-1 expression in human allergic skin before transplantation. The difference in adhesion molecule expression between the treated and untreated skin grafts is an indication for homing and recruitment possibilities in the graft after challenge. The disappearance of the ICAM-1 and ELAM-1 expression on endothelial cells after transplantation is probably due to the revascularization of the grafts by host microcirculatory bed (10).

In conclusion, this study indicates that activation of human cells in an allergic affected human skin graft 2 weeks after transplantation can be established as judged from the induction MHC-class II antigen expression. The present investigation suggests that based on expression of ICAM-1 and ELAM-1 also the homing functions will be intact. Using BNX mice, probably due to the NK-deficiency, human cells and tissues are better be preserved in the graft. As a result relatively more MHC-class II$^+$, ICAM-1$^+$ and ELAM-1$^+$ cells after allergen exposure could be observed as compared to Balb/c-nude mice. However, far less human T-cells were observed in the 2 week old allergic human skin graft 72 hours after allergen exposure as compared to human allergic affected skin before transplantation. This might be explained by the level of expression of the adhesion molecules present in the skin. It is possible that the very few ICAM-1 and ELAM-1 positive cells observed in the human skin are not able to allow homing and to recruit enough human T-cells from the circulation to the challenged skin. Therefore, extension of this study including human PBL cell-transfer after graft establishment will determine whether a higher frequency of human T-cells present in circulation increase the chance of human T-cells to home to the human skin graft after allergen application.

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Chapter 6

General Discussion

GENERAL DISCUSSION

In search of a marker to differentiate between human allergic and irritant contact dermatitis and the development of an *ex vivo* human allergic contact dermatitis response in a human-skin-onto-immune-deficient-mice model our study has focussed on cytokine analysis in human contact dermatitis and the prerequisites to establish an *ex vivo* human-skin-onto-immune-deficient-mice model that retains the possibility to develop an allergic contact dermatitis reaction.

In chapter 2 we discuss the ultimate advantage of the *in situ* approach which lies in the ability to study the localization and activity of cytokines in relation to their direct unchanged original microenvironment (including the demonstration of cell-cell interactions), in all types of tissue at any given moment, without inducing artefacts by manipulations such as cell separation and (pre) culture. A variety of immuno-histochemical methods and a variety of *in situ* hybridization methods are used in different studies to analyse cytokine production. These methods are generally applicable. However, the experimental character of various methods and the interpretation of staining patterns in specific tissue compartments requires specific training and expertise. We propose to select the method of choice on the reagents available, individual preference and the amount of cytokine produced in one specific cell. Signal amplification with a specific staining method based on a high signal to noise ratio is often a prerequisite to detect human cytokines *in situ*. The indirect two step method using biotinylated primary anti-cytokine antibody and subsequently streptavidin-HRP/AP together with the indirect three step method using unlabelled primary anti-cytokine antibody, a secondary biotinylated antibody and streptavidin HRP/AP are the most frequently used methods. This choice was mainly based on the efficient signal amplification steps. However, also the indirect two step method based on an unlabelled primary anti-cytokine antibody and an enzyme-labelled secondary antibody is frequently used in spite of an intermediate signal to noise ratio. The last choice is mainly based on its simplicity.

We found that for cytokine-mRNA detection radioactive ^{35}S labelled cRNA probes are mainly used. However, in more recent literature, this preference of using ^{35}S labelled cRNA probes has shifted to non-radioactive labelled oligonucleotide probes due to the high specific signal obtained, easy production and labelling, no need of radioactive facilities, low reagents costs and rapid processing.

To determine in which lymphoid compartment the T-B cell interactions required for antibody production take place in human lymphoid tissue we studied the localization of cytokine and antibody producing cells in human palatine tonsil. For this we

established the usefulness of the indirect cytokine detection method. The intracellular cytokines were demonstrated using specific anti-cytokine antibodies and enzyme-labelled secondary antibodies. Intracellular cytoplasmic cytokine staining was observed.

Using this staining method co-localization of cytokine producing cells and antibody forming cells in the extrafollicular area similar as described in murine spleen (1) was observed. This indicates that in human lymphoid tissue T-B cell interactions take place in the extrafollicular area. Most likely, antigens are taken up and processed by macrophages or interdigitating cells in the extrafollicular area. After this, upon contact, antigen-specific T-cells are activated and proliferate. B-cells inside the germinal centre, binding soluble or processed antigen, migrate into the mantle zone and extrafollicular area. Subsequently, these B-cells encounter the specific T-cells in the extrafollicular area, and cognate T-B cell interactions result in cytokine and antibody production, including isotype switching.

In chapter 3, we discuss the *in situ* detection of cytokine production in allergic and irritant contact dermatitis in man. We used the same assay to detect cytokine producing cells as described for the tonsil as well as a new assay to detect cytokine-mRNA producing cells. The immunohistochemical findings with respect to the T-cell derived cytokines IL-2 and IFN-γ were confirmed with the *in situ* hybridization findings. Moreover, to detect IL-4 and IL-1ß we developed two different sensitive staining methods with high signal to noise ratios. IL-4 producing cells were demonstrated using a three step method including anti-cytokine antibody, biotinylated secondary antibody and enzyme-labelled streptavidin. IL-1ß producing cells were demonstrated using biotin labelled anti-cytokine antibody and enzyme-labelled streptavidin.

It is known that T-cells, epidermal cells and macrophages are involved in contact dermatitis (2,3). Moreover, it is known that there are cytokine networks in the skin (4-11). IL-2, IL-4, IFN-γ and IL-10 are cytokines produced by T-cells. IL-1, IL-10 and TNF-α are cytokines produced by epidermal cells. IL-1 and TNF-α are cytokines produced by macrophages. These cytokines are produced during an inflammatory response and represent the cytokine profile of the response.

