IN VITRO SELECTION OF SPONTANEOUSLY TRANSFORMED STHE CELLS RESISTANT TO THE CYTOTOXIC ACTION OF MACROPHAGES AND OF HYDROGEN PEROXIDE

E. A. Vol'pe

UDC 616-006-092:612.017.1].085.2

KEY WORDS: transformed cells; natural resistance; resident and activated peritoneal exudate cells; macrophages; hydrogen peroxide; in vitro selection

Macrophages (Mph), monocytes, neutrophils, and lymphocytes (NK cells) are the principal types of effector cells of natural resistance (NR) in the defense of the host against tumor growth and dissemination of metastases. It has been shown that activated Mph can effectively destroy tumor cells in vitro and in vivo [10, 11], by producing several cytotoxic factors such as active forms of oxygen (the superoxide anion, singlet oxygen, hydroxyl radicals, and hydrogen peroxide), neutral proteases, tumor necrosis factor, and interleukins 1, 2, etc. [3, 13, 14]. Meanwhile, data have been published to show that Mph can potentiate the malignant properties of a tumor [6, 15-17]. It was shown previously that variants of cells of the STHE strain with low malignancy and spontaneously transformed in vitro, acquire malignant properties (tumorigenic and metastatic activity) during in vivo selection, and in addition, they acquire resistance to hydrogen peroxide (H_2O_2) and secrete an increased amount of prostaglandins E_2 on contact with NK cells [7, 9, 10]. We have shown that spontaneously transformed cells of the STHE strain, sensitive to the cytotoxic action (CTA) of activated macrophages, lose their sensitivity to the CTA of Mph [1]. In order to examine the possible role of Mph and other effector cells of NR in tumor cell selection in vivo, we attempted to carry out such selection in vitro, using as the selection factor, peritoneal exudate cells (PEC) of normal Syrian hamsters (resident PEC), and also PEC activated by bacterial lipopolysaccharide (LPS), and using parental cells of the STHE strain as the targets.

The aims of this investigation were: 1) to attempt to obtain variants of STHE cells resistant to CTA of Mph by co-culturing them with resident and activated PEC in vitro; 2) to study the sensitivity of variants of STHE cells, selected with the aid of PEC, to the CTA of activated Mph and hydrogen peroxide.

EXPERIMENTAL METHOD

A strain of embryonic Syrian hamster fibroblasts, spontaneously transformed in vitro (strain STHE), with a low level of malignancy, was used as the target cells (TC) [8]. Cocultivation of STHE cells was carried out with resident and activated PEC (PEC_r and PEC_a) of intact Syrian hamsters, containing 1.9 ± 0.8% of neutrophils, 56.4 ± 3.3% of macrophages, and 41.6 ± 3.5% of lymphocytes. Cocultivation cycles of newly obtained variants of STHE with PEC_r and PEC_a were repeated after 1 week. Variants of STHE cells obtained at different stages of selection with PEC in vitro were frozen in liquid nitrogen and kept until required for study. The sensitivity of the STHE variants to CTA of activated Mph was studied in the cytolytic test (CLT) [2, 4]. Mph, attracted by thioglycolate, were obtained from the peritoneal exudate of hamsters. Films of PEC were fixed with methanol and stained by the Romanovsky—Giemsa method, and at least 200 cells in each preparation were counted under the microscope. PEC obtained from animals contained 65.6 ± 1.8% of Mph. PEC were enriched with macrophages by sedimentation in 96-well flat-bottomed planchets for 2 h. According to their morpho-

Laboratory of Antitumor Immunity, Research Institute of Carcinogenesis, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Trapeznikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 8, pp. 192-195, August, 1991. Original article submitted December 29, 1990.

