DETECTION OF TUMOR CELL CYTOTOXICITY AGAINST NORMAL TARGET KILLERS

B. B. Fuks, M. S. Zedgenidze,
I. V. Spirande, I. N. Kogarko,
and A. G. Sterlina

UDC 616-006-018.1-092:612.017.4

KEY WORDS: killers; cytotoxicity; tumor cell.

During the last decade the study of the cytotoxicity of T killers [1, 2], normal killers [4], and K cells and macrophages [5, 9, 15] relative to the tumor cell has attracted considerable attention.

The action of tumor cells on the cells and tissue structures of the host has received comparatively little study in connection with the problem of tumor invasion and metastasization. It is still not known exactly how malignant cells invade the host's tissues [10]. Active and passive movements of the tumor cell [14], and the secretion by the tumor cell of neutral proteinases, lysosomal hydrolases, and enzymes destroying basement membranes, glycoproteins, collagen, and elastin of the matrix have been suggested as the possible causes of invasion [6, 8, 10, 11]. However, the mechanism of action of these factors on the host cell has virtually not been studied. Tumors are known to have an immunosuppressive action [12, 13]. However, the action of the malignant cell on the membrane of other cells, especially lymphocytes, has not been elucidated. This is partly because of the lack of a sufficiently sensitive technique for testing damage to cell membranes.

EXPERIMENTAL METHOD

Primary mouse leukemic cells EL-4 ($H2^b$) and L-1210 ($H2^d$), methylcholanthrene sarcoma MKh-11 of C57BL/6 mice, and blood lymphocytes from five patients with chromic lymphatic leukemia, with a leukocyte count of more than 200,000/mm^3, were used as effectors. In each experiment effector cells from at least three tumor-bearing mice were used. In control experiments the effectors were spleen cells from mice of various lines.

Human K-562 erythroleukemia cells and mouse EL-4 leukemia cells, adapted to *in vitro* conditions [3], were used as target cells. Medium RPMI-1640 with 10% bovine fetal serum (Flow) was used.

The target cells were labeled with ^{3}H-uridine and the cytotoxic test was carried out by a highly sensitive technique improved by the writers [3]. The cytotoxicity index CI was calculated by the equation:

$$CI = \left(1 - \frac{\text{target cells incubated with effector cells}}{\text{target cells incubated without effector cells}}\right) \times 100.$$

As a rule in the cytotoxic test a control was used with cold inhibition of the target cells (K-562) and with a ratio of effectors : labeled target cells : unlabeled target cells of 50:1:50. The routine method of cytotoxicity testing with targets labeled with ^{51}Cr was used in the control experiments.

Isolation of the cells subcultured intraperitoneally in syngeneic mice was carried out on a Hypaque–Ficoll gradient with density 1.078-1.09 g/cm^3 on a K-23 centrifuge (Janetzki) at 3000 rpm for 15 min at 20°C. Leukemic cells L-1210, subcultured intraperitoneally in C57BL/6 mice were isolated by mass-cytolysis with anti-$H2^b$ sera and rabbit complement, followed by removal of the killed cells on Hypaque–Ficoll. Films of the fractionated cells were stained with methyl green and pyronine.

Institute of Human Morphology, Academy of Medical Sciences of the USSR. Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Blokhin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 92, No. 9, pp. 342-345, September, 1981. Original article submitted April 24, 1981.

 0007-4888/81/9209-1250$07.50

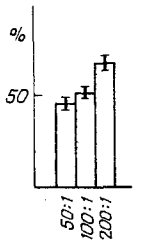

Fig. 1

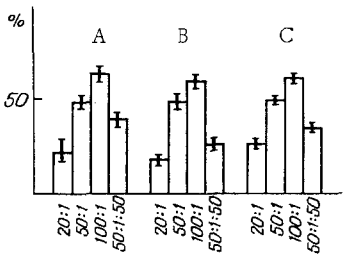

Fig. 2

Fig. 1. Cytotoxicity of L-1210 ($H2^d$) mouse leukemia cells against EL-4 ($H2^b$) mouse leukemia cells adapted to tissue culture. Abscissa, ratio of effector cells to target cells in cytotoxic test; ordinate, CI (in %).

