ENHANCED PRODUCTION OF TUMOUR NECROSIS FACTOR α (TNF-α) BY ITS PRECURSOR ON THE CELL SURFACE OF PRIMED THP-1 CELLS

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To clarify the biological significance of tumour necrosis factor α (TNF-α) precursor, we analysed its expression at the primed and triggered stages using human monocyte-like cell line THP-1. To prime them, THP-1 cells were treated with either recombinant human interferon γ (rIFN-γ) or recombinant human tumour necrosis factor α (rTNF-α). At the primed stage, transient accumulation of TNF-α, mRNA and a small amount of 26-KDa TNF-α precursor was observed, and the precursor molecule was located on the cell surface. Following treatment of the primed cells with bacterial lipopolysaccharide (LPS), augmentation of transcription of TNF-α mRNA and production of a larger amount of TNF-α precursor were observed followed by secretion of a larger amount of mature TNF-α (17-KDa) than secreted by the unprimed cells (triggered stage). This suggests that with priming THP-1 cells might be changed to a stage where they are ready for production of a larger amount of TNF-α at the triggered stage. When either primed or unprimed THP-1 cells were pretreated with anti-TNF-α antibody, augmentation of TNF-α production by primed THP-1 cells was specifically suppressed, suggesting that TNF-α precursor itself may play an important role in the enhancement of TNF-α production by the primed macrophages after treatment with LPS.

We previously studied tumour necrosis factor α (TNF-α) production during embryogenesis of mice and demonstrated that not only macrophages but also other cells from various embryonic tissues are able to secrete TNF-α in response to stimulation by bacterial lipopolysaccharide (LPS).[1] We also detected mRNAs for TNF-α and other cytokines in several mouse embryonal carcinoma cell lines[2] and in various embryonic tissues of mice during embryogenesis.[3] Spontaneous TNF-α secretion was observed only in a restricted region of embryo, however.[1] It is therefore speculated that TNF-α presents a precursor protein in a membrane integrated form[4] during embryogenesis. Osawa and Natori reported TNF-α mRNA and 26-KDa TNF-α precursor protein rather than 17-KDa TNF-α in a mature secretory form at a specific developmental stage of mice.[5]

Using rabbit alveolar macrophages which had been primed with Bacillus Calmette-Guerin (BCG) in vivo, we analysed the synthesis of TNF-α during the primed and triggered stages of its production and found that transcription of mRNA encoding TNF-α precursor and translation of TNF-α precursor protein had already occurred even in the primed macrophage with no LPS stimulation, although we could not detect any activity in the culture supernatant of these primed macrophages.[6] From these observations, it can be said that the primed stage is one where precursor TNF-α exists in a membrane-bound form on the cell surface. This expression pattern of TNF-α seems to resemble that observed in the mouse embryo and in embryonal carcinoma cell lines. Therefore, TNF-α precursor which exists on the cell surface might play a more significant role than secreted mature TNF-α (17-KDa) does in regulating differentiation and development, even taking into consideration the concept that TNF-α orchestrates the cytokine network.[7] This proinflammatory state driven by the TNF-α precursor we termed 'ontogenic inflammation',[1,2,9] because the regulatory mechanism of homeostasis during embryo-

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genesis might be regarded as a prototype of regulatory mechanisms seen in adults.

26-KDa TNF-α precursor on the cell surface is known to exhibit cytotoxic activity against certain transformed cell lines via cell-to-cell contact with no secretion of mature 17-KDa TNF-α.[4,8] Recently, Birkland *et al.* reported that $CD4^+$ T lymphocyte cell line which expressed membrane bound TNF-α precursor can activate macrophages for antileishmanial defence.[10] However, there has been little information about the biological activities of TNF-α precursor in connection with a regulatory role of primed macrophages.

In this study we examined the biological significance of cell surface-bound TNF-α precursor at the primed stage using the human monocyte-like cell line THP-1[11] as a model system of TNF-α production. The results obtained so far suggest that TNF-α precursor on the cell surface may act to augment mature TNF-α production by some stimuli such as LPS, and that it seems to be involved in a positive feedback loop of TNF-α production by primed macrophages.

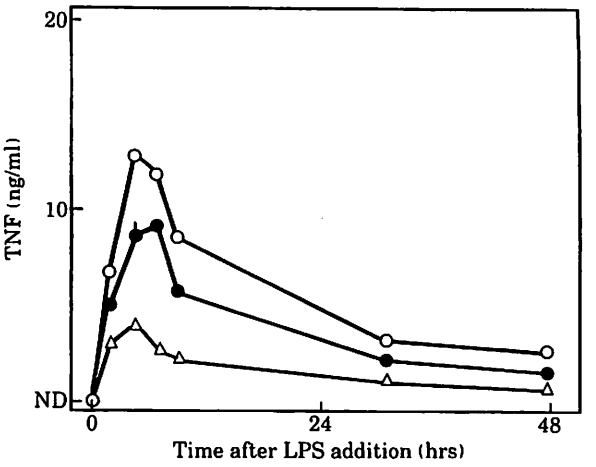

Figure 1. Kinetics of TNF-α accumulation in the culture supernatant during the triggered stage.

THP-1 cells were treated with rIFN-γ (10^3 units/ml) or rTNF-α (10^3 units /ml) for 24 h. After extensive washing and resuspending, cells were challenged with a 1 μg/ml of LPS for indicated periods of time. TNF-α amount in the culture supernatants was measured by radioimmunoassay. Each value is the mean of duplicate assays. ND means 'not detected' (less than 0.15 ng/ml). (○) primed with rIFN-γ; (●) primed with rTNF-α; (△) without priming.

RESULTS

Enhanced TNF-α Production in THP-1 Cells Primed with IFN-γ or TNF-α

We established a system to access the primed stage of macrophages by measuring the augmentation of TNF-α production in vitro using human promonocytic leukaemia cell line THP-1. Although it was difficult for us to directly evaluate the primed stage, we could quantify it by analysing mature TNF-α release from macrophages before and after the challenge with a secondary stimulus, such as bacterial lipopolysaccharide (LPS) (triggered stage).[12] THP-1 cells were treated with either recombinant human interferon γ (rIFN-γ) or recombinant human TNF-α (rTNF-α), both of which are physiological molecules and known to act as a 'primer' for TNF-α production in vitro[13–19] and in vivo as well.[12,20] The primed THP-1 cells were washed extensively, resuspended in a fresh medium, and then treated with LPS. The amount of mature TNF-α released into the culture supernatant was quantified by human TNF-α specific radioimmunoassay.

