New mutations on platelet GPIIb in Sub-Saharan African populations revealed by genotyping discrepancies

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BACKGROUND: Previous studies of platelet allele frequencies in Sub-Saharan African populations enabled us to identify discrepancies in HPA-3 typing, suggesting the presence of new mutations and of a greater polymorphism than so far described in other populations.
OBJECTIVES: To analyze these discrepancies and to assess the factors leading to potential alloimmunization in these populations.
MATERIALS AND METHODS: Samples: Maternal samples from a Beninese woman following *in utero* death and panels of blood donors from Benin, Cameroon, Congo, and Pygmies from Central Africa. Techniques: Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), PCR-sequence specific primers (PCR-SSP) and sequencing techniques.
RESULTS: Three new mutations were found on GPIIb gene: exon 26 a) 2614C>A situated between HPA-3 and HPA-9w, b) 2645C>T downstream of HPA-3, c) intron 26 IVS26+89G>A. These mutations may lead to discrepant DNA typing results, due either to a localization in the complementary sequence recognized by the primer or to the appearance of a new enzyme restriction site. Furthermore, a bilateral linkage << deletion (Δ9 bp) intron 21 and the HPA-3b allele (exon 26) >> found in Caucasian, Asian, and Oceanian populations is not found in African populations, suggesting that its appearance was prior to HPA-3.
CONCLUSION: Three new mutations have been identified, two of them potentially immunogenic through their position. Furthermore, the polymorphism found on intron 26, localized in the complementary sequence of the PCR primer, may lead to a false typing assignation. It is therefore important to diversify techniques, both genomic (PCR-RFLP and PCR-SSP), and proteomic monoclonal antibody-specific immobilization of platelets antigen (MAIPA) to ensure accurate HPA antigenic system typing.

INTRODUCTION

It is now generally accepted that some 200,000 years ago, modern man emerged in Africa.

A number of studies, on genomic DNA and mitochondrial DNA alike, reveal greater genetic diversity in African populations than elsewhere, a diversity that can only have developed over thousands of years through countless mutations and which identifies these populations as the probable forerunners of all others.[1,2] Previous studies on HLA diversity in Sub-Saharan Africa (SSA) show a high degree of heterogeneity among the populations of this continent, correlated with geographic and/or linguistic differentiations.[3]

Therefore it seemed worthwhile to investigate the platelet polymorphisms. These polymorphisms may act as clinically important alloantigens in three conditions: in fetomaternal allo-immune thrombocytopenia, platelet transfusion therapy refractoriness, and post transfusion purpura. During our previous study[4] we identified discrepant typing results, leading us to suspect new mutations on glycoprotein (GP) IIb.[4] We therefore decided to focus the present study on GPIIb, and more particularly on exon 26, the site of HPA-3 and -9w polymorphism. We extended our study to include intron 21 and exon 30 of GPIIb according to the polymorphism linkage described by Peyruchaud and Ruan[5,6] in Caucasians.

ABBREVIATIONS: GP = glycoprotein; SSA = Sub-Saharan Africa; SSP = sequence specific primer.

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TABLE 1. PCR amplification: sequences of primers

PCR	GPIIb gene		Primers	PCR products
RFLP	Intron 21	Sense	5′-CAG ACC TTC CAA GGG AGC TT-3′	279 bp
		Antisense	5′-GTG AGG ACC AAG ATT CTG GC-3′	
RFLP	Exon 26 "primer pair n°1"	Sense	5′-CCT GGG CCT GAC CAC TCC TT-3′	259 bp
		Antisense	5′-CCA CAG AGG CCC ACA GCA CA-3′	
RFLP	Exon 26 "primer pair n°2"	Sense	5′-GGA AGA AAG ACCT GGG AAG G-3′	379 bp
		Antisense	5′CAT GCT CCT CCA TGT TCA CTT-3′	
Sequencing	Exon 30	Sense	5′-TGT GCT CTG GGG CCA GCA A-3′	196 bp
		Antisense	5′-GGA GGC AAC TTG TTG GAG AA-3′	
SSP	Exon 26	3a	5′-GGG GGA GGG GCT GGG GA-3′	293 bp
		3b	5′-GGG AGG GGC TGG GGC-3′	
		3Antisense	5′-GGC CCT GGG ACT GTG AAT-3′	

MATERIALS AND METHODS

Subjects

All the samples were received after informed consent.

