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A. R. Villanueva

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A New Biological Stain for Peripheral Blood and Bone Marrow Smears

A. R. Villanueva, B.S.*

A new blood stain has been developed for the demonstration and differentiation of cell types in peripheral blood or marrow smears, parasites in blood, and tissue impression preparations. It is available as a prebuffered powder which is dissolved in 100% reagent methanol. The method of staining is simple and rapid, with no precipitate. Overstaining is not likely to occur. The tinctorial patterns closely resemble some predecessors and, as the stain solution ages the staining quality improves. It is specific, stable, reproducible and resistant to fading.

Since the introduction of neutral histologic dyes 75 years ago, there has been little development of new polychrome blood stains, perhaps because the older blood stains *trained* several generations of pathologists and technologists, and deviating from established procedures would create problems of interpretation.

This paper describes a new blood stain whose tinctorial patterns resemble certain of its predecessors, but which produces stains of better quality. It is prepared as a pre-buffer, single powder, to be dissolved in 100% reagent methanol.

Materials

1) *Smears:* Blood or bone marrow smears should be prepared fresh and air-dried. Unfixed, air-dried smears which have been kept for as long as a year will stain satisfactorily, although not as sharply as fresh preparations.

2) *Solutions Required*

(A) *Stock Buffers:*

I. Buffer mixture, pH 6.8 . . . .Content of 1 vial (Hellige Certified Reagent)
Freshly boiled and cooled distilled water . . . . . .100 ml

II. Buffer mixture, pH 5.8 . . . .Content of 1 vial (Hellige Certified Reagent)
Freshly boiled and cooled distilled water . . . . . .100 ml

(B) *Working Buffer A:*
Buffer mixture, pH 6.8 . . . . . .2 ml
Buffer mixture, pH 5.8 . . . . . .2 ml
Distilled water . . . .96 ml

(C) *Working Buffer B:*
Buffer mixture, pH 6.4 . . . .Content of 1 vial

*Orthopaedic Research Laboratory.

(Hellige Certified Reagent)
Freshly boiled and cooled distilled water . . . . . 1000 ml

3) *Composition of the Blood Stain Powder:*

The stain consists of 1 gram methylene blue chloride (C.I. No. 52015); 100 ml 1% aqueous sodium bicarbonate; 2.72 grams of sodium acetate; 100 ml 1% aqueous eosin Y (C.I. No. 45380) and 1.20 ml of glacial acetic acid.

The resultant precipitate is filtered, dried and powdered.

4) *Stock Solution:*

(A) Powdered stain 0.375 gm
Methanol, 100% (A.R.) . . . . 100.00 ml

Solution is prepared by dissolving the dye in 100% reagent methanol. Stir or shake for at least a half hour with a mechanical stirrer or shaker. Then, cover the solution tightly and incubate overnight at 45°C-50°C. Cool, filter and store in a well-stoppered dark bottle. The stock solution is then ready to use.

Note: This stain is available as the Villanueva Blood Stain either in dry powdered form or in solution from Lipshaw Manufacturing Company, Detroit, Michigan.

(B) Staining Mixture:
Stock Villanueva blood stain . . . . 20 ml
Working buffer (either "A" or "B") 42.5 ml

Because this mixture appears to deteriorate after one hour, it is best prepared fresh before staining each batch of slides.

Staining Methods

A. *Rack Method:*

1) Allow fresh smears to air dry.

2) Flood the smear with the stock solution measured from a medicine dropper and let set undisturbed for 1-2 minutes. Add to this twice the amount of working buffers, either "A" or "B", and gently agitate by drawing small amounts in and out with a medicine dropper. After 4-5 minutes, wash with distilled water, and drain.

3) Dry in air or in an incubator at 45°C-50°C.

4) Examine under oil immersion.

5) For permanent reference, mount under a cover slip in any neutral synthetic resin mountant (Before mounting remove any immersion oil with xylol).

