Radioautographic Investigations on DNA and Protein Metabolism in 2 Cases of Di Guglielmo’s Disease

By Felice Gavosto, Giovanni Maraini and Alessandro Pileri

ERYTHEMIC MYELOSIS, which was first described by Di Guglielmo in 1923, has only recently been universally recognized as a distinct entity characterized by a systemic and irreversible disorder of red cell precursors.

Most of the previous papers have dealt with the morphology and histogenesis of the erythremic cells. Reports on the functional and biochemical behavior of these cells are very scanty. Astaldi and Tolentino in 1949 and Blackburn and Lajtha in 1951, with the method of bone marrow culture in vitro, were able to demonstrate a slower maturation rate of these cells together with a qualitatively disturbed nuclear development. By means of the statmokynetic method Bernardelli, Mele and Bani in a case of chronic erythremic myelosis demonstrated that the rate of proliferation of the erythroblasts was normal. Furthermore, they confirmed that the maturation process is clearly slower than in the normal bone marrow. The presence of hemoglobin in red cell precursors was demonstrated by Cowles et al. in a case of acute erythremia.

More recently Baldini et al. expanded the investigations on the physiopathology of this disease. On the basis of the serum iron turnover and iron utilization studies, these authors demonstrated the existence of an ineffective type of erythropoiesis in this disease, “perhaps due to an acquired (neoplastic) defect in the uptake or utilization of vitamin B12 by the erythroblasts.”

The present paper deals with some aspects of desoxyribonucleic acid (DNA) and protein metabolism in two cases, one of acute and the other of chronic erythremic myelosis. DNA metabolism was studied using tritium-labeled thymidine. Thymidine is known to be a specific DNA precursor. Unlike other precursors which have been used previously (phosphorus, formate, serine, adenine, guanine), thymidine is incorporated only into the DNA molecule to form thymine. Tritiated leucine was also used to investigate protein metabolism. This amino acid is not incorporated into other cellular constituents. Moreover, it rarely undergoes exchange phenomena.

The study was carried out by a high resolution radioautographic technic. Unlike the usual chemical methods, autoradiography allows a study at the cellular level; therefore, it is particularly suitable for hematologic investigations. The use of tritium as a tracer, because of the very low range of its
electrons in photographic emulsions, has enormously increased the resolution power of the technic. In the last few years this technic has been successfully applied in hematologic investigations dealing mainly with DNA metabolism.9,13

DESCRIPTION OF THE CASES

Case 1: Chronic Erythremic Myelosis

R. A., a white housewife, age 35, was admitted for the first time to the Medical Clinic of Turin Medical School on January 20, 1959, complaining of weakness, pallor and fever of one year's duration. The past history was noncontributory. Four months prior to admission, anemia of considerable degree was detected with circulating polychromatic and orthochromatic erythroblasts. The anemia was relieved only by blood transfusions; parenteral liver extracts and vitamin B₁₂ had been ineffectual. On admission, the patient appeared well nourished, markedly pale, with no evidence of icterus or purpura. Temperature was 38.2°C, pulse rate, 96 per minute, respiratory rate, 24 per minute. Blood pressure, 130 systolic and 70 diastolic. There were no changes in the mouth and pharyngeal mucosa. The heart was moderately enlarged to the left, and a systolic murmur was audible at the apex and at the mesocardium. The spleen was felt 2 cm. below the costal margin. The liver was not palpable. There was no lymphadenopathy. The limbs and central nervous system showed no abnormality. The eye grounds were pale, but no hemorrhages were seen.

Laboratory data were as follows: Hgb., 7.8 Gm. (46 per cent); RBC, 2,05 M. per cubic millimeter; color index, 1:15; reticulocytes, 0.8 per cent; nucleated cells, 9400 per cubic millimeter. The differential count was: promyelocytes, 0.9; myelocytes, 4.5; metamyelocytes, 3.0; polymorphonuclear granulocytes, 11.0; monocytes, 12.6; lymphocytes, 14.0; proerythroblasts, 3.6; basophilic erythroblasts, 30.6; polychromatic erythroblasts, 15.3; orthochromatic erythroblasts, 3.6; reticulum cells, 0.9; (all in per cent). Erythrocytes showed marked aniso- and poikilocytosis. Platelets were 100,000 per cubic millimeter.

