Cancer Immunol Immunother (1984) 17: 195–199

Cancer Immunology Immunotherapy

Effect of levamisole on cytotoxic T-cell-mediated immune resistance to L1210 murine leukemia in hyperimmune mice

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Summary. *The effect of levamisole (LMS) on T-cell-mediated antitumor immunity was examined in adult and aged mice hyperimmune to L1210 leukemia. The immune resistance of aged mice was depressed compared with that of adult mice, which almost completely rejected 5 × 10^7 L1210 cells inoculated IP. A significant level of tumor-specific cytotoxicity was detected in the spleen cells of adult hyperimmune mice by the ^{51}Cr-release assay after in vitro sensitization with mitomycin C-treated L1210 cells. This was mediated by cytotoxic T cells, since in vivo administration of antithymocyte serum or in vitro treatment of the spleen cells with anti-Thy 1.2 antibody and complement abrogated the cytotoxicity completely. In aged mice, however, cytotoxic T-cell activity was lower although the animals were immune to L1210.*

Administration of LMS (0.38 mg/kg) restored the depressed cytotoxicity of aged mice to the level seen in adult mice. Furthermore, in adult hyperimmune mice LMS augmented T-cell-mediated cytotoxic activity and restored the reduced cytotoxicity caused by in vivo administration of antithymocyte serum. These results indicate that LMS was effective in augmenting T-cell-mediated tumor immunity in immunologically competent or deficient hosts.

Introduction

The depression of cell-mediated immunity with aging has been found in many investigations to be related to the mitogen response of spleen cells [1, 5], helper T cells [12], or cytotoxic T cells against alloantigens [7, 20]. Levamisole (LMS), a synthetic immunomodulator, was shown to be effective in restoring depressed antibody production against sheep red blood cells in aged mice [3, 15]. This activity might be attributable to the stimulatory effect of LMS on the differentiation or maturation of T cells [16–18].

L1210 leukemia, a chemically induced transplantable tumor that has frequently been used for the screening or evaluation of antitumor agents [6], has been considered to be a low-immunogenic tumor, since the inoculation of a single cell can lead to death in syngeneic hosts [21]. Although L1210 leukemia has been also used in investigations of immunotherapy, the postulated mechanisms of immunity to L1210 leukemia are neither consistent nor well documented. A humoral factor [13, 19] and peritoneal exudate cells [2, 19] have been variously proposed as the responsible effectors. We previously reported that T cells in the spleen and peritoneal exudate were responsible for the immunity of hyperimmune mice to L1210 leukemia according to an in vitro growth inhibition assay [10]. This result suggested that effector T cells in hyperimmune mice might be cytotoxic against L1210 cells.

The present study was carried out to examine whether the immune resistance of hyperimmune mice to L1210 leukemia was mediated by cytotoxic T cells. Furthermore, the influence of aging on the immune resistance of hyperimmune mice and the effect of LMS were investigated.

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Materials and methods

Cells and animals. L1210 and P388 murine leukemic cells were collected from ascites of male DBA/2Cr mice, while for the experiments male BALB/c × DBA/2CrF$_1$ (hereafter called CD2F$_1$) mice were used. The animals were supplied by Simonsen Laboratories, Gilroy, Calif, and Laboratory Supply Co., Indianapolis, Ind, respectively. CD2F$_1$ mice were rendered immune to L1210 cells by the procedures reported previously [8, 9]. Briefly, mice sensitized IP with concanavalin A-bound L1210 vaccine cells alone or combined with immunopotentiators were challenged IP with 10^2 or 10^3 live L1210 cells. Resistant mice were subsequently challenged IP with increasing numbers of live L1210 cells at 4- to 8-week intervals. Unless specifically stated, mice inoculated four times with 10^7 L1210 cells were used as hyperimmune mice 1 month after the final inoculation. Adult and aged hyperimmune mice were 13–15 and 21–23 months old, respectively.

Drug and agent. LMS (Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan) and rabbit anti-mouse thymocyte serum (Microbiological Associates Inc., Bethesda, Md) were dissolved in sterile 0.9% NaCl solution.

