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HUman MicroNucleus Project: International Database Comparison for Results With the Cytokinesis-Block Micronucleus Assay in Human Lymphocytes: I. Effect of Laboratory Protocol, Scoring Criteria, and Host Factors on the Frequency of Micronuclei

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Micronucleus (MN) expression in peripheral blood lymphocytes is well established as a standard method for monitoring chromosome damage in human populations. The first results of an analysis of pooled data from laboratories using the cytokinesis-block micronucleus (CBMN) assay and participating in the HUMN (HUman MicroNucleus project) international collaborative study are presented. The effects of laboratory protocol, scoring criteria, and host factors on baseline micronucleated binucleate cell (MNC) frequency are evaluated, and a reference range of "normal" values against which future studies may be compared is provided. Primary data from historical records were submitted by 25 laboratories distributed in 16 countries. This resulted in a database of nearly 7000 subjects. Potentially significant differences were present in the methods used by participating laboratories, such as in the type of culture medium, the concentration of cytochalasin-B, the percentage of fetal calf serum, and in the culture method. Differences in criteria for scoring micronuclei were also evident. The overall median MNC frequency in nonexposed (i.e., normal) subjects was 6.5‰ and the interquartile range was between 3 and 12‰. An increase in MNC frequency with age was evident in all but two laboratories. The effect of gender, although not so evident in all databases, was also present, with females having a 19% higher level of MNC frequency (95% confidence interval: 14–24%). Statistical analyses were performed using random-effects models for correlated data. Our best model, which included exposure to genotoxic factors, host factors, methods, and scoring criteria, explained 75% of the total variance, with the largest contribution attributable to laboratory methods. Environ. Mol. Mutagen. 37:31–45, 2001.

Key words: cytokinesis-block micronucleus assay; peripheral blood lymphocytes; pooled reanalysis; biomarkers; DNA damage

INTRODUCTION

The use of micronuclei (MNi) as a measure of chromosome damage in peripheral blood lymphocytes (PBL) was first proposed by Countryman and Heddle [1976] and subsequently improved with the development of the cytokinesis-block micronucleus (CBMN) method [Fenech and Morley, 1985a,b], which allowed MNi to be scored specifically in cells that had completed nuclear division. As a consequence the assay has been extensively used to evaluate the presence and the extent of chromosome damage in human populations exposed to genotoxic agents in various occupational settings, in the environment, or as a consequence of lifestyles. Subgroups of the general population considered at risk because of their genetic make-up or because they are affected by certain diseases have also been evaluated, to validate this biomarker as a predictor of adverse health effects [Ban et al., 1993; Fenech, 1993; Benner et al., 1994; Tucker and Preston, 1996; Duffaud et al., 1997; Hagmar et al., 1998].

The micronucleus (MN) test is gaining increased attention among laboratories active in the field of environmental mutagenesis, and the number of published studies based on this biomarker is increasing rapidly [Surrallés and Natarajan, 1997; Fenech et al., 1999]. The expanded use of the MN assay is mostly explained by two reasons: (1) the PBL MN test provides a reliable measure of chromosome breakage and loss, at lower cost and with less work than chromosomal aberrations; and (2) the recent availability of the cytokinesis-block technique has removed the potential confounding caused by effects on cell division kinetics. As with other in vitro cytogenetic assays, there is inter- and intralaboratory variability, and limited information on the effects of the laboratory method, subject lifestyle, and the role of individual susceptibility on this variability. The long-term health significance of PBL MN is also a major issue to be clarified.

One of the principal issues to be addressed is the effect of different protocols on MN frequency, because many variations of the original protocol are in use. Based on reports in the literature, information on the effect of the protocol on the final outcome is limited, and sometimes contrasting. A study specifically designed to compare different concentrations of cytochalasin B (cyt-B) revealed that 6 μg/ml, though more effective than the standard concentration of 3 μg/ml in arresting cytokinesis, revealed a lower frequency of MNi [Surrallés et al., 1994]. In contrast, Prosser et al. [1988] did not find any differing effects of the two concentrations on MN frequency. More recently, Fenech [1998] reported that a comparative study of two different culture media, RPMI 1640 and McCoy, did not reveal differences in the MN frequencies. Other studies have addressed issues dealing with blood sampling, such as alternatives to venipuncture [Lee et al., 1997] or the effect of storage [Lee et al., 1999]. A number of articles have been published that recommended changes to the standard methods, although there has been no coordinated evaluation of the effects of different methodological aspects on the outcome frequency.

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The methodological aspect that, according to many authors, introduces most nonsampling-dependent variability in MN frequency is the interscorer variability [Radack et al., 1995; Brown et al., 1997].

To address and to try to resolve these issues, and to provide a network of laboratories working with this assay, the international collaborative HUMN (HUman MicroNucleus) study was launched in September 1997. The main goals of HUMN were: (1) to compare the protocols currently in use to assess whether, and to what extent, such variations might affect baseline MN frequencies; (2) to compare baseline MN frequencies from the various laboratories and to determine the relationships of these frequencies to subject factors; and (3) to perform a prospective study linking the accumulated MN frequency data from each laboratory to provide a powerful cohort in person-years, to determine associations with common diseases, such as cancer, aging, and selected genetic syndromes. The first two comparison goals are expected to provide information on the extent of variation of MN frequency ranges for different laboratories and protocol factors, and on the extent to which some key subject variables (e.g., age, gender, lifestyle, exposure to genotoxic agents) affect baseline MN frequencies in peripheral blood lymphocytes. More detail on the HUMN project and on the CBMN assay can be found in Fenech et al. [1999].

The collection of individual data from different studies, to perform pooled reanalyses, has been the object of recent discussions in the literature [Gordon et al., 1998; Taioli, 1999]. The advantages of such an approach are quite evident, especially for the availability of a study group of large size and for the wide extent of available information. On the other hand, the data in these individual studies were collected for different purposes and are varied with respect to quality and completeness of subject information, imposing serious limits for statistical analyses. A recent study has specifically noted that the creation of international databases of background values for various biomarkers has the great advantage of orienting standardization studies and contributing to biomarker validation [Albertini, 1999]. These data could then be used to create biomarker maps, which would be extremely useful for hypothesis generation and area-specific population monitoring.

In the study presented here, a database of nearly 7000 subjects, from 25 laboratories representative of many countries and populations, has been compiled and analyzed. The study has been performed to verify the importance of age, gender, and protocol as variables affecting the observed micronucleated cell (MNC) frequency in the cytokinesis-block procedure and to provide a reference range of *normal* values for MNC frequency against which future studies may be compared.

Fig. 1. Geographical distribution of countries containing laboratories that participated in the HUMN project.

