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Modulation of Lymphocyte Proliferation by Murine Liver Extract

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SUMMARY **Murine liver extract (LEx) purified by ammonium sulfate (45-70% saturation) possesses a strong inhibitory effect on human lymphocyte proliferation. We have shown that the inhibitory effect of LEx is not via a cytotoxic effect and that it is proportional to the length of incubation with LEx. Mitogen-prestimulated lymphocytes are more resistant to LEx inhibition than cells not prestimulated. B cells stimulated by PWM are more susceptible to LEx-induced inhibition than PHA- or Con A-stimulated T cells. In Con A cultures, there may be a population of cells more resistant to LEx inhibition. This population is not yet identified. The degree of reversibility of LEx inhibition was different in cells prestimulated by different mitogens. The inhibitory activity of LEx decreased in the presence of an increasing number of cells in the culture.**

A factor with potent inhibitory activity against lymphocyte proliferation has been reported in liver extracts (LEx) from humans[1-4] and from various animals including rats,[5] dogs,[6] rabbits,[7] pigs,[8] and mice.[9] LEx may inhibit the proliferation of both T cells and B cells in a dose-related manner. The inhibition has been shown to be non-cytotoxic by either trypan blue dye exclusion[3] or by eosin-Y dye exclusion.[6] We further extended this investigation by ^{51}Cr-release assay with a long period of observation up to 72 hr.

The inhibitory effect of LEx has been shown to be reversible using unstimulated lymphocytes at the beginning of incubation with LEx.[3,5,9] We extended the study by using mitogen-prestimulated lymphocytes to evaluate the reversibility of the LEx inhibition. By using prestimulated lymphocytes, the experimental conditions are more similar to those of an *in vivo* immune response in which lymphocytes have been activated. The effects of cell number on the inhibitory effect of LEx were also studied.

This study is a first step in elucidating the mechanism of immune inhibition by LEx.

MATERIALS AND METHODS

Preparation of LEx

Pooled murine livers from ICR mice were homogenized with Polytron (PT 10/35, Kinematics, Switzerland) in 0.01 M Tris-HCl buffer, pH 7.0. The supernatant was collected. The protein that precipitated between 45 and 70% saturation of ammonium sulfate was redissolved and dialyzed at 4°C against three changes of 0.01 M phosphate buffered saline, pH 7.0 overnight. Protein content was measured according to the method of Lowry[10] using bovine serum albumin as reference. The LEx was aliquoted and kept frozen at −70°C until use.

Lymphocyte separation and culture

Mononuclear leukocytes were separated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation, and were washed twice with Hanks' balanced salt solution (HBSS). Cells thus prepared contained 85-90% lymphocytes, 10-15% monocytes and occasional granulocytes. Viability was over 95% as determined by trypan blue dye exclusion. All the lymphocytes were cultured in RPMI-1640 supplemented with fetal calf serum (10%), pencillin (100 U/ml),

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and streptomycin (100 μg/ml) (all from Grand Island Biological Co., USA). Lymphocytes, 1×10^5, were mixed with mitogens in a total volume of 0.2 ml in each well of Micro Test III plates (Falcon, Becton Dickson & Co., USA). The optimal mitogen concentrations for lymphocyte proliferation were predetermined:[11] 1 μg/ml phytohemagglutinin (PHA, Burrow Wellcome, Dartford, England), 9 μg/ml concanavalin-A (Con A, Difco Lab, USA), and 1 μg/ml pokeweed mitogen (PWM, Sigma, USA). These concentrations were employed in all experiments of cell cultures and cell prestimulation.

To test the effect of LEx on 72-hour mitogen-prestimulated cells, the cells in each prestimulation well were washed twice with HBSS. They were then cultured a further 72 hours in fresh media to which various concentrations of LEx were added.

To test the rate of cell recovery following two-day exposure of prestimulated lymphocytes to LEx, LEx was removed and replaced by fresh media. Then, the ^{3}H-thymidine uptake was determined every 24 hr for the next 96 hr.