In allergic contact dermatitis but not in irritant contact dermatitis the presence of low numbers of specific activated memory T-cells is described (2,12-14). Therefore, it was thought that the T-cell derived cytokines were produced in allergic contact dermatitis and not in irritant contact dermatitis. Interestingly, based on the detection of IL-2, IL-4, IL-10 and IFN-γ producing cells in the dermis, we could not establish significant differences between the frequencies of cytokine producing cells in allergic contact dermatitis as compared to irritant contact dermatitis. Furthermore, in both

reaction types IL-2 and IFN-γ producing cells as well as IL-2-mRNA and IFN-γ-mRNA producing cells were observed. This suggested that naive non-specific T-cells might be activated in the allergic as well as the irritant reactions. In addition, our results are consistent with the experiments of Thomson et al. (15) who demonstrated that Th2 cytokines could also be produced in a Th1 contact hypersensitivity response and suggests that a similar balance between Th1 and Th2 cells exists in allergic and irritant reactions. The presence of IL-4 suggests a critical role for IL-4 (16), whereas the presence of IL-10 suggests an immunosuppressive mediator role (9,17-20) in irritant contact dermatitis as already established in allergic contact dermatitis.

IL-1α and TNF-α are pro-inflammatory cytokines produced by epidermal cells and macrophages. The involvement of IL-1α and TNF-α in allergic and irritant contact dermatitis was demonstrated by *in vivo* modulation experiments in man (21,22). IL-1ß and IL-10-mRNA are produced by epidermal cells. They are produced after allergen and not after irritant application in a mouse model (4,5). These data suggest that there could be a difference between IL-1ß and IL-10 production in allergic and irritant reactions in man as found in mice. The staining patterns in the epidermis of IL-1α, IL-1ß, IL-10 and TNF-α suggest that these cytokines are produced in relatively large amounts due to allergen or irritant application as compared to normal skin. In contrast to mice, minor differences in IL-1ß and IL-10 expression between both reaction types were observed in man. It appears that the IL-10 positive cells were keratinocytes and the IL-1ß positive cells were keratinocytes and dermal Langerhans cells in both reaction types. The discrepancies between our study in man and the mouse study could be explained by the difference in species, time-point (72h versus 24h), and the techniques we used as compared to Enk et al. (4,5). In contrast to Enk et al. (4,5) who studied the primary immune response in the skin, we studied the secondary immune response.

From chapter 3 it is clear that similar frequencies of cytokine producing cells are observed in both allergic and irritant contact dermatitis. In line with this result that there are no differences in frequencies of cytokine producing cells is the observation that similar changes in Langerhans cell populations are found in irritant contact dermatitis as compared to allergic contact dermatitis. Migration of Langerhans cells to the draining lymph nodes do occur in irritant as well as allergic contact dermatitis (23-27). In addition, the cells derived from lymph vessels after irritant application possess increased antigen presenting and proliferative capacities confirming the presense of Langerhans cells and/or macrophages and also functionally active T lymphocytes (28). Similar immune-associated surface antigens are expressed in both reaction types. Intercellular adhesion molecule-1 is such an antigen, which is known to play a role in leucocyte adhesion. It is expressed by keratinocytes in both allergic and irritant contact

dermatitis, and is also upregulated on the surface of vascular epithelium (29,30). The leucocytes in the cellular infiltrates in the allergic and irritant reactions consist of similar phenotypic profiles (31). After allergen or irritant application keratinocytes are able to express MHC class II molecules (31,32). Moreover, as observed for murine antigen-carrying Langerhans cells *in vivo,* after allergen skin application cell rosettes consisting of a Langerhans cell with lymphocytes could be observed in human skin lymph derived from irritant contact dermatitis (33). Thus, the conclusion must be drawn that common inflammatory pathways are operating in both allergic and irritant contact dermatitis. As a consequence we still felt the need for further investigations to find a differentiation marker in contact dermatitis.

Cytokine based immune modulation is the result of a critical balance between actions of different cytokines. This indicates that more attention has to be paid to cytokine profiles as we show in chapter 3.3. In that chapter differences were found between the ratios of frequencies of different cytokine producing cells in the allergic and irritant affected skin biopsies. The development of a routine double or multi staining method to detect two or more cytokines in one section is crucial in determining the exact cytokine profiles. It could well be that based on the detection of Th0, Th1 and Th2 cells at an early time point using a multi staining method allergic and irritant contact dermatitis responses can be differentiated in the future.

In chapter 4 we investigated the role of CD40 ligand-CD40 interaction in contact hypersensitivity (DTH) in mice. We did this to establish the possibility to use the T-cell activation marker "CD40 ligand" as differentiation marker between allergic and irritant contact dermatitis in man. It was shown that CD40 ligand and CD40 positive cells were found in human allergic affected skin. Furthermore, the kinetics of appearance of CD40 ligand, CD40 positive cells, cytokine producing cells and CD4 positive cells observed in contact hypersensitivity in mice confirmed the observations made in man. Therefore, it was hypothesized that CD40 ligand-CD40 interaction might be essential in contact hypersensitivity responses after skin exposure to an allergen. We evaluated the cellular immune response (DTH) in a mouse model after skin exposure to the contact allergen dinitrochlorobenzene (DNCB) and anti-CD40 ligand antibody treatment.

The effect of anti-CD40 ligand antibody treatment on the local humoral immune response was investigated to determine the role for CD40 ligand-CD40 interaction on local specific antibody production after allergen application on the skin. Treatment of mice with anti-CD40 ligand specific antibody before sensibilization and after sensibilization resulted in a complete inhibition of the secondary antigen specific local antibody response after antigen skin application. These results indicate that the antibody treatment was effective. Moreover, these data indicate that a specific local

humoral immune response due to skin exposure to a contact allergen (DNCB) as was found for a specific humoral immune response after i.v. antigen immunization (TNP-KLH) (34) could be modulated by anti-CD40 ligand antibody treatment.

