Research Report

Anti–α-Fodrin Autoantibodies in Moyamoya Disease

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Background and Purpose—Moyamoya disease (MMD) is a rare entity that results in progressive occlusion of the arteries of the circle of Willis, but the pathogenesis of MMD is unknown.

Methods—MMD sera (n=32) were tested for anti-endothelial cell antibodies by enzyme-linked immunoassays and flow cytometric analysis. Apoptosis was induced in human umbilical vein endothelial cells by tumor necrosis factor-α.

Results—We found that a high proportion of MMD sera had anti-endothelial cell antibodies with apoptotic stimuli. Prominent reactivities of MMD sera (72%) with recombinant human α-fodrin were observed.

Conclusions—Our study demonstrates that MMD sera contain a high incidence of anti–α-fodrin autoantibodies, providing new insight into the mechanisms of occlusion of MMD arteries. (***Stroke*. 2003;34:e244-e246.**)

Key Words: α-fodrin ■ autoantibodies ■ moyamoya disease

Moyamoya disease (MMD) is a chronic cerebrovascular occlusive disease first reported by Japanese surgeons.[1] The disease is characterized by stenosis or occlusion of the terminal portions of the bilateral internal carotid arteries and an abnormal vascular network referred to as moyamoya vessels.[2] Although the cause of MMD remains undetermined, evidence supports an infectious origin, suggesting a role for bacterial and viral infections.[3,4] It was also reported that MMD itself has been associated with Sjögren's syndrome[5] and anti-phospholipid autoantibodies.[6]

It was demonstrated that a defined set of cytoskeletal and nuclear proteins, including α-fodrin and poly(ADP-ribose) polymerase (PARP), were selectively cleaved during apoptosis induced by various stimuli.[7] These findings suggest that different proteases act in apoptosis and that, although cell death processes result in selective cleavage of almost identical cellular proteins, they can be distinguished on the basis of their cleavage products. The purpose of the present study was to seek evidence for autoantibodies against apoptosis-related proteins in patients with MMD.

Subjects and Methods

Study Patients

This study included 32 MMD patients confirmed by cerebral angiograph, CT scans, or MRI scans (the Table). Comparative studies were performed with systemic sclerosis (SSc) patients (n=16).

Cell Culture and Induction of Apoptosis

Human umbilical vein endothelial cells (HUVECs) were purchased from Bio Whittaker. Apoptosis was induced in HUVEC by tumor necrosis factor (TNF)-α (100 ng/mL, R&D Systems) and determined by an EPICS flow cytometer (Coulter) with the Mitochondrial Apoptosis Detection Kit (Biovision).

Enzyme-Linked Immunosorbent Assay for Anti-Endothelial Cell Antibodies

Enzyme-linked immunosorbent assay (ELISA) for anti-endothelial cell antibodies (AECAs) was performed as described.[8] Optical density was measured at 495 nm in a Titertek Uniskan (Flow Labs). Absorbance values greater than the mean±3 SD in normal controls were considered positive.

Flow Cytometric Analysis for AECA With Apoptosis

Apoptotic HUVECs were incubated with sera diluted to 1:20 in bovine serum albumin/phosphate-buffered saline. Cells were analyzed on a EPICS flow cytometer (Coulter). Samples were recorded as positive if the binding index was greater than the mean+3 SD of the normal group.

Western Blot Analysis

Western blot analysis with mouse mAb to α-fodrin (AFFINITI, Mamhead), PARP (Transduction Laboratories), gelsolin (DAKO), and active caspase 3 (Transduction Laboratories) was performed and visualized with ECL Western blotting reagent (Amersham Corp). Recombinant caspase 3 was purchased from Biovision, and recombinant α-fodrin was constructed by inserting cDNA into the *Eco*RI site of pGEX-4Ts.[9]

Results

ELISA for AECAs

IgG AECAs were detected in 2 of the 32 MMD patients, not in 32 control subjects (Figure 1A). IgG AECAs were present in 8 of the 16 patients with SSc (50%) ($P<0.0001$).

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Frequency of α-Fodrin–Reactive Sera From MMD Patients and Age-Matched Healthy Control Subjects

	Patients, n	Mean Age (Range), y	Sex Ratio, F/M	Positive Sera With α-Fodrin, n (%)			
				JS-1	2.7A	3′DA	Any
MMD	32	33±17	27/5	9/32*	9/32	19/32†	23/32‡
		(12–66)	(5.4/1)	(28)	(28)	(59)	(72)
Control	32	30±11	27/5	1/32	3/32	2/32	4/32
		(13–53)	(5.4/1)	(3)	(9)	(6)	(13)

Statistically significant at *$P<0.01$, †$P<0.001$, and ‡$P<0.0001$ vs healthy control subjects (Mann-Whitney *U* test).

Flow Cytometric Analysis for AECAs With Apoptosis

Cytoplasmic staining was observed in a high proportion of SSc ($P<0.0001$) and MMD ($P<0.001$) patients positive for IgG AECAs with apoptosis (Figure 1B). Proteolysis of α-fodrin to 150- and 120-kDa breakdown products was detected in TNF-α–stimulated HUVECs (Figure 1C).

Anti-Human 120-kDa α-Fodrin Abs in MMD Sera

A high proportion of sera from MMD patients (72%) reacted with each recombinant α-fodrin compared with control subjects (13%) (Table). Serum reactivities with breakdown products of PARP were not observed. Strong reactivity of MMD sera with each recombinant human α-fodrin was observed, but not in sera from SSc patients (Figure 2A). A large proportion of MMD sera reacts with C-termini of recombinant α-fodrin protein (JS-1, 28%; 2.7A, 28%; 3′DA, 59%). Cleavage products (150 and 120 kDa) of rat brain α-fodrin were detected when treated with recombinant caspase 3, and MMD sera reacted with either 150- or 120-kDa but not with 240-kDa mature form (Figure 2B). Moreover, TNF-α–stimulated HUVECs were positive for active caspase 3 (Figure 2C), and the cleavage products of α-fodrin were entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) (Figure 2D).

Discussion

A number of studies have suggested that endothelial cell injury results in an altered distribution of surface Ag and promotes active binding of immune complexes to these cells.[10] AECAs are reported to be closely correlated with the vasculitis in Kawasaki disease and Takayasu arteritis, suggesting that AECAs could contribute to the pathogenesis of vascular injury.[11]

The new information obtained here is the presence of AECAs with apoptotic stimuli in MMD patients. ELISAs performed with conventional AECAs in the MMD patients were almost negative, indicating that no antibodies directed against endothelial cells bind primarily to membrane-bound molecules. However, sera from MMD patients contain autoantibodies against cleaved product of 150- or 120-kDa α-fodrin derived from apoptotic HUVECs. In vitro study demonstrated that MMD sera react with either 150- or 120-kDa but not with 240-kDa mature-form α-fodrin, which was cleaved by the recombinant caspase 3. This is the first report that autoantibodies cleave products of α-fodrin derived from apoptotic endothelial cells in MMD patients. It was demonstrated that the fodrin α subunit is cleaved in association with apoptosis and that the 120-kDa fragment is a breakdown product of the mature form of 240-kDa fodrin α

