Lait (1991) 71, 493-499

Original article

Microparticle-enhanced nephelometric immunoassay for immunoglobulins G in cow's milk

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(Received 26 November 1990; accepted 15 April 1991)

Summary — A microparticle-enhanced nephelometric immunoassay of bovine immunoglobulins G is reported. It is based on the nephelometric quantification of the competitive immunoagglutination of covalently coated microparticles. This new immunoassay is easy to perform (1-step, no sample pretreatment, no washing or phase separation), fast (1 h at most), sensitive (8 µg/l of immunoglobulins G significantly detected in the reaction mixture), accurate (linear recovery of immunoglobulins G in overloaded milks) and reproducible (CVs from 2.3–10% in within- and between-run precision studies). The potential of this microparticle-based immunoassay is discussed with regard to the other quantitative immunoassays (radial immunodiffusion and conventional immunonephelometry in particular) used in the determination of milk immunoglobulins G.

immunoassay / immunonephelometry / microparticle / milk / immunoglobulins G

Résumé — Dosage des immunoglobulines G dans le lait de vache par immunonéphélémétrie sur support microparticulaire. *Les immunoglobulines G sont dosées dans le lait de vache par immunonéphélémétrie sur support microparticulaire. Des microsphères spécialement synthétisées comme support de la réaction antigène anticorps et sensibilisées par des gamma-globulines bovines sont agglutinées par un antisérum anti-immunoglobulines G. La mesure de la lumière dispersée par les agglutinats, à l'aide d'un néphélémètre adapté, quantifie cette agglutination et son inhibition par des immunoglobulines G libres, permettant ainsi leur dosage. Celui-ci est simple (une seule étape sans prétraitement des échantillons, sans lavage ni séparation), rapide (1 h maximum), sensible (seuil de détection : 8 µg/l dans le milieu de réaction), exact (récupération linéaire des immunoglobulines G dans des laits surchargés) et reproductible (CVs compris entre 2,3% et 10,0%). Les potentialités de cette nouvelle méthode de dosage des immunoglobulines G dans le lait de vache sont discutées par comparaison aux autres méthodes immunologiques (notamment l'immunodiffusion radiale et la néphélémétrie classique) déjà utilisées.*

immunodosage / immunonéphélémétrie / microparticule / lait / immunoglobulines G

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INTRODUCTION

Colostrum (Larson, 1958; Sasaki *et al,* 1976), mastitis milk (Randolph *et al,* 1974; Fox *et al,* 1981), and milk from end of lactation (Wheelock *et al,* 1967; Caffin *et al,* 1983) have an immunoglobulin G concentration which is greater than normal cow's milk (Guidry *et al,* 1980). Damage to milk quality may result from presence of these secretions in herd milk and several immunochemical methods (simple immunodiffusion (Hauke, 1966), immunoelectrophoresis (Gluhovschi *et al,* 1972), radial immunodiffusion (Newstead and Ormsby, 1970; Morris and Hobbs, 1971), and conventional immunonephelometry (Joisel *et al,* 1981; Lebreton *et al,* 1981) have been proposed for bovine immunoglobulin G (BIgG) detection or measurement.

Microparticle-enhanced nephelometric immunoassay (Nephelia, Diagnostics Pasteur, Marnes la Coquette, France) has been recently perfected (Cuillière *et al,* 1991; Montagne *et al,* 1991) for the measurement of various human serum proteins, especially immunoglobulins in clinical analysis. This new method has also been used (Collard-Bovy, 1988; El Bari-Bouchikhi, 1989) for the determination of the main cow's milk proteins (α, κ, and β-caseins, α-lactalbumin, β-lactoglobulin). It is based on the agglutination, competitive with free antigen, of antigen-coated microparticles in the presence of specific antiserum. Free antigen can then be quantified by measuring the light scattered by agglutinates of coated microparticles with a nephelometer. This microparticle-enhanced nephelometric immunoassay uses monodispersed hydrophilic microspheres (MS) specifically conceived as nephelometric markers of the antigen antibody reaction (Duheille *et al,* 1982), and which can be covalently coated with proteins (MS–protein conjugate).

We describe here the synthesis of MS, the preparation of MS-bovine gamma globulins (BGG) conjugate which can be used to perform a microparticle-enhanced nephelometric immunoassay of BIgG and an evaluation of the practicability of this BIgG assay in cow's milk.

