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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

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Abstract — Previously we determined that levamisole (LMS), when stored for a period of time, breaks down to three degradation products at neutral and alkaline pH. At low concentrations (10^{-6} M), Product 1 inhibits the lymphocyte response to concanavalin A (Con A), Product 2 enhances the response and Product 3 has no effect. At higher concentrations (10^{-5} M) all three products inhibit the response. To determine if these products are formed in culture media under culture conditions (e.g. in RPMI-1640 bicarbonate buffered medium, 37°C, pH 7.0–7.5, during a 72 h culture period), we added freshly prepared LMS solutions to culture media with and without lymphocytes present and maintained the pH at 7.0, 7.25 or 7.5 by varying the amount of CO_2 present. Periodically over a 72 h period, aliquots of the media were removed and analyzed for the presence of LMS and the three degradation products. Within 4 h, two of the degradation product began to form in culture media with or without lymphocytes present. Product No. 1, 3-(2-mercaptoethyl)-5-phenylimidazolidine-2-one or *dl*-2-oxy-3-(2-mercaptoethyl)-5-phenylimidazolidine (OMPI), which inhibits the lymphocyte response to concanavalin A (Con A) at concentrations above 0.4 μg/ml, was formed at pH 7.0, 7.25 and 7.5, but the compound did not reach inhibitory concentrations in the lymphocyte cultures during the 72 h culture period. Product No. 2, 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole, which enhances the Con A response between concentrations of 0.5 and 10 μg/ml, was detected at concentrations between 2.5 and 3.5 μg/ml at pH 7.25 and 7.5. Product 2 was not detected in cultures at pH 7.0 and subsequently when we cultured lymphocytes with freshly prepared LMS and maintained the pH at 7.0, no significant enhancement of the Con A response was observed. Product No. 3, *bis*[3-(2-oxo-5-phenylimidazolidin-1-yl) ethyl] disulfide was not detected in the culture media during the 72 h period. A solution of LMS which had been stored for 2 weeks in RPMI-1640 medium at 4°C (in which Product 2 was detected) significantly enhanced the Con A response of cells cultured at pH 7.0, while a similar LMS solution which had been stored for 2 weeks at a higher temperature, 37°C (which contained both Products 1 and 2), inhibited the response. The results indicate that the enhancement of the Con A response by LMS is primarily due to an oxidation product of LMS which is formed in small, but stimulatory amounts during the 72 h culture period and suggest that the varied results reported with LMS in cell culture (enhancement, no effect or inhibition) may be due to the formation of these stimulatory and inhibitory products, the relative concentration of which may vary depending upon the culture conditions used and/or the method of preparing and/or storing LMS solutions.

Levamisole (LMS), originally developed as an antihelminthic drug, was first described as an immunomodulator by Renoux & Renoux (1971). Since that time, numerous studies with LMS as an immunotherapeutic drug have been undertaken (for review see Symoens & Rosenthal, 1977; Amery & Gough, 1981) and currently, LMS is being used in humans as an immunotherapeutic agent in cancer, rheumatoid arthritis and viral infections (Horslev-Petersen, Bentsen, Engstrom-Laurent, Junker, Halberg & Lorenzen, 1988; Veys, Mielants & Verbruggen, 1987; Klefström, Holsti, Gröhn,

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Heinonen & Holsti, 1985; Balaram, Padmanabhan & Vasudevan, 1988; Hoofnagle, 1987; Steele & Charlton, 1987; 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). LMS appears to act primarily on macrophages and T-lymphocytes influencing cell-mediated immunity but the exact mechanism of action remains undefined. In our laboratory, we observed that solutions of LMS stored 4°C consistently elevated the lymphocyte proliferative response to Con A to a greater extent than freshly prepared LMS solutions. In the previous paper (Hanson, Nagel & Heidrick, 1991), we reported that LMS was found to decompose during storage in aqueous solutions at neutral and alkaline pH to form three products. The three products were purified, identified and their effects on lymphocyte response to Con A were found to be both stimulatory and inhibitory. In this study we determined the amount of the three products formed from LMS during cell culture conditions (i.e. LMS dissolved in RPMI-1640 media with fetal calf serum and maintained at 37°C for a 72 h period in a CO_2 humidified incubator, both with and without lymphocytes present). The amount of CO_2 present was varied to produce pH measurements of 7.0, 7.25 or 7.5 in the culture media.

