GENERAL PATHOLOGY AND PATHOLOGICAL PHYSIOLOGY

Generation of Free Oxygen Radicals and Clastogenesis in Bone Marrow Cells in MRL/1 Mice

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The level of chemiluminescence induced by phorbol myristate acetate or opsonized zymosan in the bone marrow cells of 5-6-month-old male MRL/1 mice is significantly higher than that in the same cells of 1-2-month-old mice. The number of chromosome aberrations induced by the prooxidant mutagen dioxydine in bone marrow cells *in vivo* is significantly higher in 5-6-month-old MRL/1 mice than in 1-2-month-old mice. It is suggested that these effects are more pronounced in 5-6-month-old animals due to progressive development of rheumatic pathology in this mouse strain.

Key Words: *MRL/1 mice; autoimmune pathology; chemiluminescence; chromosome aberrations; dioxydine*

The evidence that reactive oxygen species are involved in the pathogenesis of autoimmune and rheumatic diseases [4,6,8,10,11] and the insufficiency of the antioxidant defense system in autoimmune diseases [10,14,15] suggest that free radical processes contribute to autoimmune and rheumatic pathologies.

On the other hand, mutagenic effects of some chemical prooxidants are associated with oxidative stress and are mediated by reactive oxygen species and lipid peroxidation products, which damage genetic structures [3,6,16]. Based on these findings and on the assumption that these pathologies are accompanied by the disturbances in the antioxidant defense system, we have hypothesized that rheumatic patients are more susceptible to the mutagenic effects of prooxidants.

MRL/1 mice with age-related rheumatism have been widely used in the investigation of rheumatic diseases [5,12,13]. We studied the production of reactive oxygen species by bone marrow cells from male MRL/1 mice of different ages in response to phorbol myristate acetate (PMA) and opsonized zymosan (OZ) and assessed the *in vivo* mutagenic effect of the prooxidant dioxydine.

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MATERIALS AND METHODS

Experiments were carried out on male MRL/1 mice aged 1-2-, 3-4-, and 5-6-months (Institute of Rheumatology, Russian Academy of Medical Sciences).

Generation of reactive oxygen species was measured by luminol-dependent chemiluminescence (CL) [1,7] induced by OZ or PMA.

Bone marrow cells were washed from the femur with medium 199 (37°C). The cells were centrifuged at 1000 rpm for 5 min in an OPN-3 centrifuge, washed three times, counted, and adjusted to a concentration of 5×10^6 cell/ml with the same medium. Measurements were carried out at 37°C in a PKhL-0.1 chemiluminometer with constant stirring with the

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TABLE 1. Spontaneous Clastogenesis in MRL/1 Male Mice of Different Age

Age, months	Number of examined cells	Gaps	Single fragments	Double fragments	Exchanges	Total number of aberrant cells, % ($M\pm m$)
		per 100 cells				
1-2	400	1.0	2.0	—	—	2.5±0.8
3-4	300	1.0	0.6	—	—	1.6±0.7
5-6	700	0.5	2.7	—	0.5	3.0±0.7

use of a buffer containing 110 mM NaCl, 10 mM Tris, 5 mM glucose, and 0.65 mM luminol (pH 7.4). The cell suspension (100 μl) in a volume of either 860 μl (stimulation with PMA) or 890 μl (stimulation with OZ) was mixed with the buffer in a measuring cuvette, and spontaneous CL was recorded for 15 min. Then 10 μl PMA dissolved in DMSO (0.1 mg/ml) or 40 μl OZ (10 mg/ml) was added to the cuvette. Zymosan was opsonized with serum from random-bred male mice. The following CL parameters were measured: maximum amplitude of spontaneous CL flash, latency (period from the addition of the stimulus to the rise of CL), time and amplitude of CL peak, and time of half-decay of stimulated CL. The results were analyzed using the Student's t test.

The clastogenic effect of dioxydine was assessed from the number of chromosome aberrations in the metaphase bone marrow cells [9]. Dioxydine was dissolved in warm physiological saline *ex tempore* and administered intraperitoneally in a dose of 300 mg/kg.

Cytogenetic specimens were prepared 24 h post-injection. The mice were injected with colchicine (2.5 mg/kg, Ftrak Berlin) for the metaphase accumulation and killed by cervical dislocation after 2.5 h. Cells with achromatic gaps, singe and double fragments, and chromosome exchanges and cells with multiple chromosome aberrations (more than 5 per cell) were counted under a Standard-20 microscope at a magnification of 1000. One hundred metaphases were analyzed from each mouse (each experimental group consisted of 5 mice). The numbers of cells with chromosome aberrations including gaps in different experimental groups were compared and analyzed statistically using ϕ test.

