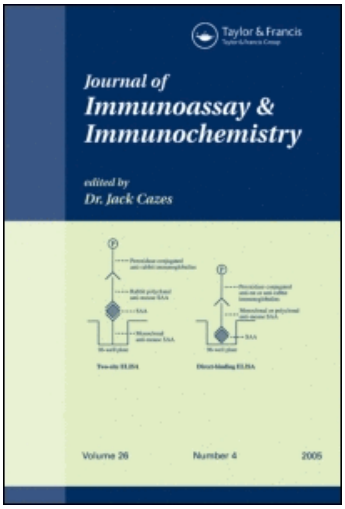

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Measurement of Immunoglobulins G, A, and M Levels in B-Lymphocytes Culture

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Abstract: Nephelometric immunoassays were developed for human IgG, IgA, and IgM quantitation in B-lymphocytes culture media. They allowed measurement of immunoglobulin (Ig) levels over a broad range of concentrations with good accuracy and precision. The kinetics of Ig production in B-lymphocyte cultures was followed and the mean amount of each Ig was determined in six different samples after three days of culture. The nephelometric immunoassays reported here could be used to study, *in vitro*, the influence of various molecules (inhibitory or amplifying effect) on B-lymphocytes' functional capacities.

Keywords: Immunoglobulin G, Immunoglobulin A, Immunoglobulin M, Lymphocytes culture media, Immunoassay, Immunonephelometry

INTRODUCTION

The analysis of B-cell differentiation and function has been a long-standing research topic. The initial demonstration of B-cells' abilities to produce immunoglobulins (Ig) came from immunofluorescent and immunochemical labelings, showing the presence of Ig in the cytoplasm of plasma cells, or cells at earlier stages of differentiation. Jerne was the first to establish the ability of single cells to produce specific antibodies in the plate assay he developed, which then became widely used.[1,2] More recently, single-cell assays for the detection of Ig-producing B-cells have rather used the

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ELISA-spot technique.[3] The two latter techniques, although very interesting for the evaluation of cell suspension's compartments, and allowing testing of both for the isotype and specificity of secreted Ig, however, do not provide information on the kinetics and amounts of production. This matter has only seldom been tackled in the literature, since the early studies demonstrating the high synthesis rate of plasma cells.[4]

In the past few years, numerous new B-cell subsets have been described at various stages of post-medullary differentiation.[5] These cells can be characterized by their topography in secondary lymphoid organs, as well as by their immunophenotype. However, studies on the functional capacities of these subsets, and their ability to respond to various stimuli, especially by producing immunoglobulins, are still almost nonexistent.

It might, nonetheless, be useful to be able to perform sensitive kinetic studies, less imprecise and time consuming than classical ELISAs, often criticized as ponderal assays. Immunonephelemetry is much more widely accepted as a reference method, allowing both accurate and fast assays which are well-suited to such studies.[6] It has been applied to a number of biological fluids, such as plasma, saliva, and milk,[7–9] but seldom to culture media. Indeed, there might be some interferences with classical methods, owing to the very different protein contents of such samples.

We report, in this study, the successful development and application of nephelometric immunoassays of human IgG, IgA, and IgM in B-lymphocytes' culture media.

EXPERIMENTAL

Lymphocyte Cultures

Palatine tonsils were obtained from 7 children, ages between 1 and 7 years old (mean 4.2 $\pm$ 2.3 years). All patients underwent surgery at a time when they displayed no sign of tonsillar inflammation or infection. All tonsil samples were forwarded to the laboratory immediately after surgery, in saline soaked gauze. Tonsil tissue was minced with scissors in sterile RPMI-1640 medium (Sigma, Saint Louis, MO, USA) and cells were further dissociated using a Medimachine and 50 μm Medicons (Dako, Golstrup, Denmark). Lymphocytes were then purified by gradient centrifugation with a Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway), and washed twice.

From each lymphocyte suspension, enriched preparations of B-cells were obtained by negative selection, using anti-CD2 metallic beads (Dynabeads M-450, Dynal, Oslo, Norway). The beads were washed four times in phosphate buffered saline (PBS) containing 0.3% bovine serum albumin (BSA, Sigma), then incubated with lymphocytes at a ratio of 10 beads for one estimated cell and gently mixed for 30 min at 4°C by tilt rotation.

