

Eosinophil-active cytokine from mononuclear cells cultured with L-tryptophan products: An unexpected consequence of endotoxin contamination

Hirohito Kita, MD,^a Arthur N. Mayeno, PhD,^a Cornelia M. Weyand, MD, PhD,^a Jörg J. Goronzy, MD, PhD,^a Deborah A. Weiler, MS,^a Steven K. Lundy, BS,^a John S. Abrams, PhD,^b and Gerald J. Gleich, MD^a
Rochester, Minn., and Palo Alto, Calif.

Background: The eosinophilia-myalgia syndrome, caused by a contaminant or contaminants in epidemiologically implicated L-tryptophan products, is characterized by eosinophilia and eosinophil degranulation. We hypothesized that immune cells are stimulated by implicated L-tryptophan and produce eosinophil-active cytokines.

Objectives: This study was designed to identify substances in L-tryptophan causing the eosinophilia-myalgia syndrome.

Methods: Peripheral blood mononuclear cells were cultured with L-tryptophan products, and supernatants were tested for their ability to enhance eosinophil degranulation and survival in vitro and for their cytokine content. Subsequently, 46 different L-tryptophan lots were analyzed for their in vitro biologic activities.

Results: After peripheral blood mononuclear cells were cultured with implicated L-tryptophan, their supernatants enhanced eosinophil degranulation and survival. These activities were blocked by anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) antibody; immunoreactive GM-CSF was measurable in the supernatants. Monocytes, but not T lymphocytes, were the responding cells. However, no correlation was observed between the in vitro biologic activity and lots of epidemiologically implicated L-tryptophan products. This biologic activity in the L-tryptophan products was characterized as endotoxin.

Conclusion: Although L-tryptophan products stimulate peripheral blood mononuclear cells to produce GM-CSF, this response is caused by endotoxin contamination of the L-tryptophan products and not by a specific L-tryptophan contaminant. Endotoxin contamination must be considered as a possible cause of eosinophil-active cytokine production by peripheral blood mononuclear cells. (*J ALLERGY CLIN IMMUNOL* 1995;95:1261-7.)

Key words: Eosinophilia-myalgia syndrome, eosinophils, cytokine, L-tryptophan, GM-CSF, degranulation, survival, endotoxin

The eosinophilia-myalgia syndrome (EMS), recognized in late 1989 as an illness associated with consumption of L-tryptophan produced by a single

From ^athe Departments of Immunology and Internal Medicine, Mayo Clinic and Mayo Foundation, Rochester, and ^bthe DNAX Research Institute, Palo Alto.

Supported by grants from the National Institutes of Health, AI 31155, AI 15231, AI 09728, AI 07047, and AI 34577, and from Showa Denko KK, and by the Mayo Foundation.

Received for publication Mar. 28, 1994; revised Nov. 28, 1994; accepted for publication Nov. 29, 1994.

Reprint requests: Hirohito Kita, M.D., Department of Immunology, Mayo Clinic and Foundation, Rochester, MN 55905.

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0091-6749/95 \$3.00 + 0 1/1/62520

Abbreviations used

EDN:	Eosinophil-derived neurotoxin
EMS:	Eosinophilia-myalgia syndrome
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IL:	Interleukin
LAL:	<i>Limulus</i> amoebocyte lysate
PBMCs:	Peripheral blood mononuclear cells

manufacturer,^{1,2} is characterized by activated T lymphocytes in blood and affected tissues during the clinically active progressive phase of disease.³

Eosinophil granule proteins were strikingly elevated in patients with EMS, and were deposited in diseased organs, suggesting activation and degranulation of eosinophils.¹ Because T cells and their products may be responsible for eosinophilia and for eosinophil activation,⁴ we hypothesized that immune cells, after stimulation by implicated L-tryptophan, could produce eosinophil active cytokines. Therefore we analyzed supernatants of peripheral blood mononuclear cells (PBMCs) cultured with L-tryptophan products for the presence of eosinophil active cytokines and compared the in vitro biologic activity and the epidemiological case implication of various L-tryptophan products.

