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N,*N*′-Diacetyl-L-cystine—the Disulfide Dimer of *N*-acetylcysteine—Is a Potent Modulator of Contact Sensitivity/Delayed Type Hypersensitivity Reactions in Rodents[1]

BENGT SÄRNSTRAND,[2] ANNE-HELENE JANSSON,[2] GIEDRE MATUSEVICIENE,[3] ANNIKA SCHEYNIUS,[3] STEFAN PIERROU,[4] and HÅKAN BERGSTRAND[4]

Department of Pharmacology, Astra Draco AB, Lund, Sweden

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ABSTRACT

Oral *N*-acetyl-L-cysteine (NAC) is used clinically for treatment of chronic obstructive pulmonary disease. NAC is easily oxidized to its disulfide. We show here that *N*,*N*′-diacetyl-L-cystine (DiNAC) is a potent modulator of contact sensitivity (CS)/delayed type hypersensitivity (DTH) reactions in rodents. Oral treatment of BALB/c mice with 0.003 to 30 μmol/kg DiNAC leads to *enhancement* of a CS reaction to oxazolone; DiNAC is 100 to 1000 times more potent than NAC in this respect, indicating that it does not act as a prodrug of NAC. Structure-activity studies suggest that a stereochemically-defined disulfide element is needed for activity. The DiNAC-induced enhancement of the CS reaction is counteracted by simultaneous NAC-treatment; in contrast, the CS reaction is even more enhanced in animals treated with DiNAC together with the glutathione-depleting agent buthionine sulfoximine. These data suggest that DiNAC acts via redox processes. Immunohistochemically, ear specimens from oxazolone-sensitized and -challenged BALB/c mice treated with DiNAC display increased numbers of $CD8^+$ cells. DiNAC treatment *augments* the CS reaction also when fluorescein isothiocyanate is used as a sensitizer in BALB/c mice; this is a purported TH2 type of response. However, when dinitrofluorobenzene is used as a sensitizer, inducing a purported TH1 type of response, DiNAC treatment *reduces* the reaction. Treatment with DiNAC also reduces a DTH footpad-swelling reaction to methylated BSA. Collectively, these data indicate that DiNAC in vivo acts as a potent and effective immunomodulator that can either enhance or reduce the CS or DTH response depending on the experimental conditions.

Oral *N*-acetyl-L-cysteine (NAC) has been used clinically as a remedy for chronic obstructive pulmonary disease for decades, and it is generally believed that its beneficial effect is due to a mucolytic activity. However, it has not been possible to demonstrate presence of the drug in epithelial lining fluid after oral administration (Cotgreave et al., 1987); this is not fully compatible with its proposed in vivo mucolytic activity. Moreover, NAC is reported to primarily reduce exacerbation rates in the disease (Boman et al., 1983). Considering this, it is possible that NAC acts in chronic obstructive pulmonary disease patients, at least in part, by enhancing their host defense.

In support of such an idea, numerous reports show that NAC treatment in vitro can modulate activities of lymphoid cells. Human T cell IL-4 synthesis and B cell IgE and IgG4 production are decreased by the compound, whereas T cell IL-2 production is enhanced (Jeannin et al., 1995). NAC inhibits apoptosis of T cell hybridomas (Jones et al., 1995) and reverses down-regulation of IL-2 mRNA and IL-2 activity in human lymphocytes (Flescher et al., 1994). In biochemical terms, NAC has been generally considered to act as a precursor of glutathione (Dröge et al., 1994; Jeannin et al., 1995; Jones et al., 1995; Yan et al., 1995), as an antioxidant (Aruoma et al., 1989), and/or as a reductant that modulates redox-sensitive transcription factors like AP-1 or NF-κB or otherwise influences transcriptional events (Schreck et al., 1991; Dröge et al., 1994; Yan et al., 1995; Xia et al., 1996). The effects of NAC, seen at close to mM concentrations,

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[2] Current address: Department of Human Pharmacology, Box 34, S 22100 Lund, Sweden.
[3] Current address: Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska Hospital and Institute, S 171 76, Stockholm, Sweden.
[4] Current address: Department of Cell & Molecular Biology, Astra Draco AB, Box 34, S 221 00 Lund, Sweden.

ABBREVIATIONS: ADA 202–718, ethylene-2,2′-bis(dithio)-bis(ethanol); AMG, aminoguanidine; BSO, L-buthionine-[S, R]-sulfoximine; CS, contact (hyper)sensitivity; CSA, cyclosporin A; DDTC, diethyldithiocarbamate; DiNAC, N,N′-diacetyl-L-cystine; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed type hypersensitivity; FITC, fluorescein isothiocyanate; HEDS, bis-(2-hydroxyethyl)-disulfide; mAbs, monoclonal antibodies; mBSA, methylated BSA; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; Oxazolone, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; RPA, RNase protection assay.

conform to the in vitro stimulatory effects of other low molecular weight thiols, like 2-mercaptoethanol and glutathione, on lymphoid cell function (Hiestand and Strasser 1985a; Dröge et al., 1994; Jeannin et al., 1995). Modulation of in vivo immune responses by NAC have also been reported (Hiestand and Strasser, 1985b; Kinscherf et al., 1994; Jeannin et al., 1995).

Reports have suggested that the efficacy of in vivo modulation by some thiols relies on the presence of an intact disulfide bridge (Hiestand and Strasser, 1985b; St Georgiev, 1988). Therefore, we examined the effects of the disulfide dimer of NAC, *N*,*N*′-diacetyl-L-cystine (DiNAC) in in vivo systems reflecting various immune responses in rodents. One such system is the delayed type hypersensitivity (DTH) reaction, an in vivo reflection of a cell-mediated immune response (Askenase, 1992). The expression DTH is often used to denote reactions induced by protein antigens as well as by contact sensitizers. However, recent reports indicate that different subsets of T cells govern classical DTH reactions to protein antigens and contact sensitivity (CS) reactions (Grabbe and Schwarz, 1998). The present report deals with effects of DiNAC in CS skin reactions induced by 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), fluorescein isothiocyanate (FITC), and 2,4-dinitrofluorobenzene (DNFB), as well as in a DTH reaction to methylated BSA (mBSA) assessed by foot pad swelling.

Materials and Methods

Synthesis and Sources of Compounds. Compounds *N*,*N*′-diacetyl-L-cystine (DiNAC; referred to as D7042) and its di-L-lysinium salt (referred to as D7193 - preferred for use over D7042 due to pharmaceutical reasons), *N*-acetyl-L-cysteine, ethylene-2,2′-bis(dithio)bis(ethanol) (ADA 202–718), L-homocystine, *N*,*N*′-diacetyl-L-homocystine, *N*,*N*′-diacetyl-cystamine, L-cystathionine, *N*,*N*′-diacetyl-L-cystathionine, racemic lanthionine, and racemic *N*,*N*′-diacetyl lanthionine, were synthesized by Astra Draco AB (Lund, Sweden) or Astra APP (Södertälje, Sweden). Aminoguanidine bicarbonate salt, L-buthionine-[S, R]-sulfoximine (BSO), diethyldithiocarbamate (DDTC), DNFB, FITC-isomer 1, and mBSA were all obtained from Sigma Chemical Co. (St. Louis, MO). Bis-(2-hydroxyethyl)-disulfide (HEDS) was from Fluka AG (Buchs, Switzerland), whereas oxazolone was obtained from either BDH Chemicals Ltd. (Poole, Dorset, England) or from Sigma. Cyclosporin A (CSA; Sandimmun) was obtained from Sandoz Pharma AG (Basel, Switzerland) and rapamycin and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Alexis Corp. (San Diego, CA). Sodium pentobarbiturate (Mebumal) was purchased from Apoteksbolaget (Umeå, Sweden) and enflurane (Efrane) was obtained from Abbott S.p.A. (Campoverde Lieti, Italy). Sources of antibodies used in immunohistochemical experiments are given below.

Animals. Male and female mice of the following strains were obtained from Bomholtsgaard (Ry, Denmark) or Charles River Breeding Laboratories (Kent, UK): BALB/c, CD-1, C57BL/6, CBA/J, NOD, MRL/Mp and MRL-l*pr*/*lpr*. The mice were used at the weight of 18 to 20 g. Female Sprague-Dawley rats were from Möllegaard (Ejby, Denmark); they were used at 150 g. The animals were caged for at least 8 days after arrival before experiments were initiated. Animals had free access to food (R3, Ewos, Södertälje, Sweden) and water. The light period in the room was 12 h (6:00 AM-6:00 PM). Rabbits of New Zealand strain of both sexes were obtained from HB Rabbit Farm, Lidköping, Sweden. All animal study designs were approved by local ethics committees.

