Int. J. Immunopharmac., Vol. 13, No. 6, pp. 655–668, 1991.
Printed in Great Britain.

0192–0561/91 $3.00 + .00
Pergamon Press plc.
International Society for Immunopharmacology.

IMMUNOMODULATORY ACTION OF LEVAMISOLE — I. STRUCTURAL ANALYSIS AND IMMUNOMODULATING ACTIVITY OF LEVAMISOLE DEGRADATION PRODUCTS

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(*Received* 15 *June* 1990 *and in final form* 30 *January* 1991)

Abstract — In our laboratory we observed that solutions of levamisole (LMS) stored at 4°C consistently enhanced the lymphocyte proliferation response to concanavalin A (Con A) more than freshly prepared solutions did. To determine if the increased immunopotentiation observed with the stored solutions of LMS was due to products formed from LMS, we assessed the stability of LMS when stored at 4 or 37°C at pH 6, 7, 7.5 and 8. Analysis of the various solutions by high pressure liquid chromatography demonstrated that LMS decomposes during storage in neutral and alkaline conditions to form three products. The formation of the products was accelerated by increasing the temperature from 4 to 37°C. The three degradation products were purified by preparative high pressure liquid chromatography and their structures determined by mass spectrometry, infrared spectrometry and homo- and heteronuclear two dimensional nuclear magnetic resonance spectroscopy. The degradation products, denoted as No. 1, No. 2 and No. 3, based on their high pressure liquid chromatography retention times, were identified as: No. 1, 3-(2-mercaptoethyl)-5-phenylimidazolidine-2-one; No. 2, 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole and No. 3, *bis* [3-(2-oxo-5-phenylimidazolidin-1-yl) ethyl] disulfide. Product 2 significantly enhanced murine lymphocyte proliferation responses to concanavalin A (Con A) at concentrations between 0.5 and 10.0 μg/ml (whereas the optimum concentration of LMS is 10–100 fold higher (50–100 μg/ml)). Products 1, 2 and 3 significantly inhibited the lymphocyte proliferative response at concentrations >2.2, 10.0 and 10.0 μg/ml, respectively.

These studies indicate that under relatively mild conditions, including physiological conditions, LMS may decompose to products which inhibit or enhance lymphocyte responses to Con A.

Although the immunomodulating effects of levamisole, [(S)-(−)-2,3,5,6-tetrahydro-6-phenyl-imidazole (2,1-b) thiazole] (LMS), were first described in 1971 by Renoux & Renoux, the mechanism of action of LMS remains undefined. Some *in vivo* and *in vitro* studies with human patients and experimental animals have demonstrated that LMS enhances the immune response, possibly by influencing the function of macrophages and T-lymphocytes (Symoens & Rosenthal, 1977; Lichtenfeld, Desner, Wiernick & Mardiney, 1976; Symoens, Rosenthal, De Brabander & Goldstein, 1979), but other studies have shown that LMS is ineffective as an immunoenhancing drug (Copeland, Stewart & Harris, 1974; Whitcomb, Merluzzi & Cooperband, 1976; Al-Safi & Maddocks, 1984; Roth & Kaeberle, 1984), or in some protocols is immunosuppressive (Otterness, Torchia & Bliven, 1979; Spreafico, 1985; Amery & Butterworth, 1983). Some recent clinical studies have shown LMS to be effective in the treatment of colon cancer (Laurie, Moertel, Fleming, Wieand, Leigh, Rubin, McCormack, Gerstner, Krook, Malliard, Twito, Morton, Tschetter & Barlow, 1989; Moertel, Fleming, MacDonald, Haller, Laurie, Goodman, Ungerleider, Emerson, Tormey, Glick, Veeder & Mailliard, 1990, while another study showed LMS to be ineffective (Arnaud, Buyse, Nordlinger, Martin, Pector, Zeitoun, Adloff & Duez, 1989). Renoux & Renoux (1974) and Renoux, Renoux & Guillaumin (1979) reported that the conflicting responses observed with LMS may be due to experimental

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factors such as sex, age, dose of the drug, and timing of administration. Otterness *et al.* (1979) concluded that the variable results obtained with LMS on thymocytes are dependent upon the state of activation of the cells. Another possible explanation is that the immunopotentiating or immunoinhibiting properties attributed to LMS are not produced by LMS itself, but are due to the action of degradation products or metabolites of LMS (Lichtenfeld *et al.*, 1976; Janssen, 1976; Goldstein, 1978), the concentration of which varies with different conditions. We observed that solutions of LMS in RPMI-1640 medium stored at 4°C for 2 weeks enhanced the proliferative response of mouse spleen lymphocytes to Con A to a greater extent than did freshly prepared solutions of the drug. This suggested that a non-enzymatic alteration in the LMS solutions was occurring. To determine if LMS was spontaneously decomposing to form products which could account for the increased enhancement, we stored LMS under various conditions of temperature and pH. Periodically, aliquots were removed and analyzed by high pressure liquid chromatography. Our results demonstrated that LMS decomposes during storage to form three products in neutral and alkaline conditions. We purified the three degradation products by semi-preparative high pressure liquid chromatography, determined their structures by mass spectrometry, infrared spectrometry and homo- and heteronuclear two-dimensional nuclear magnetic resonance and assessed the effect of each purified product individually on the proliferation response of murine lymphocytes to Con A.

EXPERIMENTAL PROCEDURES

Preparation of spleen cell suspensions

$BC3F_1$/Cum (C57BL × C3H/Anf) male mice (Cumberland View Farms, Clinton, TN, U.S.A.) (12 – 16 weeks of age) were used for the study. The mice were killed by cervical dislocation and the spleens aseptically removed and placed in Petri dishes containing RPMI-1640 medium with HEPES buffer (Hazelton Dutchland, Denver, PA, U.S.A.). The spleens were injected with this medium and the cells gently expelled from the spleen capsule with curved forceps. A single cell suspension was obtained by aspirating the spleen pulp with a syringe, first through a 20-gauge and then through a 25-gauge needle. The red blood cells were lysed by treatment with isotonic ammonium chloride in Tris buffer as described by Boyle (1968). The resulting spleen cell suspension was rinsed twice in RPMI-1640 (HEPES) medium, resuspended in RPMI-1640 with bicarbonate buffer (Hazelton Dutchland) and counted. The viability of the cells was determined by trypan blue exclusion.

Lymphocyte proliferation assay using stored and freshly prepared solutions of LMS or solutions of purified LMS degradation products

For the mitogen assay, 0.2 ml aliquots of the spleen cell suspension containing 2.5×10^5 viable cells were transferred to the wells of Micro Test II tissue culture plates (Costar, Cambridge, MA, U.S.A.), containing 10 μl media. Various solutions of LMS – hydrochloride (Sigma Chemical, St. Louis, MO, U.S.A., lot 83F-3467 and Janssen R & D, New Brunswick, NJ) at a final well concentration of 100 μg/ml and/or Con A (Sigma, lot 89C-7040) at a final well concentration of 30 μg/ml were added to the microtiter wells, each in a volume of 10 μl. These concentrations of LMS and Con A were previously determined to be the optimal concentrations for the proliferative response. After 1 h incubation, 10 μl of heat-inactivated fetal calf serum was added to each culture. The final concentration of fetal calf serum in the microtiter wells was 4.3%. The culture plates (total well volume, 240 μl) were then incubated at 37°C in a humidified CO_2 incubator. The incubator was gassed with 5% CO_2 which maintained the pH of the medium at 7.25. Forty-eight hours after adding the mitogen, the cultures were pulsed with 1.0 μCi of [^{3}H]-thymidine (Amersham, Arlington Heights, IL, U.S.A., spec. act. 2.0 Ci/mM). Sixteen hours after the addition of [^{3}H]-thymidine, the cultures were harvested with a Mash II automated cell harvester (Microbiological Associates, Bethesda, MD, U.S.A.) using glass fiber filters and double distilled water. The filters were dried, transferred to scintillation vials containing a toluene based scintillation medium and counted in a liquid scintillation counter.

