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Research Article

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ABSTRACT To evaluate whether exposure of Tn determinants at the surface of human erythrocytes, platelets, and granulocytes could arise from a somatic mutation in a hemopoietic stem cell, burst-forming unit erythroid (BFU-E) colonies, colony-forming unit granulocyte-macrophage (CFU-GM), and colony-forming unit-eosinophil (CFU-Eo) were grown from a blood group O patient with a typical Tn syndrome displaying two distinct populations (Tn^+ and Tn^-) of platelets, granulocytes, and erythrocytes. A large number of colonies was observed. Individual colonies were studied with a fluorescent conjugate of *Helix pomatia* agglutinin (HPA). A sizeable fraction of each of the erythroid and granulocytic colonies appeared to consist exclusively of either HPA-positive or HPA-negative cells, thereby demonstrating the clonal origin of those exhibiting the Tn marker. Similar results were obtained from a second patient. These findings establish that the HPA labeling of Tn cells is an accurate marker permitting assessment of the clonality of the human megakaryocyte (MK) colony assay. For the study of MK cultures a double-staining procedure using the HPA lectin and a monoclonal antiplatelet antibody (J-15) was applied in situ to identify all MK constituting a colony. Our results, obtained in studies of 133 MK colonies, provide definitive evidence that the human MK colony assay is clonal because all MK colonies were exclusively composed of Tn^+ and Tn^- MK. Furthermore, the distribution of MK within a single colony was shown to be seminormal with a mean at 6 MK, isolated MK typically being absent in culture.

Comparison of the proportion of mature Tn^+ cells in blood with their respective Tn^+ progenitors has also shown that no proliferative advantage occurs after the commitment; because Tn polyagglutinability is an acquired disorder, then the expansion of the Tn^+ clone must occur either during the proliferative stage of the pluripotent stem cell or during the commitment itself. This study therefore affords evidence that a blood group antigen plays a role in the differentiation of a pluripotent stem cell.

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INTRODUCTION

Tn polyagglutinability is an acquired disorder characterized by exposure at the erythrocyte surface of receptors that are normally hidden and become available to natural antibodies occurring in most human adult sera (1). Increasing interest in such markers is due to their frequent association with a mild hemolytic anemia or leucopenia and thrombopenia, as defined under the Tn syndrome (2). However, the Tn receptors may also be found in apparently healthy individuals.

More recently, a Tn activation was described as preceding acute leukemia or myeloproliferative disorders, suggesting its possible role in the development of the neoplastic process (3–5). Serological and biochemical evidence shows that *N*-acetyl-D-galactosamine, when covalently bound to erythrocyte glycoproteins by an

alkali-labile O glycosidic linkage, is the chief structural determinant of Tn specificity (6, 7). These "A-like" determinants exposed at the surface of Tn erythrocytes are recognized by several lectins such as *Salvia sclarea*, *Dolichos biflorus*, or *Helix pomatia*, but not by purified human anti-A antibodies (8). Investigations carried out to further characterize Tn determinants at the molecular level have shown that the erythrocytes, platelets, and granulocytes of these patients have abnormal surface glycoproteins with a low content of sialic acid and galactose (9–12). These modifications are associated with the defect of a single galactosyltransferase involved in the biosynthesis of sugar chains O glycosidically linked to cell membrane glycoproteins (10–14). On the basis of these data, it was suggested that Tn activation may originate in a mutation of a pluripotent stem cell of the bone marrow (11, 15). This hypothesis is further supported by the finding that only a sizeable fraction of erythrocytes or platelets exhibit the Tn determinants because Tn^+ and Tn^- subpopulations are identifiable in the blood of the same individual (10, 11).

In an attempt to establish the hematopoietic origin of the Tn activation, we have investigated the expression of the Tn antigen in single colonies derived respectively from burst-forming unit-erythroid colonies (BFU-E)[1], colony-forming unit-granulocyte-macrophages (CFU-GM), and colony-forming unit-megakaryocytes (CFU-MK). For this purpose we have used a fluorescent derivative of the *Helix pomatia* (HPA) lectin, which is *N*-acetylgalactosamine specific and capable of recognizing Tn determinants. We were aware that only the *Salvia sclarea* lectin is Tn specific (16) but that HPA could be safely used providing that Tn individuals of blood group O or B were selected. The results have clearly shown that expression of the Tn antigen in all erythroid (BFU-E) and granulocytic (CFU-GM) colonies is clonal. Indeed, the colonies were either completely positive or negative for the HPA marker. Because Tn was found to be a clonal marker, it was subsequently used to demonstrate the clonality of the megakaryocyte (CFU-MK) colony assay.

METHODS

Case report. The patient, P.L., is 27 yr old and an apparently healthy individual. His hematological abnormalities were discovered during a routine examination in July 1980. The leucocyte and platelet counts were respectively 2,900 and 45,000/mm^3, without erythrocyte anemia. The patient is a blood group O donor with a typical Tn phenotype (1). The erythrocytes were polyagglutinable (as seen by their agglutination with most ABO compatible human sera) and strongly agglutinated by *Salvia sclarea*, *Helix pomatia*, *Dolichos biflorus*, and *Glycine soja* lectins but unreactive with hexadimethrine bromide (Polybrene, Aldrich Chemicals, Beerse, Belgium) and *Arachis hypogea* (peanut agglutinin) lectin. As shown with other Tn samples (10, 11) a severe reduction of the UDPGal: GalNAc-β-3-D-galactosyltransferase was found in the patient's erythrocyte and platelet lysates.