Figure 1 shows kinetics of mature TNF-α accumulation in the culture supernatants during the triggered stage of primed or unprimed THP-1 cells. THP-1 cells were treated with either rIFN-γ (10^3 units/ml) or rTNF-α (10^3 units/ml) for 24 h. Cells were then washed and challenged with LPS (1 μg/ml). The amount of TNF-α released from the primed cells in the culture supernatant after treatment with LPS increased and reached a maximum level 5 h after the addition. A larger amount of TNF-α was observed than that produced in unprimed cells. We could not detect any mature TNF-α by either the primed or unprimed cells before challenge with LPS (not shown). To examine the dose response effects of the primers, THP-1 cells were primed for 24 h with various doses of rIFN-γ or rTNF-α, and were then washed and challenged with LPS. Five hours after the addition of LPS, the amount of TNF-α in the culture supernatant was measured by radioimmunoassay. As shown in Fig. 2, the TNF-α level in the culture supernatants increased dose-dependently. Unprimed THP-1 cells produced a significant amount of mature TNF-α by treatment with a relatively high concentration of LPS (1 μg/ml). LPS at a concentration of 0.1 μg/ml resulted in the most significant differences in the amount of TNF-α production between the primed and unprimed cells (Fig. 2).

We then examined optimal treatment time of the primers for TNF-α production at the triggered stage. THP-1 cells were pretreated with the primers at various times, the cells were then washed and challenged with LPS for 5 h. As shown in Fig. 3, maximum enhancement of TNF-α production was observed when the cells were pretreated with the primers for 24 h. The TNF-α level decreased significantly when the cells were pretreated for 72 h with a primer, probably due to its low cell viability.

Accumulation of TNF-α mRNA during the trig-

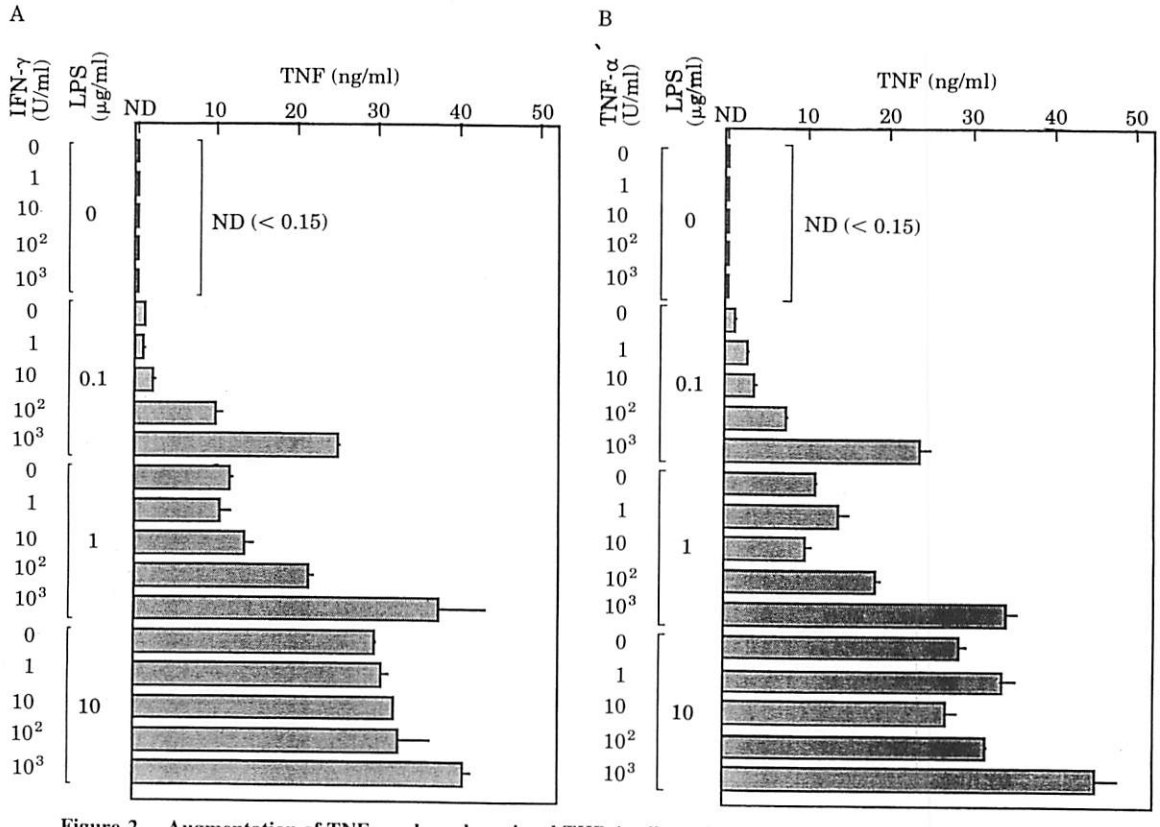

Figure 2. Augmentation of TNF-α release by primed THP-1 cells at the triggered stage.

THP-1 cells were treated with various doses of rIFN-γ (A) or rTNF-α (B) for 24 h. After washing and resuspending, cells were challenged with various doses of LPS. Five hours after LPS additions, TNF-α amount in the culture supernatants was measured by radioimmunoassay. Doses of primers and LPS are indicated. Each value is the mean of duplicate assays.

gered stage in both primed and unprimed THP-1 cells was examined by Northern blot analysis. As shown in Fig. 4, expression levels of TNF-α message in THP-1 cells primed by either rIFN-γ or rTNF-α were 2–4 fold higher than those in unprimed THP-1 cells when their peak levels were analysed by densitometric scanning. The increase in TNF-α production at the triggered stage was also confirmed at the protein level. Either primed or unprimed THP-1 cells were challenged with LPS and were then biosynthetically labelled with a radioactive methionine and cysteine mixture. The labelled cells were lysed and subjected to immunoprecipitation analysis using anti-human TNF-α polyclonal antibody. As shown in Fig. 5, the intensity of 26-KDa band corresponding to TNF-α precursor produced in primed cells was stronger than that produced in unprimed cells. These results indicate that primed THP-1 cells can newly synthesize a larger amount of mature TNF-α protein during the triggered stage than can unprimed cells, mainly due to an increase in transcription of TNF-α message for the former cells.