Induction and Assessment of CS to Oxazolone, DNFB, and FITC. Animals were sensitized (day 0) by a single epicutaneous application of 150 μL (mice), 400 μL (rats), or 1000 μL (rabbits) 3% oxazolone solution in absolute ethanol-acetone (3:1) on the shaved thorax and abdomen. Treatment with D7042, D7193, or other compounds was normally initiated by oral feeding (gavage) of 10 ml/kg body weight of an appropriate concentration of the compound immediately after sensitization. Treatment continued once daily up to and including day 6. Control animals were given the corresponding amount of vehicle (saline or water as indicated in the figure legends). Other treatment regimens with D7042 or D7193 were examined as specified in the text. In separate experiments, D7193 was administered in the drinking water (prepared fresh each day) in free access, injected either i.v. in the tail vein (20 μL) or i.p. (200 μL), or instilled intratracheally under light Efrane anesthesia. Eight days after sensitization (i.e., on day 7), mice were challenged on both sides of both ears by topical application of a total of 20 μL of 1% oxazolone dissolved in peanut oil; rats were challenged with 40 μL and rabbits with 400 μL of the same solution. Ear thickness was measured before and 24 h after challenge (in some experiments also at 48 h) using an Oditest spring caliper handled manually (van Loveren et al., 1984) or coupled to a computer-directed motor device. Challenges and measurements of ear thickness were performed under light pentobarbital anesthesia. Most experiments were performed under coded conditions.

The intensity of the CS reactions was expressed as increase in ear thickness in mm, i.e., $T_{24/48}$ -T_0 mm, where T_0 and $T_{24/48}$ represent the ear thickness before and 24 or 48 h after challenge, respectively. The figure recorded for each animal and time point is the mean of the measurements on both ears in individual tests. The results were expressed as the mean ± S.E.M from groups of 8 to 10 animals. Degree of significance for differences between means of groups was obtained by Student's two-tailed *t* test.

Mice were sensitized to DNFB or FITC, and challenges were performed as described (Tang et al., 1996). The magnitude of the CS reactions induced by DNFB and FITC were assessed as described above for oxazolone-induced reactions.

Immunohistochemical Examinations. Histological and immunohistological examinations were performed in blind manner on coded ears from BALB/c mice untreated, sensitized, and challenged with oxazolone with or without treatment with D7193. The ears were cut off at the bases and immediately snap-frozen in isopentane in liquid nitrogen and stored at −70°C. The frozen ears were processed for routine histological examinations and for immunohistochemical examinations using the ABC-technique according to principles described previously (Scheynius et al., 1996). The frozen specimens were cut through the center of the ear extending from the top to the base in a cryostat. The cryostat sections, 6 μm thick, were acetone-fixed, and incubated in 0.3% H_2O_2 in phosphate-buffered saline for 15 min at room temperature to block endogenous peroxidase. Sections were then treated with normal rabbit serum to reduce nonspecific binding and with Avidin D solution and biotin-solution blocking kit (Vector Laboratories, Inc., Burlingame, CA). This was followed by incubation with rat anti-mouse monoclonal antibodies (mAbs) (see below), biotinylated anti-rat IgG (Vector Laboratories) and avidin-biotin-peroxidase complex (Dako A/S, Glostrup, Denmark). The peroxidase reaction was developed with 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Steinheim, Germany). The sections were counterstained with Mayer's hematoxylin. Optimal dilutions of the mAbs were determined in control experiments with sections from normal mouse spleen and skin. Each ear specimen was also processed for hematoxylin and eosin staining. The following rat anti-mouse mAbs were used: anti-CD4 (L3T4, clone H129.19), anti-MHC class II I-$A^{b,d}$ (clone B21.2), anti-CD11a (the α-chain of LFA-1, clone FD441.8), anti-CD11b (the α-chain of Mac-1, C3biR, clone M1/70.15.11.5), anti-CD54 (ICAM-1, clone YNI/1.7.4), and anti-CD44 (clone IRAWB14.4) all obtained from their hybridoma cells expanded in vitro, anti-CD8 (Lyt2) obtained from Serotec Ltd., (Kidlington, Oxford, UK), and anti-CD106 (VCAM-1) from PharMingen (San Diego, CA).

Dermal cell infiltration was assessed on hematoxylin and eosin-stained sections on a semiquantitative scale from 0 to 3, where 0 = normal, 1 = small, 2 = moderate, and 3 = large dermal cell infiltrates. The immunoperoxidase-stained sections were evaluated on a semiquantitative scale ranging from 0 to 3 in dermis, where 0 = no, 1 = few, 2 = moderate, and 3 = many positive cells. The scale was adjusted for each antibody, so that the grading "3" refers to the maximal number of positive cells within all specimens. In epidermis, $CD4^+$, $CD8^+$, LFA-1^+, and C3biR^+ cells were counted at magnification 400× in ten grids or five grids, respectively, and expressed on a 0 to 3 scale, where 0 = 0 to 1 positive cell, 1 = 2 to 5, 2 = 6 to 9, and 3 = 10 or more positive cells. Keratinocytes positive for MHC class II antigens, ICAM-1, and CD44 were determined semiquantitatively. At least two sections per antibody and specimen were examined.

Multi-Probe RNase Protection Assay (RPA) Analysis of Cytokine Profiles in CS Reaction Sites. Four different groups of mice were investigated for chemokine/cytokine expression; nonsensitized mice treated either with vehicle or with 3 μmol/kg D7193 and mice sensitized to and challenged with oxazolone that were treated either with vehicle or with 3 μmol/kg D7193. Three individuals in each group sacrificed 24 h after challenge were analyzed. RNA was prepared as described (Chomczynski and Sacchi, 1987) from ears that had been cut from sacrificed mice and snap-frozen in liquid nitrogen. The ears were ground in liquid nitrogen and the resulting powder was suspended in homogenization solution (4 M guanidium thiocyanate, 0.1 M Tris-Cl pH 7.5, 1% β-mercaptoethanol). The slurry was sheared with a disposable syringe using a 0.9-mm needle. Expression of mRNAs for eotaxin, glyceraldehyde-phosphate dehydrogenase, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, IFN-γ, IP-10, L32, lymphotactin, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and TCA-3 were examined with the Riboquant multiprobe RPA system from PharMingen according to the manufacturer's protocol.

In vitro transcription of linearized DNA templates was performed for each probe in a total volume of 20 μL (10 μL [α-^{32}P]UTP, 1 μL GACU pool, 2 μL DTT, 4 μL 5X transcription buffer, 1 μL RPA template set, 1 μL RNasin, 1 μL T7 RNA polymerase) and incubated at 37°C for 1 h. The reaction was terminated by adding 2 μL of DNAse I and incubating at 37°C for an additional 30 min. Twenty six μL of 20 mM EDTA and 2 μL of yeast tRNA (2 mg/mL) were added, and the reaction was extracted once with phenol/24:1 chloroform: isoamylalcohol (1:1) and once with chloroform/isoamylalcohol alone. The aqueous phase was precipitated by adding 50 μL of 4 M ammonium acetate and 250 μL of ice-cold ethanol. After centrifugation, the pellet was dissolved in 50 μL of the hybridization buffer provided in the kit. Incorporation of radiolabel was quantified using a Bioscan Quick-count and the probe was diluted to the recommended 3.0×10^5 cpm/μL. Ten μg of total RNA was used for each incubation together with approximately 5×10^5 cpm of probe in total volume of 20 μL of hybridization buffer. The annealing reaction mixture was incubated at 56°C overnight. RNase digestion was performed by adding 100 μL of RNase mix (provided in the kit) containing 192 pg/μL RNase A, 0.6 U/μL RNase T1, and RNase buffer to the reaction. The mixture was incubated at 37°C for 45 min. To stop the digestion, proteinase K was added together with yeast RNA as carrier. The reaction mixture was extracted once with 130 μL phenol/24:1 chloroform:isoamylalcohol (1:1). The aqueous phase was removed to a new tube and precipitated with 120 μL of 4 M ammonium acetate and 650 μL of ice-cold ethanol. Reactions were precipitated at −20°C for 1 h; after centrifugation the pellet was dissolved in 5 μL of formamide-loading buffer. Dissolved precipitates were heated at 90°C for 3 min before resolution on 5%, 1X TBE, denaturing polyacrylamide gels. Gels were transferred to Whatman paper and dried in a Savant vacuum dryer. Dried gels were placed in a phospho-Imager cassette (Molecular Dynamics) and exposed overnight. The phospho-screen was scanned in a Molecular Dynamics Storm 860 scanner and analyzed with Image-Quant software (Molecular Dynamics).

