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# Antibody-mediated protection against *Brucella abortus* in BALB/c mice at successive periods after infection: variation between virulent strain 2308 and attenuated vaccine strain 19

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## SUMMARY

In BALB/c mice antibodies specific for the O polysaccharide (OPS) as well as T lymphocytes mediate protective immunity to *Brucella abortus*. We performed quantitative analyses of isotypes of OPS antibodies generated during primary infections, and tested the protective qualities of antisera at successive stages of infection against *B. abortus* strain 2308, representative of the wild type, and attenuated vaccine strain 19. IgM antibodies predominated during the first 3–4 weeks of infection. IgG3 antibodies increased slowly for the first 3 weeks but then rose rapidly and persisted at high levels ($>300\,\mu g/ml$). IgG1, IgG2a and IgG2b antibodies had increased slightly by week 4 and then remained at low to moderate levels ($<70\,\mu g/ml$). Week 2 serum pools (IgM high, IgG3 low or undetectable) transferred substantial protection against 2308 ($\geqslant 1$ log unit) which increased relatively little (to 1·2–1·5 log units) with later sera that were high in IgG antibodies. In contrast, week 2 sera conferred low levels of protection against 19 ($<0{\cdot}6$ log units), but protection was dramatically increased (to $\geqslant 2{\cdot}3$ log units) with sera obtained 1 week later that had slightly increased IgG antibodies. Monoclonal IgM antibodies also provided better protection against 2308 than 19, while monoclonal IgG3 antibodies protected much better against 19. Strain 19 opsonized with antibodies taken at any stage of infection was killed within normal macrophages, whereas comparably opsonized 2308 underwent intracellular replication. Phagocytosis of 2308 was better than of 19 when brucellae were opsonized with either polyclonal IgM or IgG3 antibodies, and the difference between strains was more extreme following IgM opsonization. The data suggest an explanation for differences in the growth curves of 2308 and 19 in spleens of BALB/c mice. Higher numbers achieved by 19 at week 2 could result from extracellular replication owing to ineffectual opsonization by IgM antibodies, while the precipitous decline of 19 beginning at week 3 could be caused by the increase in more effective IgG3 opsonins that facilitate its rapid intracellular destruction.

## INTRODUCTION

*Brucella abortus* is the principal cause of brucellosis in cattle, and vaccination with attenuated *B. abortus* strain 19 has been essential for the control and eradication of this disease.[1] Whereas virulent strains of *B. abortus*, of which 2308 is representative, produce abortions and chronic infections in cattle, 19 is almost always eliminated rapidly and rarely produces abortion.[2]

The BALB/c mouse has served as a model of infection with *B. abortus*. The prolonged persistence of 2308 and the rapid clearance of 19 in this mouse correspond to the relative virulence of these strains in cattle. Thus, in spleens of BALB/c mice 2308 plateaus at high levels for 6–8 weeks before declining slowly over a period of months.[3–5] Strain 19, in contrast, attains peak numbers at 2 weeks after infection and is then eliminated rapidly over the next 4 weeks. Notably, at week 2 post-infection numbers of 19 consistently exceed 2308.[3,4]

Protective antibodies, specific for the O polysaccharide (OPS),[6,7] were found in the bloodstream of mice 3 weeks after infection with 19, whereas protective T lymphocytes were not demonstrable even in low numbers until 1 week later.[8] This observation, coupled with the finding that 19 was more susceptible than 2308 to the protective effects of antibodies,[9]

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Abbreviations: IFN-γ, interferon-γ; KELA, kinetics-based enzyme-linked assay; NMS, normal mouse serum; OPS, O polysaccharide.

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led us to hypothesize that the rapid decline of 19 after the second week of infection resulted from a protective function of IgG antibodies that preceded the development of protective T cells.[4] It was proposed that during the first 2 weeks of infection both 19 and 2308 replicated within splenic macrophages, whereafter IgG antibodies enhanced intracellular killing through Fc receptor-mediated triggering of the respiratory burst. The advent of IgG antibodies after week 2 would thereby herald the rapid destruction of 19, but not of 2308, which persisted because it differed from 19 in being able to survive even within fully activated macrophages.[4]

