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Carcinoembryonic antigen inhibits neutrophil activation by N-formyl-methionyl-leucyl-phenylalanine

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ABSTRACT: Carcinoembryonic antigen (CEA) is a surface glycoprotein expressed in human epithelial cells and is released from their surface, especially during colorectal cancer. Frequently, colorectal cancer is accompanied by inflammation, where tumor-infiltrating neutrophils play an important role. CEA was also found to be a strong chemotactic agent for neutrophils. The purpose of this study was to find out if CEA can enhance neutrophil priming and activation. Primed neutrophils were activated by N-formyl-methionyl-leucyl-phenylalanine (formyl-MLP) and the resulting oxidative burst was measured luminometrically. Unexpectedly, *in vitro* priming of neutrophils by CEA, alone or preceded by LPS, inhibited subsequent activation of these cells by formyl-MLP. CEA may have anti-inflammatory properties *in vivo*.

Carcinoembryonic antigen (CEA or CEACAM5) is a surface glycoprotein of human epithelial cells of large intestine, esophagus and other organs (Hammarström & Baranov, 2001). The biological role of CEA is not clear, but its level significantly increases in serum during colorectal cancer, and the protein released from the cancer cells has been employed as a biological marker (Duffy, 2013). CEA is an adhesion molecule belonging to the immunoglobulin superfamily (IgSF) (Benchimol *et al.,* 1989; Oikawa *et al.,* 1989) and it can form aggregates in homophilic (CEA-CEA) and heterophilic (CEA-CEACAM1, CEA-CEACAM6) interactions (Benchimol *et al.,* 1989; Oikawa *et al.,* 1992). In addition, CEA displays strong chemotactic properties towards neutrophils *in vitro* (Ohwada *et al.,* 1995). Most chemoattractants prime and/or activate neutrophils for adhesion, triggering the process called the oxidative burst by producing reactive oxygen species (ROS) and the release of several enzymes including proteolytic ones, after cellular degranulation (El-Benna *et al.,* 2008). However, neutrophil chemotaxis and priming to the oxidative burst are not always coupled (Fumagalli *et al.,* 2007).

In this study, we tested the effect of CEA on priming and activation of human neutrophils. We chose to monitor the oxidative burst because this process can be assayed quantitatively with high sensitivity.

CEA without any preservatives was obtained from Fitzgerald Industries (North Acton, USA). *Escherichia coli* K12 LPS was kindly provided by Dr. Marta Kaszowska (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland).

Neutrophils were isolated as described by Ferrante and Thong (1980). Cell viability was greater than 98% in a Trypan blue exclusion test. Priming ability of the isolated neutrophils was tested with platelet-activating factor (PAF).

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Neutrophils were pre-incubated with the priming agent LPS (0.3 μg/ml) and/or CEA (0.05-0.3 μM) in Krebs-Ringer buffer (KRB) for 30 minutes at 37°C, then activated with N-formyl-methionyl-leucyl-phenylalanine (formyl-MLP). The priming activity is defined as the difference between levels of ROS produced by primed and non-primed neutrophils after addition of formyl-MLP.

The release of reactive oxygen species (mainly O_2-) in the oxidative burst was measured using a chemiluminescence method with luminol as described by Quinn *et al.*, (2007) with minor modifications. Briefly, a sample containing $1x10^5$ of primed neutrophils was added to individual wells in a microplate and incubated with luminol (50μM) in KRB buffer for 5 minutes at 37°C. Activation was triggered by addition of formyl-MLP (1μM final concentration), in a total reaction volume of 200 μl. Chemiluminescence was measured in counts per second (CPS) on a Victor Light 1420 Luminescence Counter (Perkin Elmer) for 30-60 minutes. All samples were run in triplicate.

The histograms represent activities of samples with CEA relative to the activity of the control (without CEA). The error bars represent propagated SEM (standard error of the mean). Statistical significance of CEA inhibitory effect was evaluated using Welch's t-test (n=3) for intra-assay analysis, and paired Student t-test (n=4) for inter-assay analysis of the chemiluminescence signals at 22 min in the second kinetic phase. IC_{50} values were calculated (using SigmaPlot 2001 v.7.0 software) by fitting the chemiluminescence data to the following equation: CPS = max/1+(x/IC_{50})^n, where max is maximal signal, x - CEA concentration, and n - Hillslope. The intra-assay standard errors for IC_{50} were in the range of 15% - 45%.

Fig. 1 (panels A and C) shows the obtained kinetic profiles of ROS generation by neutrophils. CEA alone (without addition of formyl-MLP) had no effect on neutrophils (Fig. 1A, curve -■) however, neutrophils primed with CEA produced less superoxide after the addition of formyl-MLP than non-primed neutrophils activated with formyl-MLP (Fig. 1A and Fig. 1C, curve -• and curve - ○). We focused our analysis on the second kinetic phase (18-35 min) of ROS production because it was longer and more reproducible than the first phase (0-7 min). The significant inhibitory effect

