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Inhibitory Action of Cytokine Preservatives on Chemiluminescence Assays

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Summary: Commercial tumor necrosis factor alpha (TNFα) reportedly stimulates hydrogen peroxide-mediated chemiluminescence in macrophages. However, two commercially available forms of TNFα, a sodium azide-containing recombinant human TNFα and an azide-free murine TNFα, had opposite effects on a cell-free hydrogen peroxide chemiluminescence assay. Azide-free murine TNFα stimulated peroxide-associated chemiluminescence, while the azide-containing human TNFα strongly inhibited chemiluminescence. The inhibition of hydrogen peroxide chemiluminescence by the azide-containing TNFα was attributable to small quantities of sodium azide and bovine serum albumin used as preservatives and carriers, respectively. **Key Words:** azides, bovine serum albumin, chemiluminescence, oxygen radicals, preservatives, TNFα.

Tumor necrosis factor alpha (TNFα) has been reported to stimulate generation of oxygen free radicals by alveolar macrophages as measured by chemiluminescence (CL) [1]. Conversely, other investigators have reported a free radical scavenging role for TNFα and other cytokines [2]. We used an in vitro model to investigate the early biological effects of TNFα on free radical generation during the particle-stimulated "respiratory burst" by rat alveolar macrophages. However, during the course of our investigations on CL in the presence of TNFα, we noted dramatic differences in CL, dependent on the source of TNFα. We deduced from further investigations that the inhibitory action of one type of TNFα was attributable to preservatives and carriers present in the preparation. Because cytokine preservatives and carriers alter the outcome of investigations using commercial cytokine preparations, we report the results of our study.

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MATERIALS AND METHODS

Reagents

TNFα was obtained from R&D Systems (Minneapolis, MN, USA) and was either the standard from the TNFα ELISA assay kit (recombinant human TNFα containing sodium azide) or the azide-free murine TNFα. Luminol (Sigma Chemical Co., St. Louis, MO, USA) stock solution was prepared by dissolving 2.5 mg luminol in 250 μL DMSO and bringing the total volume of the solution to 25 mL with Hepes buffer. The final concentration of luminol in each sample was 4.0×10^{-7} M. All other reagent solutions were prepared in Hepes buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Hepes, and 1 mM $CaCl_2$ (pH 7.4).

Dialysis

To investigate the role of the preservative sodium azide and carrier bovine serum albumin (BSA) present in the TNFα, we dialyzed azide-preserved human TNFα using Slide-A-Lyzer (Pierce, Rockford, IL, USA) and studied the effect of isolated fractions in CL assays. Using dialysis tubing with molecular weight cut off fractions ranging from 10 to 50 kD, we separated four fractions containing (1) sodium azide, (2) TNFα, (3) TNFα and BSA, and (4) BSA.

Chemiluminescence

Before measurement of CL, all samples were brought to a final volume of 500 μL. CL was measured in a cell-free system and integral counts collected for 15 min. CL measurements were made using a Berthold autolumat LB953 (Wallac, Gaithersburg, MD, USA). The reaction vessels contained hydrogen peroxide, HEPES, luminol (in DMSO as described above), and the product of interest (TNFα, sodium azide, and/or BSA). Results were expressed as the integral of the counts after subtraction of the negative control (containing only HEPES and luminol).

RESULTS

TNFα consistently altered cell-free hydrogen peroxide-induced CL. However, the effect of TNFα on CL was dependent on the source of the cytokine (Figure 1). While azide-free recombinant murine TNFα further enhanced hydrogen peroxide-associated CL, recombinant human TNFα (containing sodium azide) was an effective quenching agent for CL.

In the dialyzed sample of sodium azide-preserved human TNFα, the effects of the commercial cytokine could be reproduced by fractions 3 or 4, corresponding to molecular weights of >10 kD (TNFα and BSA) and >50 kD (BSA), respectively (Figure 2). Dialyzed TNFα (fraction 2) had less CL quenching effect than the sodium azide-preserved TNFα. The low molecular weight fraction (azide) of the dialyzed TNFα also quenched hydrogen peroxide-associated CL, but was less efficient than the azide-preserved TNFα or the high molecular weight fractions.