B. *Immersion Method:*

This method is used to stain numerous slides in one setting.[1]

1) Allow fresh smears to air dry. Align in a dry, removable slide tray.

2) Stain for 1-2 minutes in a staining jar containing the stock solution.

3) Transfer to the staining mixture. Lift the slide tray in and out of the mixture 20 times then let it stand in the dye for 4-5 minutes. Lift the slide tray in and out again, five to ten times, then rinse by dipping in and out three times each in two changes of distilled water.
Now follow steps 3, 4, and 5 as in Method A.

Results—Microscopic Description

Red Blood Cells. Erythrocytes are orange-pink. Nucleated red cells have dark purple nuclei and the cytoplasm is bluish-grey.

Neutrophils (Fig 1). The nuclei of immature and mature neutrophils stain reddish-purple to violet. Cytoplasm is pale blue, with fine orange-red granules. The abnormal neutrophils found in various leukemias, anemias and leucocytoses have dark purple nuclei with coarse, hyperchromic orange-red cytoplasmic granules.

Monocytes (Fig 1). The nuclei are distinguished from those of lymphocytes by their lighter, delicate reddish-purple staining. The cytoplasm is blue with fine red granules, usually finer and less numerous than those of the neutrophils.

Basophils (Fig 2). The nuclei are irregularly shaped and stain dark or light purple. Their cytoplasm is pale blue and the granules are coarse, spherical and dark violet.

Eosinophils (Fig 3). The nuclei stain like neutrophils. Their cytoplasm is pale blue and the granules are oval and bright orange-pink in color.

Lymphocytes (Fig 4). The nuclei stain reddish-purple or violet. There is a narrow rim of light to dark blue cytoplasm which sometimes contains reddish granules. In various leukemias the nuclear elements appear darkly

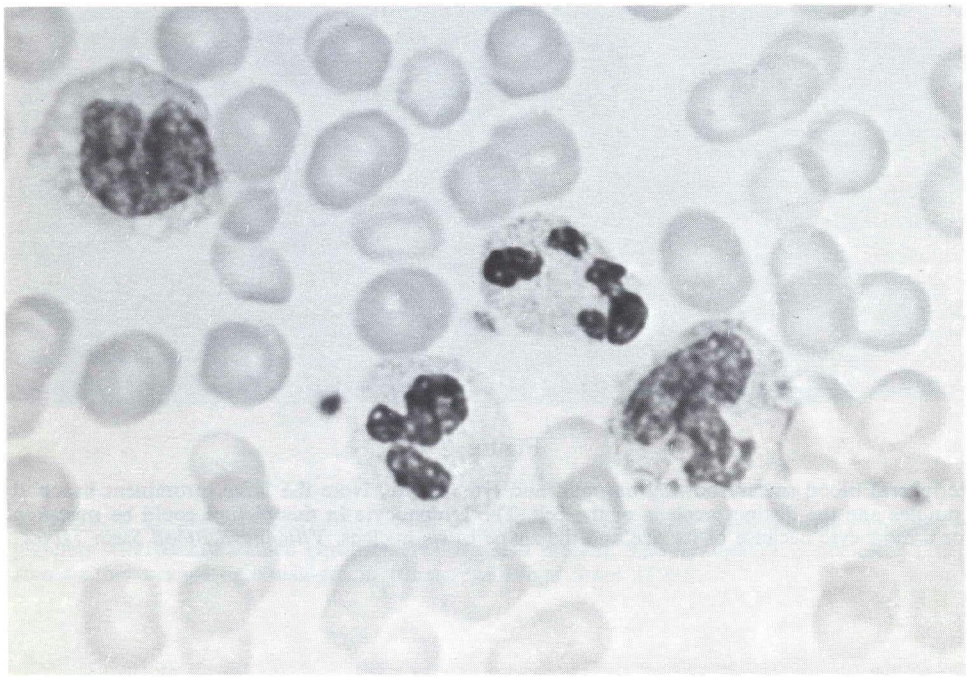

Figure 1

Peripheral blood smear showing two neutrophils lying between two monocytes. The nucleus of one of the monocytes appears horseshoe-shaped with abundant chromatin threads. Note the prominent fine granules in the cytoplasm of these cells. *Villanueva Blood Stain 1100X.* Reduced 20% for reproduction.