0007-4888/91/0008-1169$12.50

TABLE 1. Sensitivity of Parental STHE Cells and Their Variants Obtained during Cocultivation with Resident and Activated PEC in vitro to CTA of Macrophages (Mph) (aggregated data)

PEC used for cocultivation with STHE cells in vitro	Number of cycles of cocultivation	CLA[a] of macrophages (%, M ± m), activated by undermentioned agents				
		levan	n.t.[b]	MDP	PMA	inactivated (control)
Resident (PEC_r)	4	n.t.[b]	21,8[B]	24,9±3,1** (5)[2]	23,4±5,1* (5)	6,2±2,1 (6)
	6	5,7[B]	20,0±3,0* (4)	19,0±3,5* (5)	9,0[B]	3,7±1,1 (5)
	10	13,6±4,5 (3)	15,5±7,9 (3)	21,8±3,2** (10)	21,7±4,9* (4)	5,6±0,9 (10)
Activated by LPS (PEC_a)	1	—	3,6[B]	4,0±4,6 (4)	7,0±6,8 (3)	3,3±1,9 (4)
	2	—	2,8±4,6 (4)	6,7±3,6 (6)	4,1±7,1 (2)	1,3±2,0 (6)
	4	−0,4[B]	−2,1[B]	3,1±1,8 (5)	3,6±2,6 (3)	1,3±0,8 (6)
	5	1,4±1,5 (3)	0,2±1,0 (2)	3,6±0,7 (8)	−1,7±1,9 (6)	1,9±0,6 (8)
	10	0,3±0,5 (2)	3,4±2,7 (3)*	4,4±1,3 (8)	0,8±0,4 (4)	1,1±0,2 (8)
STHE parental cells (control without PEC)	—	24,2±6,5* (5)	21,9±3,0** (10)	25,3±1,9*** (22)	28,0±5,1** (9)	6,4±0,6 (23)

Legend. a) Specific cytolytic activity of Mph after incubation of Mph for 42 h with ^{3}H-thymidine-labeled STHE TC; b) n.t.) not tested; c) tested in one experiment; d) number of experiments (n) given in parentheses. Statistically significant differences (compared with inactivated control) correspond to following values: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE 2. Sensitivity of Parental STHE Cells and Their Variants Obtained by Cocultivation with Resident and Activated PEC in Vitro to Hydrogen Peroxide (H_2O_2) (aggregated data)

PEC used for cocultivation with STHE cells in vitro	Number of cycles of cocultivation	Sensitivity of TC to H_2O_2 (in %, M ± m) after treatment of surviving			Number of experiment (n)
		28	7	1,7	
Resident (PEC_r)	4	0,6±0,1	7,8±0,4*	15,2±0,1	2
	6	0,06±0,004	3,2±2,1	9,9±1,5	3
	10	1,6±1,0	16,7±7,0	24,0±7,4	6
Activated by LPS (PEC_a)	1	0,3±0,3	29,6±19,5	32,1±12,5	3
	2	0,3±0,02	25,0±9,5	30,0±8,7*	4
(PEC_a)	4	1,7±0,8	23,7±8,2	28,8±7,3*	3
	5	4,9±2,6	42,5±8,1**	50,6±7,5**	5
	10	3,0±2,3	37,6±10,3*	42,6±10,8*	4
		0,3±0,2	2,7±0,9	6,8±1,0	12

Legend. a) Incorporation of ^{3}H-thymidine label into intact target cells of each variant, not treated with H_2O_2, taken as 100%. Statistically significant differences (compared with parental STHE cells) correspond to values: *$p < 0.05$; **$p < 0.01$.

logical features 93.0 ± 3.0% of adherent cells were Mph, and the remainder were lymphocytes. To obtain complete activation of Mph, 100 μl each of the following immunomodulators was added to the wells of the planchet, the polyfructoside levan (Institute of Microbiology, Academy of Sciences of the Latvian SSR) (1 mg/ml), LPS ("Sigma," USA: 20 μg/ml), muramyl dipeptide (MDP; from "Behring Diagnostics," USA) (10 μg/ml), and phorbol-12-myristate-13-acetate (PMA; from "Sigma," USA) (2 μg/ml). Target cells, previously (18-20 h) labeled with ^{3}H-thymidine (specific radioactivity 5 Ci/mmole, dose 1 μCi/ml), washed free from unincorporated label, were added in a volume of 100 μl in three parallel tests to Mph in the ratio of 1:20. In a series of experiments, to increase the sensitivity to the CTA of Mph, the TC were treated 2 h before performance of the CLT with actinomycin D (Calbiochem, USA) 1 μg/ml [2, 5]. After incubation of TM with Mph for 42 h at 37°C in an atmosphere with 5% CO_2, 100 μl of supernatant was transferred from each well into scintillation flasks