The direct antiglobulin test was negative. Clinical examination was unremarkable. Under examination in April 1981, the leucopenia and thrombopenia were confirmed. There was no anemia but the reticulocyte count and indirect bilirubin level were slightly elevated, whereas haptoglobin was markedly reduced (<0.1 g/liter), suggesting a mild compensated hemolysis. A bone marrow specimen showed a normal cellularity with some increase in erythroid lineage.

Blood (20 ml) was obtained on three different occasions. Bone marrow cells for culture were collected by sternum puncture. Blood from two group O donors and from two group O newborns was also obtained for control purposes. A second patient, B.A., exhibiting a typical Tn syndrome (9–11) was also investigated, but few colonies could be obtained due to small amount of blood available.

Cell culture. Light density cells from blood or bone marrow were isolated by Ficoll-metrizoate (Lymphoprep-Nyegaard, Oslo, d = 1.077) density centrifugation at 400 g. The cells were washed three times in cold α-medium (Eurobio, France) and plated by two different culture techniques to grow the different commited progenitors, BFU-E, CFU-GM, and CFU-MK.

(*a*) The growth of BFU-E and CFU-GM colonies was performed by the methylcellulose procedure (17). Dulbecco's Minimum Essential Medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), as modified by Guilbert and Iscove (18), was used. The source of granulocyte macrophage-eosinophil-colony-stimulating factor (GM-CSF) was 10% Mo-conditioned medium (19). 2 U/ml of human urinary erythropoietin (Epo, 100 U/mg protein), obtained after DEAE-cellulose chromatography (20), was used to grow BFU-E colonies. The activity of this Epo was determined both by the mouse polycythemic assay and BFU-E cultures, using as reference the Connaught step III sheep erythropoietin (batch 3034.1). 2 U Epo/ml were chosen because it gives an optimal number of erythroid colonies and satisfactory erythroid maturation. 4.2×10^5 blood light density cells per milliliter were plated under these conditions. At least six cultures of each were run and incubated for 12–18 d in a fully humidified atmosphere with 5% CO_2. Colonies were scored at day 14 under an inverted microscope and were processed for immunofluorescence after having been individually picked off. After fluorescence, the type of cell constituting the colonies was determined by May-Grunwald Giemsa staining. (*b*) The culture technique for human MK colonies in plasma clot (21) was used with slight modification (22). The stimulating factor was a conditioned medium from leukocytes stimulated by phytohemagglutinin (PHA-LCM) (22, 23). 2×10^5, 4×10^5, and 6×10^5 bone marrow cells

[1] *Abbreviations used in this paper:* MK, megakaryocyte; BFU-E, burst forming unit-erythroid; CFU-Eo, colony forming unit-eosinophil; CFU-GM, colony forming unit-granulocyte-monocyte; CFU-MK, colony-forming unit-megakaryocyte; Epo, erythropoietin; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage-eosinophil-colony-stimulating factor; HPA, *Helix pomatia* agglutinin; PBS, phosphate-buffered saline (10 mM phosphate buffer pH 7.2 containing 150 mM NaCl); PHA-LCM, conditionned medium from leucocytes stimulated by phytohemagglutinin; TRITC, tetramethylrhodamin isothiocyanate.

were respectively plated, after adherence, in plasma clot. The cultures were studied at day 12, a time at which MK cultures reach their optimum. They were screened by immunofluorescence (24, 25). In addition to MK colonies this method also permits the growth of a high number of granulocyte colonies. The granulocyte colonies from bone marrow cultures were only investigated by this technique.

In contrast to the methylcellulose procedure, the plasma clot technique permits the in situ study of colonies. In addition, bone marrow BFU-E were grown in plasma clot using Epo as stimulating factor and were studied by immunofluorescence.

Immunofluorescent studies. Peripheral blood erythrocytes and granulocytes were isolated after sedimentation of the blood at 1 *g* for 3 h. Erythrocytes were obtained at the bottom of this tube and granulocytes in the buffy coat. Blood granulocytes were purified by Ficoll-metrizoate density centrifugation. The cells were extensively washed and cytospanned for staining studies. BFU-E and CFU-GM colonies were individually picked off under an inverted microscope, washed, and studied. CFU-GM colonies were studied at day 12 or 14, whereas BFU-E were investigated at days 9, 14, and 18.

