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Proliferative Responses of Central and Peripheral Rat Lymphocytes Elicited by Cord Factor (Trehalose 6,6'-Dimycolate)†

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Cord factor—a mixture of 6,6'-diesters of α,α-D-trehalose with natural mycolic acids—which is purified from mycobacteria and other microorganisms, is known to have adjuvant activity as well as to enhance nonspecific resistance to infections and tumor development. In this work, trehalose 6,6'-dimycolate (TDM) was found to induce proliferative responses in rat thymus and lymph node cells. With the thymus cells, TDM responses were greater after removal of the adherent cell subpopulation. Consistent with this observation was the finding that addition of phagocytic cells purified from peritoneal or lymph node cell suspensions to nonadherent thymocytes abrogated the response of thymocytes to TDM. With the lymph node cells, the presence or removal of adherent cells had no major consequence on the TDM-induced proliferative response, since similar increases in deoxyribonucleic acid synthesis were observed with unfractionated and nonadherent cells. The difference between the sensitivities of thymus cells and lymph node cells to regulation by adherent cells indicated the existence of more than one type of TDM responder cell in rats. TDM also displayed marked stimulatory activity on thymus and lymph node cells from germ-free rats, ruling out the possibility that TDM might have triggered a specific, secondary, in vitro immune response. Expansion of a selected cell population(s) triggered by TDM may be involved in the manifestation of adjuvant activity and possibly other immunological properties of cord factor.

Cord factor (trehalose 6,6'-dimycolate [TDM]) represents glycolipid components of mycobacteria, corynebacteria, and several other microorganisms (1, 4, 5). These glycolipids are 6,6'-diesters of α,α-D-trehalose with mycolic acids. The natural product is often a mixture of diesters and mycolic acids of various molecular sizes (C_{60} to C_{90}). Like the bacteria from which it is obtained, TDM has been shown to possess a number of striking biological properties. Cord factor has been reported to potentiate immune responses in vivo (14), to increase susceptibility to the toxic effects of bacterial lipopolysaccharides (16), to induce chemotaxis (13), and to enhance nonspecific resistance not only to bacterial (2, 18) and parasitic (A. A. F. Mahmoud, R. H. D. Civil, E. Lederer, and L. Chedid, Clin. Res., p. 380, 1977) infections but also to tumor development (3, 19). The chemical and biological characteristics of TDM have received particular attention, and current knowledge on this subject has been condensed in recent review articles (9-11). However, the underlying mechanisms of the biological activities of cord factor remain to be defined. In the present work, we explored the possibility that the immunostimulatory and perhaps other immunological activities of TDM may be due to direct interaction with cell surfaces which leads to lymphoproliferative responses. Rats were selected for this study because they do not have toxic reactions to TDM (15).

MATERIALS AND METHODS

Animals. Inbred Lewis rats used in this work were bred at our animal facilities. Germ-free Fisher rats were purchased from Harlan Industries (Indianapolis, Ind.). Animals were 10 to 15 weeks old when sacrificed to remove their lymphoid organs.

TDM. The cord factor used in this study was trehalose-6,6'-dimycolate obtained from *Mycobacterium tuberculosis* strain Peurois and was a generous gift of E. Lederer from the Laboratoire de Biochimie, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. Dispersions of TDM in RPMI-1640 medium (Flow Laboratories, Rockville, Md.) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) were prepared by mixing 1 mg of TDM in powder

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form and 4 ml of medium with the aid of an ultrasonic cleaner (Bransonic 12; Branson Co., Shelton, Conn.). The tube containing the suspension was exposed to ultrasonic vibration in water for 5 min, transferred to a water bath, and removed when the water in the bath started to boil. Appropriate dilutions of TDM were prepared in RPMI-1640 medium (Flow Laboratories) supplemented with antibiotics and 2% heat-inactivated fetal calf serum (Microbiological Associates, Bethesda, Md.). (This medium will be referred to hereafter as RPMI-S.) Amounts of TDM added to individual cell cultures were contained in 0.025 ml of RMPI-S and are described below. Microscopically dispersed TDM appeared as 0.7- to 6-μm particles. The average particle size varied among batches of TDM (2.5 $\pm$ 1.5 μm).

Lymphoid cell suspensions. An aseptic operation was maintained throughout. The thymus and lymph nodes (cervical and mesenteric) of rats were removed and collected in cold RPMI-1640 medium. Single-cell suspensions were prepared with the same medium by using a Ten Broeck tissue grinder (two strokes only) refrigerated in an ice bath. Debris was removed by filtration through nylon gauze, and the cells were washed three times by centrifugation with cold RPMI-1640 medium. The last suspension was made in RPMI-S. Cells were counted microscopically with a Neubauer hemacytometer, and the final suspension was adjusted to 10^7 viable, trypan-blue-excluding cells per ml.

