Brief Definitive Report

THYMUS-LIKE ACTIVITIES OF SULPHUR DERIVATIVES ON T-CELL DIFFERENTIATION*

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Levamisole (LMS), the levoisomer of 2,3,5,6-tetrahydro-6-phenylimidazo [2, 1-b] thiazole hydrochloride, has demonstrated immunoenhancing activities on mouse responsiveness to antigenic stimuli (1–4). This activity depended upon T-cell potentiation as evidenced by increased graft-versus-host reaction when donors were treated by LMS (5), by accelerated rejection of isogeneic skin grafts (6, 7), or by enhanced levels of delayed hypersensitivity in LMS-treated mice (7). Sodium diethyldithiocarbamate, DTC, a noncyclic sulphur drug, induced similar responses (8). LMS effect can be transferred by a serum factor obtained from animals after treatment with LMS. In transfer experiments, this factor enforced immune responses to sheep red cells (SRC) in recipient mice and induced regression of tumor size in leukemic AKR mice, even when leukemic mice have failed to respond to treatment with LMS (9, 10). On the other hand, the impaired development of T cells in *nu/nu* mice has been ascribed to lack of the maturation stimulus, or stimuli, normally provided by thymus factors (11–15).

In this report, we present evidences that LMS or DTC induce in vivo acquisition of specific T-cell markers by undifferentiated precursor lymphoid cells of thymusless nude mice and concomitantly induce functional immune activation. Also, that serum from treated *nu/nu* mice can transfer these in vivo activities and allow in vitro differentiation of precursor cells into T cells.

Materials and Methods

Healthy *nu/nu* mice from the Edinburgh strain (16), obtained from the Centre de Sélection des Animaux de Laboratoire (CNRS, Orléans, France), were maintained in an air-conditioned room and partially protected from environmental infections by Isocap filters (Iffa-Credo, l'Arbresle, France).

LMS or DTC were dissolved in pyrogen-free saline and subcutaneously administered in vol of 0.2 ml, at the doses indicated in the individual experiments. Doses of the drug were expressed in milligrams of the salt per kilogram body weight.

In in vivo assays, 5- to 6-wk-old female *nu/nu* mice were killed 4 days after a single drug administration. Any mouse showing evidence of liver disease (17) was discarded from the experiment. In vitro induction assays were performed for 2.5 h at 37°C in a humidified CO_2 incubator in the absence or presence of LMS or DTC. Amounts of the drugs varied from 10^{-9} to 10^{-3} mM/10^6 lymphocytes per ml of medium. Expression of T-cell antigens on the cell surface was tested on either in vitro or in vivo induced lymphocyte suspensions in the cytotoxicity assay (18), using Thy-1.2 and TL 1, 2, 3 antisera kindly provided by E. A. Boyse. Each cytotoxicity assay was performed in duplicate. Induction of an acquired functional (helper) activity was assayed on day 4 after immunization of *nu/nu* mice with SRC and simultaneous treatment with LMS or with DTC

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and expressed as IgM-(direct) or IgG-(indirect) plaque-forming spleen cells (PFC), using a technique previously described (1, 2, 9). Transfer of inducing activity was tested by induction assay of spleen cells or bone marrow cells of *nu/nu* mice in the presence of serum from *nu/nu* mice treated with 25 mg/kg LMS. Transfer of functional immunocompetence was assayed by the PFC test in C3H/He female mice immunized with SRC and treated with serum of LMS-treated *nu/nu* mice. The *Limulus* assay (19) (Pyrotest, Difco Laboratories, Detroit, Mich.) was used to detect contaminant endotoxin before in vitro addition or to in vivo injection, and any assay performed with a sample that resulted even questionable for trace amount of endotoxin was discarded from the experiment. Flotation of cell suspensions in bovine serum albumin (BSA) to obtain prothymocyte-enriched populations was not used since available batches of BSA were contaminated with endotoxin-like products in the *Limulus* assay.

