DETERMINATION OF LYMPHOKINE-INDUCED CYTOLYTIC ACTIVITY OF MACROPHAGES BY ^{3}H-THYMIDINE RESIDUE IN PRELABELED TUMOR CELLS

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The antitumor proteolytic activity of macrophages (Mph) in vitro is known to increase under the influence of lymphokines, which are collectively known as "macrophage-activating factor" (MAF) [3, 6]. This phenomenon lies at the basis of a series of methods revealing these mediators in cultural supernatants and biological fluids. The cytolytic activity of Mph is usually estimated from the release of the isotope into the medium from tumor cells prelabeled and cultured together with them. For this purpose a label incorporated into cytoplasm ($Na_2{}^{51}CrO_4$) or into DNA of target cells (^{125}I-IdUrd — iodo-2'-deoxyuridine; ^{3}H-dThd — tritiated thymidine) [5, 7] was used for this purpose. Destruction of cells with labeled DNA could be judged from the quantity of isotope released into the medium or remaining in the acid-insoluble fraction. In one investigation [5], for instance, the cytolytic activity of Mph, activated by BCG, was determined as the ^{125}I-IdUrd residue in tumor target cells (TC).

A method of determination of MAF-induced Mph activation by the ^{3}H-thymidine residue in prelabeled tumor cells is described in this paper. Cells of mastocytoma P815 were used as the TC. Experiments were carried out in which MAF were revealed simultaneously by this method and by the standard method with the use of ^{51}Cr-labeled TC [3, 7].

The investigation was carried out on mice aged 2-4 months, bred at the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, of the following lines: C57BL/10 (B10; H-2^b), B10D2 (D2; H-2^d), B10BR (BR; H-2^k), BALB/c (H-2^d), and CBA (H-2^k). The cells were cultured in buffered medium RPMI-1640 containing 7% fetal calf serum (FCS), 2 mM L-glutamine, and 30 μg/ml of gentamicin, in a humid atmosphere with 5% CO_2 at 37°C. The following supernatants were used as the source of MAF: 1) from spleen cells (SC) of D2 mice ($5\cdot10^6$/ml), cultured with 5 μg/ml of concanavalin A (con A, from Pharmacia, Sweden), for 48 h; 2) from a secondary mixed lymphocyte culture (MLC), in which immune (D2-anti-B10) SC ($5\cdot10^6$/ml) were cultured with B10 ($0.5\cdot10^6$/ml) or D2 SC in the control in a 24-well plate (Linbro, England) in a volume of 2 ml per well for 18 h.

Mice were immunized intravenously with B10 SC irradiated in a dose of 1500 rads ($9\cdot10^7$ cells per mouse) 13 days before setting up the MLC. The supernatants were kept at −20°C. Lipopolysaccharide B (LPS) from *E. coli* 0111:B4 was obtained from Difco, USA, and thymidine from Sigma, USA.

The P815 (H-2^d) tumor cell line was maintained by passage in vitro in medium RPMI-1640 with 10% FCS. To obtain labeled TC, tumor cells ($1.5\cdot10^6$ in 5 ml) were cultured in a glass flask for 18-20 h with 1.5 μCi/ml of ^{3}H-thymidine (specific activity 6 Ci/mmole) or they were incubated in 1 ml of culture medium containing 100 μCi of ^{51}Cr for 1 h at 37°C. Cells washed to remove unincorporated isotope were used in the test.

Peritoneal cells of mice receiving an intraperitoneal injection of 1.5 ml of 10% peptone (from Serva, West Germany) 3-4 days previously were used as the source of Mph. The cell suspension ($1.3\cdot10^6$/ml) was added in a volume of 0.1 ml to the wells of a 96-well round-bottomed plate (Linbro) and incubated for 1.5-2 h. After removal of the nonadherent cells, thrice

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washed Mph were cultured with activated agents (MAF, LPS) or without them (control) for 20-22 h in a volume of 0.16 ml per well. The Mph were then washed, TC labeled with ^{3}H-thymidine were added to them, and they were cultured together for 24 or 48 h in a volume of 0.12 ml per well. After incubation the plate was shaken and centrifuged for 5 min at 200g; the supernatent was aspirated from the wells, fresh medium or 3% TCA was added (0.1 ml per well) to the residues, the cells were resuspended, and the acid-insoluble fraction was transferred by the standard method by means of a "Harvester" apparatus to glass-fiber filters, which were dried and then placed in flasks containing scintillation fluid. Radioactivity was counted on a Beta-counter (Mark 3, USA). The results were estimated by the equation:

$$\% \text{ of specific lysis} = (1 - E/C) \times 100,$$

where E denotes the number of counts per minute in the experimental samples and C the number of counts per minute in tumor cells cultured without Mph.