For methylcellulose cultures, 46 and 45 of BFU-E and CFU-GM colonies were respectively studied. These colonies were selected on the basis that they were distinctly separated from neighboring colonies. Indeed, at the colony density at which CFU-GM were grown (70 colonies per dish), 35% do not arise from a single cell origin (26). This difficulty could be overcome by selecting well separated colonies. In addition, BFU-E colonies were recognized under an inverted microscope by their red or pink color (hemoglobinized cells). Immunofluorescent studies were performed after fixation of the cells for 10 min in pure methanol. The smears were extensively washed in cold PBS and 100 μl of 10^{-2} diluted *Helix pomatia* FITC conjugate (Industrie Biologique Franqaise, France 1 mg/ml) was added. The incubation was continued for 30 min at 4°C, and the smears were extensively washed in cold phosphate-buffered saline (PBS). Before examination under the microscope the slides were mounted with a coverslip in glycerin-buffered PBS. Similar conditions of assay were used for immunofluorescent studies of platelets and MK colonies. Therefore, platelets were first isolated from peripheral blood, washed, and resuspended in the culture medium. Aliquots of $5\text{-}10^6$ platelets were distributed in 35-mm petri dishes, and the medium was allowed to clot. From this step, a similar procedure was used for staining platelets or MK colonies. A double immunofluorescence staining was performed using an HPA-tetramethylrhodamin isothiocyanate (TRITC) conjugate to visualize the Tn antigen and a monoclonal antiplatelet antibody-fluorescein isothiocyanate (FITC) conjugate (J-15) to identify the MK. The monoclonal antibody (J-15) binds to normal human platelets but not to Glanzman's thrombasthenic platelets,[2] suggesting that the antigenic structure recognized is tightly linked to the glycoprotein IIb-IIIa complex. As shown (25), the monoclonal antibody, J-15, recognizes all the mature and immature MK without staining other cell types. Thus, by combination of the fluorescein- and rhodamin-labeled reagents, it is possible to specifically identify in situ all the MK colonies and to check them for the presence or the absence of the Tn antigen.

For this purpose, the clots were dehydrated in the petri dish with filter papers and then fixed for 5 min with 1% paraformaldehyde in PBS. The clot was washed with PBS, and 100 μl of a 10^{-2} dilution of HPA-TRITC conjugate (IBF 1 mg/ml) was applied for 90 min. After extensive washing with PBS, 100 μl of ascitic fluid (J-15 antibody) was subsequently applied at a 10^{-2} dilution for 60 min. After two additional washes in PBS, 100 μl of a rabbit anti-mouse Fab'_2 FITC conjugate (Nordic Immunology, The Netherlands, distributed by TEBU-France, 28000 Versailles), was applied at a 10^{-2} dilution for 30 min. The preparations were finally washed in PBS before examination under a Zeiss fluorescent microscope (Carl Zeiss, Inc., New York) equipped with epi-illumination and appropriate filter for fluorescein and rhodamin.

Photographs were taken with an Ektachrome ASA 200 film push-pulled to ASA 800. After fluorescent labeling, all the smears or petri dishes were respectively stained with the May-Grunwald-Giemsa or with Harris hematoxylin to identify the cells and colonies morphologically.

RESULTS

Erythroid lineage. Peripheral erythrocytes from the Tn patient (P.L., a group O individual) were first investigated by HPA-labeling with the fluorescein or rhodamin conjugate of the lectin. The erythrocytes were either strongly labeled or entirely negative, allowing an accurate scoring of each population (Fig. 1A). 572 erythrocytes were counted, 73% of which exhibited the Tn antigen (Table I). Control group O erythrocytes from adult and newborns were included, but no HPA staining was observed, confirming that the lectin recognizes only the Tn determinant, providing that blood group O individuals are selected.

BFU-E colonies were grown from the blood and bone marrow of the patient. The number of BFU-E for 1.10^6 plated cells was slightly above the normal range because we obtained $364/10^6$ cells from peripheral blood and $1,445/10^6$ cells from bone marrow cultures (normal range 40 to $200/10^6$ cells for peripheral blood; 200 to $1,000/10^6$ for bone marrow). Individual BFU-E colonies grown in methylcellulose were picked off and the Tn antigen was investigated by HPA labeling. The Tn marker was expressed in 34 of 46 (73%) colonies studied (Table I). In each colony, erythroblasts were either positive or negative with no intermediate cells (Fig. 1B). However, in three colonies of 46, a minority of cells (<5%) of one type (Tn^+ or Tn^-) were mixed with those of other types.

In methylcellulose, the cells can move more easily than in plasma clot, and may thus migrate from one colony to another during the pipetting procedure. This prompted us to perform the same investigation in plasma clot, in which case the colonies can be studied in situ. In this case, no mixture of Tn^+ and Tn^- cells could be demonstrated. The same proportion of Tn^+ and Tn^- colonies was found in cultures of peripheral and bone marrow precursors. In addition, BFU-E colonies were screened at different periods of culture

[2] Kieffer, N, G. Tobelem, A. MacMichael, J. Bastin, L. Degos, C. Ruan, and J. P. Caen. Submitted for publication.

(days 9, 14, and 18), but a mixture of Tn^+ and Tn^- cells was never detected. In patient B.A., 95% of the erythrocytes were labeled by HPA-FITC. Eight BFU-E colonies were studied, seven of them exhibiting the Tn marker, while the eighth was negative.

As an additional control, we studied erythroblasts from two blood group O adults and two blood group O newborns in culture but no HPA staining was detected in any cells of the colonies.

Granulocytic lineage. Granulocytes were isolated from the buffy coat and labeled by the HPA immunofluorescence procedure (Fig. 2a). As seen with erythrocytes, the granulocytes were either heavily labeled or negative. Granulocytes (517) were scored, of which only 29% were labeled by HPA.