All the cultures were set in quadruplicate and incubated at 37°C with 5% CO_2/air. During the terminal 4 hr of incubation, cells were pulsed with ^{3}H-thymidine (specific activity 6.7 Ci/mM, New England Nuclear Co., USA) at 0.5 μCi/well for 4 hr and were harvested with an automatic cell harvester (model M24, Brandel, USA). Isotope incorporation was measured by a standard toluene – based scintillation counting technique.[12] The percentage of isotope incorporation was calculated as: (cpm of test cultures/cpm of control cultures) × 100%, (cpm: count per minute).

Cytotoxicity studies of LEx

The possible toxic effect of LEx on lymphocytes was first investigated by the trypan blue dye exclusion method. The exclusion of 0.2% trypan blue dye was determined in lymphocytes cultured with various concentrations of LEx. Viable cells, which excluded the dye following 5 min incubation at room temperature, were enumerated.

The possible cytotoxic effect of LEx was further studied by ^{51}Cr release assay previously described.[12] Lymphocytes (2×10^6/ml) were labelled with $Na_2{}^{51}CrO_4$ (20 μCi/ml) in RPMI-1640 at 37°C for 1 hr in a shaking water bath. They were then washed twice and resuspended at a concentration of 1×10^6/ml in fresh culture medium. Aliquots of 0.5 ml of the cell suspension and equal volumes of LEx solutions of varying concentrations were mixed in test tubes. Following incubation for 48 hr or 72 hr at 37°C, the tubes were centrifuged and the supernatant of each tube was transfered to a counting vial. The cell pellet was washed once and the supernatant was combined with the previous supernatant in the counting vial (total called a). The cell pellet was then resuspended in distilled water, and the cells were disrupted by boiling for 10 min. Following centrifugation at 900 g the supernatant of the disrupted cells was removed for counting and called b. The count (a + b) was considered to represent releasible cpm. The ratio of $|a/(a+b)| \times 100$ was used as a cytoxicity index which is proportional to the degree of cell death.

RESULTS

Dose response for LEx inhibition of lymphocyte proliferation in the presence of mitogen

Mononuclear cells isolated from Ficoll-Hypaque density gradient were incubated with both mitogen and various concentrations of LEx for a total of 72 hr. Figure 1 shows that the percentage of isotope