- Patient: a woman originating from Benin, following an intrauterine fetal death at 16 weeks of gestation was referred to our laboratory for platelet immunological investigation.
- Populations: to study this new polymorphism, DNA samples from 603 subjects were available.
  - 509 individuals of Sub-Saharan African origin, previously studied in 2005 for human platelet (HPA) polymorphism[4] comprising 155 unrelated Beninese, donors from the Cotonou National Center of Transfusion and Blood Bank; 111 unrelated Aka Pygmies from the Central African Republic, both sexes included, and covering the 20 to 70 age group without common ancestry for at least three generations; 125 unrelated healthy volunteer Congolese blood donors living in Kinshasa or its immediate surroundings, and 118 unrelated healthy volunteer Cameroonian blood donors living in Duala or its immediate surroundings. Of these, 75 DNA (18 Beninese, 23 Cameroonians, 15 Pygmies, and 19 Congolese) were sequenced (sense and antisense) to identify a potential mutation responsible for discrepant results between polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and PCR-sequence specific primers (SSP).
  - 94 Sub-Saharan blood donors from our panel for anti-HLA antibodies screening, composed of: Burkina Faso (10 donors), Congo Brazzaville (10 donors), Ivory Coast (8 donors), Gabon (8 donors), Mali (10 donors), Mauritania (4 donors), Niger (5 donors), Uganda (2 donors), the Central African Republic (8 donors), Senegal (12 donors), Chad (15 donors), and Togo (2 donors).

DNA isolation

The salting-out method described by Miller and coworkers[7] was used to isolate genomic DNA from buffy coats.

PCR amplification

Using different primer pairs (Table 1), PCR was carried out in Gene Amp PCR system 9700 (Perkin Elmer, AB Applied Biosystems, Courtaboeuf, France). The presence of the amplified product was checked under ultraviolet (UV) light after electrophoresis on a 1 percent agarose gel in 1X TAE buffer with ethidium bromide staining.

HPA genotyping

Genotyping was performed by PCR-SSP and PCR RFLP (GPIIb: intron 21[5] and exon 26[8,9]) with a slight modification concerning the primer pair n°2 as described (see Table 1).

Due to the small amount of DNA available for certain subjects, we were not able to carry out the full range of tests in all cases. The number of subjects studied for each test is indicated on each table.

PCR-RFLP

PCR products, obtained with either primer pair n°1 or n°2 (Table 1), were digested with the restriction endonucleases: *Fok I* (Biolabs, St. Quentin en Yvelines, France) (5′-GGATG(N)^9/3′)[10] for HPA-3; *Bst*NI (Biolabs) (5′-CC/A TGG-3′) for HPA-9[9] and intron 21; *Sfa*NI (Biolabs) (5′ GCATC (N5)/3′) for the new mutation. Eight to 10 µL of each PCR product was digested for 2 hours at 37°C with 2 U *Fok*I *or Sfa*NI; for 2 hours at 60°C with 2 U *Bst*NI in a final volume of 20 µL containing their respective buffers. Non-digested and digested PCR products were analyzed using electrophoresis on an 8 percent polyacrylamide gel (29:1 acrylamide/bis-acrylamide) stained with ethidium bromide and visualized under UV light. The molecular weights of the restriction fragments were assessed by using 25 bp DNA step ladder (Promega, Charbonnières, France) as standard.

PCR-SSP

HPA-3 was investigated by PCR-SSP according to the method described by Klüter and colleagues.[11]

DNA sequencing

Amplified fragments (exons 26, 30, and intron 21) of GPIIb were sequenced with a sequence-based typing kit