LMS was dissolved in RPMI-1640 with bicarbonate buffer (2.4 mg/ml), sterilized by filtration, and was used either immediately after preparation or after storage for 2 weeks at 4°C. During the 2 week storage period, the pH of the stored solution increased from 7.0 to 8.0 – 8.5 due to the bicarbonate buffer.

To determine if the LMS degradation products enhanced or inhibited the lymphocytes' response to Con A, various concentrations of purified Product 1, 2 or 3 were added to the microtiter wells, each in a volume of 10 μl. Products 1, 2 and 3 were dissolved in methanol at concentrations of 2.5, 1.0 and

4.8 mg/ml, respectively. The products were then diluted with RPMI-1640 with bicarbonate buffer to final well concentrations of 0.01 – 10.0 μg/ml for Product 1, 0.02 – 20.0 μg/ml for Product 2 and 0.04 – 10.0 μg/ml for Product 3. Con A was added to appropriate wells at a final well concentration of 30 μg/ml in a volume of 10 μl. To determine if the diluent would affect the assay, methanol was tested in the cultures at the same concentrations used to dissolve the LMS degradation products.

Study of LMS stability when stored in Britton – Robinson buffer

To determine the effects of pH on the stability of LMS, the drug was dissolved in a modified Britton – Robinson buffer (0.2 M acetic acid, 0.2 M phosphoric acid and 0.2 M boric acid) titrated to the desired pH with 10 N NaOH. LMS concentration was 20 mg/ml. The LMS solutions were buffered at pH 6, 7, 7.5 and 8 and stored at either 4 or 37°C in Wheaton reaction vials under sterile conditions. At various time intervals over a 2 week period, aliquots were removed and analyzed by high pressure liquid chromatography. The pH remained constant during the 2 week incubation period.

High pressure liquid chromatography analysis of solutions for LMS and LMS degradation products

High pressure liquid chromatography analysis of LMS solutions was performed with a model 6000 A pump module (Waters Associates, Milford, MA, U.S.A.), a model 7125 injector valve (Rheodyne, Berkeley, CA, U.S.A.), a spectromonitor III UV detector (LKB Instruments, Inc., Gaithersburg, MD, U.S.A.), and a model 3390 A computing integrator (Hewlett Packard, Kansas City, MO, U.S.A.). A 250 × 4.6 mm ID, 10 μ particle size, 600 RP C18 column (Alltech Associates, Deerfield, IL, U.S.A.) was used. The mobile phase was methanol – water – ammonium hydroxide (60 + 39.9 + 0.1) and was delivered at 0.6 ml/min. Detection was at 225 nm. Chromatography was performed at room temperature. A 2 μl (40 μg LMS) aliquot of each LMS solution was injected onto the high pressure liquid chromatography analytical column. A LMS standard had a high pressure liquid chromatography retention time of 13.3 min.

Purification of LMS degradation products

A liter of LMS (Sigma) solution (10 mg/ml) was prepared in modified Britton – Robinson buffer (pH 7.5) and maintained at 37°C for 2 weeks. After 2 weeks the LMS solution was extracted three times with two liters of chloroform. The combined chloroform extracts were vacuum evaporated to dryness and the residue containing LMS and LMS degradation products was resuspended in 40 ml of methanol. The degradation products were purified by semi-preparative high pressure liquid chromatography using a Beckman model 342 gradient liquid chromatograph and a Hewlett Packard model 3390 A computing integrator. A 250 × 10 mm ID, 10 μ particle size, 605 RP RSIL C18 LL column (Alltech Associates) was used. The mobile phase was methanol – water (60 + 40) delivered at 1.2 ml/min. Detection was at 225 nm. A 20 μl aliquot was injected. Products 1, 2 and 3 had semi-preparative high pressure liquid chromatography retention times of 21, 32 and 62 min, respectively. The isolated products were recovered, vacuum evaporated at 15°C to dryness and maintained under nitrogen at 4°C. The purity of the products was assessed by an analytical high pressure liquid chromatography method using a 250 × 4.6 mm ID, 10 μ particle size, 600 RP C18 column (Alltech Associates). The mobile phase was methanol – water – ammonium hydroxide (60 + 39.9 + 0.1) and delivered at 0.6 ml/min. Detection was at 225 nm. Chromatography was performed at room temperature. Products 1, 2, 3 and LMS have analytical high pressure liquid chromatography retention times of 5.6, 8.8, 10.7 and 13.3 min, respectively.

Mass spectrometry

Mass spectra of the compounds were produced using a mass spectrometry system that consisted of a MS-9 analyser (A.E.I., Manchester, U.K.) and a MSS Series 200 electronics console (Mass Spectrometry Services, Manchester, U.K.). The samples were dissolved in methanol, transferred to a direct inlet probe, evaporated and analysed. The scan speed was 10 s/decade and resolving power was 10,000. Spectra were processed on a V.G. Series 2,000 data system (V.G., Manchester, U.K.).

Nuclear magnetic resonance spectrometry

Proton – nuclear magnetic resonance, ^{13}C nuclear magnetic resonance, proton – proton homonuclear two dimensional nuclear magnetic resonance, and heteronuclear two dimensional chemical shift correlation nuclear magnetic resonance spectroscopy were performed on a NMR 300 XL with a field strength of 7.05 tesla and 4.3 software. The proton – proton homonuclear two dimensional nuclear magnetic resonance spectroscopy analysis

Table 1. Comparison of freshly prepared* and stored† solutions of LMS on lymphocyte proliferation response to Con A

Additions to culture	Counts/min/culture ± S.E.M.‡ (stimulation index)					
	Experiment					
	1		2		3	
None	2,228 ± 254		865 ± 152		4,486 ± 355	
LMS fresh	5,979 ± 380§		1,546 ± 208§		10,117 ± 395§	
LMS 4°C	6,726 ± 16§‖		1,400 ± 169§		12,301 ± 1,027§‖	
Con A	87,734 ± 1,410		120,116 ± 2,893		120,264 ± 7,487	
Con A + LMS fresh	104,522 ± 4,329¶	(1.1)††	128,804 ± 6,460**	(1.1)††	140,569 ± 3,219¶	(1.1)††
Con A + LMS 4°C	120,038 ± 7,697¶‡‡	(1.3)§§	139,899 ± 5,029¶‡‡	(1.2)§§	146,899 ± 2,501¶‡‡	(1.2)§§

*LMS fresh, LMS was dissolved in RPMI-1640 immediately before addition to cultures.
†LMS 4°C, LMS was dissolved in RPMI-1640 and stored at 4°C for 2 weeks before addition to cultures.
‡Values are mean of four cultures.
§Significantly higher than none ($P<0.001$).
‖Significantly higher than LMS fresh ($P<0.05$).
¶Significantly higher than Con A ($P<0.001$).
**Significantly higher than Con A ($P<0.05$).
††Stimulation index = [(Con A + LMS fresh) − (LMS fresh)]/(Con A − none).
‡‡Significantly higher than Con A + LMS fresh ($P<0.05$).
§§Stimulation index = [(Con A + LMS 4°C) − (LMS 4°C)]/(Con A − none).

was based on the method of Bax, Freeman & Morris (1981). The heteronuclear two dimensional chemical shift correlation nuclear magnetic resonance spectroscopy analysis was based on the methods of Bax & Morris (1981) and Bax (1983). These methods were incorporated into the 4.3 software. Deuterated chloroform was used as the solvent for all the samples and 1% tetramethylsilane was used as the reference compound.