Culture of Mph with ^{51}Cr-labeled TC was carried out in a 96-well flat bottomed plate in a volume of 0.2 ml per well. After 18-20 h. 0.1 ml was withdrawn from each sample and the radioactivity of the specimens was estimated by means of a Gamma-counter (Mag 510). The result was calculated by the equation:

$$\% \text{ of specific lysis} = \frac{E - S}{M - S} \times 100,$$

where E is the release of isotope in the experimental samples, S the spontaneous release, and M the maximal release from TC, disintegrated by sodium dodecylsulfate.

If tumor cells labeled with ^{3}H-thymidine were cultured for 48 h with Mph, resuspended, and immediately transferred to filters, they showed only weak lysis. The use of the procedure described above revealed a high percentage of specific lysis in samples with activated Mph (Fig. 1a). Labeled DNA, escaping from the disintegrated cells, probably underwent incomplete degradation to nucleotides, and for that reason some of it could be deposited on the filter, thereby masking the lysis effect.

"Cold" thymidine, added to the medium in a concentration of 10^{-5} M, had no significant effect on cell viability and blocked reutilization of the labeled molecules. In the present experiments (Fig. 1b) addition of the above-mentioned dose did not affect release of the isotope in tests with activated Mph and increased the parameters of TC lysis under the influence of unactivated Mph to some degree. This increase was probably connected with the below-threshold toxicity of the "cold" thymidine, which is exhibited in the presence of Mph. This explanation is in agreement with data on the higher sensitivity of mastocytoma P815 cells compared with other cell populations to the toxic action of low concentrations of "cold" thymidine [8].

If LPS is present in the medium in amounts of a few nanograms, it can act as the second additional signal for triggering the lymphokine-dependent antitumor function of Mph [7]. The effect of LPS depends on the spectrum and quantity of macrophage-activating lymphokines interacting with Mph [3]. In the present experiments (Fig. 1c) the addition of LPS (10 or 100 ng) significantly potentiated the action of MAF obtained in MLC, but did not affect activation of Mph induced by supernatant from con A-stimulated SC. The range and quantity of lymphokines produced by SC in response to con A probably is sufficient to activate Mph completely in the presence of trace quantities of LPS, which are usually present in the media and sera which were used. Culture of Mph with 30% supernatant from a syngeneic lymphocyte culture of with medium containing con A did not induce their activation independently of the presence of tested concentrations of LPS (data not given).

The percentage of specific lysis in the range studied (10^4-$8 \cdot 10^4$ per well) did not depend significantly on the number of TC added to the activated Mph, and was about 60% (Fig. 2). Lysis of TC under the influence of inactivated Mph was on a very small scale.

Lysis of P815 cells labeled in different ways was investigated in the experiments whose results are given in Table 1. Mph from mice of different strains were used as effector cells. The highest values were obtained when TC labeled with ^{3}H-thymidine were cultured together with preactivated Mph for 48 h. Longer culture of Mph with TC, which was possible when the thymidine label was used, thus significantly increases the sensitivity of the method, which is particularly important when low concentrations of MAF are to be determined. No significant differences could be found in the results between Mph from the different strains of mice.

During work with a label it must be remembered that isotopes can themselves exert a toxic action on cells, thereby reducing their viability. If the toxicity induced by an isotope is