EXPERIMENTAL PROCEDURES

Quantitative determination of LMS degradation products

The three LMS degradation products were purified from LMS solutions by high pressure liquid chromatography and their structures determined by infrared spectrophotometry, proton nuclear magnetic resonance spectrometry, carbon nuclear magnetic resonance and homo- and heteronuclear two dimensional nuclear magnetic resonance (see companion paper Hanson *et al.*, 1991). Standard curves for the products were prepared by injecting 0.1 – 10 μg of the purified products onto an analytical high pressure liquid chromatography column. For all three products, there was a linear relationship between the μg added to the high pressure liquid chromatography column and the area of the peak. The amount of the three degradation products present in stored solutions of LMS and culture media was then determined by comparing the peaks from the unknown solutions with the standard curves.

Study of LMS stability during lymphocyte culture

Lymphocytes were prepared as previously described (Hanson *et al.*, 1991) and after appropriate dilution in RPMI-1640 with bicarbonate buffer, 500 μl aliquots containing 2.5×10^5 cells were transferred to the wells of tissue culture 24-well plates, containing 60 μl of medium. Four hundred microliters of a freshly prepared LMS solution were then added to appropriate culture wells [final concentration of LMS was 100 μg/ml (4.89×10^{-4} M)]. After 1 h incubation, 40 μl of heat-inactivated fetal calf serum was added to each culture (total volume of culture was 1 ml). Similar wells were prepared without lymphocytes. The cultures were maintained at 37°C in three separate humidified gas-paks (BBL Microbiology Sys., Cockeysville, MD, U.S.A.) gassed with the appropriate CO_2 – air mixture to produce a final pH measurement of either 7.0, 7.25 or 7.5. At times 0, 4, 12, 24, 48 and 72 h, the contents of three wells were removed and analyzed individually for the presence of the three LMS degradation products. Media from wells containing lymphocytes were centrifuged at 200 *g* for 15 min to remove the cells. The supernatants were extracted twice with 5 ml of chloroform, the chloroform extracts combined and vacuum evaporated to dryness. The residues were resuspended in 1 ml of methanol – water – ammonium hydroxide (60 + 39.9 + 0.1) for high pressure liquid chromatography analysis. The high pressure liquid chromatography method of analysis was the same as previously described (Hanson *et al.*, 1991) except a 100 μl (10 μg LMS) aliquot of each solution was injected onto the high pressure liquid chromatography analytical column. The concentration of the products formed was determined by utilizing standard curves prepared with known amounts of the purified products.

Study of LMS stability when stored in tissue culture media with bicarbonate buffer

To determine the stability of LMS stored in tissue culture media, LMS was dissolved in RPMI-1640 medium with bicarbonate buffer, at a concentration of 2.4 mg/ml. The solutions were stored under sterile conditions at either 4 or 37°C for 2 weeks, during which time the pH of the solutions increased from 7.0 to 8.0 – 8.5 due to the bicarbonate buffer. The medium was extracted twice with 5 ml of chloroform, the chloroform extracts were combined

and vacuum rotoevaporated to dryness. The residues were resuspended in 5 ml of methanol – water – ammonium hydroxide (60.0 + 39.9 + 0.1) for high pressure liquid chromatography analysis. Twenty-one microliter aliquots were injected onto the high pressure liquid chromatography analytical column and the concentrations of the degradation products present calculated using the standard curves prepared with known amounts of the purified products.

Lymphocyte proliferation assay

Spleen cells were prepared from $BC3F_1$ mice and cultured with Con A as previously described (Hanson *et al.*, 1991). The culture plates were incubated at 37°C in a humidified CO_2 incubator with the CO_2 flow adjusted to maintain the pH of the culture media at 7.0. LMS (freshly prepared in RPMI-1640 medium or after storage in RPMI-1640 medium for 2 weeks at 4 or 37°C) was added at a final well concentration of 100 μg/ml. Forty-eight hours after adding Con A, the cultures were pulsed with [^{3}H]-thymidine, harvested and counted as previously described (Hanson *et al.*, 1991).