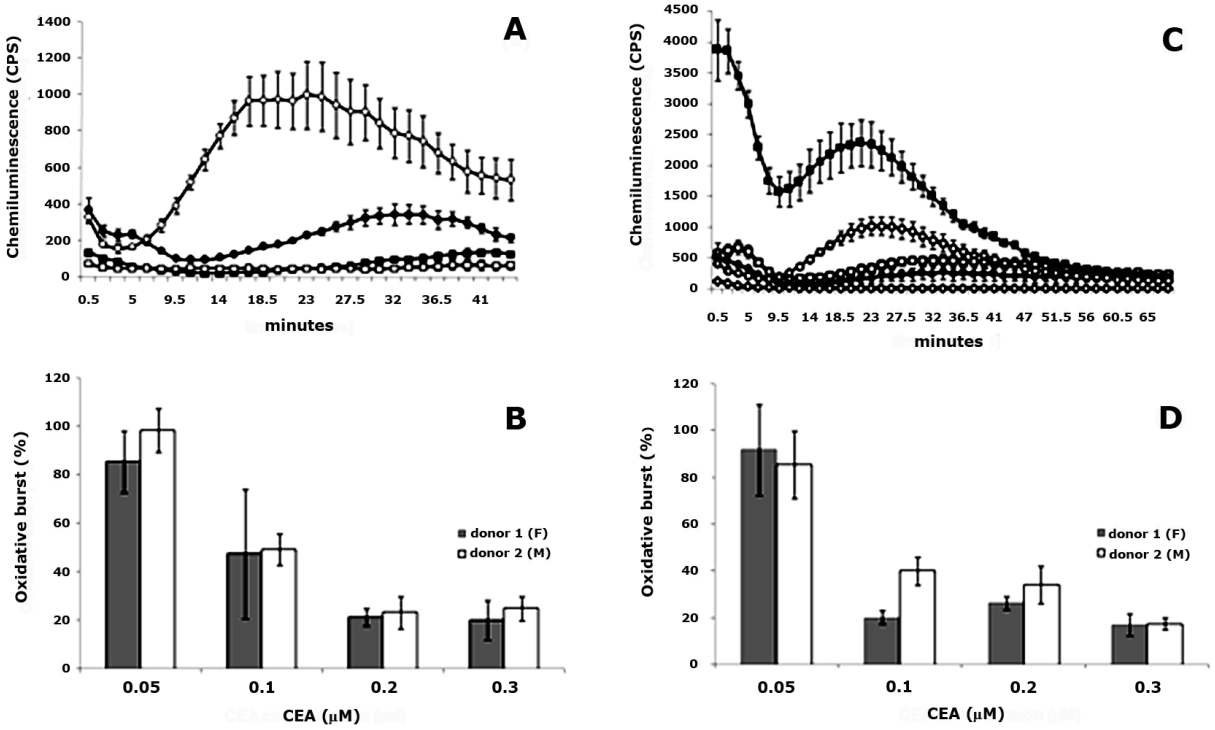

FIGURE 1. ROS generation from CEA (a, b) and LPS primed neutrophils (c, d) after exposure to formyl-MLP. (A) Representative kinetics of a few independent experiments. Neutrophils were primed with: ○ KRB buffer; • CEA (0.3μM) and then activated by formyl-MLP. In the negative control, ▫ KRB was added instead of the priming and activation agents. Neutrophils were also primed with ▪ CEA (0.3μM) without subsequent activation by formyl-MLP. The release of ROS was measured with luminol. (B) CEA concentration response data for ROS generation. Error bars represent propagated intra-assay SEM. F-female, M-male. For statistical significance see Results. (C) Representative kinetics of a few independent experiments. Neutrophils were primed with: ▪ LPS (0.15μg/ml); ○ KRB buffer; ▫ LPS (0.15μg/ml) with CEA (0.3μM); • CEA (0.3μM) and then activated by formyl-MLP. In the negative control, ◊ KRB was added instead of the priming and activation agents. The release of ROS was measured with luminol. (D) CEA concentration response data for ROS generation. Error bars represent propagated intra-assay SEM. F-female, M-male. For statistical significance see Results.

was concentration dependent (Fig. 1C) with IC_{50} values of 0.10 μM and 0.14 μM for donor1 and donor2, respectively. This observation prompted us to check if CEA added to LPS could inhibit the priming effect of the latter, and indeed that was the case. The result is presented in Fig. 1D. CEA incubated with LPS during the priming inhibited the subsequent activation of neutrophils (by formyl-MLP) with IC_{50} values of 0.14 μM and 0.16 μM for the two donors. The inhibition was statistically significant and the obtained p values from Welch's and Student paired t-tests were less than 0.05 and 0.005, respectively for CEA concentrations of 0.2μM and 0.3μM.

In this study we expected that CEA, as a chemotactic agent (Ohwada *et al.,* 1995)], would enhance priming and possibly activate neutrophils (Skubitz *et al.,* 2001). To our surprise, we observed the opposite effect, i.e., that CEA inhibited the oxidative burst, a typical indicator of neutrophil activity. Furthermore, additional experiments revealed that CEA strongly reduced the priming effect of LPS when both compounds were added together during priming of neutrophils. Other laboratories have demonstrated that neutrophil priming (with subsequent activation) and chemotaxis can be decoupled (Fumagalli *et al.,* 2007) but the inhibitory effect of CEA on the activation of the LPS-primed neutrophils (reported here) seemed unlikely.

The mechanisms of this inhibitory phenomenon should be explored. Since the IC_{50} values are the same in the presence or absence of LPS, we can hypothesize that CEA exerts its effect on signaling originating from the formyl-MLP receptor and not on signaling triggered by LPS during cellular priming. Another complementary hypothesis is that CEA, which is not expressed in neutrophils, may interact with neutrophil surface molecules such as CEACAM1 (Skubitz *et al.,* 2001; Stern *et al.,* 2005) which is known to inducing inhibitory signaling in leukocytes (Lu *et al.,* 2012).

Colorectal cancer is usually associated with inflammation in which associated neutrophils play an important role. ROS release during neutrophil activation by LPS or bacterial peptides may show genotoxic activity (Knaapen *et al.*, 2006). Activated neutrophils also release other agents (such as proteases) that play an important role in inflammation development (Wright *et al.,* 2010). Our results suggest that CEA may inhibit excessive neutrophil activation to oxidative burst under these pathological conditions, and in consequence reduce or slow down development of detrimental inflammation.

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