Culture medium. Roswell Park Memorial Institute Tissue Culture Medium 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY), 50 μ*M* 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd, Osaka, Japan). 100 U penicillin (Meiji Seika Co., Tokyo, Japan) per milliliter and 100 μg streptomycin (Kyowa Hakko Kogyo Co.) per milliliter was used as culture medium.

Preparation of spleen cells. Spleens prepared from groups of four or five hyperimmune mice were collected aseptically,

pooled on ice, and squeezed between two glass slides in culture medium. The cell suspensions were passed through a layer of four sheets of gauze to remove residual large fragments and centrifuged at 100 *g* for 5 min. Then cells were suspended in culture medium and adjusted to the stated cell concentration after viability had been scored by the trypan blue dye (Tokyo Kasei Co., Tokyo, Japan) exclusion method. Viability was over 80%.

Induction of in vitro cytotoxicity. Spleen cells were stimulated in vitro with mitomycin C (Kyowa Hakko Kogyo Co.)-treated L1210 cells. Mitomycin C-treated L1210 cells were prepared as follows. L1210 cells (10^7) were incubated with 100 μg mitomycin C per ml at 37° C for 1 h in Dulbecco's phosphate-buffered saline (Ca^{2+}-, Mg^{2+}-free). Then L1210 cells were washed three times with culture medium. Spleen cells (5×10^6) and mitomycin C-treated L1210 cells suspended in 2 ml culture medium were added to a Falcon 2051 tube (Falcon Plastics, Oxnard, Calif). Triplicate tubes were incubated for 5 days at 37° C in a humidified atmosphere containing 5% CO_2 in air.

In vitro cytotoxicity test. The cytotoxicity of spleen cells induced by in vitro stimulation with mitomycin C-treated L1210 cells was determined by the method of Brunner et al. [4] with a slight modification. Briefly, 10^7 L1210 or P388 cells in 1 ml culture medium were labeled with 200 μCi ^{51}Cr (Japan Atomic Energy Research Institute, Tokyo, Japan) at 37° C for 1 h and washed three times with culture medium. Various numbers of effector cells and 10^4 labeled target cells in 0.2 ml culture medium in Linbro 76-023-05 V-bottom microplates (Linbro Scientific, Inc., New Haven, Conn) were centrifuged at 200 *g* for 1 min, and the pelleted cultures were incubated at 37° C for 4 h in a humidified atmosphere containing 5% CO_2 in air. Then the microplates were centrifuged at 400 *g* for 5 min, and the amount of ^{51}Cr released in 0.1 ml supernatant was measured with a well-type gamma counter (Aloka Co., Tokyo, Japan). The percentage ^{51}Cr release was calculated from the counts in triplicate wells according to the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100.$$

Spontaneous release was the release (cpm) from the target cells incubated in the culture medium without effector cells; maximum release was determined by the release from the target cells incubated in saponin solution (Toa Medical Electronics Co., Ltd., Hyogo, Japan). Spontaneous release was below 20%, and maximum release was about 80% of the release from the target cells (10^4).

Treatment with anti-Thy 1.2 antibody and complement. Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.3, Grand Island Biological Co.) and 0.3% bovine serum albumin (Sigma Chemical Co., St Louis, Mo) was used for preparation of spleen cells and for dilution of antibody or complement. Equal volumes of 2×10^7 spleen cells, anti-Thy 1.2 antibody (Olac 1976, Ltd., Bicester, England) diluted 1 : 5,000, and rabbit complement (Cederlane Laboratory, Ltd, London, Canada) diluted 1 : 10 were mixed and incubated at 37° C for 1 h with gentle shaking. Spleen cells were washed twice with culture medium and used in the ^{51}Cr-release assay.

Results

Influence of aging or administration of antithymocyte serum on immune resistance of hyperimmune mice

Adult or aged hyperimmune mice that had been inoculated IP four times with 10^7 L1210 cells were inoculated IP with 5×10^7 L1210 cells 1 month after the final inoculation, and the influence of aging on the immune resistance of hyperimmune mice was examined (Table 1). The immune resistance of aged mice was significantly depressed compared with that of adult mice; most of the aged mice could not reject 5×10^7 L1210 cells, whereas the adult mice rejected this number of L1210 cells almost completely. Normal nonhyperimmunized adult $CD2F_1$ mice inoculated with 5×10^7 L1210 cells all died of tumor progression within 10 days (data not shown).