Transcription of TNF-α mRNA and Production of TNF-α Precursor during the Primed Stage of THP-1 Cells

We previously reported that TNF-α mRNA and TNF-α precursor-like molecules already existed in BCG-primed rabbit alveolar macrophages with no LPS stimulus, but we were unable to detect the release of mature TNF-α in the culture supernatants.[6] From these results, the primed stage can be regarded as the stage ready for enhanced TNF-α production where initiation of transcription of its mRNA is provoked, followed by TNF-α production with the aid of the precursor protein. Therefore, we examined expression of TNF-α mRNA during the primed stage of THP-1 cells. The cells were treated with rIFN-γ or rTNF-α and accumulation of TNF-α mRNA was analysed by Northern blot analysis. mRNA from phorbol myristate acetate (PMA) stimulated THP-1 cells was employed as a positive control in this analysis because PMA is well known as a strong inducer of TNF-α mRNA expression.[21] As shown in Fig. 6, TNF-α

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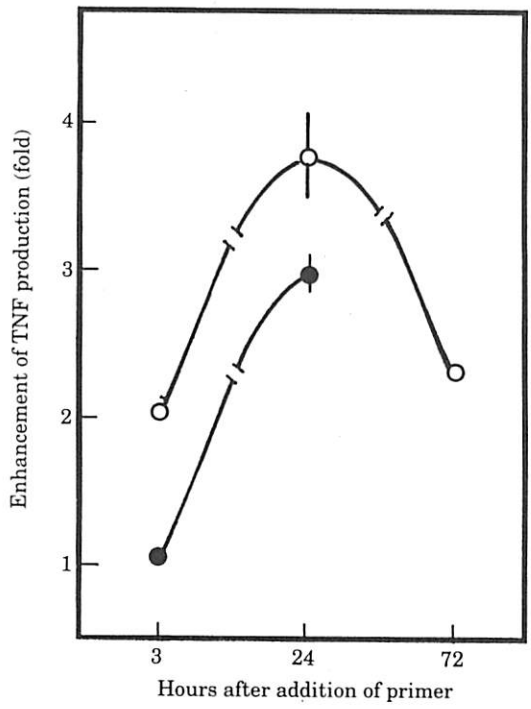

Figure 3. Determination of the optimal period of priming.

THP-1 cells were treated with rIFN-γ (10^3 units/ml) or rTNF-α (10^3 units/ml) for indicated times. After washing and resuspending, cells were challenged for 5 h with 1 μg/ml of LPS. The TNF-α amount in the culture supernatants was then measured by ratioimmunoassay. Each value is the relative enhancement of TNF-α amount compared with that produced by unprimed cells. (○) primed with rIFN-γ; (●) primed with rTNF-α.

mRNA was induced transiently and reached a maximum level 1–3 h after the addition of the primers.

Synthesis of TNF-α precursor protein at the primed stage was then analysed. THP-1 cells were treated with rIFN-γ followed by biosynthetical labelling with radioactive cysteine. Labelled cell lysates were then subjected to immunoprecipitation analysis. As shown in Fig. 7, a 26-KDa band corresponding to the TNF-α precursor was specifically immunoprecipitated with anti-human TNF-α polyclonal antibody when the cells were primed with rIFN-γ. The amount of TNF-α precursor synthesized by THP-1 cells which had been primed with rIFN-γ alone was roughly 5-fold less than that synthesized by cells stimulated with LPS by densitometric scanning.

TNF-α precursor was reported to be a biologically active transmembrane protein on the surface of plasma membrane.[4,8] Therefore, we analysed it on the cell membrane during the primed stage of THP-1 cells. After priming, the surface of intact cells was labelled with radioactive iodine. The cells were then washed, lysed, and subjected to immunoprecipitation analysis. As shown in Fig. 8, the 26-KDa band specifically immunoprecipitated with anti-TNF-α antibody was detected on the surface of the cells which had been primed with rIFN-γ (lane 5), rTNF-α (lane 7), or PMA (lane 9) and also those challenged with LPS (lane 3), although the amount of the cell surface TNF-α precursor varied. These results indicate that TNF-α mRNA is induced and the precursor protein is actually expressed on the surface of primed THP-1 cells even without the addition of LPS.

Significance of TNF-α Precursor on the Cell Surface of Primed THP-1 Cells

Possible involvement of TNF-α precursor synthesized during the primed stage in enhancing production of mature TNF-α at the triggered stage was examined. If the amount of production of mature TNF-α at the triggered stage exactly reflects the amount of accumulated precursor protein during the primed stage, inhibition of transcription or translation cannot completely suppress the release of mature TNF-α into the

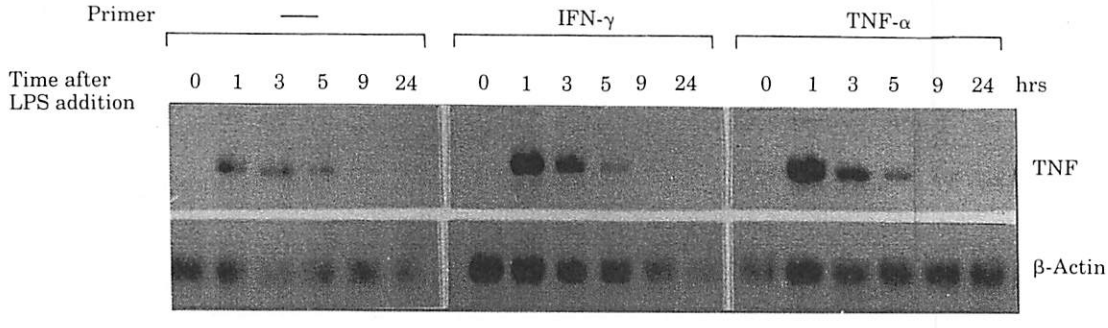

Figure 4. Kinetics of TNF-α mRNA induction during the triggered stage.

