PLASMODIUM FALCIPARUM DIHYDROFOLATE REDUCTASE ALLELES AND PYRIMETHAMINE USE IN PREGNANT GHANAIAN WOMEN

FRANK P. MOCKENHAUPT, TEUNIS A. EGGELTE, TAMARA BÖHME, WILLIAM N. A. THOMPSON, AND
ULRICH BIENZLE

Institut für Tropenmedizin, Medizinische Fakultät Charité, Humboldt Universität zu Berlin, Germany; Department of Clinical Pharmacology, Academic Medical Centre, Amsterdam, The Netherlands; Presbyterian Mission Hospital, Agogo, Ghana

Abstract. Drug resistance in *Plasmodium falciparum* affects prevention of malaria in pregnancy. In a cross-sectional study of 530 pregnant Ghanaian women, *P. falciparum* dihydrofolate reductase (DHFR) gene mutations linked with pyrimethamine resistance were assessed and associations with pyrimethamine intake were analyzed. *P. falciparum* infected 69% of women without pyrimethamine use, 59% of those who had a history of pyrimethamine consumption but a negative urine test, and 53% of individuals with a positive urine test. Eighty-one percent, 43%, and 74% of the isolates contained the mutations Asn-108, Ile-51, and Arg-59, respectively. Thr-108 occurred in 8%. Pyrimethamine use was associated with increased frequencies of Asn-108 and Arg-59 but not of Ile-51 or Thr-108. In women with prophylaxis, wild-type parasites were absent and anemia tended to be more common with an increasing number of DHFR gene mutations. Pyrimethamine appears to be not adequately effective in this part of Ghana, most likely due to the predominance of resistant parasites. Selection for resistance following insufficient prophylaxis could possibly affect the efficacy of future intermittent sulfadoxine-pyrimethamine treatment.

INTRODUCTION

In pregnancy, particularly in primigravidae, susceptibility to *Plasmodium falciparum* infection and to clinical malaria is increased giving rise to maternal anemia, low birth weight, and preterm delivery.[1–3] Chemoprophylaxis during pregnancy is, therefore, recommended in endemic regions.[4] However, the choice of drugs is restricted by adverse effects on mother and fetus, drug resistance, and by the prohibitive costs and insufficient experience of more recent substances in pregnant women. Chloroquine prophylaxis may still be useful in areas of moderate parasite resistance.[5] Although a lack of efficacy has been demonstrated in West Africa,[6] pyrimethamine is still applied, partially because of the low incidence of side effects such as chloroquine-induced pruritus[7] and the presumed better drug compliance. Increasingly, intermittent treatment with sulfadoxine-pyrimethamine in pregnancy is supported in areas of high malaria transmission.[8]

Resistance of *P. falciparum* to pyrimethamine is widespread but shows distinct differences in geographical pattern and degree.[9] This resistance is associated with a point mutation at codon 108 of the parasite's dihydrofolate reductase (DHFR) gene leading to a substitution of serine by asparagine (Ser-108→Asn-108). Further mutations, i.e., Asn-51→Ile-51 and Cys-59→Arg-59, increase the degree of resistance. An Ile-164→Leu-164 change in combination with Asn-108 and one or both of the Ile-51 or Arg-59 substitutions have been found in high-grade resistance to pyrimethamine. Resistance to cycloguanil but only a slight decrease in susceptibility to pyrimethamine is linked with an Ser-108→Thr-108 mutation in combination with Ala-16→Val-16.[10–13]

In a recent cross-sectional study on malaria in pregnancy in rural Ghana, intake of pyrimethamine as assessed by its detection in urine was associated with an only slightly lower rate of *P. falciparum* infection.[14] Here, we assessed *P. falciparum* DHFR gene alleles of isolates obtained from the study participants and analyzed the association between DHFR gene mutations and history and proof of pyrimeth-amine use. Moreover, we examined whether the clinical manifestation of resistant and sensitive parasites differed in women with and without pyrimethamine intake.

