Quality Control Program for Storage of Biologically Banked Blood Specimens in the Malmö Diet and Cancer Study[1]

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Abstract

A biological bank has been developed to extend the biochemical and molecular research base for a prospective study on diet and cancer in the city of Malmö, Sweden. The study entered individuals 45–69 years of age, of which 30,382 individuals (45%) participated. Each individual entering the bank has stored samples of viable mononuclear leukocytes (MNLs; −140°C) and granulocytes (GRANs; −80°C) or buffy coats (−140°C), erythrocytes (−80°C), and plasma/serum (−80°C). The bioassays developed to monitor the quality of storage conditions were: (*a*) viability and growth response to phytohemagglutinin for MNLs; (*b*) DNA strand breakage for GRANs; (*c*) NAD content for erythrocytes; and (*d*) thiol status for plasma/serum. The yield, purity, and storage conditions were all quality controlled, and the samples were determined to be of high standard after 137–190 weeks of storage. No differences in yield and purity were found in samples banked by different laboratory technicians. Growth responses of MNLs were severely reduced (90%) after 40 weeks of storage, which justified switching from the storage of purified MNLs and GRANs to the more cost-effective banking of buffy coats. We conclude that the quality of the banked material, based on the biochemical analysis done, indicate that the storage conditions are optimal at least up to 3.5 years, except for the growth response of MNLs.

Introduction

The Malmö Diet and Cancer Study is a population-based study including 30,382 men and women, age 45–69 years, living in Malmö, Sweden. The project uses a method for dietary assessment validated in collaboration with the IARC (1). A high autopsy rate and established cancer registries ensuring 100% identification of disease cases (2), and a quality controlled biological bank of purified and viable cells as well as plasma/serum that allows state-of-the-art development of intermediate biomarkers for identification of individuals at high risk to develop cancer (3, 4). Because the details of the Malmö biological bank have been presented elsewhere (3), only the reasons behind its design and development are covered here.

As previously pointed out (2), one major research priority of the project was to clarify the importance of oxidative stress on biomarkers of increased risk for cancer (5). Therefore, the logic used for the formation of the bank was to create a bank with the largest possible versatility, so that maximum flexibility for future use of the bank by researchers was preserved according to what methodological approaches were available at the time of its formation or may become available in the future. For example, most biological banks store serum, plasma, buffy coats, or whole blood, but this severely restricts the research options for use to only a few approaches involving molecular biology and analytical chemistry.

Specimen collection for the Malmö biological bank began in March 1991 and was completed in October 1996. There are three levels of quality control; namely (*a*) instrument variability, (*b*) yield and purity of blood cell fractions, and (*c*) storage. The first two control systems together with preliminary data have already been presented in some detail (3), but comparative data involving storage was not available at that time. Here we present the final status of the enrollment of samples into the Malmö biological bank including the yield and purity of the blood cell fractions, and we further present methodological details and the results of our quality control program for long-term storage.

Materials and Methods

Blood Sampling. About 28 ml of heparinized blood and 10 ml of blood without anticoagulant for serum preparation from each individual entering the bank were fractionated into the blood fractions indicated in Table 1 and stored in 2-ml vials, according to details presented elsewhere (3). This procedure was used to bank 16,097 individual blood samples. In August 1995, the procedure was replaced for an additional 14,285 entered individuals by banking buffy coats instead of purified MNL[3] and GRAN, whereas all other banked specimens remained the same as described previously (3). This was accomplished by centrifuging the heparinized blood sample 300 × *g* for 10 min and removing the plasma, which was centrifuged a second time at 2000 × *g* for 10 min to remove thrombocytes and then banked in 2 × 2 ml of plasma sample. The rest of the blood sample was diluted with saline (same volume as removed plasma) and

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[3] The abbreviations used are: MNL, mononuclear leucocytes; GRAN, granulocytes; PHA, phytohemagglutinin; R-10, newborn calf serum.

Table 1 Total specimens entered into the Malmö biological bank as of September 31, 1996 and the quality control of their yield and purity.