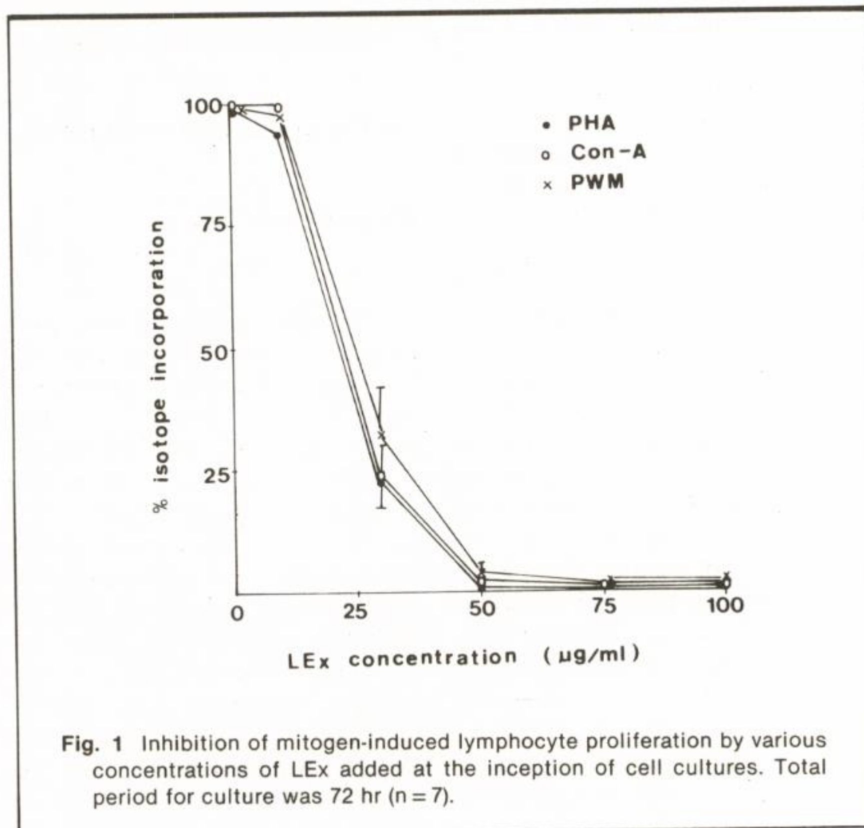

Fig. 1 Inhibition of mitogen-induced lymphocyte proliferation by various concentrations of LEx added at the inception of cell cultures. Total period for culture was 72 hr (n = 7).

incorporation decreased to 50% at around 25 μg LEx/ml for each of the three mitogens (PHA, Con A, and PWM). Complete inhibition of isotope incorporation occurred at 50 μg LEx/ml for PHA and Con A, and at 75 μg LEx/ml for PWM.

Dose response for LEx inhibition of mitogen-prestimulated lymphocytes in the absence of mitogen

Mononuclear cells were prestimulated with PHA, Con A, or PWM for 72 hr, washed twice with HBSS, and re-incubated with various concentrations of LEx for another 72 hr. Cells were pulsed with ^{3}H-thymidine in the terminal 4 hr of incubation. Figure 2 shows that the percentage of isotope incorporation decreased to 50% at 34 μg LEx/ml for PWM, at 40 μg/ml for PHA, and at 78 μg/ml for Con A. All approached a plateau at 100 μg LEx/ml. However, the inhibition was not complete, especially in Con A cultures which still showed 29% isotope incorporation even at as high as 200 μg LEx/ml.

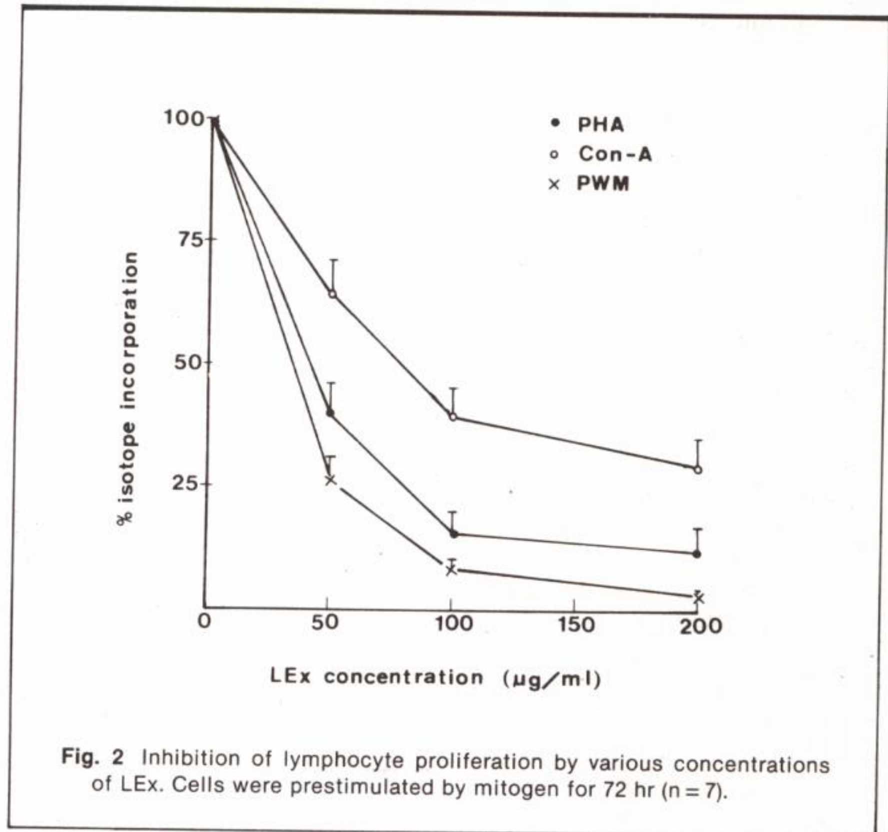

Fig. 2 Inhibition of lymphocyte proliferation by various concentrations of LEx. Cells were prestimulated by mitogen for 72 hr (n = 7).

