

## Inducible Nitric Oxide Synthase Demonstrated in Allergic and Irritant Contact Dermatitis

A. D. ORMEROD<sup>1</sup>, C. M. DWYER<sup>1</sup>, A. REID<sup>2</sup>, P. COPELAND<sup>1</sup> and W. D. THOMPSON<sup>2</sup>

Departments of <sup>1</sup>Dermatology and <sup>2</sup>Pathology, Aberdeen Royal Infirmary, Aberdeen, United Kingdom

**Eight allergic patch test reactions, eight irritant skin reactions induced by 3% sodium lauryl sulphate and six normal controls were biopsied. Biopsies were immunohistochemically stained with a mouse monoclonal antibody to inducible nitric oxide synthase (iNOS), and staining was quantified by computerised image analysis. Human chondrocytes induced to express iNOS were used as a positive control. A significant increase in iNOS was found in both irritant and allergic contact dermatitis. There were no differences in the distribution of expression of iNOS. The antibody used was confirmed by Western blotting not to cross-react with the endothelial isoform of nitric oxide synthase (NOS) but did cross-react with a 150kDa protein, which may be neuronal NOS or an isoform of neuronal NOS. Key words: cytokines; sodium lauryl sulphate; computerised image analysis; nitric oxide synthase.**

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A. D. Ormerod, Ward 29, Aberdeen Royal Infirmary, Foresterhill, Aberdeen AB9 2ZB, United Kingdom.

Nitric oxide (NO) is synthesised from arginine by isoforms of nitric oxide synthase (NOS). A constitutive neuronal isoform (type 1 or nNOS) is important in neurotransmission, while smooth muscle tone in blood vessels is regulated by a constitutive endothelial NOS (eNOS or type 3 NOS). The physiological release of NO at low levels reduces platelet adhesion to endothelium and mediates vessel relaxation responses to histamine, substance P and calcitonin gene-related peptide. The inducible isoform of NOS (iNOS or type 2 NOS) was first identified in macrophages (1). It is not produced constitutively but is induced in many cell types by lipopolysaccharide or by cytokines, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), interferon  $\gamma$  (IFN $\gamma$ ), interleukin 1 (IL-1) (2), interleukin 2 (IL-2), interleukin 6 (IL-6) and granulocyte macrophage colony-stimulating factor (GM-CSF). iNOS produces a thousand times greater level of NO than eNOS (3) and participates in the cytotoxic response to pathogens (2), in tumour cell necrosis and in apoptosis (4).

NO is thought to be pro-inflammatory in inflammatory bowel disease (5), asthma (6) and rheumatoid arthritis (7). Using intradermal injection of specific arginine analogue antagonists to NOS, especially N<sup>G</sup>-Nitro-L-Arginine-Methyl Ester (L-NAME), it has been shown that NO participates in the erythema of psoriasis (8), atopic dermatitis (9) and in irritant reactions to dithranol (10). Recently constitutive expression of nNOS has been shown in murine keratinocytes and iNOS demonstrated in cultured murine Langerhans' cells (11).

We have previously shown increased iNOS staining by immunocytochemistry in psoriasis (12) and wished to examine the presence of iNOS in allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD), in view of their differing mechanisms.

### MATERIAL AND METHODS

#### Material

Eight subjects with ACD reactions were selected from the patch test clinic (2 males, 6 females, 37–73 years). Chemotechnique standard patch test reagents were used, ethylene diamine 1% (2 pat.), and fragrance mix 8% (1 pat.), kathon CG 1.34% (1 pat.), potassium dichromate 0.5% (1 pat.), sesquiterpene lactone mix 0.1% (1 pat.), colophony 20% (1 pat) and mercapto mix 2% (1 pat.). Reactions rated ++ on the International Contact Dermatitis Research Group scale. Irritant reactions were induced by 3% sodium lauryl sulphate (SLS) in aqueous solution in 8 healthy male volunteers aged 25–40 years. All biopsies were taken 48 h after application of patch tests. Six normal volunteers provided biopsies of normal skin (4 males, 2 females, mean age 44 years). The three groups of subjects were not matched for age and sex. The protocols were approved by the Joint Ethical Committee of Grampian Health Board and the University of Aberdeen.

