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Standardization of Measurement of Immunoglobulin-Secreting Cells in Human Peripheral Circulation

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A sensitive, and at times the most sensitive, measurement of human vaccine immunogenicity is enumeration of antibody-secreting cells (ASC) in peripheral blood. However, this assay, which is inherently capable of measurement of the absolute number of antigen-specific ASC, is not standardized. Thus, quantitative comparison of results between laboratories is not currently possible. To address this issue, isotype-specific ASC were enumerated from paired fresh and cryopreserved mononuclear cell (MNC) preparations from healthy adult volunteers resident in either the United States (US group) or Egypt (EG group). Analysis of fresh cells from US volunteers revealed mean numbers of ASC per 10^6 MNC of 617, 7,738, and 868 for immunoglobulin M (IgM), IgG, and IgA, respectively, whereas EG volunteers had 2,086, 7,580, and 1,677 ASC/10^6 MNC for the respective isotypes. Cryopreservation resulted in a slight reduction in group mean IgM, IgG, and IgA ASC (maximum reduction in group mean, 14%), but in no instance were results obtained with cryopreserved cells significantly lower than those obtained with fresh cells. To determine if cryopreservation affected the number of bacterial antigen-specific ASC detected, cells from a group of US adult volunteers who received a single oral dose of a mutated *Escherichia coli* heat-labile enterotoxin (LT_{R192G}) were tested. There was no significant difference ($P > 0.05$) in the number of antigen-specific IgA or IgG ASC detected between fresh and cryopreserved MNC. The results support the views that ASC assays can be standardized to yield quantitative results and that the methodology can be changed to make the test more practical.

In vivo immunogenic stimulation of B cells triggers a series of signals which cause some of these to differentiate into a more mature state wherein they secrete antibodies specific to the inducing immunogen and emigrate via the lymph chain from the site of their induction to the bloodstream (3, 5, 17, 27). These cells achieve systemic distribution via the bloodstream and subsequently extravasate and take up residence in various lymphoid tissues, where, if additional induction signals are present, they may continue differentiation to antibody-producing cells (14, 22, 25). The passage of these cells through the peripheral circulation provides an opportunity to detect an immunogenic event relatively easily, including those which occur at sites inaccessible to routine direct evaluation (1, 12, 18). Because circulating antibody-secreting cells (ASC) are transiently (2, 18) present in peripheral blood, their presence indicates a proximal stimulus. There is evidence that stimulation of the immune system also leads to a general increase in the number of circulating ASC (13, 16), possibly from polyclonal stimulation. This observation has led to the suggestion that detection of increased levels of ASC, even in the absence of a known antigenic stimulus, is a sign of ongoing or recent stimulation to the immune system (13, 19) and may be useful as a measure of the effectiveness of therapy directed against the agent causing the immune stimulation (19). Measurement of ASC in peripheral blood is a powerful tool for the analysis of the ontogeny and control of immune responses and for measurement of the immunogenicity of vaccines, and it has potential as a rapid diagnostic method. The ASC method enables proxy measurement of immune responses occurring in the gut and other mucosal surfaces which are not available for easy direct examination (8).

Considerations contributing to the failure to realize full investigational and diagnostic benefits for the ASC procedure include the following. The most commonly used procedure for detection of ASC is cumbersome and entails the isolation and culture of mononuclear cells (MNC) immediately after their collection from a donor (2, 12); an entire assay sequence must be made at each collection interval or upon presentation of each patient. The number of ASC reported as a normal level differs widely among laboratories (1, 6, 7, 10, 11, 15, 20, 23, 24), thus making the definition of what is abnormal difficult. Additionally, experience suggests that levels of ASC in the peripheral blood of healthy individuals resident in geographically distinct areas may differ significantly (19, 28), possibly indicating requirements for regional or population-based controls.

Our purpose in the experiments reported here was to begin a process leading to standardization and simplification of ASC assays, hopefully leading to a wider and more uniform application of this powerful tool. The primary issue addressed was a direct comparison of results obtained with paired fresh and cryopreserved MNC. Both methods are known to work, but there is controversy over whether the results are comparable and whether there is a loss of sensitivity associated with cryopreservation. The use of cryopreserved cells would markedly simplify assay logistics.

