Brief Definitive Report

TWO PEAKS OF INTERLEUKIN 1 EXPRESSION IN HUMAN LEUKOCYTES CULTURED WITH TOBACCO GLYCOPROTEIN

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IL-1 is a polypeptide hormone produced primarily by activated monocytes (1, 2). Two forms of IL-1, IL-1α and IL-1β, have been identified, cloned, and shown to have similar biological properties (2). IL-1 is an immunoregulator and a mediator of inflammation (1, 2). Among its notable inflammatory properties are the ability to stimulate fibroblast (3) and smooth muscle cell (4) proliferation, to activate the production of both collagenase and collagen by fibroblasts and synovial cells (1, 2), and to induce the production of other mediators of inflammation such as IL-6, platelet activating factor (PAF) (2), and platelet-derived growth factor (PDGF) (5). Thus, IL-1 plays a role in mesenchimal tissue remodeling by affecting both the destructive and the reparative processes of connective tissue.

Tobacco glycoprotein (TGP) is a glycoprotein derived from tobacco, which is rich in polyphenols (6). TGP elicits an immediate IgE-mediated wheal and flare response in one-third of human volunteers (6), and induces a preferential IgE response in experimental animals (7). TGP stimulates proliferation of human T cells and differentiation of human B cells into Ig-secreting cells (8). It also activates factor XII-dependent pathways, leading to kinin generation (9). The multifaceted effects of TGP suggested the possibility that it might exert some of its effects by inducing IL-1 production. Here we report that human leukocytes cultured with TGP produce both IL-1α and IL-1β, and that two peaks of synthesis are observed. Thus, IL-1 production by human leukocytes exposed to TGP might be one of the cellular mechanisms underlying the pathological sequelae of cigarette smoking.

Materials and Methods

Subjects. This study was approved by The New York Hospital-Cornell University Medical Center Committee on Human Rights in Research. PBL were isolated by Ficoll-Hypaque density centrifugation of heparinized blood (8) from healthy volunteers and from leukocyte concentrations obtained from The Greater New York Blood Center.

TGP. TGP was isolated from cured tobacco leaves as previously described (6, 10).

Induction of IL-1 Production. PBL were cultured at 2.5×10^6 cells/ml medium with or without TGP as previously described (8). Supernatants were collected at various times and kept frozen. To obtain supernatants from adherent cells, PBL at 2.5×10^6 cells/well (24-well

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cluster) were pre-incubated for 1–2 h at 37°C in medium containing 2.5% FCS. The nonadherent cells were aspirated, and the adherent cells were cultured with 0.5 ml medium with or without TGP.

IL-1 Assay. The thymocyte costimulation assay was used (11). The results are presented as either mean cpm of triplicate wells or as units, calculated by probit anlaysis using monocyte-derived IL-1 as a standard. (100 U/ml) (Genzyme, Boston, MA) (12).

rhIL-1 and Antibodies to IL-1. rhIL-1α and antibodies to rhIL-1α were gifts of Drs. Peter T. Lomedico and Richard Chizzonite, respectively (Hoffman-La Roche, Inc., Nutley, NJ). rhIL-1β and rabbit polyclonal antibody to rhIL-1β were gifts from Dr. Philip Simon (Smith, Kline and French, Philadelphia, PA). Rabbit polyclonal antibodies to IL-1(α and β) was purchased from Genzyme. Rabbit anti-rhIL-1α was also purchased from Cistron Biotechnology (Pine Brook, NJ).

Isoelectric Focusing (IEF). IEF was carried out in tubes containing 1.5% agarose, 10% sorbitol, and ampholines (2.6%, pH 3.5–10 and 1.3% each of pH 4–6 and 6–8) (3). Slices were eluted into PBS, the eluates were dialyzed, and assayed for IL-1.

IL-1 Neutralization Assay. IL-1 or test sera at 10–20 U/ml were pre-incubated for 2–4 h at 37°C with or without the indicated antibodies (1 mg/ml) in a final volume of 50 μl/well. The samples were then assayed for IL-1 activity. Normal rabbit (NR) Ig was used as a control.

RNA Isolation and Analysis. RNA was isolated by the guanidinium isothiocyanate/CsCl method (13). Northern blots were made, baked, and prehybridized using standard procedures (14). Hybridization was carried out for 48 h with 2×10^6 cpm/ml probe, and labeled using the random oligomer priming reaction (15). Blots were washed using standard methodology, with a final stringency of 0.1 × SSC/0.1% SDS, at 55°C, and were exposed to Kodak X-Omat AR film. Hybridization was quantitated by densitometry; the data are presented as the ratio of IL-1 mRNA to 18S rRNA. RNA slot blots were prepared using a blotting manifold (Schleicher & Schuell, Inc., Keene, NH) and processed in the same manner as the Northern blots.