Cytotoxicity studies with LEx

The possible cytotoxic effect of LEx on lymphocyte cultures was first determined by trypan blue dye exclusion. Dye was excluded by more than 90% of the cultured lymphocytes in three experiments with no significant decrease at any concentration of added LEx up to and including 100 μg LEx/ml (Table 1).

^{51}Cr release studies were performed in triplicate in cells of five subjects (Fig.3). Exposure to 50 or 100 μg LEx/ml for 48 hr and 72 hr led to no significant increase in ^{51}Cr release over control cultures with no LEx added. In these experiments, the total releasible cpm in cells after boiling was approximately 78% of the total cellular cpm content.

Time course of lymphocyte inhibition by LEx

To study the time course of LEx inhibition, cells were incubated

Table 1 Cell number and viability determined by trypan blue dye exclusion following incubation with LEx.

Exp. number	Incubation time (hr)	LEx (μg/ml) 0	50	100
1	48	4.8 × 10^5/ml (96%)*	6.0 × 10^5/ml (94%)	5.1 × 10^5/ml (97%)
	72	4.5 × 10^5/ml (93%)	5.2 × 10^5/ml (92%)	4.9 × 10^5/ml (95%)
2	48	4.9 × 10^5/ml (90%)	4.2 × 10^5/ml (90%)	4.4 × 10^5/ml (93%)
	72	4.2 × 10^5/ml (96%)	3.9 × 10^5/ml (95%)	6.1 × 10^5/ml (95%)
3	48	4.9 × 10^5/ml (95%)	5.2 × 10^5/ml (93%)	5.4 × 10^5/ml (91%)
	72	5.6 × 10^5/ml (96%)	6.7 × 10^5/ml (94%)	4.2 × 10^5/ml (95%)

* Viability in parenthesis

with both mitogen and LEx (50 μg/ml). At various time intervals, the media were replaced with LEx-free media (Figure 4a) but the mitogens were present during the whole period of incubation. Isotope incorporation was inhibited by 50% at 36 hr of LEx incubation in Con A and PWM cultures, and at 45 hr in PHA cultures (Fig.5). It was completely inhibited by 68 hr of LEx incubation for all three mitogens.

To study the time course of LEx inhibition in mitogen-prestimulated lymphocytes, cells were prestimulated with mitogen for 72 hr, washed, and incubated with LEx (100 μg/ml) for various periods of time in the absence of mitogen (Fig.4b). At the end of LEx incubation, cells were washed and further incubated in the absence of both LEx and mitogen for a total of 72 hr (Fig.6) with ^{3}H-thymidine-pulse in the terminal 4 hr of the culture.

Isotope incorporation was 50% inhibited at 19 hr of incubation with LEx in PWM cultures, at 39 hr of incubation in PHA cultures, and at 48 hr of incubation in Con A cultures. At 68 hr of incubation with LEx, isotope incorporation in PWM cultures was nearly completely inhibited, while in PHA cultures it was 92% inhibited and in Con A cultures it was 72% inhibited.

In the experiments with mitogen-prestimulated lymphocytes, cells were incubated with 100 μg LEx/ml for various periods of time, after which proliferations were determined without further chasing with LEx free medium (Fig. 4c). Figure 7 shows that proliferation was 50% inhibited at 30 hr incubation for PHA-prestimulated cells, at 34 hr for Con A-prestimulated cells, and at 37 hr for PWM-prestimulated cells. For all, inhibition was around 75% at 48 hr of incubation. To study reversibility of LEx inhibition in later experiments, 48 hr incubation with LEx was selected.