METHODS

L-tryptophan products

Initial studies used six different L-tryptophan products: L-tryptophan capsules provided by a patient with EMS (company A, lot 1), samples of L-tryptophan tablets and bulk powder provided by the manufacturers and formulators (company A, lots 2, 3, and 4 and company B), and L-tryptophan purchased from Sigma Chemical Company (St. Louis, Mo.) (company C). EMS cases have been associated with company A L-tryptophan, lots 2 and 4, but not with company A, lot 3, company B, or company C. Subsequently, 46 different lots of L-tryptophan were tested to determine the correlation between biologic activity and case implication. The manufacturer or formulator provided these L-tryptophan products, and Dr. Michael Osterholm, Dr. Craig Hedberg, and Dr. Edward Belongia (Minnesota Department of Health, Minneapolis) provided the case association for L-tryptophan.² L-tryptophan products were dissolved at a concentration of 10 mg/ml in distilled deionized water immediately before use, and the absorbance was measured at 280 nm. To identify the contaminant or contaminants in L-tryptophan products causing activation of PBMCs, solutions of L-tryptophan were treated as follows: ultrafiltration through Centricon-3, -10, and -100 devices (Amicon, Beverly, Mass.), preincubation with polymyxin B (Sigma) for several hours, or treatment with beads coupled to endotoxin neutralizing protein⁵ (Associates of Cape Cod, Woods Hole, Mass.) for 6 hours at 22° C with gentle rotation. Endotoxin in L-tryptophan solutions (1 mg/ml) was also determined with the *Limulus* amoebocyte lysate (LAL) assay kit (Whittaker Bioproducts, Inc. Walkersville, Md.); the stated sensitivity of the assay is 0.125 endotoxin units per milliliter. Peak E, the impurity most strongly associated with EMS by epidemiologic data, was measured by high-performance liquid chromatography, as previously described.²

Preparation of protein-coated Sepharose beads

Purified human serum IgG (Organon Teknica Cappel, Malvern, Pa.) coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Inc., Piscataway, N.J.),

prepared, as described previously,⁶ was the stimulus for eosinophil degranulation. IgG was coupled at a concentration of 5 mg/ml packed beads; the same concentration of ovalbumin (Sigma) was used as the negative control.

Preparation of PBMC supernatants

PBMCs were obtained from heparinized blood of normal volunteers or patients with EMS by centrifugation on Histopaque (Sigma). PBMCs (1×10^6 cells/ml) were suspended in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mmol/L L-glutamine (Sigma), 50 U/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma), and 10% heat-inactivated selected human plasma. One hundred microliters of cell suspension was cultured with 100 µl of L-tryptophan products diluted with RPMI 1640 medium in 96-well round-bottom tissue culture plates (Falcon, Lincoln Park, N.J.) at 37° C and 7.5% CO₂. Cells cultured with medium alone were negative controls; anti-CD3-coated beads that were used as positive controls were made similarly to those described above. PBMCs were incubated for 24 hours, and supernatants were harvested and frozen at -20° C until assayed in eosinophil degranulation and survival assays. In some experiments, purified monocytes and T cells were tested in the same manner. Monocytes and T cells were isolated by plastic adherence and by rosetting with 2-aminoethylisothiuronium bromide-treated sheep red blood cells, respectively.

Eosinophil purification, degranulation assay, and radioimmunoassay for EDN

Eosinophils were purified from peripheral blood of normal volunteers or patients with mild asthma or hay fever as previously described⁶ and were greater than 85% pure and greater than 98% viable. Contaminating cells were neutrophils; no lymphocytes or monocytes were present. Eosinophil degranulation assay and radioimmunoassay for eosinophil-derived neurotoxin (EDN) were performed as described previously.⁶

Eosinophil survival assay

As described previously,⁷ suspensions of purified eosinophils were incubated with PBMC supernatants or controls. In some experiments, PBMC supernatants were pretreated with anti-interleukin (IL)-3, anti-IL-5, or anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) antibody for 1 hour at 4° C.

Assays for cytokines

GM-CSF concentrations in PBMC supernatants were assayed with a kit purchased from Genzyme (Cambridge, Mass.). GM-CSF,⁸ IL-3,⁸ IL-4,⁹ IL-5,⁸ IL-6,¹⁰ and interferon-γ¹¹ were measured in PBMC supernatants by immunoenzymetric assays, as described. IL-2 was assayed with proliferation of the murine IL-2-dependent line HT2.¹²

Statistical analysis

Data, expressed as means ± SEM, were analyzed by paired or unpaired Student's *t* test. Tests of association