Subsequent experiments provided strong support for some elements of this hypothesis. Strain 19 opsonized with a hyperimmune serum pool was almost completely eliminated by 48 hr even within normal murine peritoneal macrophages, whereas 2308 opsonized in the same fashion replicated intracellularly after 24 hr.[10] Although replication of 2308 was greatly curtailed in macrophages activated with interferon-$\gamma$ (IFN-$\gamma$), live bacteria were always present after 48 or 72 hr.[10] Jiang and Baldwin demonstrated concurrently that *B. abortus* was, in fact, susceptible to killing by reactive oxygen intermediates.[11,12]

The objectives of the present study were to analyse the temporal development of isotypes of OPS antibodies in BALB/c mice undergoing primary infection with *B. abortus*, and to evaluate the protective qualities of sera taken at successive weeks after infection against 2308 and 19. We were particularly interested in obtaining a direct test of the hypothesis that the rapid decline in numbers of 19 was attributable to the appearance of IgG antibodies.

## MATERIALS AND METHODS

*Mice*

BALB/cByJ female mice 9 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME) and were held for 1 week before use.

*Bacterial strains*

Stock cultures of *B. abortus* 2308 and 19, stored at $-70°$, were thawed and diluted to the desired concentration on the day of use. Actual numbers were determined by viable counts.[3] Stocks used for challenge infections were of 100% smooth colony morphology and were known to produce typical growth curves.[4,5,8] Stocks used for bactericidal assays were derived from a single solid agar passage of the challenge strains, were 100% smooth, and were the same ones used previously.[10]

*Yersinia enterocolitica* O:9 was obtained from G. G. Schurig (VA-MD Regional College of Veterinary Medicine, Blacksburg, VA). Stock cultures were stored at $-70°$.

*Blood sampling*

Mice were bled aseptically from the retroorbital sinus or the heart. Serum was separated after overnight incubation at room temperature and stored at $-70°$.

*Quantification of bacterial numbers in the spleen*

Mice were killed by $CO_2$ asphyxiation. Spleens were homogenized, diluted serially, and plated.[3] Colonies were counted after incubation for 3 days at 37° under 10% $CO_2$.

*Lipopolysaccharide (LPS) antigens*

LPS was extracted with hot phenol from plate grown cells of *B. abortus* strain 2308[13] and *Y. enterocolitica* O:9.[14] The OPS of *Y. enterocolitica* O:9, a homopolymer of 1,2 linked 4,6-dideoxy-4-formamido-D-mannopyranose,[15] is identical in structure to the principal (A) epitope of *B. abortus* OPS,[16] and was used to test for OPS antibodies in order to minimize detection of antibodies to the *B. abortus* LPS core, lipid A, and outer membrane proteins.

*Monoclonal antibodies*

Hybridomas producing monoclonal antibodies specific for the A epitope of *B. abortus* OPS were produced in mice by standard protocols. Clones Yst 9-1 (IgG2b),[17] Yst 9-2 (IgG3),[17] Ba-6 (IgM)[17] and 12AE6 (IgG1)[18] have been described previously. Clone M83, prepared in the laboratory of Klaus Nielsen (Animal Disease Research Institute, Ottawa, Canada), produced antibodies of the IgG2a isotype.

To purify monoclonal antibodies, alkali-treated *B. abortus* LPS[19] was bound to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ)[20] and ascites fluids (0·2 ml/ml gel) were passed through columns of the gel. Antibodies were eluted with 0·2 M triethylamine buffer (pH 11·5) and transferred to phosphate-buffered saline (PBS) through a desalting gel. Antibody protein in each eluate was quantified by optical density measurements using an extinction coefficient [E 1%/280 nm (1 cm)] of 13·7. These samples were used as standards in the antibody assay, and were stored at 4° for up to 3 weeks or at $-20°$ in a solution of 50% glycerol containing bovine serum albumin (BSA) at 2 mg/ml.