Removal of adherent cells. Cell suspensions were allowed to sediment in plastic petri dishes (Falcon Plastics, Oxnard, Calif.) while being incubated for 45 min. at 37°C in a 5% CO_2-in-air atmosphere saturated with water vapor. This procedure was repeated twice more with the nonadherent cells recovered in each preceding step. The contents of phagocytic cells, as measured by the latex particle ingestion test (F. Kierszenbaum, submitted for publication), were reduced by this treatment from 0.8 to 2% to undetectable levels in the thymocyte preparations and from 7 to 9% to 0.02 to 0.1% in lymph node cell suspensions.

Purification of adherent peritoneal and lymph node cells. Peritoneal cells from rats were removed by several lavages with RPMI-1640 medium, washed three times with the same medium, and incubated under the conditions described above. Nonadherent cells were removed by vigorous flushing with the medium, and adherent cells were scraped off of the plastic surface with a sterile rubber policeman. Adherent lymph node cells were purified by the same method. After being washed once by centrifugation, the cells were counted and adjusted to the appropriate concentration. Suspensions prepared in this manner contained 90 to 98% latex particle-ingesting cells.

Determination of cellular deoxyribonucleic acid synthesis. Cell cultures were set up in plastic plates with flat-bottom microculture wells (Linbro, Hamden, Conn.) which contained RPMI-S. Wells contained 2.5×10^5 viable cells and the appropriate amount of TDM in a total volume of 0.1 ml. Control cultures contained cell suspension only. The plates were incubated at 37°C for 48 h in a 5% CO_2 incubator saturated with water vapor. At that time, each well received 1 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.; specific activity, 2 Ci/mmol) in 0.025 ml of RPMI-S, and the plates were incubated for an additional 24 h. Cultures were processed in an automated culture harvester (MASH-II; Microbiological Associates), and measurements of the incorporated radiolabel were made with a liquid scintillation spectrometer. The results presented in this paper represent the average of triplicate determinations.

RESULTS

Lymphoproliferative responses induced by TDM. Although addition of TDM to rat thymocyte cultures usually resulted in a significant ($P < 0.05$) increase in deoxyribonucleic acid synthesis over that of the control, unstimulated cultures, this phenomenon was not observed in two of seven experiments. Furthermore, the extent of the stimulatory phenomenon, when it occurred, varied among some individual experiments despite the lack of modification of the protocol. This apparent inconsistency suggested the existence of a regulatory cell and prompted the execution of additonal experiments in which TDM responses by unfractionated thymocytes were compared with those produced by partially purified cell populations. The first attempts in this direction included the use of nonadherent thymus cells. Removal of adherent cells resulted in a marked increase in cell responsiveness to TDM (Fig. 1). A similar phenomenon was observed when the nonadherent subpopulation of previously unresponsive thymus cells was used (Fig. 2). Restoration of the cultures with purified adherent peritoneal cells abrogated the response of nonadherent cells to TDM. Although Fig. 1 shows the inhibitory effect of reincorporating adherent cells to a level of 5%, similar results were obtained in separate experiments by restoring cultures with as little as 1% adherent peritoneal cells or with 5% adherent lymph node cells (data not shown). Of interest was the finding that addition of 0.1% adherent peritoneal cells was not sufficient to reduce the response of nonadherent thymocytes to TDM (data not shown). With lymph node cells, significant stimulation was afforded by TDM whether or not adherent cells were removed (Fig. 3). Whereas a relatively larger concentration of TDM was necessary in some experiments to produce optimal responses with nonadherent lymph node cells (Fig. 3), this was not the case in other experiments in which either the shift was in the opposite direction or there was no shift at all. Although the shifting of optimal dose-response curves did not represent a systematic trend, one such case is illustrated in Fig. 3 to highlight a somewhat frequent occurrence for which we cannot provide an explanation at the present time.