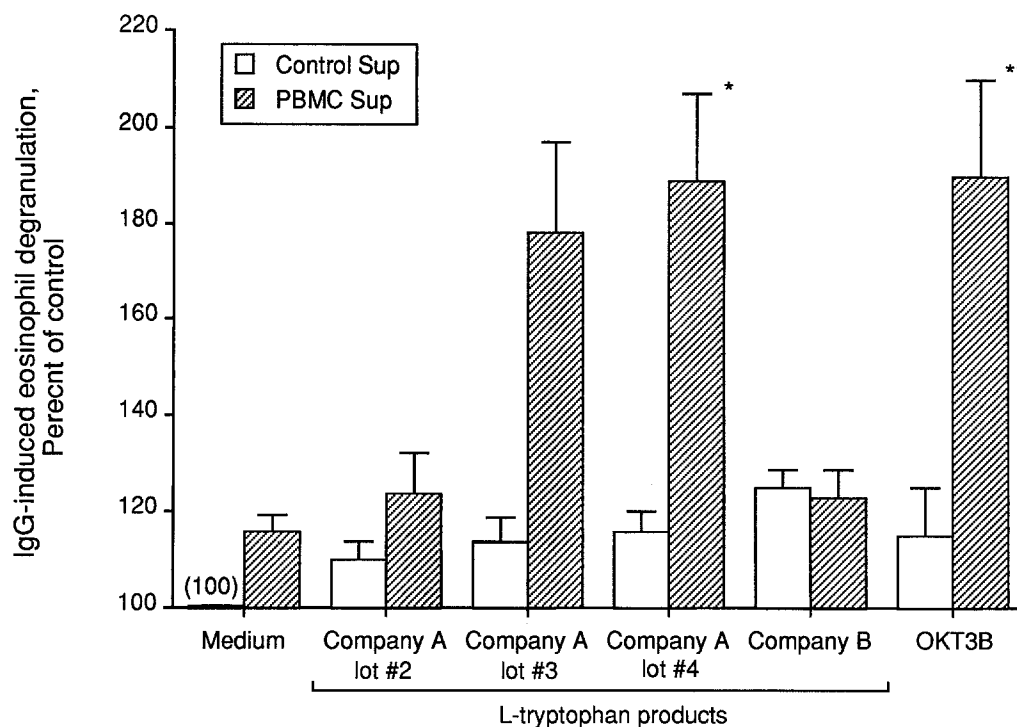


FIG. 1. Effect of supernatants from PBMCs cultured with L-tryptophan products on eosinophil degranulation. PBMC supernatants were prepared by culture of PBMCs from three normal subjects and three patients with EMS with various L-tryptophan products or anti-CD3-coated Sepharose beads (OKT3B) on five separate occasions, as described in the Methods section. Control supernatants were obtained by the same procedure, except for the absence of PBMCs. Eosinophils were preincubated with 25% (vol/vol) PBMCs or control supernatants for 1 hour at 37° C, and IgG- or anti-CD3-coated Sepharose beads were added. After 2 hours at 37° C, supernatants were collected, and the EDN concentrations in the samples were measured by radioimmunoassay as an indicator of eosinophil degranulation. Data represent percent control of IgG-induced eosinophil degranulation (mean $\pm$ SEM) in the presence of PBMC supernatant from six subjects. Eosinophil degranulation assays of duplicate samples were performed in five separate experiments with eosinophils from five different donors. IgG-induced eosinophil degranulation by medium only has been assigned a value of 100%, and the results from the five experiments have been normalized. Eosinophil degranulation in the absence of stimulant (spontaneous EDN release) and IgG-induced degranulation in the absence of test supernatant (basal IgG-induced EDN release) were 19.2 ± 7.6 and 85.4 ± 6.8 ng/10⁶ eosinophils (mean $\pm$ SEM, $n = 5$), respectively. The total eosinophil content of EDN was 2552.8 ± 200.7 ng/10⁶ eosinophils (mean $\pm$ SEM, $n = 5$). Sup, Supernatant. * $p < 0.05$; ** $p < 0.01$

were performed by chi square analyses with Yates' correction.

RESULTS

Analyses of interactions between leukocytes and L-tryptophan: Eosinophil degranulation

We exposed PBMCs from normal individuals and patients with EMS to L-tryptophan, and supernatants from these cultures were incubated with purified eosinophils. One hour later, IgG-coated beads were added to eosinophils to induce degranulation. As a control, supernatants from control cultures containing L-tryptophan, but not PBMCs, were also tested; L-tryptophan itself did

not affect IgG-induced eosinophil degranulation. In contrast, PBMC supernatants cultured with implicated L-tryptophan products from company A (lot 1) enhanced IgG-induced eosinophil degranulation in a concentration-dependent manner. Supernatants from company C L-tryptophan PBMC cultures had essentially no effect on eosinophil degranulation. Additional sources of L-tryptophan (company A, lots 2, 3, and 4 and company B) were incubated with PBMCs, and culture supernatants were tested for their eosinophil degranulation enhancement. Results with PBMCs from normal individuals and patients with EMS were similar (data not shown), and they are