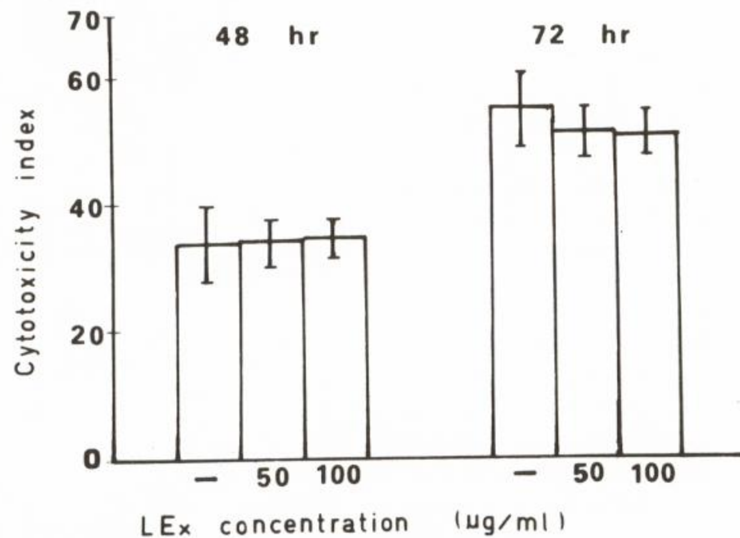

Fig. 3 Cytoxicity index as determined by ^{51}Cr release assay of lymphocytes cultured with various concentrations of LEx. (n = 5).

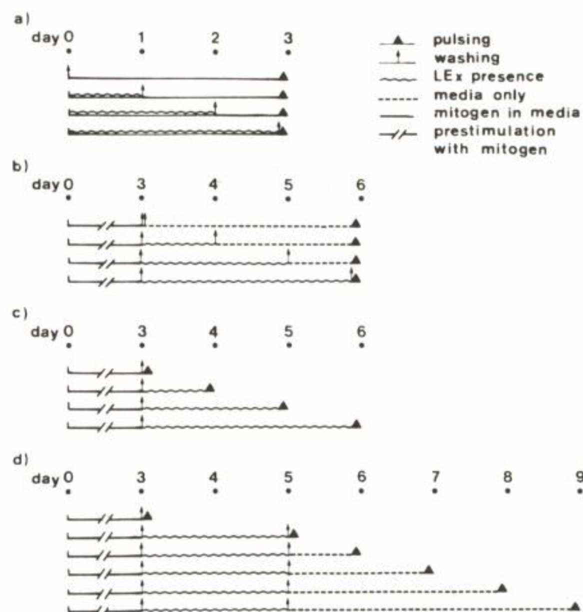

Fig. 4 Schedules for experiment design. The results of a, b, c and d, are shown in Figs. 5, 6, 7 and 8, respectively.

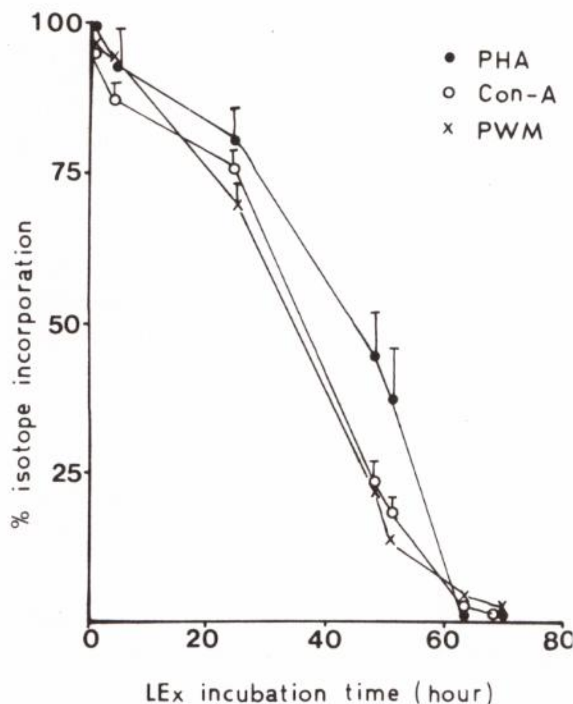

Fig. 5 Inhibition of mitogen-induced lymphocyte proliferation by LEx (50 μg/ml). LEx was removed at various time intervals and replaced with LEx-free media. Mitogens were present during the whole period of incubation. (n = 8). Please refer to Fig. 4a.