Statistical analysis of data

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

RESULTS

Standard curves for purified LMS degradation products

Various concentrations of purified preparations of Products 1, 2 and 3 were analyzed by analytical high pressure liquid chromatography and standard curves prepared by plotting the peak area vs μg of compound injected. For all three products there was a linear correlation between the peak area and the amount of product injected over the range of 1 – 10 μg (data not shown). These standard curves were then used to determine the amount of the degradation products present in unknown samples of culture media and stored solutions of LMS.

Determination of LMS degradation products formed during 72 h culture period with and without lymphocytes

Since we previously determined that decomposition of LMS occurred under conditions in which cell culture is normally done (37°C, pH 7.0 – 7.5) it is possible that the enhancement

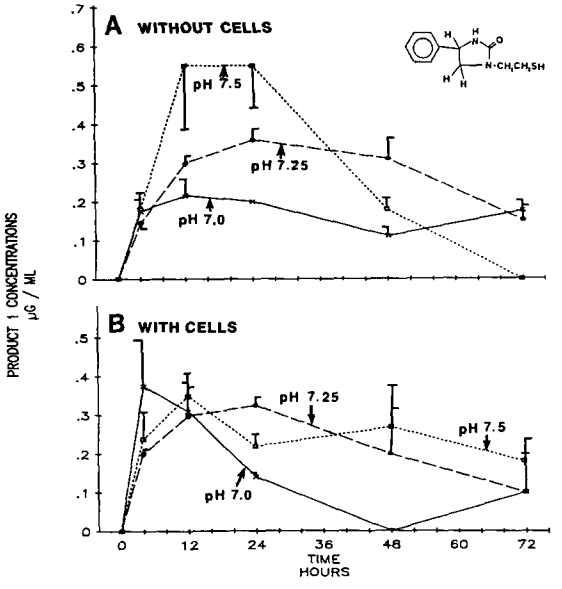

Fig. 1. Concentrations of Product 1 formed from LMS during lymphocyte culture at 37°C over 72 h. The stability of LMS was determined in the absence (A) and presence (B) of lymphocytes. The cultures were maintained at either pH 7.0, 7.25 or 7.5 as indicated. The original LMS concentration was 100 μg/ml. Values are the mean of three wells ± S.D.

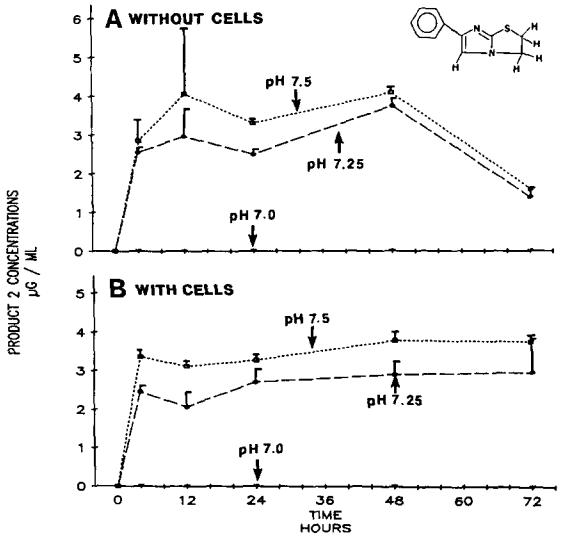

Fig. 2. Concentrations of Product 2 formed from LMS during lymphocyte culture at 37°C over 72 h. The stability of LMS was determined in the absence (A) and presence (B) of lymphocytes. The cultures were maintained at either pH 7.0, 7.25 or 7.5 as indicated. The original LMS concentration was 100 μg/ml. Values are the mean of three wells ± S.D.

