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Transformation by Simian Virus 40 of Spleen Cells from a Hyperimmune Rabbit: Evidence for Synthesis of Immunoglobulin by the Transformed Cells

(cloning/coprecipitation/autoradiography/differentiated)

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ABSTRACT **Spleen cells derived from a rabbit hyperimmunized with a Type III pneumococcal vaccine were exposed to simian virus 40 *in vitro*. After 114 days, transformed cells growing on the surface of one culture dish were observed and a cell line was established. The transformed cells had a morphology characteristic of cells transformed by simian virus 40, contained the simian virus 40-specific T antigen, and yielded infectious simian virus 40 upon cultivation with indicator cells in the presence of Sendai fusion factor. Transformed cells incorporated labeled amino acid into protein with the antigenic properties of rabbit immunoglobulin G.**

We have previously attempted to obtain a virus-transformed cell line capable of indefinite growth *in vitro* and continuous production of specific antibody (1). Lymph-node cells from immunized rats were exposed to simian virus 40 (SV40); four transformed cell lines were obtained, but none was found to synthesize immunoglobulin or immunoglobulin fragments. Attempts to establish a relationship between the transformed cells and lymphoid cells were inconclusive, and the identity of the target cell for transformation remained uncertain. In order to increase the likelihood of successful transformation of antibody-producing cells, resort was made, in the present experiments, to cells from a rabbit hyperimmunized with a pneumococcal vaccine. Rabbits injected intravenously with pneumococcal vaccines produce large amounts of antibody of restricted heterogeneity (2). Furthermore, marked plasma-cell infiltration of the spleen has been demonstrated (3). This report describes the infection with SV40 of spleen cells from one such rabbit, and the subsequent establishment of a transformed cell line capable of producing rabbit immunoglobulin of the IgG class. In the accompanying manuscript, the presence of a protein of markedly restricted heterogeneity that specifically binds the immunizing antigen is demonstrated (4).

MATERIALS AND METHODS

Immunization. Rabbit L-27 was kindly provided by Drs. J. Kimball and A. M. Pappenheimer, Jr., Harvard University. This animal had been injected repeatedly with a Type III pneumococcal vaccine according to a schedule described (5, 6). When the rabbit was killed, the serum contained 11 mg of antibody against S3 pneumococcal polysaccharide per ml. Isoelectric focusing of purified antibody showed three major and one minor species (4).

Initiation and Maintenance of Stationary Suspension Cultures. These procedures were performed essentially as described for rat lymph-node cells (1). Briefly, rabbit L-27 was killed. The spleen was removed, minced finely, and pressed through wire mesh. Cells were suspended in medium RPMI 1640, supplemented with 20% unheated fetal-calf serum, 2 mM glutamine and antibiotics, infected with virus (see below), seeded in 60-mm diameter plastic dishes, and maintained in the above medium at 37° in a humidified atmosphere of 5% CO_2 in air. Half of the medium of each plate was changed weekly, and 3 days after each medium change each plate was supplemented with $^1/_{10}$ volume of fresh medium. Bone-marrow cells were obtained by aspiration, dispersed by repeated pipetting, and treated as above.

Virus Transformation of Suspension Cultures. SV40, strain 777, was purified as described (7); the virus pool used titered 2.5×10^{10} tissue culture infective $doses_{50}$ ($TCID_{50}$) per ml on the BSC-1 line of monkey kidney cells. Spleen and bone-marrow cells were infected with SV40 at virus : cell multiplicities of 104 and 125, respectively, in suspension in a total volume of 1.0 ml at 37° for 1 hr with continuous mixing. Uninfected control cells were treated in parallel with 1.0 ml of RPMI 1640 medium. At the end of the adsorption period, 8×10^6 cells per ml were seeded in plastic dishes; the occurrence of transformation was followed by examination of both the bottom surface and the unattached cells in suspension. The transformed cells that appeared on the surface of one infected spleen culture were dispersed with 0.05% trypsin–0.2% EDTA and passed to fresh culture vessels. These cultures were maintained in Eagle's minimal essential medium (8) with four times the usual concentration of vitamins and amino acids (MEM × 4), 10% fetal-calf serum, 10% tryptose phosphate broth, 2 mM glutamine, and antibiotics (maintenance medium).

In order to detect immunoglobulin production, cells were exposed to radioactive amino acid (9). Labeling medium consisted of MEM × 4 lacking leucine, 10% dialyzed calf serum, and antibiotics, supplemented with a final concentration of 5.24 mg/liter unlabeled leucine (10% of the normal leucine concentration) and 0.3 μCi/ml of [^{3}H]leucine (specific activity

Abbreviations: SV40, simian virus 40; IgG, immunoglobulin G; TRSC-1, SV40-transformed rabbit spleen cell line 1; MA-111, normal rabbit kidney cell line 111; TRK-73 SV40-transformed rabbit kidney cell line 73.

