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To: Mr. Peter Waldman, Acquisition and Assistance Unit, Department of Energy, Chicago Operations Office, 9800 South Cass Avenue, Argonne, IL 60439

From: Profs. James V. Neel and Samir M. Hanash, Principal Investigators, Departments of Human Genetics and Pediatrics, University of Michigan Medical School, Ann Arbor, MI 48109-0618

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The progress during 1993-94 with respect to ER 60533 (J. V. Neel and S. Hanash, co-PIs) can be summarized under three headings:

1. The development of two-dimensional DNA gels for the detection of mutation.

Last year we reported on the initiation of an effort to explore the usefulness of the technique of two-dimensional DNA gels in the study of mutation. Such a gel is illustrated in Figure 1. These efforts have continued throughout this year. Last year we also reported that of the approximately 2000 DNA fragments that can be visualized on a single gel, we had found a subset of 482 spots to be suitable for the study of quantitative variation. With respect to these spots, we can distinguish with high accuracy between a spot due to the presence of two homologous DNA fragments, and a spot due to the presence of only one. The ability to make this distinction means that one can detect deletion/duplication-type mutation. This technique, including development of the algorithms facilitating the scoring of the gels, is being brought up in collaboration with Dr. J. Asakawa of the Radiation Effects Research Foundation, and the material being used in the pilot studies is the collection of 1200 cell line trios (mother/father/child) developed in Japan for the study of the genetic effects of the atomic bombs. This year we have written these results up and a manuscript, a copy of which is appended to this report, is in press with *Proceedings of the National Academy of Sciences USA* (1) (Appendix A).

The chief progress this year with respect to these gels has been a study of qualitative variation (in contrast to the quantitative variation described above). The requirements for spot quantitative reproducibility are not as strict for these qualitative studies as for the work just described, and we identified in the gels 1114 spots (of which the aforementioned 482 are a subset) that were appropriate for qualitative studies. A total

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Figure 1. A two-dimensional DNA gel; in this case, the restriction site enzymes used to obtain the first dimension were *NotI* and *EcoRV* and, for the second dimension, *HinfI*. The preparation is based on the "smaller fragment" end of the first-dimensional gel.

of 142 variable spots were identified; the heterozygosity index for these DNA fragments was 4.4%. This study has now been written up and submitted to *Genomics*; the manuscript is appended (2) (Appendix B).

On the basis of the work in progress in this laboratory, we have been asked by Dr. Y. Hayashizaki, a Human Genome Project Group Leader at RIKEN, Tsukuba Life Science Center, to contribute a description of our techniques and early findings to a Symposium on 2-D DNA Electrophoresis which will be published in the journal *Electrophoresis*. This contribution is now in preparation (2).

On the basis of our experience to date with these gels, we believe that per unit research time, these gels may represent an order of magnitude improvement in the ability to monitor human populations for an increased mutation rate. The fact that 2-D DNA monitoring is at the ultimate (DNA) level should eliminate many of the ambiguities associated with studies at the grosser phenotypic levels, such as studies of the frequency of congenital malformations. However, it is important that DNA monitoring involve DNA that ultimately finds phenotypic expression, i.e., involves working genes. It is useful in that context to recall that the *NotI* restriction enzyme involved in the DNA digestions that result in the first dimension fragments in 2-D DNA gels, cuts preferentially in the unmethylated CpG "islands" that are associated with functional genes (3-7). We therefore believe that these 2-D DNA gels are "enriched" with functional genes. This proposition will be explored as the identification of these DNA fragments progresses.

We are also exploring the possibility that these 2-D DNA patterns will be useful with respect to understanding the early and late genetic changes in cancer. The first step has been to determine the quality of the gels that can be obtained using various malignant tissues. Here the technical issue is to separate the tumor cells (and their normal precursors) from the supporting stroma. Figures 2 and 3 represent preparations from two malignancies of potential interest. Several recent studies (8, 9) identify multiple losses of fragments and fragment amplifications in 2-D DNA gels from patients with hepatocellular carcinoma; this technique may become a preferred technique for the study of the mutations underlying the oncogenic process.

2. Research on stored blood samples. During the period 1962-1976, the DoE supported an extensive series of population studies of relatively unacculturated American Indians in Central and South America. A total of 15 tribes were contacted in the course of these studies. In 1990, the stored blood samples that had resulted from these studies were divided among three laboratories and have become the basis for a series of collabor-

Figure 2. A 2-D DNA gel prepared as described in the legend for Figure 1, based on tumor cells from a bronchogenic carcinoma.