MATERIALS AND METHODS

Subjects and Laboratories

Organization

In early 1997 an invitation letter was sent by the steering committee of the HUMN project to 130 laboratories that had published studies on the MN test in human lymphocytes using the CBMN assay. The letter included a questionnaire for some basic information about the data available in each laboratory. The committee received 42 responses and, based on that information, a so-called "information package" was prepared and sent to the interested laboratories. All of these laboratories, with the exception of one (which did not subsequently participate), had used the CBMN assay in their investigations. A more sophisticated questionnaire requesting detailed information about laboratory protocol, scoring criteria, individual data of subjects in the study, and references of published studies, if any, was sent with this information package. An Excel file, to be used as a template for submitting original data to the coordinating center (INRC, Genoa), was also sent. As of December 1999, 25 databases were received from laboratories in 16 countries, mostly in Europe, but also in Asia, America, Australia, and New Zealand (Fig. 1). The laboratories that contributed their data to the study and references to the original publications of these data are listed in Table I.

The questionnaire distributed to participating laboratories about their protocols requested more information than was used in the subsequent statistical analyses. Additional details about blood collection, culture method, cyt-B use, harvest time, staining, and various aspects of the study design were obtained, although this information was not included in the statistical analyses for various reasons: (1) some items of the protocol were identical in all laboratories (e.g., all collected blood by venipuncture); (2) other questions were answered by only a limited number of participants (e.g., two questions on the coefficient of variation were answered by only six labs out of 25); and (3) other factors were removed from the analysis after a preliminary statistical evaluation, to reduce the number of parameters to be included in regression models (e.g., whether the culture vessel was plastic or glass). All parameters used in the analyses are reported in Table II.

Databases Used

The total HUMN database contains 6583 subjects, with a mean age of 44 years and a proportion of males only slightly higher (53.3%) than females. The individual database sizes range from 11 to 1637 subjects, with a mean size of 263 subjects, and each contained the results of one to seven different studies. The general characteristics of each database are reported in Table III.

Most of the studies included in the HUMN database were designed as

TABLE I. Laboratories That Contributed Data to the HUMN Project

Institution	Principal researcher	Country	Publications describing the data (where applicable)
ARN—Laboratorio Dosimetria Biologica	M. Di Giorgio	Argentina	Di Giorgio et al., 1992, 1996
CSIRO Health Sciences and Nutrition	M. Fenech	Australia	Fenech et al., 1994, 1997a,b, 1998; Fenech and Rinaldi, 1994, 1995
Belarus Academy of Sciences—Institute of Genetics and Cytology	L. Mikhalevich	Belarus	Fenech et al., 1997c
University Gent—Department of Anatomy, Embryology, and Histology	A. Vral	Belgium	Thierens et al., 1996
National Center of Hygiene, Medical Ecology, and Nutrition—Toxicology Lab.	E. Mirkova	Bulgaria	Mirkova et al., 1994, 1998a,b, 1999
Institute for Medical Research and Occupational Health	A. Fucic	Croatia	Fucic et al., 1990, 1992, 1994, 1996
Centro de Protección e Higiene de las Radiaciones	O. Garcia Lima	Cuba	Garcia Lima, 1997
Universitatsklinikum Essen—Institut für Medizinische Strahlenbiologie	W.U. Muller	Germany	Gantenberg et al., 1991; Wuttke et al., 1996; Streffer et al., 1998
Bhabha Atomic Research Centre—Cell Biology Division	A.P. Krishnaja	India	Krishnaja and Sharma, 1991, 1994, 1998
Università di Torino—Dipartimento di Biologia Animale e dell'Uomo	P.M. Bigatti	Italy	Bigatti et al., 1994; Ardito et al., 1997; Lamberti et al., 1998
Università di Pisa—Dipartimento di Scienze dell'Uomo e dell'Ambiente	R. Barale	Italy	Barale et al., 1993, 1998a,b
National Institute for Cancer Research—Toxicological Evaluation Unit	C. Bolognesi	Italy	Bolognesi et al., 1993a,b,c, 1997a
University of Bologna—Department of Pharmacology	P. Hrelia	Italy	Castelli et al., 1999
Università di Pisa—Dipartimento di Scienze dell'Uomo e dell'Ambiente	L. Migliore	Italy	Migliore et al., 1991; Franchi et al., 1994; Scarpato et al., 1996, 1997
Università della Tuscia—Dipartimento di Agrobiologia e Agrochimica	P. Mosesso	Italy	Mosesso et al., 1995; Proietti de Santis et al., 1997
CNR—IRECE	M.R. Scarfì	Italy	Scarfì et al., 1990, 1993, 1994, 1996; Franceschi et al., 1992
ISS—Laboratorio di Tossicologia Comparata ed Ecotossicologia	A. Zijno	Italy	Zijno et al., 1994, 1996a,b,c; Carere et al., 1995
Radiation Effects Research Foundation—Department of Radiobiology	S. Ban	Japan	Ban et al., 1993
Yamanashi Prefectural College of Nursing—Human and Health Sciences	Y. Odagiri	Japan	Odagiri et al., 1997; Odagiri and Uchida, 1998
University of Auckland Medical School—Cancer Research Laboratory	L.R. Ferguson	New Zealand	
Third Military Medical University—Molecular Toxicology Laboratory	J. Cao Jia	PR China	
Research Institute of Roentgenology and Radiology	I. Vorobtsova	Russia	Vorobtsova, 1997
Institute of Preventive and Clinical Medicine	E. Szabova	Slovak Rep.	
National Yang Ming Univ. Med. School—Institute of Environmental Health Sciences	W.P. Chang	Taiwan	Chang et al., 1996, 1997
ECU School of Medicine—Radiation Oncology	T.K. Lee	United States	Lee et al., 1994a,b, 1995a,b, 1997; Wiley and Lee, 1996

TABLE II. Method Variables Evaluated in the Statistical Analysis

Selected protocol variables		
1. Length of time the blood allowed to stand before being prepared for culture	<7 hr (80%)	≥7 hr (20%)
2. Temperature [°C] the blood was kept prior to preparation for culture	Room temperature (82%)	Fridge (4–5°C) (18%)
3. Culture method used: Whole blood or isolated lymphocytes	Whole blood (84%)	Isolated lymphocytes (16%)
4. Ratio of whole blood to culture medium (for whole blood)	<10% (52%)	≥10% (48%)
5. Type of medium used[a]	RPMI 1640 (60%)	F10 (24%)
6. Percentage of added fetal calf serum	≤10% (52%)	>10% (48%)
7. Time after initiation cytochalasin-B was added to cultures	<44 hr (20%)	≥44 hr (80%)
8. Final concentration of cytochalasin-B in the culture medium[b]	3 μg (44%)	6 μg (32%)
9. Time after PHA stimulation the cells were harvested	<72 hr (20%)	72 hr (80%)
10. Hypotonic treatment	No (16%)	Yes (84%)
11. Cultures per individual typically set up	1 (24%)	≥2 (76%)
Scoring criteria for identification of binucleated cells		
1. Must have two round or oval nuclei	Yes (99%)	
2. The two nuclei must be in the same condensation state	Yes (83%)	
3. The two nuclei must have similar size	Yes (99%)	
4. The two nuclei can touch each other or partially overlap	Yes (83%)	
5. The cytoplasmic boundary of the cell has to be clear and intact	Yes (100%)	
6. The two nuclei may be attached by a fine nucleoplasmic bridge	Yes (60%)	
7. The nuclei must not be in an early stage of apoptosis	Yes (70%)	
Criteria for identification and scoring of micronuclei in binucleated cells		
1. Morphologically identical but smaller than nuclei	Yes (82%)	
2. Round or oval in shape	Yes (99%)	
3. Diameter between 1/3rd and 1/16th of main nuclei	Yes (91%)	
4. Nonrefractile	Yes (95%)	
5. Not linked to main nucleus	Yes (73%)	
6. May overlap or touch main nucleus	Yes (49%)	
7. Same color as nucleus	Yes (85%)	
8. Similar staining intensity as nucleus	Yes (83%)	