and the radioactivity of the samples was determined (in cpm) on an "LKB 1219 RackBeta Wallac" liquid counter (Sweden). The specific cytolytic activity (CLA) (in %) was calculated by the standard method [4]:

$$CLA = \frac{\text{yield of label in experiment} - \text{spontaneous yield of label}}{\text{total yield of label} - \text{spontaneous yield of label}} \cdot 100,$$

where the total yield of the ^{3}H-thymidine label was obtained by addition of a 2% solution of SDS ("Serva," West Germany) to the labeled STHE TC. The sensitivity of the different variants of STHE to H_2O_2 was tested by the method described previously [7]. Briefly, the test target cells were treated for 30 min at 20°C with three different concentrations, differing by a factor of 4, of H_2O_2 (28, 7, and 1.7 mM respectively). Next, the H_2O_2-treated TC, like the intact control cells, were washed, resuspended in Eagle's medium with lactalbumin hydrolysate containing 10% bovine serum and ^{3}H-thymidine (1 μCi/2 ml medium), and transferred into;o three scintillation flasks for culture at 37°C. Incorporation of ^{3}H-thymidine into the cell nuclei was determined after 21 h as a percentage of the corresponding intact control. Each variant of the cells was tested repeatedly in two or three or more experiments. The results were subjected to statistical analysis by Student's t test, differences being considered significant at the $p < 0.05$ level.

EXPERIMENTAL RESULTS

Several (from one to 10) successive cycles of cocultivation of the parental STHE cells with PEC_r and PEC_a were used for selection. Each selection cycle was carried out in vitro for 1 week. For this purpose a mixture of STHE cells and PEC (1:10) was centrifuged for 10 min at 1000 rpm, and the residue was resuspended in Eagle's growth medium with lactalbumin hydrolysate and 10% bovine serum and grown in Carrel flasks. After incubation for 40-44 h at 37°C the monolayer was washed with Hanks' solution at 37°C to remove the LPS activator and nonadherent PEC, after which fresh medium was added. If necessary, the change of medium was repeated. After the very first cycle of cocultivation of STHE cells with LPS-activated PEC, but not with resident PEC, mass death of STHE cells was noted. The LPS activator itself had no toxic action in a concentration of 20 μg/ml on STHE cells, since addition of LPS to the parental STHE cells in the absence of PEC did not lead to death of the STHE cells. TC surviving after cocultivation with PEC proliferated and were tested for sensitivity to the CTA of Mph and to H_2O_2.

In one series of experiments the sensitivity of STHE variants selected with PEC_r and PEC_a, to the CTA of peritoneal Mph, attracted by thioglycolate and additional ly activated by levan, LPS, MDP, and PMA, was tested. The aggregated results of these investigations are given in Table 1. They show that virtually all the variants of STHE tested, selected in four, six, and 10 cycles of cocultivation with resident PEC in vitro, preserved high sensitivity to the CTA of activated Mph, just like the original cells of the parental STHE strain, not subjected to selection with PEC. After brief treatment of the test TC with actinomycin D, some increase was observed in the sensitivity of the STHE variants, which had undergone four and 10 selection cycles with PEC_r (data not given), to the CTA of Mph was observed. Only in one of 10 experiments was resistance to the CTA of activated Mph of the STHE variant observed, after 10 cycles of cocultivation with PEC_r in vitro.

Unlike these observations, when STHE variants obtained after 1, 2, 4, 5, and 10 cycles of cocultivation with LPS-activated PEC in vitro were tested, it was found that all the test variants became highly resistant to the CTA of Mph, activated by levan, as early as after the first cycle of selection. This property of TC, moreover, was found to be stable and was maintained during passage of the selected variants in vitro in the absence of PEC. Treatment of these TC with actinomycin D had no significant effect on the strengthening of cytolysis of TM by activated Mph.