DNA Probes. The IL-1α probe was the 1.7-kb Xho I insert of pαIL-1; the IL-1β probe was the 1.3-kb Pst I insert of pβIL-1. Both probes were a gift of Dr. Yu-Chung Yang (Genetic Institute, Cambridge, MA). The 18S rRNA probe was pXC-1, a rat genomic ribosomal probe.

Results

TGP Stimulates IL-1 Production by Human Leukocytes. Initially, 2.5×10^6 PBL were allowed to adhere. The adherent cells were cultured for 24–48 h in 0.5 ml medium containing 10% AB serum with 25 μg/ml TGP (8, 10). IL-1 concentrations (n = 4) ranged from 130 to 566 U/culture with an average (mean ± SEM) of 299 ± 94 U/culture. IL-1 production by TGP-stimulated cells is dose dependent: 0.1 μg/ml TGP causing detectable production (46, 58, and 131 U/culture), with maximum production (169 ± 76 U/culture; n = 5) obtained at 10–25 μg/ml. Similar levels of IL-1 were produced whether PBL or adherent cells were cultured with TGP and whether FCS, autologous, or AB serum were added to the culture media. In serum-free medium only about one-third as much IL-1 was produced by TGP-stimulated cells as was produced in the presence of serum. It should be noted that TGP is not mitogenic to thymocytes (10) and that the IL-1-like activity in these cultures is not due to IL-2, since TGP does not stimulate IL-2 production (8).

Characterization of TGP-induced IL-1. IL-1 neutralization using antibodies specific for IL-1α and IL-1β suggested that both species of IL-1 were produced by TGP-stimulated adherent cells (Fig. 1 *b*, *A* and *B*) or PBL (Fig. 1 *b*, *C* and *D*). Two roughly equal peaks of IL-1 activity with pIs of 5.3 and 7.0 were obtained when concentrated supernatants of PBL cultured with TGP were analyzed by IEF (Fig. 1 *a*). The IL-1 with the acidic pI was neutralized by antibodies to IL-1α and the second peak was neutralized by antibodies to IL-1β (data not shown). Two peaks of IL-1 activity were

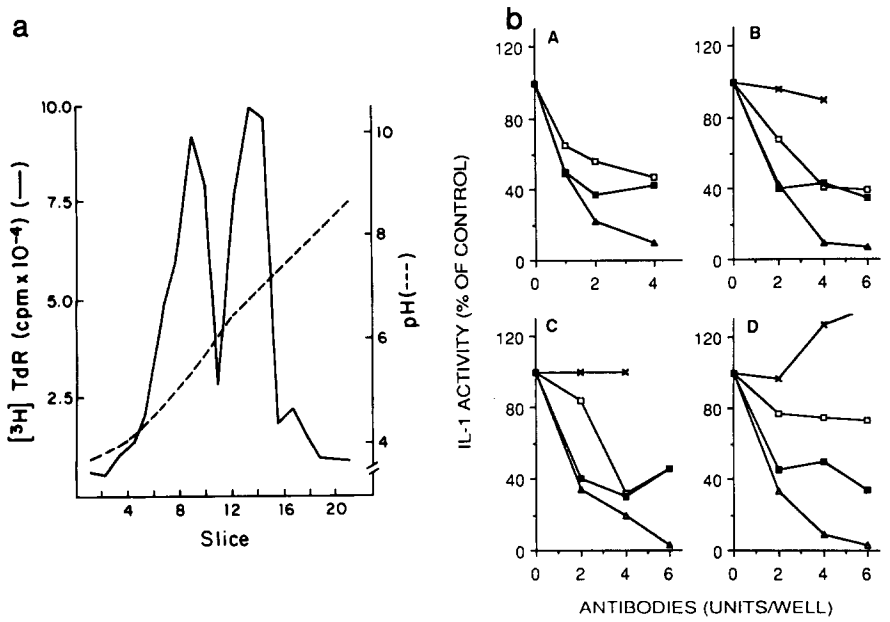

FIGURE 1. IL-1α and IL-1β production by TGP-stimulated cells. (*a*) IEF of pooled supernatants from PBL cultured for 12–48 h with TGP (15 μg/ml). The slice number is indicated on the abscissa and the pH and the [^{3}H]TdR incorporated by thymocytes is shown on the Y-axis. (*b*) IL-1 neutralization by antibodies to: IL-1 (α + β) (Δ), IL-1α ($\square$), IL-1β ($\blacksquare$), or with NRIg ($\times$), of supernatants from adherent cells (*A* and *B*), or PBL (*C* and *D*) from four individual donors cultured for 24–48 h with TGP (10–15 μg/ml). IL-1 activity in the absence of antibodies was defined as 100%.

also obtained when supernatants of adherent cells were analyzed by IEF; for these the ratio of IL-1α to IL-1β was roughly 2:3.