MATERIALS AND METHODS

Reagents

Acrolein, 2-hydroxyethylmethacrylate, methacrylic acid, Triton X-100 and ethylenediaminetetraacetic acid disodium salt (EDTA–Na_2) were purchased from Merck (Darmstadt, Germany). *N,N'*-methylenebisacrylamide came from Eastman Kodak Co (Rochester, USA). Sodium dodecyl sulfate, hydroquinone, 2-aminoethanol, boric acid, sodium azide and polyethyleneglycol (PEG) 6 000 were obtained from Prolabo Rhône Poulenc (Paris, France). Whole BGG and human serum albumin (HSA) (99% electrophoretically pure) were from Sigma Chemical Co (Saint Louis, MO, USA). BIgG (98% electrophoretically pure, estimated molecular ratio IgG1/IgG2: 3/1) and rabbit anti-BIgG (equilibrated avidity anti-heavy chains $\gamma 1 + \gamma 2$) antisera were kindly supplied by Diagnostics Pasteur (Marnes la Coquette, France). Cow's milk specimens used for recovery and precision studies were collected without specification of breed or lactation period.

Preparation of MS–BGG conjugate

MS were produced as previously reported (Montagne *et al,* 1991) by copolymerization (gamma irradiation from ^{60}Co, 25 krad/cm^2/h; 3 h) of a mixture (120 g/l) of acrylic monomers (2-hydroxyethylmethacrylate 49.7%, acrolein 47%, methacrylic acid 2%, and *N,N'*-methylenebisacrylamide 1.3%) in the presence of sodium dodecyl sulfate (0.9 g/l) as surfactant. MS were stored under argon at 4 °C in hydroquinone (1 g/l). Their size, dispersion and concentration were studied as previously described (Duheille *et al,* 1982).

BGG were bound to these MS by mixing 50 mg of MS with 1.8 mg of BGG and 6 mg of HSA in 5 ml of 0.05 M borate buffer pH 8.0. After 2 h at room temperature and 14 h at 4 °C, 0.25 ml of a 2.7 M 2-aminoethanol buffered solution pH 7.8 was added to the binding mixture that had been left to rest for 2 h at room temperature. Coated MS were then collected by centrifugation (8 000 *g*, 1 h) at the interface of a discontinuous sucrose gradient (200/800 *g*/l in 0.05 M borate buffer pH 8.0). They were stored at 4 °C in 0.05 M borate buffer pH 8.0 containing 30 mM NaN_3.

Immunonephelometric study of MS–BGG conjugate

All immunonephelometric studies were carried out with 0.05 M borate buffer pH 8.0 containing 1.5 mM EDTA–Na_2, 30 mM NaN_3, 2 g/l of Triton X-100 and 30 g/l of PEG 6 000 (milk nephelometry buffer). Immunoreactivity of the MS–BGG conjugate (25 and 50 mg/l) was studied by measuring the light scattered by MS–BGG conjugate clusters during their competitive agglutination with anti-BIgG antiserum (125–32 000-fold diluted) in the presence of free BIgG (0 to 0.5 mg/l), with the Diagnostics Pasteur nephelometer, Nephelia N600 (Cuillière *et al,* 1991). The nephelometer light source was an He-Ne laser (power 2 mW, wavelength 632.8 nm); the reagents were distributed in disposable Nephelia microcuvettes (light path 1 cm) and the scattered light was collimated at an angle of 10° on a light-sensitive silicium diode.

MS–BGG conjugate as a reagent for BIgG immunoassay

BIgG immunoassay in milk was performed following 2 procedures: 10 µl (or 40 µl) of 500-fold diluted milk (BIgG serial dilution for calibration curve) were mixed together in a reaction microcuvette with 25 µl (or 100 µl) of 100-fold diluted antiserum, 440 µl (or 310 µl) of milk nephelometry buffer and 25 µl (or 50 µl) of MS–BGG conjugate suspension (0.5 g/l). All dilutions and dispensations were carried out in the milk nephelometry buffer, with an automated dilutor-dispenser (Hamilton, Bonaduz, Switzerland) and the scattered light was measured with the nephelometer (5 s per microcuvette) after 1 h (or 12 min) at rest at room temperature.

The use of the MS–BGG conjugate as a reagent for BIgG immunoassay in milk was evaluated by analytical recovery and precision studies. Analytical recovery was tested by comparing results of BIgG determination in 6 dilutions (1/1 to 1/2.5) of a milk overloaded by 3.1 g of BIgG per l with those of BIgG determination in overloaded milk by 6 increasing amounts of BIgG (0.3–1.9 g/l). Precision was assessed by measuring low, middle, and high BIgG concentrations in milk 10 times in the same run (within-run precision) and on 5 successive runs (between-run precision).

RESULTS

Hydrophilic, polyfunctional and monodispersed MS of 235 nm (SD = 6 nm, $n = 36$) dry diameter were produced by the described copolymerization of acrylic monomers. BGG and HSA as stabilizing proteins were covalently bound to these MS by imine bonds between aldehyde groups on MS and primary amino sites on proteins (Duheille *et al,* 1982) with binding yields of 25 and 8% respectively. MS–BGG conjugate kept its stability and immunoreactivity for several months when stored at 4 °C.