The bone marrow was highly cellular. The differential count (per cent) was as follows: myeloblasts, 0.4; promyelocytes, 0.8; myelocytes, 3.0; metamyelocytes, 0.8 polymorphonuclear granulocytes, 7.8; eosinophilic granulocytes, 0.2; lymphocytes, 1.8; reticulum cells, 1.8; plasma cells, 0; proerythroblasts, 5.6; basophilic erythroblasts, 44.2; polychromatic erythroblasts, 22.0; orthochromatic erythroblasts, 6.6; cells in mitosis, 1.6 per cent. The myeloid-erythroid ratio was 0.14. Megakaryocytes were decreased in number but morphologically normal. Some megaloblastoid changes were seen in the nucleated red cells. Cells representing intermediate forms between reticuloendothelial cells and primitive red cells were numerous.

Tourniquet test, slightly positive. Plasma prothrombin content, 60 per cent. Howell test, 120 seconds. Coombs' test, negative. Erythrocyte osmotic fragility, slightly increased. Fetal hemoglobin (Singer-Chernoff method), 2.6 per cent. Electrophoretic analysis of hemoglobin revealed no abnormal components. Iron59 turnover rate revealed a T ½ of 185 minutes.

Fecal urobilinogen was 127 mg. per 24 hours. Serum iron was 85 µg. and iron binding capacity, 190 µg. 100 ml. E.S.R. (Westergren) was 80 mm. after 1 hour. Total serum protein 6.7 Gm./100 ml. with 50 per cent albumin, 5, 12.5, 12.5, and 20 per cent globulins. Serum bilirubin, 0.8 mg./100 ml. Gastric analysis showed absence of free HCl even after parenteral histamine. A biopsy of the gastric mucosa revealed no abnormality. The stools were guaiac negative on several occasions. Several blood cultures were negative. The urine was normal. X-ray examinations of the chest, pelvis and gastrointestinal tract showed no abnormalities. In the skull three small areas of bony rarefaction were observed.

The diagnosis of erythremic myelosis was made mainly on the basis of the predominance of immature forms of red cell precursors in the peripheral blood and on the megaloblastoid bone marrow picture, which was resistant to treatment. The chronicity of this form was testified by the long course of the disease and the absence of marked thrombocytopenia with hemorrhagic manifestations.
Case 2: Acute Erythremic Myelosis

G. C., a white man, age 52, was admitted for the first time to the City Hospital on March 25, 1959, complaining anemia, fever and weakness of two months' duration. Four weeks prior to admission, a severe normochromic anemia with circulating erythroblasts was discovered. Vitamin B₁₂ and parenteral liver extracts had been ineffectual. The past history was noncontributory. The patient died on April 4, 1959.

On admission, the patient appeared markedly pale and dyspneic, with no clear evidence of icterus. There was some bleeding from the gums. No petechiae were observed. Temperature was 39°C, pulse rate, 120 per minute, respiration rate, 40 per minute. Blood pressure, 125 systolic and 70 diastolic. There was no lymph node enlargement. A systolic murmur was heard over the precordium. The spleen and the liver were felt 2 cm. below the costal margin. There were no neurologic abnormalities.

Laboratory data were as follows: Hgb., 6.5 Gm. (40 per cent); RBC, 2.26 M per cubic millimeter; color index, 0.90; reticulocytes, 1.2 per cent; nucleated cells, 3000 per cubic millimeter. The differential count was: promyelocytes, 1; metamyelocytes, 1; polymorphonuclear granulocytes, 29; monocytes, 4; lymphocytes, 22; proerythroblasts, 8; basophilic erythroblasts, 7; polychromatic erythroblasts, 19; orthochromatic erythroblasts, 9 (all in per cent). Erythrocytes showed marked anisocytosis. Several macrocytes were present. Platelets were absent on the smear. The bone marrow was highly cellular and showed a striking erythroid hyperplasia. The differential count (in per cent) was as follows: myeloblasts, 0; promyelocytes, 0.1; myelocytes, 0.2; metamyelocytes, 0.7; polymorphonuclear granulocytes, 1.7; lymphocytes, 2.4; plasma cells, 0.1; reticulum cells, 2.6; proerythroblasts, 39.5; basophilic erythroblasts, 30.1; polychromatic erythroblasts, 13.0; orthochromatic erythroblasts, 9.6; cells in mitosis, 2.5 per cent, all in the erythroid series. Megakaryocytes were absent. The immature red cells showed megaloblastoid features (fig. 1). Cells representing intermediate forms between reticuloendothelial cells and early megaloblastic erythroblasts were numerous. Some multinucleated erythroblasts and atypical mitotic figures were seen. The Coombs' test was negative. Urinalysis showed only moderate urobilinuria. Fasting

