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Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin

T. C. MCGUIRE, A. J. MUSOKE & T. KURTTI *International Laboratory For Research On Animal Diseases, Nairobi, Kenya*

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Summary. Bovine immunoglobulin G subclass (IgG1 and IgG2) antibodies were found to fix bovine complement while only IgG1 fixed guinea-pig complement *in vitro*. Similar results were noted when IgG1 and IgG2 antibodies were tested by passive cutaneous anaphylaxis (PCA) in that both IgG1 and IgG2 caused PCA in bovine skin while only IgG1 mediated the reaction in rat skin. In precipitation reactions IgG1 antibodies to DNP failed to cause precipitation of DNP_{19}-ovalbumin while IgG2 antibodies to DNP precipitated DNP_{19}-ovalbumin. Both IgG1 and IgG2 antibodies to ovalbumin precipitated ovalbumin. Surprisingly, IgG2 antibodies to equine erythrocytes caused phagocytosis by bovine neutrophils and peripheral blood monocytes while IgG1 antibodies failed to cause either phagocytosis or adherence. Results with peripheral blood monocytes cultured for 7 days demonstrated that both IgG1 and IgG2 could mediate phagocytosis.

INTRODUCTION

The bovine immunoglobulin system closely resembles that of other species with respect to both physiochemical properties and nomenclature. Well characterized classes include IgG, IgM (reviewed by Butler, 1969), IgA (Mach, Pahud & Isliker, 1969; Vaerman, Heremans & Van Kerckhoven, 1969), and IgE (Hammer, Kickhofen & Schmid 1971; Wells & Eyre, 1972; Nielsen, Holmes, Wilkie & Tizard, 1976). The IgG class contains two documented subclasses, IgG1 and IgG2, which have antigenic differences in the Fc portion of the heavy chains (reviewed by Butler, 1969). A possible third IgG subclass, IgG3, has been reported (Babel & Lang, 1976), but remains unconfirmed. It is presumed that additional heterogeneity awaits discovery.

Importance of IgG subclasses in other species resides in part with their functional properties, usually associated with the Fc portion of the molecules. Receptors for Fc of some IgG subclasses are present on a number of cells including lymphocytes, monocytes-macrophages, platelets, neutrophils and basophils-mast cells (reviewed by Spiegelberg, 1974). When immunoglobulins are bound to antigen the Fc portion may interact with Clq and activate the complement system, although immunoglobulin subclasses in some species do not fix complement by the classical pathway (reviewed by Spiegelberg, 1974). Additionally, non-complement fixing antibodies in sufficient quantity will inhibit the binding of complement fixing antibodies (Johnson & Allen, 1968b; McGuire, Van Hoosier & Henson, 1971). Predominance of antibodies in a subclass unable to activate important phlogogenic

Correspondence: Dr T. C. McGuire, Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164, U.S.A.

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pathways, such as complement fixation (CF) and phagocytosis, could compromise the host in some infectious disease processes.

Experiments in this paper evaluate the ability of bovine IgG1 and IgG2 to cause phagocytosis by macrophages and neutrophils, to activate bovine and guinea-pig complement, to form a precipitate with different antigens and to cause homologous and heterologous passive cutaneous anaphylaxis (PCA).

MATERIALS AND METHODS

Specific immunoglobulin preparation

A 2 year old Hereford steer was immunized with DNP_{60}-equine IgG, serum collected, and the anti-DNP antibodies removed on a DNP-lysine-agarose column as described previously (Wofsy & Burr, 1969). Eluted antibody appeared to be primarily IgG1, IgG2 and IgM when examined by immunoelectrophoresis against rabbit anti-bovine serum. The eluate was separated on a Bio-gel A 1·5 column (Bio-Rad Laboratories, Richmond, California) to obtain IgM. The IgM fraction was purified further by isolectric focusing in a pH gradient of 5–7 (LKB-Produkter AB, Bromma, Sweden). The IgG fraction was dialysed with 0·005 M sodium phosphate buffer at pH 8 and placed on a DEAE-cellulose column equilibrated with the same buffer. The immunoglobulin fraction which failed to bind to the column was IgG2 and bound immunoglobulin was removed with a linear gradient from 0 to 0·3 M NaCl in the starting buffer. All IgG1 fractions were found to contain small amounts of IgG2. The IgG1-rich fractions were pooled, dialysed against 0·001 M sodium phosphate pH 7·5 containing 0·5% glycine and then separated on an isoelectric focusing column with a pH gradient of 6–8. Polyethylene glycol was used to stabilize the pH gradient. Those IgG1 fractions on the lower end of the pH gradient lacked IgG2.