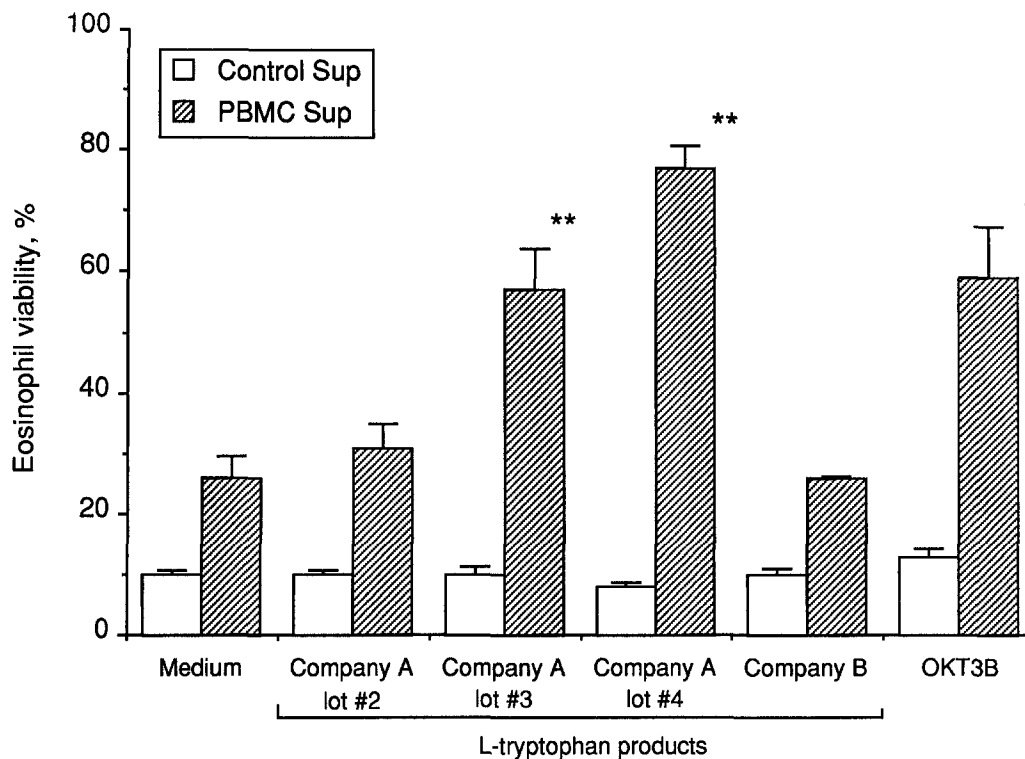


FIG. 2. Effect of supernatants from PBMCs cultured with various L-tryptophan products on eosinophil survival. Supernatants were prepared by culture of PBMCs from eight normal subjects with various L-tryptophan products or anti-CD3-coated Sepharose beads, as described in the Methods section, on three separate occasions. Control supernatants were obtained with the same procedure except for absence of PBMCs. Eosinophils were cultured with 10% (vol/vol) PBMCs or control supernatant for 4 days. Eosinophil viability (mean $\pm$ SEM) was determined as described in the Methods section. Eosinophil survival assays of duplicate samples of supernatants were performed on four separate occasions with eosinophils from four different donors. Sup, Supernatant. * $p < 0.05$; ** $p < 0.01$.

presented together in Fig. 1. Supernatants of control cultures without PBMCs had no effect on IgG-induced eosinophil degranulation, whereas supernatants from PBMCs cultured with L-tryptophan from company A (lot 4) or anti-CD3-coated beads significantly enhanced degranulation ($p < 0.05$ and $p < 0.01$, respectively). In contrast, L-tryptophan from company A, (lot 2) and from company B were inactive.

Eosinophil survival assay of PBMC supernatants

The factors enhancing eosinophil degranulation in the PBMC supernatants were further studied by means of the eosinophil survival assay. Supernatants from PBMCs of eight normal individuals cultured with L-tryptophan were tested in the eosinophil survival assay (Fig. 2). Without PBMCs, the various lots of L-tryptophan from company A and company B and anti-CD3 had no effect on

eosinophil survival. PBMC supernatants stimulated by L-tryptophan from company A (lots 3 and 4) or by anti-CD3 caused a significant increase in eosinophil survival; in contrast, L-tryptophan from company A (lot 2) and from company B had no effect on eosinophil survival. These results are consistent with the eosinophil degranulation assay. PBMCs from two patients with EMS enhanced eosinophil survival with L-tryptophan from company A (lots 3 and 4) and anti-CD3 similar to that seen in Fig. 2 and consistent with the results of the eosinophil degranulation assays.

