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Isolation, Characterization, and Synthesis of an Immunoregulatory Metabolite of Niridazole: 1-Thiocarbamoyl-2-Imidazolidinone

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SUMMARY

TRACY, J. W., E. H. FAIRCHILD, S. V. LUCAS AND L. T. WEBSTER, JR. Isolation, characterization, and synthesis of an immunoregulatory metabolite of niridazole: 1-Thiocarbamoyl-2-imidazolidinone. *Mol. Pharmacol.* **18:** 313–319 (1980).

An immunoactive substance, called niridazole immunoregulatory factor (NIF), has been isolated in chromotographically pure form from the urine of patients treated with the antihelminthic drug, niridazole. The isolate was shown to be a single compound which displayed ultraviolet, infrared, proton magnetic resonance, and mass spectral properties consistent with the structure, 1-thiocarbamoyl-2-imidazolidinone, a previously unreported metabolite of niridazole. The structural assignment was confirmed by chemical synthesis of 1-thiocarbamoyl-2-imidazolidinone from 2-chloroethylisocyanate and thiourea. Both the isolated and the synthetic compounds had identical spectral characteristics and suppressed delayed contact hypersensitivity of mice to 2,4-dinitrofluorobenzene at an optimal dose range of 10^{-10} to 10^{-11} g/kg.

INTRODUCTION

When administered to animals and man, the antihelminthic nitrothiazole drug, niridazole, has been shown to be a potent suppressor of several manifestations of cell-mediated immunity (1–4). By contrast, this drug was found to affect antibody production only minimally (5).

Certain observations suggested that the immunoregulatory effect was mediated by an agent other than niridazole itself. Thus, niridazole-induced immunosuppression of granuloma formation around *Schistosoma mansoni* eggs injected intravenously into mice persisted for periods of up to several weeks, although niridazole itself was reported to be completely metabolized within hours (2, 6). Furthermore, serum from niridazole-treated guinea pigs reversibly suppressed antigen-induced production of macrophage migration inhibition factor (MIF)[3] by sensitized guinea pig lymphocytes, whereas normal guinea pig serum to which niridazole had been added did not (3, 7). Finally, niridazole-dependent immunosuppressive activity was discovered in the urine of rats and a patient treated with the drug (8). This material, partially purified by solvent extraction and Sephadex LH-20 chromatography, suppressed lung granuloma formation around *S. mansoni* eggs *in vivo* and MIF production by sensitized guinea pig lymphocytes *in vitro*; niridazole itself was inactive *in vitro* (8).

Recently, Blumer *et al.* further purified and characterized the immunoactive Sephadex LH-20 fraction derived from the urine of niridazole-treated rats (9). A chromatographically pure material was obtained by high-pressure liquid chromatography (HPLC), which suppressed delayed contact hypersensitivity of mice to 2,4-dinitrofluorobenzene (DNFB) at doses as low as 1 pg/kg of body weight. This isolate, named niridazole immunoregulatory factor (NIF), gave an ultraviolet absorption spectrum with absorption maxima at 231 and 256 nm but no absorbance at 360 nm; absorbance at 360 nm is characteristic of the nitrothiazole moiety of niridazole. Isolation of sufficient quantities of NIF to permit chemical characterization was hampered, however, by the need to use some material at each step of the purification procedure to assay for immunosuppressive activity.

We now present evidence that the chemical identity of this potent immunoactive substance is 1-thiocarbamoyl-2-imidazolidinone.

MATERIALS AND METHODS

Patient Urine

Twenty-four-hour urine specimens were obtained from

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[3] Abbreviations used: MIF, macrophage migration inhibition factor; HPLC, high-pressure liquid chromatography; DNFB, 2,4-dinitrofluorobenzene; NIF, niridazole immunoregulatory factor; ^{1}H-nmr, proton nuclear magnetic resonance.

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two patients harboring mild *Schistosoma haemtobium* infections who were receiving niridazole orally in single daily doses of 25 mg/kg body weight for 7 days. Control urine samples were collected from each patient before drug treatment was started. The patients showed normal liver function tests and were in generally good health. Urine samples were collected without preservative and stored frozen at −20°C.