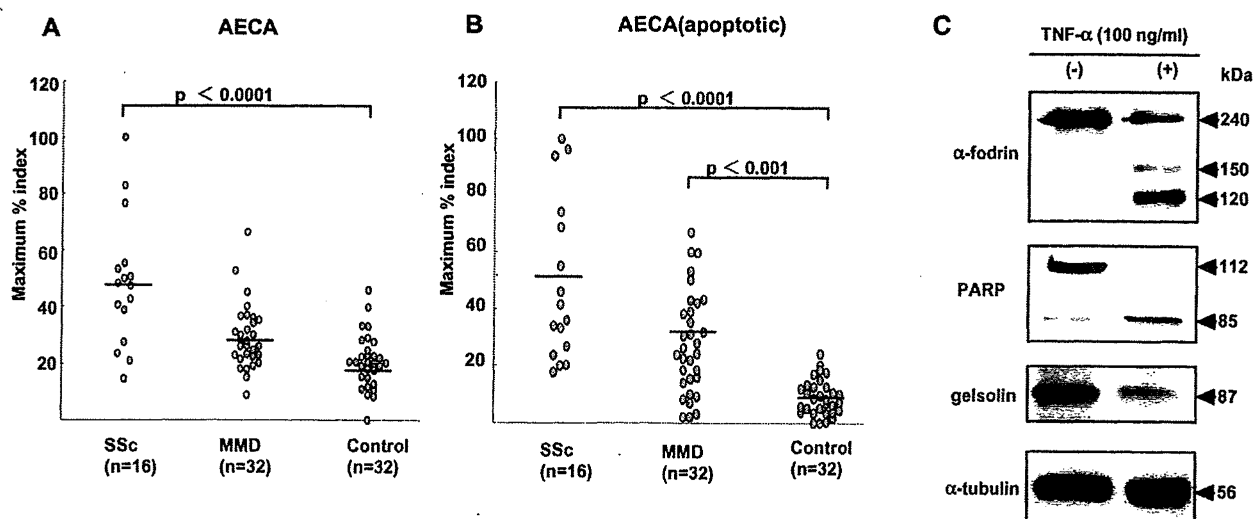

Figure 1. A, IgG AECA level was significantly higher in sera of SSC patients than control subjects ($P<0.0001$, Mann-Whitney *U* test). B, A high proportion of SSc and MMD patients were positive for IgG AECAs using apoptotic HUVECs compared with control subjects ($P<0.0001$ and $P<0.001$, respectively, Mann-Whitney *U* test). C, Proteolysis of α-fodrin to 150- and 120-kDa breakdown products was detected in TNF-α (100 ng/mL)–stimulated HUVECs. Treatment with TNF-α (100 ng/mL) affected breakdown of PARP (85 kDa) and gelsolin (cleavage product not detected).

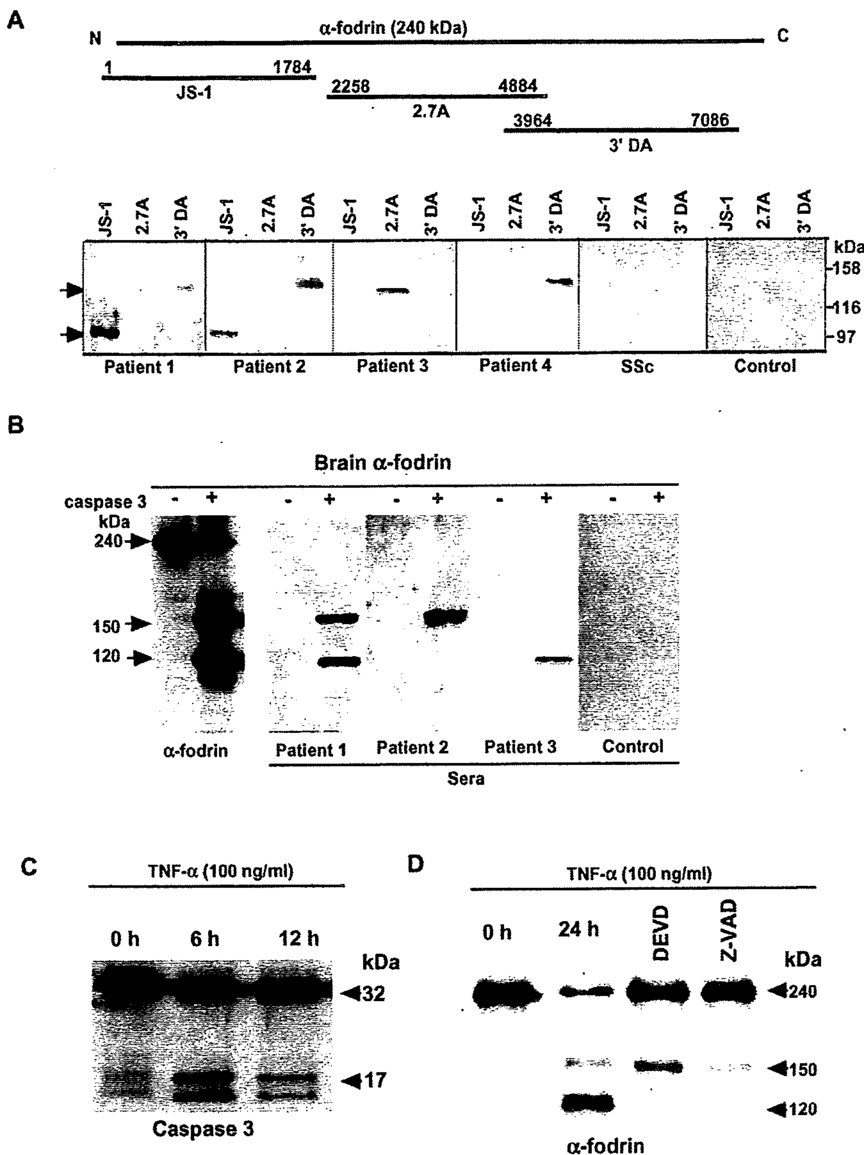

Figure 2. A, Map of cDNAs encoding human α-fodrin. MMD sera react mostly with C-termini of recombinant α-fodrin protein (JS-1, 28%; 2.7A, 28%; and 3′DA, 59%). B, MMD sera react with either 150- or 120-kDa but not with 240-kDa mature-form rat brain α-fodrin when cleaved by the recombinant caspase 3. C, TNF-α–stimulated HUVECs were positive for active-form caspase 3. D, Cleavage products of α-fodrin were entirely blocked by the preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO).

subunit.[12] A higher proportion of MMD sera reacts with C-termini of α-fodrin containing caspase 3 cleavage sites. Indeed, we detected active caspase 3 in apoptotic HUVECs, and cleavage products of α-fodrin were blocked by caspase inhibitors. The activation and injury of endothelial cells induced by TNF-α and other proinflammatory cytokines may underlie the local effects of these mediators in vivo. These data suggest that anti–α-fodrin autoantibody could contribute in part to the pathogenesis of MMD and may provide new insight into the mechanisms of occlusion of the arteries.

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REVIEW

The role of caspase cascade on the development of primary Sjögren's syndrome

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Abstract: Primary Sjögren syndrome (SS) is an autoimmune disease characterized by diffuse lymphoid cell infiltrates in the salivary and lacrimal glands, resulting in symptoms of dry eye and dry mouth due to insufficient secretion. Previously, we have identified the 120 kDa α-fodrin as an important autoantigen on the development of SS in both animal model and SS patients, but the mechanism of α-fodrin cleavage leading to tissue destruction in SS remains unclear. In murine primary SS model, tissue-infiltrating CD4$^+$ T cells purified from the salivary glands bear a large proportion of Fas ligand (FasL), and the salivary gland duct cells constitutively possess Fas. Infiltrating CD4$^+$ T cells identified significant ^{51}Cr release against mouse salivary gland (MSG) cells. *In vitro* studies demonstrated that apoptotic MSG cells result in a specific α-fodrin cleavage into 120 kDa, and preincubation with caspase-inhibitor peptides blocked α-fodrin cleavage. The treatment with caspase-inhibitors *in vivo* prevented the development of autoimmune lesions in the salivary and lacrimal glands. Thus, an increased activity in caspase cascade may be involved in the progression of α-fodrin proteolysis and tissue destruction on the development of SS.
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Keywords **:** *Sjögren's syndrome ; autoantigen ; caspase ; apoptosis*

INTRODUCTION

Primary Sjögren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La (1-4). The spectrum of presentation of the disease is broad, ranging from the organ-localized dysfunction of exocrine gland to systemic complications such as liver, kidney and lung involvement (5). Although it has been assumed that a combination of immunologic, genetic, and environmental factors may play a key role on the development of autoimmune lesions, little is known about the disease pathogenesis. Autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance (6, 7). Although the specificity of cytotoxic T lympyocyte (CTL) function has been an important issue of organ-specific autoimmune response, the mechanisms responsible for tissue destruction in SS remain to be elucidated. The histopathological changes in the minor salivary gland biopsy are characterized by focal and/or diffuse lymphoid cell infiltrates and parenchymal destruction. The majority of lymphoid cells in the salivary biopsy are CD 4$^+$ T cells with a small proportion of CD8$^+$ T cells (2). These T cells express the $\alpha\beta$ antigen receptor and cell surface antigens associated with mature memory T cells. Since it was evident a preferential use of specific variable region segments of the antigen receptor β chain by salivary gland T cells (8), it has been assumed that a unknown organ-specific autoantigen