CFU-GM and CFU-eosinophil (Eo) colonies were grown from peripheral blood and from bone marrow. The number of colonies was markedly above the normal range because we found $192/10^6$ cells in peripheral blood and $1,900/10^6$ cells in bone marrow cultures (normal range: 50 to $100/10^6$ cells for peripheral blood, maximum value $10^3/10^6$ cells for bone marrow). As mentioned above, the Tn antigen was investigated either in methylcellulose or in plasma clot. Some 183 colonies were studied and 58% were positive (Table I). Once again each colony was composed either of positive or of negative cells (Fig. 2B). Morphological investigations carried out after immunofluorescence labeling showed that most of the Tn blood colonies grown in the presence of Mo medium were composed of eosinophils, whereas the majority of bone marrow colonies grown in the presence of PHA-LCM were comprised of neutrophils and monocytes. A slight difference was observed in the distribution of Tn marker

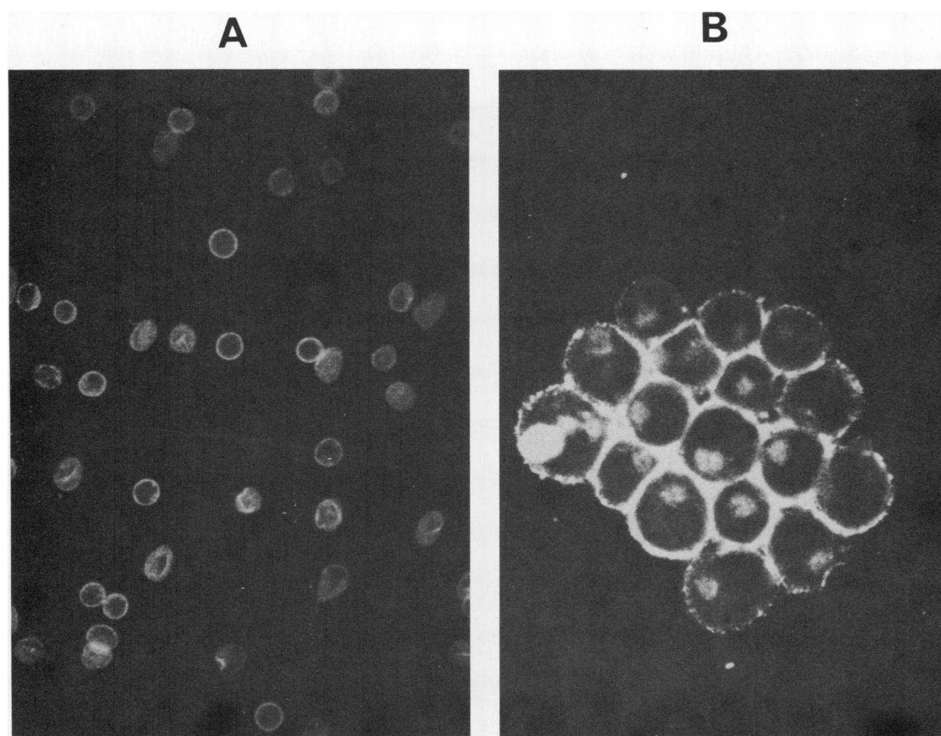


FIGURE 1 Immunofluorescent detection of Tn antigen on erythrocytes (1A) and BFU-E colonies (1B) using a FITC of the HPA lectin. (1A) Smears of washed erythrocytes from the Tn patient (P.L.) were fixed with methanol, washed in PBS, and reacted with 100 μl of HPA-FITC (10 μg/ml in PBS) for 30 min at 4°C. After extensive washings in cold PBS, the smears were examined under a fluorescent microscope equipped with epi-illumination. 73% of the erythrocytes were labeled (Tn^+ cells). Negative erythrocyte (Tn^- cells) are not visible on this picture. (1B) BFU-E colonies from the peripheral blood of the patient (P.L.) were grown by the methylcellulose technique in the presence of 2 U Epo. Colonies were picked off and cytocentrifuged and the HPA-FITC was applied after fixation with methanol. The colonies were either entirely stained (73%) or were negative (27%). A typical positive burst colony is shown. The slight difference in fluorescence intensity among cells within the colony were not obvious on smears and results from the photographic procedure.

TABLE I

HPA Labeling of Peripheral Blood Cells and Hematopoietic Progenitors from a Tn Patient (P.L.)

Cell lineage	Peripheral blood: Total number of cells examined	Peripheral blood: Proportion of HPA stained cells	Culture: Total number of colonies examined	Culture: Proportion of HPA stained colonies
Erythroid	572 (Erythrocytes)	418 positive (73%) 154 negative (27%)	46 (BFU-E)	34 positive (73%) 12 negative (27%)
Granulocytic	517 (Granulocytes)	149 positive (29%) 368 negative (71%)	200 (CFU-GM)	121 positive (60.5%) 79 negative (39.5%)
Megakaryocytic	500 (Platelets)	251 positive (50.2%) 249 negative (49.8%)	133 (CFU-MK)	65 positive (49%) 68 negative (51%)

Quantitative determination of HPA fluorescent cells in the peripheral blood and in vitro cultures of hematopoietic progenitors derived from a blood group O patient (P.L.) with a typical Tn syndrome. Only the Tn^+ cells are labeled by the lectin. No fluorescence could be detected with blood from control O or B donors.

because 75% of the 32 eosinophilic colonies were positive, while 59% of the 168 neutrophilic-monocytic colonies were Tn positive. Bone marrow granulocyte colonies were only studied by the plasma clot technique because the colony density was too high to allow for correct pipetting when the methylcellulose technique was used. In patient B.A., 82% of the peripheral granulocytes were stained by HPA-FITC. Six CFU-GM colonies were examined. All of them were Tn positive.