Rosetted cells were attracted by a magnet, and the enriched B-cell suspensions of the supernatants were then retrieved. The quality of the enrichment was assessed in an aliquot of each preparation by flow cytometry (Epics XL, Beckman Coulter, Miami, FL, USA) after labeling with, respectively, CD7 for T-cells and CD22 for B-cells. The proportion of remaining T-cells was $0.94\% \pm 0.1\%$, and B-cell purity amounted to $99.2\% + 0.1\%$.

Each cell suspension, in RPMI medium supplemented with 2% Ultroser (Gibco BRL-Life Technologies, Rockville; MD, USA), 1% HEPES (Eurobio, Paris, France), 1% of antibiotics (Penicillin $10{,}000\,\mathrm{U}\cdot\mathrm{mL}^{-1}$, Streptomycin $10{,}000\,\mu\mathrm{g}\cdot\mathrm{mL}^{-1}$) and antifungal (Fungizone $25\,\mu\mathrm{g}\cdot\mathrm{mL}^{-1}$) (Gibco), was cultured at 2×10^6 cells/mL in 12 culture well plates (Costar, Cambridge, USA) at 37°C in a water-saturated atmosphere of a 7% CO_2 incubator (Sanyo, Tokyo, Japan) from day one to day three.

Ig Immunoassays

Nephelometric immunoassays of Ig in lymphocyte culture media were performed as a one-step test in a reaction microcuvette (Nephelia® microcuvette, Sanofi-Diagnostics-Pasteur, Marnes la Coquette, France) by mixing 100 µL of pure culture medium or diluted (1/160 to 1/2560, 1/40 to 1/1280 and 1/10 to 1/160 for IgG, IgA, and IgM, respectively) calibrator (Behring Diagnostics, Marburg, Germany) with 40 µL of anti-human IgG γ-chain, IgA α-chain, or IgM μ-chain rabbit antiserum (Behring) and 160 µL of $0.14 \cdot$ M NaCl. The scattered light was measured with a Sanofi-Diagnostics-Pasteur Nephelometer Nephelia® N600 after incubation for 30 min at room temperature. The results are expressed as mg/L of culture medium.

Precision and Analytical Recovery of the Ig Immunoassays in B-Lymphocyte Culture Media

The precision of the Ig immunoassays was assessed by measuring IgG, IgA, and IgM at low, intermediate, and high concentration, 10 times, during the same assay (within-run precision) and in 5 successive assays (between-run precision).

The Ig recovery in lymphocyte culture media was tested in dilution-overloading experiments. The dilution assays were performed on dilutions of a lymphocyte culture medium sample containing 25.9, 11.9, and 27.3 mg/L of IgG, IgA, and IgM, respectively. The overloading assays were performed by adding to a lymphocyte culture medium sample with unmeasurable Ig levels (<4.7, <1.8, <7.2 mg/L for IgG, IgA, and IgM), a human serum with high Ig concentrations (12.0, 2.3, and 1.2 g/L of IgG, IgA, and IgM, respectively) in graded proportions. The slopes, calculated by linear regression analysis, for the dilution and overloading assay performed for each Ig, were compared using a Student's *t*-test. For the total recovery of

each Ig, including dilution and overloading assays, the null hypothesis H0 (intercept = 0 and slope = 1) vs. the alternative hypothesis H1 (intercept ≠ 0 and slope ≠ 1) were tested by *F*-(Fisher) and *t*-tests, respectively. The computer program, GraphPad Prism 3.00 (GraphPad Software, San Diego, CA), was used for all data analyses and statistics.

Application to Lymphocyte Culture Media

The immunoassays of IgG, IgA, and IgM were applied to follow the kinetics of Ig production by a single lymphocyte culture during four days of cultivation. Lymphocyte culture medium was collected on each day and frozen at −20°C until tested.

The developed immunoassays were also used to determine IgG, IgA, and IgM concentrations in lymphocyte culture media collected in 6 different cultures after 3 days and were also stored at −20°C.

All samples were assayed as described above. Data obtained after 3 days of culture were analyzed using the Kolmogorov-Smirnov test for normality and Anova with Newman-Keuls post-test for comparison of Ig levels in culture media.

RESULTS

Ig Immunoassays

Calibration ranges from 4.7 to 75.0 mg/L of IgG, from 1.8 to 57.5 mg/L of IgA, and from 7.2 to 116.0 mg/L of IgM in lymphocyte culture medium (Figure 1) were obtained when the assays were performed as indicated in the Experimental section.

The precision of the Ig immunoassays in lymphocyte culture media was assessed by the CVs obtained in within- and between-run studies (from 2.7 to 9.6%) over broad ranges of concentration, as shown in Table 1.