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targeted by autoreactive T cells may be present in the salivary glands. We have established and analyzed an animal model for primary SS in NFS/*sld* mutant mice thymectomized 3 day after birth (3d-TX) (9-20). When the repertoire of T cell receptor (TCR) Vβ genes transcribed and expressed within the inflammatory infiltrates was analyzed in an animal model, a preferential utilization of TCR Vβ gene was detected in these lesions from the onset of disease (10). We have previously identified a 120 kDa organ-specific autoantigen from the salivary gland tissues of this animal model (21). The sequence of the first 20 NH_2-terminal residues was found to be identical to that of cytoskeletal protein human α-fodrin (21). Furthermore, sera from patients with SS reacted positively with purified 120 kDa antigen, and proliferative response of peripheral blood lymphocytes (PBMC) from SS patients to the purified autoantigen was detected, but not from SLE or RA patients, and healthy controls. These results indicate that the anti-120 kDa α-fodrin immune response plays an essential role on the development of primary SS. Recent reports have demonstrated evidences that caspase 3 is required for α-fodrin cleavage during apoptosis (22-24). In Jurkat cells, caspase 3-like proteases have been reported to cleave α-fodrin and poly (ADP-ribose) polymerase (PARP) but with differential sensitivity to the caspase 3 inhibitor, DEVD-CHO (24). We speculate that an increase in the enzymatic activity of apoptotic proteases is involved in the progression of α-fodrin proteolysis during development of SS.

Involvement of Fas and FasL in tissue destruction

It is now clear that the interaction of Fas with FasL regulates a large number of pathophysiological process of apoptosis (25, 26). We speculate that an increase in the enzymatic activity of apoptotic proteases is involved in the progression of α-fodrin proteolysis during development of SS. To determine the possible involvement of Fas and FasL in tissue destruction of SS, we first analyzed Fas expression in the salivary gland specimens of 3d-thymectomized (3d-Tx) NFS/*sld* mouse model (10) and in the mouse salivary gland cells (MSG) isolated from non-thymectomized (non-Tx) NFS/*sld* mice. Immunohistology revealed that the majority of tissue-infiltrating lymphoid cells in the salivary glands bear FasL in SS model, and epithelial duct cells express Fas antigen on their cell surface. We found that tissue-infiltrating $CD4^+$ T cells isolated from the affected glands bear a large proportion of FasL (>85%), compared with $CD8^+$ T cells bearing FasL on flow cytometry (<23%) ($P < 0.01$) (Fig. 1A). A minor proportion of infiltrating $CD4^+$ T cells express Fas (<31%), and $CD8^+$ T cells bearing Fas were negligible (<5%). Primarily cultured MSG cells isolated from 3d-Tx, non-Tx NFS/*sld* and C57BL/6 mice constitutively express Fas with high proportion (51%-60%) on flow cytometry (Fig. 1B). Immunohistochemically, epithelial duct cells in non-Tx NFS/*sld* and C57BL/6 salivary glands are positive for Fas. RT-PCR analysis demonstrated that Fas mRNA was constitutively present in the salivary glands of SS model, non-Tx NFS/*sld*, and normal C57BL/6 mice. MSG cells isolated from these mice did not express FasL on flow cytometric analysis. A significant increase of $TUNEL^+$-apoptotic epithelial duct cells in the salivary glands was observed in SS model mice, compared with those in non-Tx NFS/*sld*, and C57BL/6 mice at all ages. We next investigated whether tissue-infiltrating T cells are responsible for tissue destruction as judged by *in vitro* ^{51}Cr release cytotoxic assay against MSG cells. Infiltrating $CD4^+$ T cells, but not $CD8^+$ T cells, identified significant ^{51}Cr release against MSG cells. These cytotoxic activities were almost entirely inhibited by incubation with anti-murine neutralizing FasL mAb (FLIM58 : 1μg/ml), indicating that the cytotoxicity by activated $CD4^+$ T cells towards salivary gland epithelial cells was Fas-based.

Participation of caspases in α-fodrin cleavage

To confirm the organ-specificity of a cleavage product of α-fodrin, we investigated various strains of mice with salivary gland destruction, such as MRL/*lpr*, nonobese diabetic (NOD) mice, in addition to 3d-TX NFS/*sld* mice. Protein immunoblot analysis demonstrated that the 120 kDa α-fodrin was detected in these affected glands, but not in normal mice. We examined the *in vitro* cleavage of α-fodrin using 240 KDa α-fodrin in MSG cells. Anti-Fas Ab-induced apoptosis was confirmed by FACS analysis using in situ TUNEL procedure, and DNA laddering and formation. We could detect the 120 kDa α-fodrin in apoptotic MSG cells on immunoblotting. We examined the *in vitro* cleavage of α-fodrin in MSG cells induced by anti-Fas mAb (*Jo2* : 300 $ngml^{-1}$). Anti-Fas mAb-stimulated apoptosis in MSG cells was con-

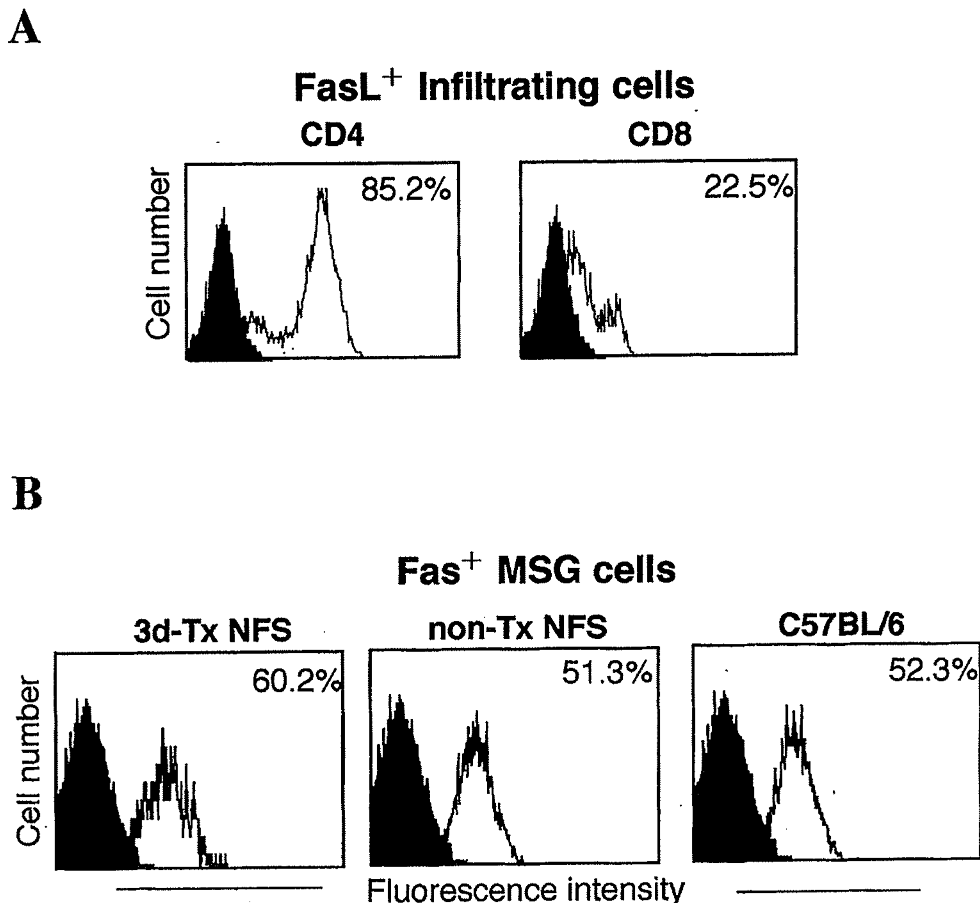
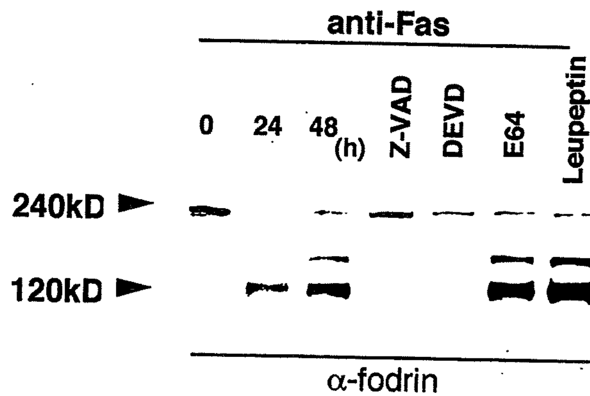