Mice

Male C57Bl/6J mice weighing between 16 and 20 g were purchased from the Jackson Laboratory, Bar Harbor, Maine. They were maintained on Purina Lab Chow and water *ad libitum* in the Animal Resource Center of Case Western Reserve University.

Chemicals

Ethyl acetate, ChromAR grade, and *N,N*-dimethylformamide, spectAR grade, were purchased from Mallinckrodt Chemical Co. Thiourea and 2-chloroethylisocyanate were obtained from Eastman Organic Chemicals. Acetonitrile, uv quality, was purchased from Burdick–Jackson Laboratories (Muskegon, Mich.). Niridazole was kindly provided by Ciba-Geigy, Summit, N.J. An authentic sample of NIF isolated from rat urine was a generous gift of Dr. Jeffrey L. Blumer. All other chemicals were of the best commercially available grade.

Determination of Niridazole Immunoregulatory Factor by High-Pressure Liquid Chromatography

High-pressure liquid chromatography was performed with a Waters Associates Model ALC/GPC 201 liquid chromatograph equipped with a Model 660 solvent programmer. The column effluent was monitored by means of a Schoeffel Model 770 dual-beam variable-wavelength spectrophotometric detector. The system was interfaced with a Columbia Scientific Supergrator 3 to measure the areas under chromatographic peaks.

Niridazole immunoregulatory factor was assayed by analytical HPLC on a reverse phase column (Waters Associates, μBondapak C_{18}, 0.4 × 30 cm) equilibrated with 7% (v/v) acetonitrile in water. The column was developed with a linear flow program of the same solvent from 0.5 to 2.0 ml/min over a period of 10 min beginning at the time of sample injection. The column effluent was monitored at 256 nm. At 25°C, under these conditions, NIF eluted with a k' value[4] of 3.5.

For NIF measurements in crude urine, the urine sample was extracted twice with an equal volume of water-saturated ethyl acetate. The organic extracts were pooled, filtered through a 0.5-μm Fluoropore filter (Millipore Corp.), and evaporated to dryness. The residue was dissolved in 7% acetonitrile in water to achieve 10–20% of the original urine volume.

Isolation of Niridazole Immunoregulatory Factor from Urine of Patients Treated with Niridazole

Concentration. Three liters of urine from patients treated with niridazole was thawed, pooled, and concentrated to 600 ml by rotary evaporation at 30°C. The dark brown precipitate which formed upon cooling to 4°C was removed by centrifugation in the cold for 10 min at 10,000*g*. No NIF was detected by HPLC in ethyl acetate extracts of this precipitate.

Solvent extraction. The fivefold concentrated urine was extracted twice with an equal volume of water-saturated ethyl acetate. The aqueous phase was discarded. The combined organic extracts were filtered through phase separating paper (Whatman PS-1) and evaporated to dryness. The amber-colored residue was taken up in 2.5 ml of distilled water. A small amount of water-insoluble material, which contained no detectable NIF, was discarded.

Sephadex G-25 chromatography. The water-soluble material from the previous step was chromatographed on a column of Sephadex G-25 (fine, 2.6 × 62 cm) which was equilibrated and eluted at 25°C with distilled water containing 0.02% sodium azide. The column effluent was monitored at 256 nm and 3-ml fractions were collected. Fractions containing NIF, as determined by HPLC analysis of 5-μl samples, were pooled and the volume of the pool was reduced to 3 ml by rotary evaporation. This material was then subjected to a second chromatographic step on the same column which had been washed with five bed volumes of 2 M NaCl and then equilibrated and eluted with distilled water. The column effluent was monitored as before and fractions containing NIF were pooled and evaporated to dryness.

Preparative high-pressure liquid chromatography. Niridazole immunoregulatory factor was further purified by preparative scale HPLC on a Waters Associates μBondapak C_{18} column (0.79 × 30 cm) equilibrated with 7% (v/v) acetonitrile in water. Chromatography was carried out isocratically at a flow rate of 2 ml/min. The column effluent containing NIF was collected and the solvent was removed by rotary evaporation.