In the same group of mice, in which we demonstrated a reduction of the frequency of antigen specific antibody forming cells, ear swelling (DTH) was not found to be influenced by anti-CD40 ligand antibody treatment. These results suggest that blockage of the interaction between CD40 ligand expression on T-cells and CD40 expression on Langerhans cells is not sufficient to prevent contact hypersensitivity. Therefore, it is concluded that the CD40 ligand-CD40 interaction is functional predominantly in the humoral immune response caused after skin exposure to a contact allergen.

It could well be that the few CD40 ligand positive cells observed in contact hypersensitivity in mice and allergic contact dermatitis in man are specific activated memory T-cells. This is supported by the knowledge of the presence of a minor fraction of specific activated memory T-cells in allergic contact dermatitis. This suggests that characteristics of these activated memory T-cells are not useful as possible differentiation markers.

Today, allergic contact dermatitis as compared to irritant contact dermatitis in man still represents a serious problem by the difficulties that arise if the dermatitis is an occupational allergic contact dermatitis. Irritant contact dermatitis can be prevented by wearing special clothes or gloves, but allergic contact dermatitis is difficult to prevent due to the low concentration of a chemical which can cause the reaction. Total avoidance of the responsible allergen represents a definite solution in allergic contact dermatitis. However, frequently the responsible allergen is unknown or difficult to avoid in cases where the allergen is abundantly present in the (occupational) environment.

If there is a suspected allergen the epicutaneous patch test is used to determine the individual sensitivity. The most important disadvantage of this test is the risk of sensitization the person during the test. A routine test to establish the predisposition of an individual to be sensitized in the future for a given allergen is not available yet. Therefore, an *ex vivo* model suitable to establish the individual sensitivity or predisposition to be sensitized in the future is of great value to occupational health practice. The introduction of a human-skin-grafted-immune-deficient-mice model that retains the challenge phase with or without the sensitization phase of an allergic contact dermatitis reaction may provide an alternative way to develop a human allergic contact dermatitis response *ex vivo*. To establish the individual sensitivity *ex vivo* the prerequisites of the model are: the persistence of human antigen presenting cells for the uptake of allergen and migration, the persistance of antigen specific T-

cells and non-specific T-cells in the graft or circulation during several weeks, and signals for homing or recruitment of human T-cells to the human skin graft after challenge. To establish the individual predisposition to be sensitized in the future also the sensitization phase has to be included in this model. Upon sensitization human allergen-specific T-cells have to proliferate in the mouse draining lymph node and become memory T-cells.

We studied the functional role of human antigen presenting cells "Langerhans cells" in human skin grafts after transplantation onto immune-deficient mice. A fluorescent allergen was painted on the human skin graft and both fluorescent microscopy as well as immunohistochemical staining techniques were used to address the question whether human Langerhans cells take up the antigen and migrate to the mouse draining lymph node. So, we extended the classical human-onto-nude-mouse-skin-grafting experiments done by Krueger et al. (35) and the contact sensitivity experiments with fluorescent allergen (36-39). Human MHC-II positive cells with a dendritic appearance and fluorescent-carrying cells were found in the draining lymph node of painted human skin grafted mice. This strongly suggests that human Langerhans cells in human skin after transplantation on immune-deficient mice are capable to take up antigen and to migrate after stimulation.

Langerhans cells migrate to the dermal compartment (challenge phase) or lymph node (sensitization phase) to present the antigen to T-cells in order to initiate an appropriate antigen-specific immune response. In case of a human-skin-onto-immune-deficient-mice it can be questioned whether it is possible to initiate the challenge phase of an allergic contact dermatitis reaction. In this thesis human skin biopsies used for transplantation were obtained from allergic affected skin 72 hours after allergen application and the effect of allergen application on the human skin grafts onto immune-deficient mice was investigated. By using allergic affected skin, in which human T-cells are present, we tried to overcome cell transfer. However, human T-cells were not observed in the human skin grafts after challenge due to the disappearance of the T-cells from the human skin graft 2 weeks after transplantation and the low frequency of human endothelial cells expressing adhesion molecules in the grafts. The few ICAM-1 and ELAM-1 positive cells observed in the human skin graft are not able to allow homing and to recruit enough human T-cells from the circulation to the challenged skin. In contrast to our results Yan et al. (40,41) demonstrated human cell recruitment into human skin after intradermal TNF-α injection and an i.v. injection of human neutrophils. They found that human cell recruitment into human skin transplanted onto severe combined immunodeficient (SCID) mice induced by TNF-α is dependent on E-selectin (ELAM-1). The grafted skin used in these experiments retained its human vasculature 4-6 weeks after grafting in contrast to our results seen in grafted Balb/c-nude and BNX-mice and other

reports describing the revascularization of human skin grafts onto nude mice (42,43). The discrepancy may be due partly to the transplantation methods or the different species of mice or the amount or type of human cells present in the circulation. Supposedly, human endothelial cells with their adhesion molecules and T-cells play an important role in the challenge phase of allergic contact dermatitis in a human skin graft. This suggests that human endothelial cells and T-cells have to be present in a human-skin-onto-immune-deficient mice to induce an efferent allergic response. Human PBL cell-transfer after graft establishment will determine whether a higher frequency of human T-cells present in circulation increase the chance of human T-cells to home to the human skin graft after allergen application.