MS–BGG conjugate suspensions were not autoagglutinated and their sedimentation rate was very slow in the milk nephelometry buffer (variation of the light scattered by the MS–BGG conjugate after 2 h at rest: 1.1%, SD = 0.6%, $n = 20$). But they were strongly aggregated by the anti-BIgG specific antiserum. The nephelometer permitted measurement of the light scattered by the MS–BGG clusters (fig 1) during competitive agglutination performed in the presence of various concentrations of free BIgG. Increase of light scattering was faster and greater for 50 mg/l of MS–BGG conjugate and 500-fold diluted anti-BIgG antiserum (fig 1A) than for 25 mg/l of con-

jugate and 2 000-fold diluted antiserum (fig 1B). However, the 50 mg/l MS–BGG conjugate agglutination was completely inhibited by free BIgG concentration > 504 µg/l, while only 126 µg/l were necessary to inhibit in a similar manner the 25 mg/l MS–BGG conjugate agglutination with 2 000-fold diluted antiserum. In the latter conditions, 7.88 µg/l was the lowest BIgG concentration to give a significant inhibition (light scattering 3 SD lower than the mean of light scattered without BIgG).

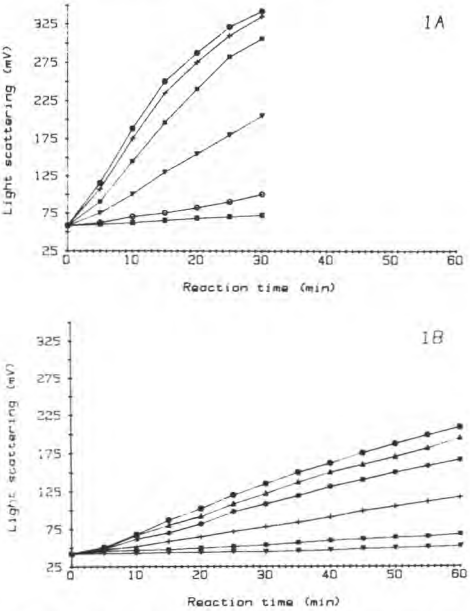

Fig 1. Kinetics of MS-BGG conjugate agglutination. MS–BGG conjugate, 50 mg/l, and 500-fold diluted antiserum (1A); MS–BGG conjugate, 25 mg/l, and 2 000-fold diluted antiserum (1B); free BIgG concentration (µg/l) in reaction mixture: 0 (●), 7.88 (▲), 15.75 (*), 31.5 (+), 63 (■), 126 (▼), 252 (○), and 504 (⧗).

Cinétique de l'agglutination du conjugué MS-BGG. Conjugué MS-BGG, 50 mg/l, avec antisérum dilué au 1/500 (1A); conjugué MS-BGG, 25 mg/l, avec antisérum dilué au 1/2000 (1B); BIgG libres dans le milieu de réaction (µg/l) : 0 (●), 7,88 (▲), 15,75 (*), 31,5 (+), 63 (■), 126 (▼), 252 (○), and 504 (⧗).

Figure 2 shows sigmoidal inhibition curves obtained after a reaction time of 12 min for 50 mg/l of MS–BGG conjugate with 500-fold diluted antiserum and after 1 h for 25 mg/l of MS–BGG conjugate and 2 000-fold diluted antiserum. Observed inhibition range (from 31.5–504 µg/l of BIgG in reaction mixture for the first system and from 7.88–126 µg/l for the second) showed that BIgG could be measured between 0.20 and 3.15 g/l in cow's milk, with respectively a 6 250-fold and 25 000-fold sample dilution, by a microparticle-enhanced nephelometric immunoassay using these MS–BGG conjugates as reagent.

Evaluation of this immunoassay was carried out by recovery and precision studies using the 25 mg/l MS–BGG conjugate agglutination with 2 000-fold diluted antiserum, as this procedure required the greatest dilution of milk sample (1/25 000).

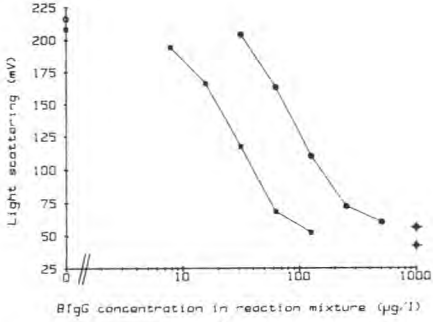

Fig 2. Inhibition of MS-BGG conjugate agglutination by free BIgG. MS-BGG conjugate, 50 mg/l, antiserum diluted 500-fold, reaction time, 12 min (●); MS-BGG conjugate, 25 mg/l, antiserum diluted 2000-fold, reaction time, 1 h (■); agglutination without BIgG (○, ❑); light scattered by MS-BGG conjugate alone (+,+).