Analytical recoveries of the Ig immunoassays in lymphocyte culture media (Figure 2) were linear ($n = 10$, $r^2 = 0.9787$, $P < 0.001$), for the range of IgG concentrations tested in the dilution-overloading assay (10.4–60.0 mg/L); $n = 11$, $r^2 = 0.9947$, $P < 0.001$, for IgA (3.0–46.0 mg/L); and $n = 8$, $r^2 = 0.9883$, $P < 0.001$, for IgM (8.6–124.7 mg/L). Mean percentages of recovery of 101%, 108%, and 91% were obtained for IgG, IgA, and IgM, respectively. The slopes of dilution (0.9324, 0.8124, and 1.166, for IgG, IgA, and IgM) and overloading (0.9458, 1.059, and 0.9987, for IgG, IgA, and IgM) curves obtained for each Ig were not significantly different ($P > 0.05$). The slopes (0.9530, 1.005, and 0.9389, for IgG, IgA, and IgM) and the intercepts (1.1040, 0.4208, and −0.8168 mg/L, for IgG, IgA, and IgM) of the total recovery curves, including dilution and overloading assays performed for each Ig, were not significantly different ($P > 0.05$) from 1 and 0, respectively.

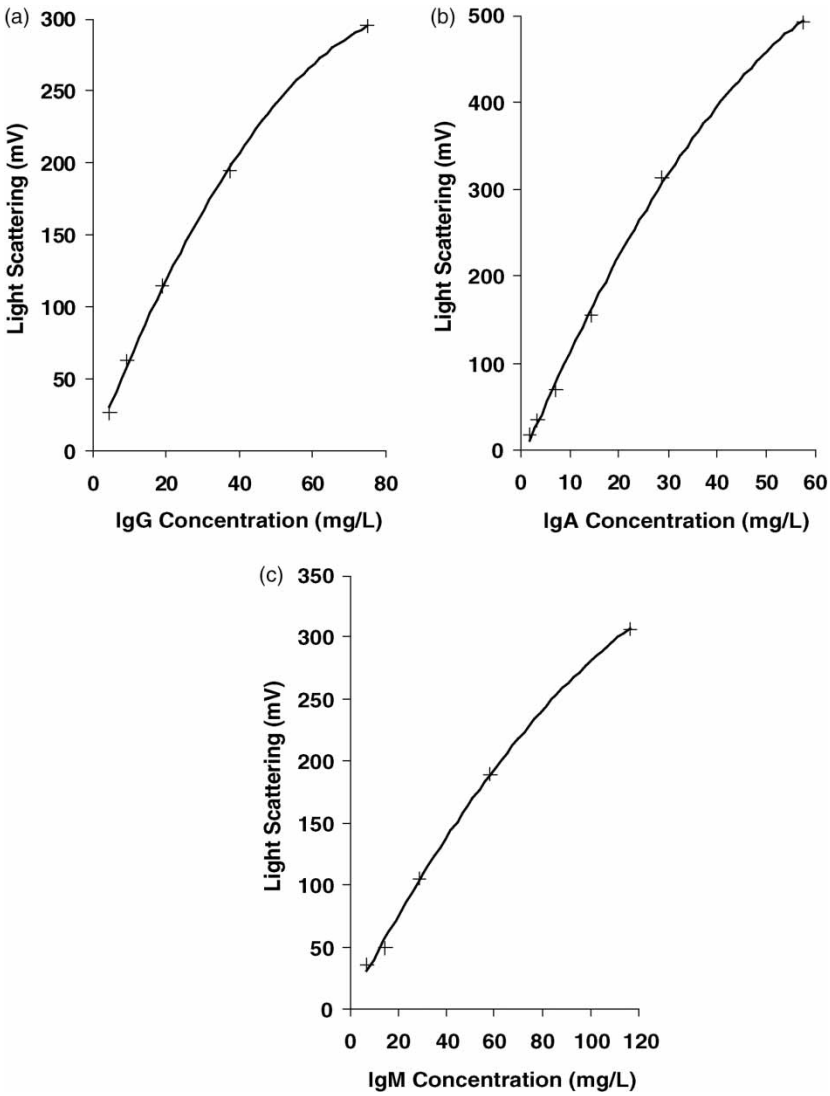

Figure 1. Calibration curve for the IgG (a), IgA (b), and IgM (c) immunoassays in B-lymphocyte culture media. The assay procedure is described in the text.