Storage conditions	Entered individuals (*n*)	Missed individuals (*n*)	Yield (%)[a] mean ± SD	Purity[a]			
				MNL	GRAN	PLT[b]	ERY[b]
MNL, −140°C, 3 vials	16040	57	54 ± 15	–	0.04 ± 0.05	7.6 ± 8.8	2.0 ± 3.5
GRAN, −80°C, 1 vial	15922	175	45 ± 16	0.10 ± 0.08	–	1.8 ± 2.1	n.d.[b]
WBC, Buffy coat, −140°C, 3 vials	14228	57	68 ± 12	n.d.	n.d.	n.d.	n.d.
ERY, −80°C, 2 vials	30289	93	2 ml[c]	0.06 ± 0.09	0.06 ± 0.09	0.5 ± 1.2	–
Plasma, −80°C, 2 vials	30277	105	4 ml	0	0	3 ± 5[d]	0
Serum, −80°C, 2 vials	30256	126	2 ml	0	0	0	0

[a] Yield and purity criteria presented includes >93% of the total sampled individuals in comparison with published standard procedures ± 2 SD. Yield, % cells recovered/cells present in blood) × 100; Purity, contaminant/cell except for the purified ERY samples which are given as contaminant/1000 cells.
[b] PLT, platelets; ERY, erythrocytes; n.d., not determined.
[c] Representative samples were taken 2/week from September 1992 until September 1996, $n = 278$. Yield equaled 11.3 ± 2.1 × 10^9 cells/ml of packed ERY.
[d] PLT × 10^6/ml plasma.

centrifuged at 2000 × *g* for 10 min, after which the buffy coat layer was removed and cryopreserved in 50% autologous serum and 10% DMSO. The alteration in banking procedures was motivated partly by financial constraints from the major grant supplier and partly by our data showing that MNL proliferative responses could not be maintained for more than one year (Fig. 2), which did not justify the additional cost of banking purified MNLs and GRANs.

In addition to quality-control storage conditions for the bank we initially recruited 10 blood donors. The various blood fractions were generated as described elsewhere (3), and the oxidative-sensitive storage bioassays reported on below for monitoring the quality of long-term storage at −80°C and −140°C were performed on fresh and freshly frozen samples, which were in turn compared with long-term stored frozen samples. The donated samples were divided into the blood fractions described previously (3), and then each aliquoted into 10 portions and stored in the biological bank at either −80°C or −140°C. Periodically, over 190 weeks, representative samples were thawed and the oxidative-sensitive storage bioassays were performed to assess the quality of the banked specimens.

Oxidative-sensitive Storage Bioassays. One of the primary laboratory research aims of the Malmö Diet and Cancer Study is to evaluate if endogenous oxygen metabolism on an individual basis can be influenced by diet and detected as biological intermediate end points in the development of cancer and cardiovascular disease. This orientation has resulted in the following assays being used to quality control the influence of oxidative stress and DNA damage on the storage over time of biological samples in the bank: (*a*) plasma/serum, the levels of reduced/oxidized protein and nonprotein thiols; (*b*) MNL, mitogenic (proliferative) response to growth induction by PHA; (*c*) GRAN, DNA strand breakage estimated by nucleoid sedimentation; and (*d*) erythrocytes, the level of NAD pools estimating hydrolysis by NADase and oxidative stress. The appropriateness and utility of using these biomarker end points to quality control biologically banked specimens have been presented (4–13).

Plasma/Serum Storage Assay. The stability of plasma stored at −80°C was assayed by estimating any change in the amount of reactive thiol material over time. Each plasma sample was thawed and centrifuged at 2000 × *g* to sediment any precipitated fibrin. Plasma (2 ml, 20%) in water was prepared and 30 μl of 5,5′ dithiobis-(2-nitrobenzoic acid) were added as a 9.5 mg/ml solution dissolved in 0.1 M K_2HPO_4, 17.5 mM EDTA, pH = 7.5. The mixture was left to react at room temperature for 1 h, at which time the absorption at 412 nm (A_{412}) was measured. Chloramine T (Sigma Chemical Co., St. Louis, MO) dissolved in water was then added at a final concentration of 40 μM, and the A_{412} again was read after 30 min. The difference in absorption was calculated and used as a quality control analysis of Chloramine T-sensitive thiols that occur in stored plasma and might vary with storage time. Variation in the assay procedure itself was periodically evaluated over time by using fresh serum as a reference sample.

