ORIGINAL REPORT

A NEW METHOD FOR CYTOTOXICITY *IN VITRO* TEST OF CELLULAR IMMUNITY

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INTRODUCTION

Development of cytotoxic test *in vitro* has considerably contributed to the progress of immunology against tumor cells from experimental animal and from several types of human neoplasia. Tumor specific transplantation antigen has been found in all experimental tumors. Chemical carcinogen induced and viral induced tumors have been mostly used as models of autochthonous tumors.

Spontaneous mammary tumor (SMT) may provide a model useful in such investigations since they have been a convenient model for the investigations between tumor and host defenses. Both *in vivo* (Morton,[1] Vaage,[2] Irie[3]) and *in vitro* (Hellström,[4] Heppner,[5] Le Francois[6]) studies have shown the specific nature of the immunological response to such tumors. In most of these investigations SMT from different lines of C3H mice have been employed. In the present study, SMT from DBA/2Ha-DD mice have been used. A quantitative *in vitro* cytotoxicity test which based on colony inhibition (7) by immune lymphocytes have been used to study the host response against primary spontaneous mammary tumors (4, 5). The use of an *in vitro* test for the direct measurement of cytotoxicity on cultured SMT cells is the subject of the present report. In this assay, the cytotoxic activity of syngeneic and allogeneic lymphocytes has been tested against tumor cells in confluent and complete monolayer.

MATERIALS AND METHODS

Tumors and Mice: 2–3 month old mice of following strains have been used; DBA/2Ha-DD, DBA/2J and C57Bl/6Ja. The incidence of SMT in DBA/2Ha-DD mouse is about 70%. Most tumors appear at the age of 7–12 months. SMT can be transplanted from the

primary host to syngeneic DBA/2Ha-DD mice. In this study, both immunizing and target cells were from either SMT or first transplant generations of SMT in DBA/2Ha-DD mice.

Preparation of Tumor and Kindney Cell Suspensions: Tumors were excised aseptically, and freed of obviously necrotic portions. Small tumor pieces (2–3 mm^3) were placed in a 1 : 1 mixture of 0.2% trypsin solution and Eagle's minimum essential medium with Eagle's balanced salt solution which was enriched 3 times in its content of amino acids and vitamins (3 × E medium). Trypsinization was then carried out at 37° with gentle stirring for 60 minutes. If a lot of tumor cells would be needed, this trypsinization was repeated 2 times at 60 minutes intervals. The suspension was centrifuged and the cells were washed twice in 3 × E medium. Cell viability in the final suspension was determined by the trypan blue exclusion method and was found to be 50–70%. All calculations were in each case based upon viable cells only. Kidneys from 10–20 day old mice were treated in the same way to obtain suspensions of normal cells. In some comparative experiments tumor cell suspensions were obtained by shaking and mechanical teasing without trypsinization, and no differences in experimental patterns could be discerned with respect to trypsinized cells.

Immunization and Preparation of Effector Cells: For immunization tumor cells suspensions were injected intraperitoneally (i.p.) into mice of each of the three lines used, taking advantage of the fact that SMT cells do not grow rapidly in the peritoneal cavity of syngeneic DBA/2Ha-DD mice. Otherwise specified immunizing cells were injected 3 times at 7 day intervals. Spleen cells were obtained 7 to 14 days after last immunization. Spleen cell suspensions were prepared as follows. The spleen capsule was cut with fine scissors and the cells teased gently into cold 3 × E medium with blunt forceps. After one washing, the red blood cells were removed by hypotonick shock; 2 volumes of distilled water were added to 1 volume of cell suspension in medium for 20 seconds and isoosmolarity was then restored by adding medium 10 times in excess. After washing, the cells were centrifuged for 5 minutes at 1500 rpm and resuspended at the desired concentration.