PARTICIPANTS, MATERIALS, AND METHODS

Participants. Five hundred and thirty pregnant women presenting for routine antenatal care at the Presbyterian Mission District Hospital in Agogo, Ghana, were subsequently enrolled into a cross-sectional study of malaria in November and December 1998.[14] Malaria transmission in that area is holoendemic.[15] The study protocol was reviewed and approved by the Ministry of Health, Accra, Ghana, and the Ethics Committee, Charité, was notified about this approval. Informed consent was obtained from all participants. The women originated from Agogo (n = 335) and from surrounding rural villages (n = 195). Sociodemographic data and information about the present and previous pregnancies were documented. Iron and folate supplementation and pyrimethamine prophylaxis (25 mg weekly) are prescribed to all women attending antenatal care at Agogo Hospital and purchase of the drugs is registered in their antenatal care files. Information on pyrimethamine prophylaxis was obtained from the women and from these files. Trained midwife nurses estimated the trimester of pregnancy by abdominal/pelvic examination. Venous blood was drawn into EDTA, and urine samples were collected.

Laboratory examinations. Hemoglobin (Hb) concentrations were measured using a HemoCue® photometer (Ångelholm, Sweden) and anemia was defined as an Hb level < 11 g/dl.[16] White blood cell (WBC) counts were performed on a Cell Counter (HC555, Clinicon, Germany). Malaria parasite densities were counted per 500 WBC on Giemsa-stained thick films and, based on the individual WBC number, calculated as parasites per microliter. Submicroscopic plasmodial infections and parasite species were assessed by nested polymerase chain reaction (PCR) assays after extraction of DNA from blood (QIAmp Blood Kit, Qiagen, Hilden, Germany).[17]

The presence of chloroquine and pyrimethamine in urine

TABLE 1
Baseline data grouped according to history of pyrimethamine (PYR) intake and its detection in urine

	Study subgroups		
	I. No history of PYR intake; no PYR in urine	II. History of PYR intake; no PYR in urine	III. PYR in urine
No.	279	152	92
Educational index (mean, range)	1.03 (0–3)	1.12 (0–3)	1.27 (0–3)*
Gravidity (mean, range)	3.1 (1–12)	3.6 (1–11)	3.4 (1–11)
Gestational week (mean, range)‡	23.2 (12–44)	28.3 (12–44)*	28.2 (12–44)*
No. of current visits to ACC (mean, range)	1.2 (1–5)	2.4 (1–9)*	2.2 (1–7)*
% of individuals with CLQ in urine	55 (154/279)	62 (94/152)	66 (61/92)
% *Plasmodium falciparum*-infected	69 (192/279)	59 (89/152)†	53 (49/92)†
Geometric mean parasite density (95% CI)¶	288 (205–406)	303 (170–540)	442 (193–1,010)
% submicroscopic of *P. falciparum* infections	44 (84/192)	54 (48/89)	59 (29/49)†
% anemic (Hb < 11 g/dl)	59 (164/279)	48 (73/152)†	45 (41/92)†

Educational index encoding: no education = 0, primary school = 1; secondary school = 2; and tertiary school = 3.
ACC = antenatal care clinic; CLQ = Chloroquin; 95% CI = 95% confidence interval.
* Difference in comparison to group I: P (Mann-Whitney U) $\leq$ 0.05.
† P (χ^2) $\leq$ 0.05.
‡ n = 517.
¶ n = 169.

was demonstrated by an ELISA dipstick coated with monoclonal antibodies against these drugs. The detection limits are in the range of 120 nmol/L for chloroquine and 250 nmol/L for pyrimethamine. The drugs can be detected for at least 3 months (chloroquine) and 3 weeks (pyrimethamine) after intake.[18,19]

In samples positive for *P. falciparum,* the parasites′ DHFR gene alleles Ser-108, Asn-108, Thr-108, Asn-51, Ile-51, Cys-59, Arg-59, Ala-16, Val-16, Ile-164, and Leu-164 were assessed by mutation specific restriction digestion of nested PCR products of DNA sequences containing the respective codons.[20] To increase the sensitivity of the outer PCR assays particularly in submicroscopic *P. falciparum*-infections, the protocol was slightly modified. Briefly, the PCR buffer consisted of 67 mM Tris-base, pH 8.8; 16.6 mM ammonium sulfate; 10 mM β-mercaptoethanol; 2.5 mM $MgCl_2$; 0.01% bovine serum albumin, and 5% DMSO. Outer PCR assays were run with an initial denaturation at 99°C for 7 min after which 1.5 U of *Taq* polymerase (Amersham Pharmacia, Freiburg, Germany) were added at 85°C before the continuation of the original amplification scheme. Amplified and digested fragments were separated on 3.5% GTG® agarose gels (FMC BioProducts, Rockland; ME) or on 2% Separide® matrix gels (Gibco BRL, Karlsruhe, Germany) and visualized by ethidium-bromide staining and UV translumination.