The molecular mass of the IL-1 was determined to be 14–15 kD by gel filtration chromatography on a Sephacryl S200 SF column. The IL-1 eluted from the column showed an IEF pattern similar to that in Fig. 1 *a*.

Kinetics of IL-1 Activity in Cultures of TGP-stimulated Cells. PBL cultured with TGP proliferate between days 5 and 10 of culture (8, 10). To determine whether the IL-1 production correlates with proliferation, supernatants from PBL or adherent cells cultured with TGP for various times were assayed for IL-1 (Fig. 2). IL-1 activity was present by 4 h of culture, peaked between 16 and 24 h, and decreased thereafter.

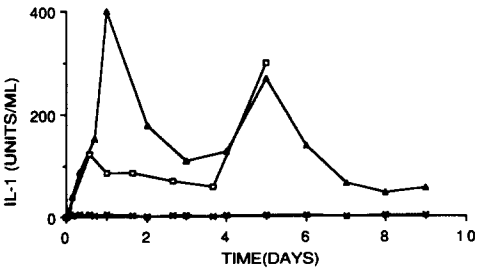

FIGURE 2. Kinetics of IL-1 activity in supernatants of PBL ($\blacktriangle$) or adherent cells ($\square$) cultured with TGP (15 μg/ml) or media alone ($\times$). Supernatants were collected at the indicated times, and their IL-1 activity was measured. This figure represents six independent experiments, all of which gave similar results.

A second peak of activity was observed between days 4 and 5 of culture (Fig. 2). Similar kinetics were observed with both PBL and adherent cells cultured with TGP (Fig. 2).

Two Peaks of IL-1 mRNA. To determine whether this secondary peak of IL-1 activity was concomitant with an increase in the steady state level of IL-1 mRNA, RNA isolated from TGP-stimulated PBL was subjected to Northern blot (Fig. 3 *A–C*) or slot blot (Fig. 3 *D*) analysis. IL-1β mRNA was detectable by 1 h of culture (Fig. 3 *C*). The steady state level of the IL-1β mRNA peaked at ~16 h (Fig. 3, *A* and *B*), decreased, and then increased again on day 5 of culture (Fig. 3, *A* and *B*). Hybridization to RNA from PBL cultured with TGP for up to 9 d confirmed the appearance of a second peak of IL-1β mRNA in TGP-treated cells (Fig. 3 *D*).