Fig. 1.—Bone marrow of the case of acute erythremic myelosis.
blood sugar and BUN were within normal limits. Serum bilirubin was 1.5 mg./100 ml. (direct reacting, 0.55). Gastric analysis showed the presence of free HCl.

The patient was treated with blood transfusions, parenteral liver extracts and vitamin B₁₂ without effect. The course was progressively and rapidly downhill, and the patient died 10 days after admission. Necropsy was not obtained.

The diagnosis of the acute form of Di Guglielmo's disease was made on the basis of the very rapid course, the acute onset of severe, progressive anemia, the high grade fever, the absence of platelets with hemorrhagic phenomena, and the bone marrow picture showing striking erythroid megaloblastoid hyperplasia, mainly due to the most immature red cell precursors.

**MATERIAL AND METHODS**

Studies were carried out in 4 normal individuals and in 2 patients with erythremic myelosis. The bone marrow was obtained by sternal puncture, aspirated into a heparinized syringe and immediately diluted with the same volume of culture medium (Hank's solution + lactalbumen hydrolysate, 5 per cent). One ml of the bone marrow suspension was transferred into each of two siliconized test tubes. Tritium-labeled thymidine (specific activity, 890 mc. mM) was added to the first tube, and tritium-labeled L-leucine (specific activity 29.1 mc./mM) to the second one to a final concentration of 2.5 μc. per milliliter and 10 μc. per milliliter, respectively. The procedure was carried out under sterile conditions. The tubes were incubated in a rotating system at 37 C. Samples were taken after 1 and 3 hours, and smears were made on gelatine-coated slides. The smears were fixed in Carnoy's solutions, and after several washings in distilled water, radioautographic preparations were made according to the technic described by Pelc.¹⁹ The films were developed after an exposure of 6 days for H³-thymidine and 16 days or more for H³-L-leucine (figs. 2 & 3). The smears were stained through the stripping film with May-Grünwald-Giemsa. About 200 cells for each cell type were observed, and the number of grains was counted. For tritiated thymidine the grains over the cell nucleus were counted. For H³-L-leucine, grain counts, both over nucleus and cytoplasm, were

![Fig. 2.—H³-Thymidine incorporation in a cell of acute erythremic myelosis.](image)
Fig. 3.—H\textsuperscript{3}-DL-leucine incorporation in the cells of chronic erythremic myelosis.

For the cells with a low incorporation rate, it was necessary to employ longer periods of exposure of the autoradiographs in order to achieve good conditions for the grain counting. Obviously, when the amount of labeling in the elements of the different cell types was compared, consideration of the fact that the number of grains was proportional to the time of exposure was made, since all the other experimental conditions were constant.

RESULTS

The results obtained are summarized in tables 1 to 4 and in figures 4 and 5. Table 1 shows the percentages of bone marrow cells which incorporated tritiated thymidine, both from normal individuals and from the two cases of Di Guglielmo's disease. Only small differences in the percentage of labeling for each cellular type were observed with the 4 normal marrows. The percentages of labeled cells decreased progressively from the less to the more mature cellular types, and no labeled cells were found at the level of the orthochromatic erythroblasts.