MNL Storage Assay. The viability assay used was based on the ability of the T-lymphocytes present in the MNL fraction to respond to the mitogen, PHA (11, 12). The cryopreserved MNLs (1 ml) were thawed in water at 37°C, immediately placed on ice, diluted with 10 ml of cold RPMI 1640, sedimented in a refrigerated centrifuge (300 × *g*), and washed again with 10 ml of cold RPMI 1640. After the second sedimentation, the cells were suspended in 20% autologous plasma-supplemented RPMI 1640 and counted. The recovery after thawing (average yield of 10 samples) varied between 68% and 81% of the original number of stored cells, and the average cell viability estimated by trypan blue exclusion varied from 87–99%. The cell concentration was adjusted to 2 × 10^6 cells/ml, and 12 cultures were set up in a microtiter plate containing 100 μl of cell suspension (200,000 cells) + 100 μl of RPMI 1640 including 12 μg/ml PHA. The cultures were incubated in 5% CO_2 atmosphere at 37°C for 44 h, then given [^{3}H]-labeled thymidine (2 Ci/mmol) at 1 μCi/ml and 50 μM unlabeled thymidine. After an additional 48-h incubation at 37°C, the cultures were frozen at −80°C, thawed, and harvested on glass fiber filters using a cell harvester. The incorporation of [^{3}H]-thymidine/200,000 cells gave an estimate of the growth response to PHA. Variation in the PHA assay itself was routinely evaluated against fresh MNLs at all sampled time points.

Erythrocyte Storage Assay. Erythrocytes have an ectoplasmic location of NADase (6), so if there is any lysis of these cells during storage it would cause a decrease in NAD content, which would be detected by this procedure. Frozen erythrocyte pellets (500 μl) were thawed on ice in the presence of 1 ml 1.8 M perchloric acid and an internal standard thymidine, dThd. After centrifugation at 14,000 × *g*, the supernatant was neutralized on ice with 2 M K_2CO_3. After another centrifugation at 14,000 × *g*, the supernatant was ready for analysis by high-performance liquid chromatography (*i.e.*, at a 4.15 × dilution). The yield, estimated from extraction of erythrocytes with known amounts of NAD, nicotinamide, and dThd added was 83 ± 5%, $n = 7$. An isocratic high-performance liquid chromatography method for separation of nicotinamide, NADP, NAD, and dThd has been developed. The separation was performed with a 3-μm C18 column (30 mm × 3 mm I. D.; Perkin-Elmer Corp., Norwalk, CT) using a four-pump Perkin-

Elmer (410 LC) system equipped with a variable UV detector (LC-95) and an integrator (LCI-100). Baseline separation of nicotinamide, NAD, and dThd within < 5 min was obtained when the general operating conditions were as follows: flow rate, 1.5 ml/min; elution buffer was 150 mM potassium phosphate, pH 6, containing 1–2% methanol (v/v); temperature, 20°C–25°C; recycling time between runs, 5 min; and detection, 254 nm. A standard curve was prepared from frozen erythrocyte samples that were incubated for 1.5 h at 37°C before the addition of 0–40 μM NAD, followed by extraction with perchloric acid. The NAD concentration in the samples was determined as a function of the peak height of NAD divided with the peak height of the internal standard, dThd, which in turn corrected for chemical assay variability.