Results

Our studies show that healthy *nu/nu* mice had few or no cells bearing T-cell markers in the absence of activation by an overt or remote administration of endotoxin (20) or by mouse hepatitis virus infection (17). Under these conditions, T cells could be induced in vivo in healthy *nu/nu* mice by an injection of LMS or DTC (Table I). In vivo induction of spleen prothymocyte differentiation by LMS or by DTC gave rise to cells of the TL^- Thy 1^+ phenotype. This is the phenotype of peripheral T cells and of the medullary thymocyte. This fast induction of functional T cells takes place only in the spleen, suggesting prothymocytes are absent in lymph nodes (Table I). The acquisition of a cytotypic T-cell surface marker after a single treatment with LMS or DTC compared favorably with the effects of repeatedly administering preparations from thymus (17, 20). In contrast to the effects of thymopoietin (17, 21), precursor cells from spleen of *nu/nu* thymusless mice incubated with LMS or DTC did not differentiate into T cells (data not tabulated, as similar to controls).

Administration of LMS or DTC evoked in SRC-immunized *nu/nu* mice significant numbers of IgG-PFC which were not present in immunized but untreated control animals (Table II). An early switch from IgM to IgG antibody synthesis is a known effect of activated T lymphocytes (22). Therefore, present data confirm that LMS and DTC are in vivo able both to recruit and to activate the T-cell lineage (5–10). Inducing or immunostimulating in vivo activities of LMS or of DTC are likely associated with their sulphur moiety (8, 23), since DTC is devoid of the imidazole ring.

Nu/nu mice treated with LMS produced a factor that was absent from serum of untreated mice. This factor allowed both in vitro induction of thymocyte differentiation and transfer of immune stimulation. Indeed, when precursor cells from spleen or bone marrow of *nu/nu* mice were incubated in the presence of serum from LMS-treated nude mice, they express thymocyte markers (Table III). The inducing activity of serum from LMS-treated *nu/nu* mice could be compared to the in vitro efficiency of thymus extracts (17, 18, 20, 21) or of thymic serum factors (14, 15). Functional activation was evidenced by a twofold enhancement of the immune response to SRC evoked by administering to C3H/He mice as little as 0.2 ml of serum from *nu/nu* mice treated with LMS (Table IV).

Discussion

Sulphur derivatives, LMS or DTC, administered to *nu/nu* mice, can induce in vivo differentiation of prothymocytes into mature T cells, in number and quality

TABLE I

Proportions of Thy-1$^+$ and TL$^+$ Cells in Spleen or Lymph Nodes of nu/nu Mice 4 Days after a Single Administration of LMS or DTC

Treatment	Percent of T-antigen-bearing cells in:		
	Spleen		Lymph nodes Thy-1$^+$ or TL$^+$
	Thy-1$^+$	TL$^+$	
Saline	3, 4, 6, 6,	<5, <5, 6, 7	<5, <5, <5
LMS 2.5	7, 10, 10, 11, 14	0, 5, 5, 6, 9,	<5, <5, <5
25	19, 28, 38, 43	9, 9, 12, 16	
DTC 2.5	15, 17, 17, 25, 29	3, 5, 7, 7, 8	<5, <5, <5
25	17, 16, 21, 22, 23	4, 5, 5, 7, 9	<5, <5

Cell suspensions, made separately from spleen and from pooled axillary, mesenteric, and inguinal lymph nodes, were adjusted at 5 × 10^6 nucleated viable (trypan blue assay) cells in RPMI 1640 medium (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 1% IgG-free calf serum. The percentage of in vivo induced cells was calculated according to the formula $100 \times (a-b)/a$, where a = percent live cells after incubation with rabbit complement and b = percent live cells after incubation with antiserum and complement. All data are values for individual mice.

TABLE II

In Vivo Helper Effects of Treatment with LMS or DTC on nu/nu Mice Responsiveness to Intravenous Immunization with 10^8 SRC

Treatment (mg/kg drug)	PFC/10^6 spleen cells	
	IgM	IgG
None	0, 0, 0	0, 0, 0
LMS alone, 25	0, 0, 0	0, 0, 0
SRC alone	61, 75, 75	0, 0, 0
SRC + LMS, 2.5	74, 101	88, 116
SRC + LMS, 25	60, 72, 75	245, 190, 215
SRC + DTC, 25	95, 107	35, 63

Acquired functional activity was assayed on day 4 postimmunization and simultaneous s.c. treatment. All data are values for individual mice. Negative *Limulus* assays indicated less than 0.5 ng endotoxin per ml of injected suspensions. Note that, healthy *nu/nu* mice, not contaminated by endotoxin or disease, did not demonstrate "background" PFC.