#### Methods

Human cartilage chondrocytes, known to produce iNOS, were incubated for 18 h with IL-1 $\beta$  10U/ml and TNF $\alpha$  50 mg/ml in Dulbecco-Vogt Eagle's medium (DMEM, nitrite-free), containing 10% fetal calf serum, glutamine, penicillin and streptomycin, for 18 h and demonstrated to be synthesising NO by the presence of nitrite (detected by the Griess reagent). With this positive control, also serving as validation of the sensitivity of the anti-mouse antibody for the human isoform of iNOS, the staining protocol was optimised.

Frozen sections were fixed in acetone and were incubated at 40°C overnight with 5  $\mu$ g/ml monoclonal antibody to the unique terminal amino acid sequence 961–1144 of mouse macrophage iNOS (Transduction laboratories), which includes the NADPH binding sites of the enzyme. This antibody has been shown to detect human iNOS by Western blotting, and not to react with eNOS from human endothelial cells or nNOS from rat brain (data provided by Transduction laboratories). Recently, it has also been shown to cross-react with a hitherto unidentified 150 kD NOS from osteoarthritis cartilage (OANOS) (13) and possibly rat nNOS, although this does not agree with the manufacturer's quality controls. Detection was by a streptavidin biotin complex followed by horseradish peroxidase, giving a brown reaction product from diaminobenzidine. A darker reaction product was obtained by final rinsing in copper sulphate 0.5% in saline for 5 min to enhance contrast for image analysis. Omission of the primary antibody and normal mouse serum served as the negative controls. Under these conditions negative controls showed minimal background staining, while the positive control was strongly positive. All samples were treated identically in a single session.

Computerised image analysis (Seescan, Cambridge) of the sections was used to quantify staining. Constant lighting was applied to all sections. A standardised computer-assisted image analysis routine applied equally to all sections. Thresholds were adjusted, by two observers, to highlight the areas judged to be positively stained. The percentage of area staining positive (above the standard threshold) was quantified. The brightness of the overall image was also measured, to provide a global measure of staining.



### Western blotting

In view of recently identified potential cross-reactivity between the iNOS antibody and nNOS, the specificity of the antibody was tested by Western blots. Extraction of NOS isozymes was by a modification of the technique used by Amin et al. (13). Three-millimetre punch biopsies (10 mg) were homogenised by hand in 0.5 ml buffer (50 mM Tris HCl/0.9% NaCl, pH 7.4, containing 1 mM PMSF, 2 mM N-ethylmaleimide and 0.025 mg/ml leupeptin as protease inhibitors). The tissue was held over a bed of dry ice and ground throughout 3 freeze thaw cycles, after which it was microwaved for 15 s and ultrasonicated for 10 min. Prior to electrophoresis the extracts were centrifuged for 5 min at 13,000 rpm. Fifty microlitres of each skin lysate, 10 TI of the control lysates (stimulated mouse macrophage and human endothelial cells, supplied by Transduction laboratories) and 10 TI of kaleidoscope pre-stained standards (Bio-Rad) were separated on 4–20% gradient SDS polyacrilamide gels, using a discontinuous system at a constant current of 30 mA per gel. The gels were immunoblotted overnight on a Biorad Trans Blot Cell with cooling and stirring at a constant 35 V onto immobilon membrane (millipore). Before immunostaining the membranes were blocked with a 5% solution of bovine serum albumin (Sigma) to prevent non-specific interactions between the antibodies and the membrane. The same primary anti iNOS monoclonal antibody (above) was used at a concentration of 1/250 and the bands were demonstrated by incubation with the various antisera, followed by an alkaline phosphatase-conjugated antoglobulin. The secondary antibody was Sigma rabbit anti-mouse IgG (whole molecule) alkaline phosphatase conjugate supplied by Sigma. Visualisation of the bands was by staining in a carbonate buffer, pH 9.8, containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Bands were identified by position relative to the standard molecular weight markers and the lysate standards.

### Statistics

The area of staining was skewed towards lower values, which was largely corrected by transforming the area to the natural Log ( $\text{Log}_e$ ), followed by a two-sided, two sample *t*-test using the SPSS statistical package.