Footpad DTH Responses in Mice Sensitized to mBSA. A DTH response to mBSA was induced in BALB/c mice as described (Tarayre et al., 1990). Briefly, mBSA emulsified in Freund's complete adjuvant was injected intradermally on day 0 (a total of 0.25 mg of mBSA in a total volume of 0.1 ml of emulsion was given at three injection sites). Challenge was performed by injection on day 7 of 0.025 ml mBSA solution (25 mg/mL) in the right hind paw; the left hind paw, injected with the corresponding volume of the vehicle, served as a control. Twenty-four hours later, the animals were deeply anesthetized and both hind paws were cut. The DTH reaction was estimated from the difference in weight between the two hind paws.

DTH Granuloma Reaction Induced by mBSA. A s.c. DTH reaction induced by mBSA resulting in a quantifiable chronic granulomatous lesion (Dunn et al., 1989) was used to assess effects of treatment with D7193 on a chronic cell-mediated inflammatory reaction. Briefly, mice were sensitized to mBSA (a total of 25 mg of mBSA and 500 mg of Dextran in a total volume of 0.1 ml of emulsion was given at a total of three injection sites). The animals were challenged 3 weeks later by s.c. implantation of Millipore filters (10 mm diameter) soaked in 25 μL of mBSA solution (3 mg/mL). After 7 days, filter implants were dissected away from the lesions and weighed (wet/dry) for quantification of inflammation. The results were expressed as the mean ± S.E.M from groups of 8 to 10 animals. Degree of significance for differences between means of groups was obtained by Student's two-tailed *t* test.

Pharmacological Modulation of the CS Skin Reactions to Oxazolone in Mice. Mice sensitized to oxazolone as described above were treated orally with D7193 or vehicle and simultaneously with either freshly prepared NAC (3 μmol/kg or 30 μmol/kg orally), CSA (30 μmol/kg orally), rapamycin (3 μmol/kg orally), aminoguanidine (AMG; 813 μmol/kg i.p. once daily from day 2 before sensitization up to and including the day of challenge, day 7), L-NAME (115 μmol/kg orally by gavage from day 5 before sensitization to day 6), or BSO (2 mmol/kg twice a day i.p. and 20 mM in drinking water from day 5 before sensitization up to and including the day of challenge, day 7). The results were expressed as the mean ± S.E.M. from groups of 8 to 10 animals. Degree of significance for differences between means of groups was obtained by Student's two-tailed *t* test.

Results

Influence of DiNAC (D7042/D7193) on CS Skin Reactions to Oxazolone in Mice. The capacity of D7042 to modulate the CS skin reaction to oxazolone is illustrated in Fig. 1, which shows the results of measurements of ear thickness 24 h after challenge in animals treated with D7042 compared with those from animals treated with NAC. Treatment with both compounds causes a dose-dependent increase of ear thickness; however, the log dose-response relations indicate that DiNAC is 100 to 1000 times more potent than NAC and twice as effective at the highest examined comparable dose.

Separate experiments showed that the capacity of D7042 to enhance the CS reaction at either 24 or 48 h after challenge did not differ if treatment was terminated on day 6 as described above or if it continued until evaluation (i.e., treatment for days 0–9, challenge day 7, evaluation day 8 and day 9; data not shown).

Experiments comparing the effects of D7042 (salt-free form of DiNAC) to those of D7193 (the lysine salt form of DiNAC) at 0.03 or 3 μmol/kg did not reveal any difference in activity between the two preparations. Doses of lysine corresponding to those present in the administered doses of D7193 did not affect the CS reaction (data not shown).

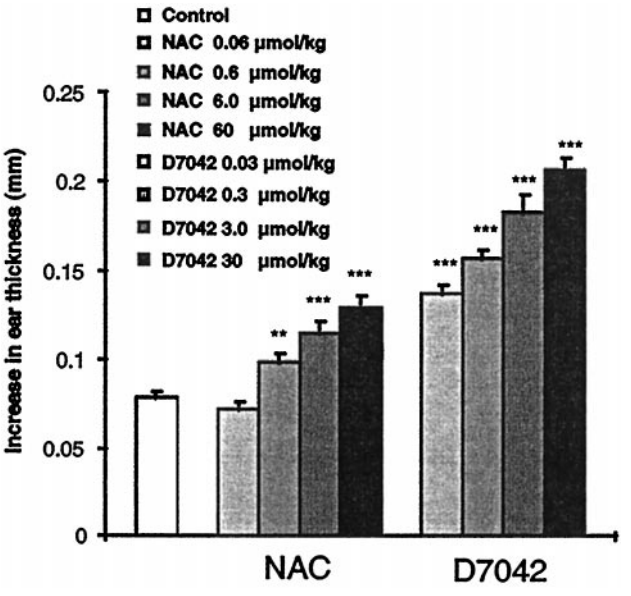

Fig. 1. Effects of treatment with *N*,*N*′-diacetyl-L-cystine (D7042) or NAC, both given by oral gavage once daily during days 0 to 6, on the CS response to oxazolone in BALB/c mice sensitized to oxazolone on day 0 and challenged on day 7. The response was measured as the increase in ear thickness (mean ear swelling) ± S.E.M. 24 h after challenge (day 8). The response of control animals that received saline is illustrated by the open left column. $n = 10–15$, $^{**}p < .01$, $^{***}p < .001$.

The effect of D7193 is highly reproducible from experiment to experiment. Analysis of data from experiments performed during a period of 6 years shows that the *group* mean increase in ear swelling 24 h after challenge in oxazolone-sensitized control animals is 0.09 mm ($n = 55$) compared with a group mean increase of 0.16 mm in animals treated with 3 μmol/kg D7193 ($n = 55$; $p < .001$).

Separate kinetic experiments showed that a minute degree of ear swelling seen already at 2 h after challenge (the early phase of the CS reaction; cf. Askenase, 1992) is not enhanced in animals treated with D7193 (results not shown).

Dose-response assessments of the effects of D7193 after oral, i.p., i.v., or intratracheal administration (Fig. 2) reveal that 0.03 μmol/kg as well as 3 μmol/kg of the compound stimulated the CS reaction to a similar degree irrespective of route of administration whether the reactions were read 24 h or 48 h (not shown) after challenge. In these experiments, even the 0.0003 μmol/kg dose of D7193 induced a significant enhancement of the CS reaction. Mice given D7042 in the drinking water displayed increased CS reactivity of the same degree as those given the compound by gavage treatment once daily (results not shown).

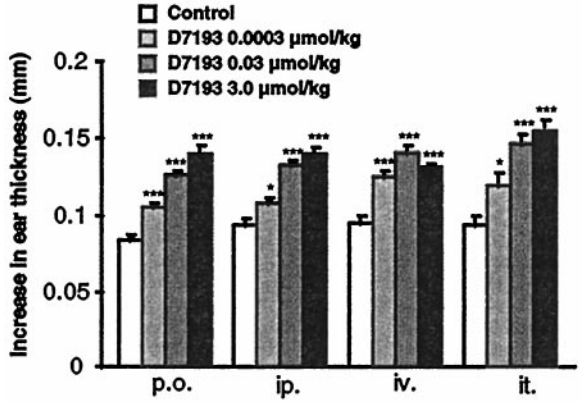

Fig. 2. The influence of the route for administration on effect of D7193. The compound or the vehicle (saline) was given to BALB/c mice sensitized to oxazolone on day 0 at the indicated dose either p.o., i.v., i.p., or intratracheally (it) as indicated once daily for days 0 to 6. The response to challenge with oxazolone on day 7 was measured as increase in ear thickness 24 h after challenge (day 8). Controls received saline. $n = 10$, $^{*}p < .05$, $^{***}p < .001$.

Optimal enhancement of the CS response to oxazolone by D7193 required that treatment was performed during the whole sensitization period, although animals treated for shorter periods of time (i.e., for days 0–4 or days 4–6) also express significantly enhanced CS responses (Fig. 3). Treatment every second day is as effective as daily treatment, but treatment each third day is not effective (results not shown).

We examined whether the effect of treatment with D7193 on the CS skin reaction in oxazolone-sensitized animals 24 h after challenge was strain-dependent. As shown in Fig. 4, mouse strains differ in their CS response to oxazolone. However, the relative degree of enhancement induced by D7193 treatment was similar in the strains examined whether they can be considered a TH1 phenotype (CD-1, C57BL/6, CBA/J), a TH2 phenotype (BALB/c) based on responses to *Leishmania* infection (see Bogdan et al., 1993), or carries an autoimmune phenotype (MRL-*lpr*/*lpr*, MRL-Mp or NOD).