The effect of antithymocyte serum on immune resistance was examined in adult hyperimmune mice (Table 2). Immune resistance to L1210 cells in adult hyperimmune mice was almost completely lost following antithymocyte serum, and mice that received antithymocyte serum could hardly reject 5×10^7 L1210 cells.

These results indicate that T cells are essential for immunity in hyperimmune mice and that the function of T cells might be depressed with advancing age,

Characterization of effector cells in hyperimmune mice

In vitro cytotoxicity was tested by the ^{51}Cr-release assay (Table 3). The spleen cells of adult hyperimmune mice immunized with 10^7 L1210 cells twice or four times became cytotoxic following in vitro stimulation with mitomycin C-treated L1210 cells. Without in vitro stimulation no cytotoxicity was induced. The cytotoxicity induced after in vitro stimulation became more pronounced with increasing concentrations of stimulator cells, reaching a maximum at 2×10^5 stimulator cells. The addition of over 5×10^5 stimulator cells did not enhance cytotoxicity and in some cases reduced it (data not shown). The cytotoxicity of the spleen cells of mice immunized four times with 10^7 L1210 cells seemed to have the maximum level of host immunological capacity which was only slightly, and not significantly, more marked than that of mice immunized twice with L1210 cells. In subsequent studies, mice immunized four times with L1210 cells were used as hyperimmune mice.

The cytotoxicity of adult hyperimmune spleen cells was compared with that of adult normal spleen cells (Table 4). Whether or not normal spleen cells were stimulated with mitomycin C-treated L1210 cells, they exhibited no cytotoxicity at all. On the other hand, hyperimmune spleen cells became cytotoxic after in vitro resensitization with mitomycin C-treated L1210 cells. This cytotoxicity increased in proportion as the effector : target ratio was increased from 25 : 1 to 200 : 1.

Effector cells of the cytotoxicity of hyperimmune spleen cells were characterized (Table 5). The cytotoxicity of spleen cells from adult hyperimmune mice was completely lost when these cells were treated with anti-Thy 1.2 antibody and complement after in vitro stimulation just before the cytotoxicity test. Treatment with antibody or complement alone did not affect the cytotoxicity. It was thought that the cytotoxicity was specific for L1210 cells, because it was not effective against P388 cells histocompatible with L1210 cells. These results strongly suggest that the immune resistance of hyperimmune mice was mediated by cytotoxic T cells and was at a maximum level of host immunological capacity.

Table 1. Immune resistance to L1210 leukemia in adult or aged hyperimmune mice

No. of L1210 cells inoculated[a]	No. of 1-month survivors/total	
	Adult mice	Aged mice
5×10^7	15/17[b]	4/12[b]

[a] Hyperimmune mice were inoculated IP with 5×10^7 L1210 cells
[b] Significant at $P < 0.01$ (Fisher's exact test)

Table 2. Loss of immune resistance of hyperimmune mice following in vivo administration of antithymocyte serum

No. of L1210 cells inoculated[a]	No. of 1-month survivors/total	
	Untreated	Antithymocyte serum[c] (0.25 ml/mouse)
5×10^7	13/14[b]	1/13[b]

[a] Hyperimmune mice were inoculated IP with 5×10^7 L1210 cells
[b] Significant at $P < 0.01$ (Fisher's exact test)
[c] Administered IP 4 days before inoculation of L1210 cells

Table 3. Influence of frequency of inoculation on cytotoxicity of spleen cells

Stimulator[a] (cells)	Cytotoxicity (%)[b]	
	Frequency of inoculation of L1210 cells[c]	
	2	4
–	1.7 ± 1.6	5.4 ± 1.0
2×10^4	9.0 ± 1.4	7.5 ± 0.9
5×10^4	20.1 ± 0.3	24.5 ± 2.3
2×10^5	33.2 ± 1.9	42.2 ± 1.7

[a] Spleen cells (5×10^6) were stimulated in vitro with each number of mitomycin C-treated L1210 cells for 5 days
[b] Effector : target ratio was 100 : 1. Mean ± SD
[c] Adult mice resistant to the inoculation of 10^6 L1210 cells were inoculated IP with 10^7 L1210 cells twice or four times. Spleen cells were prepared 1 month after the final inoculation of 10^7 L1210 cells