## RESULTS

We found a significant increase in staining in the dermis in both ICD (20%) ( $p=0.008$ ) and ACD (16.4%) ( $p=0.028$ ) reactions, compared to normal (1.6%). In the epidermis there was a significant increase in staining in the ICD reactions (6.0%) ( $p=0.027$ ), which also approached significance in the ACD reactions (4.5%) ( $p=0.075$ ). Fig. 1 shows the results of staining in positive control cartilage, normal skin and in ACD and ICD reactions.

The Western blot (Fig. 2) shows positive identification of 130 kDa band, corresponding to iNOS, and confirms no reactivity with eNOS but shows cross-reactivity with a skin-derived 155 kDa band, which may represent nNOS or an isoform like OANOS. However, the iNOS and 155 kDa bands are stronger in samples extracted from allergic and irritant reactions and also in psoriasis, compared with normal skin. Other bands at 170 kDa and 95 kDa are not expected but could represent membrane binding of NOS and proteolytic NOS derivatives. They are unlikely to represent homologous enzymes of the cytochrome p450 system, which have molecular weights of 48–57 kDa.

### Controls for image analysis

Controls were designed to exclude observer bias in the application of image analysis. The overall intensity of the image of

each section was lower (darker) in those exposed to SLS ( $p=0.022$ ) and allergen ( $p=0.029$ ) than in normal controls. This confirms the increased staining (darker image) in ICD and ACD independently of any observer-selected thresholds.

With the Seescan system, increasing thresholds progressively defines more of the grey scale as stained areas. The intensity of the images correlated with the threshold applied to the image ( $r=0.74$ ,  $p=0.001$ , Pearson's coefficient): the brighter the image, the higher the threshold used.

If various thresholds were applied to random images, those with a higher threshold would have a greater area of staining. In this study there was no correlation between the area of staining and the threshold set  $r=0.13$ , indicating that the results were independent of the threshold. Nor was there any significant difference between thresholds applied to the three groups—irritant, allergic and normals.

## DISCUSSION

We have demonstrated increased staining using this antibody to iNOS in irritant and allergic contact dermatitis. This frequently used iNOS antibody showed cross-reactivity with nNOS or a skin-derived nNOS isoform in the Western blot. The Western blot may be more sensitive, as we have separately shown totally different staining patterns with an nNOS-specific antibody and the same iNOS antibody, in psoriasis (data submitted for publication). This implies that the iNOS antibody is more specific in tissue sections and that it is detecting iNOS.

The induction of iNOS can be explained by the cytokines present and probably contributes to the oedema, erythema and induction of cytokines in contact dermatitis. The cytokines TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$ , IL-2, IL-6 and GM-CSF (2, 14, 15, 16) are all potential co-stimuli for the induction of iNOS. In experimental ICD using SLS, Larsen et al. (16) have shown TNF $\alpha$  and IL-6 to be increased up to ten times and IL-1 $\beta$ , IL-2, and GM-CSF up to three times. In a study of ACD and ICD reactions in the mouse, mRNA for IL-1 $\beta$ , IL-6, IL-10, and GM-CSF were upregulated (21) with a relatively greater increase in TNF $\alpha$  in ICD. Similarly, GM-CSF, TNF $\alpha$ , IFN- $\gamma$  were upregulated soon after allergen and irritant contact (17). According to Enk et al., IL-1 $\alpha$ , IL-1 $\beta$ , IFN induced protein 10 and macrophage inflammatory protein 2 (MIP-2) were upregulated only after antigen exposure (17). Some other reports also contrast with the above, suggesting IL-1 to increase only in ACD (15, 18), while IL-8 has been demonstrated in ICD and ACD (19) and as a direct response of cultured keratinocytes to provocation with SLS (20).

Both ICD and ACD lead to the accumulation of activated T lymphocytes (21, 22), differing only in the early infiltration of memory T cells in allergic reactions. Activated lymphocytes also induce iNOS (23). Although different pathways, cytokines and timing are involved in ICD and ACD reactions both induce iNOS. It is also possible to induce keratinocyte NO production through ligation of the low affinity IgE receptor (CD23). Such activation could participate in ACD and would lead to release of TNF $\alpha$  and IL-6 from the keratinocytes (24), leading to further autocrine induction of NO.