Megacaryocytic lineage. Platelets were isolated from peripheral blood; 52% were labeled by the HPA lectin (not shown). CFU-MK were grown in plasma clot from bone marrow and their number was in the normal range (90 CFU-MK/10^6 cells).

To assess the clonality of the human MK colony assay, double staining by HPA-TRITC and monoclonal platelet antibody (J–15) was performed. This procedure permitted an easier identification of all the MK colonies and accurate recognition of all the MK within each colony. Indeed, the monoclonal J-15 antibody is highly specific for the MK lineage and recognizes mature as well as immature MK, allowing elimination of other types of cells that may be intermingled with MK in the colony. Some 133 colonies were investigated at different cellular concentrations of platelet cells (from 2×10^5 to 6×10^5 cells); 49 colonies were Tn positive as in the other three myeloid lineages and again, all the colonies consisted exclusively of either positive or negative MK (Table I, Fig. 3).

It was also possible to demonstrate by this technique that the average number of MK per colony was six and that there was no difference in the distribution of MK per colony in Tn^+ and Tn^- colonies (Fig. 4, Student's t test = 0.2, $P > 0.10$). One third of the colonies were constituted of two MK. However, these are true colonies because they have a homogenous Tn type (either Tn^+ or Tn^-), and we have shown (25) that no MK can be detected in culture before day 5, therefore demonstrating that such colonies really arise as a result of the differentiation of a progenitor. With patient B.A., only the platelets were studied, and 81% were Tn positive.

DISCUSSION

Because the presence of a dual population of cells is well established within Tn bloods (12), we have investigated the level of cell differentiation at which the Tn antigen might be expressed. The presence of Tn determinants was therefore explored by HPA labeling of BFU-E, CFU-GM, and CFU-MK colonies derived from blood and/or bone marrow progenitors from a group O, Tn patient. Two possible results were expected. Firstly, a mixture of Tn^+ and Tn^- cells might coexist within each colony. Secondly, a dual population of colonies might be found, one entirely positive and the other entirely negative for the Tn marker.

The present study clearly demonstrates that the second eventuality occurred, indicating that two different populations of stem cells are present in the bone marrow of the two Tn patients investigated: one is normal (Tn^- progenitors), and the other exhibits the somatic mutation (Tn^+ progenitors). The expression of the Tn antigen on the cells contained in each colony was independent of their stage of maturation. In fact, in BFU-E colonies, the maturation of erythroblasts is not entirely synchronous, whereas the Tn antigen was similarly expressed on all cells of the colony. The Tn marker was found both at early periods of culture

when the erythroblasts are mostly immature and at late periods, when they are fully mature. These results clearly establish that Tn cannot be considered as an antigen of maturation.

The clonal expression of Tn antigen in erythroid and granulocytic colony assays was confirmed with a blood sample from a second O patient (B.A.). We found that this patient had numerous Tn^+ circulating cells because 95, 81, and 82% of peripheral erythrocytes, platelets, and granulocytes, respectively, were labeled by HPA-FITC.

As compared with the results obtained with the first patient, P.L., (Table I), it is obvious that the proportion of Tn^+ cells in peripheral blood varies over a rather wide range from one individual to another. The nature of the mechanism that controls the phenomenon is unknown.