Fig. 2. Cytotoxicity of MKh-11 sarcoma cells (A), and of EL-4 (B) and L-1210 (C) mouse leukemia cells against K-562 human erythroleukemia cells cultured *in vitro*. Legend as to Fig. 1.

The anti-$H2^b$-serum was obtained by immunizing BALB/c mice intraperitoneally with a suspension of spleen cells from C57BL/6 mice ($10 \cdot 10^6$) in Freund's complete adjuvant weekly for 6 weeks. Anti-HB^b-serum obtained by immunizing M523 mutants of CBA ($H2^k$) mice with C57BL/6 mouse cells, and BLOD2 ($H2^d$) mice with BLO ($H2^b$)[1] mouse cells.

EXPERIMENTAL RESULTS

First, cytotoxicity of L-1210 leukemic cells isolated from the peritoneal cavity of BALB/c mice against EL-4 leukemic cells subcultured *in vitro* (Fig. 1) was detected. In this experiment (as in those which followed) the suspension containing L-1210 cells, before the cytotoxic test, was adsorbed on plastic Petri dishes (Flow, No. 6120307) for 45 min at 37°C in medium No. 199 with 10% fetal serum (Flow) to remove macrophages, and then divided on Hypaque–Ficoll. Small lymphocytes were present as impurities in the original suspension to the extent of 15-17%, but only 3-5% in the interphase. EL-4 leukemia cells growing *in vivo* were cytotoxic against the same cells growing *in vitro* (CI 38%).

In the next experiments cytotoxicity of leukemia and tumor cells L-1210, EL-4, and MKh-11, isolated from the peritoneal cavity, against K-562 cells was detected. Heating the EL-4 and L-1210 cells for 20 min at 56°C virtually abolished their cytotoxicity (CI 8-9%). An appreciable difference was found in the cytotoxicity of the original suspensions of tumor cells and interphase cells. With a ratio of effector to target cells of 100:1, CI had the following values respectively: MKh-11 (initial suspension) 36%, interphase 72-78%; EL-4 (initial suspension) 39-48%, interphase 60-78%; L-1210 (initial suspension) 30-40%; interphase 75-80%. Mainly the large tumor cells remained in the interphase. Later, the initial suspensions of peritoneal tumor cells were fractionated on Hypaque–Ficoll with a density of 1.078. Under these circumstances only large tumor cells remained in the interphase, and contamination by small lymphocytes was reduced to 1%. As Fig. 2 shows, cytotoxicity was clearly dependent on dose of interphase tumor effector cells. Labeled target cells inhibited cytotoxicity by 50-80%. A separate experiment showed that with a ratio of effector to target cells of 200:1, CI in the presence of RNase was 61% and in its absence 33%.

Although contamination of the tumor cells by macrophages and lymphocytes could be substantially reduced by adsorption on plastic and centrifugation on Hypaque–Ficoll, the presence of blast forms of lymphocytes as impurities still remained possible. Accordingly experiments were carried out in which tumor cells were subcultured in an allogeneic environment. L-1210 ($H2^b$) cells were subcultured intraperitoneally in C57BL/6 ($H2^b$) mice, followed by removal of the allogeneic ($H2^b$) cells by mass cytolysis with anti-$H2^b$-serum and complement and by centrifugation on Hypaque–Ficoll.

In the thrice reproduced experiment described below L-1210 cells were isolated after three or four subcultures in C57BL/6 mice. After adsorption of macrophages and removal of drying cells by centrifugation on Hypaque–Ficoll, the viability of the cells in interphase was over 99% in the test with trypan blue and eosin. Mass cytolysis was carried out in 1 ml

of medium. For 30 min the cells were treated with anti-$H2^b$-serum at 4°C, then for 60 min in the presence of complement at 37°C. The number of cells which died was 12%. In parallel control tests (without serum and complement or with complement alone) viability still remained at about 99%. The cells subjected to mass cytolysis were washed twice and centrifuged on Hypaque—Ficoll. The viability of the cells remaining in interphase was over 99%. In this experiment, in a 14-h test and with the ratio of effector to target cells of 20:1, 50:1, and 100:1, CI before mass cytolysis was 20, 43, and 56% respectively, but after mass cytolysis and removal of the killed cells on Hypaque—Ficoll it was 18, 43, and 60% respectively (cold inhibition amounted to 82%).