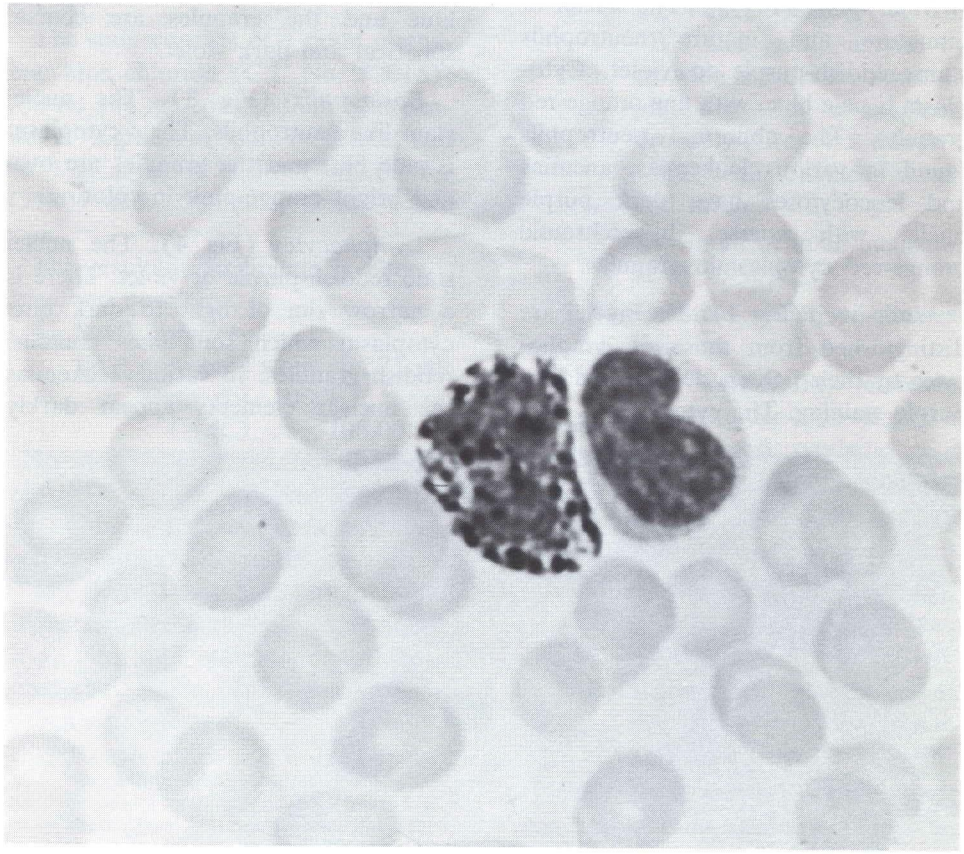

Figure 2

Peripheral blood smear showing basophil and lymphocyte. Note the large, prominent basophil granules and the distinct nucleus of the cell. The lymphocyte in this picture could be mistaken for a monocyte because of its size and the shape of its nucleus. *Villanueva Blood Stain 1100X.*