To determine which cytokine was produced by PBMCs stimulated with L-tryptophan, supernatants were treated with specific monoclonal antibodies to IL-3, IL-5, and GM-CSF.⁷ PBMC supernatants from six normal individuals cultured with L-tryptophan (company A, lots 3 and 4) had their eosinophil survival enhancement completely blocked by anti-GM-CSF antibody, whereas anti-

TABLE I. Cellular requirements of cytokine induction by L-tryptophan products

Cell	Stimulating L-tryptophan products	Eosinophil survival (%)		
		Exp 1	Exp 2	Exp 3
		10%*	5%	5%
PBMCs (1×10^5 /well)	Company A, lot 4	87.9 ± 1.0	66.6 ± 5.5	70.9 ± 3.4
	Company B	5.4 ± 1.7	4.4 ± 1.4	1.3 ± 0.3
T cells (1×10^5 /well)	Company A, lot 4	8.5 ± 1.7	3.8 ± 4.2	0.0 ± 0.0
	Company B	4.8 ± 2.3	1.0 ± 0.6	0.0 ± 0.0
Monocytes (2.5×10^4 /well)	Company A, lot 4	87.9 ± 2.2	39.7 ± 22.8	84.8 ± 6.8
	Company B	12.7 ± 6.2	0.0 ± 0.0	6.9 ± 1.9
T cells + Monocytes	Company A, lot 4	87.6 ± 1.4	52.0 ± 3.2	70.9 ± 12.5
	Company B	31.1 ± 32.1	10.0 ± 3.8	16.6 ± 8.6

Various subpopulations of PBMCs were cultured with L-tryptophan products for 24 hours.

Supernatants were tested in the eosinophil survival assay as described in the Methods section.

*Indicates concentration of supernatant used in eosinophil survival assay.

IL-3 or anti-IL-5 antibody had essentially no effect (results not shown). Similarly, GM-CSF was also identified in the PBMC supernatants from two patients with EMS (results not shown). These results show that PBMCs from both normal individuals and patients with EMS are stimulated in vitro by specific lots of L-tryptophan products to produce GM-CSF.

Immunoassay for cytokines

As determined by immunoassay, PBMC supernatants from five donors stimulated on three occasions with L-tryptophan from company A (lot 4) contained increased GM-CSF concentrations (results not shown). In contrast, medium controls and PBMC supernatants cultured with L-tryptophan from company A (lot 2) or from company B did not contain increased concentrations of GM-CSF. Analyses of PBMC supernatants for other cytokines including interferon- γ , IL-2, IL-3, IL-4, IL-5, and IL-6 showed increased levels of IL-6 from cells incubated with company A (lot 4); the other cytokines were not detected.

Cellular requirements of cytokine production

PBMCs, purified T cells, purified monocytes, and purified T cells reconstituted with monocytes from healthy volunteers were stimulated with L-tryptophan products. According to the results of the eosinophil survival assay (Table I), the monocyte-enriched cell population produced most of the cytokine activity. T cells alone did not produce any appreciable activity. Similar results were obtained with PBMCs from patients with EMS (data not shown).

Identification of the factor in L-tryptophan causing PBMC stimulation

As determined by ultrafiltration, the molecular mass of the PBMC activating factor was greater than 100,000 (data not shown). Activity was only slightly reduced by boiling L-tryptophan (data not shown), indicating that the factor is relatively heat-insensitive. These observations plus the cytokine production by monocytes led us to suspect endotoxin contamination in the L-tryptophan products. The antibiotic polymyxin B binds and inhibits endotoxin.¹³ Therefore we preincubated L-tryptophan with polymyxin B before addition to PBMCs, and then incubated with PBMCs for 24 hours before assay for GM-CSF. Polymyxin B inhibited GM-CSF production by L-tryptophan-stimulated PBMCs in a concentration-dependent manner (results not shown). Treatment of L-tryptophan with endotoxin-neutralizing protein⁵ attached to beads also substantially reduced (by ~80%) GM-CSF production from L-tryptophan-stimulated PBMCs (results not shown). The LAL assay for endotoxin showed that untreated L-tryptophan samples were positive (more than 0.125 endotoxin units/ml), whereas the treated samples were negative (results not shown). Therefore the biologic activity in L-tryptophan products is endotoxin.

