REVERSIBLE INJURY TO THE NORMAL KILLER MEMBRANE ON CONTACT WITH A TUMOR CELL

L. V. Van'ko, I. V. Bogdashin,
and B. B. Fuks

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An investigation has been reported [5] which showed that normal human killer cells, in contact with target cells (K-562), are temporarily inactivated. The present writers showed previously [4] that tumor cells injure the target cells usually used for testing normal killers, and also lymphocytes with a reduced level of RNA and protein synthesis. A modified method of Hamaoka [1] was used for testing reversible cell membrane damage. The modification was that pancreatic ribonuclease was added to the culture medium in a concentration of 1 μg/ml. Reversibility of injury to the membrane was judged from hydrolysis of [^{3}H]-RNA due to penetration of ribonuclease molecules inside the target cells. In previous experiments purified suspensions of effector tumor cells were used. However, the possibility that minimal contamination by other cytotoxic cells may have been present among the tumor target cells was not completely ruled out.

It was accordingly desirable to investigate the process of membrane injury to normal killer cells under conditions allowing visual control of the cells in contact, i.e., on isolated conjugates of effector tumor cell with the lymphocyte identifying the tumor cell and acting in this case as target cell, and details of the investigation are given below.

EXPERIMENTAL METHOD

The target cells were splenocytes of A/Sn mice labeled with [^{3}H]uridine in a concentration of 4 μCi/ml per 3×10^6 cells (specific activity 24 Ci/mmole) for 1 h at 37°C. The same lymphocytes treated *in vitro* with actinomycin D (from Serva, West Germany) in a concentration of 1 μg to 1 ml medium RPMI-1640 (Flow Laboratories, England) with 10% embryonic calf serum for 6 h at 37°C also were used as target cells. The effector tumor cells were YAC mouse plymphoma cells obtained from Dr. Petranyi (to whom the author is deeply grateful), and maintained *in vivo* in A/Sn mice. Cells of the same lymphoma maintained *in vitro* (YAC-1) also were used.

Tumor cell—lymphocyte pairs (conjugates) were tested by the writers' modification of Bonavida's method [6]. Effector cells and target cells were mixed in the ratio of 1:1 or 1:2, incubated for 5 min at 37°C, and centrifuged at 200g for 5 min. The supernatant was poured off, the residue resuspended, and added to 0.5% solution of liquid agarose, cooled to 37°C. Fragments of coverslips (10 × 10 mm) were dipped into the solution of agarose with the cells. Coverslips with a layer of cells in agarose were placed in the wells of a cell culture plate (3 cm^3 × 24), to which 0.5 ml of medium was added after gel formation. Pancreatic ribonuclease (from Serva) in a concentration of 1 μg/ml was added to the cell mixture after conjugate formation or to the medium after gel formation.

After incubation for 1.5 h at 37°C the preparations were fixed with ethanol for 10 min, glued to slides by means of dammar resin, and after drying they were treated with 5°C for 20 min. The preparations were coated with type M photographic emulsion and exposed for 10 days in darkness at 4°C. After development, the specimens were stained with methyl green and pyronine. The number of grains of reduced silver above the labeled target cells

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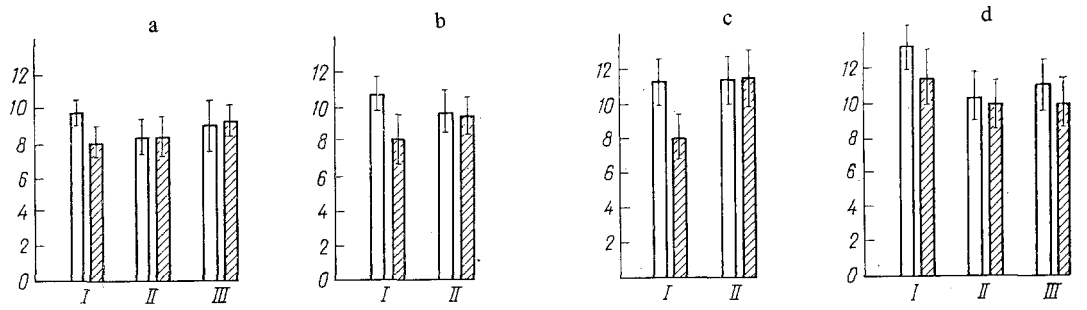