Antisera were made in goats to purified IgG1, IgG2 and IgM anti-DNP antibodies. Antiserum to IgG1 was absorbed with IgG2 coupled to agarose while anti-IgG2 was absorbed with IgG1 coupled to agarose. Anti-IgM serum was absorbed with both IgG1 and IgG2 coupled to agarose. Specificity of the antisera was checked by immunodiffusion, immunoelectrophoresis and finally by double antibody radioimmunoassay using ^{125}I-labelled IgG1, IgG2 or IgM (Barbet & McGuire, 1978). Reactivity of antisera to IgG1, IgG2 and IgM were also compared with those previously described (McGuire, Pfeiffer, Weikel & Bartsch, 1976) and with those from a commercial laboratory (Miles Laboratories, Inc., Elhart, Indiana).

Three Hereford cows (2 years old) were immunized with horse erythrocytes and sera collected (Banks & McGuire, 1975). Specific antibody was separated from serum by absorption to horse erythrocytes, washing and subsequent elution at 56° (Landsteiner & Miller, 1925). Eluates contained IgG1, IgG2 and IgM and these were separated and evaluated as described for the DNP antibodies.

A Hereford cow (2 years old) was injected with ovalbumin and the serum collected and passed through an ovalbumin-agarose column. Attached antibodies were eluted with glycine–HCl 0·2 M, pH 2·5 and immediately brought to neutral pH. Subsequently, IgG1, IgG2 and IgM fractions were purified and evaluated as described for the DNP antibodies.

Preformed immune complexes

Fractions containing various amounts of IgG1 or IgG2 antibodies were centrifuged at 9650 ***g*** for 5 min and incubated with ovalbumin at equivalence for 1 h at 37° and for 48 h at 4°. The precipitates were recovered by centrifugation and washed three times with 0·005 M veronal buffered saline, pH 7·4. Immune complexes of IgG2 and DNP were also prepared. The precipitates were dissolved in 1 M NaOH and the protein concentration determined by a described method (Lowry, Rosebrough, Farr & Randall, 1961). Since IgG1 and DNP failed to produce any visible precipitates, various antibody to antigen ratios (μg/μg) between 30:1 and 200:1 were used.

Complement fixation

To measure CF, immune complexes were incubated for 90 min at 37° with bovine or guinea-pig serum diluted to provide approximately 10 CH_{50} units/ml. Antibody or antigen alone was included as control. After incubation the precipitates were removed by centrifugation and the complement activity remaining in the supernatant determined (Rapp & Borsos, 1970). Rabbit erythrocytes sensitized with sheep haemolysin were used as the indicator system for bovine complement (Barta & Barta, 1972). When guinea-pig serum was used as the source of complement, the procedure was similar to that described except that sheep erythrocytes sensitised with rabbit haemolysin were used as the indicator system.

Passive cutaneous anaphylaxis

Homologous short term PCA was performed in normal Freisian calves weighing about 75 kg. The calves were shaved on the flank and several concentrations of ovalbumin antibodies in 0·2 ml quantities were injected intradermally. The antibodies were heat inactivated at 56° for 1 h and centrifuged at 9650 ***g*** for 5 min before use. Two and four hours later the calves were challenged intravenously with 40 ml PBS containing 80 mg of ovalbumin and 1% (w/v) Evans blue dye. The reactions were read 15–30 min after challenge and graded 0 to 4+. Positive reactions varied from small areas of intense blueing (1+) to raised blue circular zones 2 cm or greater in diameter (4+).

Heterologous PCA was carried out with adult female rats. The rats were shaved and 0·1 ml of antibody dilutions injected intradermally over the back. Two and four hours later the rats were challenged with 0·5 ml PBS containing 1 mg of ovalbumin and 1% Evans blue dye. Reactions were evaluated as described for the homologous PCA.

Peripheral blood monocytes and neutrophils

Blood from cattle was collected in heparin (10 units/ml) and a buffy coat of leucocytes obtained by centrifugation for 10 min at 200 ***g***. Leucocytes were diluted in 2 parts minimal essential medium (MEM), layered onto Hypaque–Ficoll (Boyum, 1968) having a specific gravity of 1·077 g/ml and centrifuged at 4° for 35 min (325 ***g***). Leucocytes at the interface of Hypaque–Ficoll and MEM were collected, washed once for 5 min at 225 ***g*** and two additional times for 5 min at 170 ***g***. The washed cells were composed predominantly of lymphocytes with the remainder being monocytes. Neutrophils were obtained from the bottom of the Hypaque–Ficoll separation tube and washed as described for the mononuclear cells.