Figure 1. FasL and Fas expression in the salivary gland tissues from 3d-Tx NFS/*sld* mice. (A) Flow cytometric analysis of FasL expression on tissue-infiltrating lymphocytes isolated from salivary glands of 3d-Tx NFS/*sld* mice gated on CD4, and CD8. FasL expression on tissue-infiltrating CD4+ T cells was prominent compared with that on CD8+ T cells. Five mice in each group were analyzed at 8-, and 12-wk-old of age. (B) Flow cytometric analysis of Fas expression on MSG cells from 3d-Tx, non-Tx NFS/*sld*, and normal C57BL/6 mice. Fas expression was constitutively observed on MSG cells from each group of mice. Five mice in each group were analyzed.

firmed by flow cytometry of DNA content of nuclei with PI and Annexin V. Western blot analysis demonstrated that the 240 kDa α-fodrin in apoptotic MSG cells was cleaved to smaller fragments into 120 kDa on time-dependent manner, and the cleavage was entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) (Fig. 2A). Protease inhibitor cocktails, cysteine protease inhibitors (E 64), and serine protease inhibitor (Leupeptin) had no significant effect on 120 kDa α-fodrin cleavage in apoptotic MSG cells (Fig. 2A). The 113 kDa PARP in apoptotic MSG cells was not cleaved to smaller fragments. We next investigated whether cysteine proteases are involved in α-fodrin cleavage on apoptotic MSG cells. The caspase 1- and caspase 3-like activities in anti-Fas mAb-stimulated MSG cell extracts were determined using fluorescent substrates (27), and caspase inhibitors (z-VAD-fmk, DEVD-CHO) inhibited these activities at different dose (0.2, 2, and 20 μM) (Fig. 2B).

Preventive effect of caspase inhibitors in vivo

We next examined whether α-fodrin cleavage to 120 kDa fragment on apoptotic human salivary gland cells (HSG) (28) could be blocked by preincubation with specific protease inhibitors. In apoptotic HSG cells, calpain inhibitor peptide and caspase inhibitor (Z-VAD-fmk) had partially blocked 120 kDa α-fodrin formation. Moreover, a combination of calpain inhibitor peptide and caspase inhibitors (Z-VAD-fmk and Z-DEVD-fmk) almost entirely inhibited the formation of 120 kDa α-fodrin. Protease inhibitor cocktails, other cysteine protease inhibitors (E64), and serine protease inhibitor (Leupeptin) had no effect on 120 kDa α-fodrin cleavage in apoptotic HSG cells. By immunohistochemistry using polyclonal Ab against synthetic 120 kDa α-fodrin, a cleavage product of α-fodrin was present exclusively in epithelial duct cells of the labial salivary gland biopsies from SS patients, but not in control individuals. Protein



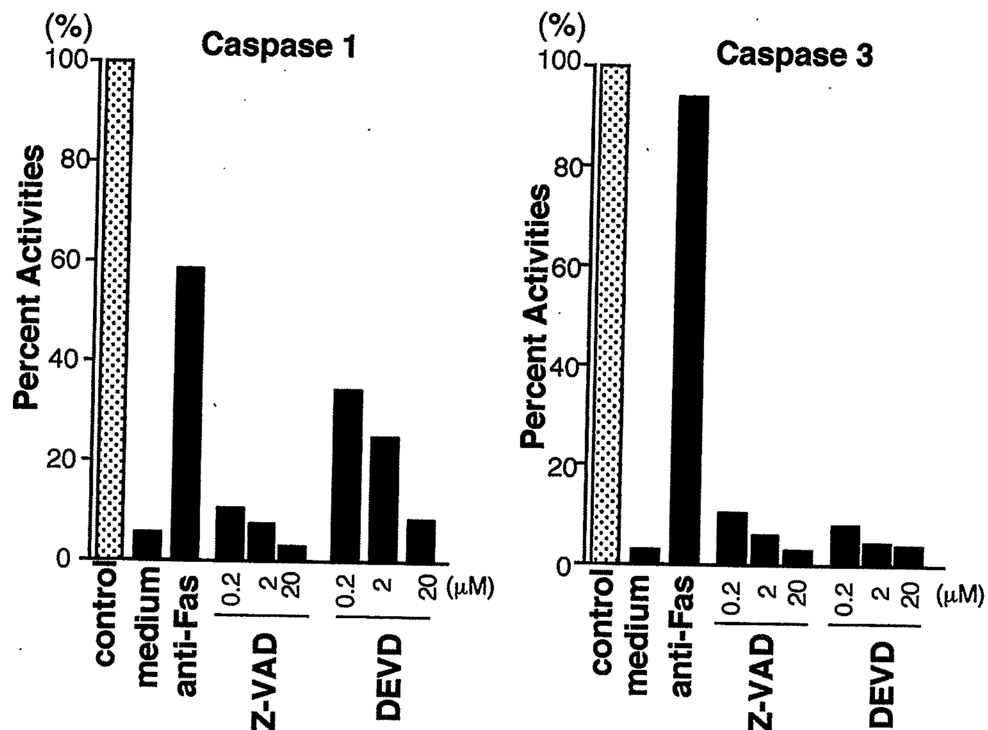


Figure 2. (A) Western blot analysis demonstrated that the 240 kDa α-fodrin in apoptotic MSG cells was cleaved to smaller fragments into 120 kDa on time-dependent manner, and the cleavage product was entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) measured at 24 h. Protease inhibitor cocktails, cysteine protease inhibitors (E64), and serine protease inhibitor (Leupeptin) had no significant effect on α-fodrin cleavage. (B) Activation of caspase 1-like and caspase 3-like proteases was detected during anti-Fas-induced apoptosis, and caspase inhibitors inhibited these activities. Cytosolic extracts were prepared from MSG cells (1×10^7 cells) which were treated at 37℃ with 300 $ngml^{-1}$ of Jo-2. 100% activity as control was calculated using the values that 300U/ml recombinant caspase 1 or caspase 3 was added to each substrate (200 μM MOCAc-YVAD(dnp)-NH_2 and MOCAc-DEVD (dnp)-NH_2).

immunoblot analysis confirmed the same results. This indicates that a cleavage product of 120 kDa α-fodrin is present in the diseased glands with human SS, but not in control glands. We further investigated whether the i.v. injection of caspase-inhibitors protects SS animal model against the development of autoimmune lesions. The both treatment with i. v. injection of z-VAD-fmk and DEVD-CHO (3 times per week) ($P<0.005$) prevented the development of autoimmune lesions in the salivary and lacrimal glands. The average saliva and tear volume of the treated SS animal model was significantly higher than that of the control group. A significant decrease of autoantigen-specific T cell proliferation was observed in spleen cells from treated mice. In addition, serum autoantibody production against

120 kDa α-fodrin was clearly inhibited by the treatment with caspase-inhibitors. The treatment of murine SS model with i.v. injection of z-VAD-fmk and DEVD-CHO prevented the development of autoimmune conditions, resulting in restoration of saliva and tear secretion. These results suggest that increased activity of caspase cascade is involved in the progression of α-fodrin proteolysis during the initial stages on the development of primary SS.