[a]Four laboratories used other media.
[b]Six laboratories used other concentrations.

occupational or environmental surveys of subjects exposed to genotoxic agents such as ionizing radiation, aromatic hydrocarbons, pesticides, cytostatic drugs, metals, or air pollution. Other studies were designed to study diet, either as supplementation of chemopreventive agents, or to evaluate the effect of some nutrients (or food) on the MNC frequency. For the most part, each database is composed of individuals living in the same country of the laboratory; however, four databases included data from people (primarily children) monitored because of their exposure to ionizing radiation as a result of the Chernobyl accident. The inclusion of data from other collaborative studies on cytogenetic biomarkers performed in Europe, and especially in Italy, has probably led to an overrepresentation of this continent in the study.

Exposure to Genotoxic Agents

Subjects in the HUMN database were originally classified as exposed or not exposed on the basis of the questionnaire filled in by the researchers. On the first evaluation of the data, a few databases had mean frequencies of MNC considerably higher than those of the majority of other databases. These few databases included individuals heavily exposed to occupational or environmental genotoxins. There was an obvious difference in the criteria of classification among laboratories and, therefore, all subjects were reclassified using an exposure matrix prepared by a panel of occupational hygienists, epidemiologists, and cytogeneticists from the European Study Group on Cytogenetic Biomarkers and Health (ESCH) [Bonassi et al., 2000]. Because an important goal of this study was to describe baseline frequencies of MNC, we applied the quantitative criteria provided by the ESCH matrix to the available material (questionnaires and published studies), and identified a group of 665 subjects exposed to known genotoxic agents. These subjects were removed from the analysis describing the baseline occurrence of MNCs, whereas they were included in all multiple regression models, since the inclusion of a specific term for exposure provided estimates adjusted for this factor. In the same way, individuals exposed to atomic bomb radiation in Hiroshima or living in high radiation background areas (n = 1084) [Ban et al., 1993; Fenech et al., 1997c; Garcia Lima, 1997; Vorobtsova, 1997] or carriers of genetic alterations (n = 15) [Krishnaja and Sharma, 1994] were not considered in the evaluation of the baseline occurrence of MNCs because their conditions may have led to a certain degree of genomic instability.

Statistical Methods

Measures of MN frequency

MN frequency measures are generally reported as MNi or MNCs per 1000 binucleate cells (‰). In this study, four laboratories expressed their data as number of MNi per 1000 binucleated cells, nine as the frequency of MNCs per 1000 binucleated cells, and the other 11 laboratories reported both indexes. These figures are in agreement with other surveys [Surrallés and Natarajan, 1997] and show that the frequency of MNC is a preferred index; we therefore decided to use it in all analyses. To use all available information we estimated the frequency of MNCs in the laboratories that had not used this index, applying the ratio MNi/MNCs, estimated from the mean values of the 11 laboratories that reported both indexes, which was 1:1.12. Using this value, an estimated frequency of MNCs was obtained for the four laboratories that scored only the frequency of MNi (Table III).

TABLE III. Principal Characteristics of the 25 Databases Included in the HUMN Project

Laboratory code	Subjects	MNCs[a]		MNi[b]		Cells scored per subject		NDI[c] Mean (SD)	Mean age	Males (%)	Exposed[d] (%)
		Mean (SD)	Min-max	Mean (SD)	Min-max	Median	Min-max				
AM1	66	5.89 (3.82)[e]		6.59 (4.28)	0–18	500	265–1155		11	53.0	71.2
AM2	130	12.41 (6.79)	2–37	13.55 (7.89)	2–42	1641	981–3587	1.79 (1.34)	58	67.7	
AM3	65	16.94 (10.49)	1–43			1007	846–5812		36	56.9	12.3
AS1	296	12.11 (10.53)	1–55			1000	1000		30	38.5	44.6
AS2	124	10.42 (9.76)	1–61			1000	1000		39	29.8	
AS3	110	10.75 (6.28)	1–35	11.88 (7.28)	1–40	1000	1000–3000	1.87 (0.18)	38	80.9	13.6
AS4	11	17.16 (3.85)	13–24	18.84 (3.88)	13–24	1000	1000–1056		33	100.0	
AS5	941	52.22 (14.78)	18–124	60.40 (17.81)	19–149	1000	1000		62	39.9	100.0
EU1	56	1.75 (1.45)[e]		1.95 (1.62)	0–5	2000	2000		42	64.3	37.5
EU2	1637	3.47 (2.99)	0–25			2000	1000–2000		45	48.3	
EU3	404	5.96 (3.62)	1–24			2000	902–5703		41	79.2	
EU4	223	7.11 (4.91)	0–42			2000	2000–3000		41	82.5	28.3
EU5	39	7.43 (5.85)[e]		8.31 (6.55)	1–29	2000	2000	1.38 (0.14)	50	74.4	
EU6	69	9.64 (6.80)	0–39	10.11 (7.48)	0–41	1500	1000–3973		17	56.5	69.6
EU7	59	8.69 (6.28)	1–31	9.61 (7.38)	1–35	1000	1000	1.95 (0.26)	48	40.7	
EU8	98	9.56 (6.95)	1–28	10.20 (7.48)	1–29	1000	1000–3000	1.71 (0.31)	43	80.6	
EU9	200	9.70 (4.55)	3–25			1433	1000–5304	1.78 (0.24)	32	54.0	
EU10	126	11.27 (7.34)	1–35	12.57 (9.06)	1–46	1000	1000–3400		25	44.4	
EU11	58	14.27 (11.30)	0–42			1000	1000–1500	1.92 (0.18)	60	44.8	
EU12	116	39.27 (33.86)	1–132	53.77 (51.00)	1–198	1500	1000–2000	1.66 (0.31)	40	54.8	50.0
EU13	212	14.35 (6.88)[e]		16.05 (7.69)	2–43	1000	1000		36	100.0	
EU14	98	19.05 (11.05)	2–60	23.99 (17.97)	2–126	500	268–1500		14	70.4	75.5
EU15	153	53.61 (39.58)	6–206	59.57 (45.61)	6–247	1000	1000		36	70.6	100.0
AU1	121	8.98 (4.15)	2–30			2000	1879–4000		35	16.5	8.3
AU2	1171	16.15 (12.38)[e]		18.09 (13.85)	0–107	1000	1000–2000		43	47.8	9.1
Total	6583					1000	265–5812	1.67 (0.79)	44	53.3	25.5

[a]Number of micronucleated cells per 1000 binucleated cells.
[b]Number of micronuclei per 1000 binucleated cells.
[c]Nuclear Division Index.
[d]Previously exposed to mutagenic chemicals or radiation, as described in the text.
[e]Estimated value (from MNi using a ratio of 1:1.12 as described in Statistical Methods).

