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ANAMNESTIC CHEMILUMINESCENCE OF MURINE SPLEEN CELLS

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Abstract

The oxidative response of murine spleen cells to secondary exposure to antigen was determined by luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) amplified chemiluminescence, CL. BALB/cj and CBA/J mice were immunized with saline or an antigen solution of saline, luminol, and bovine serum albumin. Spleen cells were obtained from mice two and four days after immunization, and the CL response to *in vitro* antigenic exposure was measured for 35 minutes. At two days post-immunization, there was no difference in the CL of control and antigen-primed cells. By day four, the antigen-primed CL response differed significantly in both magnitude and time course from the primary antigen-stimulated response of the controls. This early development of differential CL response to antigenic challenge suggests a role for oxidative metabolic activity in the expression of the anamnestic immune response.

Introduction

The production of peroxides and other active oxygen species is one of the earliest measurable signals of immunogenic stimulation (1,2). Alterations in oxidative activity have long been correlated with the phagocytic and bactericidal activity of macrophages and polymorphonuclear neutrophils, although CL has also been associated with the activation of lymphocytes (2) and thymocytes (3). Increased peroxidase activity of mouse splenic lymphocytes (4,5) and human peripheral lymphocytes (6) in response to antigenic exposure *in vitro* has been reported. *In vivo* exposure to antigen also results in enhanced activity of the enzyme (1). That oxidative metabolic activity plays an early role in immune cell function is apparent. This paper reports the rela-

tionship of murine splenocyte in vitro CL with previous in vivo exposure to antigen.

Materials and Methods

Reagents. Hank's Balanced Salt Solution (HBSS) buffered with 20 mM HEPES, pH 7.4, was the medium for all experiments. The luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione) mixture for immunizations was prepared by suspending 3 mg/ml luminol (Baker Chemical Co., Phillipsburg, NJ) and dissolving 3 mg/ml Bovine Serum Albumin, BSA (Sigma Chemical Co., St. Louis, MO) into phosphate buffered saline, PBS, pH 7.4. The luminol indicator for the CL assay was a 1:5 dilution of a freshly prepared solution of 1 mg/ml luminol, 1 mg/ml BSA in PBS, pH 6.9. The luminol mixtures were prepared at room temperature, filtered (Gelman Acrodisc, 0.2 μ m) to remove undissolved luminol and stored at 4°C in the dark until use.

Animals. Male CBA/J or BALB/cj mice (Jackson Laboratories, Bar Harbor, ME), 25-30 g., were maintained on a 12/12 light/dark cycle with food and water ad libitum. Control animals were immunized with 0.5 ml saline (subcutaneous or intraperitoneal) and experimental animals received 0.2 ml luminol-BSA antigen (subcutaneous). CL was assayed 2 or 4 days after immunization.

Spleen Cell Preparation. Mice were killed by rapid cervical dislocation and their spleens removed. The spleens were gently teased in room temperature HBSS (20 mg spleen/ml HBSS). The suspension was centrifuged at 6 x g for 3 minutes to remove residual tissue. Cell viabilities were determined by exclusion of trypan blue dye, and the cell concentration was adjusted to $8-12 \times 10^3$ viable cells/mm³. Differential counts made of stained fixed slides showed suspensions to be 94-96% lymphocytes. One ml samples in dark-adapted glass scintillation vials were incubated for 25 min at 37°C in a shaker water bath.

Assay of Chemiluminescence. After the incubation period, 100 μ l of the luminol-antigen mixture was added to each vial. CL was measured before, at, and every 5 minutes after the addition of luminol, on a Beckman model LS230 liquid scintillation counter in the "out of coincidence" mode (gain 350, preset 0.2 min, window 0-100). Cells were maintained at 37°C between CL readings.

Statistical Analysis. A repeated measures analysis of variance was used to determine differences in the time dependent variation of the CL response between groups. The average CL value for each group, determined from the total of all CL measurements between T=5-35 min, was compared using Duncan's multiple range test.

Results

The CL response of murine splenocytes to in vitro primary and secondary exposure to antigen is illustrated in Figure 1. As no differences were seen in the responses of the two strains of mice, the data were pooled. At two days post-immunization, the oxidative activity was higher, although not sig-

