

Evaluation of FITC-Induced Atopic Dermatitis-Like Disease in NC/Nga Mice and BALB/c Mice Using Computer-Assisted Stereological Toolbox, a Computer-Aided Morphometric System

Malene Hvid^{a, c} Helene Kofoed Jensen^c Bent Deleuran^{b, c} Kaare Kemp^d
Christina Andersson^d Mette Deleuran^a Christian Vestergaard^a

Departments of ^aDermato-Venereology and ^bRheumatology, Aarhus University Hospital, ^cInstitute of Medical Microbiology and Immunology, University of Aarhus, Aarhus, ^dLEO Pharma, Ballerup, Denmark

Key Words

NC/Nga mice • Atopic dermatitis • Computer Assisted Stereological Toolbox • CD4 • CD8

Abstract

Background: The NC/Nga mouse spontaneously develops eczematous atopic dermatitis (AD)-like skin lesions when maintained under conventional conditions, but not when kept under specific pathogen-free (SPF) conditions. Hence, there is a need for an AD model in mice housed under SPF conditions, as this is mandatory for research animals in many countries. **Methods:** We evaluated the use of the hapten FITC as an inducer of AD-like disease in NC/Nga and BALB/c mice maintained under SPF conditions. Mice were either untreated or treated with tacrolimus or betamethasone. Using the software Computer Assisted Stereological Toolbox as a stereological method, the mice were sensitized to FITC and the histological efficiency of disease induction with regard to inflammation and CD4+ and CD8+ lymphocytes, in addition to mast cells, was evaluated. The method was validated by comparison to a conventional semiquantitative observer-dependent method. **Results:** Our findings prove that FITC does indeed induce AD-like lesions in NC/Nga mice with regard to the histological appearance of the mice. However, when evaluating the immunological response in the affect-

ed areas of the mice with regard to the CD4/CD8 ratio and the effect of treatment, we found that the immune response in the NC/Nga mice differed from AD skin lesions in humans in certain aspects. **Conclusions:** These results emphasize the importance of an assessment of not only the histological but also the immunological appearance of the skin when evaluating AD-like disease in mice as a model for AD in humans.

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Introduction

Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin disease estimated to affect 10–20% of children and 1–3% of adults in the Western world [1]. The underlying immunological mechanisms are not fully elucidated, although some features have been described. The lesions in acute and chronic AD are characterized by a cellular infiltrate consisting primarily of CD4+ T lymphocytes, but also mast cells and eosinophils. The main cytokines expressed are IL-4, IL-5 and IL-13 [2, 3], suggesting the importance of Th2 cytokines in the development of disease, although a more diverse Th1/Th2 cytokine profile is observed in the chronic phase of the disease. Elevated serum IgE is detected in approximately 80% of patients [4]. Therapeutic options for treatment in-

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Correspondence to: Dr. Malene Hvid
Department of Dermato-Venereology, Research Laboratory S
Aarhus University Hospital
P.P. Ørumsgade 11, DK-8000 Aarhus C (Denmark)
Tel. +45 8942 1741, Fax +45 8619 6128, E-Mail malenel@medmicro.au.dk

clude tacrolimus, a topical calcineurin inhibitor, or corticosteroids, e.g. betamethasone [5].

Several animal models exist for AD, including mice, cats and dogs. One murine model, the NC/Nga mouse, attracts attention as these mice spontaneously develop the skin condition when kept in a conventional, air-controlled environment [6]. However, due to laboratory animal welfare regulations in many countries, it is not possible to maintain mice under conventional conditions; hence, there is a need for the establishment of an NC/Nga mouse model kept under specific pathogen-free (SPF) conditions. When maintained under these conditions, the mice do not develop disease spontaneously; however, an AD-like disease can be induced in the NC/Nga mice by application of haptens, e.g. 2,4,6-trinitrochlorobenzene (TNCB) [7].

The clinical symptoms exhibited by the spontaneous NC/Nga mouse model mimic symptoms of AD in human patients, e.g. edema, erythema, lichenification, desquamation and dryness [6]. The local immune response in the lesions and the responsiveness towards treatment with corticosteroids and tacrolimus are similar in NC/Nga mice and humans [8, 9].