Infrared spectrophotometry

Infrared spectrophotometry was performed on a Beckman IR9 and a Beckman IR 18A spectrophotometer. Infrared spectra of LMS products were obtained in potassium bromide pellets. The spectra were recorded at a speed of 40 cm^{-1} from 600 to 4,000 cm^{-1} using a double beam.

Quantitative determination of LMS degradation products

The amount of the three degradation products present in the solutions of LMS stored at various pH and temperature was determined by comparing the high pressure liquid chromatography peaks from the solutions with standard curves prepared from known amounts of the purified products [see companion paper (Hanson & Heidrick, 1991)].

Statistical analysis

Data were analyzed by Student's *t*-test with a $P<0.05$ chosen as the criterion for significance.

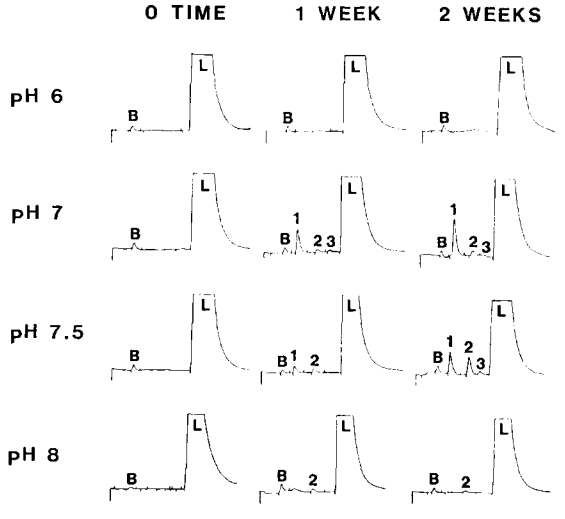


Fig. 1. High pressure liquid chromatography analysis of LMS solutions stored at 37°C in Britton – Robinson buffer at various pH for 2 weeks. (B) Buffer elution peak; (L) LMS peak; (1) Product 1 peak; (2) Product 2 peak; (3) Product 3 peak. In order to detect the presence of the decomposition products, it was necessary to increase the attenuation of the integrator to the point that the LMS peak appeared offscale.

RESULTS

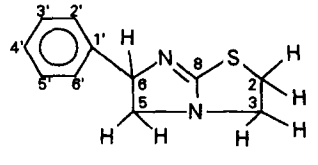
Comparison of stored vs freshly prepared LMS on lymphocyte proliferation

To determine if storing LMS altered its ability to enhance mitogenic responses, the effects of freshly

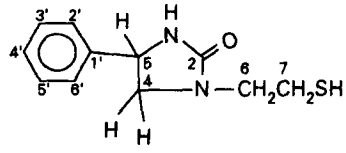
Table 2. Concentration (μg/ml) of Products 1, 2 and 3 formed from LMS in Britton–Robinson buffer (20 mg/ml) at 4 and 37°C after 2 weeks

	4°C			37°C		
	pH 7.0	pH 7.5	pH 8.0	pH 7.0	pH 7.5	pH 8.0
Product 1	0*	0	2 (0.01%)	396 (2.0%)†	208 (1.0%)	0
Product 2	0	0	52 (0.3%)	163 (1.0%)	720 (4.0%)	138 (0.9%)
Product 3	0	0	0	120 (0.5%)	244 (1.0%)	0

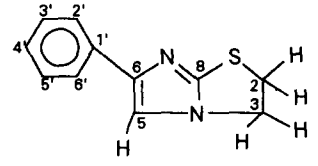
*Values are the mean of two separate experiments, each assayed in duplicate.
†Percent of original levamisole concentration.



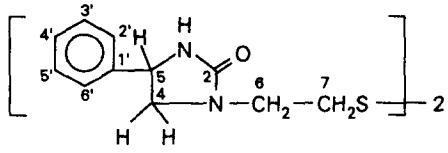
L-2,3,5,6-tetrahydro-6-phenylimidazo (2,1-b) thiazole hydrochloride
LEVAMISOLE



3-(2-mercaptoethyl)-5-phenyl imidazolidine-2-one
PRODUCT 1



6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole
PRODUCT 2



bis (3-(-2-oxo-5-phenylimidazolidin-1-yl) ethyl) disulfide
PRODUCT 3

Fig. 2. Structure of LMS and proposed structures of LMS degradation Products 1, 2 and 3.

Table 3. ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of Products 1, 2 and 3

	Product 1		Product 2		Product 3	
	ppm	J (Hz)	ppm	J (Hz)	ppm	J (Hz)
H-2*			3.83	7.4, 7.0		
H-3			4.20	7.4, 7.0		
H-4	3.28†	8.8, 8.8			3.18	8.8, 8.8
	3.88	8.8, 8.8			3.79	8.8, 8.8
H-5	4.78	8.8, 8.8	7.23	n.r	4.68	8.8, 8.8
H-6	3.33†	14.2, 7.0			3.37	14.1, 6.6
	3.47	14.2, 7.0			3.49	14.1, 6.6
H-7	2.68	7.0, 8.2	7.0		2.77	6.6, 6.6
H-1′			7.23	7.2, 1.4		
H-2′ + H-6′			7.72	7.2, 1.4		
H-3′ + H-5′			7.35	7.2, 1.4		
H-1′ thru H-6′	7.37	n.r.			7.27	—
NH	4.68	—			5.23	9.5
SH	1.42	8.2				

*Indicates carbon number.
†Non-equivalent protons.
n.r. Not resolved.

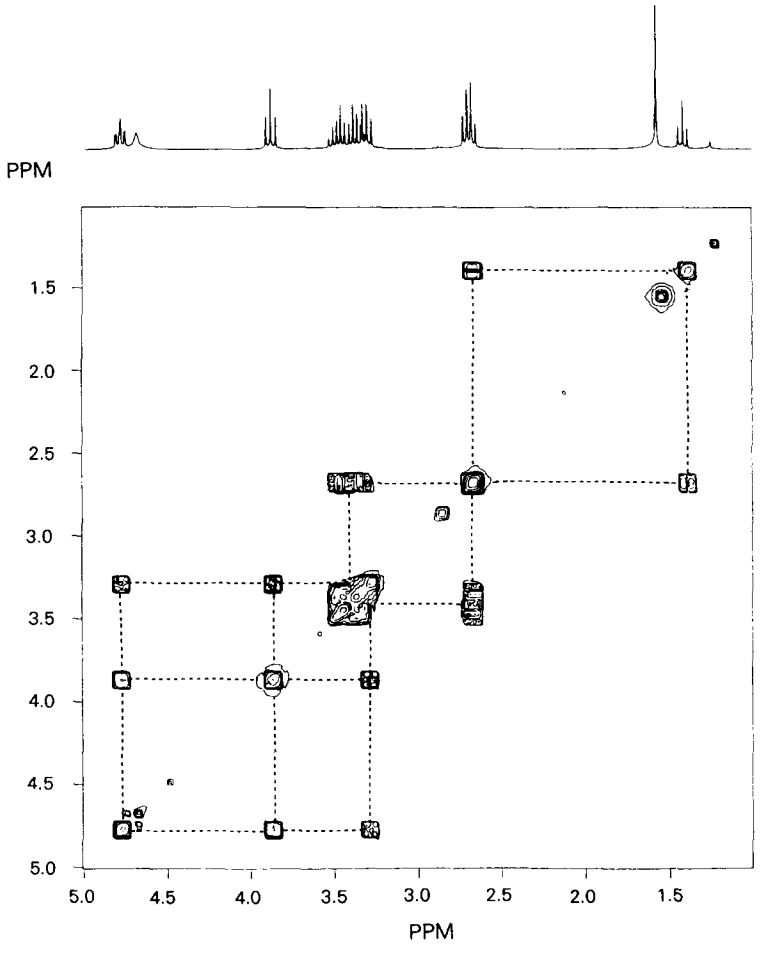


Fig. 3. Contour plot of two dimensional correlated proton – nuclear magnetic resonance spectrum at 300 MHz recorded in c. 3 mmolar solution of Product 1 in deuterochloroform, the internal lock solvent. The spectral width was 2100 Hz. Only the aliphatic portion of the spectrum is shown. The data set consisted of 512 points in both the t_1 and t_2 dimensions. Sixteen transients were accumulated for each value of t_1. The total accumulation time was c. 1.3 h. Before Fourier transformation, the free induction decays were sine bell weighted. An absolute value plot is shown.

