EFFECT OF ACTINOMYCIN D ON CYTOTOXIC ACTIVITY OF NORMAL LYMPHOCYTES IN TISSUE CULTURE

B. D. Brondz

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Lymph gland cells of inbred lines of normal mice, treated with actinomycin D and thoroughly washed, possessed a cytotoxic action on monolayer cultures of syngenic and allogenic macrophages when incubated together for 16-20 h at 37°. Macrophages of lines CC57BR, C3H, and A were more resistant to this effect than macrophages of lines C57BL/10 and B10.D2. The toxicity was due to a substance secreted into the culture medium from living lymphocytes treated with actinomycin D, which combines with the culture cells and destroys them.

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Normal mouse and human lymphocytes possess a cytotoxic effect in vitro on allogenic target cells in the presence of substances (phytohemagglutinin, streptolysin S, antilymphocytic serum, etc.) causing blast-transformation of lymphocytes [5-8]. This effect is found only in the presence of an antigenic difference between the lymphocytes and target cells and cannot be detected if they are antigenically identical (in a syngenic system). However, it has been reported that normal lymphocytes may have a cytotoxic action if the medium contains substances (interferon, methotrexate) producing neither agglutination nor, apparently, blast-transformation of lymphocytes. In these cases the lymphocytes destroyed both allogenic and syngenic target cells.

The cytotoxic activity of normal lymphocytes treated with actinomycin D was investigated in the present study.

EXPERIMENTAL METHOD

Mice of inbred lines C57BL/10Sn, CC57BR, B10.D2, C3H/Sn, and A were obtained from the nursery of the N. F. Gamaleva Institute of Epidemiology and Microbiology.

Actinomycin D (AD) (Mann Res. Lab., USA) was diluted in 96° ethanol (2 mg/ml) and kept at 4°. Further dilutions in medium No. 199 were carried out on the day of the experiment.

The lymphocytes were obtained from lymph glands of normal mice, washed three times, the number of living cells counted, and suspended in a concentration of 5×10^7/ml in medium No. 199 containing or not containing AD. After incubation in a water bath at 37° for 60 min with periodic agitation, the cells were washed four times with Hanks' solution, suspended in medium No. 199, counted, and introduced in a concentration of 20×10^6/ml into cultures of peritoneal macrophages of mice belonging to different lines [1]. Treatment with AD had no toxic effect on the lymphocytes. After incubation at 37° for 16-20 h, the culture medium with the lymphocytes was removed and the number of living macrophages counted

TABLE 1. Cytotoxic Action of Normal Lymphocytes Treated with AD on Syngenic and Allogenic Macrophages

Source of lymphocytes	Source of macrophages	AD (in μg/ml)	Cytotoxic effect (in %)
CC57BR	CC57BR	1	33,5±1,2 [1]
		2	69,7±1,5 [1]
		3	76,9±2,0 [1]
C3H	C3H	1,5—3	24,2±1,8 [1]
B10.D2	C57BL/10	0,25	4
		0,5	14
		1	64
		2	83±7 [1]
	CC57BR	1,5—3	52,6±3,1 [1]
	A	2,5	48
	C3H	3	34,6

[1] Mean of 2-8 experiments ± standard error.

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TABLE 2. Cytotoxic Effect of Various Materials in Vitro after Treatment of Normal Lymphocytes with AD

Substrate with which target cells were incubated	Cytotoxic effect (in %)				
	Expt. no.				
	1	2	3	4	5
Lymphocytes AD[1]	78.7	60	70.4	79	
AD (2 mg/ml)[2]		59.8	86		
Sup.[3]		84.2	83.7		
Wash.[4]		11.4[9]	22.8[10]		
Lymphocytes AD (heated)[5]			0[9]	12.3[9]	15.3[9]
Lymphocytes AD (freezing–thawing)[6]		18.9[10]	8.6[9]		
CM of lymphocytes AD[7]		28.8[10]	87	81.5	84.7
CM of lymphocytes AD + macrophages[8]	0[9]	2.2[9]	33.4		