Other blood group antigens are present in erythroid

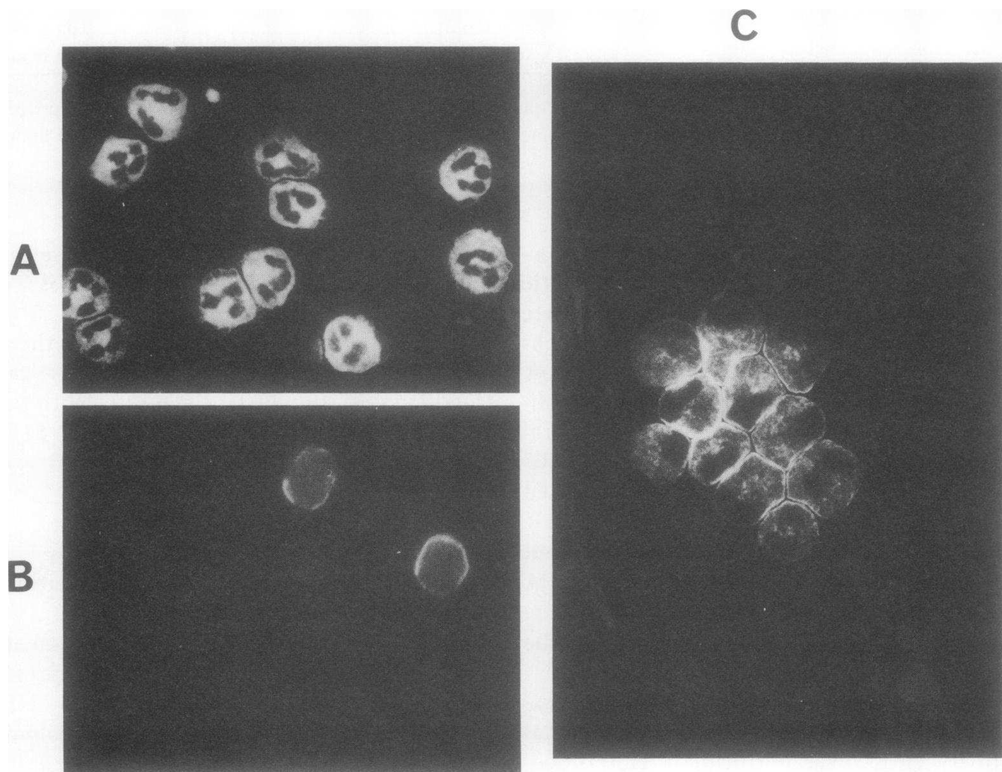


FIGURE 2 Immunofluorescent detection of Tn antigen on granulocytes (2A, B) and CFU-GM colonies (2C) using HPA-FITC labeling. (2A). An EDTA anticoagulated blood sample (patient P.L.) was sedimented at 1 g for 3 h. The buffy coat was carefully aspirated, washed in PBS, and processed for immunofluorescence. The smears were fixed with methanol, washed in cold PBS, and reacted for 30 min at 4°C with 100 μl of HPA-FITC (10 μg/ml in PBS). (2B). The granulocytes were purified by Ficoll-metrizoate (d = 1.077) density centrifugation for 25 min at 1,600 g. The polymorphonuclears were collected at the bottom of the tubes and the residual erythrocytes were eliminated by hypotonic lysis in 0.2% (wt/vol) NaCl. The isolated granulocytes were treated for 5 min at 20°C with 1% (wt/vol) paraformaldehyde in PBS, washed, and further incubated with 0.1 ml HPA-FITC (10 μg/ml in PBS) for 30 min at room temperature. After extensive washings in PBS, the smears (2A) or free erythrocyte suspensions (2B) were mounted in glycerine-buffered PBS and examined for fluorescence. As shown from this picture, cells fixed with methanol exhibit a cytoplasmic fluorescence (2A), while a peripheral labeling was observed with free cells in suspension (2B). Both patterns of labeling were specific because control group O cells were not labeled and identical percentage of Tn^+ granulocytes (29%) were found for a same patient. The Tn^- granulocytes are not visible on these preparations as they are not labeled. (2C) Blood BFU-GM and CFU-Eo were grown in methylcellulose using Mo-medium as stimulating factor. The colonies were individually picked off, cytocentrifuged, and stained with HPA-FITC after fixation in methanol. Colonies were made of either Tn^+ granulocytes (60%) or Tn^- granulocytes (40%). A Tn^+ eosinophil-granulocyte colony, with a typical cytoplasmic fluorescence, is shown.