Frequencies were compared by χ^2 tests and χ^2 test for trend (χ^2_{trend}) as applicable. For continuous variables, the Mann-Whitney U test, Student's *t*-test, and one-way analysis of variance were used. Because in many cases the age provided by the participants was unreliable, age was not regarded for statistical analysis.

RESULTS

Pyrimethamine prophylaxis. Information on antimalarial chemoprophylaxis was available for 521 women. Of these, 53% (n = 277) reported that they had not taken any drugs to prevent malaria; 5% (n = 24) and 42% (n = 220) reported being on chloroquine and pyrimethamine prophylaxis, respectively. Pyrimethamine was detected in the urine of 17% (92 of 530) of all women, in 7% (22 of 301) of those re-

portedly not using the drug, and in 31% (68 of 220) of those claiming to take pyrimethamine regularly. Among women with zero, one, and more than one previous visit to the antenatal care clinic, the history of pyrimethamine use was positive in 19% (62 of 327), 78% (76 of 98), and 85% (82 of 96), respectively (χ^2_{trend} = 176.3, P < 0.0001), whereas pyrimethamine in urine was detected in 11% (38 of 334), 23% (23 of 99), and 32% (31 of 97; χ^2_{trend} = 25.0, P < 0.0001). For subsequent analysis, the women were classified into three groups (including two individuals lacking information on drug history but with positive urine tests): Group I, women without a history of pyrimethamine intake and no pyrimethamine in urine (n = 279); Group II, positive history of pyrimethamine intake but no pyrimethamine in urine (n = 152); and Group III, women with pyrimethamine in urine (n = 92). Women in the latter two groups had been attending the antenatal care clinic more frequently and were further advanced in gestation than women without malaria prevention (Table 1). The prevalence of *P. falciparum*-infection and of anemia, and the proportion of microscopically-detectable parasitemia but not the geometric mean parasite densities were lower in women with history or proof of pyrimethamine intake than in women lacking both (Table 1). Severe anemia (Hb < 7 g/dl) occurred in 5 women (1%). They all belonged to the group of individuals without history or proof of pyrimethamine intake (Fisher's exact test, P = 0.06, in comparison to women with a history of or proven pyrimethamine consumption).

DHFR gene mutations. Malaria parasites were found by microscopy in 32% (172 of 530) of the women; parasite densities ranged between 11 and 31,988 parasites/μl (geometric mean parasite density [GMPD], 304/μl; 95% confidence interval [231–401/μl]). By PCR, *P. falciparum* infection was detected in 63% (335 of 530) of the women. In women of gravidity one, two, three and four, and ≥ five, the prevalences of *P. falciparum* infection were 73% (95 of 130), 70% (79 of 113), 65% (94 of 145), and 47% (67 of 142) (χ^2_{trend} = 20.0, P < 0.0001), respectively. The GMPD declined from 745 [500–1,108] parasites/μl in primigravidae to 69 [40–117] parasites/μl in gravidae ≥ five (F = 13.9, P < 0.0001).

Typing of the *P. falciparum* DHFR gene alleles was suc-

TABLE 2
Dihydrofolate reductase (DHFR) gene alleles, pyrimethamine (PYR) use and manifestation of infection in pregnant women

DHFR allele	No.	% prevalence in subgroups of PYR use			Geometric mean parasite density [95% CI]* in subgroups of verified PYR use		% anemic in subgroups of verified PYR use	
		History − Urine test −	History + Urine test −	Urine test +	No PYR detected	PYR detected	No PYR detected	PYR detected
Wild type (Ser-108)	47	20.5	9.5	0	178 [97–327]	–	57	–
Ile-51 only	1	0.5	0	0	0†	–	100	–
Arg-59 only	11	5.3	1.2	0	235 [67–820]	–	64	–
Ile-59 + Arg-59	4	1.1	2.4	0	793 [9–70,858]	–	50	–
Asn-108 only	20	6.3	3.6	10.4	299 [59–1,518]	238 [4–15,630]	67	40
Asn-108 + Ile-51	17	4.2	9.5	2.1	166 [55–498]	0†	75	0
Asn-108 + Arg-59	108	25.8	39.3	54.2	319 [180–566]	370 [127–1,076]	66	46
Asn-108 + Ile-51 + Arg-59	114	36.3	34.5	33.3	366 [229–583]	644 [154–2,687]	62	69