Autoimmune lesions induced by immunization with autoantigen

To examine the autoimmune nature of 120 kDa α-fodrin, recombinant α-fodrin protein identical to an autoantigen was administerred subcutaneously (s.c.) into normal NFS/*sld* mice at 4 wks. Organ-specific autoimmune lesions similar to SS developed at 8 wks after the injection in almost all mice immunized with autoantigen, but not in all groups of control (19). No inflammatory lesions were observed in other organs. A majority of infiltrating cells were $CD4^{+}$ and $FasL^{+}$, and the epithelial duct cells express Fas on their cell surface. A specific cleavage of α-fodrin into 120 kDa was detected in the salivary glands of immunized mice, but not in controls. Mice injected with recombinant autoantigen showed a significant increase of autoantigen-specific T cell proliferation in spleen cells. A high titer of serum autoantibodies against 120 kDa α-fodrin was detected in immunized mice, compared with control mice by ELISA. These data demonstrated evidences that a cleavage product of 120 kDa α-fodrin is pathogenic autoantigen on the development of murine primary SS.

Concluding remarks

There is increasing evidences that the cascade of caspases is a critical component of the cell death pathway (29-31), and a few proteins have been found to be cleaved during apoptosis. We provided evidence that α-fodrin is cleaved by one or more members of caspases during apoptotic cell death in SS salivary glands. Fodrin cleavage by caspases can potentially lead to cytoskeltal rearrangement, and it is of interest to point out that α-fodrin binds to ankylin, which contains a cell death domain (32). It has been shown that cleavage products of α-fodrin inhibit ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles (33), supposing that a cleavage product of 120 kDa α-fodrin could be a novel component of an unknown immunoregulatory networks such as cytolinker proteins (34). These results are strongly suggestive of essential roles of caspase cascade for α-fodrin cleavage leading to tissue destruction in autoimmune exocrinopathy of primary SS.

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—Note—

Differences in Responsiveness of Mouse Strain against p-Benzoquinone as Assessed by Non-Radioisotopic Murine Local Lymph Node Assay

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Abstract: *The non-radioisotopic modification of murine local lymph node assay (LLNA) by using 5-bromo-2'-deoxyuridine (BrdU) was conducted to investigate the strain-related difference of the responsiveness of mice to* p*-benzoquinone (PBQ) with BALB/cAnN, CBA/JN and CD-1 mouse strains. Strain and dose related differences were analyzed by two-way analysis of variance (two-way ANOVA). CBA/JN was considered to be the highest responsive strain to PBQ, and interaction was detected between CD-1 and each of the other inbred strains. These results support the recommendation in the OECD test guideline 429 and the skin sensitization test guideline of US-EPA with regard to the selection of mouse strain for LLNA.*

Key words: *local lymph node assay, responsiveness,* p*-benzoquinone*

Contact dermatitis caused by chemicals is a serious health problem, and a prediction of the skin sensitizing potential of chemicals is necessary to secure safe handling of chemicals. The guinea pig maximization test and the Buehler test have been widely used for predicting the skin sensitizing potentials of chemicals for regulatory purposes for a long time [1, 6]. Recently the murine local lymph node assay (LLNA) has been recognized as a new stand-alone sensitization test which can be used for regulatory purposes [3–5], and it is based upon consideration of the induced proliferative responses in lymph nodes draining the site of topical exposure to the test chemical. In the standard LLNA, cell proliferation is measured using the incorporation of radiolabeled thymidine or uridine into draining lymph node cells, and this requires specific facilities and handling conditions. We previously developed a non-radioisotopic alternative method for the LLNA which uses 5-bromo-2'-deoxyuridine (BrdU) incorporation in place of radioisotopes [9, 10]. The responsiveness of mouse strains against antigen is known to vary with their H-2 haplotypes. We report here the difference of responsiveness of three mouse strains in the modified murine local lymph node assay against *p*-benzoquinone, a known potent contact allergen to human.

p-Bebzoquinone (BZQ, Lot No. 012D2294, Kanto Chemical Co., Tokyo, Japan) was dissolved in acetone:olive oil (AOO; 4:1). 5-Bromo-2'-deoxyuridine (BrdU, Nacalai Tesque, Kyoto, Japan) was dissolved in

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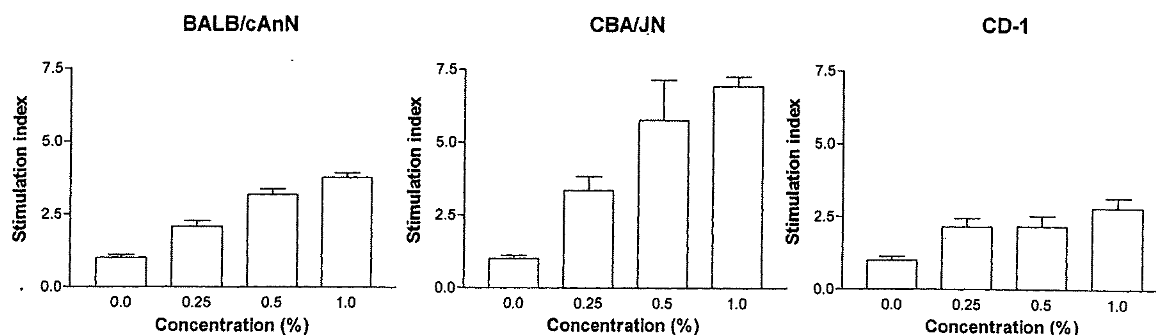

Fig. 1. Dose responses in the modified local lymph node assay with *p*-benzoquinone.
*Data are represented as the mean ± standard error.

physiological saline at a concentration of 10 mg/ml. Female CBA/JN, BALB/cAnN and CD-1 mice were obtained from Charles River Japan Ltd. (Kanagawa, Japan). Mice were housed in animal rooms maintained at a temperature of 23 ± 2°C and relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10 to 15 cycles/h, and lighted artificially for 12 h daily.

Mice were randomly allocated to 4 groups (4 mice/group) per each strain of mouse. A 25 μl volume of PBQ in AOO was applied to the dorsum of both ears of the mice daily for three consecutive days. A single intraperitoneal injection (5 mg/mouse/injection) of BrdU was made on day 4. On day 5, auricular lymph nodes were removed, weighed, and stored at −20°C until analysis by an ELISA to measure BrdU incorporation.

BrdU incorporation into the lymph node cells was determined using a commercial cell proliferation assay kit (Boehringer Mannheim Corp., Indianapolis, IN, USA, Cat. No. 1647229). Lymph nodes were crushed, and after passage through a #70 nylon mesh, the cells were suspended in 15 ml of physiological saline. The cell suspension (100 μl) was added to the wells of a flat-bottom microplate (Coster 3595, Corning Inc., NY, USA) in quadruplicate. After centrifugation (3000 × g, 10 min), the supernatants were removed, 200 μl of Fix-Denat solution was added to each well, and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 μl, Boehringer Mannheim Corp.) was added to each well, and after rinsing 3 times with washing buffer (phosphate-buffered saline), 100 μl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined as the BrdU labeling index with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. Means and standard errors for the labeling indices were calculated for each treatment group, and the stimulation index (SI) was calculated by dividing the labeling indices in each test group by that in the concurrent vehicle control group. Then, two-way analysis of variance (Two-way ANOVA) was performed with regard to dose and strain as factors.

Dose responses against PBQ for each mouse strain are shown in Fig. 1. The SI values for 0.25%, 0.5% and 1% PBQ were 3.4, 5.8 and 6.9 in CBA/JN, 2.1, 3.2, and 3.8 in BALB/cAnN, and 2.1, 2.2 and 2.8 in CD-1, respectively. The SI values increased in a dose dependent manner in all mouse strains. Positive responses (SI>3) were noted ≥0.25% in CBA/JN, ≥0.5% in BALB/cAnN and >1.0% in CD-1. As the results of two-way ANOVA, CBA/JN showed the highest responsiveness to PBQ, and interaction was noted between CD-1 and each of the other inbred strains (Table 1). Consequently, CBA/JN was considered to be the highest responder strain to PBQ, and CD-1 is not a preferable strain for LLNA. In the OECD and US-EPA guidelines [2, 8], and the ICCVAM validation report for LLNA [7], CBA/Ca or CBA/J mouse are recommended for selection of animal species. In this study, the CBA/JN mouse showed the highest responsiveness to PBQ among three mouse strains tested. Our result supports the animal selection described in the test guidelines and the review article mentioned above.