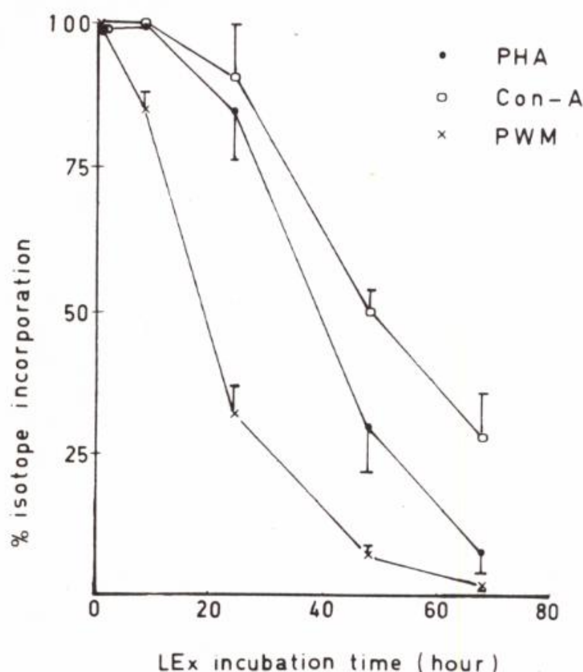

Fig. 6 Inhibition of mitogen-prestimulated lymphocytes by LEx (100 μg/ml) which was removed at various time intervals. (n = 4). Please refer to Fig. 4b.

Reversibility of proliferation inhibition

In order to study the reversibility of LEx inhibition, mitogen-prestimulated lymphocytes from eight donors were incubated with 100 μg LEx/ml for 48 hr (yielding 75% inhibition). Then, cells were washed and chased with LEx-free medium for various periods of time up to 96 hr (Fig. 4d and Fig. 8). For PHA-prestimulated cells, proliferation returned to the pre-LEx level at 24-48 hr, and then declined when the cells became crowded. For Con A-prestimulated cells, proliferation did not return to normal, but increased only slightly before declining rapidly until the end of the 96 hr-observation period. For PWM-prestimulated cells, proliferation approached normal after 48 hr-incubation, and then declined.

Thus, PHA-prestimulated cells may recover to achieve normal proliferation levels, whereas both Con A- and PWM-prestimulated cells may recover only enough to achieve subnormal proliferation levels and then decline.

The effect of cell number on LEx-induced lymphocyte inhibition

In the presence of LEx, PHA-prestimulated lymphocytes from nine donors were cultured at various cell concentrations for 72 hr (Fig.9). At high cell concentrations, there was less of a LEx inhibitory effect. This result indicates that the effect of LEx can be overcome by the presence of an increased number of cells.

DISCUSSION

LEx possesses a potent inhibitory activity on lymphocyte proliferation. This has been reported in various studies.[1-9] However, whether or not this effect is due to a cytotoxic effect on lymphocytes has received little attention. Chisari[3] reported using