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MATERIALS AND METHODS

Study populations. The purpose and risks of the study were explained to all candidate volunteers, and written informed consent was obtained from all who participated. The study was reviewed and approved by the Committee for Protection of Human Subjects of the U.S. Navy Medical Research Unit Number Three, Cairo, Egypt; the Committee for Protection of Human Subjects of the Naval Medical Research Institute, Bethesda, Md.; the U.S. Army Surgeon General's Human Subject Research Review Board; and Alexandria University, Alexandria, Egypt.

Short-term (1- to 8-week) cryopreservation experiment. Fifty-five adults were included in the short-term cryopreservation experiments: 25 residents of the Washington, D.C., area (US group) (mean age, 34.5 years; standard deviation [SD], 8.6; range, 20 to 50; mode, 25; 10 females) and 30 adults from Alexandria, Egypt (EG group) (mean age, 30.2 years; SD, 8.9; range, 20 to 50; mode, 20; 19 females). Inclusion criteria were self-reported healthy status, confirmation of healthy status by a study physician, no reported acute disease within 1 month prior to enrollment, absence of known chronic disease, and completion of the informed consent process.

Long-term (64-week) cryopreservation experiment. MNC from 16 (mean age, 33.7 years; SD, 8.7; range, 20 to 50; mode, 25; 6 females) of 25 US volunteers were available for evaluation after 16 months in liquid nitrogen.

Experiments to detect *Escherichia coli* LTB-specific ASC. Sixteen adult residents of the Washington, D.C. area (mean age 28.9 years; SD, 9.7; range, 19 to 50; mode, 28; 5 female) were included in an experiment to detect *E. coli* heat-labile enterotoxin B subunit (LTB)-specific ASC. Volunteers were asked to fast for 90 min before immunization and to drink 120 ml of a bicarbonate buffer (a 0.35 M solution of $NaHCO_3$) in order to neutralize gastric acid. Five minutes after ingestion of $NaHCO_3$, they were given 5 ml of saline solution containing 5 to 100 μg of LT_{R192G} (6), followed immediately by an additional 30 ml of $NaHCO_3$. Blood samples for ASC assays were collected just prior to and 7 days after immunization. The LT_{R192G} administered in this study was prepared by the Swiss Serum and Vaccine Institute, Bern, Switzerland, in conformance with Good Manufacturing Practices guidelines. This mutated heat-labile enterotoxin molecule is currently undergoing clinical evaluation as an oral mucosal adjuvant under investigational new drug permit BB-IND-6346. Volunteers for this study were selected from the ongoing study of 36 individuals. Inclusion criteria for the present study were immunization with LT_{R192G} (not placebo) and a detectable ASC response to LTB by fresh MNC.

Isolation and cryopreservation of peripheral blood MNC. Peripheral blood was collected directly into Vacutainers containing EDTA (Becton Dickinson Vacutainer Systems, Rutherford, N.J.), and MNC were isolated by a Ficoll (Organon Teknika Corp., Durham, N.C.) density gradient (2). Cryopreservation of MNC was accomplished by suspending isolated MNC at a concentration of 4×10^6 cells/ml in RPMI 1640 (JRH Biosciences, Lenexa, Kans.) containing 50% fetal calf serum (Intergen, Purchase, N.Y.), 2 mM glutamine (JRH Biosciences), and 50 μg of gentamicin (Goldline Laboratories, Fort Lauderdale, Fla.)/ml and holding these at 4°C for 10 min before slowly adding (one drop at a time, with continuous mixing) an equal volume of ice-cold freezing medium containing RPMI supplemented with 2 mM glutamine, 50 μg of gentamicin/ml and 20% dimethyl sulfoxide (Fisher Scientific, Fair Lawn, N.J.). One-milliliter aliquots of the cell suspension were distributed into 2-ml cryovials (Nunc Inc., Naperville, Ill.) held in a 4°C wet-ice bath. The cryovials were placed, one vial per well, into a Styrofoam test tube rack designed to hold 15-ml centrifuge tubes. This test tube rack was then moved into a Styrofoam box which had been preplaced in a 4°C refrigerator. The cell suspensions were held at 4°C for 10 to 15 min, after which the box and its contents were placed in a −70°C freezer overnight. The following morning, the vials were moved to a liquid nitrogen freezer and stored there until assays were performed.

