Brief Definitive Report

ADHERENT CELL FUNCTION IN MURINE T-LYMPHOCYTE ANTIGEN RECOGNITION

IV. Enhancement of Murine T-Cell Antigen Recognition by Human Leukocytic Pyrogen

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Soluble products of cultured adherent cells of mononuclear phagocyte-macrophage (Mϕ) lineage have been shown to be involved in the in vitro activation of thymus-derived (T) lymphocytes by mitogen (1–5) and more recently, by antigen (6, 7). Although the function of such products is undefined in vivo, it is tempting to speculate that such factors play a significant role in the viability, differentiation, and immunologic reactivity of the lymphoid organ of the intact animal.

Leukocytic pyrogen (LP), a soluble product of bone marrow-derived phagocytic cells has been shown to have a precise in vivo activity, namely, the mediation of the febrile response (8). In addition, recent advances have allowed for the ready acquisition of highly purified human LP derived from peripheral blood monocytes (9–11). In this report, by utilizing a Mϕ-dependent, antigen-specific murine T-cell proliferation assay, we have examined the ability of human LP to partially replace intact murine peritoneal exudate cells (PEC) as well as soluble products of murine PEC in the T-cell proliferative response to antigen.

Materials and Methods

Animals. B10.A/SgSn J (B10.A), and C57BL/6 (B6) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Where possible, only female mice between 8 and 24 wk of age were used.

Antigens. The dinitrophenyl (DNP) conjugate of egg albumin (OVA) was prepared as described elsewhere (12). The conjugate contained 13 moles of DNP per mole of protein ($DNP_{13}OVA$). Crude keyhole limpet hemocyanin, (KLH, Bio Marine Industries, Inc., Malvern, Pa.) was purified by repeated ultracentrifugation and used in the associated form.

Immunization. Mice were immunized by injections of 0.05 ml per hind foot pad of an emulsion of antigens and complete Freund's adjuvant (CFA) containing 1 mg/ml of killed *Mycobacterium tuberculosis* (H37Ra, Difco Laboratories, Detroit, Mich.); 10 μg of $DNP_{13}OVA$ or of KLH were present in 0.1 ml of emulsion. Boost immunization of equivalent amounts of antigen in CFA was performed 2–3 wk after priming.

Cell-Culture Technique. The method for assaying Mϕ-dependent, antigen-specific T-cell proliferation has been described elsewhere (6, 7). Briefly, naive mice are primed with antigen in CFA in the hind foot pads and boosted with equivalent antigen in CFA 14–21 d later. 1–4 wk after that, the popliteal and femoral lymph nodes are removed, teased, and pressed through a No. 200 wire-mesh screen and incubated on a nylon-wool adherence column for 60 min. On elution, a T-cell enriched, adherent cell-depleted population of immune lymph node lymphocytes (LNL) is obtained. This population contains 90–95% T cells, 1–5% B cells, and 0.1–0.5%

Mϕ. The immune LNL are then incubated with or without antigen, or reconstituting PEC (sterile, mineral oil-induced, mitomycin C-treated cells from nonimmune donors) as a source of Mϕ. In some experiments, other accessory additions to culture than PEC are used. Cultures are carried out in flat-bottom microtiter plates with 2×10^5 LNL and 5×10^4 PEC per well in a vol of 200 μl of RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with fresh L-glutamine (0.3 mg/ml), gentamicin (10 μg/ml), penicillin (200 U/ml), 2-mercaptoethanol (2.5×10^{-5} M), 10 mM Hepes buffer, and 7.5% fetal calf serum (FCS). The cultures were carried out at 37°C in humidified atmosphere of 5% CO_2 and 95% air for 96 h. 16–24 h before harvesting, 1 μCi of tritiated thymidine ([^{3}H]TdR, 6.7 Ci/mM New England Nuclear, Boston, Mass.) was added per well. [^{3}H]TdR incorporation was measured after harvesting, by liquid scintillation spectrometry and the results reported as mean counts per minute ± SEM for triplicate determination or as the number of counts above control (Δcpm); e.g., experimental (aggregated) − control (no aggregation). All washing procedures were done in Hanks' balanced salt solution (Grand Island Biological Co.).