Modulation of Effects of D7193 on CS Skin Reactions to Oxazolone in Mice by Selected Agents. To assess the influence of NAC on the effect of D7193, CS reactions in BALB/c were assessed in mice given either D7193 at the indicated doses, *freshly prepared* NAC at the indicated doses, or both agents (administered separately by gavage once daily according to the standard schedule treatment). The results show that simultaneous treatment with NAC reduces the enhancement of the D7193-induced CS reaction to levels that are even lower than those recorded in animals treated with NAC alone (Fig. 5A). Oxazolone-sensitized mice treated with the glutathione-depleting agent BSO (2 mmol/kg twice a day i.p. and 20 mM in drinking water (Leeuwenburgh and Ji, 1995) express markedly increased CS reactions, which are

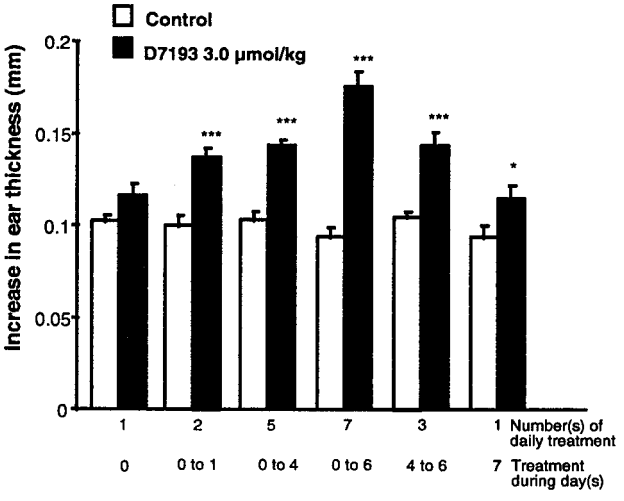

Fig. 3. The influence of treatment regimen on the capacity of D7193 to enhance the CS response to oxazolone in sensitized BALB/c mice. The experimental design followed the standard procedure except for the treatment periods. The figures given below the *x*-axis refer to the time period of oral treatment once daily with 3 μmol/kg D7193. Day 0 refers to the time of sensitization. n = 10, $^{*}p < .05$, $^{***}p < .001$.

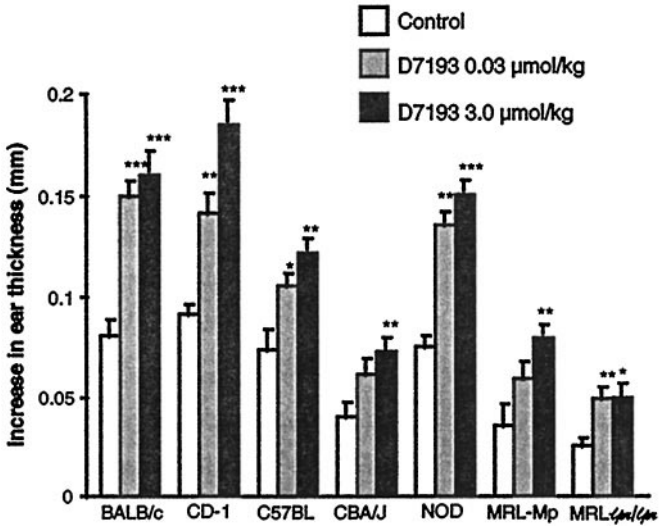

Fig. 4. The effect of oral treatment with 0.03 μmol/kg or 3.0 μmol/kg of D7193 day 0 to 6 on the CS response to oxazolone 24 h after challenge in animals of different mouse strains sensitized to oxazolone on day 0. n = 10 for each group, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

even further augmented into very large reactions by simultaneous D7193 treatment (Fig. 5B).

The immunosuppressive agent CSA at a dose of 30 μmol/kg slightly reduces the CS reaction to oxazolone when given by gavage once daily from days 0 to 6 to BALB/c mice sensitized to oxazolone (Fig. 6A). In animals treated with CSA in this way, simultaneous treatment with 3 μmol/kg D7193 leads to a partly reduced CS reaction compared with that seen in animals treated with D7193 alone (Fig. 6A). Oxazolone-sensitized mice treated with rapamycin to inhibit the p70 S6 kinase pathway (Proud, 1996) from days 0 to 6 do not display significantly reduced CS reactions. Simultaneous treatment with rapamycin and D7193 leads to effects similar to those seen with treatment with CSA; rapamycin blocks the enhancement recorded with the low dose of D7193 and partly blocks that induced by treatment with 3 μmol/kg D7193 (results not shown). Oxazolone-sensitized mice treated with the nitric oxide (NO) synthase inhibitor AMG (Tracey et al., 1995; Brenner et al., 1997) display slightly reduced CS reactions. Still, the CS reactions are enhanced in mice simultaneously treated with AMG and D7193 (Fig. 6B). In one of two experiments, animals simultaneously treated with AMG and D7193 expressed a significantly enhanced CS reaction compared with that in animals treated with D7193 alone (data not shown). Oxazolone-sensitized mice treated orally with another NO synthase inhibitor, L-NAME, at a dose which is effective in colonic inflammation in rats (Kiss et al., 1997)–a species less sensitive to L-NAME than mice (Tracey et al., 1995)–displayed slightly increased CS reactions; despite this, simultaneous treatment with L-NAME tended to reduce the enhancement of the CS reaction brought about by D7193 treatment (Fig. 6C).

Histological and Immunohistochemical Assessment of the Enhancement of the Skin CS Reaction Induced by D7193 in BALB/c Mice. Histological and immunohistochemical examinations were performed on ear specimens from six normal nontreated mice, six mice sensitized to and challenged with oxazolone, and eight mice sensitized to and challenged with oxazolone that were also treated with 3.0

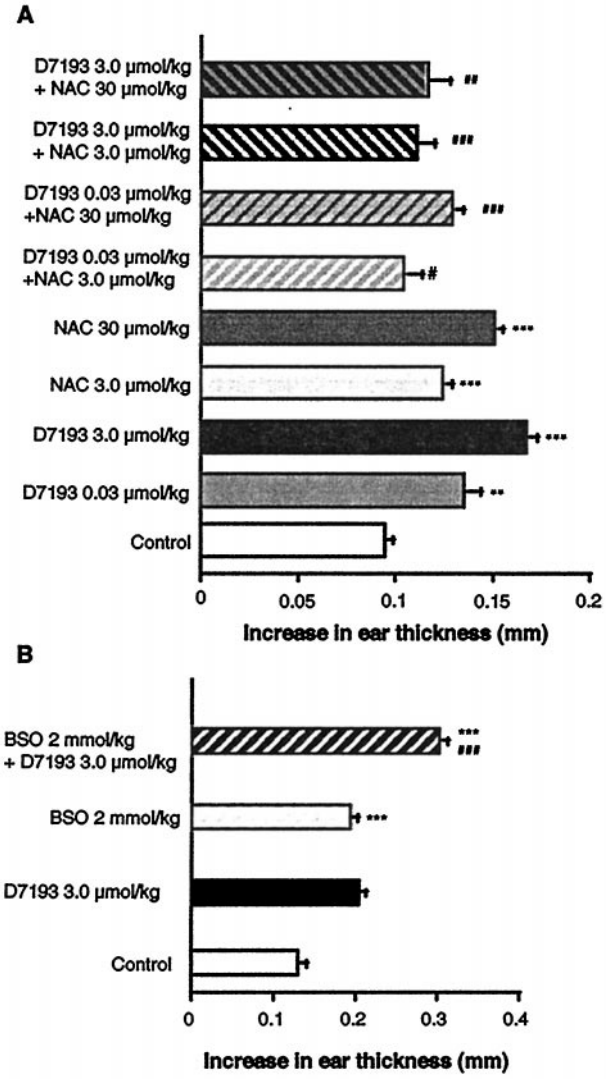

Fig. 5. A, the effect of oral treatment with 0.03 μmol/kg or 3.0 μmol/kg of D7193 separately, 3 μmol/kg or 30 μmol/kg of NAC separately, or the indicated dose of both compounds simultaneously on days 0 to 6 on the CS response to oxazolone in sensitized BALB/c mice (n = 10). Controls received saline. $^{**}p < .05$, $^{***}p < .001$ (experiments with single drug treatment compared to controls), $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ (animals treated with NAC compared to animals treated with a combination of NAC and D7193). B, the effect of treatment with BSO [2 mmol/kg twice a day i.p. and 20 mM in drinking water from day 5 before sensitization up to and including the day of challenge (day 7)], D7193 (3.0 μmol/kg) day 0 to 6 or a combination of the two drug regimens on the CS response to oxazolone in sensitized BALB/c mice (n = 10). $^{***}p < .001$ (experiments with single drug treatment compared to controls), $^{\#\#\#}p < .001$ (animals treated with D7193 compared to animals treated with a combination of BSO and D7193).