For use in assays, vials containing the required number of MNC were thawed by immersion in a 37°C water bath. Immediately after the contents of the vial thawed, 1 ml of RPMI containing 10% fetal calf serum, 2 mM L-glutamine, and 50 μg of gentamicin/ml (complete medium) was slowly added with a 1-ml pipette (one drop at a time, with continuous stirring). The contents of the cryovial were then transferred to a 15-ml polypropylene tube containing 13 ml of RPMI, mixed, and centrifuged for 15 min at $500 \times g$ at 4°C. After a second wash under the same conditions in 5 ml of complete medium, the cells were suspended in 1.5 ml of complete medium and viability was determined by trypan blue exclusion (20 μl of the cell suspension was mixed with 40 μl of 0.4% trypan blue [JRH Biosciences] in one well of a 96-well U-bottom plate [Nunc Inc.]). No more than four samples were tested for viability at one time. Immediately after mixing, the suspension was placed into a hemocytometer chamber. Cells were counted by using 10× ocular and 10× objective lenses.

ASC assays. ASC were enumerated by using a modification of the enzyme-linked immunosorbent spot-forming assay (2, 18, 19). Nunc immunoplates (MaxiSorp; Nunc Inc.) were separately coated with 100 μl (20 μg/ml) of a primary antibody in carbonate coating buffer (pH 9.6) for 1 h at 37°C, followed by overnight incubation at 4°C. Control wells were similarly coated with 10 μg of bovine serum albumin/ml. Plates were washed three times with phosphate-buffered saline (PBS)–0.1% Tween 20 (PBS-T) and blocked with 5% fetal calf serum in RPMI (for 1 h at 37°C). For detection of immunoglobulin A (IgA) and IgM ASC, MNC were adjusted to 2×10^5 viable cells/ml, and for detection of IgG ASC, they were adjusted to 2×10^4 viable cells/ml; 100-μl portions of the respective suspensions were added to separate wells. Plates were incubated for 3 h at 37°C in a humidified incubator in which the atmosphere contained 5% CO_2. Plates were then washed five times with PBS-T. The antibodies secreted during this time were detected with alkaline phosphate-conjugated immunoglobulins (see above), diluted in PBS-T supplemented with 1% fetal calf serum and 0.05% Tween 20, and incubated for 2 h at 37°C. After the plates were washed five times with PBS-T, 100 μl of barbital buffer containing 10 μg of Nitro Blue Tetrazolium, 5 μg of BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate, 4 mM $MgCl_2$, and 0.6% agarose (50°C) was applied to each well. Plates were held at room temperature for 15 min to allow the agarose to solidify and then were placed at 4°C overnight. The following day, the plates were equilibrated to room temperature and the wells were observed with a dissecting microscope (StereoZoom 7; Leica) at a magnification of ×30. Dark blue spots were counted as ASC. The numbers of spots found in wells with identical contents were summed (triplicate wells for LTB antigen-containing wells; duplicate wells for all other conditions) and adjusted to number per 10^6 MNC. In most instances, no spots were seen in control wells with bovine serum albumin. In a few instances, one or two spots were found. In these cases, the number of spots per control well was subtracted from each of the corresponding test wells.

LTB-specific ASC were detected with a slight modification of the above procedures. Plates were coated with 5 μg of LTB (provided by J. D. Clements, Tulane University, New Orleans, La.)/ml, and triplicate assays were prepared for each isotype. MNC were used at 3.3×10^6 cells/ml. The other steps in the assay were as presented above.