Application to Lymphocyte Culture Media

Application of the Ig immunoassays developed to follow the kinetics of Ig production by one lymphocyte culture over four days (Figure 3a) shows that the three isotypes were measurable as early as the first day. Concentrations of Ig in culture medium increased on the second day, then remained steady.

Table 1. Precision of Ig immunoassays

Ig	Within-run precision (n = 10)[a]			Between-run precision (n = 5)[a]		
IgG						
Mean (mg/L)	5.2	21.5	48.5	6.8	19.5	47.0
SD (mg/L)[b]	0.3	0.6	2.3	0.5	0.9	3.0
CV (%)[c]	5.4	2.7	4.7	7.2	4.7	6.5
IgA						
Mean (mg/L)	3.6	12.2	32.1	4.1	11.6	35.6
SD (mg/L)[b]	0.2	0.5	2.8	0.4	0.7	3.3
CV (%)[c]	6.4	4.5	8.9	8.9	6.0	9.1
IgM						
Mean (mg/L)	14.5	29.1	60.2	15.7	27.8	59.3
SD (mg/L)[b]	0.7	0.9	4.6	1.1	2.2	5.7
CV (%)[c]	4.5	3.1	7.6	7.1	7.9	9.6

[a]Number of assays.
[b]Standard deviation.
[c]Coefficient of variation.

The Ig immunoassays in lymphocyte culture media at three days of cultivation yielded the Ig levels observed in Figure 3b. Mean levels $\pm$ SEM were 14.6 ± 3.7, 4.6 ± 1.2, and 13.0 ± 2.2 mg/L for IgG, IgA, and IgM, respectively. Concentrations of IgA in culture media were lower ($P < 0.05$) than both IgG and IgM concentrations.

DISCUSSION

The nephelometric immunoassays reported here, that were developed for the determination of Ig levels in lymphocyte culture media, are easy to perform (one-step assay, without washing or phase separation) and rapid (30 min), allowing the measurement of Ig concentration over large ranges (4.7 to 75.0 mg/L of IgG, from 1.8 to 57.5 mg/L of IgA, and from 7.2 to 116.0 mg/L of IgM), with high reproducibility (CVs lower than 10% in within- and between-precisions) and accuracy (linear recovery in dilution-overloading assays).

These immunoassays allow the determination of human IgG, IgA, and IgM levels in B-lymphocytes culture media to follow the kinetics of Ig synthesis and to compare Ig levels.

Only a few reports deal with attempts at determining, quantitatively, Ig levels after B-cell cultures.[10] They used cumbersome radioimmunoassay, ELISA, reverse passive haemagglutination, or reverse hemolytic plaque assay[11–14] and showed the same isotypic partition as we did. Comparisons