In the case of chronic erythremic myelosis, the percentage of labeling for all the different elements of the erythroid series did not significantly differ from that observed with normal bone marrows. On the contrary, the bone marrow from the case of acute erythremic myelosis showed a significantly lower percentage of labeled cells; the cells characteristic of the disease (see

<table>
<thead>
<tr>
<th>Bone marrow cells</th>
<th>Normal bone marrows</th>
<th>Erythremic myelosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. 1*</td>
<td>C. 2</td>
</tr>
<tr>
<td>Prierythroblasts</td>
<td>58 %</td>
<td>62 %</td>
</tr>
<tr>
<td>Basophile erythr.</td>
<td>51 %</td>
<td>45 %</td>
</tr>
<tr>
<td>Polychrom. erythr.</td>
<td>12 %</td>
<td>9</td>
</tr>
<tr>
<td>Orthochr. erythr.</td>
<td>0 %</td>
<td>0</td>
</tr>
</tbody>
</table>

*C. 1 = Case 1.
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Table 2.—Cells Incubated with H3-Leucine—Grain Counts per Cell of Cellular Surface

<table>
<thead>
<tr>
<th>Bone marrow cells</th>
<th>Erythremic myelosis</th>
<th>Normal bone marrows</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1h</td>
<td>3h</td>
<td></td>
<td>1h</td>
</tr>
<tr>
<td>Proerythroblasts</td>
<td>15.4 ± 3.2</td>
<td>49.8 ± 16.5</td>
<td>19.9 ± 10</td>
<td>82.9 ± 40.5</td>
</tr>
<tr>
<td>Basophilic erythro.</td>
<td>5.8 ± 3.2</td>
<td>13.3 ± 8.7</td>
<td>1.2 ± 1.6</td>
<td>3.5 ± 4.2</td>
</tr>
<tr>
<td>Polychromatic erythro.</td>
<td>1.1 ± 1.5</td>
<td>2.9 ± 3.5</td>
<td>23.1 ± 8.7</td>
<td>7.5 ± 5.2</td>
</tr>
</tbody>
</table>

Table 3.—Cells Incubated with H-Leucine—Grain Counts per 100 sq.μ

<table>
<thead>
<tr>
<th>Bone marrow cells</th>
<th>Erythremic myelosis</th>
<th>Normal bone marrows</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1h</td>
<td>3h</td>
<td></td>
<td>1h</td>
</tr>
<tr>
<td>Proerythroblasts</td>
<td>7.2</td>
<td>18.9</td>
<td>8.7</td>
<td>31.4</td>
</tr>
<tr>
<td>Basophilic erythro.</td>
<td>4.8</td>
<td>11.7</td>
<td>7.6</td>
<td>28.3</td>
</tr>
</tbody>
</table>

Table 4.—Cells Incubated with H3-Leucine—Mean Grain Counts per 100 sq.μ of Nuclear and Cytoplasmic Surface

<table>
<thead>
<tr>
<th>Bone marrow cells</th>
<th>Erythremic myelosis</th>
<th>Normal bone marrows</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleus</td>
<td>Cytoplasmin</td>
<td>Nucleus</td>
<td>Cytoplasmin</td>
</tr>
<tr>
<td>Proerythroblasts</td>
<td>8.3</td>
<td>20.5</td>
<td>3.5</td>
<td>16.6</td>
</tr>
<tr>
<td>Basophilic erythro.</td>
<td>10.2</td>
<td>16.4</td>
<td>0.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>

description above) showed a percentage of labeling fourfold lower than normal proerythroblasts and threefold lower than normal basophilic erythroblasts. An even greater decrease in the incorporation of tritiated thymidine (more than tenfold) was observed in the polychromatophilic erythroblasts when compared to that observed in the same elements from normal bone marrow. The percentage of labeling after 1 hour of incubation did not significantly differ from that detected after 3 hours, both in normal and pathologic cases.

Tables 2 to 4 and figures 4 and 5 summarize the results obtained studying tritiated leucine incorporation. Practically all the nucleated elements of the bone marrow showed leucine uptake. A wide range of values of incorporation was found between cells of the same type. This was clearly reflected in the high value of the standard deviations obtained. Nevertheless, in the 4 normal bone marrows, the same cellular types showed very similar degrees of incorporation, and statistical analysis did not show significant differences between the same elements of the different normal bone marrows. The incorporation of tritiated leucine decreased progressively from the less to the more mature elements. In the first 3 hours, the uptake increased linearly with the time of incubation.