Cycloheximide treatment. To confirm that the immunoglobulins detected with previously frozen MNC were actively synthesized in vitro, MNC were thawed and then treated with 50 μg of cycloheximide (Sigma Chemicals, St. Louis, Mo.)/ml in bulk suspension for 1 h at 37°C, and thereafter during 3 h of incubation in microtiter plates (9). Spots were enumerated as described above.

Other reagents. Affinity-purified rabbit immunoglobulins specific for human IgM or IgA (Sigma Chemicals) were used to capture IgM or IgA secreted by MNC. A rabbit antibody specific for the $F(ab')_2$ fragment of human IgG (Cappel; Organon Teknika, West Chester, Pa.) was used as the solid-phase reagent to detect IgG ASC. These antibodies were used at a concentration of 20 μg/ml. Second antibodies included alkaline phosphate-conjugated goat anti-human IgA (0.25 μg/ml) and IgM (0.25 μg/ml) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Alkaline phosphate-conjugated goat antibody specific for the $F(ab')_2$ fragment of human IgG (Cappel; Organon Teknika) was used at a final dilution of 1:12,000 to detect IgG ASC. The following chemicals were purchased from Sigma Chemicals: Tween 20, agarose type I low EEO, barbital buffer, Nitro Blue Tetrazolium tablets, BCIP tablets, and bovine serum albumin.

Statistical analysis. Wilks-Shapiro analyses, analyses of variance (unpaired), *t* tests, confidence intervals, and box and whisker analyses were applied as indicated in Results. Statistix software (Analytical Software, Tallahassee, Fla.) was used.

RESULTS

Viability of MNC. Table 1 presents the levels of viability of the MNC determined immediately before ASC assays were performed. Fresh cells were essentially 100% viable. Freezing cells and maintaining them frozen for 1 to 8 weeks resulted in about 96% of cells being viable. Cells maintained frozen for 64 weeks had a mean viability of 92.5%. Cells stored frozen for 4 to 5 weeks (for LTB-specific ASC) were, on average, 99.5% viable.

Normality of data. To determine if data from ASC assays were normally distributed, counts of ASC were analyzed in natural form and following transformation to natural logs. In 21 (64%) of 33 comparisons, log-transformed data were more normally distributed, at times substantially more normally, than their linear counterparts. Linear data were slightly more normally distributed than log-transformed data in 11 (33%) comparisons; in 1 (3%) instance, both forms of data were equally normally distributed. Because we wanted to employ parametric statistics to define normal values and to test for effects of freezing and geographic differences, the analyses that follow were made with log-transformed data.

Effect of cryopreservation. Slightly fewer ASC were found for freeze-thawed MNC than for their corresponding fresh preparations; the frozen US samples gave on average 95% of the number of ASC found for fresh samples, and the frozen EG samples yielded 86% on average. In no instance was the group mean number of ASC after cryopreservation statistically significantly different from the number obtained when fresh

TABLE 1. Viability of MNC used for ASC assays

Site	% MNC excluding trypan blue[a]		
	Fresh	Frozen (no. of wks)	
		1–8[b]	64
United States	99.8 ± 0.55 (97.8–100.0)	96.0 ± 2.58 (86.3–100.0)	92.5 ± 5.81 (81.0–100.0)
Egypt	100.0 ± 0 (100.0)	96.4 ± 7.15 (60.0–100.0)	ND[c]

[a] Expressed as mean ± SD (range).
[b] Data from samples tested at 1, 2, 4, and 8 weeks of cryopreservation are combined.
[c] ND, not done.

cells were evaluated. Further, similar numbers of ASC were identified in aliquots kept in the freezer from 7 to 448 days.

Isotype distribution among study sites. Healthy EG volunteers had significantly higher numbers of IgM and IgA ASC than did their US counterparts. IgG ASC levels did not differ between these groups (Fig. 1).