*Kinetics-based enzyme-linked assay (KELA)*

KELA was used to minimize the variation that occurs in single-read ELISA. OPS antibodies were measured with a computer-assisted assay described previously.[21] Wells of polystyrene plates (Nunc-Immuno Module Maxisorb F16, Krackler Scientific Inc., Albany, NY) were coated with *Y. enterocolitica* O:9 (0·5 $\mu$g LPS/ml carbonate buffer, 0·1 M, pH 9·6). After 3 hr at 37° plates were sealed and stored at 4°. Immediately before use, plates were washed four times (0·05% Tween-20 in PBS, pH 7·4) and test serum samples (1:100 unless noted otherwise, in Tris buffer containing 0·05% Tween-20 and 2% powdered milk) were added in duplicate. After 1 hr at 37°, isotype-specific goat anti-mouse horseradish peroxidase conjugates having $<1\%$ cross-reactivity (Southern Biotechnology Associates Inc., Birmingham, AL) were added (100 $\mu$l/well) at a dilution of 1:500 in the same buffer and the plates were incubated at 23° for 30 min. After washing, 200 $\mu$l of substrate solution [0·4 mM 2,2-azino-di-(3-ethylbenzthiazoline sulphonic acid) (Sigma Chemical Co., St Louis, MO) and 2 mM $H_2O_2$ in 0·05 mM citrate buffer (pH 4·0)] was added to each well. Absorbance at 410 nm was recorded in a plate reader (Bio-Tek EL 312, Winooski, VT) three times at computer-timed 2-min intervals. Plates were agitated continuously by the reader for 2 min prior to the first reading and between each of the other readings.

The rate of the reaction between substrate solution and enzyme, expressed as slope, is directly proportional to the amount of antibody in the sample.[22] Slopes were determined from the linear regression of time versus absorbance ($r^2 \geqslant 0{\cdot}999$). For each isotype, eight dilutions of the monoclonal OPS antibody were included in every plate as standards.

The plots of slope versus concentration (μg/ml) gave sigmoidal curves that were essentially symmetrical about their inflection points. Therefore log logit transformations were performed to provide more accurate estimates of antibody concentrations at high and low ends of the scale.[23] The correlation coefficients ($r^2$) of the resultant standard curves for IgM, IgG3, IgG1, IgG2b, and IgGa were 0·997, 0·988, 0·988, 0·996 and 0·999, respectively. Concentrations of antibodies in test samples were deduced from the transformed standard curves. The lowest detectable concentrations (μg/ml) of OPS antibodies were 1·0 for IgM and IgG2a, 2·4 for IgG2b, and 4·4 for IgG1 and IgG3.

*Experimental design*

*Time–course of infection.* Mice were bled retroorbitally and then inoculated intravenously (i.v.) with $5 \times 10^4$ brucellae in 0·1 ml PBS. In some experiments five mice were exsanguinated at selected intervals and spleens were cultured for quantitative counts. Alternatively, a single group of animals ($n = 10$) was bled at sequential intervals. Times post-infection at which animals were tested ranged from 1 to 20 weeks.

*Antiserum pools.* Equal volumes of serum were pooled from representative samples ($n = 3$–8) taken at individual times after infection. Other pools, prepared from samples chosen for highly enriched content of IgM or IgG3 antibodies, yielded 'pure' working solutions in which only a single isotype was detectable by KELA. Physical and chemical purification procedures were avoided because elution of OPS antibodies from solid matrices sometimes resulted in substantial denaturation.

*Passive transfer experiments.* Recipient groups ($n = 5$) were given 50 μl of antiserum (unless otherwise noted) because a preliminary trial had demonstrated that this volume sufficed to transfer protection. Injections were made i.v. in a total volume of 100 μl, using a diluent of PBS + 20% normal mouse serum (NMS). The mice were inoculated 1 hr later with $5 \times 10^4$ brucellae. Prior studies had demonstrated that even 100 μl of NMS exerted no protective effect *B. abortus* in BALB/c mice, in comparison with a PBS control.[3] In experiments with monoclonal antibodies, ascites fluids were diluted to 100 μl with PBS. Brucellae in spleens were enumerated 1 week after infection.

*Bactericidal assays.* Procedures of Jones and Winter[10] were followed exactly. *Brucella abortus* [$1 \times 10^8$ colony-forming units (CFU)/ml] was opsonized for 30 min at 37° with concentrations of antibodies fourfold lower than the agglutination titre. In comparisons with IgM antibodies, NMS preserved for complement activity was added to a final volume of 10% at 5 min before the end of the opsonization period. Opsonized brucellae were added to monolayers of murine peritoneal macrophages on glass coverslips and incubated for 30 min at 37° under 5% $CO_2$. Extracellular organisms were removed by incubation for 1 hr in medium containing 50 μg gentamicin/ml. One group ($n = 5$) of coverslips was harvested and counted at this time to establish the number of bacteria phagocytozed, which provided the baseline (100%) values for calculating per cent survival at 24 or 48 hr post-phagocytosis. Numbers of intracellular organisms were determined by plate counts after monolayers had been disrupted by three rapid cycles of freezing and thawing. Extracellular growth was controlled by inclusion of 12·5 μg/ml gentamicin in the culture medium, and represented < 1% of intracellular growth at 24 or 48 hr.