Table 1. Concentrations of Products 1, 2 and 3 formed from LMS stored in RPMI-1640 with bicarbonate buffer for two weeks at 37 and 4°C*

	Product concentration μg/ml	
	4°C	37°C
Product 1	0.0	13.3 ± 1.4†
Product 2	7.5 ± 0.1	15.0 ± 0.1
Product 3	0.0	0.0

*LMS concentration, 2.4 mg/ml.
†Values are the mean of three aliquots analyzed separately ± S.D.

observed with freshly prepared solutions of LMS is due to the formation of Product 2 in the culture medium. Therefore, we investigated the stability of LMS under actual cell culture conditions. Freshly prepared LMS was added to culture media and incubated for various time periods up to 72 h with and without lymphocytes present. Periodically, media from three wells was removed and analyzed individually for the presence of the LMS degradation products. The results showed that within 4 h, two of the degradation products (Products 1 and 2) were detectable in the media. The amount of Product 1 measured in the media is shown in Fig. 1. In the culture media without cells present (Fig. 1A) the formation of Product 1 was pH dependent with greater degradation occurring as the pH increased. The pH effect was not as apparent in the cultures with cells present. The concentration of Product 1 did not reach inhibitory concentrations (0.4 μg/ml) in the cultures with the cells present at any time during the 72 h period and a decline in the amount of the compound present was observed after 12 – 24 h. In cultures maintained at pH 7.5 without cells, the concentration of Product 1 reached 0.54 μg/ml.

The formation of Product 2 in the culture media with and without cells present is shown in Fig. 2. Product 2 was detectable within 4 h at pH 7.25 and 7.5, but was never detected at pH 7.0 throughout the 72 h period. In the presence or absence of lymphocytes, the concentration of Product 2 formed during the first 48 h was in the range of 2 – 4 μg/ml, well within the stimulatory range of 0.5 – 10 μg/ml. In the absence of lymphocytes, the concentration of Product 2 began to decrease after 48 h. In the presence of lymphocytes (Fig. 2B), the concentration of Product 2 remained fairly constant for the 72 h culture period. In the presence or absence of lymphocytes, the formation of Product 2 from LMS was dependent on pH, with greater degradation occurring at pH 7.5 than 7.25.

Analysis of LMS solutions stored in RPMI-1640 at 4 or 37°C for 2 weeks

Our original observation was that LMS solutions in RPMI-1640 stored at 4°C were more stimulatory than were freshly prepared solutions of LMS. To determine if this observation could be attributed to formation of stimulatory concentrations of Product 2, we stored solutions of LMS in RPMI-1640 at 4 and also at 37°C and after 2 weeks analyzed aliquots on the high pressure liquid chromatography for the presence of the degradation products. The results (Table 1) show that at 4°C, Product 2 but not Product 1 is formed. The concentration of Product 2 detected (7.5 μg/ml) when diluted (0.01 ml to 0.240 ml) would be 0.31 μg/ml, which is in the stimulatory range based on our assessment of the stimulatory activities of this compound (Hanson *et al.*, 1991). Analysis of the solution stored at 37°C showed that both Product 1 and Product 2 were formed at the higher temperature and were present at concentrations of 13.3 and 15.0 μg/ml, respectively. When diluted in culture, the concentrations would be 0.554 μg/ml for Product 1 (inhibitory) and 0.625 μg/ml for Product 2 (stimulatory).

Comparison of freshly prepared and stored solutions of LMS on lymphocytes cultures at pH 7.0

The observation that the stimulatory degradation product, Product 2, was not formed during culture at pH 7.0, allowed us to address the question of whether the stimulation observed with freshly prepared LMS was entirely due to formation of Product 2 or if undegraded LMS has enhanced activity. Four separate experiments were set up with lymphocytes cultured at pH 7.0. To these cultures were added (1) freshly prepared LMS, from which no Product 2 would be formed and the amount of Product 1 formed would be neither stimulatory nor inhibitory, so any stimulation observed should be due to undegraded LMS; (2) LMS stored at 4°C, which when diluted contained 0.31 μg/ml of Product 2, but no Product 1 and should be stimulatory; or (3) LMS stored at 37°C, which when diluted contained 0.554 μg/ml of Product 1 (inhibitory) and 0.625 μg/ml of Product 2 (stimulatory). The results (Table 2) showed that slight (but not significant) enhancement was observed with freshly prepared LMS in three of the four experiments, significant stimulation was observed in all four experiments with LMS stored at 4°C and inhibition was observed with LMS stored at 37°C.