Monocyte cultures

Leucocytes for culture were isolated from the peripheral blood of Freisian cattle using the erythrocyte lysis method. Each 25 ml of heparinized blood was mixed with 45 ml of sterile distilled water. After 30 s 5 ml of a ten-fold concentrated solution of Earle's balanced salt solution (calcium- and magnesium-free) (Gibco Bio-Cult,Glasgow, Scotland) was added. The leucocyte suspension was then centrifuged at 100 ***g*** for 10 min. The cell pellet was washed three times in Earle's balanced salt solution and then resuspended in culture medium. Cell viability was determined with the trypan blue dye-exclusion test and generally ranged from 90 to 99%. Aliquots of 10 ml containing 5×10^6 cells/ml were inoculated into 25 cm^3 Falcon flasks and the cultures incubated at 37°. After 24 h the unattached cells were removed and the cell layer washed with culture medium. The medium was replaced every 2–4 days. The medium was RPMI-1640 with 25 mM of HEPES buffer (Associated Biomedic Systems, Inc., Buffalo, N.Y.) and 20% of gamma globulin-free, mycoplasma-tested and virus-screened foetal calf serum (Gibco, Grand Island, N.Y.). Prior to use, the serum was heat-inactivated at 56° for 30 min. These manipulations enabled us to initiate and maintain cell layers composed predominantly of monocytes that remained attached to the culture flask.

Phagocytosis and adherence assay

Chambers were made with Lucite rings having an 8 mm inner diameter and coverslips (Berken & Benacerraf, 1966). Washed leucocytes (0·1 ml of 1×10^7/ml) from the Hypaque–Ficoll separation were added to the chambers, incubated for 30 min at 37° and then washed three times with MEM containing 2% foetal calf serum. The cells attached to glass at this stage were over 95% monocytes as determined by Giemsa staining of fixed coverslips and by the accumulation of dye in nuclear areas when stained live with 0·002% neutral red solution. These monocytes were also phagocytic when presented with optimally sensitized erythrocytes. Erythrocytes were sensitized with various amounts of antibody for 30 min at 37°, washed three times and resuspended to a 1% solution in MEM (Banks & McGuire, 1975). Sensitized erythrocytes (0·1 ml) were added to the chambers for 1 h at 37°, washed three times and examined. Viability was determined by trypan blue dye (0·5%) exclusion. Three hundred monocytes were examined microscopically for either phagocytosis or adherence. Cells were scored as phagocytosing when one or more erythrocytes were internalized while adherence required three or more erythrocytes attached to a monocyte.

RESULTS

Precipitation

All the antigen–antibody combinations tested caused visible reactions in tube precipitation and double immunodiffusion with the exception of IgG1 antibodies to DNP reacted with DNP_{19}-ovalbumin. In the later case an extensive range of antibody and antigen con-

centrations were tried with negative results. Comparison of quantitative precipitin curves of IgG1 antibodies to ovalbumin with those of IgG2 antibodies to ovalbumin yielded almost identical results.

Complement fixation

Both IgG1 anti-DNP and IgG1 anti-ovalbumin antibodies fixed guinea-pig complement when complexed with their respective antigen (Table 1). It should be noted that the IgG1 anti-DNP antibodies and DNP_{19}-ovalbumin complexes were soluble. In comparison to IgG1-antigen complexes neither IgG2 anti-DNP and DNP_{19}-ovalbumin complexes nor IgG2 anti-ovalbumin and ovalbumin complexes were able to fix guinea-pig complement (Table 1). When bovine complement was incubated with the same four antigen–antibody complexes all were capable of fixation (Table 2). The IgG1 anti-ovalbumin and ovalbumin complexes fixed more bovine complement than an equivalent amount of IgG2 anti-ovalbumin and ovalbumin complexes (Table 2).

Table 1. Fixation of guinea-pig complement by bovine antibody–antigen complexes

Antibody	Antigen	Amount of complexes (μg)	Amount of complement fixed (%)
Anti-ovalbumin IgG2	Ovalbumin	51	<1
		24	<1
		15	<1
Anti-DNP IgG2	DNP_{19}-ovalbumin	230	<1
		130	<1
		40	<1
Anti-ovalbumin IgG1	Ovalbumin	130	57
		81	50
		40	38
Anti-DNP IgG1	DNP_{19}-ovalbumin	(13)*	38
		(50)*	25
		(200)*	25

* Since no precipitate occurred in this situation a constant amount of antibody was added (100 μg) along with varying amounts of antigen. The numbers given in parenthesis are the ratios of antibody to antigen.

