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Antigenic stimulation with goat and cow milk by oral and parenteral route in guinea pigs

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The aim of this study was to examine the sensitisation capacity of goat milk (GM) and cow milk (CM) by the oral or the parenteral route, and thus determine whether the serologic responses developed are different, identify the proteins targeted by the antibodies thus formed, and determine whether these antibodies depend on the immunisation route employed. Enzyme-linked immunosorbent assay was used to determine the specific antibodies of the isotypes IgG1 and IgG(Fc) developed against GM and CM. The sera from the animals immunised parenterally developed a stronger serologic response than did those immunised by the oral route. When CM was used as the immunising agent, the specific isotopic response was stronger than that obtained with GM. There were no differences in the antigen profile revealed by the sera of the animals immunised by the oral or parenteral routes. A marked degree of cross-reactivity between the two types of milk was found.

Keywords: goat milk antibodies; cow milk antibodies; oral route; parenteral route; ELISA; Western blot

Introduction

The proteins of cow milk (CM) are among the first antigens with which an infant comes into contact; normally, this is the first non-homologous antigen received in significant quantities. Cow milk protein allergy (CMPA) is the predominant food allergy in babies (Wood, 2003). Milk allergy is due to the absence of oral tolerance to CM proteins. Nowadays it is known that CM proteins, in some circumstances, are responsible for hypersensitivity reactions, not only among babies but also in children and adults. The reported prevalence of CMPA in infants and adults varies between studies, in part due to the difficulties in accurate diagnosis, differences in the age of study populations and the clinical assessment criteria used. However, it is clear that CMPA is most prevalent in early childhood, with generally reported figures of 2–6% (García-Ara, Boyano-Martínez, Díaz-Peña, Martín-Muñoz, & Martín-Esteban, 2004; Hill et al., 1997), and decreases in adulthood to 0.1–0.5% (Bindels & Hoijer, 2000; Woods, Thien, Raven, Walters, & Abramson, 2002).

It has been suggested that allergenic activity may be related to β -lactoglobulin, α -lactalbumin, seroalbumin, gammaglobulin and casein, but none of the proteins in CM in particular have been identified as being its principal allergen. A recent review

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by Wal (2001) concluded that no structural or functional characteristic within any of the CM proteins could be considered to be associated with allergenicity. Koritz, Suzuki, and Coombs (1987) found evidence of the presence of β -lactoglobulin in the gastrointestinal content of guinea pigs and rats that had been sensitised with whole CM by oral route. They also detected the presence of caseins, but only in the duodenum in the guinea pigs. Suzuki, Koritz, and Coombs (1987), using the same sensitisation route and the same animal model, detected antibodies against β -lactoglobulin in the serum of guinea pigs but not in that of rats.

One of the various proposals for ceasing of CMPA is the replacing of CM by GM. Park (1994) highlighted the importance of this substitution for patients with CMPA, recommending it on the grounds that, in his opinion, tolerance was practically 100% among patients with CMPA. Restani (2004) observed that GM seems to present a lower allergenic power than CM, but also cited various cases in which there had occurred an allergic reaction among people of different ages following the consumption of either GM or products derived from it. Moreover, anaphylactic reaction to GM in a CM allergic patient has been observed (Pessler & Nejat, 2004), and allergy to GM can occur in a CM tolerant patient, involving mainly caseins (Ah-Leung et al., 2006).

Taking into account this background and its importance, we believe it is necessary to continue investigating this subject. The aim of this study is to determine whether sensitisation to GM or CM, by the oral or parenteral route, produces a different serologic response, and also to establish against which protein or proteins the antibodies are directed, and whether the target molecules against which they are formed depend on the sensitisation route employed. Our experimental animal was the guinea pig, and the level of antibodies in the IgG1 and IgG(Fc) isotypes was determined using the ELISA technique. The Western blot test was applied to determine the antigen profile of the two types of milk in the sera of the animals sensitised by each of the two routes.

Materials and methods

Milk proteins

The GM was from Granadina goats and the CM was from Holstein Friesian cows. Table 1 shows the fractional composition of the GM and CM used, this being determined by means of the near infrared spectroscopy (NIRS) method (Burns & Ciurczak, 2001; Gómez-Ruiz, Miralles, Agüera, & Amigo, 2004). The protein concentration of GM and CM was determined using the bicinchonic acid method (Smith et al., 1985), and the aliquots obtained were subsequently stored at -80°C until required.