A requirement of antibodies for opsonization of smooth *B. abortus* has been reported previously.[10,24–26] In a representative experiment, the increase in phagocytosis obtained by opsonization of 19 and 2308 with PBS + 10% NMS preserved for complement activity, over that in PBS alone, was < 175 CFU, while opsonization of the same bacteria with subagglutinating concentrations of OPS antibodies produced increased uptakes over PBS of > 60,000 and > 130,000 CFU with 19 and 2308, respectively.

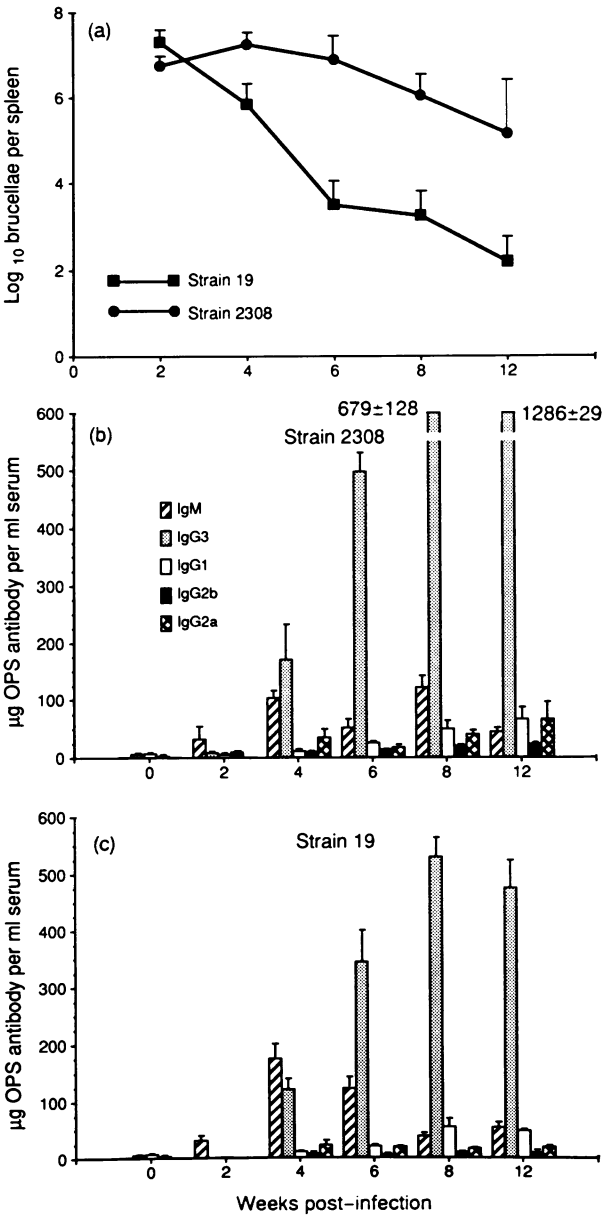


**Figure 1.** (a) Growth curves of *B. abortus* 2308 and 19 in BALB/c mice following i.v. inoculation of $5 \times 10^4$ CFU. The isotype distribution of OPS-specific antibodies was determined by KELA assays in the same groups of mice infected with 2308 (b) and 19 (c). There were five mice per treatment group. Bars: (a) means ± SD; (b) and (c) means ± SE.

*Statistics*

A geometric mean value for each spleen count was obtained by averaging the triplicate values following $\log_{10}$ conversion.[3] Results of passive transfer experiments are reported as log units of protection, calculated by subtracting individual counts of the principal group from the mean count of the corresponding control group.[5] Statistical comparisons between experimental groups were carried out by Student's *t*-test.[27]

## RESULTS

### Isotypes of OPS antibodies in mice infected with *B. abortus*

Splenic growth curves of strains 19 and 2308 (Fig. 1a) were typical.[3–5,8] Numbers of 19 peaked at 2 weeks post-infection and dropped more than 1000-fold during the next 4 weeks. Strain 2308 plateaued at high levels between weeks 2 and 6 and then declined gradually. In accord with prior observations,[3,4,8] numbers of 19 at week 2 were significantly higher than 2308 ($P < 0{\cdot}01$).