Determination of population-specific normal values. Because the populations studied had been determined to be healthy by the inclusion screening process, the values obtained for these individuals were used to establish definitions of normal levels of ASC. Normal values were determined separately for fresh and frozen samples, each population studied, and each isotype. For samples tested without freezing, the natural log of the single result for each individual was entered into the calculations. For samples tested after freezing, for each individual, the mean of the natural-log results from the intervals between 1 and 8 weeks of freezing was determined and used in the normal-value calculations. Pooling of the data for an individual for samples maintained frozen for 1 through 8 weeks was done because previous analyses (see above) established that these values were not statistically significantly different. The group mean values and data on variance around these means are presented by study site in Fig. 1.

Evidence that cryopreservation does not adversely affect the detection of bacterial antigen (LTB)-specific ASC. MNC collected from volunteers before LT_{R192G} immunization showed no detectable LTB-specific ASC (data not shown). Table 2 presents data for LTB-specific responses measured on day 7 after immunization. Freezing did not adversely affect the detection of LTB-specific ASC; both the numbers of individuals having responses and the magnitudes of the responses were similar for fresh and frozen samples.

Evidence that ASC assays performed on cryopreserved MNC detect immunoglobulins synthesized during the assay interval. In parallel experiments, cycloheximide treatment re-

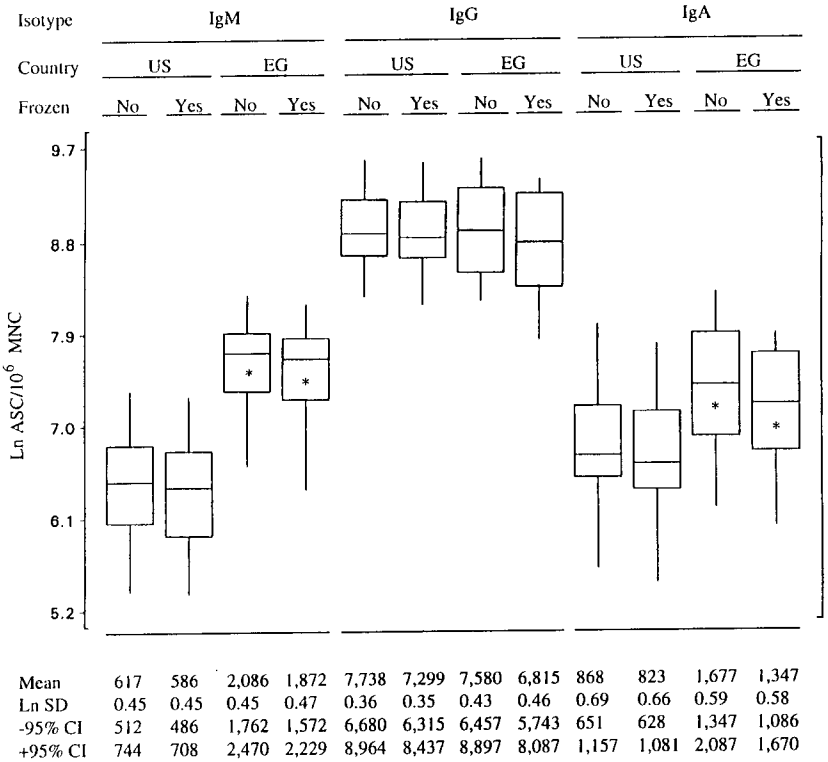

FIG. 1. Presented are numbers of ASC per 10^6 MNC by immunoglobulin isotype, country of MNC origin (United States [US] or Egypt [EG]), and frozen versus nonfrozen status. The boxes enclose the middle half of the data (data between the first and third quartiles), the lines bisecting the boxes are at the values of the medians, and vertical lines (whiskers) indicate the range of typical data. Boxes marked with asterisks denote EG groups which differ from the paired US group at P values of ≤ 0.001.

TABLE 2. Normality of data and descriptive statistics for concentration of LTB-specific ASC in fresh or frozen MNC

Isotype	Wilks-Shapiro statistic for MNC		No. of ASC/10^6 MNC[a]	
	Fresh	Frozen	Fresh	Frozen
IgG	0.949	0.931	93 ± 1.904 (1–1,327)	106 ± 1.895 (1–1,250)
IgA	0.978	0.984	83 ± 1.747 (5–1,250)	86 ± 1.629 (5–1,250)

[a] Expressed as back-transformed mean ± log SD (range).

sulted in a 94.1% ± 3.62% or 92.6% ± 2.99% reduction in LTB-specific or total ASC, respectively.