To investigate whether the hapten-induced AD-like condition in NC/Nga mice maintained under SPF conditions is indeed representative of human AD in regard to the histopathological changes in addition to its distribution of CD4+ and CD8+ T cell infiltrates, we examined skin lesions from NC/Nga mice with FITC-induced AD-like disease.

Our results revealed that although the NC/Nga mice develop a skin condition which histologically mimics human AD under these conditions, the CD4+ and CD8+ T cell ratio in the infiltrates is different to the ratio observed in human AD lesions.

A semiquantitative method and the software Computer Assisted Stereological Toolbox (CAST-grid), an unbiased stereological technique, were applied to evaluate the histological changes in the skin of the mice with respect to the presence of CD4+ and CD8+ T lymphocytes. The results obtained by the 2 methods were compared in order to evaluate CAST-grid as an accurate method for analysis of the number of positive cells in immunohistochemistry.

Materials and Methods

Animals

The animals used in this study were 4-week-old female NC/Nga TNDCrj mice (Charles River Laboratories Japan, Kanagawa,

Japan) and BALB/c mice (Taconic M&B, Ry, Denmark). The mice were allowed to acclimatize for 2–3 weeks before entering the experiment and were kept in Scantainers (5–7 mice per cage) under SPF conditions (21–25°C, 30–60% humidity and 12-hour light/dark cycle). Five mice were included in each group.

Induction of Disease

For disease induction, the hapten FITC (Sigma-Aldrich, St. Louis, Mo., USA) was applied. Dibutyl phthalate (LEO Pharma, Ballerup, Denmark) mixed 1:1 with acetone was used as vehicle. The mice were sensitized on day 0. The abdomens of the mice were shaved and 102 µl of 1.4% FITC was applied to each mouse. Additionally, each footpad was sensitized using 12 µl of 1.4% FITC, giving a total amount of 150 µl of 1.4% FITC/mouse. The mice were challenged once a week for 8 weeks with 150 µl of 0.5% FITC applied to the shaved back skin. The same area was used for the duration of the experiment. A control group of mice was given vehicle only. All animal procedures complied with the national law and guidelines and were approved by the Danish Animal Experiments Inspectorate (2006/561-1117).

Steroid and Tacrolimus Treatment

Mice kept under SPF conditions with fully developed skin lesions after FITC challenge were treated with either betamethasone valerate (7 µg/ml; Sigma-Aldrich) in acetone or 0.1% tacrolimus ointment (Astellas Pharma Inc., Tokyo, Japan), which was applied to the involved regions. Acetone was used as vehicle for topical treatments. Mice were treated 3 times a week after the 5th challenge.

Skin Biopsies

At the end of the experiments, the mice were killed by cervical dislocation. The hair on the back was removed by shaving, upon which an area of approximately 25 mm² was excised, and the subcutaneous fat and blood vessels were carefully dissected away. Each sample was divided into 2 and used for histology and immunohistochemical staining, respectively.

Histology

Skin biopsies for histology were fixed using 10% neutral buffered formalin and subsequently embedded in paraffin. The samples were cut into 2-µm sections, deparaffinized and stained with hematoxylin and eosin or toluidine blue and analyzed by light microscopy. The histological evaluation was performed blinded using a grading scale ranging from 0 (no disease) to +++ (severe disease). The following histopathological changes were evaluated: epidermal hyperplasia, fibrosis, spongiosis, epidermal crusts and hyperkeratosis. Additionally, the sections were examined for mast cell infiltration in the subepidermal area by determining the number of mast cells in 5 fields of vision at ×200 magnification. The obtained results were divided into 3 groups according to the average number of mast cells per field of vision: 0 = 0–5 intensely stained cells; + = 6–15 intensely stained cells, and ++ = more than 15 intensely stained cells per vision field (×20 objective; Leica Microsystems GmbH, Wetzlar, Germany).