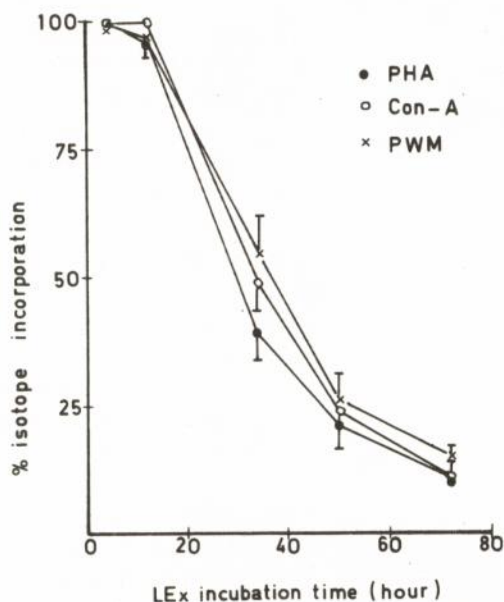

Fig. 7 Time course of lymphocyte inhibition of mitogen prestimulated cells by LEx (100 μg/ml). (n = 4). Please refer to Fig. 4c.

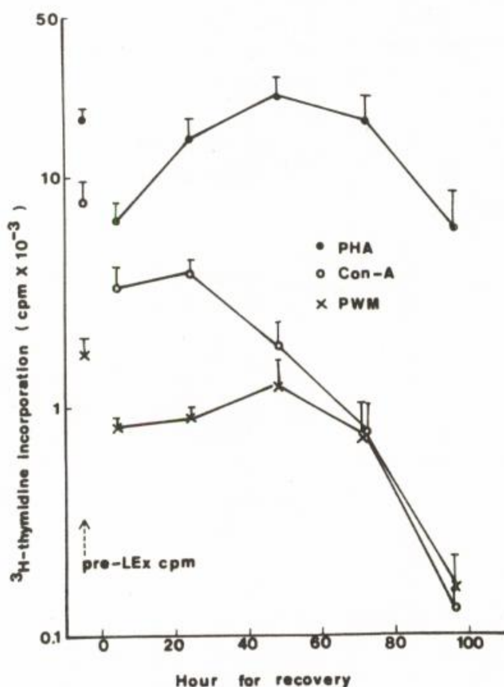

Fig. 8 Reversibility of LEx inhibition of lymphocyte proliferation. The mitogen-prestimulated lymphocytes were incubated with 100 μg LEx/ml for 48 hrs. Then, cells were washed and chased with LEx-free medium for various periods of time up to 96 hr (n = 8). Please refer to Fig. 4d.

trypan blue dye exclusion and Burleson *et al*[6] eosin-Y dye exclusion to demonstrate that the inhibitory effect of LEx is not due to cytotoxicity. However, a more precise determination of cytotoxicity using ^{51}Cr release has only been reported by Vogelfanger *et al*[8] where no data was included.

We carefully studied the possible cytotoxic effect of LEx by measuring ^{51}Cr-release in lymphocytes from five normal volunteers. We proved there was no cytotoxicity in cells cultured with LEx for both 48 hr and 72 hr (Fig.3). Thus, the LEx-induced inhibition of cell proliferation is not due to cytotoxicity.

In other reports, unstimulated lymphocytes separated from whole blood have been used to study LEx inhibition of cell proliferation. However, in ongoing immune responses as frequently encountered in a real situation, the lymphocytes are activated in some way. In order to study the effect of LEx on activated lymphocytes, cells were prestimulated with mitogens for 72 hr prior to reaction with LEx (Figs.2, 6, 7, 8, 9).

The results revealed that cells prestimulated by mitogens were more resistant to LEx inhibition than cells not prestimulated. In dose-response experiments, cells not prestimulated by mitogens could be inhibited by more than 90% at 50 μg LEx/ml (Fig.1). However, for prestimulated cells, even as high as 100 μg LEx/ml did not inhibit 95% of cell proliferation (Fig.2).