* Parasitemia only microscopically detectable.
† Submicroscopic infection.
History − = negative history of pyrimethamine intake; history + = positive history of pyrimethamine intake; 95% CI = 95% confidence interval.

cessful in 97% (326 of 335). Overall, 71.5% (233 of 326) of the isolates exhibited the Asn-108 core mutation, 9.2% (30 of 326) contained mixed alleles (Asn-108/Ser-108), and wild-type parasites (Ser-108) were present in 19.3% (63 of 326). Further DHFR variants (including mixed alleles) were observed in 43% (139 of 326, Ile-51), 74% (240 of 326, Arg-59), and 8% (27 of 326, Thr-108). No Val-16 or Ile-164 mutations were found. Combinations of the various DHFR mutants were more frequent than single mutations; triple mutants (Asn-108/Ile-51/Arg-59) predominated, and occurred in 36% (116 of 326) of the isolates.

The distribution of the parasite genotypes was influenced by pyrimethamine consumption (Table 2) ($\chi^2 = 37.0$, $P = 0.0007$). The resistant Asn-108 variant was observed in 73% (138 of 190) of infected women without a history of pyrimethamine intake, in 87% (73 of 84) of those reporting routine use of the drug but urine-negative, and in all (48 of 48) of the infected women with demonstrable pyrimethamine in their urine ($\chi^2_{trend} = 21.2$, $P < 0.0001$). A similar trend was
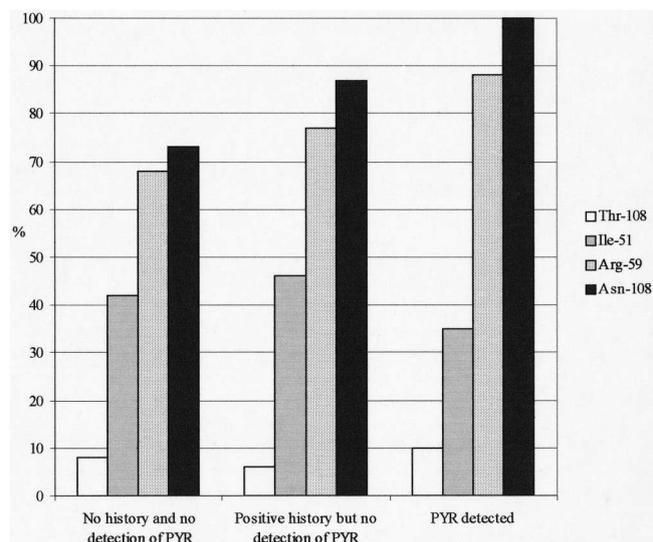

FIGURE 1. Prevalence of *Plasmodium falciparum* dihydrofolate reductase (DHFR) gene alleles grouped by pyrimethamine (PYR) use in pregnant women. The *P. falciparum* DHFR variants Asn-108 ($\chi^2_{trend} = 21.2$, $P < 0.0001$) and Arg-59 ($\chi^2_{trend} = 8.0$, $P = 0.005$) increase in frequency with reported and even more with verified use of PYR.

observed for parasites containing the Arg-59 mutation but not for Ile-51 or Thr-108 (Figure 1). Resembling the association of pyrimethamine intake with the number of antenatal care visits, the Asn-108 variant occurred more frequently with compliant antenatal care, i.e., in 73% (163 of 222), 97% (56 of 58, $\chi^2 = 14.4$, $P = 0.0001$), and 96% (44 of 46, $\chi^2 = 10.7$, $P = 0.001$) of women with zero, one, and more than one previous visit, respectively. This was also seen in women without pyrimethamine in urine (no previous visit, 71% [145 of 204]; one previous visit, 95% [40 of 42], $\chi^2 = 10.9$, $P = 0.001$; and ≥ 1 previous visit, 94% [30 of 32], $\chi^2 = 7.4$, $P = 0.007$).