Immunohistochemistry

Skin biopsies from each animal were embedded in Tissue-Tek OCT compound (Sakura, Torrance, Calif., USA), snap-frozen in liquid nitrogen and stored at –80°C until use. The sam-

ples were cut into 6- μ m sections on a cryostat (HM 500; Micron, Waldorf, Germany) and air dried overnight. The sections were acetone-fixed and blocked in 10% standard rabbit serum (X0902; Dako, Glostrup, Denmark), followed by blocking for endogenous biotin (X0590; Dako) in accordance with the manufacturer's descriptions. Then the sections were incubated with primary antibodies for 30 min at room temperature (RT). The applied primary antibodies were rat anti-mouse CD4 (MAB554; R&D Systems, Minneapolis, Minn., USA) and rat anti-mouse CD8 (MAB116; R&D Systems) at a concentration of 0.3 μ g/ml. The primary antibody was detected by incubation with biotin-conjugated rabbit anti-rat antibody (1:800; Vector Lab Inc., Burlingame, Calif., USA) at RT for 30 min followed by incubation with streptavidin-alkaline phosphatase (K0391; Dako) for 30 min at RT, in accordance with the manufacturer's descriptions. Positive cells were visualized using New Fuchsin substrate (K0624; Dako). Coon's/1% BSA was used as washing buffer following each incubation step. Slides were finally counterstained with Mayer's hematoxylin and rinsed in tap water. The slides were fixed in Aquamount (Gurr BDH Chemicals Ltd., Poole, UK). Images of representative fields were acquired using an Olympus Provis AX70 microscope (Olympus, Lake Success, N.Y., USA) equipped with a Leica DFC 320 digital camera system (Leica Camera AG, Solms, Germany).

Immunohistochemical Evaluation

Staining for CD4+ and CD8+ lymphocytes was assessed by a semiquantitative as well as a stereological method. The semiquantitative scoring was assessed in a blinded fashion by 2 observers, with consideration to both the intensity and distribution of specific staining: 0 = 0–5 intensely stained cells; + = 6–15 intensely stained cells, and ++ = more than 15 intensely stained cells per vision field ($\times 40$ objective; Leica Microsystems).

The stereological evaluation was performed using a morphometric system consisting of an Olympus AH-3 microscope equipped with a motorized stage, which was controlled by a computer for manual interactive counting on the computer screen. The software CAST-grid version 2.0 (Olympus, Albertslund, Denmark) was used to generate counting areas. Necrotic areas, artifacts and fibrous areas were omitted manually. A counting frame was placed randomly and systematically moved throughout the encircled counting area until 30% of the entire area was sampled using a $\times 20$ objective. Only cells with staining restricted to the plasma membrane, a visible nucleus and located within the counting frame were considered positive. The mean number of positive cells per square millimeter of tissue was assessed for each mouse. Staining was analyzed in a blinded fashion by 2 observers.

Statistics

The correlation between the semiquantitative scoring system and the stereological method was evaluated by a one-way analysis of variance. The results obtained using the stereological method CAST-grid were defined as the continuous variable, whereas the results from the semiquantitative method were used as the categorical grouping variable (0, +, ++).

Data were log-transformed and normality was checked by QQ plots and the assumption of variance homogeneity by Bartlett's test.

Results

The NC/Nga as well as the BALB/c mice developed histological signs of AD-like disease following sensitization and challenge with FITC.

Histopathology

In the NC/Nga mice, sensitization and challenge resulted in a profound inflammation with a mono- and polynuclear cellular infiltration of the dermis. Furthermore, the dermis appeared denser and thicker compared to the negative controls. Hyperproliferation, hyperkeratosis and some exocytosis characterized the epidermis, mimicking AD lesions in humans. Treatment with tacrolimus reduced the cellular infiltrate moderately, but resulted in an increased epidermal thickness. In general, the NC/Nga mice did not respond to the steroid treatment. A profound cellular infiltrate in addition to epidermal hyperproliferation was observed following betamethasone treatment.

When BALB/c mice were subjected to similar sensitization and challenge, inflammatory changes resembling the previously described reaction in the NC/Nga mice were observed, with the exception that only a slight hyperproliferation of the epidermis was present. Treatment with neither tacrolimus nor betamethasone had an effect on the proliferation of the epidermis. Application of tacrolimus ointment lowered the cellular infiltration of the skin, whereas betamethasone did not have any visibly beneficial effect on the inflammatory reaction in the skin of the mice (results are summarized in table 1).