Table 2. Effect of LMS stored at various temperatures on lymphocyte proliferative response

Additions to culture	Counts/min/culture (stimulation index) Experiment 1		2		3		4	
None	1358		1192		945		1532	
LMS fresh	3181*		2672*		3465*		3254*	
LMS 4°C	4040*		3168*		3116*		3647*	
LMS 37°C	5629*†		4166*†		4643*†		4806*†	
Con A	189,016		113,552		157,797		148,856	
Con A + LMS fresh	191,136	(1.0)‡	115,892	(1.0)	140,751	(0.9)	154,754	(1.0)
Con A + LMS 4°C	203,575§	(1.1)	141,677§	(1.2)	172,147§	(1.1)	170,872§	(1.1)
Con A + LMS 37°C	146,458‖	(0.7)	101,095‖	(0.8)	127,136‖	(0.8)	106,440‖	(0.7)

*Significantly higher than none ($P<0.001$).
†Significantly higher than none, LMS fresh and LMS 4°C ($P<0.05$).
‡Stimulation index = [(Con A + LMS) − LMS alone]/(Con A − none).
§Significantly higher than Con A ($P<0.05$).
‖Significantly lower than Con A ($P<0.05$).

Table 3. Effect of various combinations of Product 1 and Product 2 on lymphocyte proliferation response to Con A

Product 1 concentration (μg/ml)	Product 2 concentration (μg/ml) 0.0	0.5		1.0		2.0		3.0		4.0	
0.0	1.00*	1.11†		1.13†‡		1.19†‡		1.25†‡		1.29†‡	
0.1	1.01	1.05	(+.01)§	1.06	(+.01)	1.04†	(+.06)	1.17†‡	(+.04)	1.21†‡	(+.06)
0.2	0.97	0.98	(−.06)	1.02	(+.03)	1.23†	(+.15)	1.16†	(+.05)	1.18†‡	(+.05)
0.4	0.87†‡	0.88	(−.11)	0.88	(−.12)	0.90	(−.13)	0.82	(−.24)	1.01	(−.07)
0.6	0.83†‡	0.79†‡	(−.18)	0.84†‡	(−.14)	0.85†‡	(−.16)	0.88	(−.16)	1.00	(−.06)
0.8	0.81†‡	0.78†‡	(−.18)	0.84†‡	(−.13)	0.74†‡	(−.26)	0.87†‡	(−.16)	0.94	(−.11)

*Results are expressed as stimulation index:

$$\text{Stimulation index} = \frac{(\text{Con A} + \text{Products}) - \text{Products alone}}{(\text{Con A} - \text{none})}.$$

The values are the average stimulation indexes of two separate experiments (triplicate wells). Average counts/min/culture of Con A stimulated cultures for Exp. No. 1 were 143,655 ± 9928 and for Exp. No. 2, 155,379 ± 3621.
†Significantly different from Con A stimulated culture in Exp. No. 1 ($P<0.05$).
‡Significantly different from Con A stimulated culture in Exp. No. 2 ($P<0.05$).
§Difference between SI if additive (SI of Product 1 + SI of Product 2/2) and actual SI (SI of Product 1 + Product 2).

Effect of various combinations of Product 1 and Product 2 on lymphocyte proliferative response to Con A

To further investigate why the LMS solution stored at 37°C was markedly inhibitory although stimulatory concentrations of Product 2 were present, we investigated the effect of various combinations of purified Product 1 and Product 2 on the proliferation assay. The results (Table 3) indicate that concentrations of Product 1 greater than 0.4 μg/ml abolished the stimulatory effect of Product 2.

DISCUSSION

In the previous paper (Hanson *et al.*, 1991) we found that LMS non-enzymatically decomposed when stored under mild conditions to form three products. The three products were purified, their structures determined and their individual effect on Con A stimulated lymphocyte proliferation was assessed. Products 1, 2 and 3 were identified as 3-(2-mercaptoethyl)-5-phenylimidazolidine-2-one, 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole and *bis* [3-(2-oxo-5-phenylimidazolidin-1-yl) ethyl] disulfide, respectively. Product 2 enhanced the lymphocyte proliferative responses to Con A at concentrations between 0.5 and 10.0 μg/ml, but inhibited the responses at concentrations greater than 10.0. Products 1 and 3 did not enhance the response to Con A at any of the concentrations tested, but inhibited the responses at concentrations >0.4 and 10.0 μg/ml, respectively.