The isotype distribution of OPS antibodies at sequential periods after infection was very similar in mice infected with 2308 and 19. Representative experiments are depicted in Fig. 1b and c. These experiments were repeated twice with each strain, and results were closely comparable whether mice were bled sequentially or terminally. IgM antibodies at levels above background (mean background values = 1 μg/ml) were present at week 1 post-infection (data not shown), increased up to week 4, and remained at low to moderate levels thereafter (Fig. 1b and c). Increases in IgG3 antibodies above background (mean background values 6 to 7 μg/ml) were low or absent at week 2 (Fig. 1b and c) but by week 3 increases had occurred in each of three experiments for which data were available (mean values 26 to 131 μg/ml). Thereafter concentrations of IgG3 antibodies rose steeply and from week 6 onward ranged between 300 and 700 μg/ml, although higher levels were sometimes attained (Fig. 1b and c). Increases over background levels of IgG1, IgG2a and IgG2b antibodies were marginal or absent until week 4 (Fig. 1b and c). During the first 12 weeks after infection, maximum mean concentrations attained in all six experiments by IgG2a (67 μg/ml) and IgG1 antibodies (66 μg/ml) exceeded IgG2b antibodies (24 μg/ml), but were far below IgG3 antibodies. IgA antibodies were never detected.

### Passive transfer experiments

Passive transfer experiments with serum pools from donors infected with 2308 2 weeks and 3 weeks previously are presented in Table 1. Protection provided against 2308 differed by 0·5 log units between sera taken at 2 and 3 weeks post-infection (group 1 versus group 5), whereas sera taken at week 3 conferred 1·8 log units more protection than week 2 serum against 19 (group 2 versus group 6). Protection with the week 2 pool was significantly greater against 2308 than against 19 (group 1 versus group 2) ($P < 0{\cdot}05$), while the week 3 pool conferred significantly more protection against 19 than against 2308 (group 5 versus group 6) ($P < 0{\cdot}001$). Reciprocal experiments, performed with serum pools derived from donors infected with 19, yielded the same results and identical statistical comparisons. In the latter experiments increases in antibody concentrations between week 2 and week 3 serum pools (increases per mouse dose in IgM of 0·7 μg, IgG3 of 0·8 μg, and IgG1 of 0·6 μg) resulted in differences in protection against 2308 of 0·26 log units (1·22 versus 1·48) and against 19 of 2·02 log units (0·35 versus 2·37). Protection against 2308 or 19 provided by week 3 serum pools was equivalent to that conferred by serum pools taken at later periods (weeks 4–20), even though IgG3 antibody levels in some of these later pools were in excess of 60 μg/dose (data not shown). A titration experiment confirmed that in this passive transfer system 4 μg

**Table 1.** Protective capacity of sera taken at 2 and 3 weeks after infection from donors infected with *B. abortus* strain 2308

| | Donors† | Recipients‡ | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Quantity of OPS antibody transferred (μg)§ | | | | |
| Experiment no. | Week post-infection when bled | Group ($n = 5$) | IgM | IgG3 | Challenge strain | $\text{Log}_{10}$ brucellae in spleen ($\bar{x} \pm$ SD) | $\text{Log}_{10}$ units of protection ($\bar{x} \pm$ SD)¶ |
| 1 | 2 | 1 | 1·4 | ND | 2308 | 5·49 ± 0·20 | 0·99 ± 0·21*** |
| | | 2 | 1·4 | ND | 19 | 6·08 ± 0·37 | 0·58 ± 0·37* |
| | | 3 | None | None | 2308 | 6·48 ± 0·23 | |
| | | 4 | None | None | 19 | 6·66 ± 0·18 | 2 |
| | 3 | 5 | 8·8 | 4·1 | 2308 | 4·65 ± 0·17 | 1·48 ± 0·17*** |
| | | 6 | 8·8 | 4·1 | 19 | 4·30 ± 0·09 | 2·37 ± 0·09*** |
| | | 7 | None | None | 2308 | 6·13 ± 0·19 | |
| | | 8 | None | None | 19 | 6·67 ± 0·22 | |

† Week 2 and week 3 sera were from pools of seven and three mice, respectively.