Clustered Nature of the Data

A total of 26 variables referring to protocol and scoring criteria were included in the statistical models described below (Table II), together with main host factors and exposure to exogenous genotoxic agents. A group of 108 individuals with missing data were removed from this part of the analysis, which therefore considered a total of 6475 subjects.

The major problem encountered in the statistical analysis was the clustered nature of the data, since most of the variables concerning technical aspects of the protocol were strongly correlated within each laboratory. The presence of this within-cluster (laboratory) correlation of observations leads to a poor fitting of the statistical models and to an underestimate of standard errors of model parameters; this pattern of events is known as *overdispersion* [Lindsey, 1997]. A recommended approach to these data is to apply the negative binomial regression model [Diggle et al.,1995]. The probability density function for this model is

$$\Pr(Y = y) = [\Gamma(y + \mu\phi)\phi^{\mu\phi}]/[y!\Gamma(\mu\phi)(1 + \phi)^{y+\mu\phi}]$$

where Y is a count or a rate, i.e., the number of MNCs with respect to the scored cells; μ is the expected value of Y; ϕ is a scale parameter, to be estimated from the data, influencing the variance of Y, i.e., $\mu(1 + \phi)/\phi$; Γ is the Gamma function.

The expected value of Y can be modeled by a linear predictor of explanatory variables

$$\mu = e^{\alpha+\beta_i X_i}$$

where the parameter α is the baseline rate of binucleated cells and β_i is a vector of unknown parameters to be estimated from the data, representing the effect of the X_i variables. The concept underlying this regression model is that overdispersion rises because the parameters themselves are random variables and so the total variability inside the observations depends on two different probability density functions referring to the Y and the parameters, respectively. Since the Y's are distributed according to the Poisson distribution, while the parameters are assumed to follow the Gamma distribution, the resulting mixed distribution is the negative binomial. The estimate of the parameters can be accomplished via maximum likelihood; they are interpretable as frequency ratios and the scale parameter ϕ takes into account the overdispersion, permitting the correct estimate of the standard errors of the parameters. Alternative analyses performed using the generalized estimating equation (GEE) [Diggle et al.,1995] and multilevel (ML) [Goldstein and McDonald, 1988] models yielded similar results.

To assess the goodness of fit of a generalized linear model it is possible to estimate the so-called pseudo-R^2 in the following way: [(Residual deviance of the null model) − (residual deviance of current model)]/(Residual deviance of the null model) × 100. This statistic falls between 0 and 100 and represents the proportion of variability explained by the current model compared to the null model [Kleimbaum et al., 1988].

Another problem affecting these data was the presence of multicollinearity, since many variables considered in the analysis also tend to correlate among laboratories. This phenomenon has the effect of inflating the variance of the model and, in extreme cases, could alter the point estimates of single variables. The analysis was carried out with the GLIM statistical package [Frances et al., 1993]. The negative binomial regression model was fitted to the data with a macro developed by Hinde [1996].

RESULTS

Methodological Aspects

The questionnaire distributed to all participants provided the opportunity to investigate in detail the protocols of a number of laboratories that, because of their geographical distribution, expertise of researchers, and sizes of databases, are considered to be representative of all laboratories currently performing the CBMN assay in human peripheral lymphocytes. All laboratories used blood collected by venipuncture and treated with heparin as anticoagulant. Blood was generally kept at room temperature (82%) for the shortest time possible (80% less than 7 hr, and 64% of laboratories processed samples within 4 hr). The total volume of each culture ranged between 0.5 and 10 ml, with 5 ml being the most frequent choice (11 laboratories). The large majority of laboratories (21 out of 25) used whole-blood cultures, and among these, the ratio of whole blood to culture medium ranged from 4 to 12% (one laboratory used a ratio of 20%). RPMI 1640 was the medium used in 15 laboratories, but Ham's F10 was used by six and two laboratories used McCoy's medium. The concentrations of phytohemagglutinin (PHA) ranged from 20 to 240 μg/ml, with no value frequent enough to be considered as the standard. This is not surprising because seven different manufacturers provided the PHA (of different activities and purities) to 25 laboratories. The percentage of fetal calf serum added was another heterogeneous parameter. The most common quantity was around 10% (12 laboratories), but 15% (five labs) and 20–25% (seven labs) were also used; one laboratory used 2.5%. The most common concentration of L-glutamine added was 200–300 mg/L, but the amount of L-glutamine normally present in most media induced about half of the laboratories not to add it to the culture. Only seven laboratories mentioned the use of antibiotics or antimycotics, but because this item was not addressed by a specific question it was collected through the notes, and may therefore be underestimated.

Cyt-B use is similar across laboratories, because all but three obtained the reagent from the same source (Sigma, St. Louis, MO), and all laboratories used dimethylsulfoxide (DMSO) as the solvent. Most laboratories (15) used about 0.2–0.3% DMSO in the culture; the concentrations in the other laboratories was between 0.4 and 0.75%. Cyt-B was added to cultures 44 hr after PHA stimulation in the large majority of laboratories. As shown in Table II, most laboratories used 3 μg/ml of cyt-B, but concentrations of up to 6 μg/ml were also used.

Cells were harvested 72 hr after PHA stimulation in 80% of the laboratories, but harvesting at 69, 68, and 66 hr is also described, and hypotonic treatment was widely used (84%). Cells were generally fixed with methanol and acetic acid (64%) at 3:1 (sometimes 5:1 or 6:1) before being transferred to slides (16% of laboratories reported fixing cells after transferring them to slides). Cells were usually transferred by dropping or gently smearing the cell suspension, although four laboratories used cytocentrifugation for this purpose. Giemsa was the standard staining method.