The resulting white residue was dissolved in 2 ml of distilled water and subjected to a second preparative HPLC step designed to remove minor contaminants and minimize solvent impurities. The reverse phase column was exhaustively washed with acetonitrile and then equilibrated and eluted with water which had been purified by passage through a Milli-Q-System (Millipore Corp.). In this elution system, NIF chromatographed with a k' value of 16. The solvent flow rate was 4.0 ml/min and the effluent was monitored as before. At the appropriate time, the column effluent was collected and its volume reduced to 4 ml by rotary evaporation. After lyophilization, NIF (3.2 mg) was recovered as a fine white powder.

Spectroscopic Characterization of Niridazole Immunoregulatory Factor

Ultraviolet absorption spectra were recorded at 25°C by a DW2 uv-visible spectrophotometer in the split-beam mode. Proton magnetic resonance spectra were determined with a Bruker Wh-270 NMR spectrometer. Samples were run in acetone-d_6 (Merck, 99.96% D) with tetramethylsilane used as the internal standard.

Mass spectrometry was performed with a Kratos MS-30 dual-beam mass spectrometer equipped with a Kratos DS-50 S computer data system. The sample was intro-

[4] $k' = (V_e - V_0)/V_0$, where V_e is the elution volume of the sample and V_0 is the void volume of the system.

duced by means of a direct insertion probe and spectra were obtained in electron impact mode at an ionizing potential of 70 eV and at a resolving power of 3000. Samples were pelleted in KBr for infrared analysis, and infrared spectroscopy was performed with a Perkin–Elmer Model 337 infrared spectrophotometer.

Synthesis of 1-Thiocarbamoyl-2-Imidazolidinone

1-Thiocarbamoyl-2-imidazolidinone was synthesized from 2-chloroethylisocyanate and thiourea according to the scheme depicted in Fig. 1. To a 1-liter three-neck flask, fitted with stirrer, reflux condenser, and adding funnel, were added 19.0 g (0.25 mol) of thiourea dissolved in 400 ml of dry acetone. The solution was heated to 45°C and 12.5 g (0.09 mol) of anhydrous potassium carbonate were added. Next, 5 g (0.05 mol) of freshly distilled 2-chloroethylisocyanate, diluted in 100 ml of acetone, were added dropwise over a 90-min period. After an additional 2 h at 45°C, the reaction mixture was cooled to 25°C and filtered to remove the insoluble salt. The filtrate was evaporated to dryness under reduced pressure.

The intermediate product, *N*-[[(2-chloroethyl)amino] carbonyl]carbonylthiourea, was not isolated but instead was dissolved in 400 ml of *N,N*-dimethylformamide and heated to 90°C. Fourteen grams (0.05 mol) of potassium acetate were added at one time. After stirring for 30 min at 90°C, the solvent was removed by rotary evaporation at 90°C.

The solid residue was dissolved in 100 ml of boiling distilled water, filtered, and cooled to 4°C. Upon standing in the cold, large white crystals formed which were collected by filtration and washed briefly with ice-cold distilled water. The mother liquor was concentrated twofold and returned to the cold, when a second crop of crystals formed. Recrystallization from water gave 1.9 g of white crystals, which melted at 203–204°C (uncorrected m.p.). The overall yield was 24% relative to 2-chloroethylisocyanate. Accurate mass determination of the product gave a molecular ion m/e = 145.0313 (calculated for $C_4H_7N_3OS$, m/e = 145.0310). The compound displayed a molar extinction coefficient, E_m = 16,900 M^{-1} cm^{-1}, at λ_{max} = 256 nm.

Contact Hypersensitivity to DNFB

Contact hypersensitivity of male C57Bl/6J mice to DNFB, as measured by ear swelling, was done by the method of Phanuphak *et al.* (10). Mice sensitized by two daily applications of DNFB to the shaved abdomen were challenged with hapten 4 days later and ear swelling was determined after another 24-h period as described in detail by Blumer *et al* (9). Test materials (NIF or 1-thiocarbamoyl-2-imidazolidinone dissolved in 0.2 ml of water) were injected intraperitoneally 48 h before sensitization.