No association between the presence of the Asn-108 variant with residence in Agogo (79%, 156 of 197) or in the rural villages (83%, 107 of 129) was found. A trend for less-resistant parasites (Asn-108, Asn-108/Ser-108) with increasing gravidity (primigravidae, 84%; secundigravidae, 82%; gravidae 3 and 4, 80%; and gravidae ≥ 5, 75%) did not reach statistical significance ($\chi^2_{trend} = 1.8$, $P = 0.18$).

All isolates from anemic women on verified pyrimethamine prophylaxis contained the Asn-108 mutation. However, an association of individual DHFR alleles with parasite density or anemia was not observed in either women with detected pyrimethamine use or in those without (Table 2). The isolates were grouped according to the presence of the Asn-108 core mutation and the postulated degree of drug resistance:[10–13] i.e., low, Asn-108 only; intermediate, Asn-108 + Ile-51 and Asn-108 + Arg-59; and high resistance, Asn-108 + Ile-51 + Arg-59. In women without pyrimethamine in urine, the prevalence of anemia was not influenced by antifolate resistance (low, 67% [10 of 15]; intermediate, 67% [67 of 100]; high, 63% [63 of 100]). Among women with verified pyrimethamine consumption, a non-significant trend for more anemia with increasing grade of resistance was seen (low, 40% [2 of 5]; intermediate, 44% [12 of 27]; high, 69% [11 of 16]; $\chi^2_{trend} = 2.3$, $P = 0.13$).

DISCUSSION

Due to the spread of chloroquine-resistance,[9,21] sulfadoxine-pyrimethamine can be anticipated to become the first-line treatment for uncomplicated malaria in many parts of Africa. Moreover, intermittent treatment during pregnancy

with this compound constitutes a promising alternative to continuous chemoprophylaxis.[8]

In the present study in rural Ghana, the prevalences of *P. falciparum* infection and of anemia were 16% and 14% lower, respectively, in women with confirmed pyrimethamine use than in those without pyrimethamine in urine and without a history of drug intake. Most *P. falciparum* strains exhibited mutations linked with pyrimethamine resistance and these were selected by insufficient drug use. Although the study was not designed to assess the prophylactic efficacy of pyrimethamine, this suggests that the drug is not appropriate for the prevention of malaria in pregnancy in this part of Ghana.

Several circumstances are likely to account for the small differences in parasitologic and hematologic indices in women with and without pyrimethamine use. Firstly, blood stage parasites were not eradicated before the commencement of weekly pyrimethamine prophylaxis. Prophylaxis aimed against primary hepatic tissue forms may be more effective than suppression of blood stage parasites, although this could not be confirmed in Nigeria.[6] Secondly, participants' adherence to prophylaxis was poor. The validity of information on drug consumption is reputedly low,[22] but, in our study, it was increased by cross-checking with the individual antenatal care files. The qualitative pyrimethamine dipstick assay can demonstrate drug intake within a period of approximately three weeks but cannot verify whether the drug was consumed one day or three weeks ago.[18] Thus, an unknown proportion of positive urine tests may reflect subeffective drug concentrations. Nevertheless, as can be concluded from the low rate of positive urine tests in women with several antenatal care visits, regular weekly prophylaxis was rarely performed. Thirdly, folate supplementation may have influenced the efficacy of pyrimethamine as has been demonstrated in sulfadoxine-pyrimethamine treatment.[23] Finally, and perhaps most importantly, pyrimethamine resistance of *P. falciparum* is frequent and intense in the study area.

In 1991, treatment with sulfadoxine-pyrimethamine suffered a failure rate (RII/RIII) of 37% in the coastal region of Ghana.[24] In the present study, the prevalence of three mutations in the *P. falciparum* DHFR gene (Asn-108 + Ile-51 + Arg-59) was 36%, and 81% of the isolates exhibited the gene core mutation Asn-108. The association between the DHFR gene alleles with the response to antifolates is less straightforward *in vivo* than *in vitro* possibly due to interfering factors such as pharmacokinetics and semi-immunity.[10–13,25–30] Nevertheless, in two recent studies, the triple DHFR gene mutation has been found to be reasonably predictive of the clinical failure of sulfadoxine-pyrimethamine treatment.[25,30]