Table 4. ^{13}C NMR chemical shifts (ppm) of levamisole and Product 2

Compound	Carbon number								
	C2	C3	C5	C6	C8	C1′	C2′,6′	C3′,5′	C4′
Levamisole	34.13	48.98	58.25	76.73	174.29	142.9	126.49	128.39	127.15
Product 2	34.61	46.21	112.30	118.37	150.20	134.2	124.70	128.55	126.77

prepared or stored solutions of LMS on lymphocyte proliferative responses to Con A were compared. Both freshly prepared solutions of LMS and the solutions of LMS stored for 2 weeks at 4°C in RPMI-1640 bicarbonate media (pH estimated to be 7.5 – 8.5 for majority of the storage time) elevated the lymphocyte proliferative response to Con A with a P value <0.001 (Table 1). The lymphocyte proliferative response was significantly higher (4 – 15%) (P<0.05) with the stored solutions of LMS than with the freshly prepared solutions. Although both LMS solutions alone were mitogenic, a synergistic effect was seen in combination with Con A (see stimulation indexes). These data demonstrate

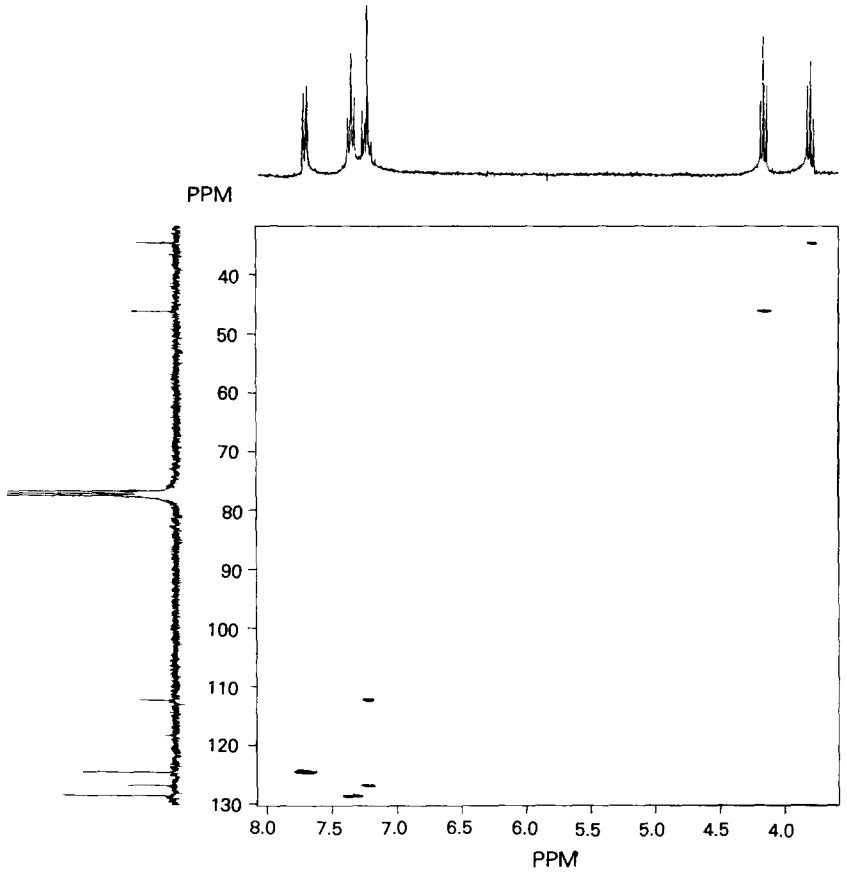


Fig. 4. Contour plot of $^{13}C/^{1}H$ shift correlated nuclear magnetic resonance spectrum recorded in c. 0.3 molar solution of Product 2 in deuterochloroform, the internal lock solvent. The spectral widths in the t_1 and t_2 dimensions were 7430 and 1350 Hz, respectively. Two hundred and fifty transients were accumulated for each of the 64 values of t_1. The data set consisted of 1024 points in t_1 and t_2. Sine bell weighting was used. Total accumulation time was 11.5 h.

that solutions of LMS stored at 4°C for 2 weeks are more active than fresh preparations.

LMS stability during storage

The stability of LMS during storage was assessed in Britton – Robinson buffer in which the exact pH could be strictly maintained. The high pressure liquid chromatography chromatograms of LMS solutions stored for various times at 37°C and at pH 6, 7, 7.5 and 8 in the Britton – Robinson buffer are shown in Fig. 1. The high pressure liquid chromatography data demonstrate that at pH 7, 7.5 and 8.0, LMS decomposed to form three products referred to as Product 1, Product 2 and Product 3 with high pressure liquid chromatography retention times of 5.6, 8.8 and 10.7 min, respectively. LMS did not decompose to form products at pH 6 and only Product 2 was formed at pH 8. The high pressure liquid chromatography analysis of analogous LMS solutions stored at 4°C demonstrated that only Product 1 and Product 2 were formed by 2 weeks and only at pH 8.0. Thin-layer chromatography analysis supported the high pressure liquid chromatography results (data not shown). The concentrations of Products 1, 2 and 3 formed at 4 and 37°C after 2 weeks are shown in Table 2. The optimal pH for Product 1 formation appeared to be pH 7 while the optimal pH for formation of Products 2 and 3 appeared to be 7.5. These data indicate that the decomposition of LMS is temperature and pH dependent.

Purification and structural identification of LMS degradation products

To obtain a large quantity of the degradation products for purification and identification, a liter of