DISCUSSION

This study shows that numbers of immunoglobulin isotype- or antigen-specific ASC detected after cryopreservation of MNC do not differ significantly from those detected when fresh MNC are tested. Further, we show that the normal level of isotype-specific ASC differs between geographically isolated populations.

The capacity to use cryopreserved MNC yields several practical advantages. Because facilities for cryopreservation are more widely distributed and more transportable than are facilities capable of performing ASC assays, the use of cryopreserved cells allows for the extension of ASC technology to any study site where MNC can be cryopreserved. An additional benefit is that cryopreservation enables direct comparison (the same assay performed with the same reagents on the same day) of numbers of ASC (both total and antigen-specific) present in an individual at different times—for example, before and after vaccination or at increasing intervals from an infection or clinical illness. Because the number of ASC detected remained relatively stable even when cells were held in liquid nitrogen for more than a year, it may be possible to measure precisely over long intervals the ontogeny of ASC responses and the factors regulating them.

Using the same reagents and techniques in two widely separated laboratories, one in North America and one in Africa, we showed that normal levels of IgM and IgA ASC in healthy individuals differ for geographically separated populations. While a definitive explanation for the increased ASC in the EG population is not at hand, there is an increased frequency of infection with enteric pathogens in Egypt compared with the United States (4, 26, 29), and others have shown that isotype-specific ASC are increased in response to such infections (13, 21). It seems reasonable to suggest that the increased numbers of IgM and IgA ASC found in the EG population may reflect a more frequent stimulation of their mucosal immune systems by enteric, respiratory, or other mucosal-surface pathogens.

ASC methodology has proven, at times, the most sensitive measure of immunogenicity for a number of enteric vaccines (2, 12, 28) and thus may play an important role in protocols designed to determine the optimal formulations and delivery regimens for these. Our demonstration of different basal levels of ASC in US and EG populations raises the caution that vaccine-induced levels of antigen-specific ASC may differ between populations. The cryopreservation methodology presented in this report provides a means to address this issue directly.

As shown in Table 3, a review of the literature reveals that ASC results may vary greatly between laboratories. However, in the present study this was not the case. The numbers of isotype-specific ASC measured for healthy individuals were highly reproducible within a population and a laboratory and were reasonably reproducible between the collaborating US and EG facilities. The present result indicates that the ASC assay can be standardized to allow quantitative comparison of results between laboratories.

TABLE 3. Comparison of results of the present study with results published by others

No. of subjects	Study site	Mean ASC/10^6 MNC			Reference
		IgM	IgG	IgA	
25	United States	617	7,738	868	This study
30	Egypt	2,086	7,580	1,677	This study
32	United States	75	396	454	15
48	United States	302	1,220	792	15
39	United States	41	247	112	10
16	Sweden	500	500	4,000	7
10	Sweden	150	500	500	23
4	United Kingdom	213	NR[a]	1,288	16
37	Finland	850	1,566	1,800	11
9	Finland	1,000	7,000	2,000	20

[a] NR, not reported.

ASC methodology has proven useful in the study of the ontogeny of immune responses (12, 16), in the measurement of immunogenicity of candidate vaccine preparations (2, 8, 23), and in the detection of immune responses to clinically apparent (18, 20, 21) and asymptomatic (21, 28) infections, as well as to allergens linked to hypersensitivity reactions (10, 11). ASC methods have been particularly useful in characterizing immune responses initiated at mucosal surfaces which are not easily subject to direct observation. The procedure also has potential as a rapid diagnostic method (19) and could yield new information for epidemiologic studies. We believe that the results reported here represent an initial step towards the further refinement and standardization of this method that should reduce the technical and logistical barriers that have hampered its wide-scale application.

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