Tissue Culture: Primary cultures were prepared for each experiment. 3 × 10^7 viable tumor cells or kidney cells were added to 30 ml of 3 × E medium supplemented with 10% heat inactivated fetal calf serum and incubated at 37°C. The medium was usually changed on the second day to discard dead cells and cell debris. When the growth of the cells reached the stage of confluent monolayer (5th or 6th day of culture), the primary culture was used as a source of cells for secondary cultures on which performed the cytotoxicity tests. The secondary culture of tumor cells thus prepared was found to be homogeneous and suitable for the test.

Cytotoxicity Test: Cell from primary cultures were collected after trypsinization (0.2% trypsin). After two washings in 3 × E medium, 2 × 10^4 viable cells in 0.2 ml 3 × E medium were plated on each well of a Micro Test II Plate (Falcon Plastics No. 3040) as shown in Fig. 1. Only roughly half of these cells could attach on the well surface and divide. After 24–48 hours incubation at 37°C in 5% CO$_2$ in air, the cells formed a confluent monolayer on the whole surface of the well bottom. The medium was removed and these monolayer were washed once with fresh medium. Spleen lymphocyte to target cell ratio of either 400 : 1 or 200 : 1, or 100 : 1. This ratio was established based on the observation that the number
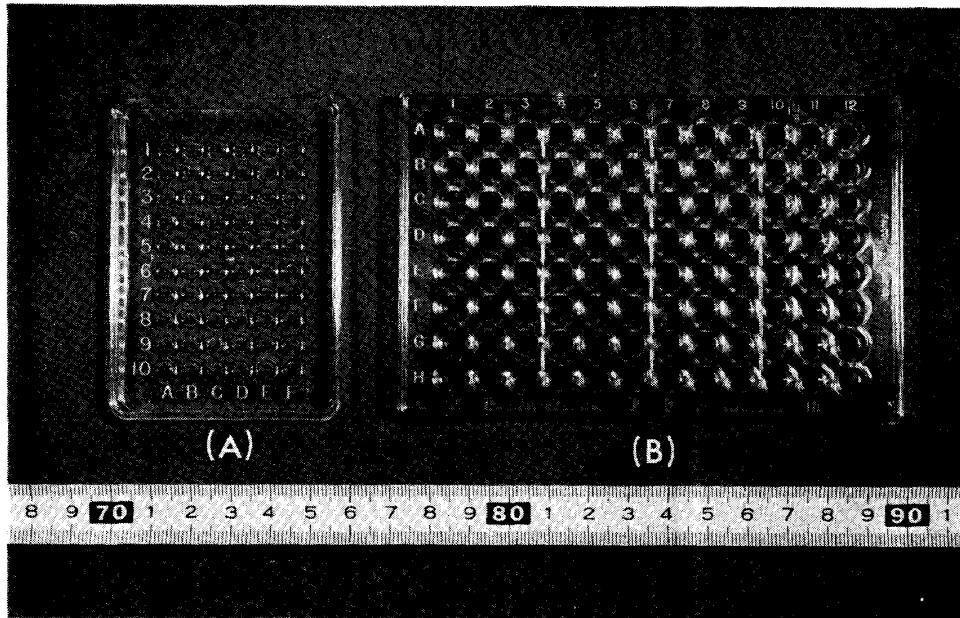
Fig. 1. Micro-plates for cytotoxic test.
A (left side) Terasaki Plate. B (right side) Micro Test II Plate (Falcon Plastics No. 3040).

of SMT cells at the time of lymphocytes addition, as found in control wells, was approximately 2 to 3×10^4 cells/well. After 48 hours of further incubation under the same conditions, lymphocytes were removed and the attached target cells were fixed in absolute methanol and stained by Giemsa. A semi-quantitative method was used for the evaluation of monolayer damage. In double-blind checks by two investigators, the monolayer was evaluated according to five categories as follows;

  (—)...intact monolayer in the well.

  (+)...scattered defects (holes) in the monolayer.

  (++)...confluent destructed areas.

  (+++)...extensive damage, with islands of intact monolayers.

  (++++)...completely destructed monolayer.