THP-1 cells were treated for 24 h with or without primers as described in Fig. 1. After washing and resuspending, cells were challenged with 0.1 μg/ml of LPS for indicated periods of time. Extraction of total cellular RNA and Northern blot analysis were carried out as described in 'Materials and Methods'. Total RNA (5 μg/lane) blotted onto membrane was probed with ^{32}P-labelled 4th exon fragment human TNFα genomic clone. The same membrance was rehybridized with ^{32}P-labelled β-actin cDNA probe after removal of the TNF-α probe.

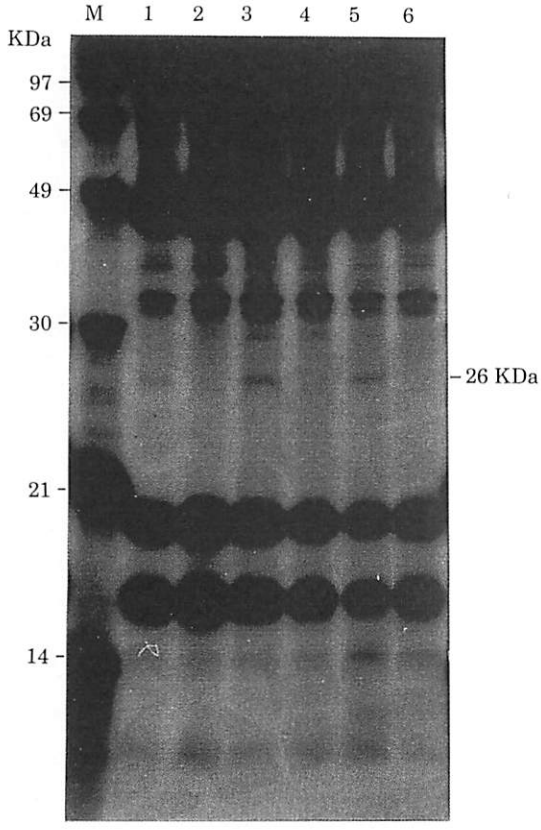

Figure 5. Immunoprecipitation analysis of 26-KDa TNF-α precursor synthesis during the triggered stage.

THP-1 cells were treated with or without primers for 24 h as described in Fig. 1. After washing, cells were challenged with 0.1 μg/ml of LPS for 30 min and then biosynthetically labelled for a further 30 min using the Trans^{35}S-label. Immunoprecipitation experiment of ^{35}S-labelled cell lysate using anti-human TNF-α polyclonal antibody was carried out as described in 'Materials and Methods'. Immunoprecipitated materials were subjected to SDS-PAGE followed by fluorography. Lanes 1, 3, 5 are immunoprecipitate with anti-TNF-α antibody and lanes 2, 4, 6 are with control rabbig IgG. Cell lysate in lanes 1 and 2 is without priming, in lanes 3 and 4 it is primed with 10^3 units/ml of rIFN-γ, and in lanes 5 and 6 it is primed with 10^3 units/ml of rTNF-α, respectively. Lane M shows molecular weight standard.

culture supernatant at the triggered stage. Primed or unprimed THP-1 cells were challenged with LPS in the presence or absence of a transcriptional inhibitor actinomycin D (ActD) or a protein synthesis inhibitor cycloheximide (CHX). As shown in Fig. 9, both the inhibitors of either transcription or protein synthesis suppressed almost all of the mature TNF-α accumulation irrespective of cell priming. These results suggest that precursor TNF-α on the cell surface at the primed stage may concern *de novo* synthesis of mRNA corresponding to augmentation of TNF-α production at the triggered stage. Although the extent of contribution of the TNF-α precursor at the primed stage was quite small, the enhanced TNF-α production by primed THP-1 cells at the triggered stage can be explained by augmentation of transcription and translation during the triggered stage.

To determine a possible role of TNF-α precursor on the cell surface in view of augmentation of newly synthesized TNF-α at the triggered stage, we examined the effect of anti-TNF-α antibody on the augmentation of TNF-α production from the THP-1 cells which had been primed with IFN-γ. THP-1 cells were treated with rIFN-γ in the presence or absence of either anti-human TNF-α monoclonal antibody or control mouse IgG. The cells were then washed and challenged with LPS. To examine the specific effect of anti-TNF-α antibody on priming, THP-1 cells were also pretreated with anti-TNF-α antibody or control IgG without priming by rIFN-γ. As shown in Fig. 10, augmentation of TNF-α production by THP-1 cells primed with rIFN-γ was abolished, apparently by treatment with anti-TNF-α antibody. The same amount of mouse IgG did not show such an effect. The TNF-α level in unprimed cells was not affected by these antibodies. Therefore, abolition of augmentation of TNF-α production at the triggered stage by the addition of anti-TNF-α antibody at the primed stage indicates that TNF-α precursor produced at the primed stage could be involved in the mechanism of augmentation of TNF-α production at the triggered stage.

DISCUSSION

We proposed that the inflammation-like state which we originally called 'ontogenic inflammation' might be a regulatory mechanism to normalize embryogenesis, and that ontogenic inflammation might be a prototype of a regulatory mechanism which is converted into a mechanism of the nervous, endocrine, and immune systems in adult to maintain homeostasis.[9] We then proved the existence of an inflammation-like state which is characterized by the primed stage of TNF-α production during mouse embryogenesis and in teratocarcinoma cell lines.[1–3] Furthermore, we indicated that TNF-α precursor-like proteins as well as TNF-α mRNA can be detected even in the primed alveolar macrophages from rabbit immunized with BCG.[6] These observations suggest that macrophages in the primed stage having TNF-α precursor on their cell surface are more dominant in the physiological state of the body than in the triggered stage accompanied with the secretion of mature 17-KDa TNF-α. Thus, such macrophages may have a crucial biological significance in the restoration of various distortions of homeostasis. This notion led us to analyse in more detail the existence of TNF-α mRNA and TNF-α precursor protein during the