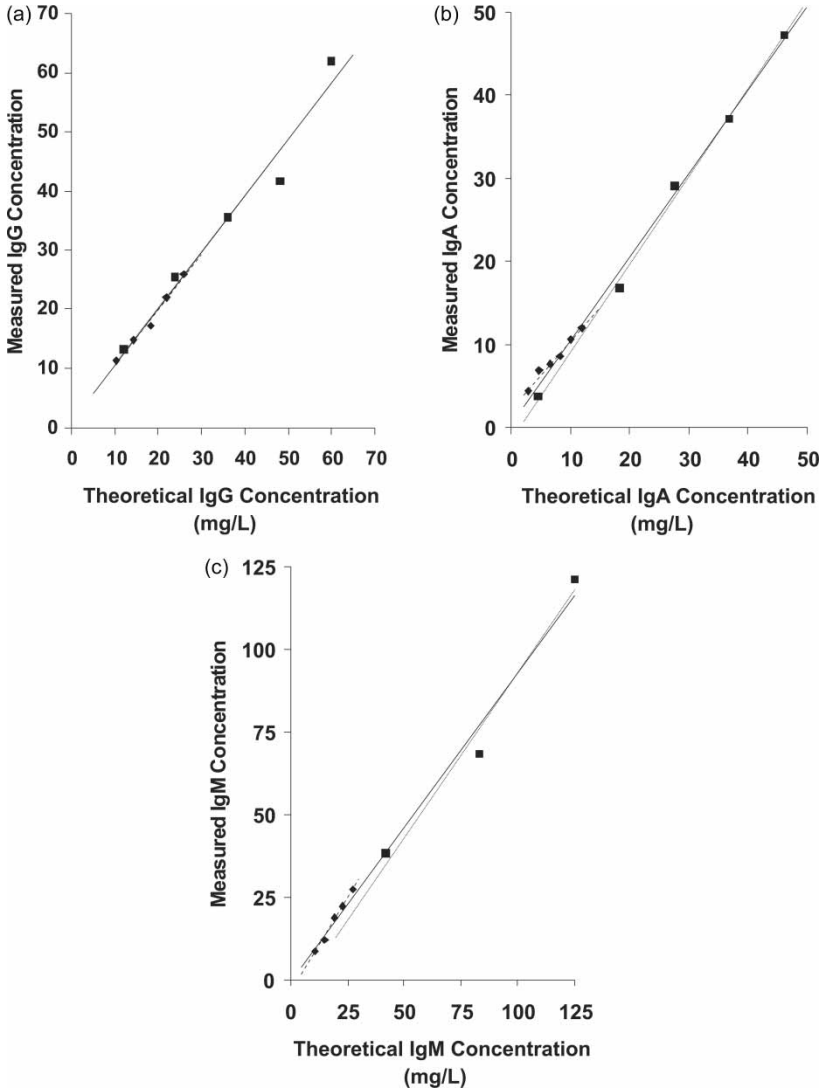

Figure 2. Analytical recovery of IgG (a), IgA (b), and IgM (c) in lymphocyte culture media: ◆ and – – –, dilution assay; ■ and ·····, overloading assay; ——, linear regression for total recovery. Experimental details and linear parameters are provided in the text.

are difficult to achieve, since these assays were performed in different culture conditions and with varying cell concentrations. Here, we used the purified B-cell compartment of tonsils, a secondary lymphoid organ with active Ig synthesis. The data obtained are consistent with the isotypic partition observed among plasma cells in tonsil sections.[15] B-cell enrichment using

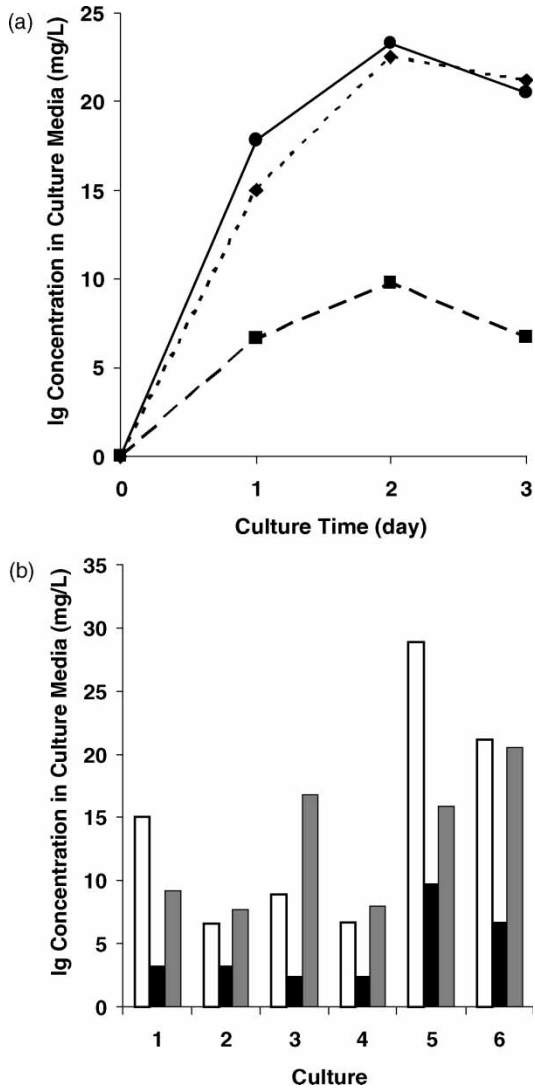

Figure 3. Immunoglobulin G, A, and M concentration in lymphocyte culture media. (a) Kinetics of Ig synthesis, (b) Ig concentrations in six lymphocytes cultures on day 3. IgG: ◆ and white bars; IgA: ■ and black bars; IgM: ● and striped bars.

microbeads had no deleterious effect on the kinetics of Ig production, which suggests that similar assays could be performed after further selection of specific B-cell compartments from secondary lymphoid organs.

The nephelometric immunoassays reported here could, thus, be used to study, not only the secretory capacities of B-cells at various stages of

activation, but also to test the *in vitro* influence of various molecules on Ig synthesis such as inhibitory or amplifying effects, owing to the large range of measurable Ig concentrations.

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