# EFFECT OF LEVAMISOLE ON THE CYTOTOXIC ACTIVITY OF NORMAL LYMPHOCYTES IN TISSUE CULTURE

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Preliminary incubation of normal nonimmune splenic lymphocytes from C57BL/6j and BALB/c mice for 45 min at 37°C with levamisole ($1 \cdot 10^{-4}$ to $1 \cdot 10^{-5}$ M) followed by washing potentiated the cytotoxic effect against allogeneic target cells (L cells). When lymphocytes and levamisole were added to the culture of target cells simultaneously no cytotoxic effect occurred.

KEY WORDS: levamisole; lymphocytes; cytotoxic effect; target cells.

Recent investigations have confirmed that levamisole [1,2,3,5,6-tetrahydro-6-phenylimidazo-(2,1-b)thiazole hydrochloride] is an immunostimulant and not an antianergic agent. Data have been described on stimulation of normal immunity in healthy subjects [3,5,10-12,14,15]. The stimulating action of levamisole on phagocytosis [7], mobility and chemotaxis of neutrophils [3], monocytes, and macrophages [8], and activity of membrane receptors toward IgG and the C'3 component of complement [12] has been established. In low concentrations levamisole potentiated blast transformation of lymphocytes [5, 11] and lymphokinin production in response to suboptimal doses of mitogens [6, 14]. The stimulating action of levamisole on the suppressor function of lymphocytes from the healthy human thymus and spleen has been demonstrated [11]. Under the influence of levamisole there is an early switch of immunoglobulin synthesis from IgM to IgG [9], a property of activated lymphocytes of T-helpers [13]. Levamisole, injected into mice simultaneously with transplantation of allogenic tumor cells, stimulated T-cell cytotoxicity within a narrow range of relative doses of antigen and levamizole [4]. The object of the present investigation was to study the effect of levamisole on induction of nonspecific cytotoxicity of lymphocytes relative to allogeneic target cells using a model of transplantation immunity *in vitro*.

## METHODS

Healthy inbred C57BL/6j and BALB/c mice weighing 18-20 g, from the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR, were used. The mice were killed, autopsied with sterile precautions, and the cervical, submandibular, paratracheal, axillary, subclavian, and inguinal lymph nodes were removed. Lymphocytes were obtained from the lymph nodes and spleens in a Potter's homogenizer. The suspension was filtered through two layers of gauze and washed by centrifugation at 1000 rpm for 10 min. The lymphocytes were diluted to a concentration of $5 \cdot 10^7$ cells/ml. Levamisole (from Jansenn Pharmaceuticals) was diluted in medium No. 199 and used in concentrations of $1 \cdot 10^{-4}$ (24.1 μg/ml) and $1 \cdot 10^{-5}$ M (2.41 μg/ml). The lymphocyte suspension with levamisole solution in the ratio of 1 : 1 was incubated for 45 min at 37°C in an atmosphere containing 5% $CO_2$. They were then washed 3 times by centrifugation in medium No. 199 at 100 rpm for 10 min. To tubes containing a 24-h culture of target cells $4 \cdot 10^6$ live lymphocytes were added. Living and dead cells among the lymphocytes were counted beforehand by Schreak's method in Brondz's modification [1]. Surviving fibroblasts undergoing malignant change (L-cells), obtained initially by Earle from C3H mice, were used as target cells. The cells were cultured in medium No. 199 with the addition of 10% inactivated bovine serum. For the experiment the L cells were removed from the glass with 0.25% trypsin solution, transferred to test tubes in a volume of 1 ml containing $1 \cdot 10^5$ cells, and cultured for

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TABLE 1. Interaction of Allogeneic Lymphocytes, Incubated with Levamisole and Washed Free from It, with L-Cells

| Number of living L-cells (×1000) after incubation for 48 h | | | | | | | |
|---|---|---|---|---|---|---|---|
| with lymph node lymphocytes, incubated with medium No. 199 | with lymph node lymphocytes, incubated with levamisole | percentage cytotoxicity | P | with splenic lymphocytes, incubated with medium No. 199 | with splenic lymphocytes, incubated with levamisole | percentage cytotoxicity | P |
| 297,0±8,6† | 300,0±10,1 | n.d. | >0,05 | 238,0±5,5† | 22,25±4,3 | 90,65 | <0,001 |
| 170,0±7,5† | 137,3±13,1 | 19,3 | >0,05 | 74,9±3,5† | 43,3±4,2 | 42,2 | <0,001 |
| 191,0±4,2 | 194,0±6,0 | n.d. | >0,05 | 150,5±8,6 | 98,5±5,7 | 34,6 | <0,001 |
| 175,5±12,9 | 174,5±4,06 | n.d. | >0,05 | 56,5±3,16 | 28,0±1,3 | 50,4 | <0,001 |
| 194,0±10,0* | 198,0±9,8* | n.d. | >0,05 | 209,0±1,24* | 38,0±3,9* | 81,8 | <0,001 |
| 178,0±14,7* | 179,5±5,8* | n.d. | >0,05 | 226,0±7,2† | 40,0±4,7 | 82,3 | <0,001 |
| 56,9±8,25* | 34,5±5,4* | 38,9 | >0,05 | | | | |

Legend: 1) n.d. — no difference. 2) *$1 \cdot 10^{-5}$M levamisole, in all other cases concentration used was $1 \cdot 10^{-4}$M; †lymphocytes of BALB/c mice; in all other cases lymphocytes of C57BL/6j mice were used.