Animals and experimental protocol

Forty-eight male *Dunkin Harley* guinea pigs with a mean body weight of 220 g at weaning and free from specific pathogens, including ectoparasites and endoparasites (Harlan Ibérica, Barcelona, Spain) were used throughout this study. They were housed, individually, in stainless steel wire-bottomed cages in ecologic chambers maintained under controlled conditions ($21\text{--}24^{\circ}\text{C}$, 50–60% relative humidity, diurnal

Table 1. Protein fraction composition (g/100g total protein) of goat milk (GM) and cow milk (CM).

	GM	CM
Total casein	81.7	82.0
Total whey protein	18.3	18.0
α_{S1} -casein	16.9	32.6
α_{S2} -casein	11.5	8.5
β -casein	47.4	30.2
κ -casein	5.9	10.7
Serum albumin	2.3	1.5
α -lactalbumin	5.5	6.4
β -lactoglobulin	10.5	10.1

light cycle of 12 h). Two assays were designed: in the first one, animal sensitisation was carried out by the oral route and in the second one was by the parenteral route. All work was carried out following European Union and Spanish rules and guidelines regarding the ethical treatment of laboratory animals (Jefatura del Estado, 2007).

First assay: oral sensitisation

Three groups of eight guinea pigs were used. Two of them were for oral sensitisation to GM or CM proteins (Groups II and III, respectively). The other one (Group I) was the control group. All animals were given a standard diet free from milk proteins (Harlan Ibérica, Barcelona, Spain), and water was also provided. In addition, the following test products were given in liquid form: GM and CM (Groups II and III, respectively). The milk protein content was 32.6 g/l. For both types of milk, the protein concentration was standardised to that measured in the milk with the lowest such content. The standard diet, water and milk were administered *ad libitum*. The milk intake was maintained for 15 days, and from days 16 to 22, the only liquid provided was water. Sera samples were taken from the orbital sinus at Day 0 and by cardiac puncture under anaesthesia, at 22 days after initiating the experiment. Subsequently, the animals were killed by intraperitoneal administration of sodium pentothal. Sera aliquots were stored at -80°C until needed for serologic study.

Second assay: parenteral sensitisation

Three groups of eight guinea pigs were used. Eight animals were given injections of GM (Group II) and another eight, CM (Group III) at a protein concentration of 32.6 g/l. The other group (Group I) was used as control. The protein solution (1 ml) was injected intraperitoneally on Day 0, and a second, booster, injection (300 μl) was given on Day 15 (Bevilacqua et al., 2001). Sera samples were taken from the orbital sinus at Day 0 and by cardiac puncture under anaesthesia at 25 days after initiating the experiment. Subsequently, the animals were killed by intraperitoneal administration of sodium pentothal. Sera aliquots were stored at -80°C until needed for serologic study.

Serologic study*ELISA test*

The specific antibodies (IgG1 and IgG(Fc)) against GM and CM were detected by a modified ELISA (Gómez García et al., 2003). The plates (Nunc MaxiSorp Surface, Roskilde, Denmark) were coated with 100 µl/well of 0.05 µg/µl of GM and CM, in 0.1 M carbonate buffer (pH 9.6), sealed and incubated for 30 min at 37°C. Unbound antigen was removed by decanting. The plates were washed three times for 5 min in a wash solution (tap water, 0.05% Tween-20; Merck, Darmstadt, Germany). This procedure was used between each step. Then, individual serum samples, 1:4 or 1:512 and two-fold serial dilutions to the endpoint, first and second assay, respectively, were added to the plates, and sealed and incubated for 30 min at 37°C. Goat anti-guinea pig IgG1 or goat anti-guinea pig IgG(Fc) conjugate to peroxidase (Serotec) diluted to 1:5000 and 1:10,000, respectively, in PBS/0.05% Tween-20 (Merck), was incubated for 30 min at 37°C. The plates were developed by H₂O₂ as substrate and chromogen 2,2'-azino-bis (3-ethylbenzyl-thiazoline-6-sulfonic acid; ABTS; Sigma-Aldrich), kept for 15 min at room temperature in the dark. The reaction was stopped with 1% SDS (Merck). The plates were read at 405 nm in a Bio-Rad Microplate Manager version 4.0 program. A limiting optical density (OD) value was calculated by adding to the mean of the OD values of three sera replicates from Day 0 for each animal, the results of multiplying the standard deviation by three. The OD values above this limit were considered positive.

