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Quantitative *In Vitro* Measurement of Simian Virus 40 Tumor-Specific Antigens

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ABSTRACT **Humoral antibodies, specific for the SV40 tumor-specific transplantation antigen, can be detected by an *in vitro* cytotoxic assay. ^{51}Cr-labeled SV40-transformed target cells are selectively killed in the presence of specific antibody and rabbit complement. Inhibition of cytotoxicity can be used to measure the antigen concentration and specific activity on different SV40-transformed cell lines. The assay described has several advantages when compared to other methods currently available to detect the specific SV40 antigen: it is simple, rapid, objective, reproducible, and quantitative.**

The SV40 tumor-specific transplantation antigen (TST antigen) was initially demonstrated by resistance to SV40 tumor transplants in adult hamsters immunized with the virus (1–3) or with SV40-transformed cells (4). Subsequently, several features of the SV40 TST antigen have been defined:

(*a*) It is specific: hamster and human cells transformed by SV40 virus share a common antigen capable of stimulating specific transplantation resistance to SV40-induced tumors (4, 5).

(*b*) It is distinct from the SV40 T or S antigens (6), and from certain SV40 virus-induced enzymes (7).

(*c*) It is widely thought to be coded for by the viral genome, but its synthesis as a result of derepression of cellular (host) genes has also been suggested (8).

(*d*) It is probably associated with cell membranes: activity is retained in cell "ghosts" prepared by gentle lysis of cells (9–11).

(*e*) It may be unstable: its activity has been destroyed by freeze-thawing and by formalin treatment (4).

It is generally conceded that further progress in the analysis and characterization of the SV40 TST antigen has been limited by the lack of a reliable *in vitro* method to detect the antigen (12, 13). The present paper describes a cytotoxic assay with ^{51}Cr-labeled SV40-transformed target cells that is simple, rapid, objective, and quantitative; inhibition of cytotoxicity allows precise measurement of surface antigens on different SV40-transformed cell lines.

MATERIALS AND METHODS

Animals

Mice used in these experiments were inbred AL/N and BALB/c mice, maintained by the NIH breeding unit.

Tissue culture cell lines

Two transformed cell lines, SV·AL/N and Py·AL/N (clone 4), established by exposing normally contact-inhibited AL/N cells to SV40 virus and polyoma virus, respectively, in tissue culture, were generously provided by Dr. K. K. Takemoto (NIAID). Both cell lines have been shown by appropriate tests to express their respective TST antigen and T antigens (ref. 14 and unpublished observations). The SV·AL/N cells are uniformly rejected in syngeneic AL/N animals (Tumor Dose 50 > 10^8 live transformed cells, subcutaneously) and will grow progressively as tumors only in immunosuppressed (anti-lymphocyte serum-treated or sublethally irradiated) hosts. The PY·AL/N cells produce tumors in normal AL/N mice ($TD_{50} \sim 10^5$). The mKS·A cell line, derived from BALB/c kidney cells transformed in primary culture by SV40-virus clone 307L, was obtained from, and previously characterized by, Dr. Saul Kit (15). mKS·A cells are readily transplantable in BALB/c hosts ($TD_{50} \sim 2 \times 10^5$ cells).

Antisera

A pooled, hyperimmune serum (designated anti-SV·AL/N), specific for the SV40 TST antigen, was obtained after 7–12 weekly subcutaneous injections, of approximately 10^6 SV·AL/N cells, in groups of AL/N recipients. A pooled (A.BY × WB/Re) anti-A·SW serum (designated C3) directed at H-2 alloantigenic specificities 1 and 3, purchased from the Jackson Laboratory, was used as a positive marker to detect strain-specific histocompatibility antigens expressed on AL/N and BALB/c target cells.

Cytotoxic assay

A cytotoxicity assay with ^{51}Cr-labeled target cells was devised. This is a modification of a method previously described by Sanderson (16) to measure mouse alloantibodies. The SV·AL/N, PY·AL/N, and mKS·A lines to be used as target cells were grown in monolayer culture under standard conditions [Minimal Eagles Medium (Grand Island Biological Co. no. 109) containing 10% fetal calf serum (GIBCO no. 651) and 2% penicillin–streptomycin (GIBCO no. 507)]. 16–18 hr before labeling, the monolayers were trypsinized, gently dissociated to single cells, and maintained on a rotary shaker overnight. Immediately prior to labeling, the cells were washed once in 0.01 M Tris-buffered saline medium (NIH Media Unit), pH 7.4, supplemented with 10% fetal serum (GIBCO no. 651). The remainder of the assay, and all further cell or serum dilutions, was done in this medium. About 20×10^6 target cells (volume 1 ml) were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham/Searle > 150 Ci/g) for 45 min at 37°C with constant shaking. The labeled cells were washed four times with large volumes of chilled, fresh medium and brought to a final concentration of 10^6 cells/ml.