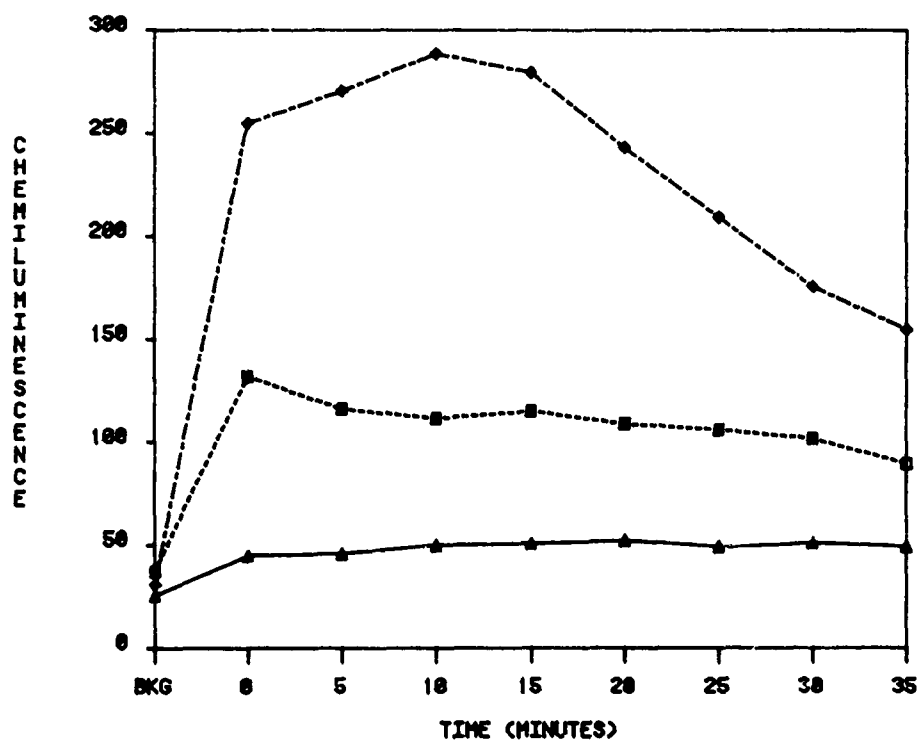


FIGURE 1.

Chemiluminescent response of murine spleen cells to primary and secondary antigenic exposure. The oxidative response to antigen was determined for unprimed cells (Δ - Δ) and cells taken from mice two days ($\square$ - $\square$) and four days ($\diamond$ - $\diamond$) after immunization. Luminol amplified CL is expressed in 10^3 cpm. Points represent the mean of 3 or 4 animals.

nificantly different, from that of unprimed cells. By four days, however, the response was greatly enhanced; the time course of CL differed at the $P=0.0001$ level, and mean overall CL was significantly greater (Table I) than for either control or two-day groups.

Discussion

The CL responses shown here demonstrate the enhancement of antigen stimulated oxidative metabolic activity which results after prior in vivo immunologic exposure. Whether the change in oxidative activity, as measured here by CL, is a cause or by-product of the anamnestic response is unclear; however, it is certain that alterations in oxidative activity play a role in the very early immune response to secondary antigenic challenge. Both the production of peroxides per se and the resulting peroxidation of membrane surface macromolecules may be involved in the anamnestic immune response.

The increase in CL activity seen at 4-days post-immunization supports previous reports of enhanced oxidative capacity in activated lymphocytes. CL and H_2O_2 production have been described in antigen and mitogen stimulated

TABLE I
Total and average chemiluminescent responses of murine
splenocytes following in vivo exposure to antigen

Group	Total CL ^a (cpm x 10 ³)	Average CL ^b (cpm x 10 ³)
Control (4) ^c	169.4 ± 68.7 ^d	24.2 ± 25.9 (28) ^e
2 Day (3)	484.7 ± 155.6	69.2 ± 58.8 (21)
4 Day (3)	1405.8 ± 439.8	200.8 ± 166.2 ^f (21)

^acpm above background from T=5-35 min

^bMean of all CL measurements from T=5-35 min

^cNumber of animals

^dMean ± S.D.

^eNumber of measurements

^fMean differs from both control and 2 Day; $p<.01$, Duncan's multiple range test

mouse (3) and rat (2) lymphocytes. Paul et al (4) reported peroxidase activity in murine splenocytes in the presence of particulate antigens. The splenic peroxidase (SPO) increased in activity after animals were immunized with soluble or particulate antigens, with maximal enzyme activity preceeding the appearance of antibody producing cells. In the presence of H_2O_2 , this SPO is bactericidal in vitro (5) and also mediates the deamination of amino acids and production of CO_2 and aldehydes (7).

Peroxidation of the cell surface resulting from the increased CL activity seen here may serve in lymphocyte activation and immuno-amplification. Cell surface modification by sodium periodate or neuraminidase and galactose oxidase has been shown to induce blastogenesis and nonspecific cytotoxicity in normal mouse lymphocytes (8,9,10) and antigen specific cytotoxicity in "memory" cells which had been previously exposed to antigen in vivo or in vitro (9,10). Additionally, $NaIO_4$ or NAGO modified cells can indirectly stimulate secondary cytotoxicity from syngeneic cells in mixed lymphocyte culture (11). In rat lymphocytes, indirect NAGO stimulation selectively activates cytotoxicity rather than blastogenicity (12). The stimulatory effects of membrane peroxidation are dependent upon the presence of induced free surface aldehydes (8,11) which may crosslink membrane macromolecules (via a Schiff base) on the same cell or between cells (8).

It is possible, then, that initial antigenic exposure gives rise to a population of "memory lymphocytes" containing enhanced SPO activity, which may be localized at the cell membrane (1). Upon secondary antigenic challenge, these cells produce or induce the anamnestic oxidative response we report here; with the generated H_2O_2 (in the presence of SPO) inducing free aldehydes (7) on cell surfaces. Direct peroxidation of nearby normal lymphocytes results in blastogenesis and some nonspecific cytotoxicity (8,10); peroxidation of memory lymphocytes results in antigen specific cytotoxicity (9,10) and indirect stimulation of normal, surrounding lymphocytes selectively stimulates cytotoxicity (12). In such a way, enhanced oxidative response to secondary antigenic challenge and the resulting peroxidation of cell surface macromolecules may orchestrate the amplification of specific and nonspecific anamnestic immune activity.

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