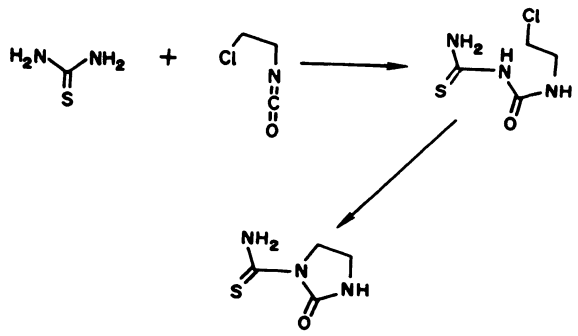

FIG. 1. *Scheme of chemical synthesis of 1-thiocarbamoyl-2-imidazolidinone from 2-chloroethylisocyanate and thiourea*

RESULTS

Determination of Niridazole Immunoregulatory Factor by High-Pressure Liquid Chromatography

A sample of purified NIF, isolated and shown to be immunosuppressive by the procedure of Blumer *et al.* (9), was used as a standard to establish chromatographic conditions for the direct determination of NIF by HPLC (see Materials and Methods). Crude urine could not be assayed directly because of interfering substances. Therefore, NIF was measured after extraction into ethyl acetate. This material partitioned from urine into ethyl acetate with a distribution coefficient of about 1.8 such that two extractions with an equal volume of water-saturated ethyl acetate removed 93% of the total NIF present. After the solvent was evaporated the residue was dissolved in 10–20% of the original urine volume of 7% acetonitrile for injection onto the HPLC column. Typical chromatograms of ethyl acetate extracts of control pretreatment urine and of urine obtained on the third to fifth days of treatment are shown in Fig. 2. A 256

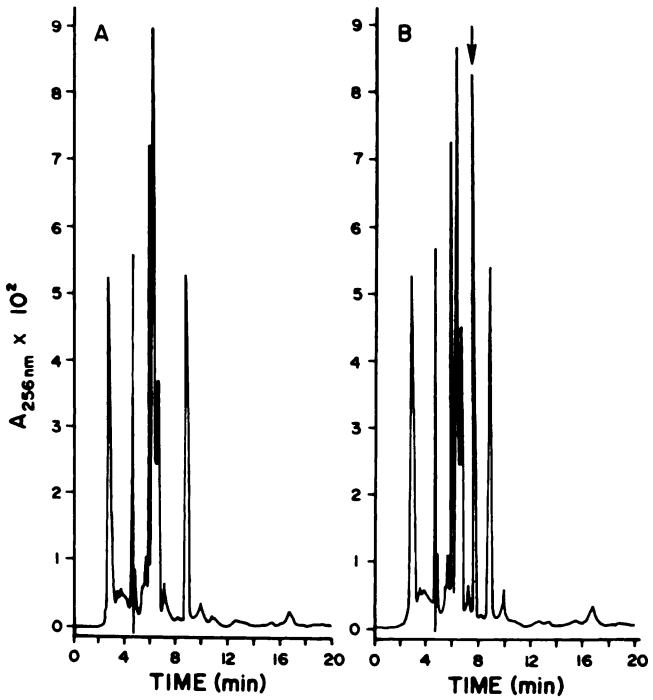

FIG. 2. *Reverse phase HPLC chromatograms of 10 μl of an ethyl acetate extract of (A) pretreatment control urine and (B) urine collected 4 days after beginning niridazole therapy*

Two-milliliter urine samples were extracted twice with an equal volume of water-saturated ethyl acetate. After removing the solvent by rotary evaporation, each residue was dissolved in 250 μl of 7% acetonitrile in water. Chromatographic conditions are described in Materials and Methods. The arrow indicates the position of NIF.

nm-absorbing peak at $k' = 3.5$ is seen in the extracted niridazole treatment urine which is not present in the extract of control urine. The use of a linear solvent flow gradient instead of isocratic elution gave better resolution of NIF from other 256 nm-absorbing material. The more nonpolar components of the extract, including the oxidative metabolites of niridazole (11), were eluted at 2 ml/min by an injected 1-ml bolus of acetonitrile.