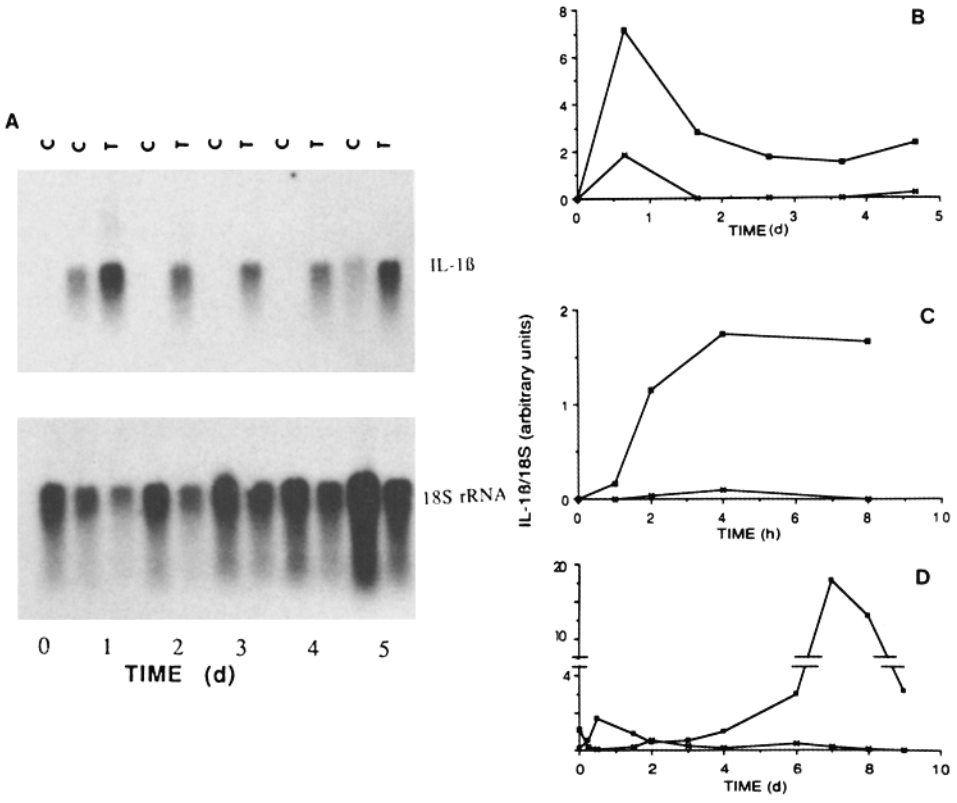

FIGURE 3. Analysis of IL-1β mRNA in PBL cultured with or without TGP (25 μg/ml) for the times indicated on the *x*-axis. (*A*) Northern blot of pooled RNA from PBL of three volunteers that were cultured with (*T*) or without (*C*) TGP for the indicated times. (*B–D*) Ratio of IL-1β mRNA to 18S rRNA in RNA of PBL cultured with (■) or without (×) TGP. Arbitrary units were determined from densitometry scans of x-ray film of Northern blots (*B* and *C*) and a slot blot (*D*). (*B*) Densitometry tracing of the Northern blot in (*A*). (*C*) RNA from PBL of an individual donor stimulated with TGP for short periods of times. (*D*) Pooled RNA from PBL of three donors, which were cultured for up to 9 d.

Discussion

In this study we show that TGP, a rutin-rich glycoprotein purified from tobacco and present in tobacco smoke, induces human mononuclear cells to produce IL-1. Two aspects of this response are of particular interest. First, about one-third of the IL-1 secreted is IL-1α, which contrasts with the preferential secretion of IL-1β by human leukocytes cultured with other inducers of IL-1 (2), including *Chlamydia trachomatis* (Rothermal, C. D., J. Schachter, P. Lavrich, E. C. Lipsitz, and T. Francus, manuscript submitted for publication). Second, the initial peak of IL-1 activity occurs by 24 h of culture and is followed by a second peak of IL-1 activity 4–6 d thereafter. Analysis of RNA from these cells shows that the second increase in IL-1 is concomitant with increased steady state levels of IL-1β mRNA. Preliminary data suggest a similar pattern of IL-1α expression, although the steady state level of the IL-1α mRNA is much lower. TGP has some unusual effects on the immune system. It stimulates the proliferation of human T cells in an IL-2-independent manner (8). TGP is a B cell mitogen in mice (10) and it preferentially elicits IgE production (7). The polyphenol groups seem to play a regulatory role in the preferential IgE response (7). Induction of both IL-1α and IL-1β by TGP supports the view that TGP activates cells of the immune network via a different pathway than other B or T cell mitogens.

Whether the second peak of IL-1 mRNA and of IL-1 activity in TGP-stimulated cells is unique to this system is under study. The second peak could result from the reactivation of the same cells or the stimulation of a different subpopulation of cells by TGP, by the IL-1 (2), or other factors produced by TGP-stimulated cells. These results suggest that the cells initially stimulated to make IL-1 do not become refractory to restimulation, and/or that a more quiescent cell population can ultimately be stimulated. When taken in the context of the continuous exposure of the smoker to TGP-like products, and the broad biological effects of IL-1, the role of IL-1 in the pathology of cigarettte smoking should be considered. IL-1 enhances leukocyte binding to human vascular endothelial cells, increases their procoagulant activity, their production of plasminogen activator inhibitor, and the release of PAF (2) and PDGF (6). Thus, IL-1 could initiate a cascade of events that bring about a decrease in blood flow and an accumulation of leukocytes and platelets, resulting in clot formation and vascular damage. In this respect, it is worth noting the recent studies showing a decrease in the production of IL-1 and TNF by PBL of volunteers who added ω-3 polyunsaturated fatty acids to their diet (16). Since a low incidence of cardiovascular and inflammatory diseases has been associated with diets including ω-3 fatty acids (reviewed in reference 16), the reduced production of IL-1 in these volunteers is consistent with the hypothesis that IL-1 production in vivo plays a role in the cardiovascular disease associated with cigarette smoking.

Summary

We have previously shown that tobacco glycoprotein (TGP), a polyphenol-rich glycoprotein isolated from tobacco or from cigarette smoke, affects the immune system. In this study we show that TGP induces human PBL and adherent cells to produce IL-1α and IL-1β. Two peaks of IL-1 activity were observed; one at 18–24 h, the second at 4–6 d after initiation of culture. A similar pattern was observed for the steady state level of IL-1 mRNA. These data suggest that the production of IL-1 by cells

stimulated with TGP might be a factor in cardiovascular disease associated with cigarette smoking.

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