Presence of the Tn Antigen on Hematopoietic Progenitors from Patients with the Tn Syndrome

William Vainchenker, Giovanna Vinci, Ugo Testa, Annie Henri, Antonio Tabillo, Marie-Pierre Fache, Henri Rochant, and Jean-Pierre Cartron

INSERM U.91, Hôpital Henri Mondor, 94010 Creteil, France; Laboratoire de Cytofluorométrie Analytique & Séparative, 94270 Villejuif, France; and Laboratoire de Biochimie Génétique, INSERM U.76, Centre National de Transfusion Sanguine, 75015 Paris, France

Abstract

The Tn syndrome is an acquired clonal disorder characterized by the exposure of a normally hidden determinant, the Tn antigen, on the surface of human erythrocytes, platelets, granulocytes, and lymphocytes. Two distinct populations, Tn positive (Tn⁺) and Tn negative (Tn⁻), of mature hemopoietic cells are present in Tn patients. To determine whether the Tn antigen is already expressed on erythroid, myeloid, and pluripotent progenitors, light-density mononuclear blood cells from two patients with this syndrome were separated by fluorescent-activated cell sorting and by affinity chromatography into Tn⁺ and Tn⁻ fractions, using their binding properties to Helix pomatia agglutinin (HPA). Burst-forming-unit erythroid (BFU-E), colony-forming-unit granulocyte/macrophage (CFU-GM), and mixed colony-forming-unit (CFU-GEMM) cells were assayed in plasma clot cultures. After 12–14 d of culture, colonies were studied by a double fluorescent labeling procedure. First, a fluorescein-conjugated HPA permitted evaluation of the presence or absence of the Tn antigen at the surface of the cells composing each colony, and second, the binding of a murine monoclonal antibody against either glycoporin A (LICR-LON-R10) or against a myeloid antigen (80H5), revealed by an indirect fluorescent procedure, was used to establish the erythroid or myeloid origin of each cell. The Tn⁺ fraction obtained by cell sorting gave rise to nearly 100% Tn⁺ colonies composed exclusively of cells bearing this antigen. The reverse was observed for the Tn⁻ cell fraction. These results demonstrate that in the Tn syndrome, BFU-E, CFU-GM, and CFU-GEMM of the Tn⁺ clone express the Tn antigen at this early stage of differentiation.

Introduction

The Tn syndrome is an acquired disorder first identified by the polyclonality of erythrocytes following exposure of the cryptoantigen Tn at the surface of red cells (1, 2). This disorder may occur in apparently healthy individuals or may be associated with a mild hemolytic anemia, leukopenia, or thrombocytopenia, but has also been described in some acute leukemias or myeloproliferative diseases (3, 4, 5).

The biochemical basis of Tn activation is now well char-

acterized and corresponds to the exposure of an N-acetylgalactosamine residue carried by cell surface glycoproteins (6), which arise from a selective loss of a 3-β-D-galactosyltransferase (T transferase) activity in Tn positive (Tn⁺) cells (7, 8, 9). The Tn antigen has been detected at the cell surface of erythrocytes and more recently on granulocytes, monocytes, platelets, B lymphocytes, and T lymphocytes by the binding of a fluorescent Helix pomatia agglutinin (HPA), a reagent known to interact strongly with N-acetylgalactosamine (10, 11). Interestingly, two distinct populations of peripheral blood cells, Tn⁺ and Tn⁻ (Tn⁻), are typically observed in patients with the Tn syndrome. Recently we have shown that the Tn condition was a clonal disease since a sizeable fraction of erythrocytic, granulocytic, or megakaryocytic colonies grown from the hematopoietic precursors seemed to consist exclusively of either Tn⁺ or Tn⁻ cells (10). It was therefore proposed that this syndrome was a clonal disorder in which a somatic mutation had occurred at the level of a pluripotent stem cell common to the myeloid B and T lymphoid lineages (11).

The purpose of this study was to investigate whether the Tn antigen was already expressed at very early stages of differentiation, i.e., on the erythroid, myeloid, and pluripotent progenitors that give rise to Tn⁺ mature cells.

Methods

Patients. After informed consent was obtained, peripheral venous blood (20–100 ml) was collected on preservative free heparin (3 U/ml) on one to four occasions from two patients, Pl. and Ba., with a typical Tn syndrome, who are known to belong to blood group O. These patients have been extensively studied before (7, 8, 9, 10, 11), and we have previously reported no differences in the percentage of Tn⁺ and Tn⁻ colonies between bone marrow lineages and peripheral blood colonies (10). Therefore, for the convenience of the patient, only the peripheral blood was studied. Peripheral blood was also obtained from normal blood group O volunteers at the Centre National de Transfusion Sanguine, Paris, France.