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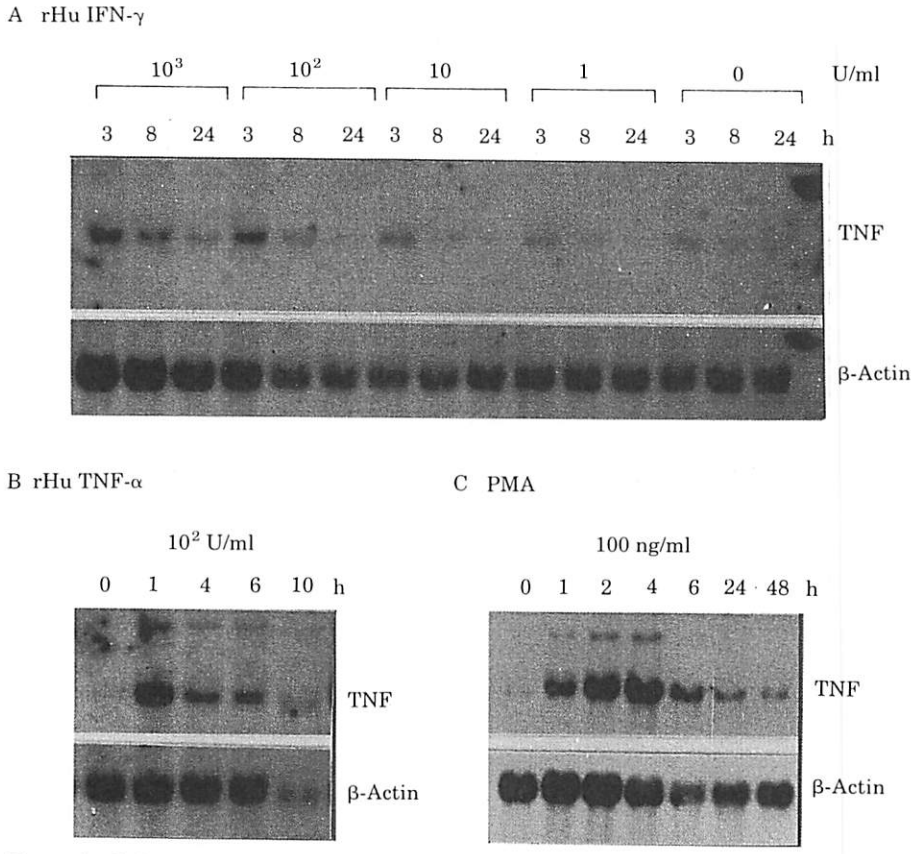

Figure 6. Induction of TNF-α mRNA expression during the primed stage of THP-1 cells.

THP-1 cells were primed with rIFN-γ, rTNF-α, and PMA at the indicated doses. Total cellular RNA was extracted from cells treated for indicated periods of time with various primers. Northern blot analysis was performed as described in the legend of Fig. 4.

primed stage, and also to study the possible biological function of the TNF-α precursor at the primed stage using a macrophage-like cell line (THP-1).

Certain cytokines such as IFN-γ or TNF-α, which are intrinsic factors, are known to have the ability to enhance endogenous TNF-α production in vivo in response to various foreign stimuli of bacterial origin.[12,20] Similar 'priming' phenomena have also been observed in TNF-α production by cultured macrophages in vitro.[13–19] However, there have been few reports on the significance of primed macrophages carrying the TNF-α precursor on their surface.

We studied in more detail the existence of TNF-α precursor through the primed stage and the triggered stage using a system of THP-1 cells in vitro, in which the primed stage is separable from the triggered stage. It is known that THP-1 cells, a human monocytic leukaemia cell line, are able to produce TNF-α[22,23] and that the expression pattern of various markers of differentiation closely resembles those of monocyte-macrophages.[11] Therefore, this cell line can be considered a good model of macrophage, as a main producer of TNF-α in vivo. In this connection, we confirmed that transcription of TNF-α mRNA and production of 26-kDa TNF-α precursor occurred during the primed stage (Figs 4–6). These facts are also supported by the observation that membrane-bound TNF-α exists on the cell surface of primed mouse peritoneal macrophages and was induced by a low dose of LPS (T. Okutomi, personal communication). Therefore, it is likely that during the primed stage THP-1 cells may gain an ability to enhance production of TNF-α in response to LPS.

What kind of biological significance does the TNF-α precursor have on the surface of primed cells? It is quite interesting that all of the primers used in this experiment can induce differentiation of promyelocytic or monocytic leukaemia cell lines into mature macrophage-like cells.[24,25] This acquired phenotype of cells which produce a larger amount of TNF-α at the triggered stage might therefore be greatly involved with differentiation of immature macrophages into mature macrophages. Witsell and Schook reported that differentiation of bone marrow-derived macrophages induced by GM-CSF was inhibited by the blockade of endogenous TNF-α message by an

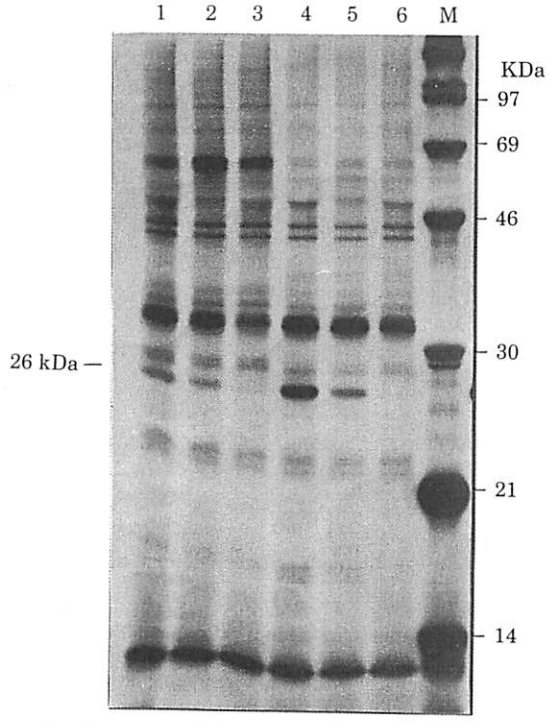

Figure 7. Immunoprecipitation analysis of 26-KDa TNF-α precursor synthesis during the primed stage.