GRAN Storage Assay. The GRAN fraction is the main source of DNA in the biological bank. The nucleoid sedimentation assay is a sensitive, fast, and reproducible method to measure changes in the nuclear structure and DNA organization caused by small amounts of DNA single- and DNA double-strand breaks (7), which may have been introduced during freezing and long-term storage at −80°C. Frozen GRANs (2 ml) were thawed at 37°C and 600-μl aliquots were immediately transferred to ice, followed by the addition of 2 × 1 ml of RPMI medium with 10% R-10. After 1 min, a 4-ml aliquot of R-10 was added and then 1 min later another 12 ml was added. The GRAN suspension was immediately centrifuged at 400 × *g* for 10 min at 4°C. The pellet was stored on ice and then again resuspended in R-10 before adjusting the cell density to 2×10^6 cells/ml. The yield of GRAN after storage at −80°C was 88 ± 5% (n = 4). Nucleoids were formed according to a procedure originally developed by Cook and Brazell (8) and modified by Romagna *et al.* (9), where 300 μl of a lysis solution (2 M NaCl, 10 mM Tris, 10 mM EDTA, 0.5% Triton X-100 (v/v), pH 8 at 4°C) were carefully layered on a continuous gradient containing 2 M NaCl, 10 mM Tris, 10 mM EDTA, pH = 8 at 4°C, and 15–30% sucrose solution (w/v) was formed in a 5-ml ultracentrifuge tube (Beckman Instruments) using a gradient maker. To detect the nucleoid band, the gradient solution contained 1 μg/ml DNA dye Hoechst 33258, which has been shown not to influence the sedimentation rate of the nucleoids at this concentration (10). A 100-μl GRAN suspension representing 2×10^5 cells was carefully added to the lysis solution at the top of the gradient, and after 30 min lysing time at 4°C the gradients were placed in a SW 50.1 rotor (Beckman Instruments) and centrifuged for 30 min at 4°C at 60,000 × *g* (25,000 rpm). The nucleoid band was detected by the visible fluorescence of the DNA Hoechst dye complex using a long-wave UV lamp (Black ray, 366 nm). The sedimentation distance is an estimate of the degree of DNA strand breakage, and it was calculated from the top of the gradient to the middle of the nucleoid band. The sedimentation rate of GRAN nucleoids was expressed as the percentage of control nucleoid sedimentation (*i.e.*, nucleoids from fresh MNLs that were also controlling biochemical assay variability).

Statistics. The time points for the individual groups of biomarker end points were compared by Student's *t* test.

Results

The status of the Malmö biological bank is presented in Table 1. In August 1995 the biological bank switched from banking purified MNLs and GRANs to storing buffy coats, based on the data reported in Fig. 1. The data show that the proliferative responses of the purified MNL fraction could not be cryopreserved for more than 40 weeks at −140°C without significant loss of proliferative viability. However, the average yield of WBCs from 28 ml of blood was only positively influenced by these changes in banking because: MNL = 54 ± 15% (equivalent to $36 \pm 15 \times 10^6$ cells); GRAN = 45 ± 16% (equivalent to $48 \pm 25 \times 10^6$ cells); and buffy coats (MNL + GRAN) = 68 ± 12% (equivalent to $125 \pm 42 \times 10^6$ cells). The other blood fractions were produced in excess and only aliquots were entered into the bank as erythrocytes ($11.5 \pm 2.0 \times 10^9$ cells/ml), plasma (4 ml), and serum (2 ml).