Consequently, removal of foreign cells (allogeneic lymphocytes, macrophages, etc.) did not abolish and did not reduce the cytotoxicity of the L-1210 cells against K-562 target cells.

To assess whether contamination with normal killers (lymphocytes or peritoneal macrophages) can persist for a long time during repeated passage of L-1210 cells through C57BL/6 mice the following control experiments were set up. L-1210 cells subcultured intraperitoneally in C57BL/6 mice were transplanted intraperitoneally into CBA ($H2^k$) mice, and contamination with $H2^b$ cells of the $H2^b$ haplotype was tested by means of the cytotoxic test (with anti-HB^b-serum). On the 4th day of tumor growth in the peritoneal cavity of the CBA mice, cells sensitive to anti-$H2^b$-serum were absent from the tumor cell suspension. A similar result was obtained in another experiment. L-1210 cells, grown intraperitoneally in DBA/2 mice (77%) were mixed with peritoneal lymphocytes (23%) of intact C57BL/6 mice and injected intraperitoneally into CBA mice. Five days later, no cells of the $H2^b$ haplotype were found in the tumor cell suspension in the cytotoxic test.

The results of experiments with cold inhibition of cytotoxicity show, first, the need for contact between effector cell and target cell and, second, they are indirect evidence against the possible cytotoxic action of effector cell products secreted into the culture medium. Finally, they are evidence also against death of labeled K-562 target cells as a result of worsening of the conditions for their nutrition in the presence of a large number of effectors. However, to obtain a direct answer to the question of whether products of tumor effector cells secreted into the medium may have a cytotoxic action, the following experiment was carried out. EL-4 cells, after adsorption on plastic and centrifugation on Hypaque—Ficoll, were incubated for 14 h with unlabeled K-562 target cells (100:1) in the presence or absence or ribonuclease (10 µg/ml). A 0.1 ml sample of the incubation medium was then taken and labeled K-562 cells (10^4) were incubated in it for 4-14 h at 37°C in the presence of ribonuclease (10 µg/ml). CI in this experiment did not exceed 1%.

The possibility of potentiation of the cytotoxicity of the tumor effector cells on account of ribonuclease present in the incubation medium also was tested. For this purpose, ribonuclease (1-10 µg/ml) was added to the incubation medium in three experiments with target cells labeled with ^{51}Cr. CI was increased by 1-2% (1 µg RNase/ml) and by 4-8% (10 µg RNase/ml) compared with its level in the test without addition of ribonuclease. Peritoneal cells isolated from the peritoneal cavity of BaLb/c mice immediately after rejection of the allogeneic EL-4 leukemia cells (11th day) were weakly toxic against K-562 cells in the ratios of 2:1, 5:1, and 10:1, and CI was 5, 7, and 9% respectively, i.e., they were present in a number of equivalent to those present as contamination of the tumor cells (about 12%).

Blood lymphocytes from five patients with lymphatic leukemia, with a high blood leukocyte count, proved to be cytotoxic against K-562 cells. With effector-target cells ratios of 20:1, 50:1, and 100:1, CI in these patients was 33, 37, and 43%; 35, 40, and 55%; 8, 11, and 15%; 32, 55, and 61%; and 34, 54, and 63% respectively.

As was shown above, mouse tumor cells of strains EL-4, L-1210, and MKh-11, and also blood leukocytes from patients with chronic lymphatic leukemia were cytotoxic against the targets of normal killers — K-562 cells. EL-4 and L-1210 cells also were cytotoxic against EL-4 cells adapted *in vitro*. The cytotoxicity was linked with injury to target cell membranes because it became permeable to the enzyme ribonuclease. RNase itself does not act on target cells and virtually does not potentiate the cytotoxic effect when the routine technique for testing toxicity with ^{51}Cr is used. In experiments with sarcoma MKh-11 a cytotoxic effect against K-562 cells (33%) was obtained without the addition of pancreatic RNase to the incubation medium.