The agreement between laboratories in the scoring criteria for selecting binucleated cells and identifying a micronucleus seems to be very good (Table II). The agreement in

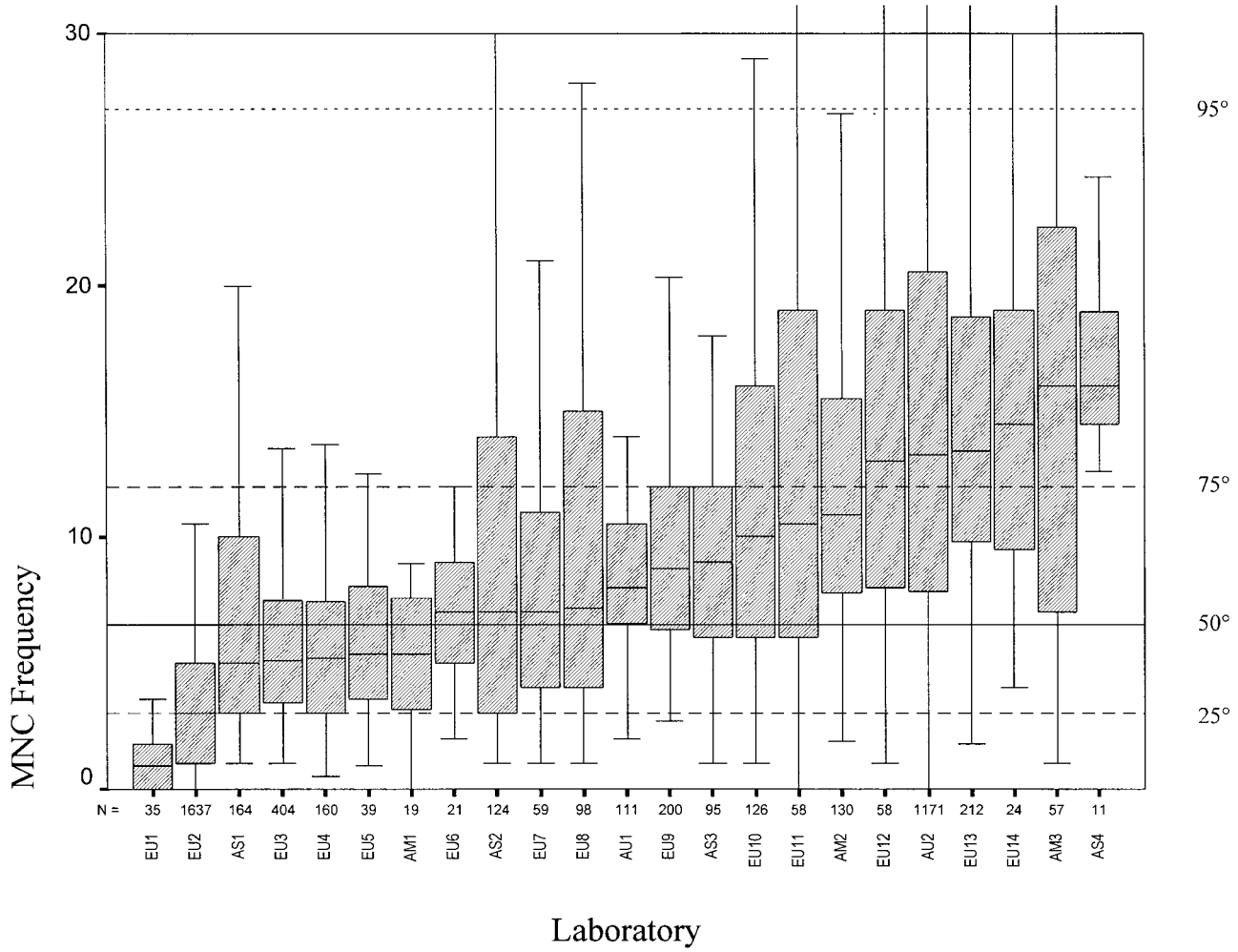

Fig. 2. Baseline MNC frequencies among 23 HUMN project databases. Only data for nonexposed subjects are shown. Horizontal lines represent percentiles of the MNC frequency distribution in the whole database.

the criteria for identification of binucleated cells is excellent for the first five items in Table II, whereas a slight disagreement is present for criterion 6 (*The two nuclei may be attached by a fine nucleoplasmic bridge*), adopted by only 15 laboratories, and for criterion 7 (*The nuclei must not be in an early stage of apoptosis*), adopted by 17 laboratories. The criteria for scoring MN are essentially the same in all laboratories, apart from criterion 6, which has divided the laboratories in two nearly equal groups—those accepting a micronucleus that may overlap or touch the main nucleus and those that do not.

Baseline MNC Frequency

The main characteristics of the 25 databases evaluated in this study and the overall frequencies of MN obtained in each laboratory are reported in Table III. The distribution of MN frequencies in the 4899 nonexposed subjects from all individual databases is presented in Figure 2. Databases have been ranked by the median value of MNC per 1000 binucleated cells, and each box shows the median, the interquartile range, and extreme values within a laboratory. The heterogeneity resulting from exposure to genotoxic agents has been removed, although some variability remains. From this subset of data we get a whole median value of 6.5‰, which could be considered as a reference frequency for the general population. Figure 2 also shows that 75% of the database is below a frequency of 12.0‰ and 95% is below 26.8‰. The interquartile range that defines the most frequent values is between 3 and 12‰. Median baseline frequencies were higher in females than in males (7.0 vs. 6.3‰) and in subjects aged 40 years and over relative to those aged less than 40 years (7.6 vs. 5.9‰). To take into account the different size and therefore the unbalanced contribution of databases to these figures, we also estimated a median MNC frequency from the median of each laboratory. The resulting value of 7.5‰ was higher, although the interquartile ranges largely overlapped (5.3–12.5‰).