To establish the predisposition of a person to be sensitized in the future both the sensitization phase as well as the challenge phase have to be included in the human-skin-onto-immune-deficient-mice model. In view of the fact that human Langerhans cells in the skin graft are capable to take up antigen and migrate to the lymph node in a limited period (window) 2-3 weeks after transplantation (chapter 5.1) we expect that the induction of an *ex vivo* human allergic contact dermatitis response (including the sensitization and challenge phase) in a human-skin-onto-immune-deficient-mice model reconstituted with human PBL's would be difficult to achieve. Firstly, the allergic contact dermatitis response is based on a sensitization and challenge phase, and therefore needs several weeks to develop in a human-skin-onto-immune-deficient-mice. Secondly, our results and results from other studies suggest that adhesion molecules have to be present on human endothelium in the skin graft for homing of (antigen specific) human T-cells to the human skin graft (44,45). Based on the first point it could be easier to develop a human-skin-onto-immune-deficient-mice model to establish the individual sensitivity, because only the challenge phase has to be included for this purpose.

Further investigations may contribute to the development of a model to establish the *ex vivo* individual sensitivity or to predict the individual predisposition to be sensitized in the future.

GENERAL CONCLUSIONS

Different successful antibody and probe based *in vivo* human cytokine detection methods are available and generally applicable. Signal amplification is important for human cytokine detection.

T-B cell interactions occur in the extrafollicular area of human palatine tonsils.

Similar frequencies of cytokine producing cells are present in human allergic and irritant contact dermatitis *in vivo*. The study of cytokine profiles may be very useful.

CD40 ligand (gp39) and CD40 positive cells are present in contact hypersensitivity in mice and allergic contact dermatitis in man. CD40 ligand-CD40 interaction does play a functional role in the local humoral immune response but not in a contact hypersensitivity response after skin exposure to a contact allergen in mice.

Human Langerhans cells present in the human skin graft on immune-deficient mice are capable to take up antigen and to migrate to the murine draining lymph node after allergen application on the graft

Homing and recruitment of human T-cells do not seem to occur in a human allergic skin graft onto immune-deficient mice without cell-transfer after challenge .

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SUMMARY

The aim of the studies presented in this thesis was to find a differentiation marker between human allergic and irritant contact dermatitis, and to develop an *ex vivo* model to establish the individual sensitivity or predisposition to be sensitized in the future for a specific allergen.

Special emphasis was put on the development of antibody and probe based *in situ* detection methods (Chapter 2). We analysed the existing literature with respect to cytokine detection in human tissue and we presented these different methods, their advantages and disadvantages. Furthermore, the ultimate advantage of the *in situ* approach and the essential specificity controls for the antibody and probe based detection methods are listed and described. Studies using the *in situ* cytokine detection approach to analyse the presence of cytokine producing cells in human palatine tonsil and in both allergic and irritant contact dermatitis are described in chapter 2.2 and chapter 3 respectively. Since similar cytokines are produced in both the allergic and irritant affected skin sites, we wondered whether CD40 ligand (gp39) a T-cell activation marker, could be used as a differentiation marker between allergen and irritant contact dermatitis. Therefore, we studied the functional rol of CD40-CD40 ligand interaction in DTH reactions in mice in *in vivo* modulation experiments using anti-CD40 ligand monoclonal antibody treatment (chapter 4).

To develop an *ex vivo* model to establish the individual sensitivity or predisposition to be sensitized for a specific allergen we first questioned whether the functional role of antigen presenting cells "Langerhans cells" within the human skin graft grafted onto immune-deficient mice is preserved (chapter 5.1). Secondly, the possibility to imitate the efferent phase in a human-skin-onto-immune-deficient-mice model was studied using human allergic skin grafts and specific allergen application on the skin graft (chapter 5.2).

In chapter 2 the evident advantages of the *in vivo* approach, which allow the localization, activity, and interactions of various cells and cell products, antibodies or cytokines, in relation to their direct unchanged original micro-environment, were described. It is concluded that the *in vivo* approach can be used with all types of tissue at any given moment, provided biopsies can be taken, without inducing artefacts by manipulations like cell separation and culture. A variety of immunohistochemical methods and a variety of *in situ* hybridization methods could be found in different studies to analyse cytokine production. We concluded that selection of the method of choice will depend on availability of reagents, individual preference and the amount of cytokine produced in one specific cell. To amplify the signal a staining method which resulted in a high signal to noise ratio was necessary. To test the specificity of the

anti-cytokine antibody or the correctness of the staining procedure several different controls have to be incorporated in the assay. The indirect two step method using biotinylated primary anti-cytokine antibody and subsequently streptavidin-HRP/AP together with the indirect three step method using unlabelled primary anti-cytokine antibody, a secondary biotinylated antibody and streptavidin HRP/AP are the most frequently used methods mainly based on the amplification steps included. Although less sensitive, the indirect two step method based on an unlabelled primary anti-cytokine antibody and an enzyme-labelled secondary antibody is also frequently used due to its simplicity.

Considering cytokine-mRNA detection we concluded that mainly radioactive ^{35}S labelled cRNA probes were used. However, recent reports show that this preference of using ^{35}S labelled cRNA probes has shifted to non-radioactive labelled oligonucleotide probes due to the high specific signal obtained, no safety problems, no need of radioactive disposal facilities, no high reagent costs, easy production and labelling, and rapid processing.

The usefulness of the *in situ* immunohistochemical approach to detect cytokine producing cells is shown in the human tonsil. To determine which cytokines are important in the humoral immune response *in vivo* and in which anatomical compartment(s) the T-B cell interactions are localized we studied the localization of cytokine producing cells together with antibody forming cells in human palatine tonsil after recurrent tonsillitis. Co-localization of cytokine producing cells and antibody forming cells in the extrafollicular area was found. This points out that in human lymphoid tissue T-B cell interactions take place in the extrafollicular area.