Isolation of Niridazole Immunoregulatory Factor from Urine of Patients Treated with Niridazole

The immunoactive factor was isolated in chromatographically pure form from human niridazole treatment urine by a series of steps which included extraction into ethyl acetate, repetitive chromatography on Sephadex G-25, and preparative scale HPLC. A summary of a typical purification is shown in Table 1, while details of individual steps are given in Materials and Methods. Overall, NIF was recovered in a 74% yield from the fivefold concentrated urine sample.

The purified material chromatographed in the standard HPLC system as a single uv-absorbing species when the column effluent was monitored at 205, 230, and 256 nm; no 360-nm material was detected under these conditions. A single 256 nm-absorbing peak was also observed when pure NIF was chromatographed by reverse phase HPLC in 30% (v/v) methanol in water and by normal phase HPLC on a silica column (μPorasil, Waters Associates) in chloroform:methanol (70:30).

Spectral Characterization of Niridazole Immunoregulatory Factor

Several lines of evidence suggested that the structure of isolated NIF was 1-thiocarbamoyl-2-imidazolidinone. These were as follows.

Ultraviolet-visible absorption spectrum. The absorption spectrum of NIF taken at neutral pH was identical to that previously reported (9). Maximal absorption was seen at 256 nm, but there was no absorption at 360 nm, the region characteristic of the nitrothiazole moiety of niridazole. This was consistent with both conservation of the imidazolidinone structure and modification of the nitrothiazole moiety in NIF as compared to niridazole (9).

^{1}H-nmr spectrum. The proton magnetic resonance spectra of NIF and niridazole are shown in Figs. 3A and B, respectively. The singlet (8.1 ppm) due to the olefinic proton of the nitrothiazole ring of niridazole is notably absent from the NIF spectrum, providing more evidence

TABLE 1

Purification of niridazole immunoregulatory factor from urine of patients treated with niridazole

Step	Total NIF[a]	Total solids[b]	NIF recovery
	mg	*mg*	*%*
Fivefold concentrated urine	~4.34 [c]	58200	100
Ethyl acetate extract	4.06	812	93
First Sephadex G-25 step	3.78	ND[d]	87
Second Sephadex G-25 step	3.37	ND	78
Preparative HPLC in 7% CH_3CN	3.26	ND	75
Preparative HPLC in water	3.19	3.19	74

[a] The amount of NIF obtained at each step of the purification was estimated from the HPLC peak area of 256 nm-absorbing material eluting from the μBondapak C_{18} column at $k' = 3.5$ (see Fig. 2). Peak area was related to amount of NIF using the final isolated product as a standard.

[b] Gravimetric determination after lyophilization.

[c] The total NIF content in the fivefold concentrated urine was estimated from the amount of NIF extracted into ethyl acetate after four extractions with an equal volume of the organic solvent.

[d] ND = not determined.