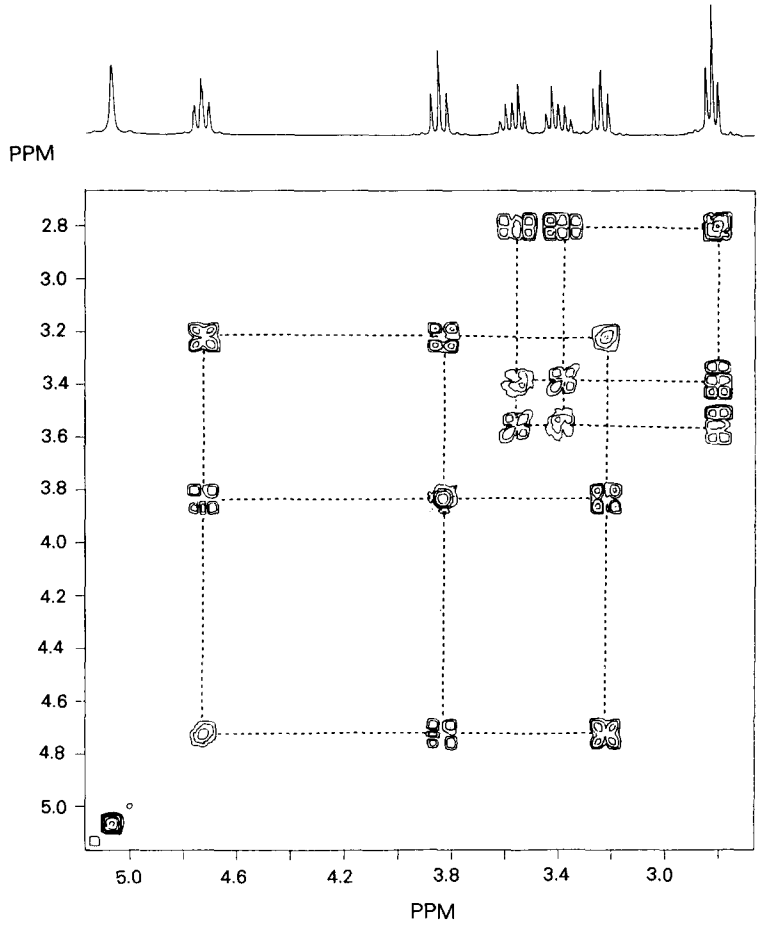


Fig. 5. Contour plot of two dimensional proton – nuclear magnetic resonance spectrum at 300 MHz recorded in c. 0.3 molar solution of Product 3 in deuterochloroform, the internal lock solvent. Only the aliphatic portion of the spectrum is shown. The data set consisted of 512 points in both the t_1 and t_2 dimensions. Four transients were accumulated for each value of t_1. The total accumulation time was c. 0.3 h. Before Fourier transformation, the free induction decays were sine bell weighted. An absolute value plot is shown.

LMS (10 mg/ml) was prepared in modified Britton – Robinson buffer at pH 7.5 and maintained at 37°C for 2 weeks. The three degradation products were purified by semi-preparative high pressure liquid chromatography from a chloroform extract of this solution. After purification by semi-preparative high pressure liquid chromatography, each of the products gave a single peak on the analytical high pressure liquid chromatography column. Product 1 was determined to be 3-(2-mercaptoethyl)-5-phenyl-imidazolidine-2-one (Fig. 2) based on the following spectral information. The high resolution mass spectrometry exhibited a molecular ion at *m/z* 222 ($C_{11}H_{14}N_2OS$). Loss of the C_2H_3S radical produces an ion at *m/z* 175 ($C_{10}H_{11}N_2O$). Subsequent cleavage in the imidazole ring and loss of NHCO produced the base peak at *m/z* 132 ($C_9H_{10}N$). The infrared spectrophotometry data demonstrated the presence of sulfhydryl (2,550 cm^{-1}) and carbonyl (1,680 cm^{-1}) functional groups. The complete proton – nuclear magnetic resonance assignments of Product 1 are given in Table 3 and were verified by proton – proton homonuclear two dimensional nuclear magnetic resonance spectroscopy (Fig. 3). The triplet at 4.78 ppm (H-5) is coupled to the non-equivalent protons H-4 (3.88 and 3.28 ppm). These data also demonstrated that the non-equivalent H-6 protons (3.47 and 3.33 ppm) are coupled to the H-7 protons (2.68 ppm) which in turn are coupled to the sulfhydryl proton (1.42 ppm).

Table 5. The effect of Product 1 (sulfhydryl) and Product 2 (oxidation) on the lymphocyte response to Con A

Additions to culture	Counts/min/culture ± S.D.*			
	Product 1		Product 2	
None	1,920 ± 591		1,869 ± 936	
Product				
20.0 μg/ml	ND		1,311 ± 601	
10.0	268 ± 190†		1,933 ± 764	
2.2	7,025 ± 318‡		2,004 ± 1,071	
1.0	5,893 ± 1,423‡		1,924 ± 948	
0.2	1,950 ± 518		1,592 ± 740	
0.1	1,923 ± 793		1,900 ± 1,177	
0.02	2,045 ± 732		1,935 ± 1,102	
0.01	2,086 ± 480		ND	
Con A	127,400 ± 12,098		150,616 ± 16,776	
Con A + Product				
20.0 μg/ml	ND		144,611 ± 27,573	(1.0)
10.0	3,429 ± 3,143§	(0.03)‖	189,073 ± 21,525**	(1.3)
2.2	115,177 ± 8,399¶	(0.86)	187,194 ± 17,216**	(1.3)
1.0	117,898 ± 10,232	(0.89)	173,093 ± 16,919††	(1.2)
0.2	124,966 ± 12,792	(0.98)	159,233 ± 13,188	(1.0)
0.1	128,174 ± 12,214	(1.00)	151,071 ± 11,849	(1.0)
0.02	125,981 ± 13,548	(0.99)	159,324 ± 16,485	(1.0)
0.01	123,646 ± 10,591	(0.97)	ND	

*Mean of 24 wells (three from each of eight individual animals).
†Significantly ($P<0.001$) lower than none.
‡Significantly ($P<0.001$) higher than none.
§Significantly ($P<0.001$) lower than Con A.
‖Stimulation index $= \frac{[(\text{Con A + Product}) - \text{Product}]}{(\text{Con A} - \text{none})}$.
¶Significantly ($P<0.05$) lower than Con A.
**Significantly ($P<0.001$) higher than Con A.
††Significantly ($P<0.05$) higher than Con A.

The structure of Product 2 was determined to be 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole (Fig. 2) based on the following spectral data. The high resolution mass spectrometry exhibited a very strong molecular ion at *m/z* 202 ($C_{11}H_{10}N_2S$). This corresponds to loss of two hydrogens from LMS. Mass spectrometry also gave minor peaks at *m/z* 174 ($C_9H_6N_2S$), 147 (C_8H_5NS) and 103 (C_7H_5N). Using LMS as a reference compound, we assigned the ^{13}C nuclear magnetic resonance spectrum of Product 2 (Table 4). The signals at 118.37, 112.3 and 150.2 ppm of Product 2 spectrum, which are not similar to those of LMS's ^{13}C nuclear magnetic resonance spectrum, were assigned to carbons 4, 3 and 5, respectively. Using the heteronuclear two dimensional chemical shift correlation nuclear magnetic resonance spectroscopy we were able to determine that the protons at the signal 7.23 ppm are bonded to carbons 5 (112.3) and 4′ (126.77) (Fig. 4). The two protons at 3.83 ppm are attached to carbon 2 (34.61 ppm) and the two protons at 4.2 ppm are attached to carbon 3 (46.21 ppm). The proton–nuclear magnetic resonance assignments were verified by proton–proton homonuclear two dimensional nuclear magnetic resonance spectroscopy and appear in Table 3.