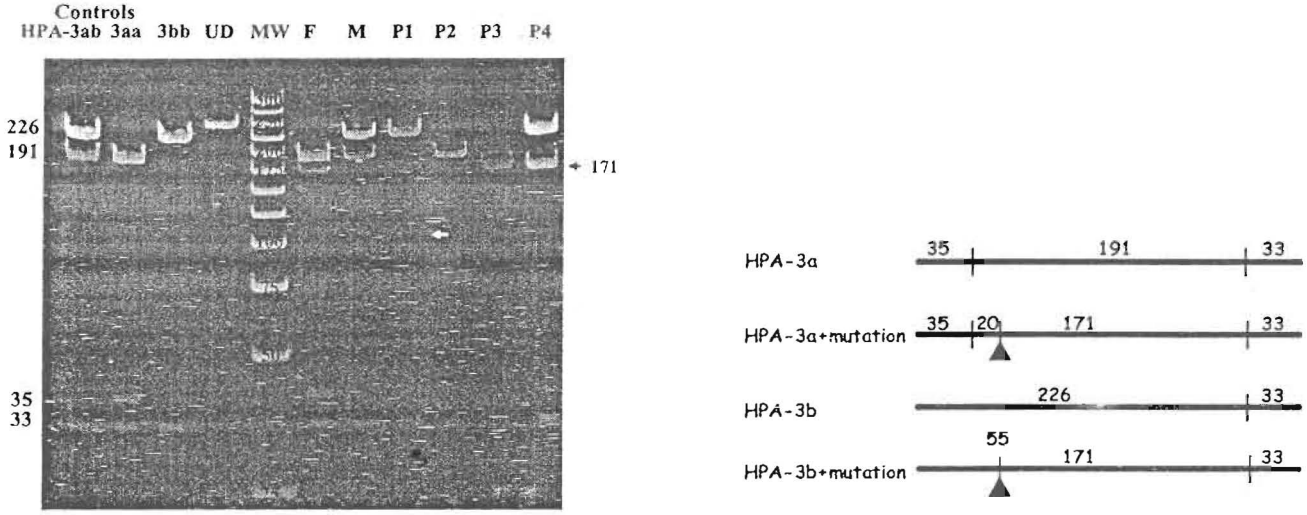


Fig. 1. A_k mutation and HPA-3 typing. Analysis of *Fok*I digested PCR products from DNA of 3 blood donor controls, A_k family (F = father; M = mother), and African blood donors P1 to P4. UD = undigested PCR product; MW = molecular weight.

(Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequence alignment was carried out using MultAlin software[12] and analyses were carried out using software package Chromas (Technelysium Pty Ltd, Shannon, Ireland).

Nomenclature

The HPA nomenclature was used according to the recommendations of the Platelet Nomenclature Committee[13] updated on the website http://www.ebi.ac.uk/ipd/hpa/table2.html and for mutations according to the recommendations of the Committee on Mutation Nomenclature.[14]

Statistical analyses

Statistical analyses were carried out using software package HLAStat2000 (Busson M. Programme HLAStat INSERM U 396, Immunogénétique Humaine 1996, Hôpital Saint Louis, Paris, France). Allele frequencies were calculated using the gene counting method of maximum likelihood.[15] Linkage disequilibrium was calculated from phenotypes according to Mattiuz.[16] Comparisons of allele frequencies between populations were assessed by chi-square test with an automatic correction of the "p" value by the Bonferroni method "pc" value.

RESULTS

1) A_k mutation (GPIIb 2614C>A)

Index case

An intrauterine fetal death was diagnosed in a Beninese woman (A_k) after 16 weeks' gestation, leading us to perform platelet immunological investigation. Despite a single incompatibility in the HPA-5 system, the mother being HPA-5aa and the father HPA-5ab, no maternal anti-HPA-5b alloantibody was detected. Maternal serological investigation with the monoclonal antibody-specific immobilization of platelets antigen (MAIPA) technique[17] showed only a weak specific reaction to the GPIIb-IIIa complex of the father's platelets. However, the paternal genotyping revealed a new pattern for HPA-3 ("F" on Fig. 1) by PCR-RFLP (primer pair n°1 and *Fok*I enzyme), showing an additional fragment of 171 bp which did not correspond to any HPA-3 allele. The mother ("M" on Fig. 1) was genotyped as HPA-3ab.

Sequencing of the paternal GPIIb exon 26 revealed a 2614C>A mutation in a heterozygous state (Fig. 2) situated 7 bases upstream from the HPA-3 site causing the substitution of a methionin by a leucine (M841L) on the mature protein.