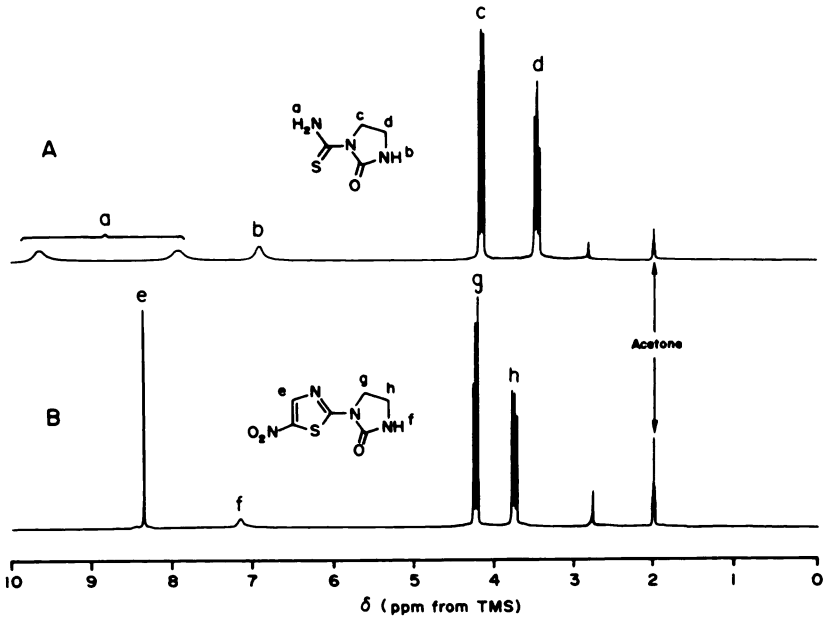

FIG. 3. *270-MHz proton magnetic resonance spectra*

(A) NIF (1-thiocarbamoyl-2-imidazolidinone)—2 mg/0.3 ml acetone-d_6. (a) δ9.6, δ7.99; (b) δ6.87; (c) δ4.17; (d) δ3.48. (B) Niridazole—1 mg/0.3 ml acetone-d_6. (e) δ8.1; (f) δ7.15; (g) δ4.25; (h) δ3.77.

for modification of nitrothiazole ring in NIF. Two additional broad downfield signals (7.9 and 9.6 ppm) also appeared in the NIF spectrum that were absent from the niridazole spectrum; the exchangeable nature of the protons associated with these signals was shown by their total disappearance upon the addition of D_2O. These signals were tentatively assigned to two nonequivalent amide protons in NIF. The upfield AA′ BB′ system due to the ethylene portion of the 2-imidazolidinone ring was essentially unaltered in the NIF as compared to niridazole spectrum, again indicating that this ring was conserved in NIF. A broad singlet at 6.87 ppm assigned to the amide hydrogen of the 2-imidazolidinone ring was shifted upfield slightly in NIF as compared to the same resonance in niridazole (7.15 ppm).

Mass spectrum. The electron impact mass spectrum of NIF (Fig. 4) shows a molecular ion at *m/e* 145.0312 (calculated for $C_4H_7N_3OS$, *m/e* 145.0310). The fragmentation pattern was consistent with an imidazolidinone ring bearing a primary thioamide substituent. Assignment of the fragments at *m/e* 85 ($C_3H_5N_2O$) and *m/e* 60 (CH_2NS) to the imidazolidinone and thioamide moieties, respectively, were borne out by accurate mass determinations. The major fragments observed at *m/e* 102 (16.3%), 85 (36.4%), 60 (53.8%), and 43 (56.9%) were also consistent with the proposed structure for NIF.

Infrared absorption spectrum. Characteristic bands were seen in the infrared spectrum of NIF which corresponded to key functions in 1-thiocarbamoyl-2-imidazolidinone. Figure 5 shows prominent features for the following:

(1) C = S stretching vibrations at 1095 cm^{-1}, consistent with those in thiourea and imidazolidinone (12).

(2) C = 0 stretching at 1710 cm^{-1}, characteristic of five-membered ring lactams.

(3) N–H bending at 1590 cm^{-1}, consistent with a primary amide moiety.

(4) N–H stretching at 3140 and 3320 cm^{-1}, typical also of primary amides.

Chemical Synthesis of 1-Thiocarbamoyl-2-Imidazolidinone

The proposed structure for NIF was confirmed by chemical synthesis of 1-thiocarbamoyl-2-imidazolidinone from thiourea and 2-chloroethylisocyanate according to the reaction shown in Fig. 1. The synthesized compound had an uncorrected melting point of 203–205°C and cochromatographed in three HPLC systems with authentic NIF isolated from human urine. The ultraviolet, ^{1}H-nmr, infrared, and mass spectra of the two substances were also identical.

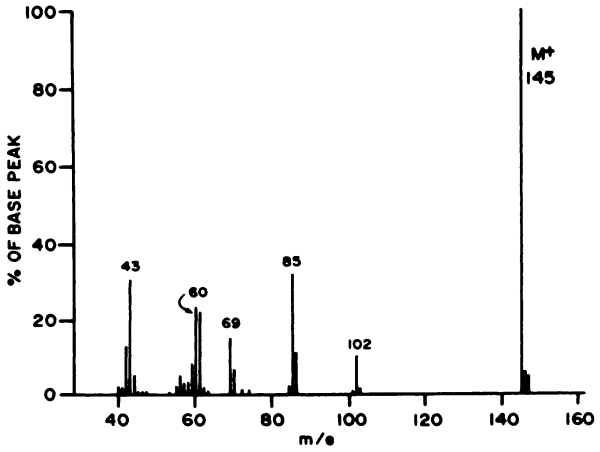

FIG. 4. *Electron impact mass spectrum of NIF*
The sample was introduced by a direct insertion probe. Source temperature was 200°C, accelerating voltage was 4000 V, ionizing voltage was 70 eV, and resolving power was 3000.