Death of the target cells was not connected with worsening of the conditions of their metabolism in the presence of a large number of tumor cells. On the addition of an excess of unlabeled target cells (cold inhibition experiments) cytotoxicity was reduced by 50-80%. The culture fluid obtained by incubating tumor cells (alone or with unlabeled target cells) was not toxic for ^{3}H-uridine-K-562 cells. These two facts are arguments in support of the need for direct contact between effector tumor cell and target cell for the latter to be injured.

The increase in cytotoxic effect with an increase in the ratio of effector tumor cells to target cells is a paradoxical fact. One possible explanation for it is that only a certain proportion of tumor cells can exert a cytotoxic action on the target.

However, to eliminate the role of contamination (by normal killers or macrophages) the tumor cells were isolated from the suspension by fractionation on Hypaque—Ficoll, after adsorption of macrophages on plastic. Contamination of the tumor cells by lymphocytes was reduced from 15-17 to 1% (morphological criterion), and with macrophages from 16-20 to 1%. This did not affect the cytotoxicity of the tumor cells. Removal of more than 90% of contaminating cells of the $H2^b$ haplotype in L-1210 cells during passage through C57BL/6 mice with the aid of anti-$H2^b$-serum and complement followed by sedimentation of the killed cells on Hypaque—Ficoll did not affect the cytotoxicity of the L-1210 cells against K-562 cells.

Peritoneal lymphocytes isolated immediately after death of the ascites tumor were weakly toxic (9%) against K-562 cells in the ratio of 10:1. This corresponds to the ratio of 100:1 in experiments with an unfractionated suspension of effector tumor cells (CI 50-60%).

This analysis thus shows that the cytotoxicity of leukemia and tumor cells against K-562 and EL-4 targets is independent of contamination by macrophages or normal killers. The question of the nature of the cytotoxic action of the tumor cells is not yet answered. The possibility cannot be ruled out that the effector tumor may secrete cytotoxic substances and that a high local concentration of them may be created in the intermembranous space. This possibility has been discussed with respect to a model of interaction between T-killers and a target [2]. Unlike in known models (T killer — target, NK — target, K cell — target), no hypothesis has yet been put forward at this stage on the method whereby the target cell is "recognized" by the effector tumor cell. Perhaps "recognition" does not take place at all, merely random contact.

LITERATURE CITED

1. B. D. Brondz and O. V. Rokhlin, Molecular and Cellular Bases of Immunologic Identification [in Russian], Moscow (1978).
2. S. N. Bykovskaya, A. F. Bykovskii, A. V. Sergeev, et al., Byull. Éksp. Biol. Med., No. 10, 443 (1977).
3. R. B. Herberman, J. Y. Djeu, H. D. Kay, et al., Immunol. Rev., 44, 43 (1979).
4. T. Igarashi, D. Rodrigues, and C. Ting, J. Immunol., 122, 1519 (1979).
5. P. A. Jones and Y. A. De Clerck, Cancer Res., 40, 3222 (1980).
6. L. A. Liotta, S. Abe, R. P. Gehron, et al., Proc. Am. Assoc. Cancer Res., 20, 235 (1979).
7. J. R. Oehler, D. A. Campbell, and R. B. Herberman, Cell. Immunol., 28, 355 (1977).
8. G. Poste and G. J. Fidler, Nature, 283, 139 (1980).
9. P. Strauli and L. Weiss, Eur. J. Cancer, 5, 1 (1977).
10. O. Stutman, Adv. Cancer Res., 22, 261 (1975).
11. C. C. Ting and D. Rodrigues, Proc. Natl. Acad. Sci. USA, 77, 4265 (1980).
12. J. P. Trinkaus, in: The Cell Surface in Animal Embryogenesis and Development, ed. G. Poste and G. Nicolson, Amsterdam (1976), pp. 225-329.
13. W. H. West, R. B. Boozer, and R. B. Herberman, J. Immunol., 120, 90 (1978).