Distribution of Mast Cells in the Dermis

In the unsensitized control NC/Nga and BALB/c mice, a few scattered mast cells were detected in the dermis and the subcutis. After sensitization and challenge with FITC, the number of mast cells invading the skin increased considerably. Treatment with tacrolimus did not result in a reduction of the number of mast cells in the skin of NC/Nga mice; however, in some of the BALB/c mice, a limited reduction could be detected. Betamethasone markedly decreased the number of mast cells in the skin of the mice (results are summarized in table 1). These results are consistent with similar investigations in humans and in NC/Nga mice kept under conventional conditions [10].

Distribution of CD4+ and CD8+ T Cells in Skin

As the histopathological changes in the untreated mice were similar to AD, we extended the studies to examine the distribution of CD4+ and CD8+ T cells.

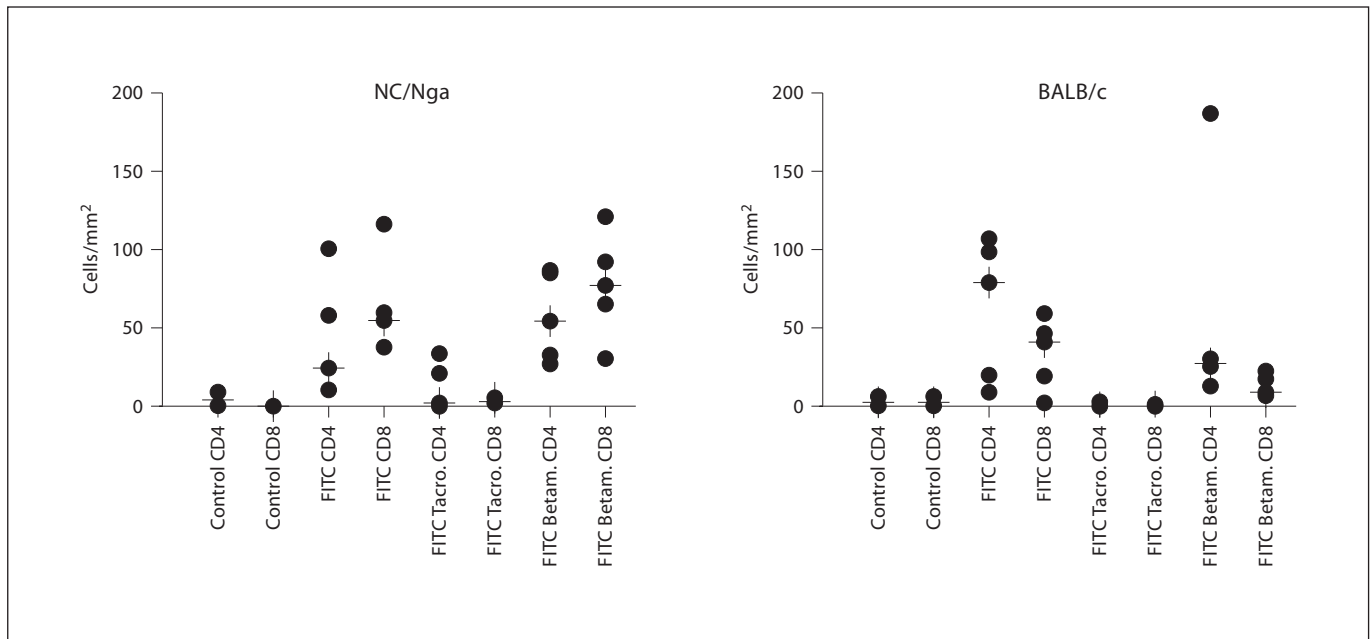


Fig. 1. Stereological evaluation of the number of CD4+ and CD8+ T cells per square millimeter of affected tissue using CAST-grid, a morphometric system. Five mice were included in each group. The closed circles represent the measurement for each mouse and the crosses represent the median value in each group. Tacro. = Tacrolimus; Betam. = betamethasone.