Both the Asn-108 and the Arg-59 variants were selected in women with either verified or reported pyrimethamine use. Selection of DHFR mutants by sulfadoxine-pyrimethamine treatment and in areas of intense pyrimethamine usage has been demonstrated previously.[26,29–32] The half-life of pyrimethamine is in the range of four days,[33] and its consumption can be demonstrated for approximately three weeks with the dipstick test.[18] Because *P. falciparum* can persist for months,[34] the selection of resistant parasites in study participants may have taken place weeks before the cross-section. This view is supported by the increasing frequency of resis-

tant parasites seen with increasing compliance to antenatal care, irrespective of the actual presence of pyrimethamine in urine. However, the Asn-108 mutant was also seen in almost three of four women without a history of pyrimethamine use. Furthermore, in accordance with a study in Mali,[29] the Ile-51 variant was not selected by pyrimethamine consumption nor was the Thr-108 mutant. In Nigeria, a high prevalence of DHFR variants has been found in an area of low pyrimethamine usage indicating that selection by the drug is unlikely to be the exclusive factor responsible for the emergence of resistant mutations.[35]

In a given transmission area, parasites with specific receptors to placental tissue have an increased chance to establish themselves in the otherwise semi-immune pregnant host.[36–38] With successive pregnancies, immune mechanisms against these particular strains develop; infections become rarer and may increasingly comprise parasite genotypes also present in nonpregnant individuals.[38,39] If drug consumption propagates resistance in parasites able to adhere to placental ligands, it seems conceivable that this resistance is further selected within the group of pregnant women. Corresponding to improving immune mechanisms against placenta-binding parasites, a decline in the proportion of resistant strains with successive pregnancies would then be expected. From our data, this hypothesis cannot be confirmed. However, it is noteworthy that resistant parasites tended to become fewer although antimalarial drugs were more commonly taken with increasing gravidity.[14]

Parasite densities and the prevalence of anemia did not differ with the individual DHFR alleles. The mere absence of wild-type parasites in women with confirmed pyrimethamine use excluded the possibility of analyzing the impact of the core mutation on the clinical manifestation of infection. In women with confirmed pyrimethamine consumption, the trend toward more anemia as the number of DHFR gene mutations increased did not reach significance, possibly due to the small number of samples.

In areas of stable and intense transmission, primigravidae in particular benefit from antimalarial chemoprophylaxis. Yet, the choice of drug and regimen needs to follow tolerability, accessibility, compliance, and the local degree of resistance.[4,40] In this rural setting in Ghana, resistance to pyrimethamine as assessed by DHFR gene mutations was frequent and intense, and it appears that pyrimethamine prophylaxis is not adequately efficient in the suppression of parasitemia or prevention of anemia. Increasing prophylaxis compliance might be useful, but it remains doubtful that it alone can improve the prevention of malaria. In addition, compliant prophylaxis with pyrimethamine as a monosubstance might select for more resistant parasites. Considering the relatively low rate of parasites exhibiting the Thr-108 mutation, the combination of chloroquine and proguanil may be an alternative. However, recent data indicate that resistance to proguanil via its metabolite cycloguanil is not solely associated with Thr-108 but also with two or three DHFR gene mutations including the Asn-108 variant.[41] Finally, due to the selection of pyrimethamine resistant parasites in the study population it appears possible that the efficacy of intermittent sulfadoxine-pyrimethamine treatment is compromised even before its implementation. Yet, there are no clinical data from the study area to support or reject this as-

sumption. The synergistic effect of sulfadoxine and, most important, the possibility of ascertaining drug intake at antenatal care visits argue in favor of sulfadoxine-pyrimethamine rather than maintaining the use of pyrimethamine.

Authors' addresses: Frank P. Mockenhaupt, Tamara Böhme, and Ulrich Bienzle, Institut für Tropenmedizin, Spandauer Damm 130, 14050 Berlin, Germany; Tel.: +49-30-30116-750, Fax: +49-30-30116-888. Teunis A. Eggelte, Department of Clinical Pharmacology, Academisch Medisch Centrum, Universiteit van Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; Tel.: +31-20-566-7049, Fax: +31-20-696-5976. William N. A. Thompson, Presbyterian Mission Hospital Agogo, P. O. Box 27, Agogo, Ashanti-Akim, Ghana; Tel./Fax: +233-51-20201.

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