The GPIIb 2614C>A mutation creates a specific restriction site revealed by the enzyme SfaN I (5'GCATC (N_5)/3' and 3'.CGTAG N_9/ . . . 5') inducing a new digestion profile: 226 + 33 bp (PCR with primer pair n°1) confirming the mutation and 259 bp for those subjects not carrying the mutation. Routinely, we used Fok1 for HPA-3 typing, which gives the additional possibility of detecting the mutation from fragment 171, but if any difficulty arises, this mutation can be confirmed by the enzyme *Sfa*NI.

Unfortunately, we could not pursue this study as we had no further contact with this family and no samples were available for the fetus.

The A_k (GPIIb 2614C>A) mutation and false typing assignation

The PCR-RFLP profile obtained with Fok1 (Fig. 1) reveals the presence of A_k mutation "2614C>A" in African

individuals (P1, P2, P3, P4). Subject P3, with the homozygous mutation, presents a genotyping discrepancy: he was genotyped HPA-3aa using PCR-SSP, but atypical HPA-3a using PCR-RFLP, identifiable only from the presence of the 35 bp fragment (191 bp absent). Furthermore, another error may occur if the time allowed for migration on polyacrylamide gel is not sufficient, giving rise to a confusion between band 191 (allele HPA-3a) and band 171 of the mutation.

Study of this A_k (GPIIb 2614C>A) mutation in SSA populations

This mutation is found with a gene-frequency of 0.0825 in the Aka Pygmy population, including two homozygous individuals. The decreasing frequency from the center of SSA towards the West should be noted, suggesting that this mutation could have originated in the center of SSA (Table 2). Finally, it should be noted that we observed this mutation in our African panel, in subjects originating in Chad, the Central African Republic, and the Republic of Congo (Brazzaville). In spite of the small number of samples per population, we were able to observe that this mutation is indeed present in SSA.

Genotyping was carried out on the members of the family of Pygmy 578, whose samples are still available, using PCR-RFLP with the enzyme *Sfa*NI. This mutation (Fig. 3) was transmitted by his father (Py557), and is found in other members of the family: cousins, aunts, all of them descendants of two brothers, "M." and "Py558"; for other members, no DNA was available. These families substantiated our demonstration that the A_k mutation segregates in a Mendelian inheritance.

two subjects. Moreover this mutation creates a specific new *Hpa*II restriction site useful for its detection. The mutation GPIIb 2645C>T leads to the presence of a leucine instead of a proline (P851L) in the mature protein.

These Cameroonian subjects (D35, D142), with no family link, belong to the Bamileke ethnic group.[4] This mutation, showing up with a frequency of 2.5 percent in the 80 Bamileke subjects tested, has not been observed in any other Cameroonian ethnic groups, nor in any other SSA population.

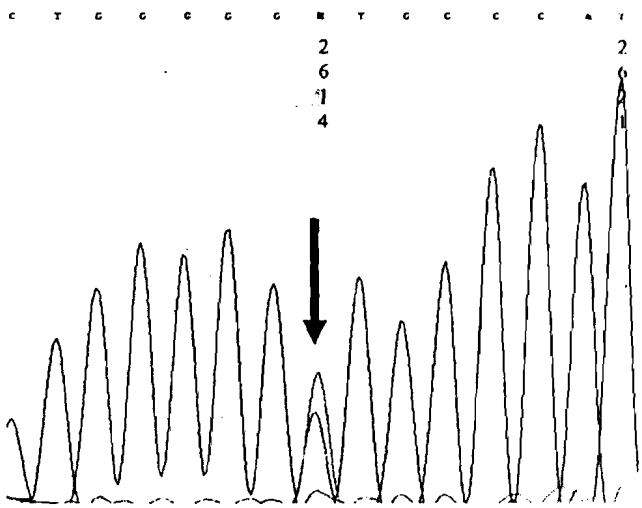


Fig. 2. A_k mutation sequencing analysis. Father (A_k) with a mutation in the A/C position 2614; the mutant alloform ATG encoding an M in comparison with the wild-type CTG encoding an L.