Fig. 1. Effect of ribonuclease on [^{3}H]-RNA content in splenic lymphocytes in contact with tumor cells. Ordinate, number of grains of reduced silver per lymphocyte. I) Intensity of labeling of lymphocytes when ribonuclease added before junction formation, II) when ribonuclease was added after junction of formation, III) when cells were incubated in the absence of ribonuclease. Shaded columns denote mean number of grains of silver per lymphocyte in contact with tumor cell, unshaded columns denote mean number of grains of silver per lymphocyte not in contact with tumor cell. a) Interaction between lymphocytes not pretreated with actinomycin D and YAC tumor cells maintained *in vivo*; b) interaction between lymphocytes pretreated with actinomycin D and YAC tumor cells maintained *in vivo*; c) interaction between lymphocytes treated with actinomycin D and SA-1 tumor cells maintained *in vivo*; d) interaction between lymphocytes treated with actinomycin D and YAC-1 tumor cells maintained *in vitro*.

(lymphocytes) in contact with effector cells (at least 80-90 cells) and above target cells not in contact with effector cells (100-110 cells) was counted. The results of counting were subjected to statistical analysis by Student's test. The confidence interval was calculated with a level of significance $P > 0.95$.

EXPERIMENTAL RESULTS

When YAC cells transplanted into A/Sn mice were used as tumor effector cells and mouse splenocytes not treated with actinomycin D were used as target cells, no difference was found in the intensity of labeling between splenic lymphocytes in contact or not in contact with the effector cells if the cells were incubated without the addition of ribonuclease or if ribonuclease was added to the medium after conjugate formation (Fig. 1a). A small but significant difference in the intensity of labeling was discovered between target cells in contact and not in contact with effector cells when the enzyme was added to the test system before conjugate formation. The mean level of label in target cells in contact with the effector was 13% lower.

In the next experiment YAC-1 cells maintained for a long period *in vitro* were used as effector cells. It will be clear from Fig. 1d that there was a small but significant decrease (by 14%) in the content of [^{3}H]-RNA in lymphocytes in contact with a tumor cell. This difference was not present if the ribonuclease was added to the medium after conjugate formation.

Mouse spleen cells forming junctions with tumor cells maintained *in vivo* and *in vitro* can be conveniently regarded as normal killers. According to data in the literature [7] on human lymphocytes, about 20% of cells binding target cells kill them during incubation for 3 h at 37°C. This percentage can be increased to 30-35 by treating the lymphocytes with interferon before conjugate formation [6, 7]. On the basis of the facts described above, in future we shall speak of lymphocytes capable of forming conjugates with tumor cells.

These experiments showed that without pretreatment of lymphocytes with actinomycin D there was a small but significant decrease in the [^{3}H]-RNA content in these cells if they had been in contact with tumor cells, provided that ribonuclease was added to the medium before conjugate formation. If the same lymphocytes treated with actinomycin D were used to obtain pairs, the fall in the level of labeling in target cells which had been in contact with tumor cells became more marked. Treatment with actinomycin D was adopted because of data in the literature [8] and our own results [3] showing an increase in sensitivity of actinomycin D-treated target cells relative to cytotoxic cells, evidently due to a reduction in protein synthesis and disturbance of membrane repair.

Similar results were obtained when different effector cells were used, namely sarcoma SA-1 cells. Cells of a YAC-1 tumor, cultured *in vitro*, had a weaker harmful action. In all experiments penetration of pancreatic ribonuclease (as could be judged from the decrease in labeled RNA) into lymphocytes forming a junction with a tumor cell took place if the enzyme was added to the system before the formation of an effector cell—target cell conjugate. Hence it follows that molecules of ribonuclease present in the zone of direct contact between cell membranes penetrate into the cytoplasm of the labeled lymphocyte.

A change in the properties of the lymphocyte membrane was thus demonstrated in isolated conjugates of a tumor cell and lymphocyte, firmly bound with a tumor cell, in the zone of contact with the tumor cell membrane, as shown by increased permeability of the lymphocyte membrane for a fairly large protein molecule (mol. wt. 11.5 kilodaltons), and an effect previously described as "membrane toxicity" could be demonstrated in the cytological preparations. The writers showed previously [2] that such changes in the membrane of target cells not penetrated with actinomycin D by no means always terminate in death. However, intensive pretreatment of target cells with actinomycin D can convert "membrane toxicity" into cytotoxicity, which can be tested by the release of ^{51}Cr from labeled target cells.

The physiological importance of this reversible injury to the membrane of a leukocyte in contact with a tumor target cell requires further analysis. In particular, an answer must be found to the question whether the phenomenon observed is linked with the temporary inactivation of normal killers described previously [5] and what is the relationship between membrane toxicity of the tumor cell and the immunosuppressive action of the tumor on the animal or human organism.

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