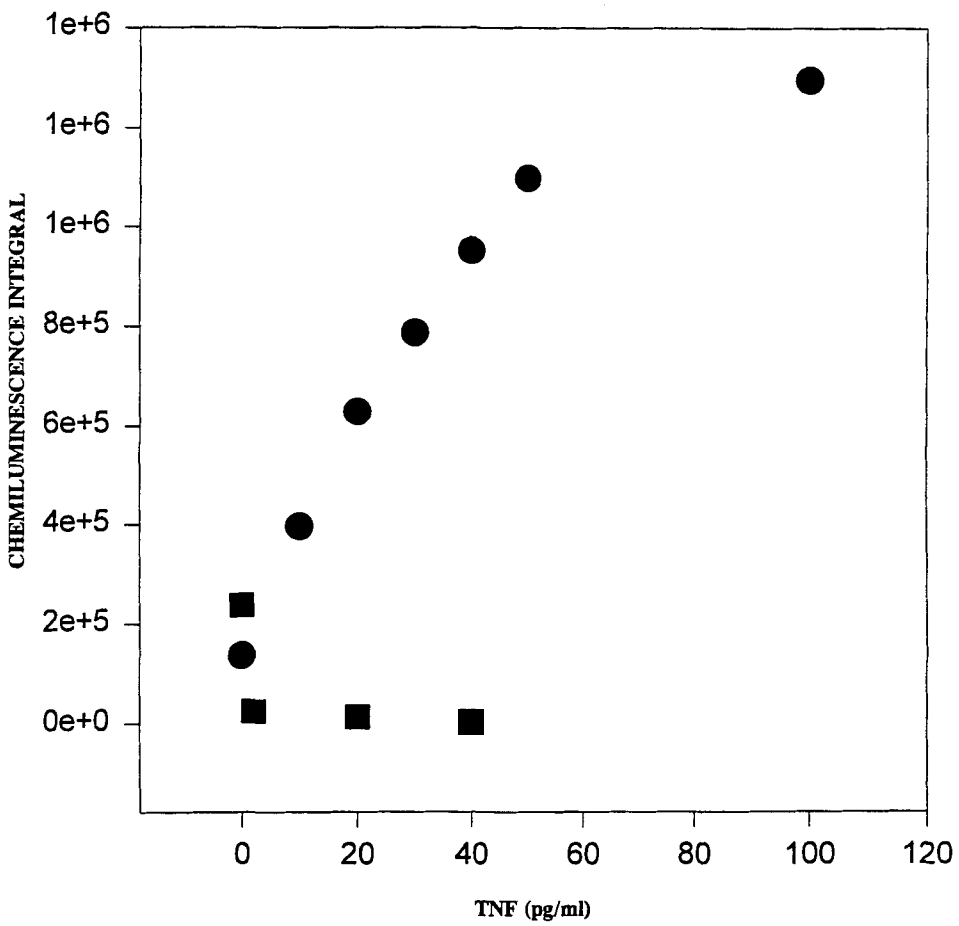

FIG. 1. Cell-free hydrogen peroxide chemiluminescence is altered by the presence of two different types of TNFα: ●, azide-free murine TNFα; ■, azide-containing human TNFα.

DISCUSSION

CL is a sensitive technique for monitoring free radical production in biological samples [3,4]. However, the results of our studies show that small amounts of sodium azide and/or bovine serum albumin may interfere with these assays. While the quantities of these preservatives in the azide-containing human TNFα used in this study remain unknown because of proprietary concerns, we have found that small amounts of azide and BSA significantly inhibit CL; thus this interference would be expected in reaction vessels when cytokines are used in experimental protocols. Our results further extend the findings of previous researchers who have noted azide-induced inhibition of CL [5,6]. In our study, not only did minute concentrations of sodium azide or BSA interfere with the CL assay, but this interference was not completely eliminated when the TNFα was dialyzed.