Preparation of PEC Supernates. Murine PEC were cultured at a concentration of $1.0–2.0 \times 10^6$/ml in supplemented RPMI-1640. After 24-h incubation at 37°C, supernates were collected, centrifuged at 1,000 *g* for 20 min, and filtered through a 45-μm Millipore filter (Millipore Corp., Bedford, Mass.) to remove any contaminating cells. These PEC supernates were then used as an accessory addition to murine-LNL culture.

Preparation of Human Adherent Cells (Mϕ). The method for preparing human Mϕ has been described elsewhere (13). Briefly, venous blood was obtained from healthy adults taking no medications at the time of study. The mononuclear cell population was obtained from heparinized whole blood by centrifugation on Hypaque-(Winthrop Laboratories, New York) Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) cushions. Resultant peripheral blood mononuclear cells (PBM) consisted of 70–85% lymphocytes and 15–30% monocytes. PBM were then cultured on sterile glass Petri dishes in complete medium for 60 min at 37°C. After this incubation, the nonadherent cells were discarded. The cells adherent to glass were then reincubated in complete medium for 30 min at 37°C after which they were vigorously washed. The adherent cells remaining after these washings were then bathed in chilled phosphate-buffered saline (PBS), chilled to 4°C, and dislodged from the Petri dishes with a sterile rubber policeman. After washing, the cells were suspended in medium, exposed to 40 μg/ml mitomycin C for 60 min at 37°C and washed four times. More than 90% of the resulting cell population was judged to be monocytes and macrophages by morphologic or phagocytic criteria. Trypan-blue viability was >95%. These human Mϕ were then added as accessory cells to cultures of murine LNL or they were placed in culture for 24 h and supernates prepared in a manner analogous to the method of preparation of supernates from murine PEC.

Preparation of Human LP. Crude supernates were prepared from mononuclear cells as described previously (8). Crude supernates containing LP were concentrated in autoclaved dialysis tubing in front of a high-speed fan. Volumes were reduced 1/20 and dialyzed against PBS with 0.02% sodium azide. Concentrated LP was passed over an immunoadsorbent rabbit anti-human LP antibody attached to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.). LP was eluted from this material in citric-acid buffer, pH 3.2 (9), neutralized with 0.1 M NaOH and stored in 0.02% sodium azide. LP eluted from the anti-LP immunoadsorbent was concentrated and chromatographed over Sephadex G-50 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc. [fine]; 165 × 5.6 cm) at 4°C in PBS with 0.02% sodium azide. The 15,000-mol wt LP peak was isolated, dialyzed against H_2O and lyophilized: this material gave a single staining band on 7.5% sodium dodecyl sulfate polyacrylamide gels (11). This material was used as the source of human LP described in the experiments in Table I. About 20 ng of purified pyrogen protein were added to each well containing adherent cell-depleted LNL and/or antigen.

Further purification of human LP was carried out using ^{125}I-labeled LP (10, 11). Approximately 25% of the LP eluting from both the immunoadsorbent and gel filtration was labeled with ^{125}I (11). After this, LP was chromatographed over Sephadex G-15 (40 × 1.5 cm) in PBS. Further purification of the LP isolated from Sephadex G-15 was carried out using ion-exchange chromatography on DEAE-cellulose in 0.02 M Tris, pH 8.1, at 4°C. Labeled LP eluted during a salt gradient at 3.5 milliohm and when this peak was focused on 4.5% polyacrylamide gels in

TABLE I

*Proliferative Response of Immune B10.A LNL Reconstituted by Various Accessory Additions to Culture**