Again, in the time course study, cells not prestimulated were completely inhibited by 50 μg LEx/ml at 68 hr of incubation (Fig.5). However, the prestimulated cells were not completely inhibited at 68 hr of incubation by as much as 100 μg LEx/ml (Fig.6). The PWM-prestimulated cells were more sen-

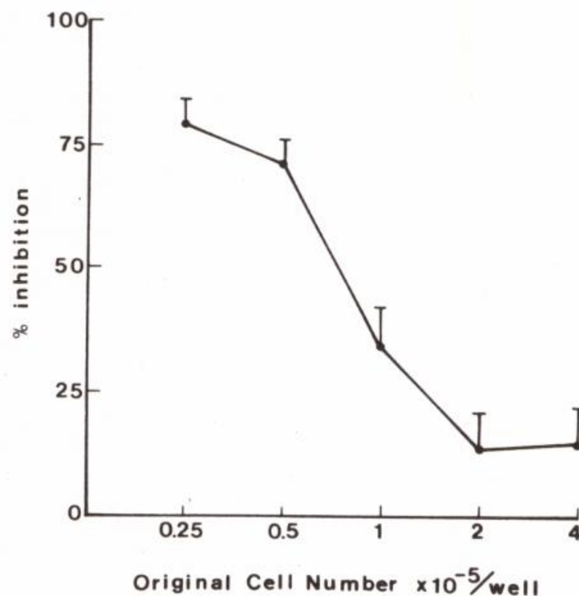

Fig. 9 Inhibition by LEx (50 μg/ml) *versus* various cell concentrations. Cells were prestimulated by mitogen for 72 hr and then further incubated with LEx for another 72 hr in the absence of mitogen. The percentage of inhibition was calculated as: 1 – (cpm of test cultures/cpm of control cultures) × 100%. (n = 9).

sitive to LEx inhibition in the earlier stage (24-48 hours) of incubation. The reason is unclear.

The resistance of prestimulated cells to LEx inhibition is more prominent in Con A stimulated cells (Fig.2 and Fig.6). Probably, the Con A stimulated cells contain a population resistant to LEx inhibition. This population has not yet been identified.

For cells not prestimulated by mitogen, LEx inhibition of proliferation was reversible. The cells were incubated with LEx for various periods of time, washed, and further incubated in the absence of LEx for a total of 72 hr. The degree of LEx inhibition was proportional to the duration of incubation with LEx (Fig.5). The inhibition was reversible after the removal of LEx from the incubation fluid. This was also true for prestimulated cells, although the degree of inhibition was less (Fig.6).

For mitogen-prestimulated lymphocytes, reversibility of LEx inhibition of proliferation was variable. Cells were treated with mitogen, washed, and then cultured with LEx in mitogen-free media for 48 hr (Fig. 4d). They were then washed and incubated further in LEx- and mitogen-free media for up to 96 hr. Cells prestimulated by PHA recovered proliferation ability in 48 hr whereas cells prestimulated by both Con A and PWM did not make a complete recovery. After the initial recovery period, cell proliferation declined (Fig.8) probably because of aging or crowding of cells. Thus, the degree of reversibility of LEx inhibition depended upon the prestimulating mitogens.

Both T and B lymphocytes can be inhibited by LEx as shown in PHA, Con A as well as PWM cultures. However, it seems that B cells are more susceptible to LEx inhibition as shown by the pronounced inhibition of cell proliferation in PWM cultures (Fig.2 and Fig.6). The mechanism is unclear.

The inhibitory activity of LEx decreased when the lymphocyte number increased in cultures (Fig.9). This may be explained in two ways. First, it has been reported by us[13] and others[14] that LEx possesses arginase activity. If the inhibitory effect is due to the arginase activity of LEx, then, the LEx activity would tend to decrease in the presence of increasing substrate represented by increasing cell numbers. Second, if the LEx exerts its activity by binding to cell surface receptors, then, increased numbers of cells would dilute the LEx available for each cell, decreasing inhibitory activity. This possibility can only be confirmed by studies using radioactive LEx or by fluorescent antibody techniques. These are in progress.

We have shown the inhibitory effect of LEx is not via a cytotoxic effect. The inhibitory effect is reversible and is proportional to the length of LEx incubation. Mitogen-prestimublated lymphocytes are more resistant to LEx inhibition than those not prestimulated. B cells stimulated by PWM are more susceptible to LEx-induced inhibition than PHA- or Con A-stimulated T cells. In Con A cultures, there may be a population more resistant to LEx inhibition. This population has not yet been identified.

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