TABLE III

In Vitro Generation of T Cells from Spleen or Bone Marrow Precursor Cells by the Action of Serum of LMS-Treated nu/nu Mice

Treatment	Percent of T-antigen-bearing cells			
	Spleen		Bone marrow	
	Thy-1$^+$	TL$^+$	Thy-1$^+$	TL$^+$
None	<5, <5	<5, <5	<5, 6	<5, <5
NMS (1:5)	0, 2	0, 0	2	0
LMS-tS				
(1:5)	18, 10	8, 8	17, 18	
(1:10)	10, 12	9, 6	NT	

Induction assays were performed on 5 × 10^6 cells suspended in 1 ml of 1640 medium and incubated for 2.5 h at 37°C in a humidified CO_2 incubator in the presence or absence of serum to be tested which was replaced in controls ("None" treatment) by 1% of IgG-free fetal calf serum. Normal mouse serum (NMS) was sampled on normal healthy 6-wk-old female *nu/nu* mice. LMS-tS was a pool of sera from female *nu/nu* mice collected 24 h after a treatment with 25 mg/kg LMS. Negative *Limulus* assays indicated less than 0.5 ng endotoxin per ml of nude mouse serum. Figures between brackets indicate final concentrations of NMS or of LMS-tS in induction assays. Proportions of Thy-1$^+$ or of TL$^+$ cells were calculated from the cytotoxicity test as in Table I. All data are values for individual mice.

TABLE IV
Serum Transfer of Immune Stimulation from LMS-Treated nu/nu Mice to Untreated Recipient C3H/He Mice

Treatment	PFC/10^6 spleen cells	
	IgM	IgG
None	10 ± 5	0
SRC	264 ± 41	19 ± 2
SRC + NMS	196 ± 37	13 ± 3
SRC + LMS-tS		
0.5 ml	466 ± 53	36 ± 4
0.2 ml	531 ± 65	49 ± 4

Pooled serum (LMS-tS), collected from healthy *nu/nu* mice 24 h after a treatment with 25 mg/kg LMS, was administered i.p. to 6- to 8-wk-old female C3H/He mice, at doses of either 0.2 or 0.5 ml, simultaneously to an i.v. immunization with 10^6 SRC. Normal mouse serum (NMS) was sampled from untreated healthy *nu/nu* mice. *Limulus* assays were negative, indicating less than 0.5 ng endotoxin in 1 ml of these sera. IgM- and IgG-antibody PFC were evaluated as described for Table II. Means of 4 ± SE.

sufficient to restore a T-cell-mediated immune function. However, LMS or DTC are unable to replace thymopoietin (17, 20), ubiquitin (24), Trainin's thymic humoral factor (25), Bach's circulating thymic factor (26), poly A:U (20), or endotoxin (20) for in vitro inducing activity.

LMS or DTC exert their activity through an indirect pathway. They induce a nonthymic cellular system to produce a factor released in the serum. This factor, in turn, induced T-cell differentiation and activation. In current studies, hepatocytes or macrophages are candidates for the release of such a factor.

Our results are compatible with a function of epithelial thymus which could be, in essence, the release of a hormone allowing a still unidentified cell system to synthesize and excrete a factor that supplies the signal for T-cell differentiation. Thymectomy or genetic deficiency of thymus development will suppress the production of the thymic hormone, therefore suppressing excretion of the so-called "thymic-factors." In vivo treatment with sulphur derivatives, LMS or DTC, can substitute in immune events for an alleged thymic hormone.

Summary

Levamisole and sodium diethyldithiocarbamate can induce in vivo thymocyte differentiation from precursor spleen cells of *nu/nu* mice and evoke indirect plaque-forming cells in nude mice immunized with sheep red cells. These sulphur drugs induce in thymusless mice the production of a serum factor which transfer in vivo immune enhancement and in vitro thymocyte differentiation. *In vivo* treatment with sulphur derivative can substitute for an alleged thymice hormone.

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