‡ Recipients were inoculated i.v. with $5 \times 10^4$ CFU *B. abortus* 19 or 2308 at 1 hr after i.v. injection of 50 μl serum + 50 μl diluent. Mice were killed 1 week later for spleen cultures.

§ Antibodies of isotypes other than those listed were not detected (ND).

¶ In comparison with the corresponding diluent (PBS + 20% NMS) control group, ***$P < 0{\cdot}001$, *$P < 0{\cdot}05$.

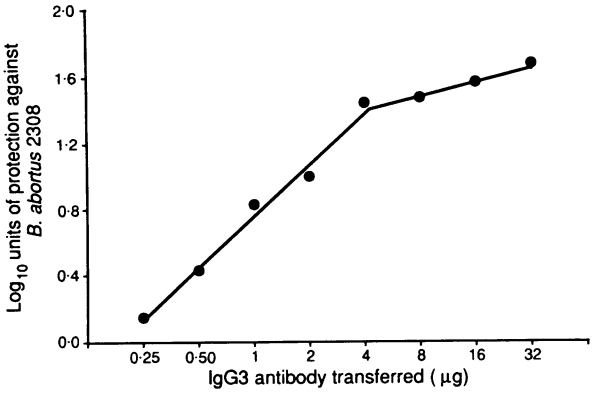


**Figure 2.** Passive protection against *B. abortus* 2308 was titrated by testing twofold dilutions of a pure preparation of polyclonal OPS-specific IgG3 antibodies. Antibodies were from pooled sera of six mice that had been infected with 2308. Only IgG3 antibodies were detected in the most concentrated solution by KELA. Levels of protection were significant in every assay ($P < 0{\cdot}05$ to $< 0{\cdot}001$). Standard deviations (not shown) did not exceed 0·20. Regression lines were fitted to two separate portions of the titration curve because regression analyses demonstrated that the first five data points best defined the ascending phase of the curve ($r^2 = 0{\cdot}887$) while the last four points defined the plateau phase ($r^2 = 0{\cdot}988$).

of IgG3 antibody provided a level of protection against 2308 that approached maximum (Fig. 2).

These data suggested that IgM antibodies, which predominated at week 2, were more protective against 2308, while IgG3 antibodies, which had always increased by week 3 and predominated thereafter, protected better against 19. To test this hypothesis we compared protection against 2308 and 19 by the OPS-specific monoclonal antibodies of IgM and IgG3 isotypes (Table 2). IgM antibodies provided significantly more protection against 2308 than against 19 (group 1 versus group 2, $P < 0{\cdot}05$), whereas IgG3 antibodies conferred over 1 log unit more protection against 19 than 2308 (group 3 versus group 4, $P < 0{\cdot}001$) (Table 2). The same differences in protection between 2308 and 19 were obtained in mice injected with pure preparations of polyclonal IgM or IgG3 antibodies (data not shown).

### Phagocytosis and killing assays

Earlier studies had demonstrated that 2308 was phagocytozed better than 19 by murine peritoneal macrophages following opsonization by hyperimmune sera taken at week 17 post-infection.[10] An experiment was performed to compare phagocytosis following opsonization with pure preparations of polyclonal IgM and IgG3 antibodies. Numbers of internalized 19 were approximately fourfold and twofold lower than 2308 (both comparisons $P < 0{\cdot}01$) when the two strains had been opsonized with IgM and IgG3 antibodies, respectively (Fig. 3). Moreover, opsonization with IgM antibodies resulted in significantly lower levels of ingestion compared to IgG3 antibodies with both 19 ($P < 0{\cdot}001$) and 2308 ($P < 0{\cdot}05$). A repetition of this experiment yielded the same results (data not shown).

Bactericidal assays performed with pooled sera taken at 2 and 4 weeks post-infection with 2308 are depicted in Table 3. Survival of 19 was 1% or less at 24 and 48 hr post-phagocytosis regardless of the quantity of IgM and IgG3 antibodies in the sample. In contrast, survival of 2308 opsonized by the same sera was between 11% and 19% at 24 hr and above 200% at 48 hr. A reciprocal experiment with serum pools from mice infected 2 and 4 weeks previously with 19 yielded the same results (data not shown). The same patterns of survival of 2308 and 19 were obtained with serum pools derived from later periods (weeks 6–20; data not shown).