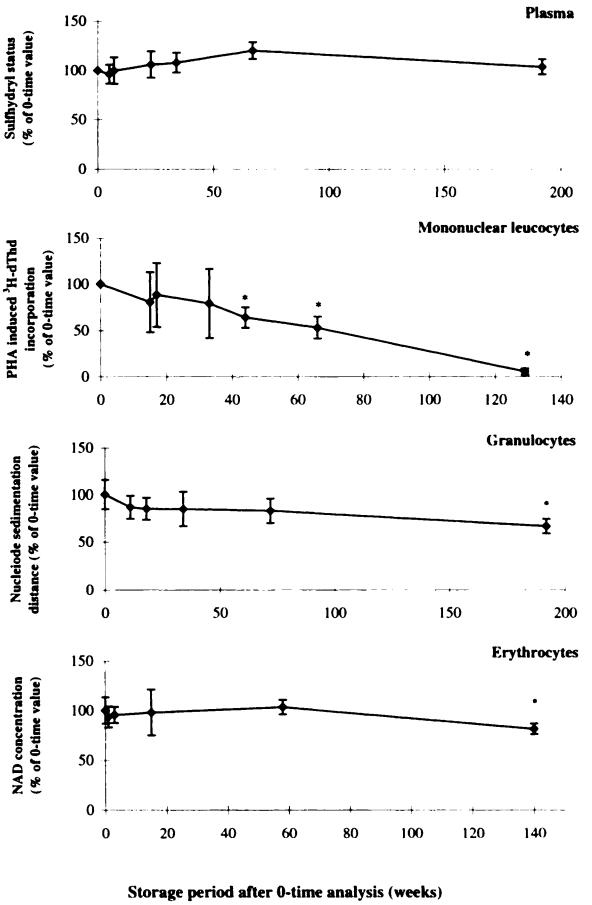

Fig. 1. The quality of samples stored in the Malmö biological bank. Ten donors contributed 10 replicate samples as identified in the figure which were in turn stored identically to the samples entering the Malmö bank and periodically assayed as indicated. Means ± SD are shown. *, statistically significant difference compared with 0 weeks control value (*i.e.*, mean ± > 2 SD).

The yield and purity criteria of the various blood fractions for the entered individuals are also presented in Table 1 as measures of the quality of the stored samples. We have analyzed the biologically banked specimens by direct quantitative analysis of cell types (Table 1) by sorting according to nuclear volume using a Sysmex K 1000 system (TOA Medical Electronics Co., , Japan; Ref. 3) and by comparison with published procedures for blood cell fractionation (Table 2). More than 93% of the total samples were within ± 2 SDs of the mean for published procedures for purity, and the yield was also comparable with state-of-the-art commercially available cell isolation procedures (Tables 1 and 2; Ref. 14 and 15). There was no sacrifice in yield when the banking program switched from entering purified MNL and GRAN samples to entering buffy

Table 2 Direct comparison of yield and purity of blood cell fractions in paired heparinized blood samples generated by the conventional state- of-the-art procedure or by the single step procedure developed for the Malmö project (3). Data are mean ± SD, $n = 6$.

Blood cell fraction	Yield from blood (%)	Purity (contaminant/cell fraction ratio)			
		MNL	PLT[a]	GRAN	ERY[a]
Conventional procedures[b]					
MNL	58 ± 6	–	10.7 ± 7.1	0.02 ± 0.01	0.05
Malmö diet and cancer—single step procedure:					
MNL	54±9	–	17.3 ± 12.6	0.06 ± 0.03	2.0 ± 3.5

[a] PLT, platelet; ERY, erythrocytes.

[b] Details of the conventional procedures were first presented by Boyum (14) and more recently supplied with purity criteria by commercial suppliers of density gradient solution. Criteria published for Lymphoprep (Nycomed AS): yield MNL fraction 70%, GRAN in MNL fraction 1%, and ERY in MNL fraction 1%; and for IsoPac Ficoll (Pharmacia Biotech, Uppsala, Sweden): yield MNL fraction 60 ± 20%, GRAN in MNL fraction 3 ± 2%, and ERY in MNL fraction 5 ± 2%.

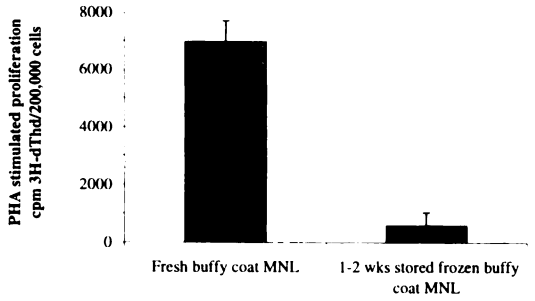

Fig. 2. The MNL from fresh (unfrozen) and frozen (<1 month) buffy coats prepared and isolated as described in "Materials and Methods" were treated with PHA for 4 days to induce a proliferative response. Growth was estimated by low activity [^{3}H]dThd labeling during the 2 last days (50 μM, 1 μCi/ml). Frozen nonstimulated cells incorporated <50 cpm [^{3}H]dThd/200,000 cells.