RESULTS

I. Development of the Cytotoxicity Test.

The optimal conditions for culturing SMT cells were established with reference to the medium used. Comparing the results obtained with the media tested, namely RPMI1640, Eagle's minimum essential and $3 \times E$, it became apparent that the best growth rate was achieved in $3 \times E$ medium. The neoplastic nature of the cultured cells was repeatedly demonstrated by injecting these cells back into syngeneic, irradiated (250 R) or non-irradiated, DBA/2Ha-DD mice; tumor growth was achieved in 100% of the animals after the subcutaneous injection of 10^5 cultured cells.

The survival of the DBA/2Ha-DD spleen lymphocytes in culture was studied as a function of the type and amount of serum supplement in the medium (Fig. 2). Similar results were obtained regardless of whether fetal calf serum or syngeneic mouse serum was
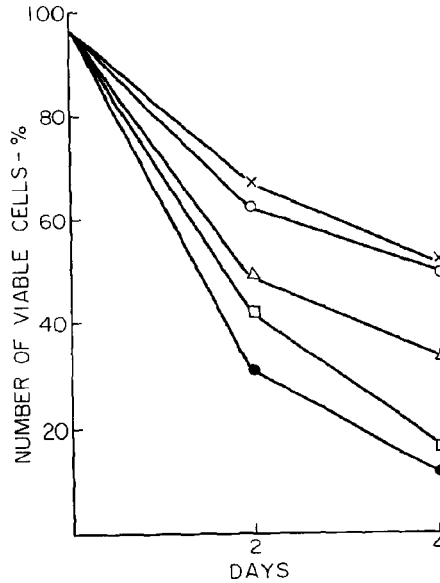
Fig. 2. Viability of DBA/2Ha-DD spleen cells $(4 \times 10^7/\text{ml})$ in $3 \times$ Eagle's medium containing serum at different concentrations.
X——X 10% DBA/2Ha-DD serum, O——O 10% fetal calf serum, △— △ 1% DBA/2Ha-DD serum, □- -□ 1% fetal calf serum, ●——● no serum.

used as a serum supplement. With 10% serum supplement, more than 60% of the lymphocytes were viable, according to the trypan blue exclusion test, 48 hours after plating, which corresponds to the time required for completion of the cytotoxicity test.

It is known that lymphocytes in vitro occur the blastic transformation spontaneously. The effect of fetal calf serum content in the culture medium on the rate of blastic transformation was, therefore, studied in this experiment. Number of large transformed lymphocytes was increasing with the percentage of the fetal calf serum in medium (Fig. 3). As the blastic transformation can appear even in the serum-free medium, the rate of blastic
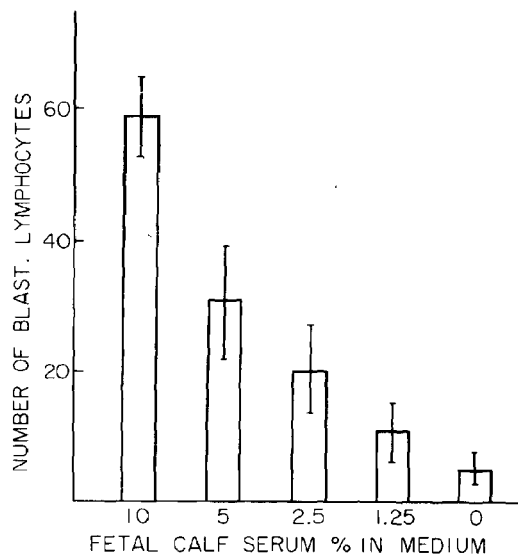