Table 4. Influence of effector : target ratio on the cytotoxicity of normal or hyperimmune spleen cells

Effector : target ratio	Cytotoxicity (%)[a]			
	Spleen cells[b]			
	Normal		Hyperimmune	
	Stimulator[b]			
	Without	With	Without	With
25 : 1	−1.6 ± 1.1	0.1 ± 1.1	0.6 ± 1.6	27.7 ± 2.8
50 : 1	−1.0 ± 0.4	−0.2 ± 1.4	1.0 ± 0.4	31.4 ± 3.0
100 : 1	−0.5 ± 1.8	−0.3 ± 2.2	0.2 ± 0.8	44.8 ± 5.4
200 : 1	1.0 ± 3.0	0 ± 0.6	0.0 ± 1.3	49.5 ± 2.8

[a] Mean ± SD
[b] Spleen cells (5×10^6) of adult normal or hyperimmune mice were cultured without or with 2×10^5 mitomycin C-treated L1210 cells for 5 days

Table 5. Effector cells of cytotoxicity of hyperimmune spleen cells

Treatment of spleen cells[a]	Stimulator[a]	Target	Cytotoxicity (%)[b]
None	–	L1210	0.5 ± 2.1[c]
None	+	L1210	42.7 ± 0.4[c, d]
Anti-Thy 1.2	+	L1210	51.0 ± 1.8
Complement	+	L1210	47.7 ± 2.1
Anti-Thy 1.2 + complement	+	L1210	5.4 ± 0.5[d]
None	–	P388	0.4 ± 1.3
None	+	P388	0.5 ± 0.7

[a] Spleen cells (5×10^6) of adult hyperimmune mice were stimulated in vitro with 2×10^5 mitomycin C-treated L1210 cells for 5 days. Treatment was performed after in vitro stimulation, just before the ^{51}Cr-release assay
[b] Effector : target ratio was 100 : 1. Mean ± SD
[c, d] Significant at $P < 0.01$ (Student's t-test)

Table 6. Effect of LMS against the cytotoxicity of spleen cells of adult hyperimmune mice

Fifth inoculation[a] of 10^7 L1210 cells	LMS[b] (0.38 mg/kg)	Cytotoxicity (%)[c]
–	–	55.0 ± 3.3[d]
–	Days −3, −2, −1	81.2 ± 2.9[d]
Day −14	–	65.1 ± 2.7[e]
Day −14	Days −17, −16, −15	76.8 ± 5.0[e]

[a] Adult hyperimmune mice were inoculated IP with 10^7 L1210 cells on day −14
[b] Administered IP
[c] Spleen cells (5×10^6) prepared on day 0 were stimulated in vitro with 2×10^5 mitomycin C-treated L1210 cells for 5 days. Effector : target ratio was 100 : 1. Mean ± SD
[d, e] Significant at $P < 0.05$ (Student's t-test)

Table 7. Effect of LMS against decreased cytotoxicity following administration of antithymocyte serum

Antithymocyte serum[a] (0.25 ml/mouse)	LMS[a] (0.38 mg/kg)	Cytotoxicity (%)[b]
–	–	55.0 ± 3.3[c]
Day −4	–	4.8 ± 0.7[c, d]
Day −4	Days −3, −2, −1	18.0 ± 1.4[d]

[a] Adult hyperimmune mice received serum or LMS IP
[b] Spleen cells (5×10^6) prepared on day 0 were stimulated in vitro with 2×10^5 mitomycin C-treated L1210 cells for 5 days. Effector : target ratio was 100 : 1. Mean ± SD
[c, d] Significant at $P < 0.01$ (Student's t-test)

Table 8. Effect of LMS against the depressed hyperimmunity following in vivo administration of antithymocyte serum

Antithymocyte serum[a] (0.25 ml/mouse)	LMS[a] (0.38 mg/kg)	No. of 1-month survivors/total
–	–	13/14[b]
Day −4	–	2/16[b, c]
Day −4	Days −4, −3, −2, −1	6/17[c]

[a] Adult hyperimmune mice received serum or LMS IP. On day 0, 5×10^7 L1210 cells were inoculated IP
[b] Significant at $P < 0.01$ (Fisher's exact test)
[c] Not significant by Fisher's exact test ($P = 0.13$)