## DISCUSSION

The isotype distribution of OPS antibodies in mice during a primary infection with *B. abortus* resembled that in mice immunized with *B. abortus* endotoxin, in which IgG3 antibodies also predominated.[28,29] Antibodies detected prior to infection were presumed to have arisen in response to cross-

**Table 2.** Protective capacity of OPS-specific monoclonal antibodies of IgM and IgG3 isotypes against *B. abortus* strains 2308 and 19

| Recipient group ($n = 5$)† | Monoclonal antibody transferred‡ | | Challenge strain | $Log_{10}$ brucellae in spleen ($\bar{x} \pm$ SD) | Log units of protection ($\bar{x} \pm$ SD)§ |
|---|---|---|---|---|---|
| | Isotype | Quantity (μg) | | | |
| 1 | IgM | 49 | 2308 | 5·51 ± 0·28 | 0·74 ± 0·28** |
| 2 | IgM | 49 | 19 | 6·21 ± 0·14 | 0·43 ± 0·14** |
| 3 | IgG3 | 391 | 2308 | 4·86 ± 0·18 | 1·39 ± 0·18*** |
| 4 | IgG3 | 391 | 19 | 4·00 ± 0·21 | 2·64 ± 0·22*** |
| 5 | | None | 2308 | 6·25 ± 0·12 | |
| 6 | | None | 19 | 6·64 ± 0·17 | |

† Recipients were inoculated i.v. with $5 \times 10^4$ CFU *B. abortus* strain 19 or 2308 at 1 hr after i.v. injection of antibody. Mice were killed 1 week later for spleen cultures.

‡ Mice were injected with 10 μl ascites fluid in PBS (final volume 100 μl). Ascites fluids were derived from hybridomas Ba-6 (IgM) and Yst 9-2 (IgG3). Control groups received PBS.

§ In comparison with the corresponding control group, ***$P < 0{\cdot}001$, **$P < 0{\cdot}01$.

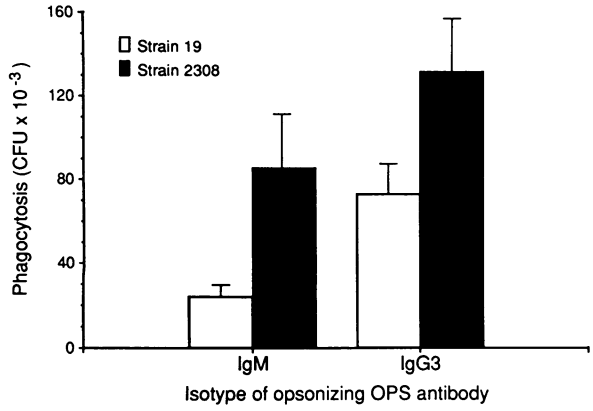


**Figure 3.** Phagocytosis of *B. abortus* 2308 and 19 following opsonization with pure preparations of polyclonal OPS-specific IgM or IgG3 antibodies. Antibodies were from pooled sera of separate sets of three mice that had been infected with 2308. The designated isotype was the only one detected by KELAs performed on the working solutions used for opsonization, without further dilution. Normal mouse serum preserved for complement activity (final volume 10%) was added to all samples. Bars = SD.

reactive antigens. These natural antibodies made no detectable contribution to protective immunity in the test systems that we employed, as NMS conferred no protection passively and provided no enhancement of phagocytosis. Moreover, concentrations of antibodies measured prior to infection were at the lowermost limits of detection, so interpretation of these data must be cautious.

Antibody-mediated protection against virulent *B. abortus* 2308 was substantial ($\geqslant$1 log unit) at week 2, when IgM antibodies predominated and levels of IgG antibodies were very low or undetectable. In fact, at limiting concentrations IgM antibodies were more protective than IgG3 antibodies. Thus, 1 log unit of protection against 2308 was obtained with 1·4 $\mu$g IgM antibody (Table 1, group 1), whereas 2 $\mu$g of IgG3 antibody were required to produce an equivalent effect (Fig. 2). A small increment of protection against 2308 was observed when larger quantities of polyclonal or monoclonal IgG3 antibodies were given alone or in combination with IgM antibody. One explanation for this could be the modestly increased uptake of 2308 by macrophages following opsonization with IgG3 antibodies over that with IgM antibodies. The ability of 2308 to replicate in normal murine macrophages, and to survive even in IFN-$\gamma$-activated macrophages,[10] probably accounts for the plateau at around 1·5 log units in protection provided by IgG3 antibodies, regardless of the quantity of antibody injected.