RESULTS

Typical curves of PMA-stimulated CL in the bone marrow cell suspension from 1-2- and 5-6-month-old MRL/1 mice are shown in Fig. 1, *a*. On the 12th-15th min, the level of spontaneous luminol-dependent CL in cells of 1-2-month-old animals was 400-500 mV. After the addition of PMA, the intensity of CL was 1000-1300 mV, the time of half-decay being 2.5 min.

The same intensity of spontaneous CL was observed in cells of the 5-6-month-old mice: on the 14th-15th min it was 400-500 mV. After the addition of PMA, the intensity of CL rose to 3000-6000 mV, which was 4- to 6-fold higher that the maximum CL in the younger animals ($p<0.001$). The activation lasted 73-90 sec, and the amplitude decreased 2-fold after 2-3 min.

PMA is a metabolic stimulator which acts via protein kinase C [2]. Since stimulation of phagocytes is usually receptor-mediated [2], we applied OZ which interacts with membrane receptors.

In the cell suspensions from 1-2- and 5-6-month-old mice, the OZ-induced CL flash reached the maximum during 4-5 min and practically did not decay over the recording period (Fig. 1, *b*). However, the intensity of CL response to OZ was significantly lower in young than in senescent mice: 900-1300 *vs.* 2700-4600 mV ($p<0.001$). Thus, the amplitude of spontaneous CL was practically the same in the two age groups, while the CL response to both stimuli was significantly higher in 5-6-month-old mice ($p<0.001$).

The same CL response to OZ and PMA was observed in cells of 1-2-month-old animals (Fig. 1), while the response of cells of 5-6-month-old mice to PMA was more intense than that to OZ ($p<0.05$). In cells of 1-2-month-old mice, the intensity of CL response to both stimuli was equal, whereas with age the sensitivity to the stimulating effect of PMA increased to a higher extent. This effect is probably related to the age-dependent sensitization of cells and low activity of the antioxidant system.

Our findings suggest that the magnitude of the mutagenic effect of the prooxidant dioxydine in mice is also age-dependent.

There are no published data on the level of spontaneous mutagenesis in MRL/1 mice. Therefore, we performed cytogenetic analysis of bone marrow metaphases from intact MRL/1 mice of different ages.

The analysis revealed 2.5±0.8% aberrant cells in 1-2-month-old mice, which does not differ signi-