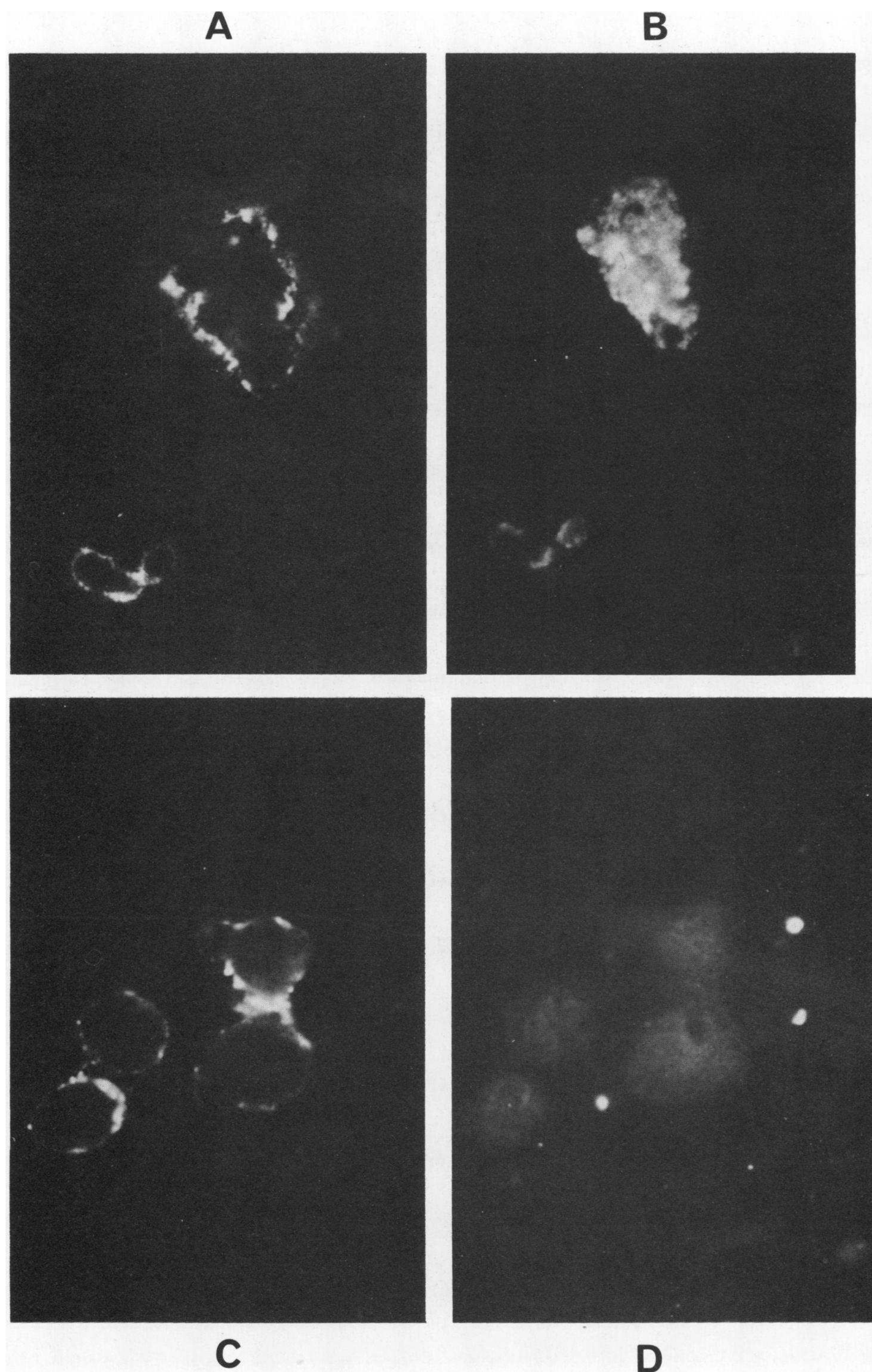
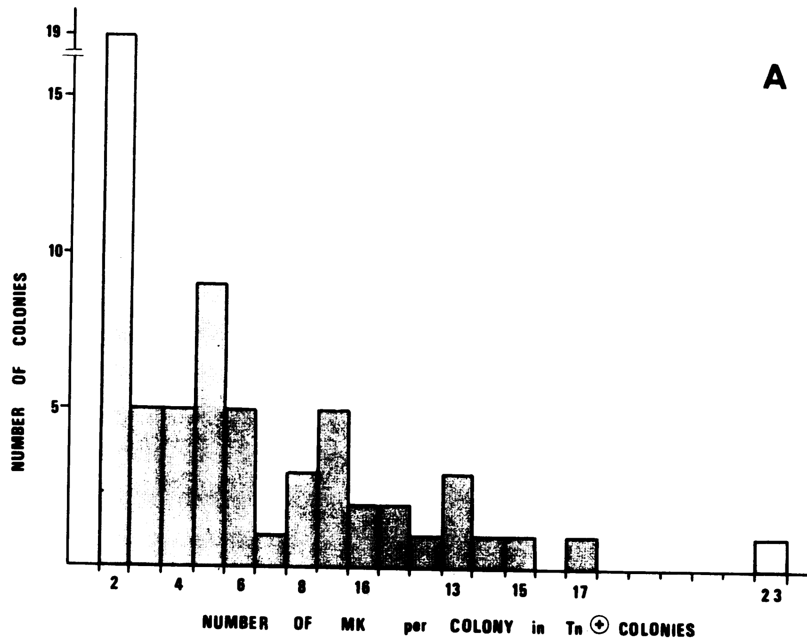


FIGURE 3 Detection of Tn antigen on CFU-MK colonies using a double immunofluorescent labeling. The colonies of MK were grown from bone marrow aspirate of the patient (P.L.) in plasma clot using PHA-LCM as stimulating factor. Because MK colonies are indeed clusters of loosely aggregated cells, a double immunofluorescent assay was applied *in situ*. The aim was to demonstrate the clonality of the MK colony assay, using the Tn antigen, revealed by



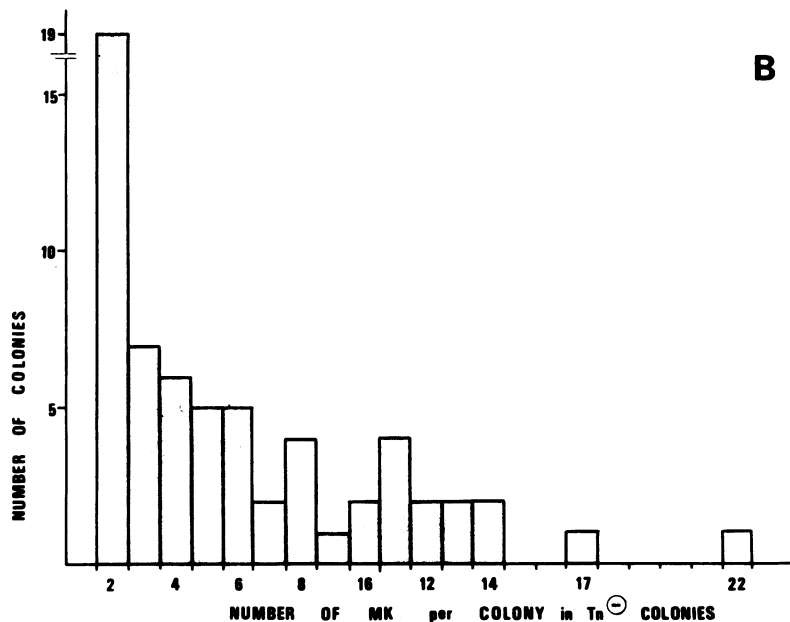


FIGURE 4 Distribution of MK in Tn^+ (4A) and Tn^- (4B) colonies identified according to the method described in the legend of Fig. 3. The distribution is similar in both types of colonies suggesting that the Tn marker does not give any proliferative advantage during the differenciation process of the CFU-MK.