THP-1 cells were treated with rIFN-γ (10^3 units/ml) or LPS (10 μg/ml) for 2 h. After washing, cells were biosynthetically labelled for 30 min with L-^{35}S cysteine. Immunoprecipitation was carried out as described in Fig. 5. Lanes 1–3 are the cell lysate of rIFN-γ primed cells and lanes 4–6 are the cell lysate of LPS treated cells. These lysates were immunoprecipitated with 5 μg (lanes 1, 4) or 1 μg (lanes 2, 5) of anti-TNFα antibody. Lanes 3 and 6 are inmunoprecipitate with 5 μg of control rabbit IgG. Lane M shows molecular weight standard.

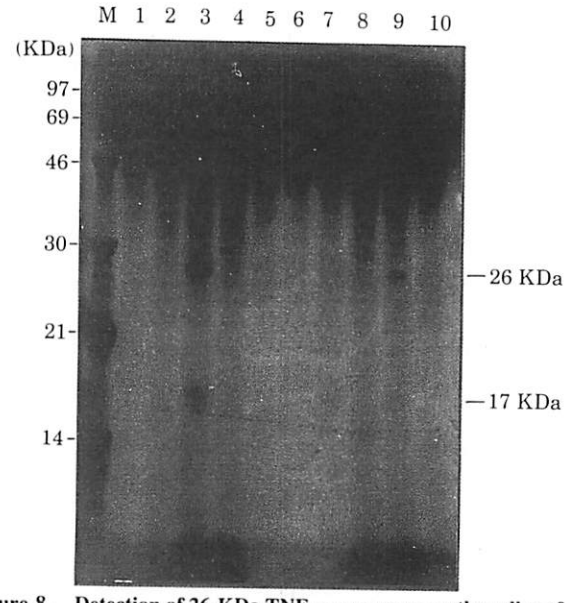

Figure 8. Detection of 26-KDa TNF-α precursor on the cell surface of primed THP-1 cells.

THP-1 cells were treated for 3 h with or without various primers. Cell surface labelling with Na^{125}I was carried out as described in 'Materials and Methods'. After the labelling, cells were extensively washed, lysed, and subjected to immunoprecipitation analysis as described in Fig. 5. Lanes 1, 3, 5, 7, 9 are immunoiprecipitate with anti-TNF-α antibody and lanes 2, 4, 6, 8, 10 are with control rabbit IgG. Cell lysate in lanes 1 and 2 is without priming; in lanes 3 and 4 it is treated with 10 μg/ml of LPS; in lanes 5 and 6 it is primed with 10^3 units/ml of rIFN-γ; in lanes 7 and 8 it is primed with 10^3 units/ml of rTNF-α, and in lanes 9 and 10 it is primed with 100 ng/ml of PMA.

antisense oligonucleotide.[26] The membrane integrated form of 26-KDa TNF-α precursor exposes its carboxyl terminus corresponding to the biological active part of mature 17-KDa TNF-α to the extracellular environment.[4,8] This means that the precursor TNF-α can bind its receptors via cell-to-cell contact. Two types of TNF receptors (p55 and p70) were reported[27–30] to mediate various biological activities of TNF-α as well as cytotoxic activities. Therefore, primed macrophage having 26-KDa TNF-α precursor on the cell surface should have some biological functions in addition to its cytotoxicity. We speculate that TNF-α precursor produced during the primed stage may act as a positive regulator of enhanced production of mature TNF-α during the triggered stage because it specifically disappeared when THP-1 cells primed with IFN-γ were treated with the anti-TNF-α monoclonal antibody, and because unprimed cells were not affected by treatment with this antibody. These results indicate that the TNF-α precursor expressed on the cell membrane is necessary to make the cell gain a phenotype that can produce a larger amount of TNF-α in response to LPS, although LPS itself is a totally foreign substance for animals. As for the mechanism of augmentation of TNF-α production by the TNF-α precursor, enhanced production by primed macrophages seems to be mainly a result of augmentation of transcription and translation of its gene at the triggered stage (Figs 4, 5), because TNF-α production at this stage was largely suppressed in the presence of ActD or CHX (Fig. 9). Therefore, TNF-α precursor might be involved in the signal transduction pathway of enhanced TNF-α gene expression, and would play a role in autocrine positive feedback mechanisms.

There are few reports which deal with the biological activities of membrane-bound form of TNF-α precursor except those by Kriegler *et al.*[4,8] where cytotoxic activities of membrane-bound form of TNF-α precursor were explained in terms of an effector molecule. As described at the beginning of this report, it seems that cells are at the primed stage for TNF-α production throughout the period of embryogenesis,[1–3] when there should not be any infectious state or cancer seen in adult. Furthermore, during the

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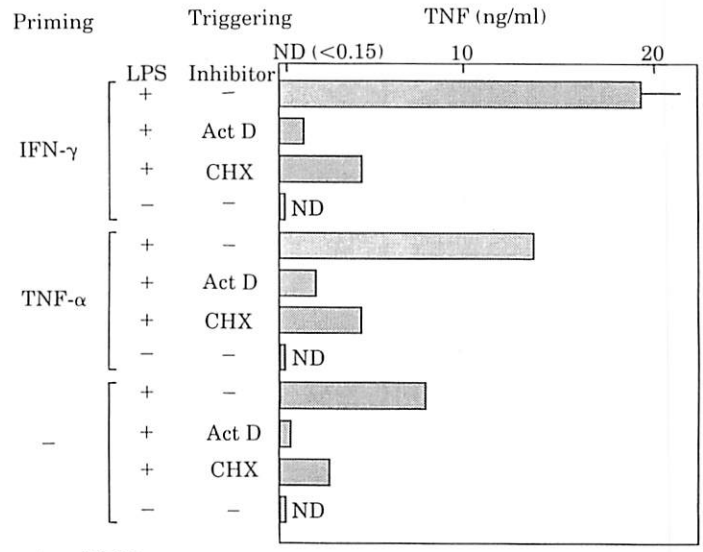

Figure 9. Suppression of TNF-α production by actinomycin D or cycloheximide at the triggered stage of primed or unprimed THP-1 cells.