Fig. 3. The effect of fetal calf serum content in medium on the lymphocyte transformation.
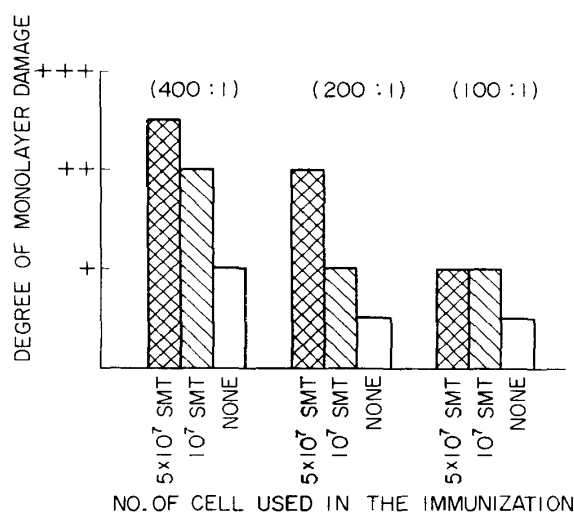
Fig. 4. The effects of numbers of immunizing cells on monolayer damage. ▨ immune lymphocytes from mice which were repeated 3 times injection of 5×10^7 SMT cells intraperitoneally. ▨ immune lymphocytes from mice which were repeated 3 times injections of 10^7 SMT cells intraperitoneally. ☐ lymphocytes from mice which were non-injected.

transformation is rather the expression of nutritional requirement of metabolically active lymphocytes during the process of transformation. Though $3 \times E$ medium supplemented with 10% fetal calf serum has been used in all these cytotoxicity experiments, it must be taken into consideration in this point if the blastic transformation of lymphocytes willl be measured.

The effects of numbers of immunizing cells on monolayer damage have studied. Intraperitoneal injection of SMT cells can be used for immunization of syngeneic hosts, because in this system it does not grow when SMT cells are transplanted intraperitoneally. After repeating 3 times injections of 10^7 or 5×10^7 SMT cells into DBA/2Ha-DD mice, the activity of spleen lymphocytes against SMT monolayer was cell number dependant as shown in Fig. 4.

II. Study of Cell-Mediated Responses to SMT.

The cytotoxic effects of spleen lymphocytes on SMT cells in confluent monolayer were studied with effector cells obtained under conditions where the immunization was by SMT inoculation into allogeneic mice across H_2 (C57Bl/6Ja), into allogeneic mice with H_2 (DBA/2J) or into syngeneic mice (DBA/2Ha-DD).

The cytotoxic activity of sensitized lymphocytes from each of the three mouse strains used was found to be considerably higher against SMT cells in monolayer than against mouse kidney cells. In the experiment summarized in Fig. 5, 10^7 SMT cells were inoculated intraperitoneally 3 times at 7 day intervals, and effector spleen cells were obtained 7 day after the last immunization. In each case 2 sets of differences were compared, namely a) the difference between the cytotoxicity of immunized and non-immunized spleen lymphocytes against SMT cells in monolayer, and b) the difference between responses to SMT and kidney cells in monolayer. The best results in terms of specificity of antitumor cytotoxicity were
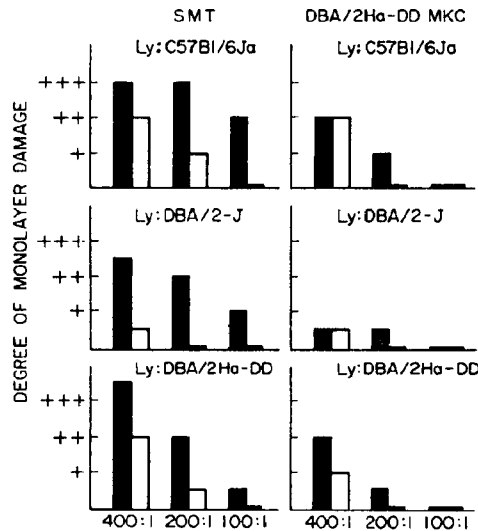
Fig. 5. Cytotoxic effect of spleen cells from C57Bl/6Ja (upper set), DBA/2J (middle set) or DBA/2Ha-DD (lower set) mice on either SMT (left side) or DBA/2Ha-DD mouse kidney cell monolayers (MKC). The donors of sensitized cells were immunized with 10^7 SMT cells 3 times at weekly intervals. Effector cells were harvested 1 week after the last immunization (solid bars). Open bars represent cells from non-immunized donors. The effector/target cell ratios were as indicated on the abscissa.