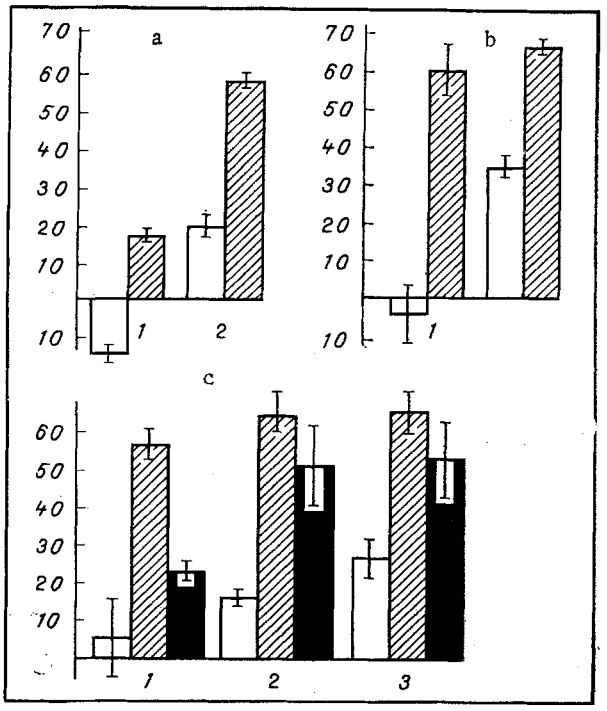

Fig. 1

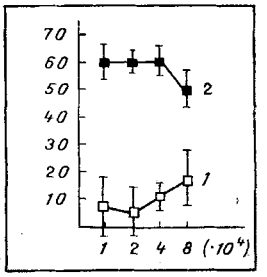

Fig. 2

Fig. 1. Effect of different conditions on detection of lytic activity of Mph. Mph cultured with 10% supernatant from con A-stimulated lymphocytes (shaded columns), with 30% supernatant from secondary MLC (black columns), or with medium (white columns). After 20-22 h, $2 \cdot 10^4$ ^{3}H-thymidine-labeled TC were added to the washed Mph and cultured together for 48 h. a) Acid-insoluble part transferred from well to filter without any change (1) or after preliminary change (2) of medium, mean results (M ± m) of five parallel determinations, constituting each test; b: 1) control (without cold thymidine), 2) Mph and TC cultured together in the presence of 10^{-5} M cold thymidine. Mean results (M ± m) of three experiments; c: 1) control (without LPS), 2) before addition of TC, Mph were cultured in the presence of 10 ng/ml, 3) the same, in the presence of 100 ng/ml of LPS. Mean results (M ± m) of three experiments. Here and in Fig. 2: ordinate, % of specific lysis. M ± m calculated by Student's test.

Fig. 2. Dependence of lysis on number of TC added to Mph. Mph cultured with medium (1) or with 10% supernatant from con A-stimulated lymphocytes (2), after which the number of TC indicated along the abscissa was added to them, and they were cultured together for 48 h. Incorporation of ^{3}H-thymidine per 10^4 labeled TC, cultured without Mph, amounted to 1000-2000 cpm. Mean results (M ± m) of three experiments.

TABLE 1. Depedence of Values of MAF-Induced Cytolytic Activity of Mph (% of specific lysis) on Label, Source of Mph, and Duration of Their Combined Incubation with TC

Series of experiments	Mph, strains of mice	TC label					
		^{51}Cr		^{3}H-thymidine			
		duration of combined incubation of Mph with TC, h					
		18		24		48	
		medium	MAF	medium	MAF	medium	MAF
I	B10D2	—3±2,4	14±2,4	—3±9,2	18±10	19±8,8	68±1,4
	B10BR	—7±1,3	9±1,5	—13±1,6	23±2,3	10±8,0	52±5,7
	BALB/c	1±2,3	12±1,0	23±7,7	22±2,3	27±6,1	71±0,5
	CBA	—6±2,3	11±1,2	27±7,1	27±2,7	0±11,0	39±3,3
II	B10.D2	—2±1,3	12±1,4	n.t.	n.t.	7±7,2	25±8,0
	BALB/c	—2±0,7	9±1,0	n.t.	n.t.	1±2,5	35±2,3
	CBA	—3±0,8	8±1,0	n.t.	n.t.	—13±7,6	22±2,8

Legend. To Mph in each well was added $2 \cdot 10^4$ TC·MAF — 10% supernatant from con A-stimulated lymphocytes (different specimens in two experiments); n.t.) not tested. Spontaneous release of ^{51}Cr from TC labeled with it was 23%. Mean results (M ± m) of three parallel tests are shown.

great, the TC labeled with it become sensitive to exposures at the subthreshold level for unlabeled cells, and in turn this makes interpretation of the results more difficult [2]. Of all the radioactive labels used, ^{3}H-thymidine is least toxic for cells [1, 4] and, consequently, it is the most suitable label for the study of the genuine antitumor properties of Mph, unconnected with the effect of the isotope. The main disadvantage of labeling with ^{51}Cr, besides its toxicity, is the high spontaneous release of the isotope into the medium, which makes the estimation of lysis impossible when labeled TC are cultured in vitro for longer than 20 h.

It can be concluded from the facts described above that the technique for detection of lysis of ^{3}H-thymidine-labeled tumor cells, in the version described above, possesses high sensitivity for determination of MAF-induced cytolytic activity of Mph. This method can be used in other systems for analysis of tumor cell destruction under conditions requiring long-term culture in vitro.

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POSSIBILITIES OF PAIRED COMPARISON OF RECEPTOR BINDING PARAMETERS OBTAINED IN A SINGLE EXPERIMENT

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Radioligand methods of analysis have now achieved wide popularity. The concrete aim of many of the investigations conducted by these methods is to compare different groups of animals with respect to receptor binding parameters. In order to analyze the results by the use of existing statistical methods, both parametric and nonparametric, a definite number of values of the parameter to be studied must be obtained in each group. The most adequate approach is to determine these values in every animal. However, this is impossible in some cases, due mainly to the insufficiency of biological material obtained from each animal, when small brain structures are used, for example. The only way of carrying out the measurement in such a case is to pool the biological material in the group [6, 7]. This method can be used and its use is justified also in preliminary investigations.

With such an approach, to obtain the necessary number of values of parameters to be studied, the procedure often adopted is to repeat experiments of a similar kind, using the same pooled material. In that case, however, not every new experiment yields additional information about the object studied, but simply enables the error of the method to be assessed.

In experiments of this type, to obtain one or two extrapolation characteristics, many measurements are required. For instance, to determine two equilibrium parameters of receptor binding (K_D and B_{max}), traditionally done on a Scatchard plot, several scores of measurements

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