In order to obtain a quantitative evaluation of the differences in the production of systemic antibodies, with respect to the different immunising solutions, we used the OD values obtained from three replicates of each serum sample, diluted to 1:64 or 1:4096 (first and second assays, respectively).

Western blot test

This test was applied to detect and identify the different antibodies developed against GM and CM in guinea pigs after oral and parenteral immunisation (Laemmli, 1970; Towbin, Staehelin, & Gordon, 1979). Forty micrograms of protein per lane from each solution were used. The protein was separated in 17% reducing SDS polyacrylamide gels (SDS-PAGE; Bio-Rad). After electrophoresis (400 V at 4°C), the proteins were transferred to nitrocellulose sheets (Protran Ba 85, 0.45 µm, Whatman GmbH, Dassel, Germany) in a Mini Trans Blot cell (Bio-Rad) for 12 h at 30 V and 4°C. After transfer, the membrane was rinsed three times (5 min each time). This procedure was used between each step. Strips 0.4 mm thick were cut and incubated at a dilution of 1:10 (first assay) or 1:50 (second assay) of guinea pig individual serum samples in PBS/0.3% Tween-20 for 1 h. After washes in PBS/0.3% Tween-20 (Merck), a 1:500 or 1:1000 dilution of a goat anti-guinea pig IgG1 or IgG(Fc) peroxidase conjugate, respectively, (Serotec) in PBS/0.3% Tween-20 (Merck) was added for 1 h. After a new washing cycle, peroxidase was developed with hydrogen peroxide (Sigma-Aldrich) and 4-chloro-1-naphthol (Sigma-Aldrich). A prestained molecular weight marker (precision plus protein standards; Bio-Rad) and caprine β-casein isolated by HPLC (Technical Services, Granada University, Spain) were included. The antigen profiles obtained were analysed using Quantity One 4.4.0 (Bio-Rad).

The cross-reactivity among the milk proteins was determined by blotting, following the protocol described above. After the electrotransfer of GM and CM had been performed, the nitrocellulose sheet was tested against the individual serum samples from the animals immunised by the oral route with CM and GM, respectively. The same procedure was followed for the parenteral route.

Statistical analysis

In the serologic tests, differences between mean OD values at dilutions of 1:64 or 1:4096 (first and second assays, respectively) were assessed by an ANOVA with Duncan's multiple range test at a 5% level of significance.

Results

The nutritional status of the guinea pigs in the different assays was satisfactory, and there were no significant differences in the weights of the animals in the different groups at the end of the assays, or in the consumption of the corresponding immunising solutions (data not reported here).

Serologic study

ELISA test

In the oral route, all sera were found to have specific antibodies against GM and CM, for the IgG(Fc) isotype. For the IgG1 subclass, six were positive against GM, and eight against CM. In the sera of the control animals, the reaction was negative. With the parenteral route, all the animals were positive for IgG1 and IgG(Fc). The systemic antibodies developed by the animals immunised intraperitoneally, for both isotypes, were stronger than those developed by the oral route, with titres of up to 1:16,384 for the parenteral route vs. 1:512 for the oral route.

Figures 1 and 2 (oral and parenteral immunisation, respectively) show the mean OD values for the dilutions assayed for the two isotypes. In every case, the OD values were higher in the animals sensitised with CM than with GM.

Tables 2 and 3 (oral and parenteral immunisation, respectively) show the mean OD values corresponding to the dilutions of 1:64 (oral route) and 1:4096 (parenteral route), for the specific antibodies of the IgG1 subclass and the IgG(Fc) class, and the results of the statistical analysis. For both isotypes (Table 2), the values corresponding to the animals given CM were higher than those given GM, and the corresponding differences presented a level of significance of $P=0.08$ for IgG1 and $P<0.05$ for IgG(Fc). In the case of the animals that were immunised with GM, the OD values obtained for both isotypes were very similar ($P=0.99$), and it was also found for the animals that had been immunised with CM ($P=0.89$).