2) GPIIb 2645C>T mutation

Population studies

Among the 603 SSA subjects, two, D35 and D142, presented an unexpected HPA-9 PCR-RFLP pattern with the enzyme *Bst*NI. As shown in Fig. 4a the pattern for the controls obtained for HPA-9a is of two restriction fragments of 230 and 29 bp (C1 and C2) and for HPA-9b is of one restriction fragment of 259 bp (C3, C4), while unexpected fragments of 185 bp and 45 bp were found for these two individuals.

In these two subjects, sequencing of exon 26 of GPIIb revealed a mutation 2645C>T in a heterozygous state (Fig. 4b). This mutation generates an additional CC/TGG *Bst*NI restriction site, altering the digestion profile of the

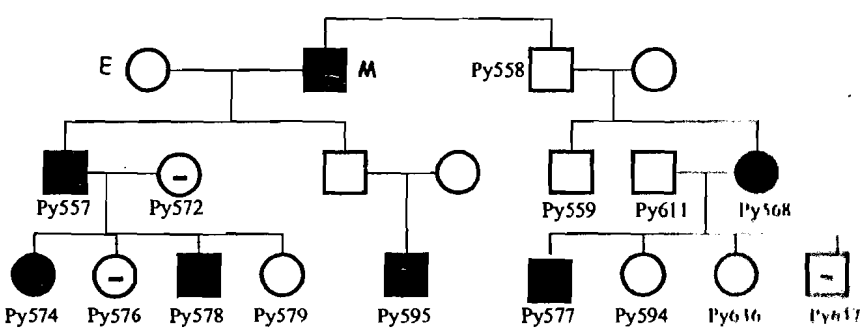
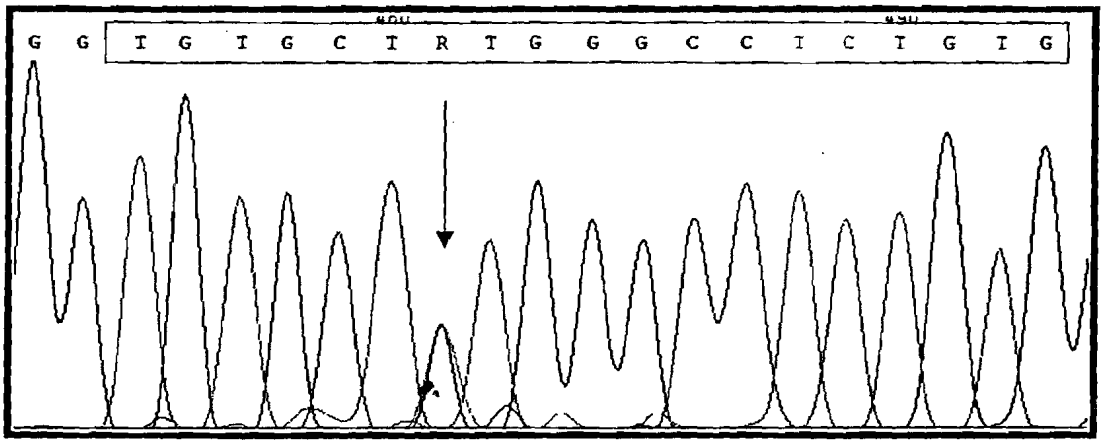


Fig. 3. Transmission of the A_k mutation in family of Pygmy 578. In black: subjects carrying the mutation; hyphen: absence of mutation; blank: not tested.

TABLE 2. Mutation GPIIb 2614C>A: gene frequencies in SSA populations

	Aka Pygmies $N = 103$	Congolese $N = 135$	Cameroonians $N = 118$	[illegible] N [illegible]
GPIIb 2614C>T	0.0825	0.0444	0.0438	[illegible]



GAAGAAAGACCTGGGAAGGCGGCCCCAGACCAACCACCGGGGCACCTCTGTGGGCTGGGGTTCGGGGGAGACCTGGGC

TGACCACTCCTTTGCCCCCCCCAGGTGGACTGGGGGCTGCCCATCCCCAGCCCCTCCCCCATTCACCCGGCCCATCACAAGCG

GGATCGCAGACAGATCTTCCTGCCAGAGCCCGAGCAGCCCTCGAGGCTTCAGGATCCAGTTCTCGTAGTGAGCAGGCTCTCT

GGTCTCGGGCCCGGCCTCCCCGGGACCCACGGGGCAGAGGGGATGGGAGGAGGGAGAGGGGTCCGGGTGTGCTGTGGGCCT

CTGTGGGCCACGCTTGGTCCCTGGGAGCACTTCAAGTGAACATGGAGGAGCATG

Fig. 5. IVS26+89G>A mutation and PCR-RFLP primers. Bold and single underlining = routine PCR-RFLP primer pair n°1; bold and double underlining-new primer pair n°2.