The protective effects of OPS antibodies differed sharply between 2308 and 19. Sera at week 2 provided relatively meagre protection against 19, whereas by week 3 and at all intervals thereafter protection against 19 was about 10-fold greater than against 2308. The data suggest that in contrast to 2308, IgM antibodies were relatively ineffectual against 19, whereas small increases in IgG antibodies between weeks 2 and 3 caused very large increases in protection. The trial with monoclonal antibodies confirmed that IgM antibodies were less protective and IgG3 antibodies were much more protective against 19 than they were against 2308. It is likely that the lower level of protection provided by IgM antibodies against 19 in comparison with 2308 was causally related to the correspondingly lower level of phagocytosis of 19 when opsonized with IgM antibodies. Moreover, as 19, once internalized, was killed just as effectively when opsonized by week 2 serum as with serum taken at any interval thereafter, the possibility arises that the high splenic numbers achieved by 19 at week 2 were a direct consequence of its ability to evade phagocytosis.

Based on these findings, we now hypothesize that it is the extracellular replication of strain 19 that enables it to reach higher numbers than 2308 by week 2. With the increase in IgG3 opsonins above a threshold level between weeks 2 and 3, extracellular 19 is more efficiently internalized and is then readily destroyed within phagocytes. IgG3 antibodies may also facilitate destruction of extracellular or cell-adherent brucellae

**Table 3.** Survival of antibody-opsonized *B. abortus* strains 2308 and 19 in murine peritoneal macrophages

| | | % survival ($\bar{x}$ ± SD)* | | | |
|---|---|---|---|---|---|
| | | Strain 19 | | Strain 2308 | |
| Antibody source† | OPS antibody in opsonizing solution ($\mu$g/ml)‡ | 24 hr§ | 48 hr | 24 hr | 48 hr |
| 2308—2 weeks | IgM—0·2<br>IgG3—0·1 | 0·6 ± 0·2 | 0·9 ± 0·5 | 19·1 ± 10 | 344 ± 47 |
| 2308—4 weeks | IgM—0·3<br>IgG3—0·6 | 0·4 ± 0·1 | 0·9 ± 0·5 | 11·5 ± 2·6 | 206 ± 30 |

*Opsonized *B. abortus* 19 or 2308 were incubated for 30 min at 37° with monolayers of glass-adherent murine peritoneal macrophages. Bacterial survival was determined at 24 and 48 hr after phagocytosis. Per cent survival was determined by dividing the number of bacteria on a coverslip at 24 or 48 hr by the mean number present at 1 hr post-phagocytosis, and multiplying by 100. Extracellular bacteria represented < 1% of the total in any of the samples.

† Strain of *B. abortus* used to infect donors and week after infection that serum was obtained. The week 2 and week 4 sera were from pools of seven and four mice, respectively.

‡ KELAs were performed directly on the working dilutions used for opsonization, without further dilution.

§ Hours post-phagocytosis at which samples were cultured.

by natural killer cells, following interaction with the FcγRIII receptor.[30] Contrary to our earlier belief[4] our current results indicate that concentrations of IgG3 opsonins sufficient to trigger the respiratory burst may sometimes be present even at 2 weeks post-infection. Moreover, the oxidative burst may also be generated by non-antibody ligand–receptor interactions such as binding of the mannosyl/fucosyl receptor, which serves both to opsonize micro-organisms and to stimulate a strong oxidative burst.[31,32]

We can offer no explanation for the inefficient phagocytosis of 19. KDO analyses on purified LPS preparations indicate that O side chains are of comparable length in smooth strains of 19 and 2308,[19,33] and no differences were detected in the major outer membrane proteins of the two strains.[34,35] It may be hypothesized that 19 nevertheless possesses cell-surface properties that hinder the binding of OPS antibodies or of C3b.

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