The structure of Product 3 was determined to be *bis* [3-(2-oxo-5-phenylimidazolidin-1-yl) ethyl] disulfide, the disulfide of oxidized Product 1, based on the following spectral data (Fig. 2). The high resolution mass spectrometry gave a molecular ion at *m/z* 442 (M+, $C_{22}H_{26}N_4O_2S_2$) and principal peaks at 221 ($C_{11}H_{13}N_2O$), 175 ($C_{10}H_{11}N_2O$) and 132 ($C_9H_{10}N$). Infrared spectrophotometry data demonstrated the presence of a carbonyl (1,680 cm^{-1}) functional group. The proton–nuclear magnetic resonance spectral

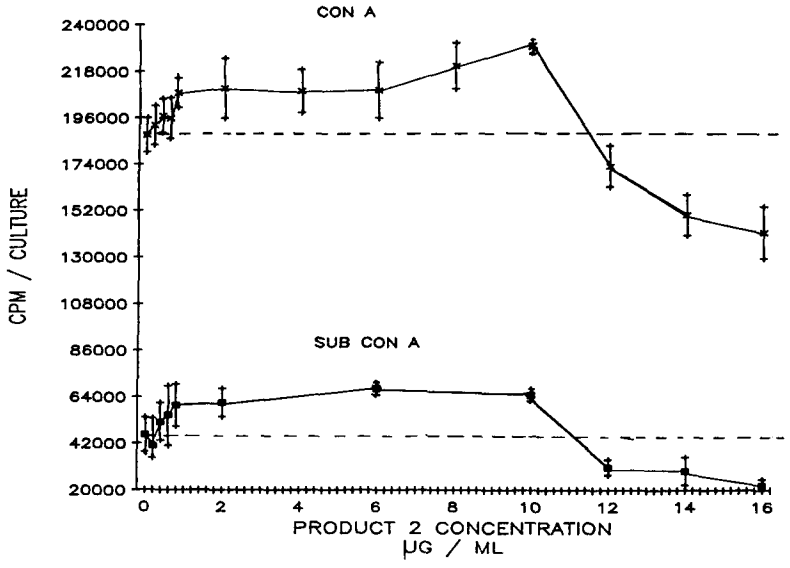


Fig. 6. The effect of Product 2 at various concentrations on Con A (optimal and suboptimal) stimulated lymphocyte proliferation. (-----) is response of Con A alone.

assignments of Product 3 are shown in Table 3. The complete assignment of Product 3 proton – nuclear magnetic resonance spectrum was determined by proton – proton homonuclear two dimensional nuclear magnetic resonance spectroscopy (Fig. 5). The data demonstrated that the triplet at 4.68 ppm (assign to H-5) is coupled to the two protons at 3.79 and 3.18 ppm (assign to H-4). These data also confirmed that the two protons on carbon 6 (3.49 and 3.37 ppm) are coupled to the two protons on carbon 7 (2.77 ppm).

Effect of purified degradation products on lymphocyte proliferation assay

The effect of Product 1 (sulfhydryl product) and Product 2 (oxidation product) on the lymphocyte proliferative response to Con A is shown in Table 5. Product 1 inhibited the lymphocyte proliferative response to Con A at a concentration of 0.2 – 10.0 μg/ml (significant at 2.2 μg/ml ($P<0.05$)). Product 1 is mitogenic (in the absence of Con A) at concentrations of 1.0 and 2.2 μg/ml. Product 2 significantly ($P<0.001$) elevated the lymphocyte proliferative response to Con A at concentrations between 1.0 and 10.0 μg/ml, but was not mitogenic by itself. Although, our results demonstrated that Product 2 was immunopotentiating, a dose – response effect was not apparent at these concentrations. Therefore, we determined the immunoenhancing effect of Product 2 at lower concentrations and using Con A at the optimal (30 μg/ml) and at a suboptimal (10 mg/ml) concentration (Fig. 6). Our results showed that Product 2 at concentrations between 0.1 and 1.0 μg/ml with optimal or suboptimal Con A resulted in a linear response. Between concentrations of 2.0 and 10.0 μg/ml, the curve plateaued and no further enhancement was observed with either optimal or suboptimal Con A. Concentrations greater than 10.0 μg/ml produced inhibition.

The effect of Product 3 (disulfide product) on the lymphocyte proliferative response to Con A is shown in Table 6. Since a higher concentration of methanol was required to maintain Product 3 in solution, the effect of methanol on the lymphocyte proliferative response to Con A is included. Methanol added alone to the lymphocyte cultures did not have any effect but enhancement of the Con A response was observed at the higher methanol concentrations. Product 3 significantly inhibits the lymphocyte proliferative response to Con A at a concentration of 10 μg/ml when compared with the appropriate control of methanol + Con A. Product 3 is mitogenic at a concentration of 2.0 – 10.0 μg/ml.

DISCUSSION

Our results indicate that LMS non-enzymatically decomposes under mild conditions during storage and that at least three degradation products are

Table 6. Effect of Product 3 (disulfide) on the lymphocyte proliferative response to Con A

Additions to culture	Counts/min/culture ± S.D.*	
None	2,611 ± 987	
Methanol 1.4%	3,042 ± 435	
Product 3		
10.0 μg/ml	8,450 ± 1,882†	
4.4	9,139 ± 1,377†	
2.0	5,552 ± 1,665†	
0.4	3,131 ± 1,009	
0.2	2,787 ± 766	
0.04	2,562 ± 873	
Con A	86,326 ± 19,145	
Con A + methanol		
1.4%‡	122,366 ± 7,700§	
0.6	109,493 ± 15,251	
0.3	96,691 ± 14,784	
0.06	85,041 ± 14,677	
0.03	88,727 ± 17,400	
0.006	89,169 ± 12,799	
Con A + Product 3		
10.0 μg/ml	93,324 ± 7,143‖	(0.7)¶
4.4	88,054 ± 3,697	(0.9)
2.0	94,894 ± 10,159	(1.0)
0.4	86,838 ± 16,062	(1.0)
0.2	85,939 ± 14,188	(1.0)
0.04	84,575 ± 19,425	(1.0)

*Mean of 24 wells (three from each of eight individual animals).

†Significantly greater ($P<0.001$) than none.

‡Methanol concentration corresponds to the amount present as diluent.

§Significantly greater ($P<0.05$) than Con A.

‖Significantly lower ($P<0.05$) than Con A + methanol.

¶Stimulation index $= \frac{[(\text{Con A + Product 3}) - \text{Product 3}]}{[\text{Con A + methanol} - \text{none}]}$.

formed. The decomposition is temperature and pH dependent. Our spectral data indicate that Product No. 1 formed from LMS is 3-(2-mercaptoethyl)-5-phenylimidazolidine-2-one or *dl*-2-oxy-3-(2-mercaptoethyl)-5-phenylimidazolidine (OMPI), known to be a metabolite of LMS *in vivo* (Janssen, 1976). Product No. 2 is 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole. Formed by oxidation of LMS, Product 2 is the major metabolite of LMS detected *in vivo* (Graziani & De Martin, 1977). Product No. 3 is *bis* [3-(2-oxo-5-phenylimidazolidin-1-yl] ethyl, the disulfide of oxidized Product 1.

Non-enzymatic degradation of LMS has been studied by others under more harsh conditions. Dickinson, Hudson & Taylor (1971a,b), studied the decomposition of LMS at 100° C under nitrogen and oxygen and they obtained four degradation products. Their structural analysis of these products (by proton – nuclear magnetic resonance and infrared spectrophotometry only) led them to propose structures for three of the products which are the same as our Products 1, 2 and 3. We did not observe their fourth product, 3-(β-aminophenethyl)-2-thiazoldinone in our study. From kinetic studies of LMS decomposition at 100°C at pH 4 – 8, Dickinson *et al.* (1971b) predicted that LMS would be grossly unstable at pH 7 – 9 at 25, 30 and 37°C. Kumar, Dobbs, Weiss & Chirigos (1980) demonstrated that LMS could be degraded to OMPI in a non-enzymatic system by reacting LMS with ascorbate. Their mass spectral data for OMPI are similar to the mass spectral data we obtained for our Product 1.