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40 Ci/mmol) or [^{14}C]leucine (specific activity 20 mCi/mmol); the medium was changed every 3 days.

Single Cell Cloning was performed by a modification of Puck's procedure (10, 11). The cloning medium consisted of MEM × 4, 10% fetal-calf serum, 5% tryptose phosphate broth, and maintenance medium previously conditioned by growth of the SV40-transformed spleen cells; the latter medium had been filtered through a 0.22-μm Millipore filter before use.

Immunofluorescence Staining for T Antigens. The presence of the SV40-specific T antigen was determined by the indirect immunofluorescence method as described (12, 13).

Virus Assays. The presence of infectious virus in culture fluids and extracts of transformed cells was determined by plaque assay as described (14). In addition, attempts were made to rescue SV40 from the transformed spleen cells by treatment with mitomycin C and cocultivation of transformed cells with indicator (BSC-B) cells with and without Sendai fusion factor as described (14).

Transplantation of Transformed Cells. Ten outbred weanling male and 20 newborn (<24-hr old) hamsters were inoculated subcutaneously in the interscapular region with 10^7 transformed cells. Animals were examined at weekly intervals.

Processing of Tissue Culture Fluids and Cell Suspensions. Tissue culture fluid in which cells had been maintained in the presence or absence of labeled amino acid, from initial transfer until growth had reached about 75–90% confluence, and fluid in which cells had been maintained during growth to complete confluence, were collected separately. Two to four × 10^8 unlabeled or 10^7–10^9 labeled cells were harvested, washed, and disrupted by five cycles of freezing and thawing. The cell extract was clarified by repeated centrifugation. Culture fluids and cell extracts were dialyzed, lyophilized, and reconstituted in 5–10 ml or 0.5 ml of phosphate-buffered saline (pH 7.2), respectively.

Immunodiffusion, Immuno- and Agarose Gel Electrophoresis, and Autoradiography. Immunodiffusion (15), immunoelectrophoresis (16), agarose gel electrophoresis (17), and autoradiography (16) were performed as described. The antisera used in immunodiffusion and immunoelectrophoresis included specific antisera against rabbit γ, α, and μ heavy chains prepared in goat obtained from Miles Laboratories, Inc., Kankakee, Ill., as well as antisera against rabbit IgG prepared in sheep and goat produced in our laboratory. The antiserum against rabbit IgG prepared in goat was capable of detecting both Fc and Fab immunoglobulin fragments (18).

Estimation of Labeled Rabbit IgG by the Method of Coprecipitation to Relatively Constant Radioactivity. This procedure was performed as described (19). Culture fluids or cell extracts from cultures of labeled transformed cells were combined with normal rabbit IgG and precipitated with antisera against rabbit IgG prepared in sheep. Extracts of SV40-transformed rabbit kidney cells (TRK-73) (13) were also examined. In order to avoid nonspecific adsorption of labeled proteins to the immune precipitate, coprecipitation was repeated until relatively constant specific activity was achieved (19).

RESULTS

Isolation of SV40-Transformed Rabbit Spleen Cells. Twelve cultures from rabbit spleen cells and 10 from rabbit bone-marrow cells were established; half of the cultures of each group were exposed to SV40. Fifteen (70%) of these cultures, equally distributed among the groups, survived and were observed for 146 days. There was no evidence of proliferation of suspended cells in any of the cultures, as determined by periodic cell counts. On day 114 after infection, a small focus of cells was observed growing on the surface of one infected spleen-cell culture; the cell line derived from this focus was designated TRSC-1 (see *Methods*).

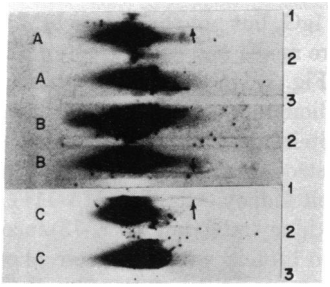

FIG. 1. Radioautography of immunoelectrophoresis of labeled transformed cell cultures. Growth to 90% confluence (wells *A*); growth to complete confluence (wells *B*); cell extract (wells *C*). Precipitin lines developed with antirabbit γ- (troughs *1*), α- (troughs *2*), and μ- (troughs *3*) heavy chain antisera. Duration of exposure, 6 months. Labeled amino acid was incorporated into rabbit IgG only (*arrows*) (anode to left).

Characteristics of TRSC-1 Cells. The TRSC-1 cells are polygonal in shape. Many multinucleated giant cells containing from 4 to 15 nuclei are present. A unique feature of these cells is the frequent occurrence of curved or indented nuclei. No collagen or reticulum was detected in TRSC-1 cells stained with the Masson or Wilder technique, respectively, and these cells did not have the characteristics of mast cells on staining with toluidine blue. The SV40-specific T antigen was detected in nearly 100% of the nuclei of the TRSC-1 cells by indirect immunofluorescence staining.