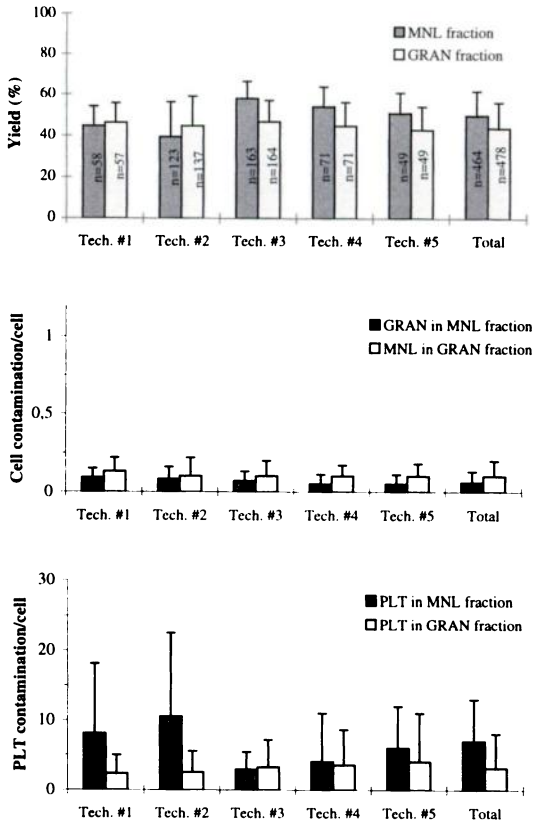

Fig. 3. The intertechnician variability determined during the routine operation of the Malmö biological bank. The five technicians were blinded to the knowledge that they were being monitored for the number of individuals indicated. Means ± SD are shown. All variations were within ± 2 SDs of the overall mean for the five technicians.

coats (Table 1). Because buffy coats routinely contained erythrocytes, MNLs, and GRANs, no meaningful purity criteria could be established for these fractions. It was established that if the buffy coats were cryopreserved for <1 month at −140°C they could be thawed and isolated onto Lymphoprep gradients (Lymphoprep; Nycomed AS, , Norway). MNLs isolated from buffy coats in this manner gave comparable viability (>65% by trypan blue exclusion) to long-term cryopreserved purified MNLs (>87%). Although evaluated only after <1 month of −140°C storage, isolated MNLs from buffy coats were still able to illicit a proliferative response comparable with cryopreserved purified MNLs stored for >100 weeks (calculated from Fig. 1 and Fig. 2). In such a manner, MNLs isolated from buffy coats could still be compared with the cryopreserved purified MNL fractions that justified the cryopreservation of buffy coats.

Any contribution of technician performance to our ability to isolate purified blood cell fractions was assessed by comparing the intertechnician variability of five technicians during 1 month of routine operation of the bank. All of the technicians were blinded to the knowledge that they were being monitored. The results reported in Fig. 3 show that, when yield or purity of cells are used to monitor cell isolation and storage procedures, intertechnician differences were within two SDs of each other (nonsignificant).

The effect of storage conditions in the Malmö biological bank on replicate samples from 10 donors bioassayed over time up to 190 weeks after storage is presented in Fig. 1. The results indicate that there has not been any degradation of the stored samples of plasma/serum or erythrocytes, during the evaluation period when using the oxidative-sensitive storage bioassays presented here as the quality control monitors (*i.e.*, mean ± < 2 SD). However, the proliferative capacity of long-term stored MNLs (*i.e.*, > 40 weeks; Fig. 1) decreased about 95% ($P < 0.05$) and GRANs at 190 weeks were slightly outside the ± 2 SD range ($P < 0.05$). To better understand why long-term stored MNLs could not illicit a growth response to PHA stimulation, we have determined recovery and viability (determined by trypan blue exclusion test) of fresh (not frozen), freshly frozen (<1 month), and long-term (128 weeks) banked MNLs (Fig. 4). Although about 30% of the frozen MNLs were not viable after thawing, there was no further decrease of viability