Since OMPI is known to be a major metabolite of LMS *in vivo* and other thiol containing compounds (e.g. 2-mercaptoethanol) are known to enhance the proliferation response to mitogens, it has been speculated that OMPI is responsible for the immunoenhancing activity of LMS. Otterness & Bliven (1980) reported that OMPI at a concentration of 10 μg/ml significantly enhanced the Con A response of thymocytes, but they reported that LMS and OMPI by themselves were not mitogenic. In contrast, we and others (Woods, Siegel & Chirigos, 1974; Hiestand & Strasser, 1985), have found LMS to be mitogenic on non-mitogen stimulated lymphocytes and we found OMPI to have mitogenic activity of its own. In our study, when the mitogenic effect of OMPI was taken into account, no enhancing activity of the Con A response could be attributed to OMPI. In agreement with our results, Otterness & Bliven (1980) found OMPI to be inhibitory at concentrations greater than 10 μg/ml. Other investigators have found OMPI to have a beneficial anti-oxidant effect in cell cultures. De Brabander, Van Belle, Aerts, Van de Veire & Geuens (1979) demonstrated that OMPI and LMS protects fibroblast culture cells against auto-oxidative necrosis by glutathione depletion. Anderson, Oosthuizen & Grabow (1981) demonstrated that OMPI and LMS prevented peroxidase-mediated inhibition of lymphocyte transformation *in vitro*. In both cases, OMPI on a molar basis was a more potent antioxidant than LMS. In addition, Anderson *et al.* (1981) reported that another thiol compound, aurothiomalate, which protects lymphocytes from a peroxidase/H_2O_2/halide system also caused a

progressive dose-dependent inhibition of mitogen-induced lymphocyte proliferation.

Our results (Table 5 and Fig. 6) demonstrated that Product 2 (oxidation product) significantly enhanced the lymphocyte proliferative response to Con A at concentrations between 1 and 10 μg/ml ($10^{-5} - 10^{-6}$ M). To our knowledge this is the first study of the immunomodulating effects of 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole (Product 2). How the structural change produced by oxidation of LMS to form Product 2 (loss of two hydrogens and introduction of a double bond) could result in greater enhancement of the Con A response is not known. Ogawa, Nakayama & Tsubura (1983) have reported that LMS binds to thymocytes, lymphocytes, and granulocytes, perhaps by means of a cell receptor. Goldstein (1978) has proposed a possible mode of action for LMS to be the imidazole ring acting like thymopoietin through cell surface receptors to modulate cyclic nucleotide levels. If LMS or active metabolites do act via cell receptors, then a small structural change could influence binding of the compound to a cell receptor. Sunshine, Lopez-Corrales, Hadden, Coffey, Wanebo, Hadden & Rojas (1977) and Renoux (1986) have also suggested that the imidazole moiety of LMS is responsible for the immunomodulatory activity rather than the thiol metabolite, possibly through its influence on the second messenger nucleotide, cGMP. A recent study by Redondo, López-Guerrero & Fresno (1987) determined the effects of LMS, imidazole, imidazole-containing compounds and thiol-containing compounds on the potentiation of IL-2 activity. The imidazole and the other imidazole-containing compounds, but not the thiol-containing compounds had effects similar to those of LMS in potentiating IL-2 activity. Our results are in agreement with these investigators, that the imidazole moiety rather that the thiol group is important for the enhancing activity on lymphocytes.

Our results (Table 6) demonstrated that Product 3 (disulfide product) added to lymphocyte cultures with Con A is inhibitory at concentrations greater than 10 μg/ml as indicated by the lower stimulatory index. We are not aware of any other studies of this compound, however, the immunomodulatory activity of other disulfides has been studied and conflicting data have been reported. Field, Gallo, Beck & Whitehouse (1978) reported that eight different disulfides and thiosulfonates significantly inhibited (>50%) ^{3}H-thymidine incorporation into lymphocytes in graft-vs-host reactions. Camus, Jaffe, Creuset, Perier, Stephan & Leveaut (1977) also demonstrated that the disulfide of 5-thio-pyridone inhibited the proliferative response of human lymphocytes. In contrast, Ohmori & Yamamoto (1983) and Ohmori, Yamauchi & Yamamoto (1985) found that the disulfide form of 2-mercaptoethanol augmented the antibody response to sheep erythrocytes as effectively as reduced 2-mercaptoethanol and that two disulfide compounds, 2-hydroxyethyldisulfide and 2-amino-ethyldisulfide augmented the lymphocyte response to lipopolysaccharide. Hiestand & Strasser (1985) demonstrated that a disulfide compound, ethylene-2-2′-*bis*(dithio)*bis*(ethanol), enhanced the allogenic response in a mixed lymphocyte reaction.

The effect of LMS in the treatment of malignancy and a variety of autoimmune disorders has been inconsistent. However, the recent comprehensive study by Moertel *et al.* (1990) showing impressive effects of LMS with 5-fluorouracil in the treatment of colon cancer, has prompted these authors to recommend the use of LMS as a standard therapy. Further studies of the metabolism of LMS and the possibility of both stimulating and inhibiting effects of its metabolites are warranted.

Acknowledgments — We thank Dr Phillip Issenberg for the mass spectra interpretations, Dr Michael Fisher for his nuclear magnetic resonance technical expertise, Dr Sidney Mirvish for the use of his high pressure liquid chromatography system, and Janssen R & D, Inc. for providing us with LMS during the early phases of this study. The nuclear magnetic resonance facilities supported by NIH grant No. RR01968.

REFERENCES

AL-SAFI, S. A. & MADDOCKS, J. L. (1984). Effect of levamisole and azathioprine on the human mixed lymphocyte reaction (MLR). *Br. J. clin. Pharmac.*, **18**, 83 – 85.

AMERY, W. K. & BUTTERWORTH, B. S. (1983). The dosage regimen of levamisole in cancer: is it related to efficacy and safety? *Int. J. Immunopharmac.*, **5**, 1 – 9.

ANDERSON, R., OOSTHUIZEN, R. & GRABOW, G. (1981). Prevention of peroxidase mediated inhibition of neutrophil motility and lymphocyte transformation by levamisole, OMPI, sodium aurothiomalate, indomethacin and tolmetin *in vitro*. *Int. J. Immunopharmac.*, **3**, 123 – 132.

ARNAUD, J. P., BUYSE, M., NORDLINGER, B., MARTIN, F., PECTOR, J. C., ZEITOUN, P., ADLOFF, A. & DUEZ, N. (1989). Adjuvant therapy of poor prognosis colon cancer with levamisole: results of an EORTC double-blind randomized clinical trial. *Br. J. Surg.*, **76**, 284 – 289.

BAX, A. (1983). Broadband homonuclear decoupling in heteronuclear shift correlation NMR spectroscopy. *J. Magn. Reson.*, **53**, 517 – 520.

BAX, A., FREEMAN, R. & MORRIS, G. A. (1981). Correlation of proton chemical shifts by two-dimensional fourier transform NMR. *J. Magn. Reson.*, **42**, 164 – 168.

BAX, A. & MORRIS, J. (1981). An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. *J. Magn. Reson.*, **42**, 501 – 505.

BOYLE, W. (1968). An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. *Transplantation*, **6**, 761 – 764.

CAMUS, J. P., JAFFE, I. A., CREUSET, J., PERIER, A., STEPHAN, S. & LEVEAUT, C. (1977). La pyrithioxine, nouveau traitement de fond da la polyarthrite rhumatoi'de Etude ouverte de soixantedouze cas avec un recul de six mois. *Ann. Med. Intern.*, **128**, 907 – 911.

COPELAND, D., STEWART, T. & HARRIS, J. (1974). Effect of levamisole (NSC-177023) on *in vitro* human lymphocyte transformation. *Cancer Chemother. Rep.*, **58**, 167 – 170.

DE BRABANDER, M., VAN BELLE, H., AERTS, F., VAN DE VEIRE, R. & GEUENS, G. (1979). Protective effect of levamisole and its sulfhydryl metabolite OMPI against cell death induced by glutathione depletion. *Int. J. Immunopharmac.*, **1**, 93 – 100.