With respect to the animals sensitised parenterally with CM and GM, the specific serologic responses were stronger, for both isotypes, than those developed by oral sensitisation. For both isotypes (Table 3), the values corresponding to the animals injected with CM were higher than those injected with GM. However, the corresponding differences were not statistically significant ($P>0.05$) for IgG1 and IgG(Fc) ($P=0.51$ and $P=0.26$, respectively). In animals injected with CM or GM,

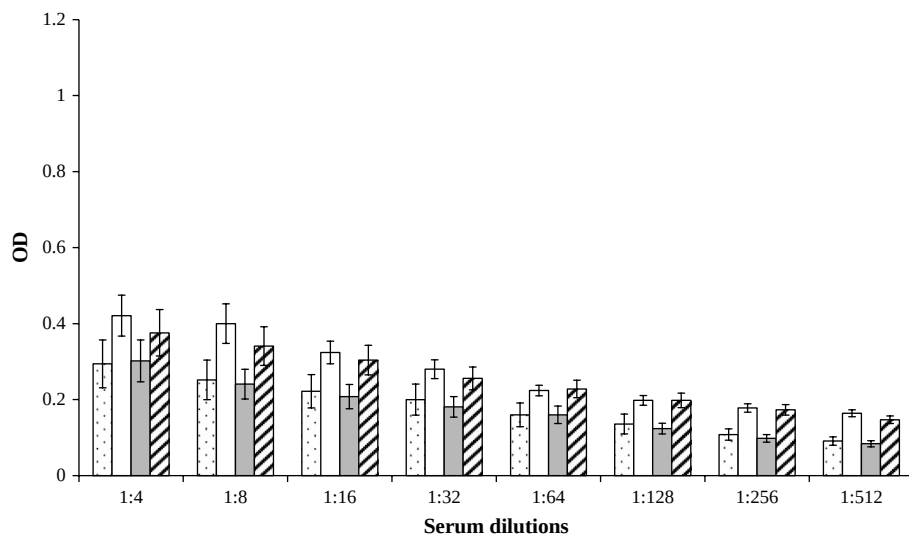


Figure 1. Mean optical density (OD) values of specific immunoglobulin obtained by ELISA in each serum dilution of guinea pigs ($n=8$) sensitised by the oral route: $\square$ goat milk antigenic solution incubated with sera of animals sensitised against goat milk and revealed with anti-guinea pig IgG1; $\square$ cow milk antigenic solution incubated with sera of animals sensitised against cow milk and revealed with anti-guinea pig IgG1; $\blacksquare$ goat milk antigenic solution incubated with sera of animals sensitised against goat milk and revealed with anti-guinea pig IgG(Fc); $\hatched$ cow milk antigenic solution incubated with sera of animals sensitised against cow milk and revealed with anti-guinea pig IgG(Fc); vertical lines represent standard errors of the means.

the OD values obtained for IgG1 were higher than those detected for IgG(Fc) ($P < 0.01$). This result was different from that obtained for sensitisation by the oral route, which produced similar OD values for both isotypes.

Western blot test

Figure 3 shows the antigen polypeptide profile (homologous reaction). In Lanes 1 and 2, bands correspond to markers of molecular weights and β -casein, respectively, while in Lanes 3, 4, 5 and 6, it refers to GM and CM by the oral route, and GM and CM by the parenteral route, respectively. The polypeptide profiles were similar for each of the two immunisation routes. In general, the apparent molecular weights of the antigen molecules that were revealed were in the range of 13–84 kDa. In the case of oral sensitisation (Lanes 3 and 4), we observed two bands in the zones of high molecular weights, both for GM and for CM, and three in the case of parenteral immunisation (Lanes 5 and 6), with the apparent molecular weights corresponding to lactoferrin, seroalbumin and Ig heavy chain. The antibodies developed by each of the two routes also revealed the casein complex, Ig light chain and β -lactoglobulin. The two isoforms of α -lactalbumin (normal and glycosylate) were only detected when the animals were immunised by the intraperitoneal route. Antigen polypeptides were more intense in the case of the intraperitoneal route than with the oral route.