Cell separation. Peripheral blood from patients Pl. and Ba. was diluted with an equal volume of Hank’s balanced salt solution (HBSS) (Eurobio, Paris, France). Light-density nonadherent peripheral blood cells were separated by centrifugation over Ficoll-metrizoate (d = 1.077, Nyegaard, Oslo, Norway). These cells were washed in cold albumin, followed by an overnight adherence to plastic. Two techniques of separation were subsequently used, both based on interaction of light-density nonadherent blood cells with HPA.

HPA recognizes terminal N-acetylgalactosamine residues (12) and was used to separate the Tn⁺ and Tn⁻ cells from patients Pl. and Ba.

1. Abbreviations used in this paper: BFU-E, burst-forming-unit erythroid; CFU-GEMM, mixed colony-forming-unit cells; CFU-GM, colony-forming-unit granulocyte macrophage; Fabβ, fragment antigen binding; HPA, Helix pomatia agglutinin; LICR-LON-R10, anti-glycoporin A; Tn⁺, Tn⁻; Tn⁻, Tn positive.

Address correspondence to Dr. Cartron, INSERM U.76, C.N.T.S., 6 rue Alexandre Cabanel, 75015 Paris, France.

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This lectin recognizes A-like determinants. In contrast to Salvia sclarea lectin, the agglutinin HPA is not strictly Tn specific (13), but unfortunately, the former lectin is not presently available in purified form. In normal blood group O subjects, however, the HPA lectin does not recognize any blood or marrow cells (10), and therefore it can be considered as specific for the Tn determinants expressed in our two group O patients.

Cellular affinity chromatography. 20 x 10^8 light-density mononuclear cells isolated from the peripheral blood of patient Pl were suspended in 2 ml of HBSS containing 0.2% bovine serum albumin and then applied to a 5-ml column of Helix pomatia Lectin-Sepharose 6 macroparticles (Pharmacia Fine Chemicals, Piscataway, NJ) pre-equilibrated in the same medium under sterile conditions. After application, the cells were incubated on the column with no flow for 15 min at room temperature. Unbound cells were then eluted with 30 ml of the same medium as above. Bound cells were eluted by washing the column with 30 ml of HBSS containing 1 mg/ml N-acetyl-D-galactosamine (Sigma Chemical Co., St. Louis, MO).

Fluorescence activated cell sorting. The nonadherent light-density mononuclear cells were labeled by HPA conjugated to fluorescein (1 mg/ml; IBF, Paris, France) using a 10^{-3} dilution at 4°C in sterile phosphate buffered saline (PBS, 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl) and held on ice until fluorescence-activated cell sorting. The sterile separation of labeled blood cells into positively and negatively stained fractions was performed on a FACS cell sorter (440; Becton-Dickinson & Co., Oxnard, CA). The argon ion laser was set to produce light at 488 nm and at an intensity of 500 mW. Fluorescence was measured through a 520 Long Pass filter. The sheath fluid was PBS, pH 7.2. Sorting was performed through a 50-μm nozzle. A 2,000-V deflection potential was selected. The sorting rate was 1,000 cells/s. Sorted cells were collected in 15-ml sterile plastic tubes (Corning Glass Works, Corning, NY) containing 2 ml of fetal calf serum.

Clonal assay. Burst-forming-unit erythroid (BFU-E), colony-forming-unit erythroid (CFU-E), and mixed colony-forming-unit (CFU-GEMM) cells were grown by a modification of the plasma clot technique (14). The medium consisted of α medium (Eurobio, France) supplemented with 10% human AB serum (Centre Départemental de Transfusion Sanguine, Val-de-Marne, France), 1% deionized bovine Cohn fraction V albumin (Sigma), and 34 μg/ml CaCl₂. Clotting was induced by addition of 10% bovine citrated plasma (Gibco Laboratories, Grand Island, NY). The stimulating activities were either 5% supernatant from the Mo-cell line (T cell line) containing both burst promoting and granulocyte-macrophage colony stimulating activities (14, 15, 16) plus 1.5 IU/ml porcine erythropoietin preparation (25 IU/mg of protein, Centre National de Transfusion Sanguine, Paris, France) for the growth of BFU-E or CFU-GEMM, or 10% supernatant from the Mo-cell line alone for the growth of CFU-GM. In these experiments, 4 x 10^8 unseparated cells, 5 x 10^6 to 1 x 10^7 Tn+ cells, and 4 x 10^5 Tn- cells were plated in 1-ml vol in 35-mm petri dishes (Falcon Labware, Div. of Becton-Dickinson & Co., Oxnard, CA). Cultures were incubated for 12-14 d at 37°C in a humidified atmosphere containing 5% CO₂ in air.