Immunosuppressive Activity of Niridazole Immunoregulatory Factor

As shown in Table 2, treatment with NIF had a profound effect on ear swelling measured 24 h after a DNFB challenge. At a NIF dose of 10^{-11} g/kg of body weight, ear swelling was 87% inhibited. In other experiments, the maximum level of suppression varied between 50 and 90%, but suppression was always observed in the same optimal dose range. Suppression was not as great at either higher or lower doses of NIF.

Immunosuppressive Activity of Synthetic 1-Thiocarbamoyl-2-Imidazolidinone

Chemically synthesized 1-thiocarbamoyl-2-imidazolidinone was tested in parallel with NIF isolated from the

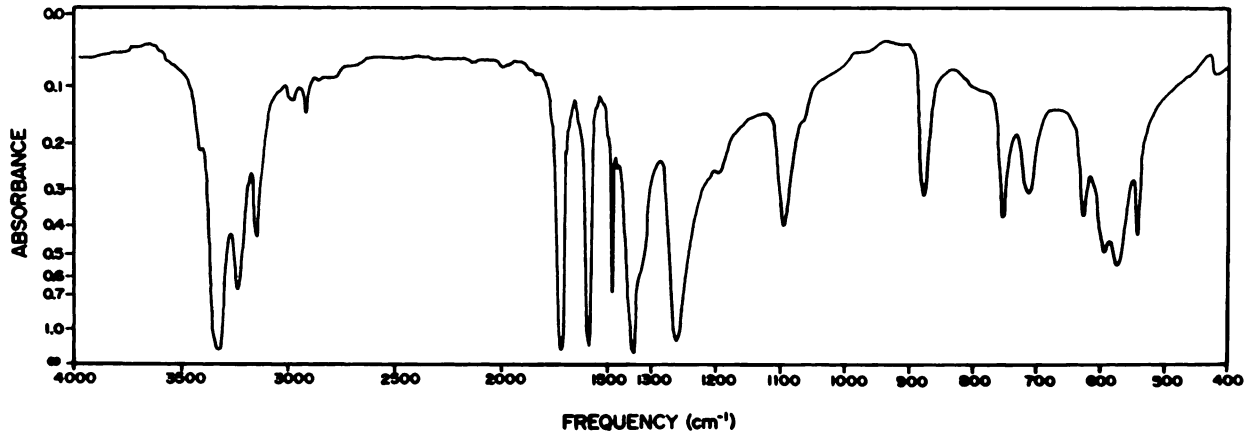

FIG. 5. *Infrared absorption spectrum of NIF*
Taken in KBr pellet (0.5 mg sample/100 mg KBr).

TABLE 2

Suppression of DNFB-induced ear swelling in C57Bl/6J mice by niridazole immunoregulatory factor (NIF)

NIF dose[a]	Swelling[b]	Relative response
g/kg	$10^{-2} \times mm \pm SD$	%
0	33.5 ± 2.2	100
10^{-8}	19.8 ± 3.0	59
10^{-9}	15.4 ± 3.9	46
10^{-10}	8.9 ± 3.3	27
10^{-11}	4.2 ± 1.4	13
10^{-12}	11.4 ± 2.2	34
10^{-13}	27.2 ± 3.2	81

[a] NIF was administered intraperitoneally to groups of five mice 48 h prior to sensitization with DNFB.