Table 1. Probabilities detected in two-way analysis of variance (two-way ANOVA)

Source of Variation	CBA/JN vs. BALB/cAnN		CBA/JN vs. CD-1		BALB/cAnN vs. CD-1	
Interaction	0.2209	ns	0.0076	**	0.0125	*
Strain	0.0385	*	0.0001	***	<0.0001	***
Dose	<0.0001	***	<0.0001	***	<0.0001	***

Asterisks indicate significance levels (*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$). ns: not significant.

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Assessment of the Skin Sensitization Potency of Eugenol and its Dimers using a Non-radioisotopic Modification of the Local Lymph Node Assay

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Key words: eugenol, local lymph node assay, non-radioisotopic, potency, sensitization.

Allergic contact dermatitis is a serious health problem. There is a need to identify and characterize skin sensitization hazards, particularly with respect to relative potency, so that accurate risk assessments can be developed. For these purposes the murine local lymph node assay (LLNA) was developed. Here, we have investigated further a modification of this assay, non-radioisotopic LLNA, which in place of tritiated thymidine to measure lymph node cell proliferation employs incorporation of 5-bromo-2′-deoxyuridine. Using this method we have examined the skin sensitizing activity of eugenol, a known human contact allergen, and its dimers 2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA) and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB). Activity in the guinea pig maximization test (GPMT) also measured. On the basis of GPMT assays, eugenol was classified as a mild skin sensitizer, DHEA as a weak skin sensitizer and DHEB as an extreme skin sensitizer. In the non-radioisotopic LLNA all chemicals were found to give positive responses insofar as each was able to provoke a stimulation index (SI) of ≥3 at one or more test concentrations. The relative skin sensitizing potency of these chemicals was evaluated in the non-radioisotopic LLNA by derivation of an EC_3 value (the concentration of chemical required to provoke an SI of 3). The EC_3 values calculated were 25.1% for eugenol, >30% for DHEA and 2.3% for DHEB. Collectively these data suggest that assessments of relative potency deriving from non-radioisotopic LLNA responses correlate well with evaluations based on GPMT results. These investigations provide support for the proposal that the non-radioisotopic LLNA may serve as an effective alternative to the GPMT where there is a need to avoid the use of radioisotopes. **Copyright © 2004 John Wiley & Sons, Ltd.**

INTRODUCTION

Allergic contact dermatitis is an important occupational and environmental health problem and there is a continuing need to identify accurately potential skin sensitization hazards and to assess effectively the likely risks to human health. Various methods have been developed for the assessment of skin sensitization potential, including those using guinea pigs, such as the guinea pig maximization test (GPMT) (Magnusson & Kligman, 1969) and Buehler's occluded patch test (Buehler, 1995), and more recently the murine local lymph node assay (LLNA) (Kimber *et al.*, 1994, 1995; Loveless *et al.*, 1996; Gerberick *et al.*, 2000). In the GPMT and the Buehler's occluded patch test the skin sensitizing potential is determined as a function of challenge-induced reactions in previously sensitized guinea pigs, whereas the LLNA is based upon consideration of induced proliferative responses in lymph nodes draining the site of topical exposure to the test chemical. In addition to hazard assessment, attention has focused more recently on evaluation of the relative skin sensitization potency as a first step in the risk assessment process. The view is that the LLNA is particularly suited to this application, not least because it is known that the vigour of lymphocyte proliferative responses induced in skin-draining lymph nodes correlates closely with the extent to which sensitization will develop.

In the standard LLNA a chemical is classified as a skin sensitizer if at one or more test concentrations it is able to induce a threefold or greater increase in lymph node cell proliferation, i.e. a stimulation index (SI) of ≥3. For the purposes of evaluating relative potency, an EC_3 value is derived mathematically from consideration of LLNA dose responses, EC_3 being the amount of contact allergen necessary to induce an SI of 3. Although the LLNA has proved to be a robust and reliable method for evaluation of skin sensitization hazards and risks, one feature that has

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sometimes limited its application is the need for a radioisotope. In the standard LLNA, lymph node cell proliferation is measured on the basis of incorporation by cells of [^{3}H]thymidine (^{3}HTdR).

We have previously explored the utility of a modified version of the assay in which, in place of radiolabelled thymidine, cell turnover is measured using the incorporation of 5-bromo-2′-deoxyuridine (BrdU) (Takeyoshi *et al.*, 2001). Here we describe investigations to explore further the value of this non-radioisotopic LLNA for the purposes of hazard identification and the determination of relative potency. To this end, responses to three chemicals have been measured: eugenol, a known contact allergen, and its dimers 2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA) and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB). For comparative purposes the activity of each of these three chemicals was also measured using the GPMT.

EXPERIMENTAL

Chemicals and reagents

Eugenol (lot no. EG0704; >95%), 2,2′-dihydroxyl-3,3′-dimethoxy 5,5′-diallyl-biphenyl (DHEA: lot no. DHEA0704; >95%) and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB: lot no. DHEB0704; >95%) were kindly donated by Kanebo Cosmetics Company (Odawara, Kanagawa, Japan) (Fig. 1). Eugenol and its dimers were dissolved in olive oil for the GPMT or in acetone–olive oil (AOO, 4 : 1) for the non-RI LLNA. 5-Bromo-2′-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg ml^{-1}.

Animals

Female Hartley guinea pigs and CBA/JN strain mice were obtained from SLC Japan Ltd (Shizuoka, Japan) and Charles River Japan Ltd (Kanagawa, Japan), respectively. The animals were housed in animal rooms maintained at a temperature of 22 ± 3 °C and at a relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10–15 cycles per hour and lighted artificially for 12 h daily. Animals were allowed free access to a laboratory diet (RC-4 for guinea pigs and MF for mice; Oriental Yeast Co., Tokyo, Japan) and tap water.

Experimental designs

Guinea pig maximization test. Guinea pigs were allocated randomly to three groups (10 animals per group). The test was conducted according to a method described previously (Magnusson & Kligman, 1969). Guinea pigs received a series of intradermal injections of eugenol or its dimers in the shoulder region to induce sensitization. After 6–8 days, sensitization was boosted by a 48-h occluded patch of the same compound placed over the injection sites. Fourteen days later, the animals were challenged on a shaved flank by a 24-h occluded patch containing the same compound. All induction and challenge concentrations were set at 5% (maximum non-irritant concentration) in olive oil for all compounds in view of preliminary dose-finding tests. All compounds elicited an apparent irritation at 10% in preliminary tests for intradermal injection and topical application, so we decided on induction and challenge concentrations of 5% for all compounds in order to compare the sensitization potency of these three compounds. Chemicals were classified by the sensitization rate for each chemical (0–8%, weak; 9–28%, mild; 29–64%, moderate; 65–80%, strong; 81–100%, extreme) according to the criteria given by Magnusson and Kligman (1969).