In a mouse model using picryl chloride to induce allergic hypersensitivity or phenol as an irritant, it was shown that inhibitors of NOS selectively inhibit allergic and not irritant reactions (25). However, irritant reactions to mustard oil in



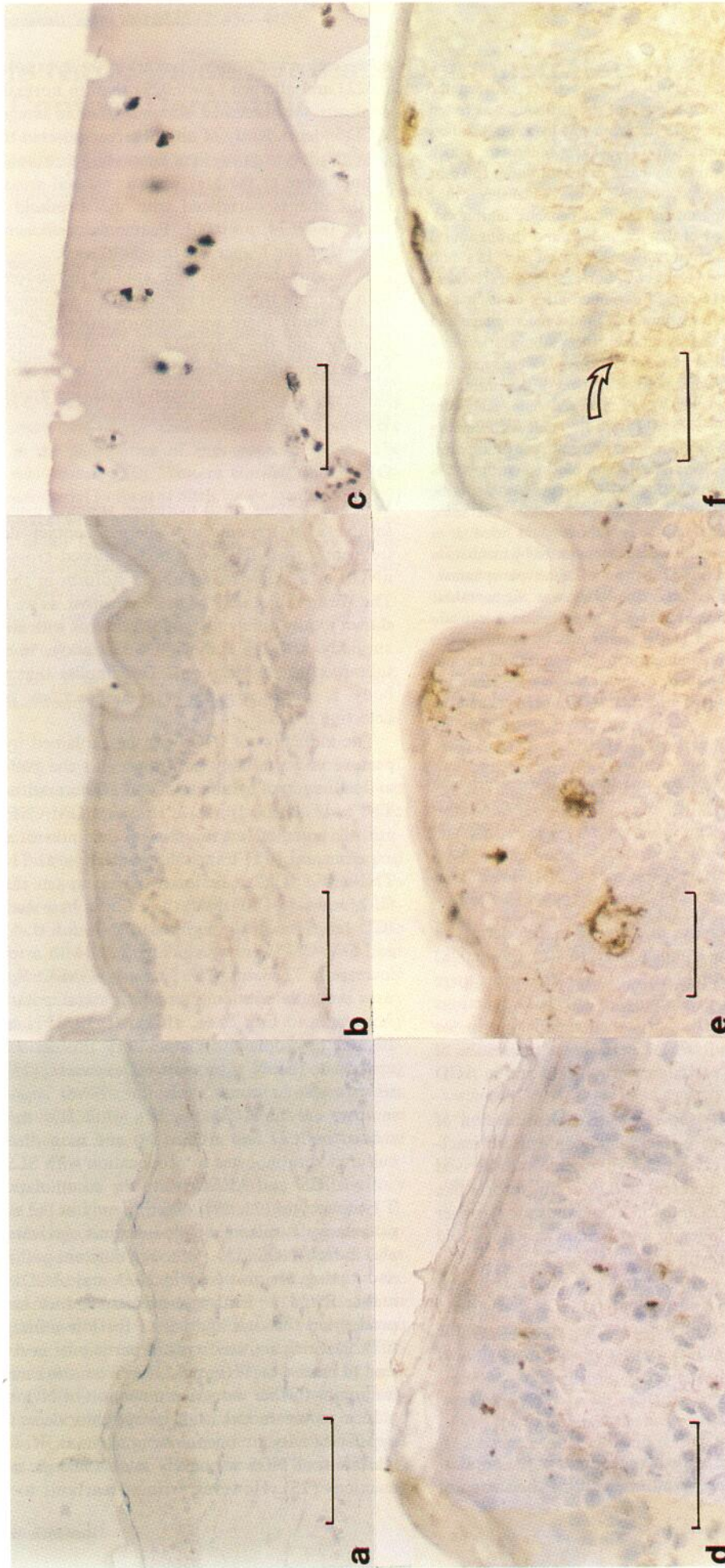


Fig. 1. Immunohistochemistry for iNOS with DAB, showing positive staining by SABC. (a) Negative control, irritant dermatitis, omitting primary antibody ( $\times 200$ , bar = 100  $\mu\text{m}$ ). (b) Normal skin ( $\times 200$ , bar = 100  $\mu\text{m}$ ). (c) Positively stained chondrocytes induced to express human iNOS ( $\times 200$ , bar = 100  $\mu\text{m}$ ). (d) Focal staining of individual cells in epidermis and dermis, irritant dermatitis ( $\times 400$ , bar = 50  $\mu\text{m}$ ). (e) Staining in allergic dermatitis staining of spongiotic areas of the epidermis, possibly including some dendritic cells ( $\times 400$ , bar = 50  $\mu\text{m}$ ). (f) Staining in the dermis; it is not possible to identify the cells stained but staining was maximal around blood vessels, suggesting that these are inflammatory cells ( $\times 400$ , bar = 50  $\mu\text{m}$ ).



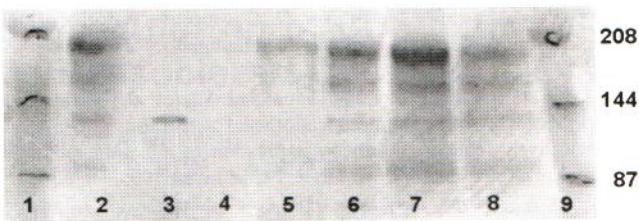


Fig. 2. Western blot stained with mouse monoclonal to iNOS. Lanes 1 and 9, molecular weight markers; lane 2, irritant contact dermatitis; lane 3, positive iNOS control-lysate of stimulated mouse macrophages; lane 4, negative control lysate of human endothelial cells containing eNOS; lane 5, normal skin; lane 6, psoriatic lesion; lanes 7 and 8, allergic contact dermatitis. iNOS bands are seen at 130 kDa and are increased in irritant dermatitis, allergic dermatitis and psoriasis. Note the presence of two bands of larger molecular weight. One approximately 155 kDa could represent a neuronal NOS or variant such as OANOS and was also positively stained with a polyclonal nNOS antibody (not shown).