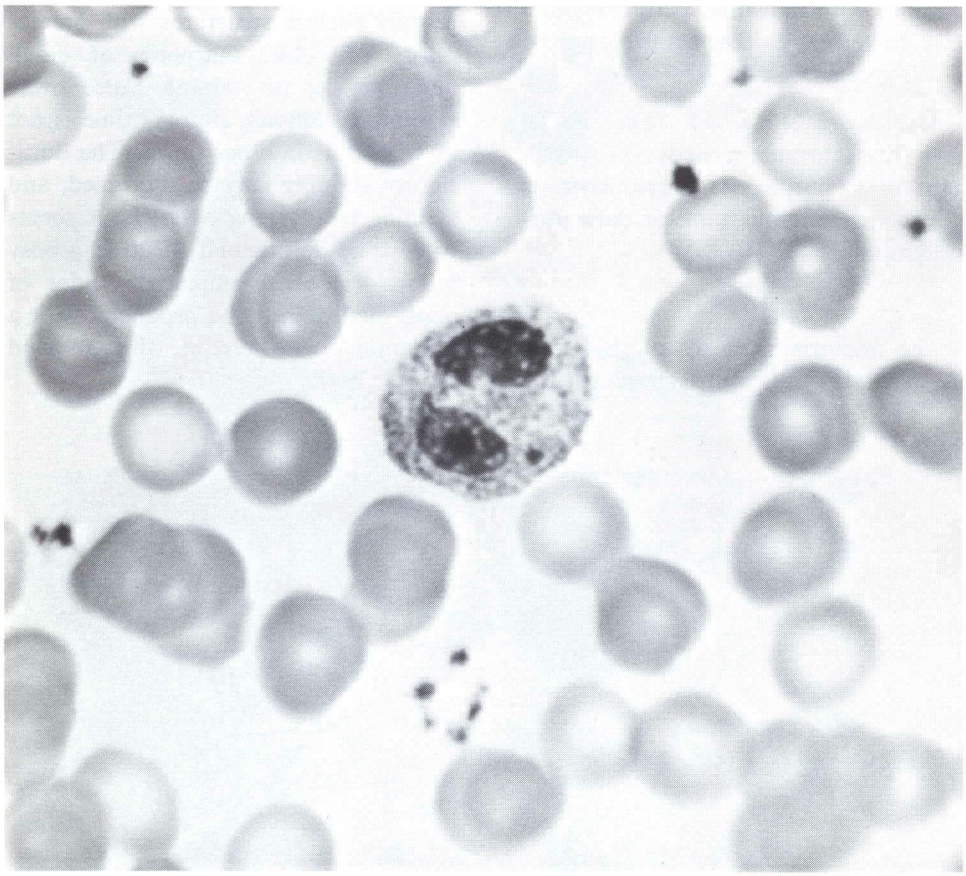

Figure 3

At center is an eosinophil. The cytoplasm contains numerous pink granules. Tiny clusters of platelets with darkly stained chromomeres are also present. The hyalomere is not clear but it stains either orange or bluish-green. *Villanueva Blood Stain 1100X.*

stained but show the finest detail of the chromatin network, as well as coarser structures of other cells.

Platelets (Fig 3). The central portions of platelets are violet to purple granules with indistinct periphery, and have frayed edges that stain orange or bluish-green.

Myelocytes (Fig 5). The nuclei have fine chromatin networks which stain dark purple. The cytoplasm is light with numerous pink or dark purple granules.

Discussion

The tinctorial patterns of this stain are almost identical to those of the Romanowsky-type stains,[2, 3] so that the problem of identification of cell types is easily mastered by physicians or technicians who are accustomed to the older stains.[4-7] However, there are certain differences. The present stain reveals nuclear structures with greater purity of color, sharpness of detail and intensity of staining. Since overstaining is difficult, staining time is not as critical as in other stains. The duration of staining may be shortened, and staining for as long as an hour seems to have no detrimental effect. In a busy laboratory where numerous slides of good quality must be produced with a minimum of attention, this method may be best.