TABLE 3. Gene frequencies (int 21 = without Δ9 bp)

	Benin $N = 155$	Cameroon $N = 118$	Congo $N = 108$	Aka $N = 106$	SSA Panel $N = 94$
HPA-9a	1.000	1.000	1.000	1.000	1.000
HPA-3a	0.6742	0.6144	0.5648	0.5142	0.6489
HPA-3b	0.3258	0.3856	0.4352	0.4858	0.3511
Int 21	0.6065	0.5466	0.5185	0.5519	0.6117
Int 21 with Δ9 bp	0.3935	0.4534	0.4815	0.4481	0.3883

N = number of subjects.

TABLE 4. Haplotype frequencies

	Predicted haplotypes				Observed
Haplotypes	Benin $N = 152$	Cameroon $N = 120$	Congo $N = 75$	Aka $N = 110$	Aka $N = 92$
HPA-9a/HPA-3a/int21	59.21	54.17	46.00	45.75	44.57
HPA-9a/HPA-3b/int21Δ9 bp	31.25	35.83	40.67	41.04	40.22
HPA-9a/HPA-3a/int21Δ9 bp	7.89	8.33	8.00	4.72	5.43
HPA-9a/HPA-3b/int21	1.64	1.67	5.33	8.49	9.78

N = number of subjects.
Frequencies (%) of predicted haplotypes (columns 2-5) using Arlequin software.[23] Column 6: haplotype frequencies (%) were estimated from parental haplotypes on 92 of the Aka subjects.

In Beninese and in Bantus (Cameroon, Congo), the frequency of subjects carrying the Δ9 bp in intron 21 is higher than the frequency of subjects typed HPA-3b; in Aka Pygmies however, the opposite is the case.

Table 4 shows the frequencies of predictive haplotypes "HPA-9, HPA-3, intron 21" in these SSA populations. As we had samples from families of 92 subjects out of the total number of 111 Aka Pygmies involved in this study, we decided to check the results using our statistical prediction tools by direct identification of the haplotypes transmitted through these families. No significant difference emerged (Table 4) between the frequencies as predicted and the frequencies actually transmitted in the Aka population. However, Table 4 shows up a significant difference in frequencies concerning haplotype HPA-9a/HPA-3b/int21 between the Aka (8.49%) as compared to Cameroonians ($p = 0.00012$ $pc = 0.0001$) and Beninese ($p = 2.5^{E}\text{-}05$, $pc = 0.0001$).

In addition to this Δ9 bp in intron 21, we used sequencing techniques to study the two other mutations in linkage disequilibrium described by Ruan[6]: a single point mutation C>G 7 bp upstream from exon 22 start codon and a C>T on the third nucleotide of exon 30. We did not observe any linkage disequilibrium 1) between the two mutations themselves, 2) with the Δ9 bp in intron 21 and HPA-3b.

DISCUSSION

The great genetic diversity of Africans is now a well-established fact, and this study adds to our previous results on HPA and HLA polymorphism.[3,4] This adds weight to the idea of an African polymorphism greater than in other populations.

Considering the role of the reported mutations in clinical conditions, these mutations have not been reported elsewhere either in database for GPIIb polymorphism involved in hemorrhagic disorders such as Glanzmann Thrombasthenia,[18] or in case of alloimmune thrombocytopenia.