Passive cutaneous anaphylaxis

Both IgG1 and IgG2 antibodies to ovalbumin caused a PCA reaction in bovine skin at several concentrations (Table 3). In contrast, only IgG1 antibodies to ovalbumin caused a similar reaction in rat skin while IgG2 antibodies to ovalbumin failed to mediate the reaction (Table 3).

Phagocytosis and adherence

Bovine peripheral blood monocytes readily phagocytosed erythrocytes coated with IgG2 antibodies (Fig. 1). Under the same conditions, IgG1 antibodies caused neither phagocytosis nor adherence. When peripheral blood neutrophils were tested the results were identical to those found with monocytes; IgG2 mediated phagocytosis while IgG1 did not. Additional studies with peripheral blood monocytes cultured for more than 7 days gave results that were different from freshly isolated monocytes. Cultured monocytes phagocytosed both IgG1- and IgG2-coated erythrocytes, but not unsensitized erythrocytes.

The peripheral blood monocytes would not interact with erythrocytes coated with IgM until the addition of equine complement. After addition of equine complement almost all of the cells bound three or more erythrocytes (Table 4). Bovine complement did not cause the binding of IgM-coated erythrocytes to monocytes.

Table 2. Fixation of bovine complement by bovine antibody–antigen complexes

Antibody	Antigen	Amount of complexes (μg)	Amount of complement fixed (%)
Anti-ovalbumin IgG2	Ovalbumin	96	22
		48	19
		30	12
Anti-DNP IgG2	DNP_{19}-ovalbumin	143	31
		69	30
		34	23
Anti-ovalbumin IgG1	Ovalbumin	72	35
		32	39
		13	15
Anti-DNP IgG1	DNP_{19}-ovalbumin	(50)*	40
		(100)*	38

* Since no precipitate occurred in this situation a constant amount of antibody was added (100 μg) along with varying amounts of antigen. The numbers given in parenthesis are the ratios of antibody to antigen.

Table 3. Passive cutaneous anaphylaxis with bovine antibodies to ovalbumin

Reaction site	Antibody subclass	Antibody amount (μg)	Incubation time* (h) 2	Incubation time* (h) 4
Homologous (bovine skin)	IgG1	300	++++	++++
		100	+++	+++
		50	++	++
	IgG2	300	++++	++++
		100	++++	+++
		50	++	+
Heterologous (rat skin)	IgG1	50	++	++
		25	–	–
	IgG2	50	–	–
		25	–	–

* The incubation time was measured from injection of antibody into the skin until the intravenous injection of antigen and Evans blue dye.

DISCUSSION

Failure of bovine IgG2 to fix guinea-pig complement has been demonstrated by several people (Murphy, Osebold & Aalund, 1966; Pierce, 1967; Plackett & Alton, 1975; Patterson, Deyoe & Stone, 1976). The same observation has been made with sheep IgG2 (Esteves, Santanna, Dos Santo 'Annes & Binaghi, 1974) and with goat IgG2 (Micusàn & Bordvas, 1977). Supplementing the guinea-pig complement with 5% bovine serum enchanced CF by some bovine antisera, especially those reacting with soluble antigens (Knight & Cowan, 1961; Boulanger & Bannister, 1960; Rice & Currière, 1961). Our preliminary experiments suggested that addition of 5% bovine serum to guinea-pig complement did not cause fixation by isolated IgG2 antibodies (data not shown). It seemed important, however, to test bovine IgG1 and IgG2 antibodies

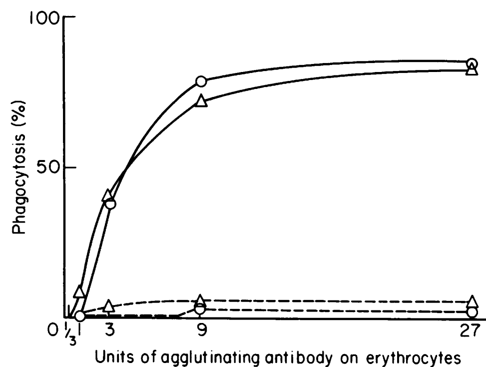

Figure 1. Evaluation of the interaction of bovine IgG1 (dashed lines) and IgG2 (solid lines) erythrocyte antibodies with freshly isolated bovine peripheral blood monocytes. o, Cow A; △, Cow B.

with completely homologous complement. We found that both IgG1 and IgG2 antibody–antigen complexes were able to fix bovine complement *in vitro* and the data indicated that IgG1 might be slightly more efficient than IgG2. The results with homologous complement are similar to findings with sheep IgG2 antibodies and sheep complement, although, the sheep data were not quantitative and the assay system were unusual (Feinstein & Hobart, 1969).