18-24 h at 37°C in the sloping position. Before addition of the lymphocytes, the tubes containing the culture were examined under the microscope. Only tubes with a uniform monolayer of cell growth were selected for the experiment. Immediately before addition of the lymphocytes, the culture fluid was removed from the tubes with the target cells. Each group consisted of six tubes. Lymphocytes from lymph nodes or spleens incubated under similar conditions with medium No. 199 served as the control material. Tubes containing cells to which medium No. 199 was added instead of lymphocytes served as the general control. The ratio of lymphocytes to target cells was 40 : 1. In the experiments in which levamisole was added directly to the cell culture, the lymphocytes were made up in a solution of levamisole.

Tubes of all groups were incubated for 48 h at 37°C. The liquid was then poured from all the tubes, 0.25 ml trypsin was added to each tube, and incubation followed. After 10-15 min 1 ml of a mixture of 0.1% eosin and 0.1% trypan blue in the ratio of 1 : 1 was added to each tube. The number of living unstained target cells was counted in two grids of a Goryaev's chamber.

## RESULTS

As Table 1 shows, preliminary incubation of lymphocytes from C57BL/6j and BALB/c with $1 \cdot 10^{-4}$-$1 \cdot 10^{-5}$ M levamisole for 45 min followed by washing endowed the splenic lymphocytes with the property of destroying allogeneic target cells. The percentage of cytotoxicity was high, namely 90.7, 82.3, and 81.8 in three experiments respectively ($P < 0.001$). In other experiments, despite the nonspecific cytotoxic activity of the lymphocytes in this system toward target cells [2], the effect of levamisole also was very clearly observed and was statistically significant. The same tendency was found after similar treatment of lymphocytes from lymph nodes of the same mice, but no statistically significant differences could be found between the groups ($P > 0.05$).

Levamisole, added in the same concentrations directly to the culture of L cells, alone or together with lymphocytes, did not cause destruction of the monolayer.

The absence of any cytotoxic action of the lymphocytes when added together with levamisole was evidently due to the effect of the latter on the lymphocytes and not to their interaction with the target cells.

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# EXPRESSION OF VIRAL ANTIGENS ON THE MEMBRANE OF NORMAL AND LEUKEMIC THYMOCYTES OF AKR MICE

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Expression of antigenic determinants of structural proteins G-MuLV (p10, p12, p30, gp14, gp17) and R-MuLV (gp69/71, p15) on thymocytes of normal and leukemic AKR mice was stuided by the membrane immunofluorescence method. A sharp difference was found with respect to this feature between normal and malignant thymocytes. The possible role of antigens of structural viral proteins MuLV, expressed on the membrane of leukemic cells, in antitumor immunity is discussed.

KEY WORDS: immunofluorescence; antigenic determinants; expression; structural proteins MuLV; thymocytes.

Mouse leukemia cells induced by oncogenic viruses of the C type contain antigenic determinants of viral structural proteins on their membrane [2, 3, 12, 13, 15]. Some of them are responsible for the reaction of the cells with hyperimmune mouse sera [6, 11, 13], and antibodies against structural proteins MuLV have been found in the sera of normal mice [8, 11]. It has recently been shown that antigenic determinants of internal structural proteins MuLV are components of glycolyzed polyprotein molecules which are themselves integral components of the cell membrane [11, 13, 15]. They may perhaps partly determine the tumor phenotype and they are evidently largely responsible for immunologic relations between the host and the tumor.

In the investigation described below an immunofluoresence method was used to study expression of antigens of viral structural proteins on the membrane of normal and leukemic thymocytes from AKR mice.

## METHODS

AKR mice were obtained from N. N. Medvedev (Hybrid Mice Nursery, N. F. Gamaleya Institute of Epidemiology and Microbiology). Thymocytes from AKR mice aged 2-4 and 8 months served as the test cells. Goat sera against individual structural proteins, products of the env gene (gp14 and gp17 G-MuLV, gp69/71 R-MuLV) and products of the gag gene (p10, p12, p30 G-MuLV and p15 R-MuLV) were obtained by J. T. August and M. Strand USA) [14]. The sera were absorbed by sheep's red blood cells and spleen cells of normal BALB/c mice and were neutralized with mouse γ-globulin as described previously [2]. The immunofluorescence test was carried out on an artificial monolayer of living cells obtained by incubation of a suspension for 10 min on a slide in a humid chamber. Rabbit antibodies against sheep γ-globulin were obtained by B. P. Bogovskii on a γ-globulin glutarate immunosorbent [4] and were conjugated with fluor-

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