Infectious virus was not detected by plaque assay in medium from TRSC-1 cells or in the corresponding cell extracts, nor was virus induced by mitomycin C or by cultivation of the TRSC-1 cells with permissive monkey kidney cells. However, fusion of TRSC-1 with monkey kidney cells by use of Sendai fusion factor, resulted in rescue of infectious SV40.

There was no evidence of tumor growth in any of the hamsters inoculated with TRSC-1; the animals were observed for 1 year.

Isolation of clones from TRSC-1 proved difficult; only a single clone has been obtained (TRSC-1-1) from over 400 isolated cells.

Detection of Rabbit IgG in Culture Media or Cell Extracts of TRSC-1 Cells. Agarose gel electrophoresis of medium and extracts of TRSC-1 cells maintained with [^{14}C]leucine showed proteins migrating with the electrophoretic mobility of serum α1 to gamma globulin; after autoradiography, it appeared that labeled amino acid was incorporated into all of these proteins. Immunoelectrophoresis was performed with culture fluids harvested from subconfluent, as well as confluent, cultures and a cell extract (combined with normal rabbit serum as "carrier"); precipitin lines were formed with goat antisera against rabbit γ, α, and μ and the plates were submitted to autoradiography. After prolonged exposure (3–6 months),

labeling of the IgG, but not the IgA or IgM precipitin arc, of both the culture media and cell extract of TRSC-1 cells were observed (see Fig. 1). In view of the minute amounts of IgG detected by radioimmunoelectrophoresis, it would appear that most of the protein migrating in the gamma globulin zone on agarose gel electrophoresis was not rabbit immunoglobulin.

Normal rabbit kidney cells (line MA-111) and SV40-transformed rabbit kidney cells (line TRK-73) incorporated labeled amino acid into protein with the electrophoretic mobility of $\alpha 1$ to β globulins; in addition, TRK-73 cells contained labeled proteins with γ-electrophoretic mobility. Neither cell line labeled proteins with the antigenic characteristics of rabbit immunoglobulin, as determined by radioimmunoelectrophoresis.

In order to quantify the total IgG present in both the labeled culture fluids and cell extracts, precipitation with sheep antisera against rabbit IgG was done. This procedure precipitated 1.2 and 1.4% of the labeled protein in extracts of TRSC-1 and TRSC-1-1, respectively; 0.7% of labeled protein was precipitated from culture fluid of confluent TRSC-1 cells.

DISCUSSION

In this paper we have described the transformation of a cell present among the spleen-cell population of a rabbit hyperimmunized with Type III pneumococcal vaccine. It is likely that the majority of cells in the spleen were producing specific antibody (3); at least four anti-S3 antibody species have been delineated (4), suggesting the presence of at least four clones of antibody-producing cells. Since the antibody produced by the transformed cells had electrophoretic properties similar to that of one of the antibody species (4), it seems likely that transformation involved a cell of the corresponding clone.

The available data indicate that the transformed cells were derived from a transformation event induced by SV40: the cells exhibit a morphology characteristic of SV40-transformed cells (20); virtually all the transformed cells contain the SV40-specific, intranuclear T antigen; and infectious SV40 virus was rescued from the transformed cell population by cultivation with indicator cells in the presence of Sendai fusion factor. Since the transformed cells produce antibody (4), it is likely that either a plasma cell or a lymphocyte was transformed, resulting in a marked change in morphology and growth characteristics.

TRSC-1 cells grown in the presence of [^{14}C]leucine or [^{3}H]leucine incorporated radioactivity into many proteins, including one with the antigenic characteristics of rabbit IgG. Based on the long period of incubation required to develop the radioautographs after immunoelectrophoresis of labeled TRSC-1 extracts and culture fluids, and the small percentage of labeled protein coprecipitated with rabbit IgG in liquid media (1.2–1.4% of the total ^{3}H-labeled proteins), it appears that the transformed cells produce only small amounts of immunoglobulin. Less immunoglobulin was detected in the culture fluid than in corresponding cell extracts. The reason for the limited production, or for the failure to secrete immunoglobulin by the transformed cell, is not known; in contrast, most myeloma cultures secrete large amounts of immunoglobulin. The low yield is not to be accounted for by heterogeneity of the culture since a clonal line produced the same amount of IgG. Other possible reasons include the selection of the cell line from a precursor cell rather than a plasma cell, the absence of antigen in the culture, or the failure to secrete antibody, which may inhibit production. Furthermore, transformation of a differentiated cell with SV40 frequently leads to a decrease in specialized function (21–26).

It seems likely that in the present experiments, SV40 transformation of an immunocompetent cell has resulted in a radically altered cell morphology, as well as a change in growth characteristics. Nevertheless, a specialized function, antibody production (4), was maintained.

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