In chapter 3 the cytokine profiles are studied in allergic and irritant contact dermatitis at the time of final reading of the epicutaneous patch test 72 hours after allergen application. Minor differences were found between the frequencies of the T-cell derived cytokine producing cells or the epidermal cell derived cytokine producing cells in the allergic group of patients as compared to the irritant group of patients. Therefore, the cytokines produced by T-cells (IL-2, IL-4, IL-10, IFN-γ) and epidermal cells (IL-1α, IL-1ß, TNF-α, IL-10) are considered to play an important role in the development of the inflammatory response in both types of contact dermatitis. These data are in agreement with data obtained in other studies in which no differences were found between allergic and irritant reactions based on the expression of adhesion molecules, cell phenotypes and other cell characteristics. However, as we show in chapter 3.3 some of these problems could be solved when differentiation is made based on the difference between the ratios of frequencies of different cytokine producing cells in allergic and irritant affected skin biopsies.

To try to find a differentiation marker we also investigated the role of CD40 ligand and CD40 in contact hypersensitivity in mice as well as in allergic contact dermatitis in man (chapter 4). CD40 ligand and CD40 positive cells were detected in allergic affected skin in both human as well as murine allergic affected skin; CD40 ligand in the dermal compartment and CD40 on epidermal and dermal cells with an evident dendritic appearance. Treatment of mice with anti-CD40 ligand antibodies before or after DNCB skin sensibilization demonstrated that the CD40 ligand-CD40 interactions are essential for the generation of secondary follicles and antigen specific antibody forming cells. But, the DTH response against DNCB after *in vivo* treatment of mice with anti-CD40 ligand antibodies was unaffected. In conclusion, these results indicate that CD40 ligand expression is not important during a specific immune response in the skin and could therefore not be used to differentiate between allergic contact dermatitis and irritant contact dermatitis.

To develop an *ex vivo* human-skin-graft-onto-mice model to establish the individual sensitivity or predisposition to be sensitized in the future for a specific allergen chapter 5.1 describes the important immunological events such as antigen-uptake by antigen presenting cells and migration of these cells to the draining lymph nodes in the human-skin-graft-onto-mice model after topical application with the fluorescent allergen RITC. Furthermore, chapter 5.2 describes the detection of human and murine MHC, human CD1a and CD3, and human ICAM-1 and ELAM-1 expressing cells in the human-allergic-skin-graft-onto-mice model after topical application with the specific known allergen.

As described in chapter 5.1 the frequencies of human and murine MHC class II positive cells in the skin grafts after transplantation and after RITC application are related to the frequencies of RITC-carrying and human MHC class II positive cells found in the draining lymph nodes before and after RITC application on the skin graft using *in situ* immunohistochemical staining methods. Grafting resulted in a time-dependent decrease of human MHC-IIDR$^+$ and CD1a$^+$ cells and an increase of mouse MHC-II-Ia$^+$ cells inside the graft. Application of RITC on a 3 week old human skin graft showed optimal migration capability as compared to 6 or 9 week old grafts. The time-dependent increase of frequencies of RITC$^+$ and MHC-IIDR$^+$ cells in the draining lymph nodes and the time-dependent decrease of MHC-IIDR$^+$ cells in the 3 week old human skin graft were concurrent. Supporting these data, human cytokine IL-1α, IL-1β and TNF-α analysis *in situ* revealed that cytokine production by keratinocytes, a property associated with dendritic cell migration, was preserved in the human skin graft. These data suggest that like dendritic cells in contact sensitization in allografted skin, dendritic cells from human xenografted skin onto nude mice are capable to migrate to mouse draining lymph nodes after allergen application. This

study revealed that induction of contact hypersensitivity is possible in a human-skin-graft-onto-nude-mice-model, although the use of this *ex vivo* model to analyse contact sensitivity is probably limited to 3 weeks after transplantation.

Analysis of the allergic human skin grafts in chapter 5.2 revealed that after grafting of human allergic affected skin onto Balb/c nude or BNX mice T-cells disappear from the graft. Moreover, less ICAM-1, ELAM-1 and MHC-class II expression was observed in the dermal compartment after grafting human allergic affected skin. However, after comparing the treated and untreated human allergic skin grafts onto BNX mice we found a difference in frequencies of MHC-class II and ICAM-1 positive cells. This is an indication for activation during the challenge phase. Human CD3 positive cells were not found in the treated skin grafts. We concluded that the level of expression of adhesion molecules in the skin and the low frequencies of human T-cells in the human skin graft or in circulation might cause the absence of T-cell infiltration in the human skin graft after challenge. This suggests that perhaps human PBL cell-transfer after graft establishment will be useful to increase the chance of human T-cells to home to the human skin graft after allergen application.

In chapter 6 the major points emerging from the studies are discussed in the light of data presented in the literature. In spite of the fact that the mechanisms between allergic and irritant contact dermatitis are different, no differences were found between these two types of inflammatory skin reactions with regard to cytokine production. These observations suggest that there are perhaps only minor differences which do not allow to discriminate between the two immune responses

SAMENVATTING (ook voor de niet-immuno/toxicoloog)

Inleiding en doel van de studie

Lichaamsvreemde stoffen kunnen interfereren met de normale functie van het afweersysteem (immuunsysteem) van de mens. Van een aantal stoffen is bekend dat zij, na enkele malen in contact te zijn geweest met de huid, verschijnselen in de huid oftewel een allergische (overgevoeligheids) reactie kunnen veroorzaken. Van andere stoffen is bekend dat zij, na verscheidene malen in contact te zijn geweest met de huid, een irritatieve (toxische) reactie veroorzaken. Er is behoefte aan betere criteria om het type contact dermatitis, allergisch of irritatief, in het laboratorium te kunnen vaststellen. Anderzijds zijn er nog geen goede gevalideerde testmethoden beschikbaar voor het bepalen van de gevoeligheid (inclusief predispositie) van een gegeven persoon om een allergische reactie te ontwikkelen als deze persoon in contact komt met een allergische stof (allergeen of ook wel antigeen genoemd).