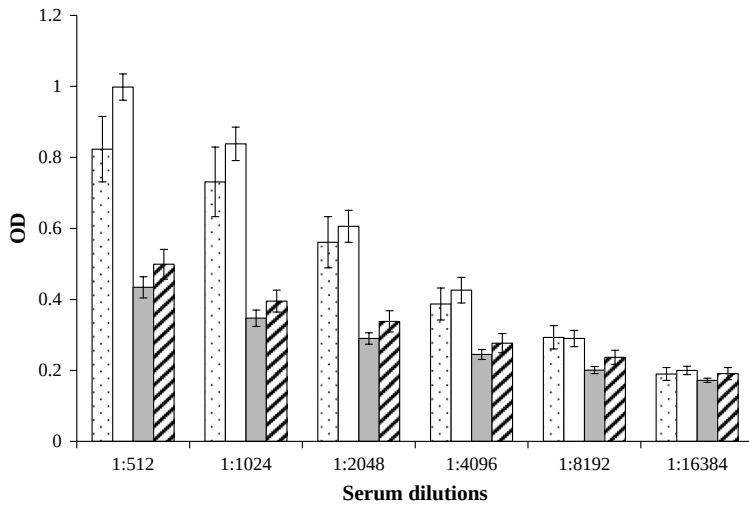


Figure 2. Mean optical density (OD) values of specific immunoglobulin obtained by ELISA in each serum dilution of guinea pigs ($n=8$) sensitised by the parenteral route: $\square$ goat milk antigenic solution incubated with sera of animals sensitised against goat milk and revealed with anti-guinea pig IgG1; $\square$ cow milk antigenic solution incubated with sera of animals sensitised against cow milk and revealed with anti-guinea pig IgG1; $\blacksquare$ goat milk antigenic solution incubated with sera of animals sensitised against goat milk and revealed with anti-guinea pig IgG(Fc); $\hatched$ cow milk antigenic solution incubated with sera of animals sensitised against cow milk and revealed with anti-guinea pig IgG(Fc); vertical lines represent standard errors of the means.

The antigen profile for the cross-reactivity (Figure 4); it is similar to Figure 3 (homologous reaction) except in the zone of high molecular weights, where only one band is revealed for the oral-route immunisation, both for GM and for CM. The rest of the antigenic profile corresponded to the casein complex, Ig light chain, β -lactoglobulin and α -lactalbumin. Once again, the two isoforms of α -lactalbumin were only apparent in the sera of the animals immunised by intraperitoneal route. In this case, too, polypeptides were more intense for the animals immunised intraperitoneally.

Discussion

To the best of our knowledge, this is the first time that a comparative study of antigenic stimulation with whole GM and whole CM, by the oral and parenteral routes, has been carried out in order to determine whether the serologic responses produced by GM and CM, with these two immunisation routes, give rise to different responses. Our study was carried out in guinea pigs, as this experimental model has been amply employed in type I allergenicity assays. It was first described by Parish, Richards, France, and Coombs (1964) as a model for studying allergy to CM proteins, and it has been widely used for allergy assays with milk formulas (McLaughlan, Anderson, Widdowson, & Coombs, 1981).

Table 2. Mean optical density (OD) values of specific IgG1 and IgG(Fc) obtained by ELISA in the serum of animals immunised by the oral route against the different antigenic solutions. Statistical analysis results.

Specific antibody	Limit values of positivity (OD)	OD ^a	Contrast	RSD ^b	Level of significance (<i>P</i>)
IgG1 GM	0.141	0.160	IgG1 GM vs. IgG1 CM	0.069	0.08
IgG1 CM	0.157	0.224	IgG1 GM vs. IgG(Fc) GM	0.078	0.99
IgG(Fc) GM	0.155	0.160	IgG(Fc) GM vs. IgG(Fc) CM	0.063	<0.05
IgG(Fc) CM	0.142	0.228	IgG1 CM vs. IgG(Fc) CM	0.055	0.89

^aSera dilution 1:64.

^bRSD, residual standard deviation.

Note: IgG1 GM, subclass of immunoglobulin against goat milk; IgG1 CM, subclass of immunoglobulin against cow milk; IgG(Fc) GM, class of immunoglobulin against goat milk; IgG(Fc) CM, class of immunoglobulin against cow milk.

It has been shown that IgE antibodies play an important role in mediating type I hypersensitivity responses in humans (Martin, Galli, Katona, & Drazen, 1989). In the case of food allergy, it is believed that food-specific IgE antibodies bind to high-affinity Fcε RI receptors on mast cells, basophils, macrophages and dendritic cells, as well as to low-affinity Fcε RII receptors on macrophages, monocytes, lymphocytes, eosinophils and platelets (Sampson & Ho, 1997). When food allergens penetrate mucosal barriers and contact IgE antibodies bound to mast cells or basophils, histamine and other mediators that induce symptoms of immediate hypersensitivity are released.