Immunofluorescent labeling of the cultures. At day 12 or 14, cultures were directly dehydrated in petri dishes with 32-mm filter papers (Whatman Laboratory Products, Inc., Clifton, NJ). A double immunofluorescent labeling was then applied in situ in the dish. Initially, 300 μl of 10^{-2} diluted HPA conjugated to fluorescein was applied to the clot for 1 h at 4°C. After extensive washings with PBS, 10^{-2} diluted acetic fluid containing a murine monoclonal antibody directed either against glycophorin A (LICR-LON-R10) (17) or against a myeloid antigen (80H5: anti-SSA-1 antigen) (18, 19) was added for 1 h at 4°C. After several washings in PBS, the preparations were incubated with a goat anti-mouse immunoglobulin fragment antigen binding (Fab') fragment (3.5 mg/ml) conjugated to tetramethylrhoda- mine-isothiocyanate (Cappel Laboratories, Cochraville, PA) at a 10^{-2} dilution. After subsequent washings, the cultures were fixed with cold methanol for 5 min. The bottom of the petri dish was then cut and the sides of the dish discarded. A 35-mm round glass coverslip was mounted with buffered glycerin on the bottom of the petri dish, which was subsequently stuck on a glass slide. Each preparation was completely scanned under a fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epillumination and appropriate filters for fluorescein, rhodamine, and phase contrast microscopy. Photographs were taken with an Ektachrome ASA 400 film (Eastman Kodak Co., Rochester, NY) push-pulled to ASA 1600.

Results

Unseparated cells from the two patients gave rise to both Tn+ and Tn- colonies in a proportion comparable to previous published results (10) (Table I). The fluorescent labeling procedure permitted detection of the Tn antigen and identification of all the colonies in each dish. LICR-LON-R10 (anti-glycophorin A) labels erythroid cells from the proerythroblast to the erythrocyte (20) and therefore stains all the BFU-E-derived colonies at day 12-14 of culture (Fig. 1). The monoclonal antibody 80H5 binds to granulocytic cells from the promyelocyte to the granulocyte (18) and to the monocyte series from the monoblast to the macrophage, and therefore stains all the CFU-GM-derived colonies (Fig. 2). Mixed colonies were individualized with the LICR-LON-R10 antibody as colonies containing both erythroid cells (glycophorin-positive cells) and nonerythroid cells (glycophorin-negative cells containing numerous granules or having the typical morphology of monocyte-macrophages by phase contrast microscopy). These mixed colonies were considered as clonal since the total number of colonies (erythroid and granulocytic) per dish was <60, therefore sustaining culture conditions for a clonal mixed colony assay (21). Scanning of the entire dish with this double fluorescence procedure permits precise determination of the proportion of the Tn+ colonies contained in each dish.

**Table I. Fraction (Percent) of Tn+ Progenitors in Colonies as Measured by HPA Labeling**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Colonies</th>
<th>Unseparated cell population</th>
<th>Separated cell populations*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pl. Experiment 1‡</td>
<td>BFU-E</td>
<td>70</td>
<td>100 (48/48)</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>60</td>
<td>99 (77/78)</td>
</tr>
<tr>
<td>Experiment 2‡</td>
<td>BFU-E</td>
<td>100 (140/140)</td>
<td>0 (0/31)</td>
</tr>
<tr>
<td></td>
<td>CFU-GM</td>
<td>100 (55/55)</td>
<td>0 (0/9)</td>
</tr>
<tr>
<td></td>
<td>CFU-GEMM</td>
<td>65</td>
<td>100 (25/25)</td>
</tr>
<tr>
<td>Ba‡</td>
<td>BFU-E</td>
<td>73</td>
<td>100 (13/13)</td>
</tr>
</tbody>
</table>

* Progenitors grown from light-density nonadherent peripheral blood cells separated by fluorescent cell sorting as described in Fig. 4.
‡ The threshold of fluorescent positivity was determined by analyzing blood mononuclear cells from a blood group O subject, with a fluorescent conjugate of HPA lectin (see Fig. 4). All the cells from the two Tn patients located above or below this threshold fluorescent intensity were respectively collected as Tn+ and Tn- fractions.
§ The threshold of positivity was determined as in ‡. However, the two channels just above this threshold were omitted for the cell collection. Accordingly, 1.5% of the cells were excluded during the sorting.
Figure 1. A BFU-E derived colony grown from the Tn' fraction. Nonadherent light-density mononuclear cells from patient P1 were separated into two fractions with a fluorescent activated cell sorter using the HPA lectin conjugated to fluorescein. $4 \times 10^5$ Tn' cells were grown by the plasma clot technique using erythropoietin and Mo-medium as the stimulating factors. At day 14, a double fluorescent procedure was directly applied on the cultures ($\times 583$). (A) The erythroblasts composing the colony are labeled with the murine monoclonal antibody LICR-LON-R10 and revealed with a goat anti-mouse immunoglobulin Fab$_2$ fragment conjugated to rhodamine. (B) All the erythroblasts are labeled by the HPA lectin conjugated to fluorescein and therefore exhibit the Tn antigen.