Abbreviation: TST antigen, SV40 tumor-specific transplantation antigen.

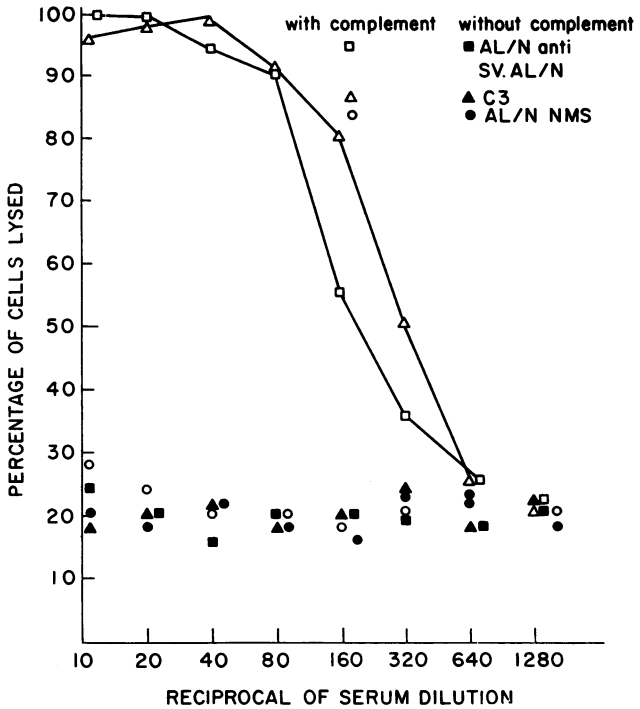

FIG. 1. Titration of serum from □ AL/N mice immunized with syngeneic SV40-transformed SV·AL/N cells, O nonimmunized AL/N controls, and △ alloimmune (A·BY × WB/Re)F_1 donors against ^{51}Cr-labeled SV·AL/N target cells. *Open figures* = complement added. *Closed figures* = no added complement.

Direct serum titration

25-μl volumes of the sera to be tested were serially diluted two-fold; 0.05 ml of labeled target cells (50,000 total cells) were added to each tube and incubated for 45 min at 37°C. 0.05 ml of fresh-frozen rabbit serum, diluted 1/4, was added where indicated, and the incubation was continued for an additional 90 min at room temperature. The reactions were terminated by the addition of 2 ml of chilled medium. Each sample was then centrifuged 5 min at 800 × *g*, and a 1-ml aliquot of the supernatant was counted. Serum titers were established at that serum dilution that gave 50% lysis of cells.

Antigen titration by cytotoxic inhibition

The routine inhibition assay involved 10 two-fold dilutions of the unlabeled cells (25-μl volumes), starting from an initial concentration of 50 × 10^6 cells/ml. A level of antiserum was chosen that would cause between 60–80% lysis of labeled target cells in the absence of any competing unlabeled target cells. Identical aliquots of the indicated serum at a fixed dilution and a constant number of labeled SV·AL/N target cells were added to each tube. The cell mixtures were then incubated for 45 min at 37°C. A standard amount of rabbit complement was added to each tube, and the assay was completed as before. A 50% lysis point was established to provide a standard reference for each cell type tested.

Calculation of % cytotoxicity

Calculation of the percentage of cells lysed was based on the expression: $P = (S\text{-}S_0)/(S_m\text{-}S_0) \cdot 100$, where P = % cells lysed, S = corrected (to total volume) supernatant counts after lysis, S_0 = corrected (to total volume) supernatant counts before lysis, and SM = corrected (to total volume) supernatant counts in the 100% lysis tubes. All counting was done in an automatic gamma well counting system (Nuclear-Chicago).

RESULTS

Cytotoxicity of anti-SV·AL/N serum

Sera from AL/N mice immunized with syngeneic SV·AL/N cells (anti-SV·AL/N), from nonimmunized AL/N controls (AL/N NMS), and from hyperimmune allogeneic donors (C3) were titrated against SV·AL/N target cells.

The assay performed in this manner allows simultaneous identification of cellular histocompatibility antigens and tumor-specific transplantation antigens. As shown in Fig. 1, the cytotoxic reaction against both types of antigens appears comparable. In the presence of added complement, target cells are killed with high efficiency by both the alloantiserum (C3) and the isoantiserum (anti-SV·AL/N). Neither serum is cytotoxic in the absence of complement.