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a fluorescent HPA staining, as marker of clonality. The first labeling was performed using the HPA-TRITC lectin. The second labeling was an indirect immunofluorescent method using a monoclonal antiplatelet antibody (J-15) followed by a rabbit anti-mouse Fab'2-FITC conjugate. The J-15 antibody produced in mice recognized all cultured MK, whatever their degree of maturity. (3A) Colony of MK labeled by the J-15 antibody. (3B) The same MK strongly stained by the HPA-FITC lectin (Tn^+ MK). (3C) Colony of 4MK labeled by J-15 antibody. (3D) The same MK were not labeled by the HPA-FITC lectin (Tn^- MK).

colonies but are expressed in a different way. For instance, the i antigen is heterogeneously found from one BFU-E colony to another in adult cells. However, within the same colony, i^+ and i^- erythroblasts coexist in different proportions (27). Thus, the i antigen displays the characteristics of a differentiation marker, whereas the Tn antigen is the consequence of a somatic mutation. As Tn abnormality is expressed in BFU-E, CFU-GM, and CFU-MK colonies, this mutation has to be considered to have occurred at the level of a pluripotent stem cell common to these lineages.

It has been extensively demonstrated that each erythroid or granulocyte-monocyte colony is derived from one cell (26, 28, 29). However, direct evidence of the clonal origin of MK colonies in man, and even in the mouse, is lacking. Assessment of the clonality of MK colonies is critical, because most MK colonies are clusters of loosely aggregated MK and are thus distinct in their aspect from the granulocytic and erythroid colonies. It could be argued that megakaryocytic colonies merely arise from the coalescence of two or more MK. In addition, because each colony includes 2–20 MK, their content of G6PD isoenzymes, which is the most reliable marker of clonality (30), cannot be evaluated.

Because two populations of hemopoietic cells do exist even at the level of BFU-E and CFU-GM in Tn individuals, the Tn antigen can be used as a reliable marker for assessment of the clonality of the human MK colony assay. Double immunofluorescent labeling using the HPA lectin and a monoclonal antiplatelet antibody was used. This latter antibody (J–15) permits the specific identification in situ of all mature or immature MK as well as normal and leukemic promegakaryoblasts (25).

The results of this study have unequivocally demonstrated the clonal nature of this assay. Indeed, each colony was composed of either Tn^- or Tn^+ MK. No significant coalescence was observed up to $6–10^5$ plated marrow cells. In addition, the Tn marker has allowed us to accurately delimit the size of MK colonies. On average, a colony was made of 6 MK. This result is in agreement with that obtained in cultures of murine bone marrow cells in plasma clot (31), but is different from those found in agar (32). However, we have also observed that the number of human MK per colony is highly variable from one colony to another, with a semi-normal distribution ranging from 2 to 23 (Fig. 4). In contrast with murine cultures (31), no single isolated MK were observed. The present study has thus afforded clear evidence that the mutation responsible for Tn reactivation has occurred in a pluripotent stem cell, and also that this mutation does not bring any significant proliferative advantage during the pathway from the commited progenitors to the mature cells. However, this study does not permit direct demonstration of the occurrence of the Tn antigen on stem cells. For the erythroid and MK lineages, the same proportion of Tn^+ cells was observed on the erythrocytes and BFU-E colonies on one hand, and in platelet and MK colonies on the other. As far as the granulocytic lineage is concerned, an even higher proportion of Tn^+ CFU-GM or CFU-Eo colonies was observed as compared with mature granulocytes (patient P.L.). This result might be explained by a peripheral destruction of Tn granulocytes by natural antibodies.

A slight difference in the proportion of Tn^+ and Tn^- colonies was observed between each hematopoietic lineage, which cannot be explained by this study. It could be tentatively ascribed either to differences in the commitment of hematopoietic precursors from the pluripotent stem cells or to changes in the rate of self-renewal from one type of commited stem cell to another.

Thus, it must be assumed that the Tn mutation has occurred in a single pluripotent stem cell followed by a clonal expansion. As no proliferative advantage was observed after the commitment, this advantage should take place either in the proliferative compartment of the pluripotent stem cell or during the process of commitment. In the near future, it will be possible to investigate these phenomena with the development of long-term bone marrow culture techniques (33) and pluripotent stem cell assays in man (34).

At the present time, we are of the opinion that Tn activation results from clonal expansion and for this reason may be considered as being equivalent to a preleukemic state. As also noticed by others (35), the Tn transformation may be compared to paroxysmal noctural hemoglobinuria because both disorders depend on a membrane abnormality, which itself results from a mutation occurring in a pluripotent stem cell (12, 36, 37). In both conditions, two subpopulations of myeloid cells coexist (12, 38), and the development of acute leukemia can be occasionally observed (3–5, 39, 40). Tn polyagglutinability is now a well characterized mutation occurring in a pluripotent stem cell, and it will be a useful model for future studies of the role of blood group antigens in the proliferation of pluripotent stem cells.

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