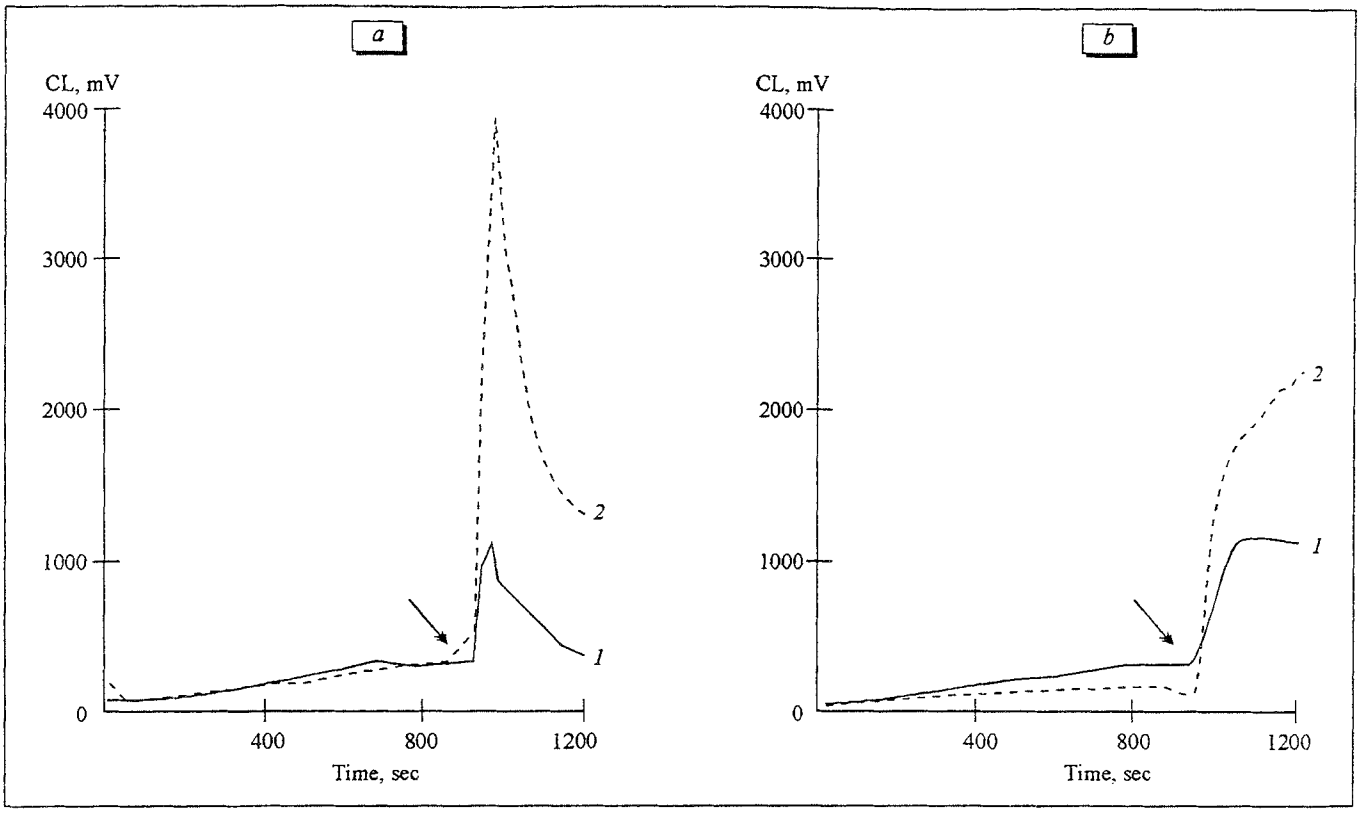

Fig. 1. Typical chemiluminescence (CL) curves for the bone marrow cells of MRL/1 mice stimulated with phorbol myristate acetate (PMA, *a*) and opsonized zymosan (OZ, *b*). Bone marrow cells from 1-2-month-old (*1*) and 5-6-month-old (*2*) MRL/1 male mice. Arrows indicate the addition of PMA and OZ.

ficantly from the mean level of mutations in mice of other strains [6]. No significant changes in the level of spontaneous mutagenesis were found in 3-4- and 5-6-month-old animals, in whom the abnormal cells constituted 1.6±0.7 and 3.0±0.7%, respectively. However, exchanges (apart from gaps and single fragments) were detected in 5-6-month-old but not in the younger mice (Table 1).

Cytogenetic analysis performed after a single intraperitoneal injection of the prooxidant dioxydine [3] in a dose of 300 mg/kg revealed a significant increase in the number of chromosome aberrations in all mice in comparison with the control: 12.2± 1.5%, 16.4±1.7%, and 32.0±2.3% in 1-2-, 3-4-, and 5-6-month-old animals respectively. These data indicate that the cytogenetic effect of dioxydine in male MRL/1 mice is age-dependent (Table 2). Older mice are more sensitive to the clastogenic effect of the prooxidant than the younger mice. It is noteworthy, that 5-6-month-old mice are more sensitive to the respiratory burst activators than 1-2-month-old mice.

Thus, with the use of two independent approaches we have demonstrated a higher sensitivity of MRL/1

TABLE 2. Clastogenic Effect of Dioxydine (300 mg/kg) in MRL/1 Male Mice of Different Age

Age, months	Number of examined cells	Gaps	Single fragments	Double fragments	Exchanges	M.A.	Total number of aberrant cells, % (*M*±*m*)
		per 100 cells					
1-2	500	—	4.8	0.2	0.2	5.2	12.2±1.5*
3-4	500	0.6	11.0	0.6	—	7.8	16.4±1.7*
5-6	400	—	18.3	0.8	0.3	18.0	32.0±2.3**

Note. M.A. — cells with multiple chromosome aberrations and gaps (>5 chromosome aberrations and gaps per cell); $p<0.001$: *compared with 5-6-month-old mice, **compared with two other age groups.

mice with advanced rheumatic pathology to prooxidants. Our findings suggest that the more pronounced effects of prooxidants in senescent animals result from reduced activity of the antioxidant defense system related to both aging and propagation of rheumatic processes.

This study proves the involvement of reactive oxygen species in the development of rheumatic diseases, suggesting that rheumatic patients are much more sensitive to environmental prooxidant mutagens than healthy individuals.

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