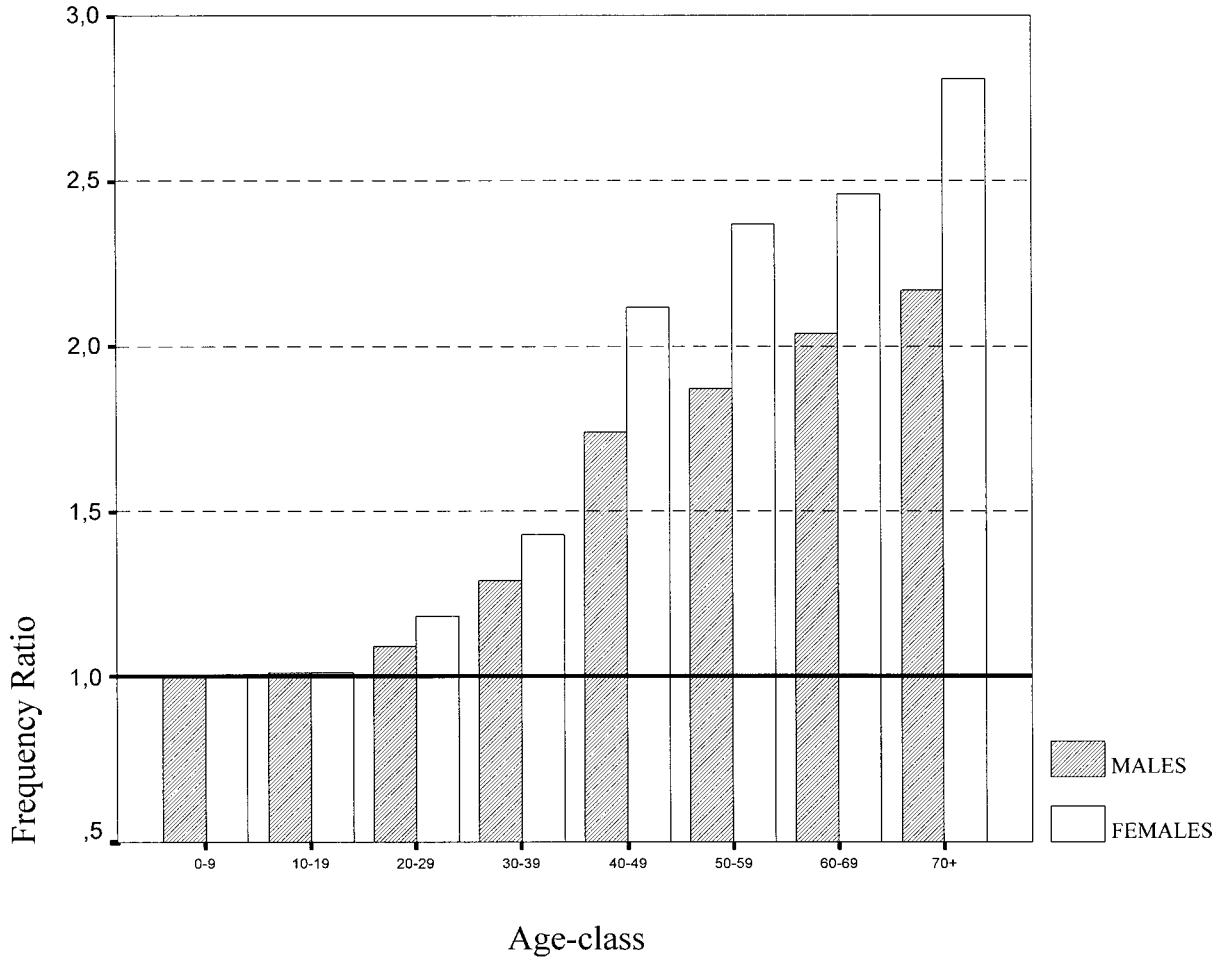

Fig. 3. Effect of age and gender on MNC frequency in peripheral lymphocytes. Frequency ratios (FRs) are adjusted by laboratory and exposure to genotoxic agents. All age-classes after 30 years were significantly higher than the reference class (0–9).

Host Factors

A more detailed evaluation of the effects of gender and age on the MNC frequency was performed using a multivariate model that estimated the frequency ratio (FR) for females vs. males, and for different ages compared with the 0–9 years age-class. These estimates are adjusted by laboratory protocol, scoring criteria, exposure, and continent, for a total of 41 covariates in the model. Estimates are rather stable because of the large size of each age-class (800 subjects on average). Figure 3 clearly shows the effects of both age and gender. MNC frequency increases monotonically with age in both genders, with the steepest increase after 30 years of age, and to a higher level in females (the adjusted FR for females vs. males was 1.19 with a 95% confidence interval [CI] of 1.14–1.24). A more pronounced effect of age in females was confirmed by different female/male FRs in age-classes below 40 years of age (FR, 1.08; 95% CI, 1.03–1.14) and in those over this limit (FR, 1.23; 95% CI, 1.18–1.28). We also calculated the effects of age and gender in single databases, to enable a direct comparison of results within each laboratory. The effect of gender was not homogeneous in the 23 data sets evaluated, because nine laboratories (although representing only 18% of subjects) showed a higher frequency of MNC in males, two of them reaching a significant difference (Fig. 4). The effect of aging, expressed as the FR between subjects of 40 years and over vs. younger subjects, was evident in all but two of the databases examined (Fig. 5).

Factors Affecting MNC Frequency

A model based on the negative binomial function of probability was used to estimate the proportion of variability of MNC frequency that can be explained by the studied parameters. To reduce the effect of the strong intra- and interlaboratory collinearity all the variables were collapsed into four general sources of variability, i.e., host factors (including age, gender, and continent [to surrogate ethnic group]), which accounted for 31% of total variability; exposure to genotoxic agents, 45%; methodological parame-