Figure 3. A 2-D DNA gel prepared as described in the legend for Figure 1, based on tumor cells from a neuroblastoma.

ative studies with these laboratories. This year we would like to describe three of these studies that have come to fruition.

a. A new mtDNA "clock" and an estimate of the time of arrive in the Americas of the first American Indians. The time at which the American Indian first reached the Americas is a long-standing, contentious issue. Our extensive past studies of the Chibcha-speaking tribes of Central America resulted in establishing a time depth for the common ancestors of these tribes of ~8000-10,000 years. We have now studied the diversity in mtDNA among these tribes, as contrasted to the diversity encountered in earlier studies of 17 other tribes widely scattered throughout the Americas and thought to be descendants of the first wave of Indians to reach the Americas (10). The results of that recently-published study (11) (that constitutes Appendix C), may be summarized as follows:

"Students of the time of entry of the ancestors of the Amerinds into the New World are divided into two camps, one favoring an "early" entry [more than approximately 30,000 years before the present (YBP)], the other favoring a "late" entry (less than approximately 13,000 YBP). An "intermediate" date is unlikely for geological reasons. The correlation of the appropriate data on mtDNA variation in Amerinds with linguistic, archaeological, and genetic data offers the possibility of establishing a time frame for mtDNA evolution in Amerinds. In this paper, we estimate that the separation of the Chibcha-speaking tribes of Central America from other linguistic groups/nascent tribes began approximately 8000-10,000 YBP. Characterization of the mtDNA of 110 Chibcha speakers with 14 restriction enzymes leads on the basis of their time depth to an estimated mtDNA nucleotide substitution rate for Amerinds of 0.022–0.029% per 10,000 years. As a first application of this rate, we consider the mtDNA variation observed in 18 Amerind tribes widely dispersed throughout the Americas and studied by ourselves with the same techniques, and we estimate that if the Amerinds entered the New World as a single group, that entry occurred approximately 22,000-29,000 YBP. This estimate carries a large but indeterminate error. The mtDNA data are thus at present equivocal with respect to the most likely times of entry of the Amerind into the New World mentioned above but favor the "early" entry hypothesis."

b. An attempt to relate variability with respect to 11 protein polymorphisms with morphological variation in American Indians. Reports by Livshits and Kobylansky (12, 13) and Kobylansky and Livshits (14) have suggested a negative correlation between biochemical heterozygosity, as measured by variation in blood groups, serum proteins, and erythrocyte enzymes, and morphological variability: the more heterozygosity, the closer the morphological measurements to the mean. This finding was interpreted as a manifestation of homeostasis in development as advocated by Lerner (15). Several subsequent studies yielded conflicting results. The data available from the previous DoE-sponsored studies on American Indians were adequate to test this hypothesis. These data consisted of 12 anthropometric measurements and 11 genetic traits (6 blood types, 2 serum proteins, 3 erythrocyte enzymes) with respect to 1025 American Indians. Multiple analyses of these data using both univariate and multivariate statistical techniques failed to reveal the negative correlation between genetic heterozygosity and anthropometric measurements previously reported. These results are in press in *Human Biology* (16).

c. The origin in Asia of the ancestors of the first wave of Amerindians to reach the New World. The question of the origin of the ancestors of the first wave of Amerindians to reach the New World has been contentious almost since the discovery of the New World. Current opinion favors the hypothesis that these most immediate ancestors were also the ancestors of the various ethnic groups now inhabiting northeastern Siberia. We have recently suggested otherwise. As described in previous reports, we have collaborated with the Wallace group at Emory University in extensive studies of the mtDNA of representatives of the various American Indian tribes from whom we have collected blood samples under past DoE sponsorship. We have also collaborated with Dr. Robert Biggar at the National Cancer Institute in studies on the frequency of Amerindians seropositive for the human T-cell lymphotropic virus, type II (HTLV-II), again using the samples collected under DoE funding. At this point, among 38 American Indian tribes studied by ourselves and others, seropositivity for HTLV-II has been encountered in 11. Similar studies on mtDNA have now been performed on the Siberian ethnic groups by the Wallace group; a major haplogroup of the American Indians is absent in Siberia. Our collaborator, Dr. Biggar, has also examined the plasma of these Siberian groups for the frequency of HTLV-II positives, finding none. Both the missing mtDNA haplogroup and the HTLV-II virus have recently been found to the south, in the Peoples Republic of Mongolia and extreme northwestern China. On that basis, we have developed the theory that the most immediate ancestors of the Amerindians originated in the area of Mongolia-Northern China, and the Siberian populations moved into Siberia after the ancestors of the first wave of American Indians

had already left for the New World. A manuscript is now in press in *Proc. Natl. Acad. Sci. USA* (17) (Appendix D). The abstract of that manuscript reads as follows:

“A commonly held theory is that the first wave of migrants into the New World were derivative from the ethnic groups then inhabiting eastern Siberia. However, these ethnic groups lack a mtDNA haplogroup (B) which is well represented in Amerindian tribes. Also, the time depth of the other three mtDNA haplogroups found in Amerindians (A, C, D) appears to be greater in the Amerindians than in the eastern Siberian ethnic groups. In this communication we demonstrate that the HTLV-II virus, present in 11 of the 38 Amerindian tribes thus far examined, is not present in any of the 10 ethnic groups of eastern Siberia that we have studied. However, the virus has just been reported in the indigenous population of the Mongolian Peoples Republic, and mtDNA haplogroup B is also represented in this region. On the basis of these facts, we propose that the ancestors of the first migrants to the New World were not derived from north and central Siberia but from populations to the south, inhabiting the regions now designated as the Mongolian Peoples Republic, Manchuria, and/or the extreme southeastern tip of Siberia.”