DICKINSON, N. A., HUDSON, H. E. & TAYLOR, P. J. (1971a). Levamisole: its stability in aqueous solutions at elevated temperatures. Part I. Isolation and identification of decomposition products formed in aqueous solutions of levamisole stored under nitrogen and oxygen at 100°C. *Analyst*, **96**, 235 – 243.

DICKINSON, N. A., HUDSON, H. E. & TAYLOR, P. J. (1971b). Levamisole: its stability in aqueous solutions at elevated temperatures. Part III. A chromatographic and polarimetric study of the kinetics of degradation. *Analyst*, **96**, 248 – 253.

FIELD, L., GALLO, A. A., BECK, F. W. J. & WHITEHOUSE, M. W. (1978). Lymphocyte surface poisons: disulfides and thiosulfonates. *Chem. Biol. Interact.*, **23**, 215 – 225.

GOLDSTEIN, G. (1978). Mode of action of levamisole. *J. Rheumatol. Suppl.*, **4**, 143 – 148.

GRAZIANI, G. & DE MARTIN, G. L. (1977). Pharmacokinetic studies on levamisole. Absorption, distribution, excretion and metabolism of levamisole in animals — a review. *Drugs exp. clin. Res.*, **2**, 221 – 233.

HANSON, K. A. & HEIDRICK, M. L. (1991). Immunomodulatory action of levamisole. II. Enhancement of concanavalin A response by levamisole is associated with an oxidation degradation product of levamisole formed during lymphocyte culture. *Int. J. Immunopharmac.*, **13**, 669 – 676.

HIESTAND, P. C. & STRASSER, M. (1985). Immunomodulating activities of ethylene-2,2′-*bis*(diothio)*bis*(ethanol) and related compounds. Effects on murine lymphocyte proliferation and functions *in vitro*. *Int. J. Immunopharmac.*, **7**, 129 – 140.

JANSSEN, P. A. J. (1976). The levamisole story. In *Progress in Drug Research* (ed. Jucker, E.) pp. 347 – 383. Birkhauser, Basel.

KUMAR, K. S., DOBBS, C. R., WEISS, J. F. & CHIRIGOS, M. A. (1980). Levamisole inhibition of microsomal lipid peroxidation as related to its sulfhydryl metabolite DL-2-oxo-3-(2-mercaptoethyl)-5-phenylimadazolidine. *J. Immunopharmac.*, **2**, 73 – 83.

LAURIE, J., MOERTEL, C., FLEMING, T., WIEAND, H., LEIGH, J., RUBIN, J., MCCORMACK, G., GERSTNER, J., KROOK, J., MALLIARD, J., TWITO, D., MORTON, R., TSCHETTER, L. & BARLOW, J. (1989). Surgical adjuvant therapy of large-bowel carcinoma: an evaluation of levamisole and the combination of levamisole and fluorouracil. *J. clin. Oncol.*, **7**, 1447 – 1456.

LICHTENFELD, J. L., DESNER, M. R., WIERNIK, P. H. & MARDINEY, M. R., JR (1976). Modulating effects of levamisole (NSC-177023) on human lymphocyte response *in vitro*. *Cancer Treat. Rep.*, **60**, 571 – 574.

MOERTEL, C., FLEMING, T., MACDONALD, J., HALLER, D., LAURIE, J., GOODMAN, P., UNGERLEIDER, J., EMERSON, W., TORMEY, D., GLICK, J., VEEDER, M. & MAILLIARD, J. (1990). Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *New Engl. J. Med.*, **322**, 352 – 358.

OGAWA, K., NAKAYAMA, T. & TSUBURA, E. (1983). Evidence for specific binding carrier of levamisole in lymphocytes and granulocytes. *Tokushima J. exp. Med.*, **30**, 59 – 64.

OHMORI, H. & YAMAMOTO, I. (1983). Mechanism of augmentation of the antibody response *in vitro* by 2-mercaptoethanol in murine lymphocytes. III. Serum-bound and oxidized 2-mercaptoethanol are available for the augmentation. *Cell. Immun.*, **79**, 186 – 196.

OHMORI, H., YAMAUCHI, T. & YAMAMOTO, I. (1985). Augmentation of *in vitro* antibody response by disulfide compounds. A comparison between intermolecular and intramolecular disulfides. *Japan J. Pharmac.*, **37**, 13 – 19.

OTTERNESS, I. G. & BLIVEN, M. L. (1980). Comparative stimulatory and anti-mitotic effects of levamisole and its metabolite OMPI on thymocytes. *Int. J. Immunopharmac.*, **2**, 23 – 29.

OTTERNESS, I. G., TORCHIA, A. J. & BLIVEN, M. L. (1979). Interaction of levamisole and mercaptans during thymocyte proliferation induced by concanavalin A. *Cell. Immun.*, **43**, 62 – 69.

REDONDO, J. M., LÓPEZ-GUERRERO, J. A. & FRESNO, M. (1987). Potentiation of interleukin-2 activity by levamisole and imidazole. *Immun. Lett.*, **14**, 111 – 116.

RENOUX, G. (1986). The ten commandments for immunotherapeutic drugs at the example of sulfur-containing agents. *Comp. Immun. Microbiol. infect. Dis.*, **9**, 121 – 129.

RENOUX, G. & RENOUX, M. (1971). Effet immunostimulant d'un imidothiazole dans l'immunisation des souris contre l'infection par *Brucella abortus*. *C. R. Acad. Sci.*, **272-D**, 349 – 350.

RENOUX, G. & RENOUX, M. (1974). Modulation of immune reactivity by phenylimidothiazole salts in mice immunized by sheep red blood cells. *J. Immun.*, **113**, 779 – 790.

RENOUX, G., RENOUX, M. & GUILLAUMIN, M. (1979). Genetic and epigenetic control of levamisole-induces immunostimulation. *Int. J. Immunopharmac.*, **1**, 43.

ROTH, J. A. & KAEBERLE, M. L. (1984). Effect of levamisole on lymphocyte blastogenesis and neutrophil function in dexamethasone-treated cattle. *Am. J. Vet. Res.*, **45**, 1781 – 1784.

SPREAFICO, F. (1985). Problems and challenges in the use of immunomodulating agents. *Int. Archs Allergy appl. Immun.*, **76** (Suppl.1), 108 – 118.

SUNSHINE, G., LOPEZ-CORRALES, E., HADDEN, E. M., COFFEY, R. G., WANEBO, H., HADDEN, J. W. & ROJAS, A. (1977). Levamisole and imidazole: *in vitro* effects in mouse and man and their possible mediation by cyclic nucleotides. In *Fogarty International Center Proceedings No. 28, Modulation of Host Immune Resistance in the Prevention or Treatment of Induced Neoplasias* (ed. Chirigos, M. A.) pp. 31 – 37. U.S. Government Printing Office, Washington D.C.

SYMOENS, J. & ROSENTHAL, M. (1977). Levamisole in the modulation of the immune response: the current experimental and clinical state. *J. Reticuloendothel. Soc.*, **21**, 175 – 221.

SYMOENS, J., ROSENTHAL, M., DE BRABANDER, M. & GOLDSTEIN, G. (1979). Immunoregulation with levamisole. *Springer Semin. Immunopath.*, **2**, 49 – 68.

WHITCOMB, M. E., MERLUZZI, V. J. & COOPERBAND, S. R. (1976). The effect of levamisole on human lymphocyte mediator production *in vitro*. *Cell. Immun.*, **21**, 272 – 277.

WOODS, W. A., SIEGEL, M. J. & CHIRIGOS, M. A. (1974). *In vitro* stimulation of spleen cell cultures by poly I:poly C and levamisole. *Cell. Immun.*, **14**, 327 – 331.