μmol/kg D7193 orally for 7 days. Hematoxylin and eosin staining of ear sections showed that in three animals treated with D7193 there was marked cell infiltration and edema. In the rest of the animals in this group, moderate to low levels of cell infiltration were observed; this effect was also seen in sensitized, challenged, but nontreated animals (Fig. 7A). In D7193-treated mice, the number of $CD8^+$ cells was increased compared with similarly sensitized and challenged, but untreated mice, with the most pronounced difference in epidermis compared to dermis (Fig. 7, B and C, respectively). Representative examples showing increases in epidermal $CD8^+$ cells in D7193-treated versus nontreated animals are shown

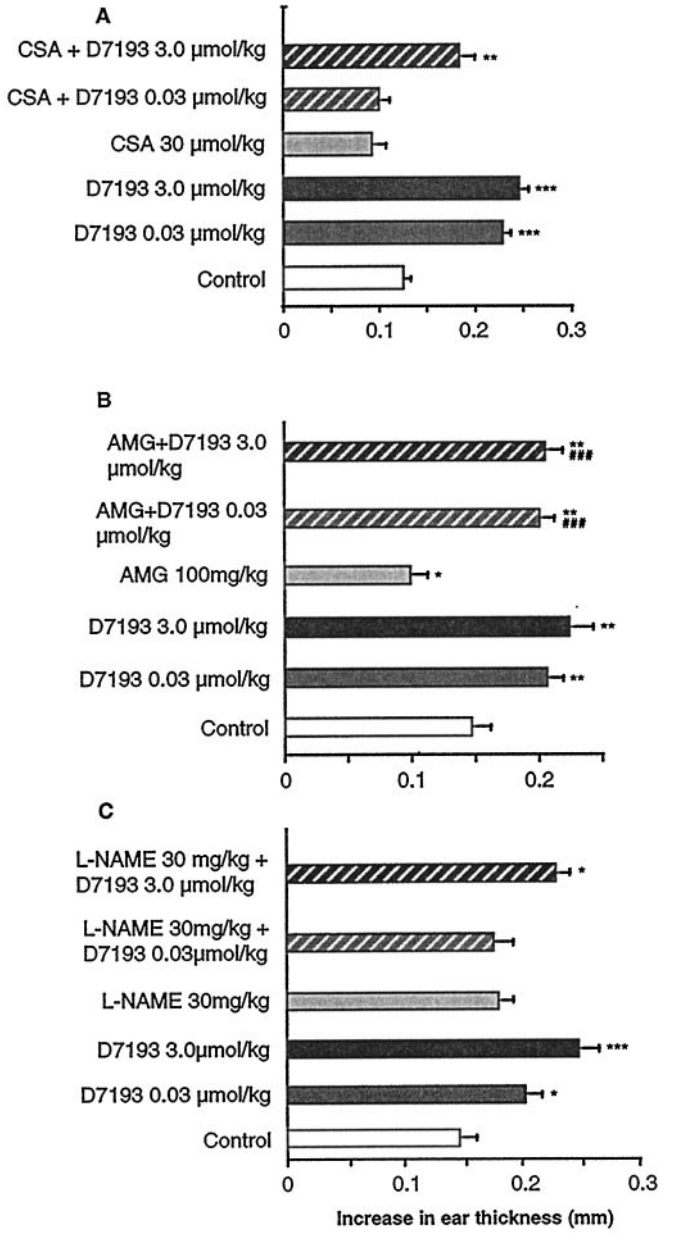

Fig. 6. A, the effect of oral treatment with CSA (30 μmol/kg), D7193 (0.03 or 3.0 μmol/kg), or a combination of the drugs (given simultaneously but separately) on days 0 to 6 on the CS response to oxazolone in sensitized BALB/c mice (n = 10). $^{**}p$ <.01, $^{***}p$ <.001 (experiments with drug treatment compared to controls), $^{\#\#}p$ <.01, $^{\#\#\#}p$ <.001 (animals treated with given dose of D7193 compared to animals treated with a combination of CSA and the corresponding dose of D7193). B, the effect of treatment by gavage with AMG [100 mg/kg (813 μmol/kg) i.p. once daily from day 2 before sensitization up to and including the day of challenge (day 7)], D7193 (0.03 or 3.0 μmol/kg), or a combination of the drugs (given simultaneously but separately) day 0 to 6 on the CS response to oxazolone in sensitized BALB/c mice (n = 10). $^{**}p$ <.01, $^{***}p$ <.001 (experiments with drug treatment compared to controls). C, the effect of treatment by gavage with L-NAME [115 μmol/kg once daily from day 5 before sensitization up to the day for challenge (day 6)], D7193 (0.03 or 3.0 μmol/kg days 0–6), or a combination of the drugs (given simultaneously but separately as indicated) on the CS response to oxazolone in sensitized BALB/c mice (n = 10). $^{*}p$ <.05, $^{**}p$ <.01.

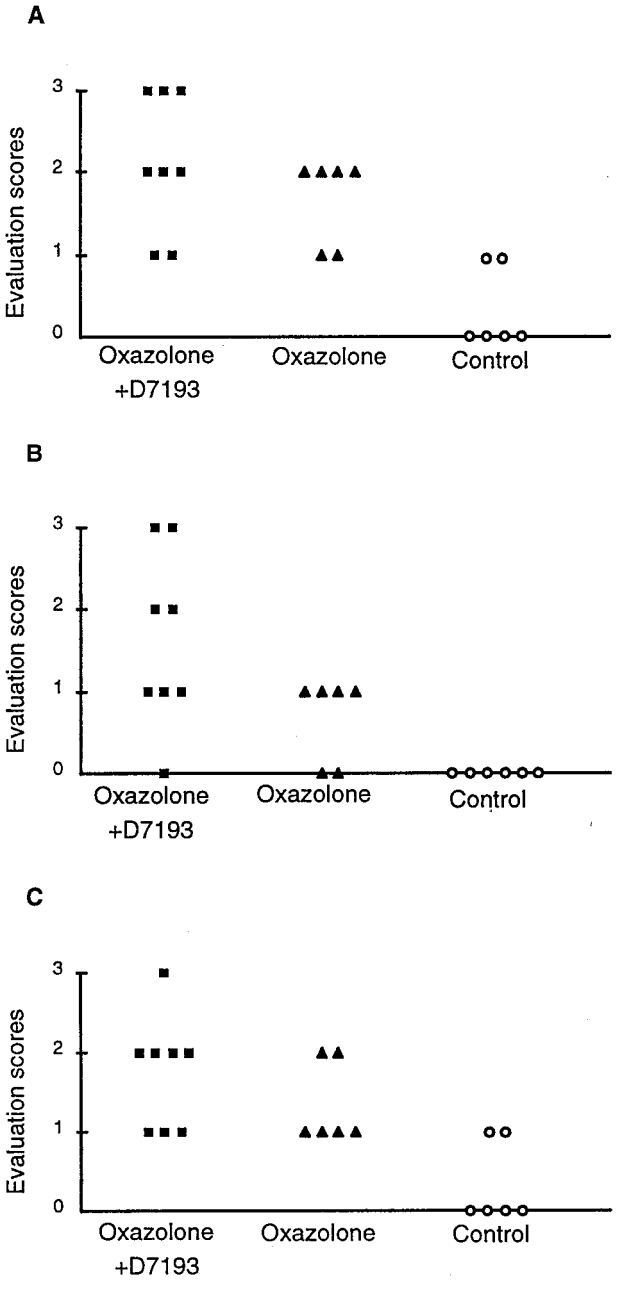

Fig. 7. Histological and immunohistochemical evaluation of ears obtained from normal nontreated (○) BALB/c mice (n = 6) and mice sensitized to and challenged with oxazolone with (■, n = 8) or without (▲, n = 6) treatment with D7193 3.0 μmol/kg orally for 7 days. Hematoxylin and eosin staining (A), presence of epidermal $CD8^+$ cells (B), and presence of $CD8^+$ cells (C) in dermis. For explanation of evaluation scores see *Materials and Methods*.

in Fig. 8, A and B. No marked differences in numbers of $CD4^+$ cells in epidermis or in dermis were observed between the animals treated or not treated with D7193. MHC class II^+ and ICAM-1^+ keratinocytes were more frequently observed in D7193-treated mice compared with sensitized, challenged, and untreated animals (data not shown). There was a slight increase of LFA-1^+, ICAM-1^+, and VCAM-1^+ cells in dermis in the D7193-treated compared with untreated animals. Other examined cell surface markers were not affected by the D7193 treatment as revealed by immunohistochemical assessment.