THP-1 cells were treated for 24 h with or without primers as described in Fig. 1. After washing and resuspending, cells were challenged with LPS (1 μg/ml) for 5 h in the presence or absence of actinomycin D (1 μg/ml) or cycloheximide (10 μg/ml). TNF-α amount in the culture supernatants was measured by radioimmunoassay. Each value is the mean of duplicate assays.

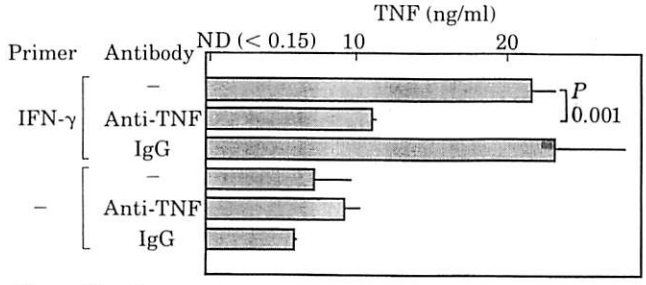

Figure 10. Suppression of priming activity of rIFN-γ by the presence of anti-HuTNF-α monoclonal antibody at the primed stage.

THP-1 cells were treated with IFN-γ (10^3 units/ml) for 24 h with or without anti-rTNF-α monoclonal antibody F5H12 (10 μg/ml). A control experiment was run parallel using non-specific mouse IgG (10 μg/ml). After extensive washing and resuspending, cells were challenged with LPS (1 μg/ml) for 5 h. TNF-α amount in the culture supernatants was measured by radioimmunoassay. Each value is the mean of triplicate assays.

primed stage, the TNF-α precursors were actually expressed on the cell membrane, showing the possibility of some biololgocal activities including a function of positive feedback regulator other than cytotoxicity. In fact, immunoreactive TNF-α that was detected during the mouse embryogenesis proved to be not mature but, rather, 26-KDa TNF-α precursor.[5] It is therefore strongly suggested that the membrane-bound TNF-α precursor may function during embryogenesis.

Recently, reduced tumorigenicity or invasiveness in vivo of tumour cells expressing TNF-α precursor was reported.[31–34] This change of phenotype is ascribable to the acquired characteristics which, by production of TNF-α, gather inflammatory cells such as macrophages or cytotoxic T lymphocytes around the tumour lesion. Therefore, in a broader sense, TNF-α precursor on the cell surface might have a potency to amplify self and/or non-self recognition locally, although membrane-bound TNF-α precursor itself seems to be insufficient to elicit the host response.[35] In this point, primed macrophages in turn may play a crucial role in restoration of the distorted situation and for maintenance of homeostasis in adult. In fact, we have demonstrated that various distorted states in host homeostasis such as seen in intractable diseases can be restored by macrophages at the primed stage, and we have proved that the biological significance of primed macrophages is involvement in the regulatory mechanisms of homeostasis.[36–42] We therefore believe that analysis of the biological significance of TNF-α precursor on the cell surface of primed cells should help clarify the mechanisms by which homeostasis can be maintained in adult and give new insights into the role of the cytokine network.

MATERIALS AND METHODS

Materials

rHuIFN-γ (GI-3, for phase study, 3×10^6 units/ml) was

supplied by Toray Industries (Tokyo, Japan). rHuTNF-α (1.7×10^6 units/mg protein) was produced and purified in our laboratory.[22] Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 was purchased from Difco Laboratory (Detroit, MI, USA). Phorbol 12-myristate acetate (PMA), actinomycin D, cycloheximide, lactoperoxidase, and glucose oxidase were purchased from Sigma Chemical Company (St. Louis, MO, USA). Polyclonal rabbit anti-human TNF-α antibody and pre-immune rabbit IgG were purchased from Endogen (Boston, MA, USA). Monoclonal mouse anti-human TNF-α neutralizing antibody was produced and purified in our laboratory (F5H12, Oshima, H. *et al.*, unpublished). Protein G Sepharose Fast Flow was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden).

Cell Culture

Human acute monocytic leukaemia cell line, THP-1,[11] was obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). This cell line was grown in RPMI-1640 (Nikken Biomedical Laboratory, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, defined grade, HyClone, Logan, UT, USA) and 60 μg/ml of kanamycin sulfate (Meiji Seika, Tokyo, Japan) and maintained at 37°C in 5% CO_2/95% air. Subculture of cells was done twice a week by 4-fold dilution. Viability of cells was usually more than 97% as judged by trypan blue dye exclusion.

TNF-α Production by THP-1 Cells

THP-1 cells were harvested and resuspended in a fresh prewarmed (37°C) medium to 5×10^5 cells/ml prior to induction, and 6 ml (3×10^6 cells) was used for each condition of induction. Various stimuli were then added and cells were incubated at 37°C in 5% CO_2/95% air. Dosage of stimuli and length of incubation time are described in the legend of each figure. Then, the cell suspensions were collected into polypropyrene centrifuge tubes and centrifuged (800 × g) for 5 min at 4°C. The cells were resuspended in 1 ml of a fresh medium, transferred to a fresh tube containing 5 ml of the fresh medium and recentrifuged. This washing procedure was repeated twice and cells were resuspended in 0.5 ml of the fresh medium. Cell density of each suspension was counted by Coulter counter (model ZM, Coulter Electronics, Ltd, Luton, England) and adjusted to 5×10^6 cells/ml. A 100 μl (5×10^5 cells) aliquot of cell suspensions was seeded onto a 96-well flat bottom cell culture plate. Then, 100 μl of medium containing LPS(2 μg/ml or 0.2 μg/ml) was added to each well and the cells were incubated for an additional 5 h (except for time course experiments) at 37°C in 5% CO_2/95% air.

Preparation of ^{125}I-labelled Monoclonal Antibody

F5H12 monoclonal antibody was labelled with Bolton-Hunter Reagent [^{125}I] kit (ICN Biomedicals Inc, Irvine, CA, USA) according to the manufacturer's instruction.