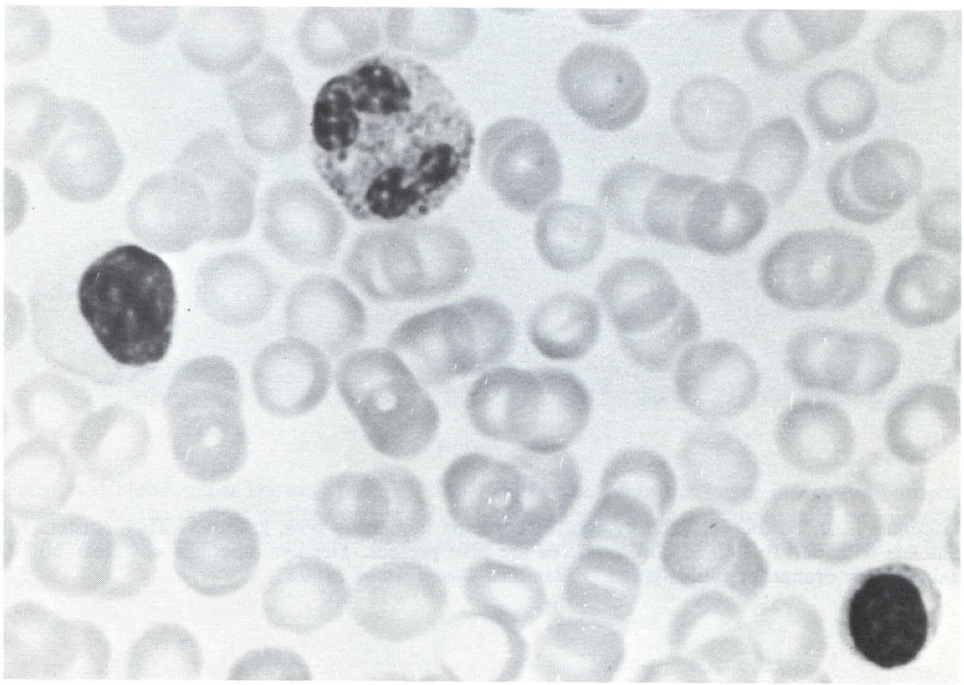

Figure 4

Blood smear shows lymphocytes at lower right and middle left. Slightly above them is an eosinophil containing large, numerous granules in the cytoplasm. *Villanueva Blood Stain 1100X.* (Reduced 20% for reproduction)