Non-radioisotopic LLNA. Mice were allocated randomly to 11 groups (four animals per group). A 25-μl volume of test chemicals at concentrations of 1%, 6%, 15% or 30% for eugenol, 1%, 6% or 30% for DEHA and 1%, 6% or 20% for DEHB was applied to the dorsum of both ears of the mice daily for three consecutive days. The concentration ranges of each test chemical were decided according to the sensitization potencies classified by the results of GPMT. A single intraperitoneal injection (5 mg per mouse per injection) of BrdU was then given on day 4. On day 5, the draining auricular lymph nodes were

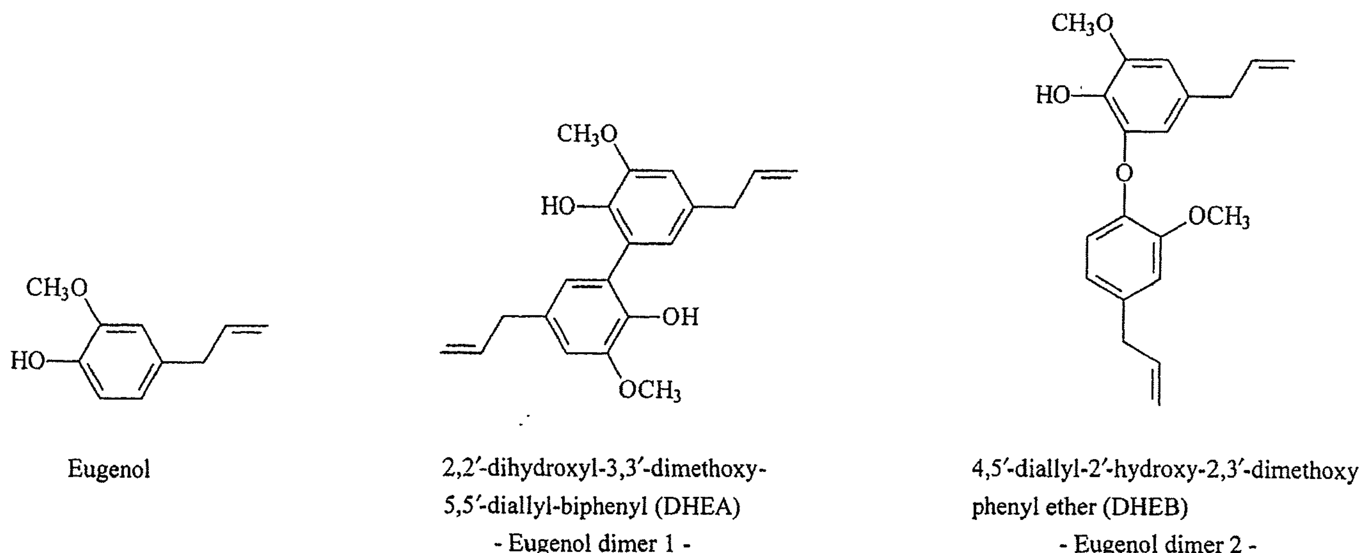

Figure 1. Chemical structures of eugenol and its dimers used in this study.

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removed, weighed and stored at –20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure BrdU incorporation. The incorporation of BrdU into lymph node cells was determined using a commercial cell proliferation assay kit (Boehringer Mannheim Corp., Indianapolis, IN, USA; Cat. no. 1647229). The lymph nodes were crushed, passed through a no. 70 nylon mesh and the lymph node cells were suspended in 15 ml of physiological saline individually. The cell suspension (100 µl) was added to the wells of a flat-bottom microplate (Coster 3595; Corning Inc., NY, USA) in triplicate. After centrifugation (3000 *g*, 10 min), the supernatants were removed. A 200-µl volume of Fix-Denat solution was added to each well and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 µl, Boehringer Mannheim Corp.) was added to each well and, after rinsing three times with washing buffer (phosphate-buffered saline), 100 µl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labelling index.

Statistical analysis

Means and standard errors were calculated for the labelling index obtained by ELISA for each treatment group. The SI values relative to the AOO-treated control value were then calculated. Data were analysed simultaneously using the Bartlett test for homogeneity of variance. If the variances were homogeneous at a level of 5% significance, a one-way analysis of variance (one way-ANOVA) was performed. If the one-way ANOVA produced a significant difference, the differences between the control group and each of the experimental groups were analysed using the Dunnett test. If the variances were not homogeneous, the Kruskal–Wallis test was employed. If this test produced a significant difference, the difference between the control group and each of the experimental groups was analysed using the non-parametric Dunnett test (Bruning & Kintz, 1997).

Measurement of EC_3 values

The estimated concentration of a chemical required to induce an SI of 3 relative to vehicle-treated controls (EC_3 value) was derived by linear interpolation as described previously (Basketter *et al.*, 2000). The EC_3 value was calculated by interpolating between two points on the SI axis, one immediately above and one immediately below the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a, b) and (c, d), respectively, then the EC_3 value may be calculated using the following equation:

$$EC_3 = c + [(3 - d)/(b - d)](a - c)$$

RESULTS

Guinea pig maximization test

In the GPMT for eugenol and its dimers, the sensitization response rates were as follows: eugenol, 20%; DHEA, 0%; DHEB, 100%. According to convention (Magnusson & Kligman, 1969) therefore, eugenol was classified as a mild skin sensitizer; DHEA as a weak skin sensitizer and DHEB as an extreme skin sensitizer (Table 1).

Non-radioisotopic LLNA

At application concentrations of 15% or greater, exposure of mice to eugenol caused a significant increase in draining lymph node weight compared with concurrent vehicle-treated controls (see Table 2). A positive response with respect to lymph node cell proliferation was obtained with 30% eugenol (SI = 3.3). Significant increases in the incorporation of BrdU were observed following treatment with both 15% and 30% eugenol, but at the lower concentration this did not translate into a positive response with respect to the stimulation index (SI = 2.3). The DHEA dimer failed to induce a positive response in the nonradioisotopic LLNA at any concentration tested, although at the highest concentration (30%) there was a significant increase in BrdU incorporation compared with vehicle-treated controls. The highest concentrations of DHEA were without effect on draining lymph node weight, although at the lowest concentration of this dimer tested there was a significant increase. Finally, the second dimer (DHEB) provoked clear positive responses in the non-radioisotopic LLNA. Treatment with 6% DHEB resulted in SI = 5.0 and treatment with 20% DHEB produced SI = 7.2. At both of these test concentrations there was also a statistically significant increase in the incorporation by lymph node cells of BrdU compared with controls. At all concentrations of DHEB examined there were significant increases in lymph node weight.

The EC_3 values for eugenol and DHEB were calculated using a standard method and were found to be 25.1% and 2.3%, respectively. Such an approach was not possible with DHEA because at no test concentration was a positive response elicited in the non-radioisotopic LLNA with respect to an SI of ≥3. An EC_3 value for DHEA has therefore been estimated, for the purposes of comparison, by linear extrapolation of the dose–response curve. Although this is somewhat unconventional, it is considered acceptable in

Table 1—Results of the guinea pig maximization test for eugenol and its dimers

Chemical name	Sensitization rate (%)	Classification[a]
Eugenol	20	Mild
2,2′-Dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA)	0	Weak
4,5′-Diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB)	100	Extreme

[a] Classified according to the criteria of Magnusson and Kligman (1969).

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Table 2—Results[a] of non-radioisotopic local lymph node assay with eugenol and its dimers used in this study

Group	Concentration (%)	Lymph node weight (mg)			BrdU labelling index ($A_{370-490}$)			
		Mean	SEM	SI[b]	Mean	SEM	SI[b]	EC_3
Vehicle control (AOO)	—	2.150	0.155	—	0.107	0.010	—	
Eugenol	1	3.325	0.485	1.5	0.187	0.031	1.7	
	6	3.325	0.578	1.5	0.161	0.031	1.5	
	15	5.175*	0.085	2.4	0.251*	0.028	2.3	25.1
	30	5.650**	0.517	2.6	0.355**	0.048	**3.3**	
2,2′-Dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA)	1	3.400**	0.426	1.6	0.182	0.018	1.7	
	6	3.050	0.393	1.4	0.183	0.039	1.7	>30
	30	2.675	0.229	1.2	0.242*	0.043	2.3	
4,5′-Diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB)	1	4.400*	0.705	2.0	0.244	0.053	2.3	
	6	5.675**	0.612	2.6	0.538**	0.073	**5.0**	2.3
	20	9.800**	0.610	4.6	0.774**	0.057	**7.2**	

[a] Results represent mean and standard errors in four mice. Significant differences from vehicle control: * $P < 0.05$ and ** $P < 0.01$ (Dunnett's test).
[b] The stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group. The cases showing three or greater SI values were defined as positive (in bold type).

the context of these comparative studies. The EC_3 value estimated for DHEA was >30%.