RPA Analysis of Chemokine/Cytokine Profiles in CS Reaction Sites 24 h after Challenge in Ears from BALB/c Mice Sensitized to Oxazolone. In ear tissue obtained from BALB/c mice sensitized to oxazolone, several

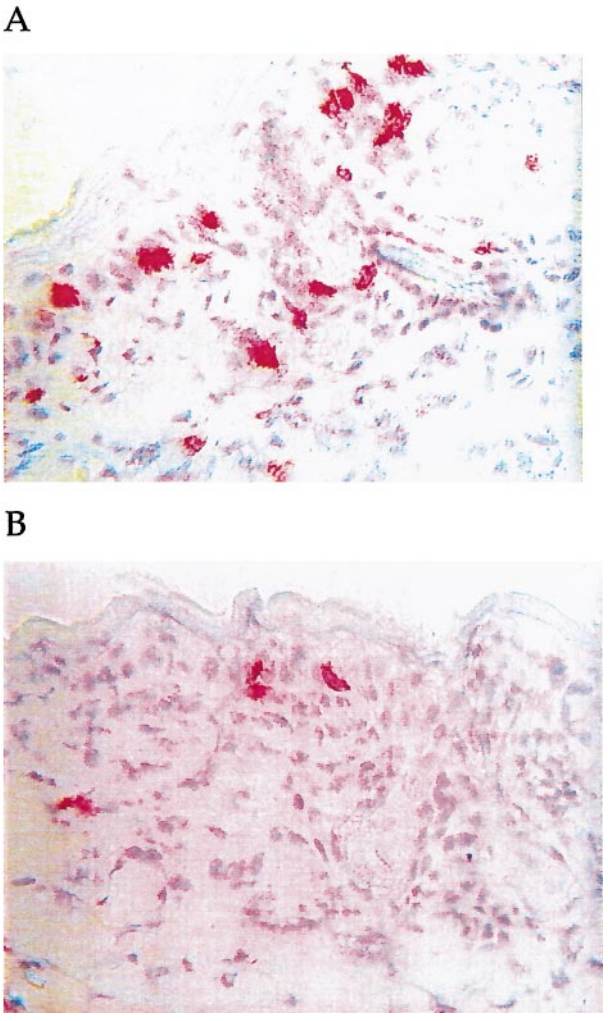

Fig. 8. Immunohistochemical staining of $CD8^+$ cells in representative examples of ear specimens from BALB/c mice sensitized to and challenged with oxazolone with (A) or without (B) D7193 treatment orally for 7 days. Magnification 370×.

chemokines/cytokines were found to be induced 24 h after challenge (Fig. 9 A and B). Thus, IL-4, IL-10, IL-13, IL-6, lymphotactin, RANTES, MIP-1β, MIP-1α, MIP-2, MCP-1, and low levels of IFN-γ can be detected in the two groups of mice sensitized to and challenged with oxazolone. IL-5, IL-9, and TCA-3 could not be detected. Expression of eotaxin, IL-2, and IL-15 was detected in tissue from control animals as well as in tissue from sensitized animals. There were no significant changes in chemokine/cytokine expression pattern between mice that were treated with D7193 or with vehicle whether or not they were sensitized to and challenged with oxazolone.

Structure Activity Studies and Effects of Reference Compounds. The effects of the disulfides ADA 202–718 and HEDS, previously reported to act as immunostimulants (Hiestand and Strasser, 1985a,b) and that of the immunomodulator DDTC, as well as those of some other compounds closely related in structure to DiNAC, were also examined in the oxazolone-induced CS reaction in BALB/c mice. Effects were expressed relative to those of DiNAC determined on the same test occasions. The data for the three compounds mentioned (Table 1) show that DiNAC is as effective as DDTC and ADA 202–718 and slightly more effective than HEDS. Effects similar to those of DDTC were also recorded with its disulfide dimer disulfiram (data not shown). A lack of effect (when examined at 0.03 or 3 μmol/kg/day) was recorded for some compounds closely related to DiNAC, e.g., L-homocystine,

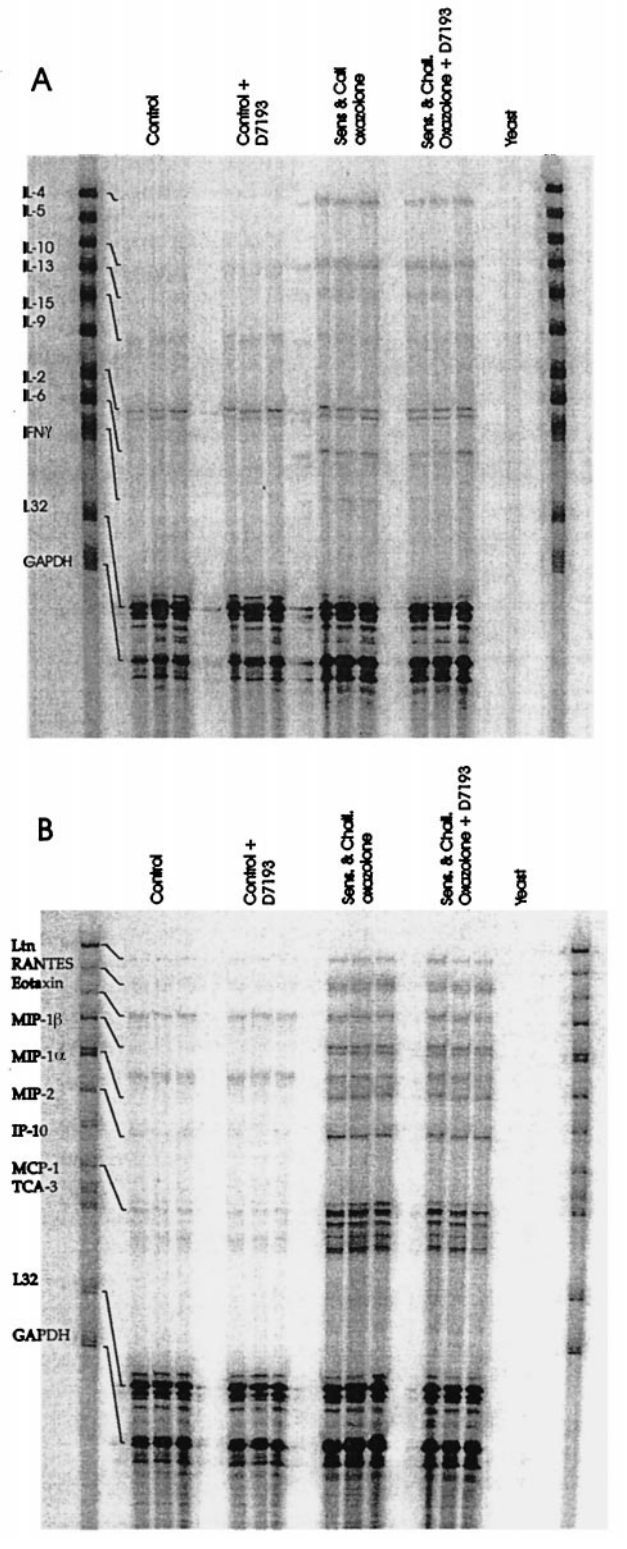

Fig. 9. Multi probe RNase protection on total RNA from whole mouse ears from nonsensitized mice treated with vehicle or D7193, or mice sensitized to and challenged with oxazolone which were also treated with vehicle or D7193. Flanking lanes contain undigested probe. For experimental details see *Materials and Methods*.

TABLE 1
Effects of selected compounds on the CS reaction in BALB/c mice
Figures given are effects (T_{24}-T_0 or T_{48}-T_0 each based on 10 individual animals) as ratios to those of DiNAC determined at the same test occasion (i.e., percent increase of examined compound divided by percent increase of D7193).