Considering all the cells in toto, it could be observed that in normal bone marrow the amount of leucine incorporated by the proerythroblasts was about 3 times higher than in basophilic erythroblasts and nearly 15 times higher.
Fig. 4.—A, H^4DL-leucine. Grain counts per cell. B, H^4DL-leucine. Grain counts corrected for a surface unit of sq. μ. Normal B.M. (— — — —); chronic trythremic myelosis ( . . . . . ); acute trythremic myelosis ( — — — — ).

Fig. 5.—A, H^4DL-leucine. Grain counts for 100 sq. μ of nuclear surface. B, H^4DL-leucine. Grain counts for 100 sq. μ of cytoplasmic surface. Normal B.M. ( — — — — ); chronic trythremic myelosis ( . . . . . ); acute trythremic myelosis ( — — — — ).
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than in polychromatophilic erythroblasts. The same pattern was observed in the pathologic bone marrows.

In order to compare the degree of incorporation in normal and pathologic bone marrows, a correction of the grain counts for surface unit was done. Tables 2 and 3 and figure 4 show the values for the cells in toto and for a surface unit of 100 sq. μ.

Both the characteristic elements of acute erythremic myelosis and the proerythroblasts and basophilic erythroblasts of chronic erythremic myelosis showed a higher leucine incorporation when compared to the normal elements. Actually, a statistically significant difference (p <0.01) of the mean grain counts was found between normal cells and the elements of acute erythremic myelosis, considering both the values for the cells in toto and those corrected for surface. The same observation was made in the proerythroblasts of chronic erythremic myelosis, whereas in the basophilic erythroblasts, the differences were significant only when the grain counts were corrected for surface.

Due to the high resolution of the technic applied, a separate evaluation of the incorporation in the nucleus and in the cytoplasm could be done. The results, corrected for a surface unit of 100 sq. μ, are represented in table 4 and figure 5. In all the elements, both normal and pathologic, a relatively higher incorporation was detected in the nucleus. This difference was more evident at the end of the first hour of incubation.

DISCUSSION

It is well known that DNA is metabolically inert in nondividing cells, and DNA synthesis is thought to be the preparatory step for mitosis. The study of DNA metabolism at the cellular level could therefore be considered a fairly adequate method for the evaluation of the proliferation rate of cells.

In recent years the thymidine uptake, both by normal and leukemic bone marrows, has been studied.9,11,15-17 It has been pointed out9,11 that there is virtually no uptake of thymidine in chronic lymphocytic leukemia and multiple myeloma, and moderate uptake in myelocytic leukemia. In previous reports from this laboratory,15,18 it was shown that, as far as thymidine incorporation is concerned, there is no significant difference in bone marrow cells from chronic myeloid leukemia and from normal individuals. However, a very significant decrease in thymidine uptake by blast cells from acute leukemia as compared to normal myeloblasts was found. A similarly low uptake in cells of acute leukemia has been recently reported by Lajtha.15

The possibility of a decreased mitotic activity in acute leukemia had already been broached by Astaldi and Mauri,19 Sacchetti and Bianchini,20 and Salera and Tamburino,21 from results obtained with bone marrow culture, utilizing the statmokinetic index. The present investigation shows that thymidine is incorporated in the erythroblasts of Di Guglielmo's disease and that there is a significant difference between the chronic and the acute form of the disease. In the latter, thymidine incorporation by red cell precursors appeared to be strikingly lower than normal while the red cell precursors of chronic erythremic myelosis behaved as normal elements. From our findings, the proliferative activity of the nucleated red cells appears to be definitely lower than normal in
acute erythremic myelosis. On the contrary, the proliferative activity of these elements in chronic erythremic myelosis does not significantly differ from that of normal red cell precursors. A definite parallelism seems therefore to be demonstrated between the behavior of nucleated red cells in acute and chronic erythremic myelosis and that of the granulocytes in acute and chronic myeloid leukemia, as Di Guglielmo himself suggested many years ago.