It became clear that cocultivation of STHE cells with activated PEC leads to mass death of the overwhelming majority of STHE cells and to selection of variants of these cells resistant to the CTA of Mph. In this connection it was interesting to study the sensitivity of the selected variants to H_2O_2 (since, as we know, hydrogen peroxide is secreted by activated Mph and neutrophils, but not by lymphocytes). Meanwhile, in our experiments on selection of STHE cel ls in vitro, an unfractionated mixture of PEC was used, which included about 40% of lymphocytes. Table 2 gives the aggregated results of a series of experiments to test the sensitivity of original parental STHE cells, three variants of STHE obtained after four, six, and 10 cocultivation cycles with PEC_r in vitro, and also five variants of STHE obtained after one, two, four, five, and 10 cocultivation cycles in vitro with PEC_a, to H_2O_2. As will be clear from these results, the original parental STHE cells, like the STHE variants selected with PEC_r as a result of four or six cocultivation cycles in vitro, preserved high

sensitivity to the damaging action of H_2O_2. After 10 cocultivation cycles with PEC_r, the STHE cells evidently became more resistant to H_2O_2, as was demonstrated in four of six tests.

By contrast with these observations, after the first cocultivation cycle of STHE cells with PEC_a in vitro, the surviving variants of STHE cells were resistant to the action of H_2O_2 and preserved (or even, perhaps, increased) this resistance during subsequent cycles of selection with the aid of PEC_a (after five and 10 cocultivation cycles with PEC_a) (Table 2).

Thus as a result of successive cocultivation cycles of original spontaneously transformed STHE cells in vitro (never with those selected in vivo) with peritoneal cells activated by bacterial LPS, we obtained stable variants of STHE possessing new characteristics, namely resistance to the CTA of activated macrophages and to hydrogen peroxide.

Unlike in our own experiments, the unsuccessful attempts of other workers to select variants of tumor cells resistant to the CTA of Mph could perhaps be explained on the grounds that, to study the possible role of macrophages in the selection of malignant variants of cells, they used as TC tumor cells obtained from an animal (i.e., in fact selected in vivo and having had contact with Mph during tumor growth) [12].

Selection of STHE variants resistant to CTA of macrophages and, simultaneously, resistant to hydrogen peroxide, with the aid of activated PEC in our experiments may be evidence of the selective role of activated macrophages (and also, perhaps, of neutrophils, also present in the composition of PEC). Meanwhile the use of unfractionated cell populations, constituting the peritoneal exudate, in the present investigation and their activation by LPS may also lead to involvement of other types of effectors of NR, and especially NK cells, in the selection of STHE cells. The study of this possibility, and also of the malignant characteristics of the selected STHE variants (i.e., their tumorigenicity and metastatic activity) will be the topic of our forthcoming research.

LITERATURE CITED

1. E. A. Vol'pe, Abstracts of Proceedings of the First All-Union Immunologic Congress [in Russian], Vol. 2, Moscow (1989), p. 29.
2. E. A. Vol'pe, Byull. Éksp. Biol. Med., **111**, No. 1, 59 (1991).
3. J. A. Badwey and M. L. Karnovsky, Curr. Top. Cell. Regulat., **28**, 183 (1986).
4. A. Benomar, D. Gerlier, and J.-F. Dore, Int. J. Cancer, **38**, 419 (1986).
5. F. Colotta, G. Peri, A. Villa, and A. Mantovani, J. Immunol., **132**, 936 (1984).
6. P. De Baetselier, A. Kapon, S. Katzav, et al., Invasion and Metastasis, **5**, 106 (1985).
7. G. I. Deichman and E. L. Vendrov, Int. J. Cancer, **37**, 401 (1986).
8. G. I. Deichman, T. E. Kluchareva, L. M. Kashkina, et al., Int. J. Cancer, **30**, 349 (1982).
9. G. I. Deichman, T. E. Kluchareva, V. A. Matveeva, et al., Int. J. Cancer, **44**, 904 (1989).
10. G. I. Deichman, Sov. Med. Rev. F, Oncol., **1**, 47 (1987).
11. R. Evans, Cancer Metastasis Rev., **1**, 227 (1982).
12. W. E. Fogler and I. J. Fidler, Cancer Res., **45**, 14 (1985).
13. W. J. Johnson, S. D. Sommer, and D. O. Adams, Contemp. Top. Immunobiol., **13**, 127 (1984).
14. C. F. Nathan, J. Clin. Invest., **79**, 319 (1987).
15. L. M. Remels and P. C. De Baetselier, Int. J. Cancer, **39**, 343 (1987).
16. J. L. Urban and H. Schreiber, J. Exp. Med., **157**, 642 (1983).
17. K. Yamashina, A. Fulton, and G. Heppner, J. Nat. Cancer Inst., **75**, 765 (1985).