obtained at an effector to target cell ratio of 200 to 1 or 100 to 1. At these ratios, the cytotoxicity of effector cells from non-immunized mice was similar on both tumor and kidney cell. In contrast, the cytotoxicity of effector cells from immunized mice on tumor target cells was significantly higher, indicating specificity of response. Reratively minor differens in cytotoxicity were seen among cells from the three mouse lines used, and were evident essentially only at an effector to target cell ratio of 100 to 1. The decreasing order of cytotoxicity with cells from C57Bl/6Ja, DBA/2J and DBA/2Ha-DD mice is consistent with expectations, based on the histocompatibility differences involved.

DISCUSSION

There are good reasons to believe that the resistance of properly immunized mice against syngeneic tumor transplants has an immunological explantation (8, 9, 10, 11, 12, 13, 14, 15). These studies carried out in inbred animals. Since transplantation techniques can not be satisfactorily employed in such work, *in vitro* tests are necessary. Several systems, namely neutralization test and tissue culture, have been developed by which cellular immune reactions against tumor antigens can be detected and quantitated;

A). Neutralization Test: so-called neutralization test were introduced by Winn (16) and used by him (17), Klein & Sjögren (10), Old et al (18), and Kitano (19) to demonstrate cell -mediated immune reaction. They are based on the finding that small lymphocytes (from lymph node or spleen) from mice which have been immunized against the H-2 antigens of another mouse strain can inhibit the growth *in vivo* of tumor carrying the respective H-2 antigens, if tumor cells and samll lymphocytes are mixed *in vitro* and inoculated into

syngeneic recipients.　The recipients are usually X-irradiated with a sublethal dose (250–400 R) in order to decrease their immunological reactivity against the transplanted cells.

B).　Tissue Culture:　tissue culture assays were introduced as a more sensitive tool than the neutralization test for studies on lymphocyte-mediated reactions to target tumor cells.

Rosenau and Moon (20) published a study of target cell destruction by immune allogeneic lymphoid cells.　Mouse L cells (of C3H origin) were seeded into culture tubes.　24 hours after the tubes had been seeded, the medium was removed and each tube received spleen cells from BALB/c mice which had been repeatedly immunized against C3H.　Immune spleen cells were found to aggregate around the target cells within approximately 18 hours after they were added to the cultures, whereas no aggregation was observed in the control.

The colony inhibition (CI) technique has been used to investigate the cellular immunity to a variety of tumors in mouse and in human.　It was introduced by Hellström (7) who studied polyoma tumors, against which a cellular immunity had already been demonstrated with neutralization test.　She found that lymph node cells from polyoma-immune mice reduced target cell colony formation.

Valuable information on such responses has been obtained by the application of *in vitro* colony inhibition procedures (4, 5, 7, 21, 22, 23).　These methods, although very useful in studies of cellular responses and of phenomena related to humoral blocking factors, provide only an indirect measure of cytotoxicity since they are based on the prevention of colony formation.　After due consideration of these points, a new method in this paper which was modified from original CI Test (7) and from MCI (23) make it a rule to measure a direct damage of target cells in a cell-mediated cytotoxicity.　In therapeutically oriented studies it seems, also, important to evaluate the interactions between immune host responces and drugs based on a direct evaluation of cytotoxic effects on target tumor cells.

Cell-mediated cytotoxicity has been studied using as a target monolayers of established tumor cell lines in culture (23) as well as primary cultures of human origin (4).　The procedure of a new method was implemented using primary cultures from primary or first transplant generation mouse mammary tumors as a source of target cells.　Cytotoxicity was evaluated based on effects on confluent monolayers.　Effector cells were obtained from normal mice or from mice immunized with cells obtained from primary tumors.　Because at the time that mammary tumor cells monolayers are confluent a reasonably constant number of cells are present in each well, defined ratios of effector to target cells could be established.　Moreover, a direct semi-quantitative comparison was made between cytotoxicity on tumor and normal cells, and between effects of sensitized and non-sensitized lymphocytes.