Table 9. Effect of LMS against the cytotoxicity of spleen cells of aged hyperimmune mice

Mice	Fifth inoculation[a] of 10^7 L1210 cells	LMS[b] (0.38 mg/kg)	Cytotoxicity (%)[c]
Adult	–	–	62.5 ± 2.0[d]
Aged	–	–	20.8 ± 1.3[d]
Aged	Day −14	–	21.3 ± 1.3[e]
Aged	Day −14	Day −17, −16, −15	60.4 ± 6.2[e]

[a] Hyperimmune mice were inoculated IP with 10^7 L1210 cells on day −14

[b] Administered IP

[c] Spleen cells (5×10^6) prepared on day 0 were stimulated in vitro with 2×10^5 mitomycin C-treated L1210 cells for 5 days. Effector : target ratio was 100 : 1. Mean ± SD

[d, e] Significant at $P < 0.01$ (Student's t-test)

Augmentation of cytotoxicity of hyperimmune spleen cells by LMS

The effect of LMS against the cytotoxicity of hyperimmune spleen cells was examined in adult mice (Table 6). The dose of LMS in this experiment was 0.38 mg/kg, which had been shown to be optimum for the potentiation of immunoprophylactic activity of L1210 tumor vaccine [11]. Administration of LMS for 3 days before the preparation of spleen cells augmented the cytotoxicity, detected after in vitro resensitization. Augmentation of the cytotoxicity by LMS was also detected when hyperimmune mice were inoculated IP with 10^7 L1210 cells 14 days before and when LMS was administered for 3 days before the inoculation of L1210 cells.

Effect of LMS in decreased cytotoxicity following administration of antithymocyte serum

The effect of antithymocyte serum on the cytotoxicity of spleen cells was examined in adult hyperimmune mice (Table 7). As already shown in Table 2, the administration of antithymocyte serum to hyperimmune mice almost completely abrogated their immune resistance to L1210 cells. This result was reflected in the cytotoxicity of spleen cells: administration of antithymocyte serum 4 days before the preparation of spleen cells abrogated cytotoxicity completely. LMS administered for 3 days after the administration of antithymocyte serum restored the cytotoxicity significantly, though only partially. This effect of LMS was thought to induce the partial, statistically not significant, restoration of hyperimmunity abrogated by the administration of antithymocyte serum (Table 8).

Effect of LMS in depressed cytotoxicity in aged hyperimmune mice

The cytotoxicity of adult and of aged hyperimmune mice was compared (Table 9). The cytotoxicity detected in the spleen cells of aged hyperimmune mice was significantly depressed compared with that of adult hyperimmune mice, and the depression of cytotoxicity with aging was confirmed. Administration of LMS to aged hyperimmune mice for 3 days before the inoculation of 10^7 L1210 cells restored cytotoxicity to almost the same level as that in adult mice.

Discussion

In this study we used the ^{51}Cr-release assay to demonstrate the development of marked cytotoxic T-cell activity in the spleen cells of mice hyperimmune to L1210 leukemia. We had already shown that effector T cells were responsible for the immune resistance of hyperimmune mice by means of the in vitro growth inhibition assay and in vivo adoptive transfer [10]. However, the results of both assays were known sometimes to disagree with those of the in vitro cytotoxicity test [14]. The present results obtained with the in vitro cytotoxicity test confirmed those of the previous studies [10] and led us to conclude that effector T cells were responsible for antitumor immunity in mice hyperimmune to L1210 leukemia. In vitro cytotoxicity was induced only when the spleen cells had been resensitized in vitro with mitomycin C-treated L1210 cells, and without in vitro resensitization no cytotoxicity was induced even in the hyperimmune mice (Table 4). This may explain the discrepancy in results between the cytotoxicity test and the other two tests in some other systems.

The in vivo resistance to L1210 leukemia in hyperimmune mice decreased with aging (Table 1) and was abrogated by administration of antithymocyte serum (Table 2). These results correlated well with the cytotoxicity of spleen cells, which was detected by the ^{51}Cr-release assay after in vitro resensitization with mitomycin C-treated L1210 cells. The cytotoxicity of spleen cells was completely abrogated by the administration of antithymocyte serum (Table 7), and decreased with advancing age (Table 9), as previously reported in allogeneic systems [7, 20]. In mice hyperimmune to L1210 cells the results of ^{51}Cr-release assay seemed to correlate well with in vivo immune resistance to L1210 cells.