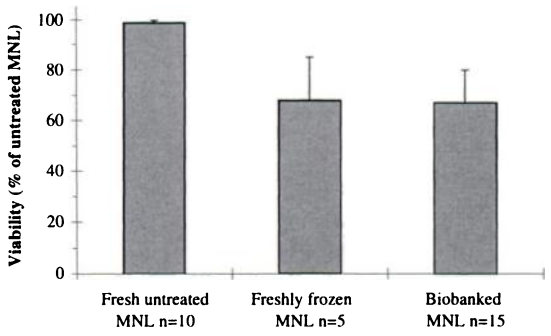

Fig. 4. Comparison between nonfrozen (fresh), recently frozen (1 week), and biobanked MNLs (128 weeks) when assayed for viability by the trypan blue exclusion test. Fresh and recently frozen MNLs were isolated on Lymphoprep, and the recently frozen MNLs were then suspended on ice in 50% autologous serum, 10% DMSO, and 40% RPMI 1640 in 2-ml cryovials and frozen in a temperature-controlled manner at −80°C freezer.

between freshly frozen and long-term frozen MNLs. Hence, we conclude that poor viability of cells could not explain the lack of proliferative activity of long-term stored MNL.

Discussion

The bank was originally built to process 36 individual blood samples/day, and because one technician can handle 12 samples/day then three technicians were needed to maintain a recruitment rate of 36 samples/day. The main objective of the design of the bank was to provide as many opportunities as possible to validate the state-of-the-art intermediate biomarker area, and also contribute to the development of other prospective biomarker areas. When the long-term storage of purified MNLs was shown to deteriorate (Fig. 1), then it was obvious that biomarkers requiring cell growth would become severely limited and other cost-benefit considerations should be motivated to bank cryopreserved buffy coats. For example, three technicians can process 48 samples/day if cryopreserved buffy coats are substituted for purified MNLs and GRANs in our overall banking procedures. Moreover, cryopreserved buffy coats could be shown to serve as a comparable source of purified MNLs by thawing and further separation on density gradients (Fig. 1 and 2). Because these data indicated no obvious advantages in terms of additional options for biomarker validation whether one banked purified MNLs and GRANs or buffy coats, the cost-effective change to banking buffy coats was made in August 1995 at participant #16155.

We have observed that phagocytic cells such as monocytes are the cell types mainly lost, and they are essential to support the PHA mitogenic response (16–18). However, even with severely reduced proliferative viability, the cryopreservation of both MNLs and buffy coats at −140°C is justified because future biomarkers to be tested with banked material, which only require intact cells but not growing cells, could use these banked specimens.

To our knowledge, there has never been reported in the scientific literature a systematic quality controlled analysis of long-term storage of human blood components at −80°C or −140°C. The data reported here demonstrate that plasma/serum, GRANs, and erythrocytes can be preserved without any oxidative degradation for at least up to 140 weeks (< mean ± 2 SD). Although MNLs could be maintained at −140°C intact and viable by trypan blue analysis for up to 202 weeks (Fig. 4), their proliferative capacity assessed by PHA mitogenic stimulation was severely reduced after 40 weeks of storage. However, these data may not accurately evaluate the proliferative viability of long-term stored MNLs. PHA responsiveness is dependent on cell surface receptors and the presence of monocytes (17, 18), both of which may have been specifically affected by the conditions of cryopreservation (*e.g.*, DMSO) altering signal transduction but not necessarily the ability of the cells to proliferate if given the right signal. For example, viral transformation and DNA transfection experiments may yield a much higher index of proliferative capacity. These possibilities are currently being investigated.

In summation, we have presented the design, feasibility, and quality control program including storage conditions for the biological banking of 30,382 individuals. Our data support the conclusion that the samples obtained by the Malmö biological bank in terms of yield, purity, and long-term storage, were collected in a reproducible and quality controlled manner. This was done in an effort to provide interested researchers who plan to biologically bank specimen in the future to have the advantage of our experience thus far.

In addition, we would like to make other investigators dealing with biomarker development to be aware of the possibilities that the Malmö biological bank can offer. The study is open to international collaboration through a program developed by a steering group responsible for the project.

We conclude that the methods used to bank blood components in the Malmö Diet and Cancer Study, seemed to be optimal except for the growth response of MNL. The bank constitutes an important resource for biochemical biomolecular research.

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