Radioimmunoassay

Human TNF-α specific radioimmunoassay system was established using F5H12 mouse monoclonal antibody against rHuTNF-α. F5H12 monoclonal antibody was prepared in our laboratory (Oshima, H., unpublished). Concentrated standard rTNF-α stock was dispensed in small aliquot and stored at −80°C. This stock was initially quantified in a commercially available radioimmunoassay kit (Medgenix Diagnostics, Fleurus, Belgium) to reconfirm the content of rTNF-α. Plastic radioimmunoassay tubes (Shionogi, Tokyo, Japan) were coated with 0.5 ml of F5H12 antibody solution (1 μg/ml) in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS(−), Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) for at least 5 h, then the tubes were washed with 0.5% tween-20 in PBS(−) and blocked with 10% Calf Serum in PBS(−) for 1 h at 37°C. They were then washed with 0.5% tween-20 in PBS(−). Serially diluted standard rTNF-α or THP-1 culture supernatants were added to the antibody coated tubes (100 μl/tube) and incubated at 4°C overnight. 300-fold diluted ^{125}I-labelled F5H12 monoclonal antibody in 0.5% tween-20 in PBS(−) was added to washed tubes (100 μl/tube) and incubated for 2 h at 37°C. Then the tubes were washed and the radioactivity of bound ^{125}I-antibody was measured by a γ-counter (ARC-300 Auto Well Gamma System, Aloka, Tokyo, Japan). This system should detect either the dimeric or trimeric form of mature TNF-α, which is thought to be a 'biologically active form' of mature TNF-α.[43–45] Detection limit of the system is 0.15–0.30 ng/ml. In several experiments, the amount of TNF-α in the culture supernatants of THP-1 cells was measured by both our radioimmunoassay system and a commercially available system (Medgenix Diagnostics), and similar results were obtained in both systems.

RNA Analysis

Total cellular RNA was extracted by a guanidium thiocyanate-acidic phenol-chloroform extraction method.[46] For Northern blot analysis, RNA was denatured with glyoxal and dimethyl sulfoxide, and was subjected to gel electrophoresis according to the standard method.[47] Size fractionated RNA was transferred onto a nylon membrane (Hybond N+, Amersham Japan, Tokyo, Japan) with 20×SSC (3 M NaCl, 0.3 M Na_3citrate) and then the membrane was fixed by UV illumination. Prehybridization was carried out at 42°C in 5×SSPE, 5× Denhart's solution, 0.5% (w/v) SDS, 50% (v/v) formamide, and 250 μg/ml denatured calf thymus DNA for at least 1 h. Hybridization with ^{32}P-labelled probe was then carried out at 42°C for at least 15 h in 5 to 10 ml of the same composition of the prehybridization buffer including heat denatured radioactive probe. Restriction fragment generated by XhoI (nearly 3′-end of 3rd intron) and PstI (3′-untranslated region of 4th exon) digestion of human TNF-α genomic clone and PstI fragment of chick β-actin cDNA clone[48] were used to detect mRNAs for human TNF-α and human β-actin,

respectively. Purified DNA probes (25 ng) were labelled by random primer extension procedure using α-^{32}PdCTP (>111 TBq/mmole, ICN Biomedicals Inc., Irvine, CA, USA) and Megaprime labelling kit (Amersham Japan, Tokyo, Japan). After hybridization, membrane was washed twice by 2×SSC containing 0.1% SDS for 15 min, once by 1×SSC containing 0.1% SDS for 30 min, and once by 0.2× SSC containing 0.1% SDS for 5 min. All washings were done at 56°C. Membrane was exposed to X-ray film (XAR-5, Eastman Kodak Co., Rochester, NY, USA) at −80°C with intensifying screen (Cronex Lightning Plus, E.I. du Pont de Nemours & Company Inc., Wilmington, DE, USA).

Biosynthetic Labelling of Newly Synthesized Protein of THP-1 Cells

Prior to labelling, THP-1 cells were washed once with a fresh prewarmed medium, once with Hanks' balanced salt solution (Nissui Pharmaceutical Co., Ltd) supplemented with dialysed FBS (dialysed against PBS(−)) to 5%, resuspended in RPMI-1640 minus methionine and/or cystine (RPMI-select amine kit, Gibco Laboratories, Grand Island, NY, USA) containing 5% dialysed FBS to 2 × 10^7 cells/500 μl and incubated for 30 min at 37°C in 5% CO_2/95% air. Next, an aliquot of 9.25 MBq of Trans^{35}S Label or L-^{35}S cysteine (ICN Biomedicals Inc.) was added to each cell suspension and incubated for a further 30 min. After labelling, cells were washed twice with ice-cooled PBS(−) and were lysed with NP-40 lysis buffer containing 1 mM PMSF, 1 μg/ml aprotinin, 5 μM (N-[N-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl] (E64), 5 μM of elastatinal, 100 μM phosphoramidon, and 20 μM of chymostatin. Resulting cell lysates were subjected to immunoprecipitation experiment. Equivalency of cell number of each preparation was reconfirmed by protein quantification.[49]

Cell Surface Labelling

Radioiodination of intact cells was performed according to the method of Kaufman *et al.*[50] Briefly, 1 × 10^7 cells treated with or without primers as described above were harvested and washed twice with ice-cooled PBS(−), then resuspended in 500 μl of PBS(−). After addition of 2.5 units of lactoperoxidase, 0.5 units of glucoseoxidase, 20 mM of glucose, and 18.5 MBq of Na^{125}I (ICN Biomedicals Inc.), cell suspensions were gently agitated for 30 min at room temperature. Next, the cells were washed 3 times with ice-cooled PBS(−) containing 0.1% sodium azide and the cell pelles were lysed with NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.05% SDS, 1% NP-40) containing 2 mM phenylmethylsulfonylfluoride (PMSF) and 1 μg/ml aprotinin for 30 min on ice. Cell lysates were centrifuged at 9000 × g for 5 min at 4°C and resulting supernatants were subjected to immunoprecipitation experiment. Equivalency of cell number of each preparation was reconfirmed by quantification of protein contents of cell lysate.[49]

Immunoprecipitation

Immunoprecipitation was carried out according to the method of Firestone and Winguth[51] except that protein G Sepharose beads were used instead of fixed staphylococcal bacterial cells. Immunoprecipitated materials were subjected to SDS-polyacrylamide gel electrophoresis.[52] For fluorography, gels were fixed and treated with an autoradiography enhancer (ENLIGHTING, New England Nuclear, Boston, MA, USA), dried, and exposed to X-ray film.

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