Accessory addition to culture	[^{3}H]TdR incorporation‡	
	200 μg/ml $DNP_{13}OVA$	Baseline§
	Δcpm × 10^{-3}	
None	0.79 ± 0.29	0.87 ± 0.62
B10.A PEC	20.88 ± 3.00	1.63 ± 1.35
B6 PEC	17.31 ± 4.01	5.48 ± 1.83
B10.A PEC supernate	10.04 ± 3.26	1.36 ± 1.06
B6 PEC supernate	9.20 ± 3.16	1.03 ± 0.81
Human Mϕ	9.58 ± 2.31	1.07 ± 0.35
Human Mϕ supernate	8.79 ± 1.36	1.89 ± 0.29
Human lymphocyte supernate‖	0.13 ± 0.06	1.22 ± 0.11
Cycloheximide-treated human Mϕ supernate‖	0.48 ± 0.21	1.51 ± 0.23
HSA (0.1%)‖	0.37 ± 0.20	2.05 ± 0.44
LP¶	7.06 ± 1.14	1.66 ± 1.30
LP**	8.10 ± 0.23	0.16 ± 0.02

* 2 × 10^5 LNL, accessory addition to culture, with or without antigen cultured per well for 4 d in flat-bottom microtiter plates.

‡ Δcpm: experimental (aggregated) − baseline (no aggregation). Data is expressed as mean Δcpm × 10^{-3} ± SE for six experiments.

§ Baseline represents incorporation of [^{3}H]TdR in mixtures of LNL with various accessory additions to culture, without antigen. Expressed as mean cpm × 10^{-3} ± SE for six experiments or for two experiments.

‖ Indicates Δcpm × 10^{-3} ± SE for two experiments.

¶ Human LP purified by immunoabsorption and gel filtration.

** Results of a single representative experiment with human LP further purified by ion-exchange chromatography and isoelectric focusing. Data are expressed as mean Δcpm × 10^{-3} of replicate samples.

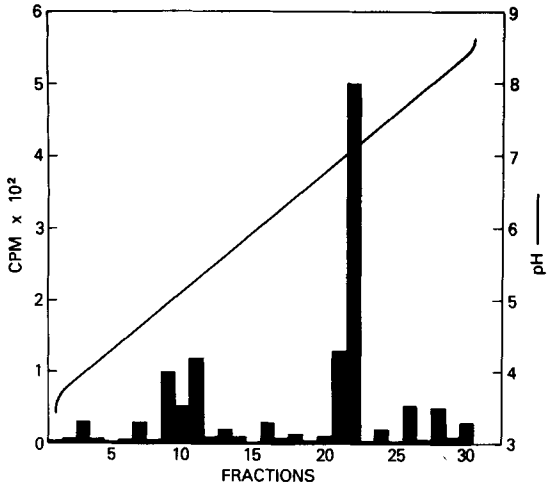

FIG. 1. Isoelectric focusing of ^{125}I-labeled human LP. 1% Ampholine (pH 3–10) and 4.5% acrylamide. Gel lengths were 2.5 × 10 mm. Constant voltage, 200 V, was applied for 18 h at 4°C. Gels were cut every 2 mm and radioactivity and pH were measured on each fraction.

1% Ampholine (LKB Instruments, Inc., Rockville, Md., pH 3–10), a homogeneous peak of radioactivity occurred at pH 7.0–7.1 (Fig. 1).

A small quantity of this radiolabeled LP (≅1,000 cpm) was added to unlabeled LP isolated from Sephadex G-50. This material was chromatographed over Sephadex G-15 followed by DEAE ion exchange and in each case, the radioactivity peak was used to indicate unlabeled LP. Because biologic activity decreased significantly during the ion exchange step, 0.1% salt-

poor human serum albumin (HSA) was added to each collection tube to prevent nonspecific adsorption. Recovery of biologic activity was between 1 and 2% of the starting concentrated crude LP.

Results

Antigen-induced Activation of Immune B10.A T Cells by Added Adherent Accessory Cells or Soluble Products of Adherent Cells. In previous reports (6, 7) an adherent cell-dependent, antigen-specific murine T-cell proliferation assay was employed to delineate at least two functions for Mϕ in the antigen-recognition process. One function may be discerned in the T-cell proliferative response to multideterminant antigens (DNP_{13}-OVA, KLH) where the response is dependent on the production of a soluble factor which is neither antigen specific nor H-2 restricted; therefore allogeneic Mϕs may reconstitute the response and supernates of cultured adherent cells may partially replace the response. A second activity is seen when the proliferation of immune T cells to the terpolymers GLT^{15} or $GL\Phi^{9}$ are examined. This response is reconstituted only with Mϕ which have identity or partial identity at H-2 with the primed T cells, suggesting a specific genetically restricted antigen-presentation function for macrophages. This latter Mϕ function has been discussed elsewhere and will not be discussed any further in this communication (7, 14).