Inhibition par BIgG de l'agglutination du conjugué MS-BGG. Conjugué MS-BGG, 50 mg/l, antisérum dilué au 1/500, temps de réaction, 12 min (●); conjugué MS-BGG, 25 mg/l, antisérum dilué au 1/2000, temps de réaction, 1 h (■); agglutination sans BIgG (○, ❑); lumière dispersée par le conjugué MS-BGG seul (+,+).

Analytical recovery was linear between 0.35 and 3.15 g/l of BIgG (mean percentage of recovery = 105.2%, recovered BIgG = 0.12 + 0.96 added BIgG, n = 12, r = 0.98). Within- and between-run precision results are given in table I; CVs ranged from 2.3–10.0%.

DISCUSSION

The nephelometric quantification of MS–BGG conjugate immunoagglutination, competitive with free BIgG, allowed the possibility of a new microparticle-enhanced nephelometric immunoassay for milk BIgG, to be considered.

The immunological specificity of such an immunoagglutination is connected with the ligand bound to the MS or with the agglutinating reagent: the anti-heavy chains γ1 and γ2 antiserum used brought this specificity. Due to the great homology between the γ1 and γ2 chains, it is difficult to obtain a polyclonal anti-γ1 (or anti-γ2) specific antiserum simply by immunisation with the purified heavy chains, and purified whole IgG1 (or IgG2) are unusable as immunogens because antisera produced in this manner react with the light chains of the other immunoglobulins. A more discriminating agglutination specificity could be obtained by using monoclonal antibodies, but the analytical specificity for the inhibition of agglutination and consequently for the described immunoassay, is principally connected with the inhibiting agent used for its calibration. Therefore, the molecular ratio IgG1/IgG2 in the standard is the main parameter for reliability of such an immunoassay.

The obtained calibration range easily covered usual (0.35–0.70 g/l) BIgG milk concentrations (Newstead and Ormsby, 1970; Mach and Pahud, 1971; Guidry *et al,* 1980) and slightly increased BIgG concentrations (Joisel *et al,* 1981) in mastitis milk (0.83 g/l) and in milk at end of lactation (1.26 g/l). In case of very increased concentrations (colostrum up to 100 g/l for the first milking (Levieux, 1978), the selected inhibition mode fully protected against the risk of analyte underevaluation by antigen excess, which may be encountered in all immunoassays based on a non competitive antigen–antibody reaction.

Microparticle-enhanced nephelometric immunoassay was particularly easy to perform. It was a 1-step assay, without washing or phase separation: diluted milk, diluted anti-BIgG antiserum, and MS–BGG conjugate were mixed together in a disposable reaction cuvette. After incubation at room temperature for 1 h at most, light scattering was automatically measured by the specially designed nephelometer at a rate of 360 samples per h. The required concentrations were calculated by compar-

Table I. Precision of the assay.
Precision du dosage.

Within-run precision				*Between-run precision*			
n	*Mean (SD)*	*g/l*	*CV (%)*	*n*	*Mean (SD)*	*g/l*	*CV (%)*
10	3.05	(0.07)	2.3	5	2.50	(0.06)	2.4
10	0.55	(0.03)	5.4	5	0.73	(0.02)	2.7
10	0.38	(0.03)	7.9	5	0.40	(0.04)	10.0

ison with a smoothed calibration curve, performed in the same run or, on account of the stability of the reagents, recalculated from stored data from a previous assay. A 24-h diffusion period is necessary to obtain reliable results (linearity and slope of the standard curve) by radial immunodiffusion (Newstead and Ormsby, 1970; Morris and Hobbs, 1971). Compared with the described microparticle-enhanced nephelometric immunoassay, radial immunodiffusion does not allow such a fast determination of BIgG in milk, and is less adapted to a large series of measurements.

Results of the precision study assessed the reproducibility of the assay performed with the 25 000-fold diluted milk, in spite of this significant dilution. Compared to conventional immunonephelometry (Joisel *et al,* 1981; Lebreton *et al,* 1981), this dilution, made possible by the sensitivity of the assay, avoids any interference and provides the possibility of work without sample blank or milk clarifying pretreatment, which decreases cost, duration and complexity of the assay.

MS–BGG conjugate, obtained by covalent binding of BGG on microparticle specifically conceived as an antigen antibody reaction support, can therefore be used as a reagent to measure milk BIgG in a microparticle-enhanced nephelometric immunoassay. This immunoassay is easy to perform, sensitive, fast and fully automatizable. Accuracy, reproducibility and the complete safety at the highest concentrations indicate that this new BIgG nephelometric immunoassay shows great promise for the routine detection of colostrum, mastitis milk and milk at end of lactation.

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