Administration of LMS augmented the cytotoxicity of spleen cells of adult hyperimmune mice (Table 6). Furthermore, the cytotoxicity abrogated by the administration of antithymocyte serum in adult hyperimmune mice was restored partially but significantly by LMS (Table 7). Considering the previous results, which showed the stimulatory effect of LMS on the differentiation or maturation of T cells [16–18], one possible mechanism of the enhancing effect of LMS on cytotoxicity in mice hyperimmune to L1210 leukemia might be stimulation of the differentiation of cytotoxic T-cell precursors. It seemed possible that the administration of antithymocyte serum might induce a systemic defect of cytotoxic T-cell activity. This defect of cytotoxic T-cell activity was thought to be restored by administration of LMS, which had the effect of stimulating the differentiation of cytotoxic T-cell precursors. This effect of LMS was thought to induce the restoration of hyperimmunity abrogated by the administration of antithymocyte serum (Table 8). However, the effect of LMS, as shown in Table 8, was statistically not significant, suggesting that the results of the ^{51}Cr-release assay were more sensitive than those obtained when immune resistance was examined by inoculating live L1210 cells.

In this study, we used hybrid $CD2F_1$ mice and not syngeneic DBA/2Cr mice, because only $CD2F_1$ mice could be supplied in sufficient quantities for the production of hyperimmune mice. Our previous results showed that hyperimmunity to L1210 cells in DBA/2Cr mice was not so strong as that in $CD2F_1$ mice, since most $CD2F_1$ mice resistant to the inoculation of 10^6 L1210 cells were also resistant to the inoculation of 10^7 L1210 cells, whereas considerable numbers of DBA/2Cr mice resistant to the inoculation of 10^6 L1210 cells could not reject 10^7 L1210 cells (data not shown). As $CD2F_1$

mice are compatible with DBA/2Cr mice in major histocompatibility antigens, it is not clear why there was this difference in the degree of hyperimmunity induced between $CD2F_1$ and DBA/2Cr mice. It seems possible that this difference in the degree of hyperimmunity was a result of the different immunological mechanisms of hyperimmunity.

Although the depression of T-cell-mediated immunity with aging has been reported in many systems, such as mitogen response [1, 5], helper T cells [12], and cytotoxic T cells [7, 20], the mechanisms suggested for this are not necessarily in good agreement. While a reduction in the number of responding T cells has been reported in some systems [1] qualitative abnormalities of T cells have been reported in another system [5]. This disagreement might be attributable to differences of the strain or age of the mice used in the experiments. Since the cytotoxic T-cell activity in adult mice hyperimmune to L1210 leukemia was at the maximum level of host immunological antitumor capacity (Table 3), the decline in cytotoxicity with age (Table 9) was thought to be caused by the reduced activity of cytotoxic T cells and not by the abnormal induction process of cytotoxic T cells. However, it was not concluded from this study whether the reduction of the cytotoxic T-cell activity was due to the reduction of the number of effector cells or to the depression of the function of effector cells. Therefore, the possible mechanisms by which LMS restored the depressed cytotoxicity in aged mice might be the increase in the number of cytotoxic T-cell precursors or restoration of the function of cytotoxic T cells. Concerning the former mechanism, it was recently reported that interleukin 2 supported the proliferation of cytotoxic T-cell precursors [22]. LMS might stimulate the interleukin 2-producing cells in vivo. Further investigations are expected in these areas.

In conclusion, immune resistance of mice hyperimmune to L1210 leukemia, a chemically induced tumor, correlated well with the cytotoxicity of spleen cells, and LMS augmented this cytotoxicity in adult mice and restored the depressed cytotoxicity in aged mice.

Acknowledgements. The authors thank Dr Y. Sakurai for encouragement in this study, F. Oh-hashi for maintenance of the hyperimmune mice, and K. Yamamoto for expert technical assistance.

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Received March 31, 1983/Accepted May 17, 1984