Table 1. Histological evaluation of AD-like lesions in BALB/c and NC/Nga mice

	Control	FITC	FITC + Tacro.	FITC + Betam.
NC/Nga				
Dermal hyperproliferation	0	++	+++	++
Cellular infiltration	0	++	++	++
Mast cell infiltration	0	++	++	+
BALB/c				
Dermal hyperproliferation	0	+	+	+
Cellular infiltration	0	++	+	++
Mast cell infiltration	0	++	+	+

Histological evaluation of skin biopsies from unsensitized (control) and FITC-sensitized in addition to tacrolimus (Tacro.)- and betamethasone (Betam.)-treated BALB/c and NC/Nga mice. For the evaluation of dermal hyperproliferation and cellular infiltration, a grading scale ranging from 0 (no disease) to +++ (severe disease) was applied. An estimation of the mast cell infiltrate was made according to the following scale: 0 = 0–5 intensely stained cells; + = 6–15 intensely stained cells, and ++ = more than 15 intensely stained cells per field of vision ($\times 20$ objective). Results represent an average of 5 mice per group.

Unsensitized NC/Nga and BALB/c mice did not develop macroscopic signs of dermatitis and only very few positive T cells could be observed (fig. 1, 2).

Upon sensitization by FITC, a pronounced infiltration of CD4+ and CD8+ T cells was observed. In NC/Nga mice, CD8+ T cells dominated the infiltrate. This finding is contradictory to previous findings in humans and NC/Nga mice kept under conventional conditions in which CD4+ T cells dominate the infiltrate. In the BALB/c mice, however, CD4+ T cells were the main subtype present in the infiltrate of the dermis (fig. 1, 2). During the acute inflammation in NC/Nga as well as BALB/c mice, it was observed that CD4+ T cells were distributed throughout the dermis, whereas CD8+ T cells were localized along the epidermal basal membrane.

Tacrolimus ointment, a calcineurin inhibitor, is widely used in the treatment of AD. To investigate the effect of tacrolimus on the composition of the cellular infiltrate, the ointment was applied to the affected areas. Tacrolimus reduced the number of CD4+ and CD8+ T cells in both NC/Nga and BALB/c mice; however, the reduction was most pronounced in the latter (fig. 1, 2). These findings are consistent with previous findings in both humans and NC/Nga mice kept under conventional conditions.