	Effect	
	24 h	48 h
HEDS 0.03 μmol/kg	0.4	<0.1
3.0	0.6	<0.1
ADA 202-718		
0.03 μmol/kg	0.7	1.0
3.0	0.9	1.0
DDTC 0.03 μmol/kg	1.0	0.9
3.0	0.8	0.6

N,*N*′-diacetyl-L-homocystine, cystamine, *N*,*N*-diacetyl-L-cystathionine, and *N*,*N*′-diacetyl lanthionine, whereas a borderline activity was recorded for *N*,*N*′-diacetyl-L-cystamine. A low, albeit significant, effect was recorded for the D-form of the DiNAC, i.e., *N*,*N*′-diacetyl-D-cystine (efficacy reduced to 50% of that of D7193 at 0.03 as well as 3 μmol/kg/day).

Effects of D7193 on CS Reactions to Oxazolone in Rats and Rabbits. The effect of D7193 treatment on oxazolone-induced CS reactions was also examined in Sprague-Dawley rats and in New Zealand White rabbits. CS responses in Sprague-Dawley rats were augmented by treatment with DiNAC according to a similar dose-response relationship as that recorded in mice (results not shown). A similar situation was observed in outbred New Zealand White rabbits, although a tendency to a bell-shaped dose-response curve with slightly reduced responses at the highest dose when compared to those at lower doses was observed in this species (results not shown).

Effects of D7193 on CS Reactions Induced by FITC and DNFB in BALB/c Mice. In BALB/c mice, FITC induces a TH2 type response, whereas DNFB induces a TH1 type response (Tang et al., 1996). Mice were sensitized to FITC and to DNFB, and challenges were performed with the corresponding agent in groups of animals treated with different daily oral doses of D7193 or the corresponding vehicle. Interestingly, in three of three experiments, D7193 enhanced the response to FITC in FITC-sensitized animals in a dose-dependent manner but markedly reduced the response to DNFB in DNFB-sensitized animals (results from representative experiments are shown in Fig. 10, A and B, respectively). DiNAC is less potent in augmenting the CS reaction in the FITC system (Fig. 10A) than in the oxazolone system (Fig. 1). Similar effects were seen when Sprague-Dawley rats were sensitized to and challenged with DNFB or FITC with D7193 (results not shown).

Effects of D7193 on Footpads DTH Reactions Induced by mBSA in Mice. Treatment with D7193 *reduces* footpads' DTH reactions induced by mBSA in mBSA-sensitized mice in a dose-dependent manner (Fig. 11).

Effects of D7193 Treatment on a DTH Granuloma Reaction Induced by mBSA. A DTH reaction to mBSA induced by the soaked filter paper method (Dunn et al., 1989) results in a quantifiable chronic granulomatous lesion. Treatment with 3 μmol/kg D7193 from days 0 to 21 reduced the development of this chronic cell-mediated inflammatory reaction (Fig. 12); treatment with 0.03 μmol/kg did not have a effect (data not shown). Treatment during days 0 to 6 did not influence the DTH granuloma reaction, whereas treatment during days 21 to 28 was as effective as treatment during days 0 to 21 (results not shown).

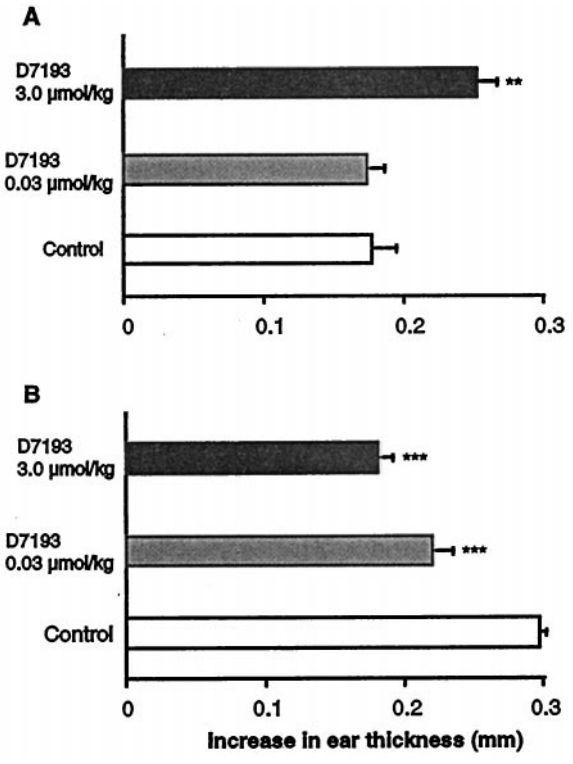

Fig. 10. The effect of treatment with D7193 on CS responses 24 h after challenge to FITC (A) and DNFB (B) in BALB/c mice sensitized to FITC and DNFB, respectively, on day 0. Treatment was performed by gavage once daily from day 0 to day 6, challenge was performed on day 7, and the CS reaction was measured on day 8. Results are shown from one representative experiment of three performed for each sensitizer ($n = 10$). $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$.

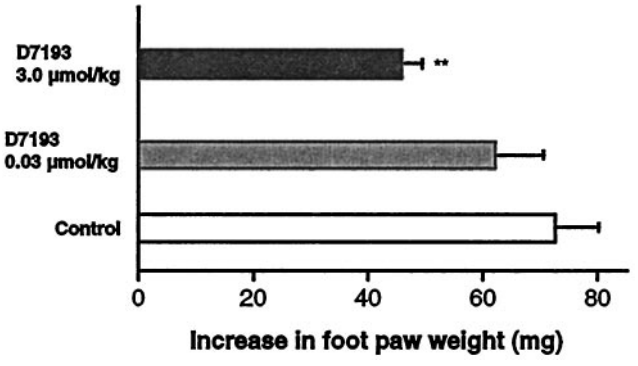

Fig. 11. The effect of treatment with D7193 on DTH responses (footpad swelling) to mBSA in BALB/c mice. Treatment was performed by gavage once daily from day 0 to day 6. Challenge was performed day 7 and the DTH reaction was measured day 8 (24 h postchallenge) as described in *Materials and Methods*. $n = 10$, $^*p < .05$.

Discussion

The present data indicate that DiNAC modulates CS and DTH responses in rodents. The cellular and molecular targets of DiNAC are not defined. Their identification is hampered by the lack of effect of the compound at relevant concentrations in in vitro systems hitherto examined (results not shown). The reason for this is not clarified; possible explanations include: 1) that the appropriate in vitro system has not

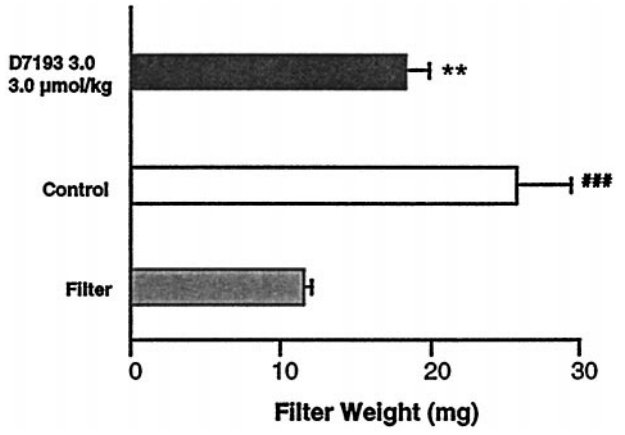

Fig. 12. The effect of treatment with D7193 on development of a granulomatous lesion to mBSA in BALB/c mice. Mice were sensitized to mBSA together with Freund's complete adjuvant. The animals were challenged 3 weeks later (day 21) by s.c. implantation of Millipore filters soaked in mBSA solution. After 1 week (day 28), lesions were dissected away from the Millipore filter implants site, dried, and weighed (wet/dry) for quantification of inflammation. Treatment with D7193 (3.0 μmol/kg) was performed by gavage once daily from day 0 to day 21. $n = 10$, $^{\#\#}p < .05$ (compared to no sensitization and no challenge), $^{**}p < .05$ (compared to vehicle treatment).

yet been examined or 2) that D7193 acts as an oxidant (see below) and that the high oxygen tension in vitro compared to that in vivo perhaps masks effects of DiNAC in the former situation.

Does DiNAC Act as an Immunomodulator? A CS reaction resulting from challenge of appropriately sensitized rodents reflects a response mediated by antigen-presenting cells and T cell subsets (Askenase, 1992; Grabbe and Schwarz, 1998). Antigen presentation in the skin is largely performed by Langerhans cells; early-acting CS-initiating T cells and mast cells are also required in the afferent phase of the response. Removal of epidermal Langerhans cells by steroid application enhances the *effector* phase, suggesting that these cells may exert a down-regulatory rather than a stimulatory role in the efferent phase (see Grabbe and Schwarz, 1998). A compound that affects only Langerhans cells would thus be expected to influence early and late phases of the CS reaction in opposite directions. The stimulatory effect observed with D7193, whether treatment is performed early or late, would suggest that it does not act solely on Langerhans cells.