Spontaneous mammary tumors were long considered nonantigenic since the transplantation methods used to detect antigens in experimentally induced neoplasms failed to reveal any immunity to spontaneous mouse mammary tumors (8, 11), until it was shown by Weiss et al (24) that such tumors have a common antigen against which transplantation immunity can be built up in mice which are mammary tumor virus-free.　The fact that mammary tumors are "spontaneous" make them particularly relevant models for human neoplasms.　Heppner and Pierce (5), therefore, applied the CI assay to mammary carcinomas which had appeared in BALB/cfC3H mice which contained MTV by foster nursing on C3H.

His conclusion was that cellular immunity can, indeed, be demonstrated against tumor specific transplantation antigen (TSTA) of spontaneous tumors and that spontaneous mammary carcinomas appear to have individually distinct TSTA in addition to the common antigens previously detected by transplantation test.

On the other hand, author applied this new method, which confluent target monolayer was usually used, to spontaneous mammary carcinomas which had appeared in DBA/2Ha-DD mice. Cultured SMT cells by bottle culture were plated into wells of Falcon Plate. 24–48 hours after plating, the medium and floating SMT cells were removed, then each wells in Falcon Plate received spleen cells from DBA/2Ha-DD, DBA/2J, and C57Bl/6Ja which had been immunized with SMT cells (10⁷) 3 times. The controls received non-immune spleen cells from those 3 strains. Another controls for target cells were, also, used kidney cells from them. 48 hours after lymphocytes were added, they were washed out and the damages of monolayer were evaluated by five categories as described in method. In this study, cellular immunity against TSTA of spontaneous mammary tumors as same as Heppner had been demonstrated as shown Fig. 5. But the presence of typical mammary tumor virus particles was noted by electron microscopy. Thus, it is likely that virus-related antigens are present on these cells and may be recongnized particularly in DBA/2J and C57Bl/6Ja mice, both of which have a low incidence of SMT. My attempt in future will be made to discriminate between different types of antigenic specificities presumably expressed on SMT cells.

The low susceptibility to chemotherapy makes it difficult to study the interrelationships between drug treatments and immune responses in vivo. Since in syngeneic systems based on established transplantable tumors the participation of immunity to the curative effects of chemotherapy is in many cases essential (25, 26, 27, 28), it seems important to study these interrelationships also in autochthonous tumor systems. The initial studies described herein led to the establishment of in vitro procedures which may be usefully employed to monitor the effects of drugs on both SMT cells and effector cells, when drugs are given either to cell donors or are added to the test culture. These applications, and the study of drug effects on possible blocking phenomena, are the subject of current investigations.

SUMMARY

A new quantitative in vitro test for the direct measurement of cytotoxicity on confluent and complete monolayer of SMT cells was designed with Micro Test Plate. The tumor used develops with high frequency in the DBA/2Ha-DD mouse, and is specific for this mouse since it grows in syngeneic host but not in DBA/2J or C57Bl/6Ja mice.

1) With 10% serum supplement regardless of whether fetal calf serum or syngeneic mouse serum, more than 60% of the lymphocytes were viable at 48 hours culture after plating.

2) Number of blastic transformation of lymphocytes was increasing with the percentage of the fetal calf serum in 3 × E medium.

3) The activity of immune lymphocytes against SMT monolayer was cell number-dependant which was intraperitoneally immunized.

4) The cytotoxicity of immune lymphocytes was greatest with cells from C57Bl/6Ja mice, less marked with cells from DBA/2J mice and least with cells from DBA/2Ha-DD mice. Moreover, its cytotoxicity was greater against SMT monolayer than against MKC monolayer.

5) Therefore, it can be concluded that cellular immunity can be demonstrated between against allo-antigen and against tumor specific transplantation antigen of SMT. This method can, also, be consider to be a good one for cytotoxicity *in vitro* test of cellular immunity.

ACKNOWLEDGEMENT

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