[b] Ear swelling = (ear thickness 24 h after DNFB challenge) − (ear thickness before challenge). Swelling measurements are corrected for the small amount of nonspecific swelling ($\leq 3 \times 10^{-2}$ mm) due to DNFB challenge of unsensitized untreated mice.

urine of niridazole-treated patients for the ability to suppress cutaneous delayed hypersensitivity in C57Bl/6J mice to DNFB (Table 3). Varying amounts of both compounds were administered intraperitoneally 48 h prior to sensitization. It is clear from Table 3 that the synthetic material was just as effective as NIF that had been isolated from urine. Although the maximal suppression seen in this particular experiment was only 70%, the optimal suppressive dose was again 10^{-11} g/kg (compare with Table 2).

DISCUSSION

The major finding presented in this report is that the chemical identity of a potent immunosuppressive fraction isolated from the urine of niridazole-treated animals and patients is 1-thiocarbamoyl-2-imidazolidinone. This structure was deduced from spectral properties of the isolated material and confirmed by chemical synthesis. The purified isolate and the chemically prepared compound had identical spectal properties and were equipotent in suppressing delayed hypersensitivity to DNFB.

It is not known whether 1-thiocarbamoyl-2-imidazolidinone is the only immunoactive material resulting from niridazole treatment *in vivo*. Recently, it was reported that three niridazole-dependent immunosuppressive fractions were found but this remains to be confirmed (13).

The loss of efficacy of high doses of chemically pure 1-thiocarbamoyl-2-imidazolidinone in suppressing the delayed hypersensitivity response to DNFB (Table 3) remains unexplained and is reminiscent of the dose–response relationship just published by Blumer *et al.* (9). Combined biodisposition and immunopharmacological studies should reveal whether this effect is produced by 1-thiocarbamoyl-2-imidazolidinone itself or a metabolite. The fact that 1-thiocarbamoyl-2-imidazolidinone is water soluble and rapidly appears in the urine after administration would suggest that this compound is not extensively metabolized *in vivo*.

The extraordinarily high potency of 1-thiocarbamoyl-2-imidazolidinone makes it very probable that this compound acts via a receptor-mediated mechanism and also raises the question of whether this or analogous compounds are involved in endogenous immunoregulation. The putative receptor-bearing target cells for 1-thiocarbamoyl-2-imidazolidinone remain to be identified, but obvious candidates are lymphocytes and accessory cells involved in the immune response. Daniels *et al.* showed that "niridazole active serum" which presumably contains 1-thiocarbamoyl-2-imidazolidinone acts reversibly in suppressing production/release of the lymphokine, MIF, when added before but not after antigen to sensitized guinea pig lymphocytes (3, 7). This observation is consistent with 1-thiocarbamoyl-2-imidazolidinone exerting its effect on the affector limb of the immune response.

1-Thiocarbamoyl-2-imidazolidinone appears to be a prototype for a new class of immunoregulatory compounds. Because studies from this laboratory now indicate that this compound suppresses a variety of cell-mediated immune systems, both *in vivo* and *in vitro*,[5] this substance should prove a powerful probe to investigate immunological responses and mechanisms at both the cellular and molecular levels. By analogy to niridazole that shows a spectrum of pharmacological effects different from known toxic immunosuppressants (2), 1-thiocarbamoyl-2-imidazolidinone, used alone or in combination with other immunoactive agents, may also be of benefit in clinical situations requiring immune modulation (14).

TABLE 3

Suppression of DNFB-induced ear swelling in C57Bl/6J mice by isolated niridazole immunoregulatory factor (NIF) and by chemically synthesized 1-thiocarbamoyl-2-imidazolidnone

NIF dose[a]	TCI dose[a]	Swelling[b]	Relative response
g/kg	*g/kg*	$10^{-2} \times mm \pm SD$	%
—	—	24.1 ± 1.8	100
10^{-9}	—	13.7 ± 2.1	57
10^{-10}	—	9.2 ± 1.0	38
10^{-11}	—	8.7 ± 1.4	36
10^{-12}	—	14.7 ± 2.3	61
—	10^{-9}	14.9 ± 2.4	62
—	10^{-10}	8.2 ± 3.0	34
—	10^{-11}	6.0 ± 1.4	25
—	10^{-12}	12.5 ± 1.9	52

[a] NIF, isolated from urine, or synthetic TCI was dissolved in water and administered intraperitoneally to groups of five mice 48 h prior to sensitization with DNFB.

[b] See footnote *b*, Table 2.

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