3. The Sellafield-Seascale “flap”. A major scare concerning the genetic effects of radiation was generated by the epidemiological studies published in 1990 by Martin Gardner and associates (18, 19) on the occurrence of leukemia and lymphoma among young people in West Cumbria, England, the site of the Sellafield Nuclear Reprocessing Facility. The subjects of this study were 52 persons with leukemia, 22 with non-Hodgkin’s lymphoma, and 23 with Hodgkin’s disease; all with onset in people born in the area between 1950 and 1983, with diagnosis under the age of 25, and 1001 controls matched for sex and age taken from the same birth registers as the patients. Associations were sought with such factors as place of residence, viral infections, child’s play habits, and parental occupational histories. The principal positive finding was that relative risks for leukemia and non-Hodgkin’s lymphoma were higher in children born near Sellafield and in children of fathers employed at the plant. For example, the relative risk was 2.4 [95% confidence interval (C.I.) of 1.0–5.7] for children of fathers employed at Sellafield at the time of their conception, and 6.4 (C.I. 1.7–26.3) for children of fathers receiving a total preconceptional (external-badge-recorded) dose of ionizing radiation of more than 100 mSv equivalents (but there were no exposures exceeding approximately 200 mSv equivalents). The authors concluded that, “This result suggests an effect of ionizing radiation on fathers that may be leukemogenic in their offspring, though other, less likely,

explanation are possible.” (18). That they believed this effect to be truly genetic is indicated by their comparison of their findings with other avowedly genetic studies.

A British law firm, Leigh, Day, and Company, advertised its willingness to file claims against the organization operating the facility, British Nuclear Fuel Plc (BNFL), and relatives of two of the children soon initiated action against BNFL, claiming damage for personal injuries and, in one of the two cases, death. A finding for the plaintiffs would challenge, worldwide, present permissible occupational exposures to ionizing radiation, as well as the validity of a major corpus of experimental data from the mouse, and, more particularly, the results of the DoE-sponsored genetic studies on the children of atomic bomb survivors with which one of the P.I.s (JVN) has been associated for many years. The Gardner study results required a genetic sensitivity to radiation of the leukemia phenotype some 4000 times greater than was compatible with the follow-up studies in Hiroshima and Nagasaki.

JVN became involved in the trial as an expert witness for the defense. This involved the preparation of multiple affidavits and three trips to England. JVN used this experience as the basis for his presidential address to the new International Genetic Epidemiology Society; a reprint of that address (20) constitutes Appendix E of this application.

Although the results of many experimental studies employing mice militated strongly against the validity of the Gardner hypothesis, probably the key item for the defense was the failure to observe a similar increase in leukemia in the children of atomic bomb survivors, despite the much larger average doses of acute ionizing radiation to the exposed survivors of the atomic bombings.

The trial extended over a period of some eight months, followed by a recess of three months, during which the judge studied the testimony. He then issued a strongly-worded verdict in favor of the defendant. As of now, this is probably the most practical and tangible application to date of data resulting from the long-time support by DoE of the genetic studies in Japan and in Ann Arbor.

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APPENDICES

Appendix A:

Asakawa, J., Kuick, R., Neel, J.V., Kodaira, M., Satoh, C., and Hanash, S.M. Genetic variation detected by quantitative analysis of end-labeled genomic DNA fragments. Proc. Natl. Acad. Sci. USA, in press.

Appendix B:

Kuick, R., Asakawa, J., Neel, J.V., Satoh, C., and Hanash, S.M. High yield of restriction fragment length polymorphisms in two-dimensional separations of human genomic DNA. Genomics, submitted.

Appendix C:

Torrioni, A., Neel, J. V., Barrantes, R., Schurr, T. G., and Wallace, D. C. A mitochondrial DNA "clock" for the Amerinds and its implications for timing their entry into North America. Proc. Natl. Acad. Sci., USA 91: 1158-1162, 1994.

Appendix D:

Neel, J.V., Biggar, R.J., and Sukernik, R.I. Virologic and genetic studies relate Amerind origins to the indigenous people of the Mongolia/Manchuria/Southeastern Siberia region. Proc. Natl. Acad. Sci. USA, in press.

Appendix E:

Neel, J.V. Problem of "false positive" conclusions in genetic epidemiology: Lessons from the leukemia cluster near the Sellafield nuclear installation. Genet. Epidemiol. 11: 213-233, 1994.

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