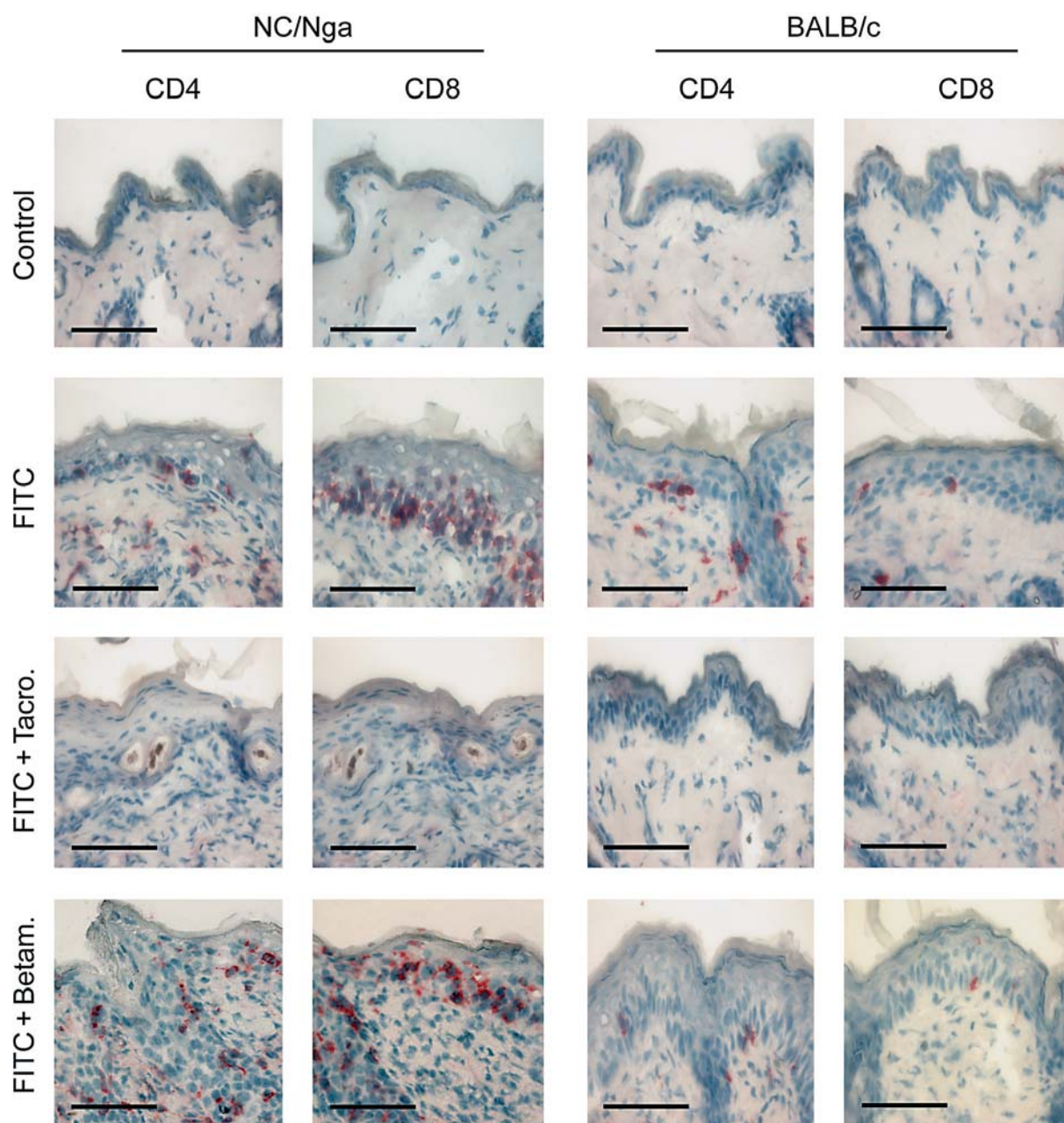


Fig. 2. Distribution of CD4+ and CD8+ T cells in the dermis of BALB/c and NC/Nga mice. The control mice in both groups showed no sign of cellular infiltrate or dermal hyperproliferation. Following FITC sensitization, a profound cellular infiltrate could be detected in both BALB/c and NC/Nga mice. The main T cell subtype present in the skin of the NC/Nga and BALB/c mice was

CD8+ and CD4+, respectively. Treatment with tacrolimus (Tacro.) lowered the number of CD4+ and CD8+ T cells in both groups, but a cellular infiltrate was still present in the NC/Nga mice, whereas treatment with betamethasone (Betam.) did not have any effect. Scale bar = 50 μ m.

Table 2. Semiquantitative evaluation of the number of CD4+ and CD8+ T cells

	Control	FITC	FITC + Tacro.	FITC + Betam.
NC/Nga				
CD4	0	++	+	++
CD8	0	++	+	++
BALB/c				
CD4	0	+	0	++
CD8	0	+	0	+

Semiquantitative evaluation of the average number of CD4- and CD8-positive T cells in the skin of control and FITC-sensitized NC/Nga and BALB/c mice. Further, the effect of treatment with tacrolimus (Tacro.) and betamethasone (Betam.) on the number of infiltrating CD4+ and CD8+ T cells was determined. 0 = 0–5 intensely stained cells; + = 6–15 intensely stained cells; ++ = more than 15 intensely stained cells per field of vision ($\times 40$ objective). Results represent the average of 5 mice per group.

In AD patients, topical treatment of the affected regions using corticosteroids is frequently used. Given that corticosteroids have been reported to have variable effects in NC/Nga mice housed under conventional conditions, we examined the effect of betamethasone treatment, a highly potent corticosteroid. In the NC/Nga mice, no reduction in the number of inflammatory cells was observed (fig. 1, 2). In the BALB/c mice, betamethasone treatment resulted in a considerable reduction in the number of CD4+ and CD8+ T cells (fig. 1, 2).

Evaluation of Counting Method

As described above, staining for CD4- and CD8-expressing T cells was assessed by a semiquantitative (table 2) as well as a stereological method (fig. 1). For both CD4 and CD8 measurements, the data were divided into 3 categories for the semiquantitative counting method: 0 = 0–5 intensely stained cells; + = 6–15 intensely stained cells, and ++ = more than 15 intensely stained cells per vision field. To evaluate CAST-grid, the correlation between the mean number of positive cells per square millimeter of tissue and each category was investigated. We found a statistically significant difference in the mean number of positive cells per square millimeter for each of the 3 categories for CD4+ and CD8+ staining ($p < 0.001$ for both measurements) and hence a correlation between the results obtained by the 2 methods. These results prove that CAST-grid is a valid and accurate method for determination of the number of positive cells.