rat skin were inhibited by the NOS inhibitor L-NAME (26). In this model, pre-treatment with capsaicin to defunctionalise afferent neurones reduced the vasodilation and NO contributing to neuronal mediator release. Following carrageenan injection in the subcutaneous rat air pouch, NOS antagonists reduced the release of prostaglandins and cellular infiltration, implying that NO activates cyclo-oxygenase enzymes (27). In mice footpads injected with carrageenan, delayed-type footpad swelling was reduced by intraperitoneal N<sup>G</sup>-Monomethyl-L-Arginine (L-NMMA), and T cells from draining lymph nodes produced significantly less IL-1, IL-2, and IL-6 after inhibition of NOS (28), suggesting that NO promotes T helper 1 lymphocyte responses. Irradiation of mouse skin with UVB light induces NO (29), which is mediated by a constitutive, calcium-dependent NOS rather than iNOS (30).

During the review of this manuscript, Rowe et al. (31) reported their findings in atopic dermatitis and allergic patch test reactions using the same antibody at a lower concentration 1.5 µg/ml compared with 5 µg/ml. They found more staining in atopic than in ACD with staining in blood vessels and perivascular inflammatory cells.

Paradoxically, NO also has some immunosuppressive properties, including the inhibition of adhesion cell molecules ICAM-1, VCAM-1 (32, 33) P-selectin (34) and leukocyte recruitment and vascular leakage in response to mast cell mediators (35). It is also capable of suppressing lymphocyte proliferation (36); especially Th<sub>1</sub> (37) could be involved in the termination of the inflammatory reaction. The results of inhibition of NO are difficult to predict. However, arthritis and diabetes in the rat, lupus and encephalomyelitis in the mouse and guinea pig colitis have all been inhibited by NOS inhibitors (38).

Our findings suggest that induction of iNOS is non-specific, occurring in a similar pattern in both ICD and ACD. Inhibitors of NOS, such as the arginine analogue L-NMMA, might inhibit dermatitis. The extent of improvement with such agents may indicate more precisely the pro-inflammatory role of NO.

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