DISCUSSION

Eugenol (a component of clove oil and nutmeg oil) has antibacterial and antioxidative effects and has utility (due to its whitening and antibacterial effects) in the cosmetic and dermatological fields (Yamazaki *et al.*, 1998, 2000). It is regarded as being a relatively weak skin sensitizer in humans and is known to cause allergic contact dermatitis in some subjects (Basketter *et al.*, 2000, 2001; Gerberick *et al.*, 2000). Eugenol has been shown previously to induce positive responses in the standard LLNA and these present investigations have shown that it is also positive in the non-radioisotopic LLNA. The derivation of EC_3 values provides an opportunity to compare the two forms of the assay with respect to sensitivity. In one series of investigations eugenol was recorded as having as EC_3 value of ca. 13% (Basketter *et al.*, 2000). In the non-radioisotopic LLNA reported here, eugenol displayed a somewhat higher EC_3 value that might be indicative of somewhat lower sensitivity (for this chemical allergen at least) compared with the standard method. Colorimetric analysis, such as an ELISA for BrdU employed in this study, has an apparently narrower dynamic range compared with the scintillation counting for radiolabelled thymidine employed in the standard LLNA. The narrow dynamic range of the endpoint in the non-radioisotopic LLNA may be a source of lower sensitivity of this alternative. Notwithstanding this difference, the data suggest that the non-radioisotopic LLNA might have the equivalent sensitivity to the GPMT.

As far as we are aware, the dimers DHEA and DHEB have not been tested in the standard assay. In the non-radioisotopic LLNA, however, they exhibited clear and marked differences in skin sensitizing activity; the former had an estimated EC_3 value of >30% (and in fact failed to elicit a positive response with respect to an SI value of ≥3) whereas the derived EC_3 value for DHEB was 2.3%. It is instructive to compare these data with the results of GPMT analyses. The same ranking order was obtained with DHEB, the chemical displaying the greatest potency (lowest EC_3 value) of the chemicals tested, being classified as an extreme sensitizer in the GPMT. In the GPMT eugenol gave a 20% response rate (classification of mild) and DHEA gave a response rate for 0% (classification of weak). In addition, the results obtained in this study showed that the non-radioisotopic LLNA could detect the sensitization potential of a chemical classified in the lowest sensitizing potency in the GPMT by using the statistical endpoint. This suggests that the statistical endpoint can achieve an improvement in the sensitivity of this alternative. Taken together, therefore, the results obtained with the non-radioisotopic LLNA reflect what can be judged from GPMT data of the relative skin sensitizing potential of these chemicals.

In conclusion, the results reported here reveal that the sensitivity of the non-radioisotopic LLNA may not be very dissimilar from the standard method and that estimations of relative potency based on EC_3 values deriving from non-radioisotopic dose responses appear to provide an accurate picture of ranking. Experience to date suggests that the non-radioisotopic LLNA may be of value in circumstances where there is a need to assess skin sensitization activity without the use of radioisotopes. Moreover, non-radioisotopic LLNA apparently has an advantage over the GPMT with regard to points of animal welfare and it can provide a rapid and cost-effective method for screening sensitizers in the same way as the standard LLNA.

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Assessment of statistic analysis in non-radioisotopic local lymph node assay (non-RI-LLNA) with α-hexylcinnamic aldehyde as an example

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Abstract

The murine local lymph node assay (LLNA) is used for the identification of chemicals that have the potential to cause skin sensitization. However, it requires specific facility and handling procedures to accommodate a radioisotopic (RI) endpoint. We have developed non-radioisotopic (non-RI) endpoint of LLNA based on BrdU incorporation to avoid a use of RI. Although this alternative method appears viable in principle, it is somewhat less sensitive than the standard assay. In this study, we report investigations to determine the use of statistical analysis to improve the sensitivity of a non-RI LLNA procedure with α-hexylcinnamic aldehyde (HCA) in two separate experiments. Consequently, the alternative non-RI method required HCA concentrations of greater than 25% to elicit a positive response based on the criterion for classification as a skin sensitizer in the standard LLNA. Nevertheless, dose responses to HCA in the alternative method were consistent in both experiments and we examined whether the use of an endpoint based upon the statistical significance of induced changes in LNC turnover, rather than an SI of 3 or greater, might provide for additional sensitivity. The results reported here demonstrate that with HCA at least significant responses were, in each of two experiments, recorded following exposure of mice to 25% of HCA. These data suggest that this approach may be more satisfactory—at least when BrdU incorporation is measured. However, this modification of the LLNA is rather less sensitive than the standard method if employing statistical endpoint. Taken together the data reported here suggest that a modified LLNA in which BrdU is used in place of radioisotope incorporation shows some promise, but that in its present form, even with the use of a statistical endpoint, lacks some of the sensitivity of the standard method. The challenge is to develop strategies for further refinement of this approach.
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Keywords: Local lymph node assay; Sensitivity; Non-radioisotopic; Statistical analysis; α-Hexylcinnamic aldehyde

1. Introduction

The murine local lymph node assay (LLNA) is a validated method for determining the sensitizing potential of chemicals in which activity is mea-

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sured as a function of induced proliferative responses in draining lymph nodes (Dean et al., 2001; Gerberick et al., 2000; Haneke et al., 2001; Sailstad et al., 2001). The standard LLNA method that employs radioisotope incorporation to measure lymph node cell (LNC) proliferation has been shown to be robust and reliable However, the use of radioisotopes requires some special containment and we have, therefore, explored an alternative strategy. We have previously developed a non-radioisotopic (non-RI) endpoint for the LLNA using 5-bromo-2′-deoxyuridine (BrdU) incorporation (Takeyoshi et al., 2001). Although this alternative method is useful in situations where there is a need to avoid the use of radioisotopes, this alternative endpoint appeared not to be as sensitive as the standard LLNA.

Here we report investigations to determine the use of statistical analysis to improve the sensitivity of a non-RI LLNA procedure based on BrdU incorporation. α-Hexylcinnamic aldehyde (HCA) a contact allergen that has been shown previously to elicit stable responses in the standard LLNA (Dearman et al., 1998, 2001) was used as the test chemical.

2. Materials and methods

2.1. Chemicals

HCA (HCA, Lot No. 0116AQ; Aldrich Chemical Company Inc., Milwaukee, WI, USA) was dissolved in acetone:olive oil (AOO; 4:1). 5-Bromo-2′-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg/ml.

2.2. Animals

Female CBA/JN mice were obtained from Charles River Japan Ltd. (Kanagawa, Japan). The mice were housed in animal rooms maintained at a temperature of 23±2 °C and a relative humidity of 55±15%. The rooms were ventilated at a frequency of 10–15 cycles/h, and lighted artificially for 12-h daily. The animals were allowed to free access to laboratory diet (MF, Oriental yeast Co., Tokyo, Japan) and tap water.

2.3. Experimental design

Mice were randomly allocated to five or four groups (four mice/group). In experiment 1, a 25 μl volume of the HCA preparation at concentration of either 0% (AOO), 3.125, 6.25, 12.5 or 25% in AOO was applied to the dorsum of both ears daily for 3 consecutive days. A single intraperitoneal injection (5 mg/mouse per injection) of BrdU was given on day 4. On day 5, the auricular lymph nodes were removed, weighed, and stored at −20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure the level of BrdU incorporation. In experiment 2, HCA preparations with concentrations of 0% (AOO), 12.5, 25 and 50% were used in the sameway.

2.4. ELISA for BrdU incorporation

The incorporation of BrdU into LNC was determined using a commercial cell proliferation assay kit (Boehringer Mannheim Corp., Indianapolis, IN, USA; Cat. No. 1647229). The lymph nodes were crushed, passed through a #70 nylon mesh, and LNC were suspended in 15 ml of physiological saline individually. The cell suspension (100 μl) was added to the wells of a flat-bottom microplate (Coster 3595; Corning Inc., NY, USA) in triplicate. After centrifugation (3000 × g, 10 min), the supernatants were removed, 200 μl of Fix-Denat solution was added to each well, and the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 μl, Boehringer Mannheim Corp.) was added to each well, and after rinsing three times with washing buffer (phosphate-buffered saline), 100 μl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labeling index.