Figure 2. A CFU-GM derived colony grown from the Tn' fraction. The colonies were grown and studied as described in Fig. 1, except that the Mo-medium alone was used as the stimulating factor ($\times 583$). (A) All the granulocytic-monocytic cells are identified by the murine monoclonal 80H5 (anti-SS-EA-1 antigen) and revealed with a goat anti-mouse immunoglobulin Fab$_2$ fragment conjugated to rhodamine. (B) All colonies are labeled by the HPA lectin (as in Fig. 1 B) and therefore display the Tn antigen.
cells could not be eluted from the column using \( N \)-acetylglalactosamine.

Cell sorting experiments were subsequently performed. In the first series of experiments, nonadherent light-density mononuclear blood cells from patients Pl. and Ba. were separated into \( Tn^+ \) and \( Tn^- \) fractions and subsequently cultured. The threshold of positivity was determined using HPA-labeled mononuclear blood cells from a normal O patient. In both patients, two subpopulations of cells were clearly present among the mononuclear blood cells. One was positively labeled with HPA, the other was not. The positive fraction represented 12 and 15% of the nonadherent light-density mononuclear cells for patients Pl. and Ba., respectively. This low percentage of HPA positive mononuclear blood cells in the two patients is explained by the high frequency (60–75%) of \( T \) lymphocytes in this cell fraction. Indeed, we have shown previously that <1% \( T \) lymphocytes from patients Pl. and Ba. exhibit the Tn antigen (11). The percentage of \( Tn^+ \) cells, therefore, reflects the proportion of \( Tn^+ \) B cells and monocytes (11).

In both patients, the \( Tn^+ \) fractions give rise to nearly 100% positive colonies (Table I). As previously demonstrated (10), these colonies were composed exclusively of \( Tn^+ \) cells (Figs. 1, 2). The \( Tn^- \) fraction gave rise to 75–81% negative colonies composed only of \( Tn^- \) cells (Fig. 3).

In patient Pl. the number of colonies per \( 10^5 \) unseparated mononuclear blood cells was 32, 18, and 5 for BFU-E, CFU-GM, and CFU-GEMM, respectively. 62% of the total number of colonies were recovered from the \( Tn^+ \) fraction while 38% were grown from the \( Tn^- \) fraction. This result agreed with the data obtained on unfractionated cells where 65% of the colonies were \( Tn^+ \) and 35% were \( Tn^- \). Furthermore, the addition of the colonies recovered in the two fractions (i.e., \( Tn^+ \) and \( Tn^- \)) was nearly equal (90–95%) to the number of colonies grown from the same number of unfractionated cells, excluding that the separation procedure by itself might have abolished the growth of one population of progenitors (\( Tn^+ \) or \( Tn^- \)). A similar result was found for patient Ba. It was obvious, therefore, that the \( Tn^+ \) and \( Tn^- \) colonies were not randomly distributed between the two fractions (random hypothesis, \( X^2 = 48.5, \text{d.f.} = 1, P < 0.0005 \)).

The presence of some \( Tn^+ \) colonies in the \( Tn^- \) fraction was considered to result from difficulty in determining the exact threshold of fluorescence positivity. In a second set of experiments (Table I, patient Pl.), cells that displayed a fluorescence intensity at the threshold of positivity, i.e., 2 channels out of 255 (Fig. 4), were omitted from the sorting procedure. \( 6 \times 10^5 \) cells were sorted as positive, while \( 58 \times 10^5 \) were \( Tn^- \). In this case, the \( Tn^+ \) and \( Tn^- \) fractions only gave rise to \( Tn^+ \) or \( Tn^- \) colonies, respectively.

**Discussion**

Previous results from our group have clearly established that the \( Tn \) syndrome is a clonal disease since the \( Tn \) abnormality is clonally expressed in colonies from the different hemopoietic lineages, i.e., granulocytic, erythroid, and megakaryocytic lineages (10). The mutation affects a stem cell common to the myeloid and lymphoid lineages including the B and T series, since lymphocytes are also involved by this disorder (11). However, a strikingly low percentage of \( T \) cells expressing the

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**Figure 3.** A BFU-E derived colony grown from the \( Tn^- \) fraction. The colonies were grown and studied as in Fig. 1. However, in this experiment, the \( Tn^- \) cells were grown at a \( 4.10^5 \)-cells/ml concentration (\( \times 583 \)). (4) The erythroblasts are identified by the antiliglycophoriin antibody (as in Fig. 1 A). (B) The colony is not labeled by the fluorescent conjugate of HPA agglutinin and therefore is considered as \( Tn^- \).
long-term bone-marrow cultures will be necessary to demonstrate this hypothesis.

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