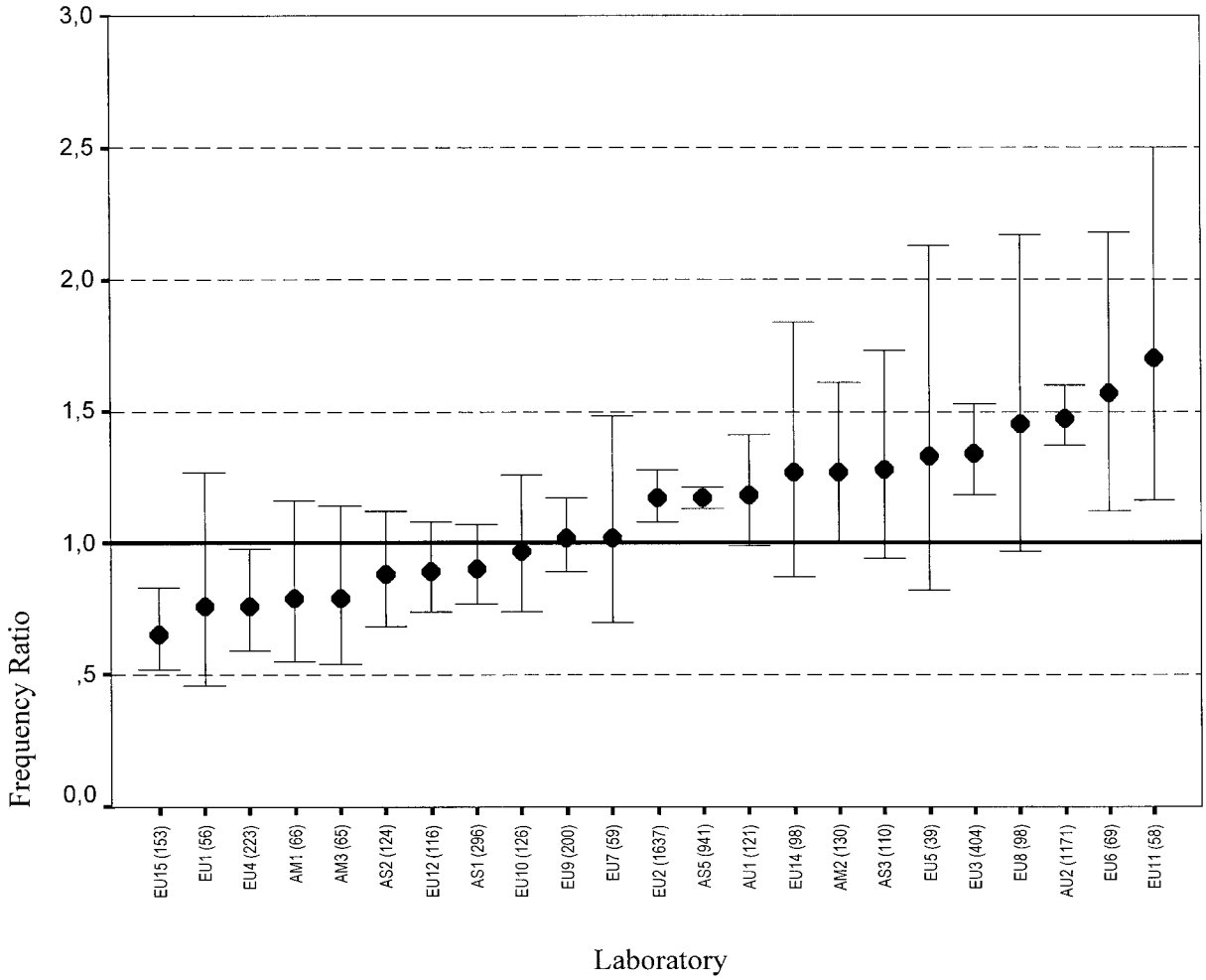

Fig. 4. Effect of gender on MNC frequency (females vs. males) by laboratory. FRs are adjusted by age and exposure to genotoxic agents. The size of each database is in parentheses.

ters (11 items, see Table II), 65%; and criteria for identification of binucleated cells and scoring of MN (15 items, see Table II), accounting for 47%. The total amount of variability explained by the complete model (which included all variables) was approximately 75%. Because of the correlation among parameters investigated, the sum of variance explained by all individual sources is largely greater than this value.

The impact of single steps of the protocol on the MNC frequency was estimated, taking into consideration the difficulties encountered in statistical methods, mentioned earlier. The effect of culture type showed a different pattern by continent. In Asia and Europe whole-blood culture yielded a higher MNC frequency: FR = 2.04 (1.81–2.22), and 2.12 (1.69–2.70), respectively, whereas, on the contrary, in Australia and New Zealand the isolated lymphocyte cultures produced the highest MNC frequencies (3.02; 2.54–3.59).

The effect of the cyt-B concentration could be evaluated only in those laboratories where both 3 and 6 μg were used. No substantial difference was found between the two concentrations, which produced mean frequencies of 3.9 and 4.4‰, respectively. Among the method variables evaluated in the statistical analysis, some determined a significant increase of MNC frequency, i.e., blood standing ≥7 hr before culture, blood kept in the refrigerator before culture, and the adoption of criterion 6 for scoring (*May overlap or touch main nucleus*), whereas other variables significantly decreased MNC frequency, i.e., hypotonic treatment, ≥2 cultures per individual, and the adoption of either criterion 4 (*Nonrefractile*) or criterion 7 for scoring (*Same color as nucleus*).

DISCUSSION

The use of analyses of pooled individual data collected from an unrelated group of similar studies has gained increasing attention in different areas of biomedical research. This study design applies very well to the field of biomarkers, and the advantages of such an approach were previously described [Gordon et al., 1998; Taioli, 1999]. Never-

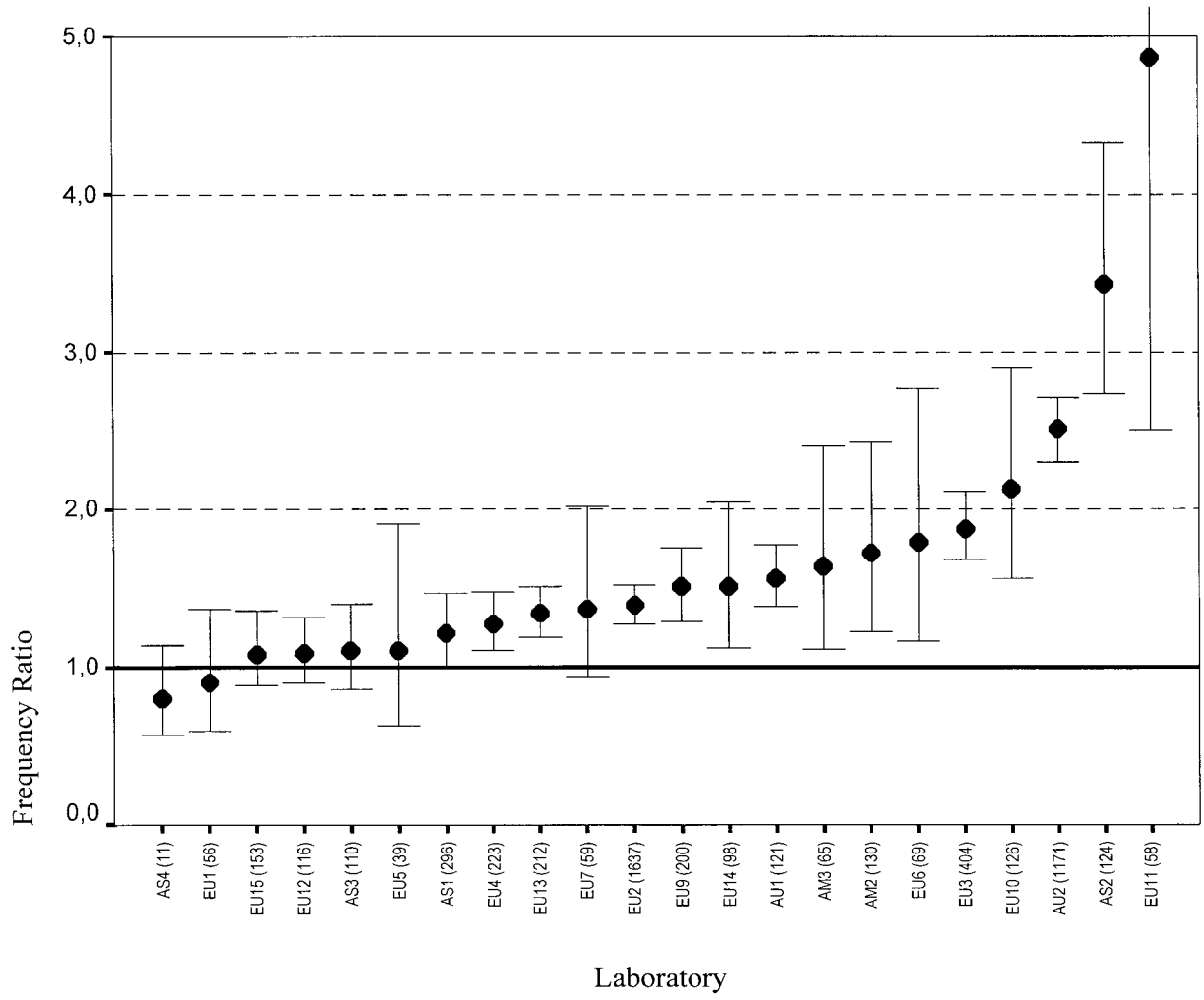

Fig. 5. Effect of age on MNC frequency (≥40 years vs. <40) by laboratory. FRs are adjusted by gender and exposure to genotoxic agents. The size of each database is in parentheses.

theless, there are some specific features of pooling biological data that should always be considered and that could affect the results of these studies. For example, the effects of the great heterogeneity in methodologies were described in a group of European laboratories using the MN assay [Surrallés and Natarajan, 1997] and confirmed by the present study, which investigated methodological aspects in a worldwide network of laboratories.