Table 3. Mean optical density (OD) values of specific IgG1 and IgG(Fc) obtained by ELISA in the serum of animals immunised by the parenteral route against the different antigenic solutions. Statistical analysis results.

Specific antibody	Limit values of positivity (OD)	OD ^a	Contrast	RSD ^b	Level of significance (<i>P</i>)
IgG1 GM	0.1923	0.387	IgG1 GM vs. IgG1 CM	0.116	0.51
IgG1 CM	0.1852	0.426	IgG1 GM vs. IgG(Fc) GM	0.095	<0.01
IgG(Fc) GM	0.1648	0.245	IgG(Fc) GM vs. IgG(Fc) CM	0.017	0.26
IgG(Fc) CM	0.1462	0.277	IgG1 CM vs. IgG(Fc) CM	0.086	<0.01

^aSera dilution 1:4096.

^bRSD, residual standard deviation.

Note: IgG1 GM, subclass of immunoglobulin against goat milk; IgG1 CM, subclass of immunoglobulin against cow milk; IgG(Fc) GM, class of immunoglobulin against goat milk; IgG(Fc) CM, class of immunoglobulin against cow milk.

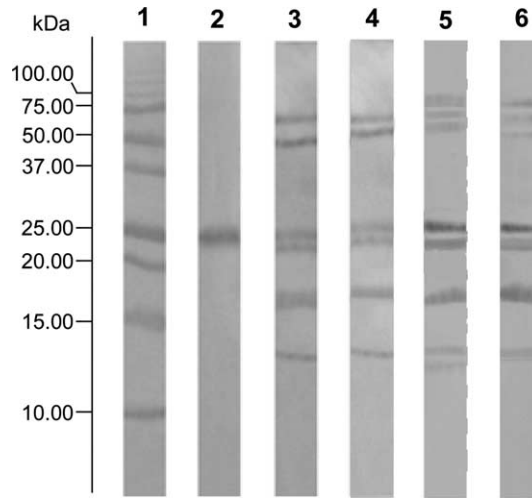


Figure 3. Immunoblot pattern IgG1 specific to both kinds of milk incubated with sera of animals sensitised against the same antigenic solutions to which they were sensitised by the oral route (goat milk, Lane 3 and cow milk, Lane 4) and by the parenteral route (goat milk, Lane 5 and cow milk, Lane 6); molecular mass Standard (Bio-Rad), Lane 1, and goat β -casein isolated by the Technical Services of Granada University and incubated with sera of animals sensitised to goat milk, Lane 2.

Various authors have found in experiments with guinea pigs that in hypersensitivity reactions mediated by IgE, IgG1 antibodies are also present, both in passive cutaneous anaphylaxis (PCA) reactions (Ovary & Warner, 1972) and in those of systemic anaphylaxis (Bevilacqua et al., 2001). We measured the levels of specific IgG1 and IgG(Fc) against antigen solutions, using the ELISA technique, for the two types of milk, as these two isotypes are present in these kinds of reactions, and because their systemic level is higher than that of the IgE, which makes their detection in serum more feasible. Indeed, Lara-Villoslada, Olivares, Jiménez, Boza, and Xaus (2004), in a study of atopy in mice, reported that antigen-specific IgE levels were too low to be detected.

With respect to sensitisation by the oral route, our results for GM and CM are in agreement with those obtained by Lara-Villoslada et al. (2004), who in the above-mentioned study found higher specific IgG1 values against CM than GM. Suzuki et al. (1987) carried out a study of antigen stimulation with whole CM, using guinea pigs and rats, and detected circulating antibodies against β -lactoglobulin by passive haemagglutination in the serum of guinea pigs, but did not detect it in that of rats. Koritz et al. (1987) reported that the production of antibodies against only β -lactoglobulin is due to the higher resistance of this fraction to gastrointestinal enzymic hydrolysis. Bevilacqua et al. (2001) studied systemic sensitisation in guinea pigs against the proteins of CM and GM, and found that the animals that consumed CM proteins developed higher levels of specific antibodies of the IgG1 subclass against β -lactoglobulin than did those given GM. Recently, Sanz Ceballos, Sanz Sampelayo, Gil Extremera and Rodríguez Osorio (2009) find higher values of IgG1