Het afweersysteem is betrokken bij het instandhouden van het evenwicht van alle functies in het lichaam. De primaire taak is de herkenning en verwijdering van infectieuse micro-organismen (bacteriën, schimmels virussen en parasieten) en kankercellen. Bovendien kunnen afweerreacties ontstaan tegen transplantaten, voedsel en lichaamsvreemde stoffen. Het afweersysteem in het lichaam kan onderverdeeld worden in twee takken: de "verkregen" of specifieke respons en de "aangeboren" of niet-specifieke respons. De laatste onderscheidt zich van de eerste doordat de weerstand niet verbetert na herhaalde infecties (geen geheugen) en door het grotendeels niet-specifieke karakter (geen herkenning). Mogelijkheden voor niet-specifieke afweer zijn bijvoorbeeld: opname van deeltjes door macrofagen en de werking van maagzuur en slijm. De specifieke afweer kan op zijn beurt weer onderverdeeld worden in: de humorale respons en de cellulaire respons. Beide responsen zijn een specifieke reactie gericht tegen een binnendringend lichaamsvreemde stof of organisme (antigeen), en zijn dus afhankelijk van herkenning.

De humorale respons bestaat uit het produceren van afweerstoffen door B-cellen die het antigeen binden en vervolgens via diverse mechanismen verwijderen. Voorafgaande aan afweerstofproductie moet het antigeen meestal eerst door een antigeen-presenterende cel (b.v. dendritische cellen, sommige macrofagen of Langerhans cellen in de huid) opgenomen en verwerkt worden om doorgaans via de T-helper-cellen aan de B-cel aangeboden te worden. De productie van mediatoren, de cytokinen, door met name T-cellen speelt hierbij een zeer belangrijke regulerende rol, zowel in de presentatie als de uiteindelijke effectorfase. Bij de cellulaire afweerrepons vindt de vernietiging van het antigeen plaats door een cel-cel interactie waarbij de T-cellen centraal staan.

Zowel T-cellen als afweerstoffen spelen een belangrijke rol in humorale en cellulaire afweerresponsen. Ook door de overlappende (één cytokine meerdere functies) en pleiotrope (één functie door meerdere cytokinen) effecten van door T-cellen geproduceerde cytokinen zijn beide responsen niet strikt te scheiden. Om het netwerk nog gecompliceerder te maken moeten we ook rekening houden met de genetische make-up van de transplantatie-antigenen (MHC/HLA) die bij iedere T-cel gemedieërde stap in de afweerrespons betrokken zijn en die bepalen of een antigeen wel of niet als lichaamsvreemd gezien wordt. Variabiliteit van MHC moleculen is een belangrijke reden voor de inter-individuele variatie in afweerresponsen die gevonden wordt in een willekeurige blootgestelde populatie.

Contact dermatitis

Huidaandoeningen door lichaamsvreemde stoffen in het bijzonder allergische en irritatieve huidaandoeningen behoren tot de meest frequent voorkomende beroepsgebonden aandoeningen. Er zijn verschillen tussen enerzijds "allergische huidreacties", die kunnen ontstaan bij het contact met lage concentraties van stoffen en alleen bij daarvoor "overgevoelige" personen en anderzijds "irritatieve huidreacties" die bij iedereen kunnen optreden indien de concentratie van de stoffen maar hoog genoeg is. Verschillende allergenen kunnen alleen een allergische huidreactie veroorzaken, maar daarnaast bestaan er ook stoffen die zowel een allergische als een irritatieve huidreactie kunnen veroorzaken. Differentiatie tussen beide typen huidreacties, nodig met het oog op te nemen maatregelen en interventies, is niet altijd goed mogelijk.

Om een bijdrage te leveren aan het ontwikkelen van een gevalideerd testsysteem met geschikte criteria bestudeerden wij het cytokine-profiel in huidbiopten van allergische en irritatieve huidaandoeningen. Naast cel-cel interacties, (die o.a. plaats vinden door CD40 ligand (gp39) en CD40 interactie), vindt regulatie van de afweerrespons plaats door cytokinen; oplosbare factoren, eiwitten met een laag molekuulgewicht (<80kD) die zeer effectief werken, vaak op korte afstand en in zeer lage concentraties. Gedurende de laatste jaren zijn niet alleen een groot aantal "nieuwe" cytokinen beschreven, maar is ook duidelijk geworden dat deze stoffen in een zeer complex netwerk functioneren. Bestudering en ontleding van dit netwerk wordt bemoeilijkt door de pleiotrope en overlappende effecten alsmede door het feit dat cytokinen zowel elkaar als de vorming van cytokine receptoren induceren. Bovendien kunnen cytokinen elkaar stimuleren, remmen of samenwerken, en deze functie is afhankelijk van de lokale situatie. Mede door toepassing van de twee nieuwe technieken immunohistochemische detectie van cytokinen (detectie van eiwitten door afweerstoffen) en in situ hybridisatie van cytokine-mRNA (detectie van cytokine-mRNA met behulp van complementaire genetische probes) zal het netwerk steeds

beter beschreven worden.

Al eerder zijn de karakteristieke kenmerken van allergische en irritatieve huidreacties bestudeerd om zo mogelijk verschillen aan te tonen tussen beide reacties. Kleine verschillen zijn aangetoond, maar tot nu toe is het nog onmogelijk om duidelijke verschillen aan te tonen waarop beide reacties kunnen worden onderscheiden.