CL assays are increasingly utilized in a variety of laboratory applications ranging

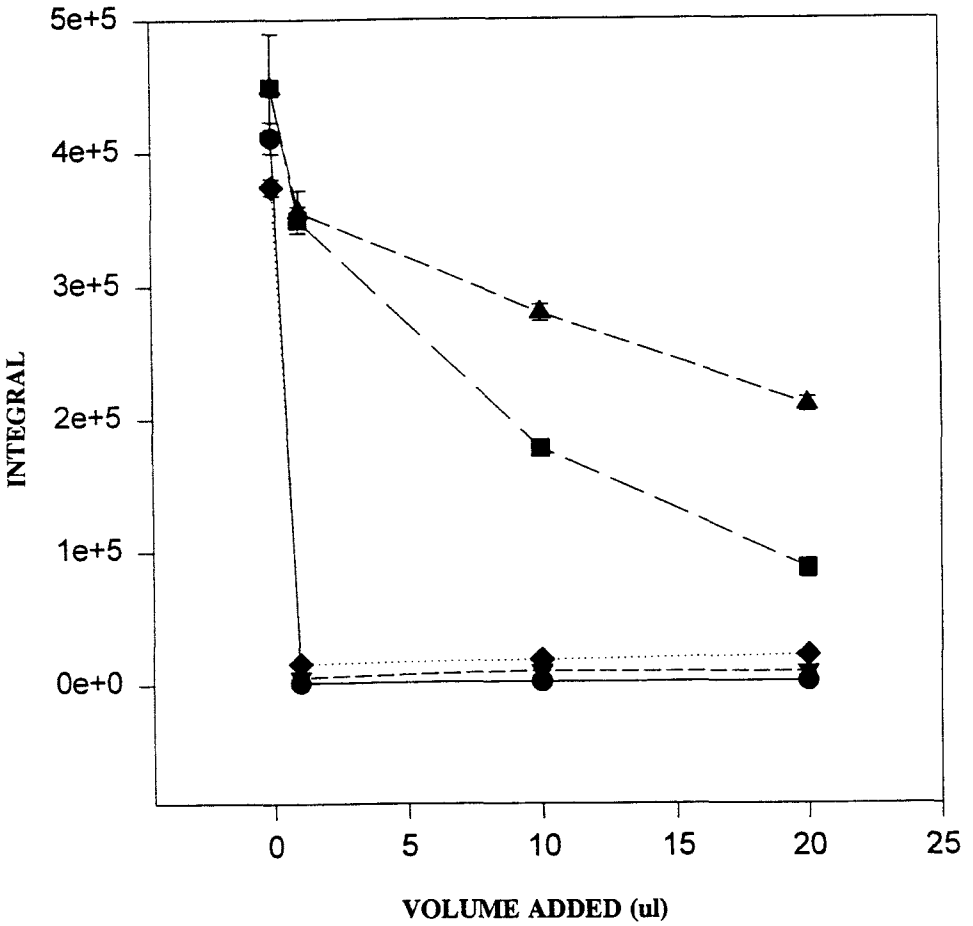

FIG. 2. Cell-free hydrogen peroxide chemiluminescence is inhibited in the presence of azide-containing human TNFα and different dialyzable fractions of this preparation: ●, azide-containing human TNFα; ■, dialyzable components smaller than 10 kD (sodium azide); ▲, dialyzable components 10–50 kD (TNFα); ▼, dialyzable components larger than 10 kD (TNFα and BSA); ♦, dialyzable components larger than 50 kD (BSA).

from cell activation studies to enzyme-linked CL immunoassays. These assays tend to be very sensitive and offer an alternative to radionuclide-based immunoassays. The interferences seen in our study with trace levels of preservatives and stabilizers are likely to be experienced by many investigators. These trace levels may explain the previously described free-radical quenching abilities of cytokines [2].

This study has several important implications. First, the effects of all reagents, regardless of their concentration, should be considered when performing CL assays; this includes trace concentrations of preservatives. Second, the concentrations of bovine serum albumin and other proteins recommended for the storage of cytokines and for preventing interactions with the reaction vessels may contribute to altered CL. Third, these interfering substances are not easily removed. Finally, detailed analyses of con-

taminating substances should be provided by manufacturers to researchers so that experiments can be appropriately controlled.

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