Correlation between GM-CSF releasing activity, LAL assay, and case association

Forty-six different samples of L-tryptophan from company A were analyzed for their ability to stimulate GM-CSF production from PBMCs, for endotoxin contamination by the LAL assay, for

TABLE II. Endotoxin activity in L-tryptophan products: Relationships with GM-CSF stimulating activity and case association

Endotoxin activity	GM-CSF stimulation		Case implication	
	+	—	+	—
+	24	1	9	16
—	1	20	7	14

Chi square (with Yates' correction) for association between endotoxin activity and GM-CSF stimulation = 34.7, $p < 0.001$; for association between endotoxin activity and case association = 1.48×10^{-2} , not significant.

peak E by high-performance liquid chromatography, and for case association. As determined by immunoassay, L-tryptophan lots that induced more than 2.0 pg/ml of GM-CSF were considered positive. L-tryptophan lots, consumed by at least one patient with EMS, were considered positive for case association. As shown in Table II, all samples (except one) that caused GM-CSF release were positive in the LAL assay, and all samples (except one) that did not elicit GM-CSF release were negative. However, contamination of L-tryptophan products with endotoxin was not correlated with the case implication of the product (Table II). These data suggest that the factor causing GM-CSF release is identical to that which is producing the positive LAL result, namely endotoxin. Because the presence of peak E in the L-tryptophan products is strongly associated with EMS by epidemiological data,² we examined the associations between peak E content, case implication, and endotoxin activity. Although peak E was associated with case implication (chi square with Yates' correction = 6.04, $p < 0.05$), it was not associated with endotoxin activity (chi square = 2.7×10^{-3} , not significant).

DISCUSSION

Although epidemiologic studies have shown that EMS is caused by a contaminant in L-tryptophan manufactured by a single company² and an animal model for EMS has been reported,^{14, 15} the pathogenesis of the disease is still obscure. We were not able to stimulate eosinophil degranulation by L-tryptophan alone. However, because eosinophilia is regulated by cytokines, such as IL-3, IL-5, and GM-CSF,⁴ and also because certain patients with EMS experienced delayed onset of symptoms after ingesting L-tryptophan,² we hypothesized that L-tryptophan products causing EMS to activate PBMCs to produce factors upregulating eosino-

phil proliferation and function. Supernatants of PBMCs incubated with L-tryptophan increased eosinophil degranulation (Fig. 1), suggesting that one or more eosinophil active cytokines are produced. Furthermore, PBMC supernatants stimulated with L-tryptophan enhanced eosinophil survival (Fig. 2). Neutralization experiments with antibodies identified the factor enhancing survival as GM-CSF. Fractionation of PBMCs indicated that the cells producing the cytokine are mainly monocytes. Furthermore, GM-CSF and IL-6 (but not interferon- γ , IL-2, IL-3, IL-4, or IL-5) could be measured by immunoassay in the supernatants.

Characterization of the L-tryptophan factor responsible for PBMC stimulation revealed that it was of high molecular weight, relatively heat-resistant, and clearly distinguishable from 1,1'-ethylidene-bis-(L-tryptophan), an implicated component in L-tryptophan products.^{2, 16} The factor is endotoxin in that its activity was neutralized by polymyxin B and by endotoxin-neutralizing protein. Further, GM-CSF stimulating activity and endotoxin activity (according to the results of LAL assay) with 46 lots of L-tryptophan showed a significant relationship (Table II). Endotoxin, a ubiquitous substance derived from the cell walls of gram-negative bacteria, is known to elicit a multitude of immunologic responses, including the activation and cytokine production of monocytes or macrophages.¹⁷ Therefore we examined the relationship between endotoxin contamination and epidemiologic implication of various L-tryptophan lots and found no correlation between them (Table II). These observations discourage belief that endotoxin activity plays a key role in pathogenesis of EMS and suggest that the observed in vitro activity reflects contamination of L-tryptophan with endotoxin.

Our results indicate that experiments showing production of eosinophil active cytokines by PBMCs must be carefully evaluated to exclude the possibility of endotoxin contamination.

We thank Dr. Michael Osterholm, Dr. Craig Hedberg, and Dr. Edward Belongia for information on the case association of lots of L-tryptophan. We also thank Ms. Linda Arneson for her skillful secretarial assistance during the preparation of this manuscript.

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