Met het toenemen van de kennis over de pathofysiologische aspecten van contact dermatitis is gebleken dat immunohistochemische en in situ hybridisatie technieken, die cytokine(mRNA) producerende cellen zichtbaar kunnen maken onder de microscoop, mogelijk hanteerbare criteria kunnen leveren.

Allergische contact dermatitis wordt veroorzaakt door een specifieke T-cel gemedieerde respons, terwijl irritatieve contact dermatitis wordt veroorzaakt door een niet-specifieke respons, op basis van een irritatieve invloed. Dit gegeven samen met de wetenschap dat Interleukine-2 (IL-2), IL-4, IL-10 en Interferon-gamma (IFN-γ) specifiek worden geproduceerd door T-cellen, en Tumor Necrosis Factor-alpha (TNF-α) en Interleukine-1 alpha en beta (IL-1α en ß) algemeen geproduceerde cytokinen zijn, kunnen mogelijk een belangrijke bijdrage leveren aan additionele criteria voor differentiatie tussen allergische en irritatieve huidreacties.

Het onderzoek beschreven in dit proefschrift toont aan dat in huidcoupes van allergische of irritatieve aangedane huid 72 uur na aanbrengen van het allergeen of irritans verhoogde expressie van IL-1α, IL-1ß, TNF-α, IL-2, IL-4, IL-10 en IFN-γ te vinden is, terwijl in de normale huid van deze patienten de expressie van TNF-α, IL-2, IL-4 en IFN-γ in de huidcoupes bijna negatief is. Er is wel expressie van IL-1α en IL-1ß in de epidermis in normale huid. Omdat na kwantificeren van het aantal cytokine producerende cellen geen significante verschillen gevonden werden tussen allergische en irritatieve contact dermatitis geeft deze ontwikkeling aan dat de betreffende cytokinen op zichzelf geen bruikbare differentiatie markers zijn. Daarentegen, laten de ratios van frequenties van verschillende cytokine producerende cellen zien (IL-4:IL-10:IL-1ß) dat het differentiatie probleem mogelijk wel oplosbaar is.

Omdat CD40 kan worden aangetoond op humane Langerhans cellen, CD40 ligand verhoogd tot expressie wordt gebracht op T-cellen in zowel humorale als cellulaire afweerresponsen en beide celtypen een belangrijke rol spelen in allergische huidreacties hebben wij de functionele rol van de CD40 ligand-CD40 interactie in allergische huidreacties in de muis bestudeerd. De resultaten van dit onderzoek geven aan dat CD40 ligand en CD40 zowel in muize als in humane allergische huidreacties tot expressie wordt gebracht, maar dat daarentegen geen functionele rol van de CD40 ligand-CD40 interactie in de allergische huidreactie is gevonden. Hieruit blijkt dat op

basis van CD40 ligand expressie in allergisch en irritatief aangedane huid waarschijnlijk geen differentiatie mogelijk is tussen beide typen.

Keuze van model

Het ideale testsysteem of experimenteel model om de gevoeligheid en predispositie van een gegeven persoon voor een bepaalde allergische stof op te sporen moet aan een aantal voorwaarden voldoen. Bij voorkeur dient met humaan materiaal of met een gehumaniseerd proefdier-systeem, dat voor de mens relevante informatie geeft, gewerkt te worden. In een ideaal model zullen alle drie de fases van de afweerrespons, herkenning van het antigeen, activatie en differentiatie van lymfocyten en de uiteindelijke effectorfase, vertegenwoordigd moeten zijn.
In een dierexperimenteel model ter voorspelling van de gevoeligheid van een gegeven persoon voor een allergeen moet het mogelijk zijn om zowel gedurende een langere periode evenals met relatief lage doses, én via de "natuurlijke route" te werken. Het grootste probleem bij validatie van dergelijke tests is de relevantie ervan voor de mens.

Nieuwe benaderingen bij de ontwikkeling van proefdiermodellen en research technieken waarmee de extrapolatieproblematiek dier-mens vooral ten aanzien van effecten van lichaamsvreemde stoffen op het afweersysteem en de gevolgen daarvan op de huid mogelijke ondervangen kan worden, zijn: immunodeficiënte muizen. De naakte muizen bezitten geen thymus en dus geen T-cellen en de SCID muizen bezitten geen functionele T en B-cellen. Dit maakt deze muizen bij uitstek geschikt voor transplantatie studies en reconstitutie met humaan lymfoïd weefsel. Het testen van de effecten van allergische stoffen op humane huidtransplantaten, bij muizen die gereconstitueerd zijn met humaan lymfoid weefsel, kan als een bijna volledig gehumaniseerd testsysteem gezien worden (full house mouse).

De studies beschreven in dit proefschrift, met betrekking tot de ontwikkeling van een contact dermatitis model, zijn grotendeels gericht op het vermogen van humane cellen in het huidtransplantaat om de allergische stof op te nemen en te transporteren naar de drainerende lymfklier. Daarnaast worden in dit proefschrift de resultaten van het transplanteren van humaan allergisch aangedane huid op muizen beschreven met betrekking tot het vermogen om in deze transplantaten opnieuw een allergische reactie op te wekken. Hoewel de humane Langerhans cellen in een humaan huid transplantaat het allergeen kunnen opnemen en dit kunnen transporteren naar de drainerende lymfklier van de muis, blijft dit vermogen beperkt tot enkele weken na transplantatie. Deze resultaten samen met de bevindingen met betrekking tot het transplanteren van allergisch aangedane huid waarin veel essentiële humane kenmerken verloren gaan bevestigen het vermoeden dat het ontwikkelen van een humaan *ex vivo* contact dermatitis model niet makkelijk is en nog veel onderzoek vereist.