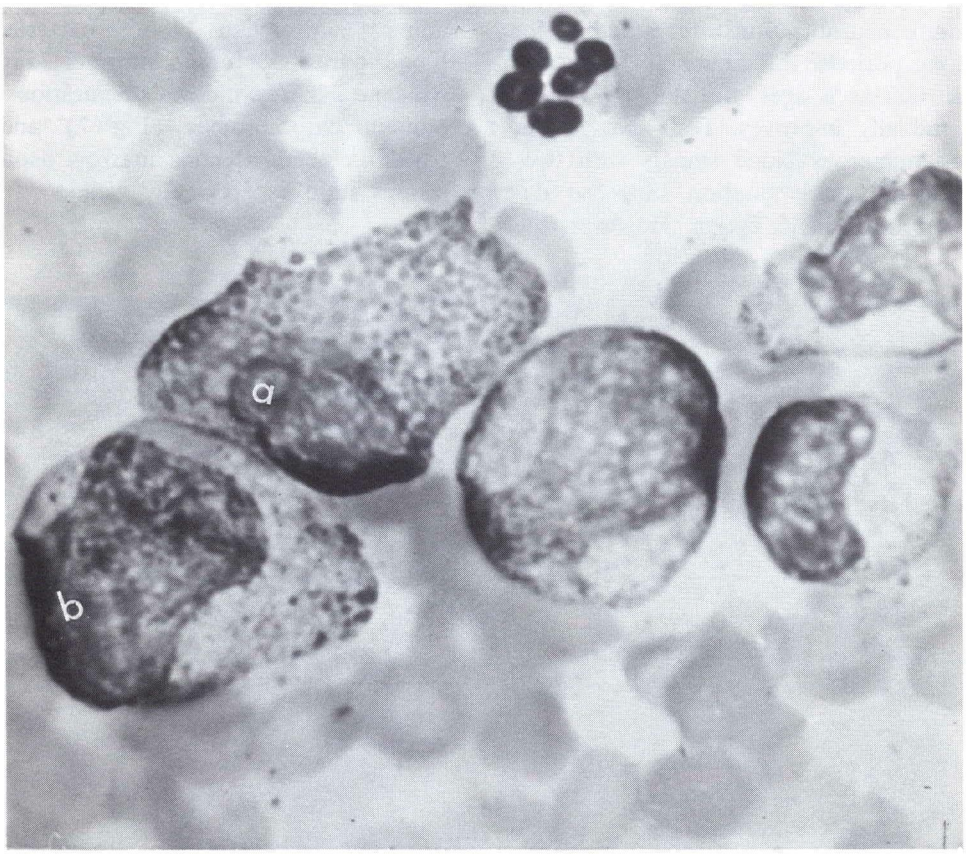

Figure 5

Bone marrow showing eosinophil and basophil myelocytes marked "a" and "b" respectively. Note the delicate nuclear chromatin network of the nuclei. At top is a normoblast undergoing karyorrhexis. *Villanueva Blood Stain 1100X.*

Incubation of the stock solution overnight at 45°-50°C improves this stain. Therefore, the stock solution keeps well in hot climates (a problem with some other blood stains).[8] While the familiar polychrome blood stains may become strongly alkaline[9] and require neutralization after standing for long periods, this new stain does not. In fact as it ages, the staining quality gradually improves. Tests carried out on numerous blood smears with two-year-old stock solution show no deterioration in its action. However, the quality of the stain provided by this method does vary with the condition of the smear. It is not optimal with year-old air-dried smears.

This stain has been favorably received by several local investigators,[10-13] who worked with human, animal and avian blood, bacteria, blood parasites, fungi, imprints (Fig 6) and skin window preparations. Human chromosomes (Fig 7) and paraffin-embedded bone marrow aspirations stain exceptionally well.[14]