The humoral immune response to a foreign antigen is a complex biological reaction occurring when an antigen is accessible to the antigen-presenting cells. In this context it is therefore important to consider the localization of the mutations we described on the GPIIb. We carried out a simulation on a model proposed by Xiong,[19] a model generated from the crystal structure of αVβ3, a structure very close to αIIb/β3 (GPIIb-IIIa) and sharing 40 percent sequence homology. The two new mutations (GPIIb 2614C>A; GPIIb 2645C>T) show up in the N-terminal calf-2 domain. The GPIIb 2614C>A (A_k) mutation is located between the HPA-9 and HPA-3 sites, thus most probably accessible; the same is true for the Cameroonian mutation GPIIb 2645C>T situated after the site HPA-3. The GPIIb 2614C>A (A_k) mutation present in the father and absent in the mother may be inferred in feto-maternal alloimmunization, the maternal serum giving a specific positive reaction with the GPIIb-IIIa paternal complex. Due to shortage of maternal sera and absence of fetal DNA for the index case A_k mutation, it was impossible to confirm the alloimmunization and thus, the potential role of this mutation in clinical conditions. To date no other case of suspected alloimmunization in SSA patients has been referred to our laboratory.

Therefore without carrying out prospective or retrospective studies in those populations, it is difficult for the time being to measure the impact of these polymorphisms in clinical conditions.

Another aspect of our study, and by no means the least, is the impact of these polymorphisms on platelet genotyping as commonly used in platelet laboratories. Whether we use PCR-SSP or PCR-RFLP techniques, discrepancies may occur caused by an unknown mutation as demonstrated here. For this reason, it is of utmost importance in these populations with a great genetic diversity to avoid false typing assignation by using complementary molecular methods or phenotyping when fresh platelets are available, as we have shown in Caucasians.[20]

Finally the generation of GPIIb allelic variants from an ancestral gene bearing HPA-3b, HPA-9a alleles should be taken into consideration.

The fact that in the SSA populations we find Δ9 bp in intron 21 associated either with HPA-3b or with HPA-3a implies that this mutation was prior to the appearance of the HPA-3a/b mutation. Nevertheless, it is interesting to examine the results obtained in the Aka Pygmy population. This population is of particular interest because it constitutes an isolate of nomadic hunter-gatherers in a primary environment. Their territory is defined by the natural borders of the Oubangui river and its two tributaries the Lobaye and the Ibanga. The study of HLA polymorphism in this particular population shows that, although included in the Bantu cluster from a linguistic point of view, the Aka Pygmies are genetically isolated from the other African populations. A considerable body of studies carried out on this population has enabled us to identify[21] a great number of differences in the erythrocyte groups, proteins, Y chromosome among others. Finding the haplotype "HPA-9a/HPA-3b/int21 without Δ9 bp" with a significantly increased frequency may suggest a retained ancestral haplotype. This would confirm the ancestral haplotype HPA-9a/HPA-3b put forward by Newman.[22] Finally, the fact of finding this haplotype with a lower frequency in the neighboring Congolese population could have originated in Aka migrations. According to the Aka Pygmies, there have been two waves of migration: the original older migrant group traveled eastward from the Democratic Republic of Congo to the Republic of Congo and the second, approximately 150 years ago, left the Republic of Congo to reach the village of Bagandu situated in the south-west of the Central African Republic. We note that records do indeed corroborate that the Aka Pygmies were originally established in the Democratic Republic of Congo.

CONCLUSIONS

We found two new mutations in exon 26, with for one of them a potential role in alloimmunization, and one in intron 26 of GPIIb (2614C>A; 2645C>T; IVS26+89G>A). Through this single exon 26 on GPIIb we confirmed that African populations present a greater polymorphism than expected, already described in other DNA regions, for example, in HLA genes. Finally, the haplotype HPA-3b/Δ9 bp within intron 21/IVS21(-7)C→G and exon 30 3063C→T is not to be found in African populations, suggesting the appearance of Δ9 bp within intron 21 prior to the appearance of HPA-3a mutation.

Taking these results into account it would be of great interest to study the clinical implication of these new polymorphisms through prospective studies. Moreover the mutations we described must be taken into account when platelet immunology laboratory investigations are performed on SSA patients.

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From 1975 to 1980, Dr G. Jaeger with the CEABH (EurAfrican Center for Human Biology) and in cooperation with the Central African government, undertook a comprehensive study of the Aka Pygmies and their environment. He welcomed this new study with enthusiasm: we would wish this article to pay tribute to a flawless partnership. We are indebted to F. Bianchi for technical assistance and G. Bertrand for helpful discussion. We thank A. Vernon-James for reading this manuscript.

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