Inhoud van het proefschrift

In hoofdstuk 1 wordt het doel van de studies in dit proefschrift besproken en wordt uitgelegd welke experimentele benadering er wordt gebruikt.

In hoofstuk 2 wordt een overzicht gegeven van de bestaande detectie methoden om cytokinen in humaan weefsel aan te tonen. De mogelijkheid om met deze techniek verschillende vraagstellingen te beantwoorden is getest in de tonsil. De nadruk ligt op de localisatie van cytokine en antilichaam producerende cellen in de verschillende compartimenten van de tonsil.

In hoofdstuk 3 worden de cytokine-profielen in allergische en irritatieve contact dermatitis beschreven.

Hoofdstuk 4 beschrijft de rol van CD40 ligand en CD40 in allergische huidreacties in de muis en in de mens en relateert de verkregen data aan de mogelijkheid om CD40 ligand als differentiatie marker tussen allergische en irritatieve contact dermatitis in de mens te gebruiken.

De resultaten verkregen met betrekking tot het humane-huid-op-immunodeficiënte-muis model zijn beschreven in hoofdstuk 5. Informatie over het vermogen tot antigeen opname en migratie van antigeen-dragende Langerhans cellen (hoofdstuk 5.1) en het vermogen van het opwekken van een allergische huidreactie (hoofdstuk 5.2) wordt gegeven.

In de algemene discussie in hoofdstuk 6 worden de belangrijkste punten die uit de verschillende studies naar voren komen besproken.

ABBREVIATIONS

ACD	allergic contact dermatitis
AEC	3-amino-9-ethylcarbazole
AFC	antibody-forming cells
AP	alkaline-phosphatase
APAAP	phosphatase-anti-alkaline-phosphatase
APC	antigen presenting cell
bp	base pair
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumine
C	crypt
CD	cluster of differentiation
cDNA	complementary DNA
cRNA	complementary RNA
CPC	cytokine-producing cell
DAB	3,3'-diaminobenzidine
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNCB	2,4-dinitrochlorobenzene
DNP	2,4-dinitrophenyl
DTH	delayed-type hypersensitivity
EFA	extrafollicular area
ELAM	endothelial cell leukocyte adhesion molecule-1
Fab	antigen-binding fragment
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
GC	germinal centre
GM-CSF	granulocyte-macrophage colony stimulating factor
gp	glycoprotein
h	hour
HEV	high endothelial venules
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish-peroxidase
Hu	human
ICAM	intracellular adhesion molecule
ICD	irritant contact dermatitis
IDC	interdigitating cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
M	microfold
MAb	monoclonal antibody
MF	macrophage
MHC	major histocompatibility complex
min	minutes
MNC	mononuclear cells
MoAb	monoclonal antibody
mRNA	messenger RNA
MZ	mantle zone
NBT	nitro blue tetrazolium
PAP	peroxidase-anti-peroxidase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PF	primary follicle
PVP	polyvinyl pyrrolidone
RAM	rabbit-anti-mouse
RITC	rhodamin b isothiocyanate
RNA	ribonucleic acid
SALT	skin-associated lymphoid tissue
SCID	severe combined immune deficiency
SD	standard deviation
SEM	standard error of the mean
SF	secondary follicle
SCLE	subacute lupus erythematosus
TBM	tingible body macrophages
TGF	transforming growth factor
Th	helper
TNF	tumor necrosis factor
TNP	trinitrophenyl

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Saskia.

CURRICULUM VITAE

Saskia Hoefakker werd op 13 januari 1966 geboren te Nijmegen. In 1985 werd het VWO-diploma behaald aan het Elshof College te Nijmegen. In datzelfde jaar startte zij met de studie (Medische) Biologie aan de Katholieke Universiteit te Nijmegen. Binnen de deelspecialisaties immunopathologie en dierfysiologie heeft zij haar stages vervuld. De stage bij de vakgroep immunopathologie op de Afdeling Pathologische Anatomie van het Radboud Ziekenhuis te Nijmegen omvatte de immunologische karakterisering van het tumorprogressie antigen PAL-M1 (onder verantwoordelijkheid van prof. dr. D.J. Ruiter en onder begeleiding van dr. G.N.P. van Muijen). Haar dierfysiologie stage werd afgerond binnen de R&D-organisatie, Organon in Oss. In het NOD-muizenmodel werd *in vivo* het effect van hormoon-afgeleide steroïden op autoimmuunparameters bestudeerd (onder verantwoordelijkheid van prof. dr. S.E. Wendelaar Bonga K.U. Nijmegen en onder begeleiding van dr. H.A.M. Verheul, Organon, Oss). Als vervolg op deze laatste stage heeft zij gewerkt binnen de groep van dr. A. Cooke aan de University College and Middlesex School of Medicine te Londen aan het effect van dezelfde steroïden in de NOD muis op de cytokine productie *in vitro*. In de periode 1990-1995 werd, gedetacheerd vanuit het Instituut Arbeid en Gezondheid van de Erasmus Universiteit te Rotterdam, bij de Divisie Immunologische en Infectie Ziekten van TNO Preventie en Gezondheid te Leiden, onder dagelijkse leiding van prof. dr. E. Claasssen en dr. W.J.A. Boersma, het onderzoek verricht dat geleid heeft tot dit proefschrift. Tijdens dit onderzoek heeft zij de Post-doctorale Opleiding Toxicologie gevolgd. Vanaf 1 september 1995 is zij wetenschappelijk-staflid bij het Secretariaat van de Gezondheidsraad te Den Haag.

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