Discussion

Corresponding to previous findings in humans as well as NC/Nga mice maintained under conventional conditions, the present investigation demonstrated that FITC could indeed induce AD-like lesions in NC/Nga mice housed under SPF conditions. However, in contrast to the results obtained in mice housed under conventional conditions [9], we found that the induced NC/Nga mouse model differs in regard to CD4+ and CD8+ T cell distribution and effectiveness of treatment.

In humans as well as in NC/Nga mice maintained under conventional conditions, the most predominant type of T cells present in the skin lesions are CD4+ T cells [4, 6]; however, CD8+ T cells were the most abundant in NC/Nga mice with FITC-induced AD-like disease. These results indicate that in the skin lesions, the immune response which is evoked by the FITC induction more closely resembles allergic contact dermatitis than AD [11]. In the NC/Nga spontaneous AD model, the likely inducer of the dermatitis is the fur mite *Myobia musculi* [12], which is supported by experiments showing that noninfested NC/Nga mice kept along with infested NC/Nga mice develop dermatitis as they themselves become infested [13]. An AD-like condition may also be induced by the house dust mite *Dermatophagoides pteronyssinus*, towards which some patients develop an allergy [14]. In light of this, it may be questioned whether the spontaneous NC/Nga model actually is a contact dermatitis towards fur mites. Matsukura et al. [7] evaluated the IgE level in NC/Nga mice kept under conventional, mite-free conditions after induction of AD-like lesions using TNCB. Under these conditions, the IgE level did not increase [7]. Furthermore, Takaoka et al. [15] found increased production of IFN- γ in NC/Nga mice with TNCB-induced atopic-like dermatitis housed under SPF conditions. Together, these results further indicate that hapten-induced disease in NC/Nga mice possibly mimics contact dermatitis more than AD.

In contrast to the findings of others [9, 16], our results showed rather unexpectedly that neither the NC/Nga nor the BALB/c mice responded to treatment with betamethasone or tacrolimus with a reduction of the cellular infiltrate. In these experiments, challenge and treatment were given at the same time; consequently, the mice may develop an immunological response to the treatment, thus explaining the inflammatory response observed following corticosteroid treatment. Another explanation could be the low frequency of treatment used in this experiment, i.e. every second day compared to daily application.

This course of treatment was chosen since earlier experiments showed a high degree of side effects when applying 0.05% clobetasol propionate once a day [9].

Human AD lesions in the dermis are characterized by a cellular infiltrate containing primarily CD4+ T cells [4]. This corresponds with the results obtained with FITC-induced AD-like disease in the BALB/c mice. These results indicate that under SPF conditions and with FITC induction, the BALB/c mice develop an inflammatory skin reaction that resembles human AD in regard to T cell distribution to a greater extent than the FITC-challenged NC/Nga mice kept under similar conditions. However, the histopathological changes in the skin of the BALB/c mice were not as pronounced as in the NC/Nga mice; for example, only limited hyperproliferation and hyperkeratosis could be observed.

Two experimental approaches were applied to evaluate the results, a semiquantitative method as well as CAST-grid, a stereological method. We found a significant correlation between the 2 techniques for determination of the number of CD4+ as well as CD8+ cells. However, CAST-grid has several advantages over conventional semiquantitative analysis. It is an unbiased method which can be performed by one person. Further, an exact value of the number of positive cells per area is determined, as opposed to semiquantitative analysis in which the sam-

ples are subdivided based on a rough estimate of the number of positive cells. The results obtained by application of CAST-grid therefore provide a more detailed description of the samples in question, leading to a more comprehensive analysis. Hence, our results show that CAST-grid can be applied as a more accurate evaluation method in future experiments.

The NC/Nga model still has its eligibility, and under different test situations, the results most likely would differ. However, the presented results emphasize the importance of a thorough evaluation of the immune response in the hapten-induced NC/Nga animal model for AD. Although the macroscopical and histopathological changes can be similar to AD, the discrepancy in the CD4+ and CD8+ infiltrates in our model should lead to caution regarding interpretation of the mechanisms involved in different test situations.

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