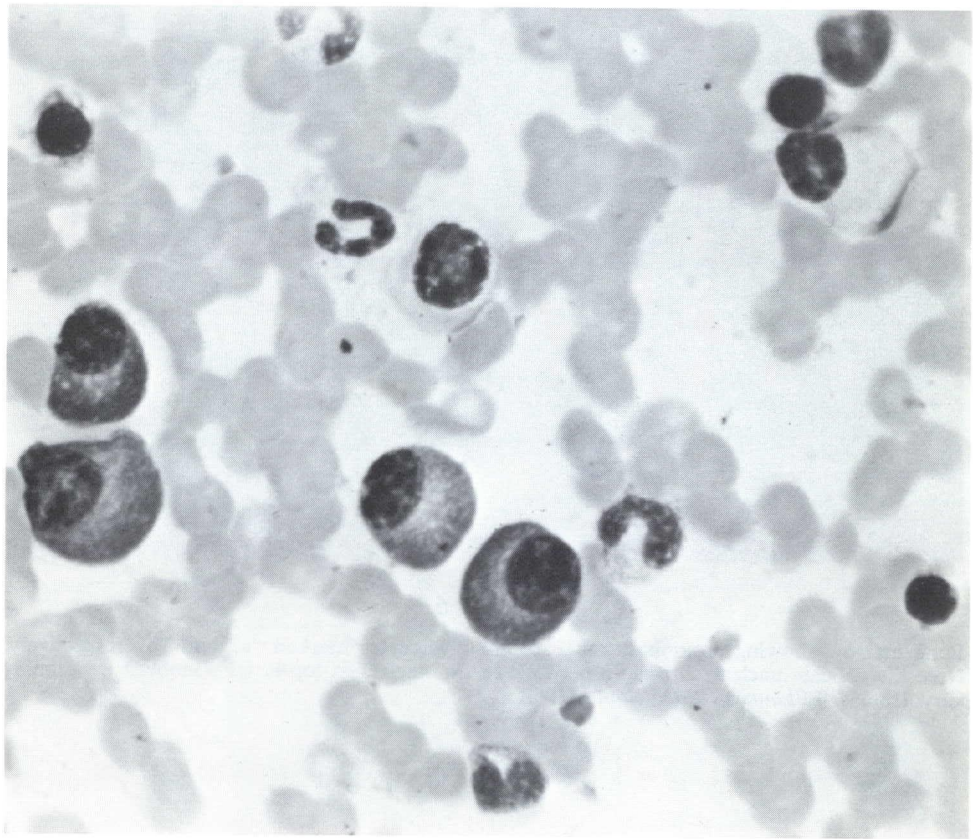

Figure 6

Photomicrograph of an imprint from a case of multiple myeloma. Note particularly the large tumor plasma cells. The eccentricity of the nuclei and the vacuolated cytoplasm show up well. *Villanueva Blood Stain 600X.*

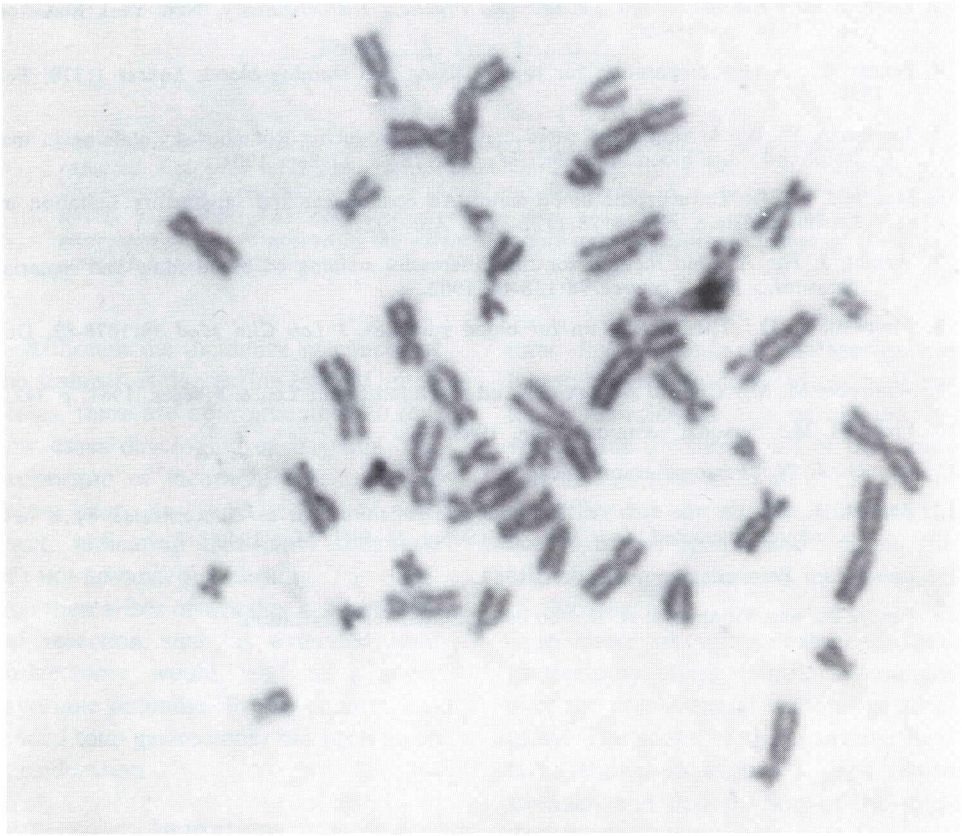

Figure 7

Human chromosomes from a culture derived from a male. Figure shows two overlapping chromosomes but a count of 46 can be readily made. Note the pronounced primary constrictions. *Villanueva Blood Stain 1100X.*

Acknowledgment

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