With respect to T cells, DTH responses to protein antigens are mediated mainly by $CD4^+$ cells, whereas CS reactions rely on $CD8^+$ effector cells (Grabbe and Schwarz, 1998). The present observation that D7193 increases the number of $CD8^+$ cells (but does not affect $CD4^+$ cells) in oxazolone-induced CS reactions suggests that $CD8^+$ T cells are direct or indirect targets of the compound.

The response to oxazolone in BALB/c mice is characterized by 1) IgG2a but not IgE antibody production and 2) IL-2 and IFN-γ production but low levels of IL-4 and IL-10 generation by mitogen-stimulated lymph node cells (Dearman et al., 1995; Dieli et al., 1997) suggesting a TH1 type of response to this sensitizer. In contrast, with respect to its response to *Leishmania*, BALB/c mice are considered to have a TH2 phenotype (Bogdan et al., 1993). The relative effect of D7193 in oxazolone-sensitized mice is neither more nor less expressed in the BALB/c strain than in other strains, which, in contrast to BALB/c based on their responses to *Leishmania,* represent primarily the TH1 phenotype mice (Bogdan et al., 1993). However, using the observation that FITC induces a TH2 response in BALB/c mice whereas DNFB induces a TH1 response (Tang et al., 1996), we observed that D7193 augments the CS reaction in the former system, but reduces it in the latter. This finding suggests that the compound may influence TH1- and TH2-mediated responses differentially in different systems. Although additional experiments are needed to clarify the effect of D7193 in the context of a TH1/TH2 concept, the contrasting effects of D7193 in the FITC and DNFB systems are unlikely to be explained by nonimmunological effects.

Does D7193 Act on Cellular Adhesion or Homing? The development of optimal CS responses relies on several types of adhesion molecules (see Grabbe and Schwarz, 1998). For example, blocking LFA-1 - ICAM-1 interactions during the afferent phase induces a state of antigen-specific nonresponsiveness (Scheynius et al., 1996), and CS reactions to DNCB are impaired in mice deficient in ICAM-1 (Sligh et al., 1993). Although expression of selectins was not examined in the present experiments, there was only a slight increase in $ICAM\text{-}1^+$ and $LFA\text{-}1^+$ cells in ear specimens from D7193 versus vehicle-treated animals. This suggests that D7193 does not primarily influence the LFA-1 - ICAM-1 interaction.

The type I transmembrane glycoprotein CD44 supports extravasation of circulating lymphocytes into lymphoid organs and is necessary for optimal CS responses (Camp et al., 1993). The present data do not reveal any change in CD44 expression with D7193 treatment.

Does D7193 Treatment Influence the Expression of Chemokines/Cytokines? Although there is a nonspecific up-regulation of TNF-α and IFN-γ in tissue exposed to irritants as compared with a more specific increase in IL-1β, MIP-2, IP-10, and MHC class II signals early in the afferent phase of allergen-specific CS reactions (Enk and Katz, 1992), TNF-α and IFN-γ are main effector cytokines in the latter (Gautam et al., 1994; Grabbe and Schwarz, 1998). Previous data also suggest that IL-12 drives the CS reaction, whereas IL-10 reduces it (see Grabbe and Schwarz, 1998). The role of IL-4 apparently differs with the CS system examined; IL-4 does not influence the CS response to oxazolone but apparently augments that to picryl chloride (see Thomson et al., 1993; Asherson et al., 1996; Grabbe and Schwarz, 1998). The present experiments detected expression of RANTES, MIP-1β, MIP-1α, MIP-2, MCP-1, IL-4, IL-6, and IL-10 in ear tissue from animals sensitized to and challenged with oxazolone. However, mRNA expression levels for none of the examined chemokine/cytokines were altered by D7193 treatment.

Precedents for the Activity of D7193? Low molecular thiols enhance various forms of the immune response in vivo and in vitro but may also reduce such responses. Disulfides like HEDS and ADA 202–718 augment allogenic responses and IFN-γ production in mixed lymphocyte reactions, and potentiate primary and secondary humoral immune responses in vivo (Hiestand and Strasser, 1985a,b; Kinscherf et al., 1994). Bell-shaped concentration-response relations in some tests for ADA 202–718 suggest a complex mode of action (Hiestand and Strasser, 1985b). Thus, the effect of DiNAC in the CS reaction has functional precedents. A stringent structure-activity relation was disclosed in the ox-

azolone system as exemplified by 1) a lack of effect recorded for some compounds closely related to DiNAC, e.g., L-homocystine, *N*,*N*′-diacetyl-L-homocystine, cystamine, *N*,*N*′-diacetyl-L-cystathionine, and *N*,*N*′-diacetyl lanthionine, 2) a borderline activity recorded for *N*,*N*′-diacetyl-L-cystamine, and 3) a low effect recorded for the D form of the DiNAC. These results, as well as the dose relation for D7193 compared with that of NAC (Fig. 1) showing that D7193 hardly acts as a prodrug of the latter, and the potency of D7193, together suggest that DiNAC acts as an oxidant at stereochemically defined site(s) of some specific target protein(s).

Possible Molecular Targets of D7193? There are several possible targets of DiNAC, some of which are presently being examined. Thus, DiNAC may interfere with oxidoreductases like thioredoxin, glutaredoxin, or protein disulfide isomerase or their corresponding oxidoreductase reductases. The importance of these systems for immune responses in general is underlined by findings that thioredoxin modulates the production of a number of cytokines in vitro (Schenk et al., 1996), is a growth factor for T cells inducing expression of the α-chain of the IL-2 receptor (Tagaya et al., 1989), and modulates activities of transcription factors such as AP-1, NF-κB, and TCF-1α (Schreck et al., 1991; Dröge et al., 1994; Schenk et al., 1996). In this context, it is interesting to note that the contact sensitizer 1-chloro-2,4-dinitrobenzene (DNCB), a glutathione-depleting agent which also affects T cell signal-transduction pathways (Kavanagh et al., 1993), is an effective inhibitor of thioredoxin reductase (Arner et al., 1995). However, the contrasting effects of D7193 on the DNFB-induced CS reaction (inhibition) and on the BSO-enhanced CS reactivity to oxazolone (further enhancement) suggest that influence on glutathione levels is not the single major effect mechanism of D7193.

The inducible NO synthase inhibitor AMG (Tracey et al., 1995) reduces autoimmune manifestations in an EAE model and induces a shift in cytokine profiles from TH1 dominance to a TH2 type (Brenner et al., 1997). The reduction of the oxazolone-induced CS reaction resulting from AMG treatment, and its reversal by simultaneous treatment with D7193, would suggest that D7193 may mimic the action of NO, i.e., by acting as an oxidizing agent. A nonselective inhibitor of NO synthase, L-NAME, did not affect the oxazolone-induced reaction and did not affect the D7193-induced enhancement of the oxazolone CS reaction. More detailed studies are needed to clarify the basis for these findings.

Disulfides like HEDS and ADA 202–718 restore a depressed DTH response in CSA-treated mice (Hiestand and Strasser, 1985b; St Georgiev, 1988). We observed that the D7193-induced enhancement of the CS reaction to oxazolone was reduced by simultaneous CSA or rapamycin treatment. Because the CSA-induced inhibition of calcineurin leads to a block in T cell IL-2 production whereas rapamycin-induced immune suppression targets IL-2-induced T cell activation, the present findings suggest that neither IL-2 production nor IL-2 action are sole targets of D7193.

As will be reported elsewhere, D7193 at appropriate doses in vivo also markedly prolongs the lifespan of MRL-*lpr/lpr* mice (B.S. et al., unpublished data), reduces development of atherosclerosis in rabbit and mice model systems (K. Pettersson et al., unpublished data), and reduces early and blocks late airway reactions in allergen-challenged sheep (B. Abraham et al., unpublished data). The immunomodulatory activity of D7193 in vivo is thus not restricted to the CS reaction.

In conclusion, DiNAC is a potent and effective enhancer of the CS reaction to oxazolone in rodents. In contrast, it reduces the CS reaction to DNFB and the DTH reaction to mBSA. From the present data, it may also be relevant to ask whether the immunological effects attributed to NAC in vivo may be due, at least partly, to the activity of a contaminating disulfide dimer molecule, DiNAC, with oxidant activity.

Acknowledgments

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Send reprint requests to: Håkan Bergstrand, Department of Cell & Molecular Biology, Astra Draco, P.O. Box 34, S-22100, Lund, Sweden. E-mail: hakan.bergstrand@draco.se.astra.com