The magnitude of the significant stimulation

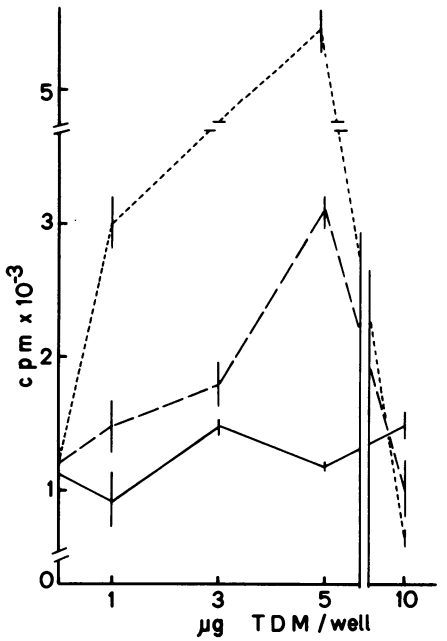

FIG. 1. *Response of unfractionated and nonadherent rat thymocytes to stimulation with TDM.* (— —) *Unfractionated thymocytes;* (- - -) *nonadherent thymocytes;* (—) *mixture containing 95% nonadherent thymocytes and 5% adherent peritoneal cells (95% of which were latex particle-ingesting cells). Points are averages of triplicate determinations, and vertical bars represent the standard errors of the means. This figure depicts results of an experiment in which a significant response to TDM was observed before removal of adherent cells. Results are representative of five experiments of identical designs.*

induced by TDM was in contrast to the normally larger lymphoproliferative responses usually produced with polyclonal activators such as phytohemagglutinin or concanavalin A. For this reason, we considered the possibility that TDM could trigger a specific response by lymphocytes primed by an ubiquitous antigen(s) conceivably present in microorganisms causing inapparent infections in the rat. Lymphoid cells from germfree rats were used to probe this possibility. Significant responses were induced by TDM when either thymocytes (Fig. 4) or lymph node cells (Fig. 5) from these animals were tested.

DISCUSSION

These results demonstrate the nonspecific stimulatory activity of cord factor on rat lymphocytes. Removal of adherent cells enhanced the stimulatory phenomenon when thymocytes were used and often permitted detection of responses that had not attained significant levels before removal. The possibility that the adher-

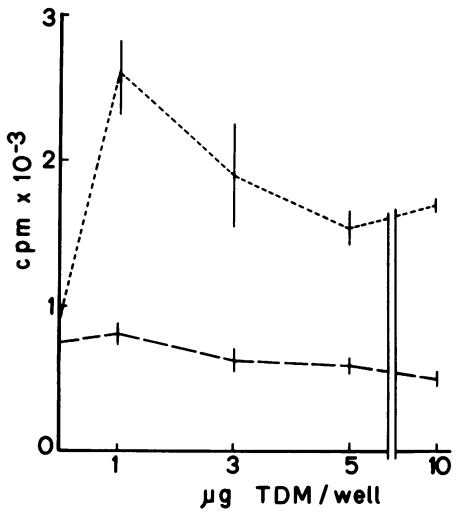

FIG. 2. *Response of nonadherent thymocytes to stimulation with TDM. Symbols are as described in the legend to Fig. 1. This figure depicts results of an experiment in which unfractionated thymus cells were not responsive to TDM before removal of adherent cells. Results are representative of two experiments of identical designs.*

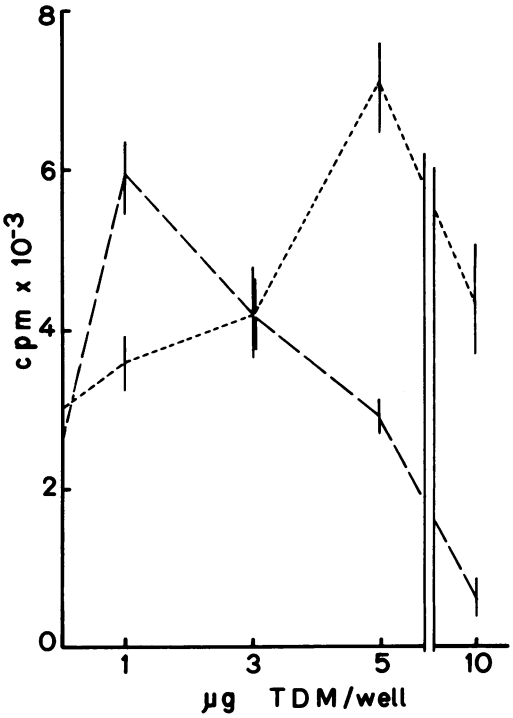

FIG. 3. *Response of adherent and nonadherent rat lymph node cells to stimulation with TDM.* (— —) *Unfractionated cells;* (- - -) *nonadherent cells. Vertical bars represent the standard errors of the means. Results are representative of three experiments of identical design.*

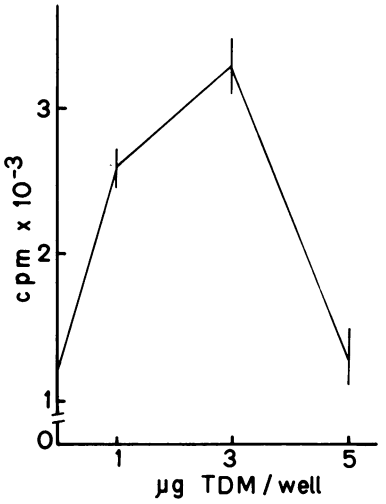

FIG. 4. *Response of germ-free rat thymocytes to stimulation with TDM. Vertical bars represent the standard errors of the means. Results are representative of two experiments of identical designs.*