Table 4. Interaction of IgM-coated erythrocytes with peripheral blood monocytes

Substance reacted with erythrocytes	Adherence (%)	Phagocytosis (%)
IgM	1	0
IgM + equine C	90	5
IgM + bovine C	2	0

The complement-fixing efficiency of bovine IgG1 antibody–antigen complexes was low when tested with guinea-pig and bovine complement (Tables 1 and 2) as was IgG2 antibody–antigen complexes when reacted with bovine complement (Table 2). It is possible that the Fc portion of the immunoglobulin molecules was damaged during the purification procedures resulting in poor complement-fixing abilities. Another explanation for the low efficiency of CF is that the alternative pathway was being measured. We feel that the classical pathway rather than the alternative pathway of CF was measured for the following reasons: (1) in order to provide 10 CH_{50} units of bovine complement serum dilutions of 1:15 or greater were required which should dilute out alternative pathway activity (Sandberg & Osler, 1971) and (2) additional experiments using 2 mM Mg and 10 mM EGTA (Fine, Marney, Colley, Sergent & Des Pres, 1972) blocked fixation of bovine complement by complexes of both IgG1 and IgG2 antibodies and DNP_{19}-ovalbumin supporting the idea that CF was by the classical pathway.

The ability of bovine IgG subclasses to cause PCA reactions in bovine skin was not clear from the literature. One paper that stated that IgG1 and not IgG2 sensitized bovine skin (Milstein & Feinstein, 1968) while another indicated that both IgG1 and IgG2 could cause short term PCA reactions while IgG1 would cause long term (48 h) reactions (Pierce, 1967). No experimental data were given in either paper. Our results show that both IgG1 and IgG2 cause short-term PCA reactions in bovine skin. Goat IgG2 (Micusàn & Bordvas, 1977) and sheep IgG2 (Esteves *et al.*, 1974) mediate homologous PCA while neither goat nor sheep IgG1 caused the reaction. Only bovine IgG1 mediates a PCA reaction in rat skin; with IgG2 being unreactive. The heterologous PCA results with bovine IgGs are the same as reported for goat IgGs (Micusàn & Bordvas, 1977).

Initial work in our laboratory indicated that erythrocytes coated with IgG1 would not cause phagocytosis by peripheral blood monocytes. During these studies work was published indicating that both IgG1 and IgG2 antibodies caused phagocytosis by monocytes cultured for more than 5 days (Rossi & Keisel, 1977b). When we cultured our monocytes for 7 days both IgG1 and IgG2 mediated phagocytosis when bound to erythrocytes giving results similar to those published (Rossi & Keisel, 1977b). Our results with freshly isolated bovine cells are comparable to those in goats in that goat IgG2 caused binding to macrophages and neutrophils while IgG1 did not (Micusàn & Bordvas, 1977). Experiments with sheep neutrophils also showed that IgG2 was cytophilic with no binding of IgG1 detected (Watson, 1975). It is not clear whether receptors for IgG1 are either uncovered by culturing or newly formed (Rhodes, 1975). It may be that cultured monocytes resemble *in vivo* activated macrophages.

The absence of IgM receptors and the presence of complement receptors on freshly isolated monocytes agree with data for cultured monocytes (Rossi & Kiesel, 1977a). Failure of bovine complement to function in this system while equine complement works well is unexplained. It may be caused by rapid degra-

dation of C3b and the subsequent binding of conglutinin (Linscott, Ranken & Triglia, 1978).

The solubility of IgG1 DNP antibodies and DNP_{19}-ovalbumin complexes was surprising and was probably caused by IgG1 molecules binding bivalently to single DNP_{19}-ovalbumin molecules (Archer & Krakauer 1977a, b). Equine IgG(T) anti-hapten antibody does not precipitate with hapten-carrier antigens (Klinman, Rockey & Karush, 1964) and floculates with protein antigens (Johnson & Allen, 1968a). Evaluation of precipitin curves of bovine IgG1 and IgG2 ovalbumin antibodies with ovalbumin revealed 'typical' precipitation with no evidence of floculation.

Differences in functional properties of bovine IgG1 and IgG2 diminish when either homologous or different systems are used. Both IgG1 and IgG2 fix bovine complement, and mediate PCA in bovine skin and cause phagocytosis by cultured monocytes. Using the criteria tested, it appears that in most infectious disease situations IgG1 and IgG2 should function similarly to rid the host of infection. If there are differences in bovine IgG1 and IgG2 function, demonstration of this will require specific evaluation of protective antibodies rather than evaluation of antibody subclass functional properties using non-infectious antigens.

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