The data in Table I examine the proliferative response of immune B10.A T cells to multideterminant antigens ($DNP_{13}OVA$, KLH) and the effect of various accessory additions to culture. As can be seen, syngeneic and allogeneic PEC reconstitute the response of the T cells to protein antigens. Additionally, murine PEC supernates partially restore the response. Quite surprisingly, intact human Mϕ as well as human Mϕ supernates, also reconstitute antigen responsiveness. However, human lymphocyte supernates, cycloheximide-treated human Mϕ supernates and HSA will not support proliferation. Human LP, purified by immunoabsorption and gel filtration clearly enhanced the proliferative capacity of macrophage-depleted B10.A LNL. Finally, in a representative experiment, LP further purified by ion-exchange chromatography and isoelectric focusing was also found to be active. In data not shown, $DNO_{13}OVA$ and human LP together will not stimulate T cells primed to another antigen.

Discussion

Soluble products of cultured mononuclear phagocytes of both mice and humans have been shown to be involved in the activation of T cells (1–5, 15, 16). In this report, we have found that partially purified human LP, an adherent cell product that has been biochemically analyzed (8–11), will enhance the Mϕ-dependent, antigen-specific activation and clonal expansion of immune T cells. Extensive purification of the human LP did not remove the T-cell stimulatory activity. Additionally, both intact human Mϕ and supernates derived from human Mϕ-enhanced murine T-cell antigen recognition. It is reasonable to assume that human Mϕ and murine PEC supernates contain human lymphocyte-activating factor (LAF) or a family of LAF-like molecules which are critical for the amplification of antigen-induced T-cell activation. Furthermore, human LP seems to be functionally identical or related to these molecules.

While a simplistic comparison of results obtained from different laboratories working on different products is not warranted, certainly conclusions may be drawn

from the numerous observations made concerning the properties of murine LAF or mitogenic factor (1–5, 17–19), human LAF (15, 16, 18, 20), or human LP (8–11, 21) which suggest biochemical or functional similarities among these molecules. The obvious pitfalls of such comparisons include differences in assay techniques, evidence that both the charge and molecular weight of some of these three products may be heterogeneous, and the possibility that there may exist cross-reactive biological function between three distinct yet similar molecules. Nonetheless, murine LAF, human LAF, and human LP are all produced by activated Mϕ monocytes, where activation implies either a phagocytic stimulus, LPS, or an interaction with activated lymphocytes or their products. The macrophage tumor cell line P388D1, has been shown to spontaneously produce LAF (4, 5) as well as pyrogen, as noted in the study of Bodel (22). Murine LAF, human LAF, and human LP all have similar if not identical molecular weights (14,000–16,000) and have, as well, similar cycloheximide sensitivity. Additionally, human LAF and human LP have essentially identical isoelectric focusing points (6.8–7.0). A recent report (19) has demonstrated a murine LAF with an isoelectric focusing point of 4.8, however, the mol wt of their LAF was in the range of 18,000–19,000.

The findings in our studies that soluble products of adherent cells may augment antigen-specific T-cell reactivity and that such LAF-like functions may be substituted for by highly purified human LP. On the basis of biochemical similarities as well as by functional characteristics it is tempting to speculate that some, if not all, LAF-like activity is due to Leukocytic Pyrogen.

Summary

A macrophage-dependent, antigen-specific murine T-cell proliferation assay was utilized to examine the role of soluble products of murine and human adherent cells in the activation of T lymphocytes. Highly purified human leukocytic pyrogen, and supernates from both murine and human mononuclear phagocytes-macrophages stimulated the immune T-cell proliferative response to the multideterminant antigens dinitrophenyl-ovalbumin and keyhole limpet hemocyanin. The implications of these studies and the relationship of leukocytic pyrogen to human lymphocyte-activating factor are discussed.

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