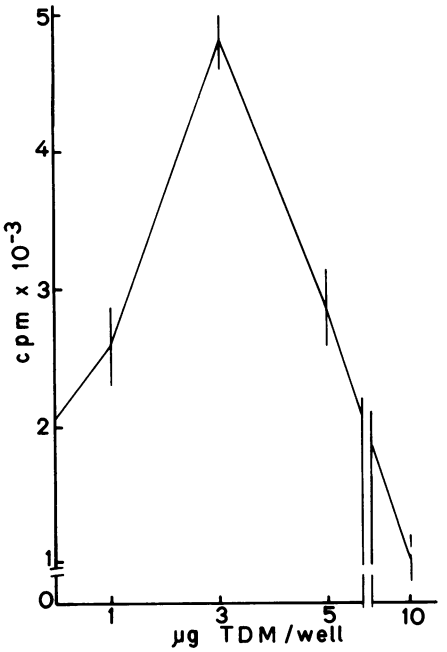

FIG. 5. *Response of germ-free rat lymph node cells to stimulation with TDM. Vertical bars represent the standard errors of the means. Results are representative of two experiments of identical designs.*

ent cells which exerted negative regulatory influence over TDM responses were macrophages was supported by the observation that adherent peritoneal and lymph node cells, 90 to 98% of which were phagocytic, were capable of suppressing TDM-induced proliferation when nonadherent thymocytes were restored to levels of 1% or greater (i.e., to concentrations comparable to the normal phagocytic cell contents of thymus cell suspensions). Thus, it is conceivable that lack of response to TDM stimulation by some of the unfractionated thymocytes may have been due to the presence of a relatively higher concentration of macrophages in the cell suspensions. Supportive of this possibility was the observation that restoration with only 0.1% macrophages failed to reduce the response of nonadherent thymocytes to TDM.

Although removal of adherent cells sometimes affected optimal dose-response relationships between TDM and lymph node cells, no systematic trend was noted when the results of identically designed experiments were compared. We have no precise explanation for the occasionally observed shifts in the optimal TDM dose, which might have been due to variations in the size of TDM particles among different preparations. Regardless of these shifts and whatever their directions, the fact remains that significant increases in deoxyribonucleic acid synthesis were induced after considerable reduction in the concentration of phagocytes.

Several lines of evidence ruled out the possibility that TDM responses could have been of a specific nature and generated by previously sensitized cells. First, significant proliferative responses were readily produced by thymus and lymph node cells from germ-free rats. Secondly, unlike antigen-induced responses, significant TDM stimulation occurred in the presence of minimal numbers of macrophages (<0.02%). An additional argument is that supraoptimal zone effects such as those produced with the higher test concentrations of TDM are atypical of antigen-stimulated lymphocyte responses and more in keeping with mitogen-induced responses. However, the two- to fivefold increases in deoxyribonucleic acid synthesis that were observed with thymus and lymph node cells suggest that the nonspecific stimulatory action of TDM is exerted on a limited number of cells, possibly representing definite cell subpopulations in the thymus and lymph nodes. Interaction of TDM with cell membranes has been established (6, 7), and it is conceivable that the proliferative responses described in this paper may have been triggered by initial binding of TDM to appropriate surface receptors.

A considerable body of evidence has accumulated in recent years for the existence in the rat spleen of macrophage-like cells with suppressive activity detectable in a variety of immunological reactions (12). The present results document the presence in the rat thymus of a cell subpopula-

tion of similar characteristics capable of modulating TDM-induced lymphocyte responses. It is interesting that Tenu et al. (17) recently reported that TDM can induce production of a mouse thymocyte mitogenic protein by mouse peritoneal macrophages. Although rat macrophages inhibited thymocyte responses to TDM in our experiments, it is not known whether this regulatory activity was actually induced by TDM. The different species from which macrophages were derived in this study and that of Tenu et al. add complexity to the comparison. It is noteworthy that marked differences in TDM toxicity for mice and rats have been reported (15), rats being considerably less or not sensitive.

These results also suggest that some of the important characteristics of cord factor, which include enhanced immunological responsiveness and the ability to induce nonspecific resistance to infection (2, 12, 18) and tumor development (3, 8), could be mediated by a cell subset(s) expanded by TDM stimulation. In this context, identification and characterization of the cells which respond to cord factor should contribute to the better understanding of the mechanisms whereby TDM-induced immunopotentiation is produced. Such studies are currently under way in our laboratory.

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