Specificity of anti-SVA·L/N serum

The specificity of the reaction is demonstrated in Fig. 2. Serum titrations were performed in an identical manner against several labeled target cells: SV·AL/N, mKS·A, Py·AL/N, and normal AL/N lymph node cells. The anti-SV·AL/N serum was specific for the SV40-transformed cell lines: SV·AL/N and mKS·A; Py·AL/N or normal AL/N lymph node cells were unaffected. As expected, the alloantiserum was cytotoxic for each of the four targets chosen. The efficiency of the cytotoxic reaction was somewhat variable,

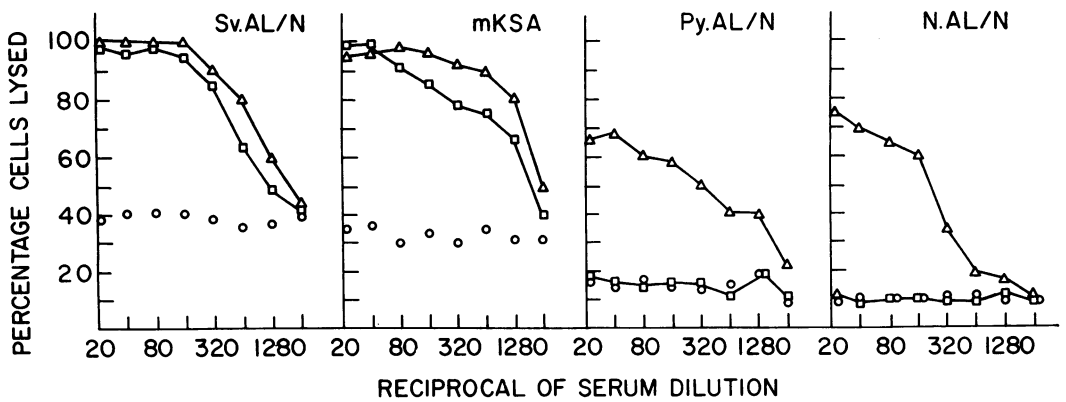

FIG. 2. Titration of sera with labeled SV·AL/N, mKS·A, Py·AL/N, and N·AL/N target cells: serum from □ AL/N mice immunized with syngeneic SV40-transformed SV·AL/N cells, O nonimmunized AL/N controls, and △ alloimmune (A·BY × WB/Re)F_1 donors.

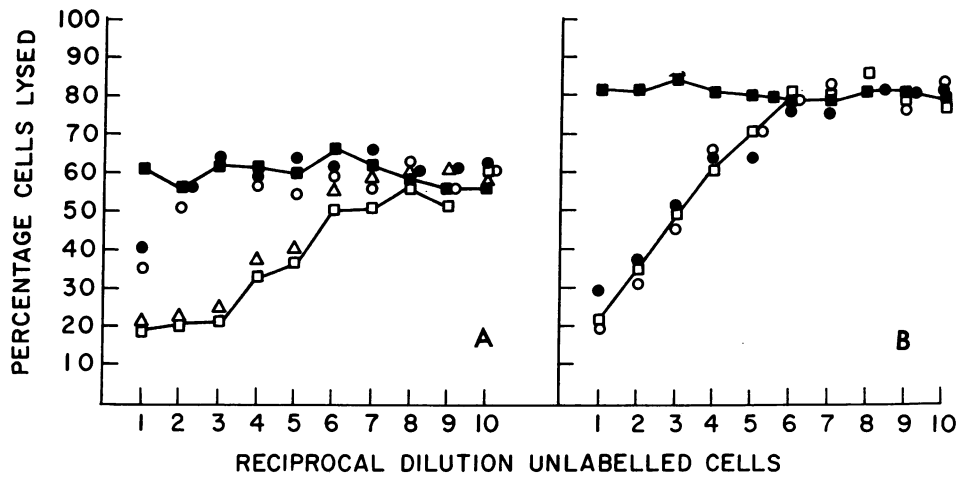

FIG. 3. Inhibition of cytotoxicity by unlabeled target cells. (*A*) Labeled SV·AL/N target cells incubated with a fixed aliquot of AL/N anti-SV·AL/N serum (serum dilution 1/160) and either no (■) or varying numbers of unlabeled (□) SV·AL/N, (△) mKS·A, (●) Py·AL/N or (○) normal AL/N lymph node cells. (*B*) Labeled SV·AL/N target cells incubated with a fixed aliquot of C3 alloantiserum (serum dilution 1/160) and various unlabeled cells as indicated above. Background lysis was 20% in both *A* and *B*.

however, presumably dependent on the intrinsic propensity of each cell type to be lysed by either specific or nonspecific factors.