In this study we found that LMS decomposes under actual cell culture conditions in a very short period of time to form two of the three products, Product 1 and Product 2, and that the amount of each product formed is influenced by slight changes in pH. The two products were found in the culture media regardless of whether or not lymphocytes were present, but the concentration of both products was relatively less in the cultures with lymphocytes. This suggests that the lymphocytes are not responsible for the degradation, but that the cells may bind or further metabolize the breakdown products, leading to a lower concentration of the products in the medium. Ogawa, Nakayama & Tsubura (1983) reported that [^{3}H] – LMS binds to lymphocytes, granulocytes and thymocytes and that the binding is saturatable and temperature dependent suggesting a receptor or binding carrier protein for LMS is present. Their use of a general label [^{3}H – LMS] does not rule out the possibility that the labeled LMS may have first been converted to Product 1 or Product 2 before binding to the cells. The studies of Hadden, Coffey, Hadden, Lopez-Corrales & Sunshine (1975); Sunshine, Lopez-Corrales, Hadden, Coffey, Wanebo, Hadden & Rojas (1977) and Anderson, Glover, Koornhof & Rabson (1976) showing that LMS increases cyclic-GMP and decreases cyclic-AMP also suggests that LMS or its degradation products or metabolites act by binding to the cell membrane.

The formation of both stimulatory and inhibitory products and the influence of pH and temperature upon their relative concentrations explains why the stored solutions of LMS produce both stimulation and inhibition of the Con A response. The greater enhancement observed with solutions of LMS stored in RPMI-1640 for 2 weeks at 4°C, can be attributed to formation of Product 2, which reaches stimulatory concentrations. The formation of Product 2 would be favored by the alkaline pH which develops with time due to the bicarbonate buffer in the medium. The formation of Product 1 (inhibitory) did not occur at this temperature. In the solutions of LMS stored at the higher temperature, 37°C, the formation of both products was greatly accelerated. The inhibitory effect of this solution can be accounted for by the high concentration of Product 1 and the fact that after Product 1 reaches the concentration of 0.4 μg/ml it appears to negate the stimulatory effect of Product 2. How this occurs is not clear but could involve competition for a receptor.

We and others (Woods, Siegel & Chirigos, 1974; Hiestand & Strasser, 1985) have found LMS to have mitogenic activity by itself on murine lymphocytes. In these studies, purified Product 1 consistently showed mitogenic activity on non-Con A-stimulated lymphocytes at concentrations of 0.4 μg/ml and above, even though the compound inhibited the Con A response at these concentrations. Product 3 was also mitogenic by itself (Hanson *et al.*, 1991) but the formation of this product was not observed under actual cell culture conditions. Thus the mitogenic activity associated with LMS is probably at least partially due to formation of Product 1.

The concept of LMS immunopotentiation and immunoinhibition being due to the action(s) of a degradation product(s) or metabolite(s) is also supported by *in vivo* data. *In vivo*, LMS is extensively metabolized with a half-life of 1 – 4 h, while LMS metabolites have a much longer half-life. Graziani & De Martin (1977) studied the metabolism of LMS in the rat and found four initial reactions. The most significant quantitative reaction was formation of an intermediate produced by the oxidative introduction of a new double bond into the imidazoline ring (i.e. Product 2), while the formation of OMPI (Product 1) was the least important pathway from a quantitative standpoint. Renoux, Kassel, Renoux, Fiore, Guillamin & Palat (1977) reported that serum taken from mice or rabbits, 24 h after injection with LMS, contained no LMS, but did contain a dialyzable, heat resistant factor which enhanced the response to SRBC of untreated recipient mice. Their studies suggest the immunomodulating effects may be due to the longer lived metabolites rather than to the parent drug.

Our overall conclusion from these studies is that the Con A-enhancing activity attributed to LMS may be due to a metabolite or degradation product of LMS, Product 2, and that the amount of enhancement observed may be influenced by the formation of an antagonistic metabolite or degradation product, Product 1. The continued use of LMS for treatment of cancer and rheumatoid arthritis justify further investigation of the actions of these metabolites.

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