A major difficulty in interpreting findings from human studies on biomarkers, including MNC frequency, is the large variability observed in absolute frequencies. The lack of reliable information on the natural background levels of many biological events makes it difficult to understand the extent and relevance of the MNC frequencies associated with the exposures or conditions under study. Therefore, one of the priorities of the HUMN project was to provide information on the baseline frequencies of MNC in human lymphocytes. The only external factor known to affect MNC frequency is occupational or environmental exposure to genotoxic agents, and therefore subjects known to be exposed to these agents were removed from this part of the analysis. The residual confounding attributable to other agents (e.g., lifestyle) that could induce chromosomal damage could be reduced further when other data are available, but, with this caveat, a range of frequencies that could be considered normal have been identified. We used the median value as the measure of central tendency since the distribution of MNC frequency values in all databases is highly right-skewed. In these conditions the mean is influenced by few high values, and it therefore gives inflated estimates of MNC frequency in the general population.

The availability of such a large amount of data representative of different human populations offers a valuable opportunity to quantitatively establish the effects of gender and age on MNC frequencies. These two host factors have been investigated in many studies, although, despite the general agreement on their effects on MNC frequencies, the extent of these effects is still controversial.

The effect of gender on MNC frequency has been described in many studies, and the few reports that show no effect have been challenged by large studies, such as those performed in Northern Europe, Australia, and Italy, which found MNC frequencies in females to be 32, 40, and 29% higher than in males, respectively [Nordic Study Group, 1990; Fenech et al., 1994; Bonassi et al., 1995]. In the present study we also found females to have higher MNC frequencies than males, but the effect was lower, i.e., 19%. However, this estimate takes into account other potential confounding factors such as exogenous exposure to genotoxins and protocol variability, which could have influenced the association between gender and MNC frequency in the other studies. The evaluation of the gender effect in all databases included here revealed a certain discrepancy, showing nine laboratories with a higher frequency in males (Fig. 4). However, apart from the evidence that the large majority of databases showed a higher frequency in females (14 laboratories accounting for 82% of the database), it should be considered that among the nine laboratories with divergent findings, two laboratories, AM1 and EU10, evaluated pediatric populations (increments in females are manifest only after 30 years of age) and two, EU4 and EU15 (those with significantly higher rates in males), evaluated occupationally exposed populations where females were underrepresented in the exposed group.

The agreement in the literature with regard to age effects is even greater. In a recent review on the effect of age on cytogenetic endpoints [Bolognesi et al., 1997b], only two out of the 25 studies examined failed to reveal a significant increase of MNC frequency with age. This ubiquitous effect of aging on the MN frequency in human populations is confirmed by our analysis, which showed the effect to be present in all but two of the databases examined (Fig. 5). An interesting aspect is the shape of the MNC frequency/age curve in the older age-classes. Two large studies (whose data are included here) described an unexpected leveling-off of MNC frequencies in the oldest age-classes, and hypothesized a selective survival of older subjects [Fenech and Morley, 1985a,b; Bolognesi et al., 1997b]. In this reanalysis the leveling-off disappeared, showing a uniform increase in MNC frequencies with age in both genders, an increase that is especially steep after 40 years. Despite the size of the whole database, available data for subjects of very old age were scanty (460 subjects over 70 and only 81 over 80). Therefore, these findings should be interpreted with caution when applied to very old age-classes. On the other hand, one laboratory (EU11) included in this database specifically evaluated a very old study group, which included some centenarians, and found that MNC frequency continued to increase in age-classes over 80 years of age, with extremely high values in subjects over 100 years of age [Proietti de Santis et al.,1997].

The proportion of variance explained by our best model reached 75% when host factors, exposure to genotoxic agents, scoring criteria, and the laboratory protocol were considered. This value is higher than estimates from other population studies [Radack et al., 1995], supporting the hypothesis that the inclusion of methodological variables can reduce the extent of the so-called sampling error (which is also known as the proportion of unexplained variance). It should be noted that the term representing the laboratory protocol ranked first in the list of factors contributing to MNC frequency variance.

The sum of the proportion of variance explained by different parameters is largely over 100%, which is not unusual in this kind of analysis because the individual contributions of different factors cannot be separated when they are strongly correlated, as they are in this case. A prudent interpretation of these findings would suggest ranking different parameters by their contributions in explaining the model variability, and using this information to set priorities for homogenization of methods. In addition, it should always be kept in mind that these studies were designed for purposes other than those evaluated here, and that pooled reanalysis is always in the realm of descriptive studies.

The attempt to evaluate the contribution of single steps of the protocol to the MNC frequency was performed, despite the above-mentioned difficulties, to identify major sources of variability to be addressed by future standardization studies. An example of these complex interactions regarding one of the most important methodological aspects is the effect of the blood culture type, which appeared to be dependent on the continent where it was made, with contrasting findings between Asia and Australia/New Zealand. This result is not easy to interpret because the covariate *continent* is a broad category, reflecting differences in lifestyle, genetic make-up, and laboratory methods not included in our survey. Similarly, the other high-priority question that concerns the role of the cyt-B concentration was addressed only in a subset of laboratories that used concentrations of both 3 and 6 μg. The results obtained here are in agreement with those from ad hoc studies [Prosser et al., 1988], which did not find any remarkable difference of MNC frequency in cultures made with different concentrations of cyt-B. The missing information about the role of the interscorer variation, which is considered one of the most important sources of variability in MNC frequency, was surrogated by applying the scoring criteria used by each laboratory. It is obvious that the contribution of this parameter to the general variability is underestimated by using this approach. The analysis of single method variables confirms that scoring is a crucial variable that needs to be controlled in a standardization study, but that other aspects, such as the presence of hypotonic treatment and the procedure for blood storage before culture, may also be important.

A comment should be made about variables such as diet or individual susceptibility, which have been shown to have the potential to affect MNC frequency [Norppa, 1997;

Fenech et al., 1998], but were not available for these analyses. The effects of these variables are not completely independent from those evaluated in our database and, therefore, their contributions to the explained variance could have been indirectly considered by our models. For example, the effect of diet could have been partially included by including the continent in our models. A future analysis of the HUMN database will try to address the contribution of these factors to the MNC frequency variance by evaluating homogeneous subgroups of the entire database.

The HUMN project has created a network of laboratories interested in increasing the understanding of the human MN assay and in its improvement, and new studies have already been planned toward these purposes. An international standardization study involving a large number of laboratories is planned; further evaluation of this database looking at specific exposures and population subgroups is ongoing; and the most ambitious project of a cohort study, aimed to evaluate the capability of the MN test to predict long-term health outcomes, is closer to the operative phase.

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