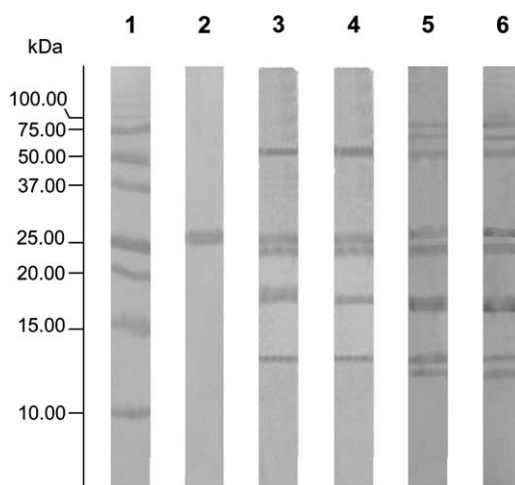


Figure 4. Cross-reaction IgG1 specific incubated with sera of animals sensitised to both kinds of milk by the oral route (goat milk, Lane 3 and cow milk, Lane 4) and by the parenteral route (goat milk, Lane 5 and cow milk, Lane 6); molecular mass standard (Bio-Rad), Lane 1 and goat β -casein isolated by the Technical Services of Granada University and incubated with sera of animals sensitised to goat milk, Lane 2.

antibodies in guinea pigs fed with CM than those fed with GM. In the same way, in the anaphylaxis tests, the responses were higher in the animals sensitised with CM than those sensitised with GM.

With respect to sensitisation by the intraperitoneal route, we obtained a greater serologic response than with the oral route, with higher OD values among the animals injected with CM than among those injected with GM. These results are in accordance with those obtained by Bevilacqua et al. (2001), who also performed intraperitoneal sensitisation with guinea pigs, using two types of GM, varying by α_{s1} -casein content, and CM. These authors reported higher antibody values for the animals injected with CM. Moreover, the antibodies developed were not only against β -lactoglobulin but also, although to a lesser extent, against α -lactalbumin and caseins. The authors concluded that this could be due to the fact that the milk proteins obtained intraperitoneally did not undergo gastrointestinal enzymatic hydrolysis, and therefore retained their natural antigenicity. Sanz Ceballos (2007) studied the possible interaction between the composition and/or nutritional utilisation of each type of milk and their respective immunogenic capacity, and found that GM protein has a higher digestibility than CM. Besides this, the digestible protein of GM is more effectively utilised at metabolic level. These facts would thus account for the differences between the two immunisation routes when either whole GM or whole CM was used.

In general, there were no differences in the antigen profile revealed by the sera of the animals immunised by the oral or parenteral routes, with GM or CM (Figure 3), except in the zone of high molecular weights, where among the animals that had been immunised intraperitoneally there were showed three bands, rather than the two that were revealed with the sera of those immunised by the oral route. In

addition, in the case of the animals that were sensitised intraperitoneally, in the zone corresponding to α -lactalbumin, there were two bands corresponding to the two isoforms. It is also noteworthy that we detected antibodies against the caseins, a result that is corroborated by the findings of other authors, who in studies of human sera have detected antibodies against this protein (Muñoz Martín et al., 2004).

The Western blot test showed that there is a marked degree of cross-reactivity between the two types of milk (Figure 4); this result is in agreement with those reported by other authors (Restani, Beretta, Fiocchi, Ballabio, & Galli, 2002). None of the proteins in CM in particular were identified as its main allergen. In a recent review of the subject by Wal (2001), it was concluded that no structural or functional characteristic present in any one of the milk proteins can be considered to be associated with allergenicity, although some homologue regions may account for the IgE type cross-reactivity.

Conclusions

From the present study, we conclude that the serologic responses obtained with the sera obtained from intraperitoneal immunisation were always greater than in the case of oral sensitisation. Similarly, and irrespective of the immunisation route employed and of the immunoglobulin isotype, the animals that were sensitised with CM presented higher OD values than those sensitised with GM. However, from a comparison of certain OD values, we conclude that this latter effect takes place to a significant degree only in the case of oral immunisation.

Furthermore, the Western blot test showed that, independently of the type of milk and of the immunisation route utilised, there is a clearly apparent band in the area of the caseins. This test also showed that there is a marked degree of cross-reactivity between the two types of milk.

Therefore, it would be of interest to continue investigating on this subject, to identify precisely which molecule or molecules are responsible for the antigenic reactivity when the oral sensitisation route is employed.

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