[1]Lymphocytes and target cells obtained from CC57BR mice.
[2]Lymphocytes treated with AD (2 μg/ml) and washed.
[3]Supernatant (Sup.) after incubation of lymphocytes with AD.
[4]Washings after 9th washing of lymphocytes treated with AD.
[5]60°–30 min followed by washing 3 times
[6]Freezing–thawing, 7 cycles, centrifugation at 2000 rpm.
[7,8]Culture medium (CM) after incubation for 18 h at 37° of lymphocytes treated with AD in empty tubes (7) and with cultures of macrophages (8).
[9]$P > 0.05$ [10] $P < 0.02$ (in the remaining cases $P < 0.01$–t-test).

in the cultures after staining them with a mixture of 0.1% solutions of eosin and trypan blue [2]. The cytotoxic effect was determined from the formula:

$$\frac{a-b}{a} \cdot 100$$

where a and b represent the numbers of living macrophages after incubation with analogous material receiving the same treatment without AD (a) or with AD (b) (mean of 4 determinations).

EXPERIMENTAL RESULTS

Normal lymphocytes treated with AD and washed had a cytotoxic action on target cells of both syngenic and allogenic origin (Table 1). This effect increased with an increase in AD concentration from 0.25 to 3.5 μg/ml. Macrophages of lines C57BL/10 and B10.D2 were more sensitive to the cytotoxic action of normal lymphocytes treated with AD than macrophages of lines CC57BR, C3H, and A. If allogenic lymphocytes, treated with AD in a concentration of 2-3 μg/ml were used, 76-90% of C57BL/10 macrophages and 26-55% of CC57BR, C3H, and A macrophages were destroyed.

How do lymphocytes treated with AD destroy the culture cells?

The following suggestions may be made: 1) inadequate removal of AD from the lymphocytes by washing; 2) the toxic effect of AD bound by lymphocytes and liberated from them through gradual destruction in culture; 3) the toxic effect of products of the reaction between AD and lymphocytes.

The results of a study of these possibilities are given in Table 2.

The first suggestion can be rejected, because the last washings from lymphocytes treated with AD had no significant toxic action on target cells. The second suggestion is evidently unlikely; both the AD itself and the supernatant obtained after treatment of the lymphocytes with AD destroyed lymphocytes to the same degree as lymphocytes treated with AD and washed. Since the AD concentration in these experiments (2 μg/ml) was only twice the minimal toxic dose, it is evident that the lymphocytes bound only a negligible quantity of AD, incapable of destroying the target cells. This is also confirmed by the fact that lymphocytes treated with AD and washed lost their cytotoxic activity if they were killed by heating or destroyed by freezing and thawing. In this case, however, destruction of AD cannot be ruled out.

To test the third hypothesis, macrophages were incubated in the culture medium obtained after incubation for 18 h of lymphocytes treated with AD and washed. This medium (Table 2) destroyed 81.5-87% of target cells (in 3 of 4 experiments). The toxicity of the medium was sharply reduced if lymphocytes treated with AD were incubated, not in empty tubes, but in cultures of macrophages. It may be supposed that the toxic substance liberated into the medium from lymphocytes treated with AD is adsorbed on to target cells.

A toxic factor, which is adsorbed on to target cells and destroys them, is thus gradually liberated into the medium from normal lymphocytes treated with AD. Although AD did not kill lymphocytes after incubation for 1 h, the percentage of living lymphocytes treated with AD and washed was reduced by 2-4.7 times after incubation for 18 h at 37° compared with lymphocytes not treated with AD. It may be supposed that intracellular enzymes diffusing into the medium from lymphocytes killed by AD nonspecifically destroy some of the target cells. This hypothesis is in harmony with results indicating the high toxicity of homogenates and extracts of lymphocytes for target cells [9]. This possibility must be remembered when a hypothesis is put forward to explain the nature of the cytotoxic effect of normal lymphocytes treated with various substances [4].

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