Investigations on the capacity of leukemic cells to incorporate amino acids have been carried out by Weisberger and al.\textsuperscript{22,23} utilizing cystine and methionine labeled with S\textsuperscript{35}. Leukemic cells showed a higher incorporation of these two amino acids than did normal leukocytes from control subjects. In 1950, Frantz and Zamecnik\textsuperscript{24} studied the in vitro incorporation of C\textsuperscript{14}-labeled DL-alanine and glycine into normal and leukemic blood cells. While in chronic leukemia, only a slight increase was found, a marked increase in the incorporation was observed in the cells from acute leukemia. Similar results were obtained by Baker and al.\textsuperscript{25} using C\textsuperscript{14}-labeled DL-leucine. All these investigations were carried out utilizing chemical methods and determining the incorporation of the isotope in the total leukocyte population. Serious criticism can be raised regarding this type of study, since it does not take into account the differences in the leukocytes in the peripheral blood populations of leukemic and normal subjects. On the contrary, study of the incorporation at the cellular level presents a more reliable picture of what occurs in the different cell types and gives a direct comparison between the metabolic behavior of each type of pathologic cell and that of their normal counterpart.

Our data on the leucine uptake, even when corrected for surface units, show that incorporation was higher in the more immature red cells of both normal and pathologic bone marrows. A similar finding (higher uptake by the more immature elements) had been observed by Ficq and al.\textsuperscript{26} and by Gavosto and al.\textsuperscript{27} utilizing glycine-l-C\textsuperscript{14}. Comparing the degree of incorporation of tritiated leucine in both acute and chronic Di Guglielmo's disease with that of normal erythroid bone marrow cells, our data demonstrated significantly higher values in erythremic myelosis. This applied to both the proerythroblasts and the basophilic erythroblasts, and it was particularly evident when the grain counts were referred to a surface unit of 100 sq. µ. The incorporation increased linearly with the time of incubation, in agreement with the findings of previous workers.\textsuperscript{23}

It is known that when an amino acid is incorporated into a cell it does not equally distribute through the whole cell, but preferentially localizes in particular subcellular structures. This has been demonstrated by ultracentrifugation and by autoradiographic methods.\textsuperscript{28-30} For this reason, we also determined separately the incorporation of tritiated leucine in the nucleus and in the cytoplasm. The grain counts were then corrected for a surface unit of 100 sq. µ. In normal as well as in abnormal erythroblasts, leucine incorporation occurred both in the nucleus and in the cytoplasm, being, however, always higher in the former. This was particularly so for the basophilic erythroblasts, being more evident after 1 hour of incubation. This relatively higher incorporation of leucine in the nuclei may be interpreted as evidence for the major role played by the nucleus of erythroblasts, particularly basophilic erythroblasts,
in protein synthesis. It is known that synthesis of globin occurs actively in these cells.31

In summary, the data presented here show that in the nucleated red cells of erythremic myelosis a disturbance in both DNA and protein metabolism exists. A lack of correlation between DNA and protein metabolism is present in the acute form of Di Guglielmo's disease.

**SUMMARY**

H3-thymidine and H3-DL-leucine incorporation in the erythroblasts of the bone marrow from a case of acute and one of chronic Di Guglielmo's disease was studied. A similar investigation was also carried out with bone marrow from 4 normal subjects. The high resolution of the radioautographic technic employed allowed a comparative study at the cellular and subcellular level.

A definitely lowered thymidine uptake was observed in the cells of acute erythremic myelosis, while increased incorporation of DL-leucine was detected in both the acute and the chronic form of this disease.

**SUMMARIO IN INTERLINGUA**

Esseva studiate le incorporation de H3-thymidina e de H3-DL-leucina in le erythroblastos del medulla ossee ab un patiente con acute e ab un alte re con chronic morbo de Di Guglielmo. Un simile investigation esseva etiam effectuate con le medulla ossee ab 4 subjectos normal. Le alte resolution del technica radioautographica empleate permetteva un studio comparative al nivello cellular e subcellular.

Un definitemente reducite captation de thymidina esseva notate in le cellular de acute myelosis erythremic, durante que un augmento del incorporation de DL-leucina esseva detegite tanto in le forma acute como etiam in le forma chronic del morbo.

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FELICE GAVOSTO, GIOVANNI MARAINI and ALESSANDRO PILERI