The specificity of the reaction of the anti-SV·AL/N serum with SV40-transformed cells can also be shown by the cytotoxic inhibition assay. Tests using the anti-SV·AL/N serum and the alloantiserum (C3) are again compared. In Fig. 3*A*, suitably diluted unlabeled SV·AL/N, mKS·A, PY·AL/N, and normal AL/N lymph node cells were incubated for 45 min at 37°C with 25 μl of anti-SV·AL/N serum, diluted 1/160. Standard amounts of *labeled* SV·AL/N cells and rabbit complement were then added to each tube, and the assay was completed as before. The two SV40-transformed cell lines, SV·AL/N and mKS·A, inhibited the cytotoxic reaction with much greater efficiency (about 16 times) than either of the polyoma-transformed or normal AL/N cells. An identical procedure was followed in the experiment shown in Fig. 3*B*, except that the alloantiserum (C3), diluted 1/160, was substituted for the anti-SV·AL/N serum. In this instance, all of the cell lines tested inhibited the cytotoxic reaction with equal efficiency.

Calculation of specific activity of SV40-TST antigen

The cytotoxic inhibition assay also allows quantitation of antigen concentration and specific activity. Units of antigen are defined as the inverse of the dilution of *unlabeled* cells that causes a reduction in cell lysis to 50%. For example, in Fig. 3*A*, a six-fold dilution of unlabeled SV·AL/N cells was required to reach the 50% lysis point. SV40-TST antigen units can be calculated as: 6/0.025 ml = 240 units/ml of the unlabeled cell population. Specific activity is given in terms of antigen units/10^6 unlabeled cells by:

$$\frac{240 \text{ units/ml}}{50 \times 10^6 \text{ unlabeled cells/ml}} = 4.8 \text{ units}/10^6\text{cells}.$$

When calculated in a similar manner, the SV40 TST antigen activity for mKS·A cells is comparable to that of the SV·AL/N cells. In contrast, the activity of Py·AL/N or normal AL/N cells is of a much lower order. The specific activities of the H-2 histocompatibility antigens, determined from the data shown in Fig. 3*B*, are nearly identical for each of the cell types tested.

DISCUSSION

The rapid recent advances in attempts to solubilize and characterize histocompatibility antigens in the mouse have depended on a cytotoxic assay using ^{51}Cr-labeled target cells (15). Cytotoxic inhibition has been extensively utilized as a standard quantitative measure for surface antigens on intact cells (17) or on various subcellular fractions (18–25).

Quantitation and biochemical characterization of tumor-specific transplantation antigens have lagged behind the histocompatibility antigen work for lack of a rapid *in vitro* method for their detection. Most work describing the SV40 TST antigen has employed tedious, costly, time-consuming, and imprecise *in vivo* transplantation assays. Coggin and Ambrose have discussed a 5-day *in vivo* method that employs immunodiffusion chambers to detect transplantation immunity against SV40 tumors in hamsters. Their technique, which is considerably more rapid than conventional assays, is nevertheless unfortunately cumbersome and nonquantitative (26). Pekarek *et al.* have demonstrated that the migration of spleen cells from hamsters resistant to SV40 tumors was selectively inhibited on monolayers of SV40-transformed cells. Their technique, although rather quick and simple, allows at best a qualitative determination of SV40 TST antigen activity (27). Smith *et al.* have recently described a cytotoxic assay for the SV40 TST antigen (11), adapted from the microdroplet lymphocyte toxicity test developed by Terasaki to study human leukocyte antigens (28). In experienced hands, this technique, based on exclusion by cells of vital dyes, has been shown to be reproducible and has been successfully applied on a large scale (29). Nevertheless, it has certain disadvantages when compared to the isotope techniques used in cytolytic systems. The dye test is subjective, and quite commonly the distinction between a living or dead cell is difficult to discern. Because the cells being tested will all die within a matter of a few hours, the assay must be read promptly, thus limiting the number of tests that can be performed at any one time. Most importantly, the dye test has not been adapted to allow

quantitative cytotoxic inhibition studies, which are required for precise measurement of antigen concentration and specific activity.

The cytotoxic test, using ^{51}Cr labeled SV40-transformed target cells, discussed in the present paper overcomes many of the disadvantages of techniques previously available to detect the SV40 and other TST antigens: it is simple, sparing of materials, rapid, objective, sensitive, reproducible, and quantitative. This technique should facilitate rapid progress in further biological and biochemical characterization of the tumor-specific transplantation antigens.

The specificity of the anti-SV·AL/N serum was further demonstrated in tests with the embryonic hamster cell lines of Diamadopoulos *et al.* (31–33). The serum was cytotoxic only for those cell lines that expressed both the SV40 tumor (T) and surface (S) antigens. A cell line that contained the S antigen but lacked the T antigen, and a line that lacked both S and T antigens were unreactive. These results were confirmed by appropriate absorption experiments.

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