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(54) Title: A POLYSACCHARIDE VACCINE TO ENHANCE IMMUNITY AGAINST BRUCELLOSIS

(57) Abrégé/Abstract:
A vaccine comprising purified outer-polysaccharide (OPS) is effective for protection against brucellosis. The vaccine is derived from Brucella or a variety of cross reactive bacteria. The vaccine can be administered by different routes (intramuscularly, subcutaneously, intraperitoneally, orally). The vaccine is effective in protecting against other infectious bacteria, aside from Brucella. It is likely that the vaccine can be given after infection to reduce illness.

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A Polysaccharide Vaccine to Enhance Immunity Against Brucellosis

Abstract

A vaccine comprising purified outer-polysaccharide (OPS) is effective for protection against brucellosis. The vaccine is derived from *Brucella* or a variety of cross reactive bacteria. The vaccine can be administered by different routes (intramuscularly, subcutaneously, intraperitoneally, orally). The vaccine is effective in protecting against other infectious bacteria, aside from *Brucella*. It is likely that the vaccine can be given after infection to reduce illness.

A Polysaccharide Vaccine to Enhance Immunity Against Brucellosis

Background of the Invention

Brucellosis is a debilitating disease that can cause abortions and weight loss in animals, "undulating" fevers, "night sweats", incapacitation and arthritis in humans. It is very hardy to environmental factors, easily aerosolized and infectious through skin abrasions, ingestion and the pulmonary route. It is difficult to treat with antibiotics and often persists as a life-long infection. Brucellosis is a disease endemic to most countries, especially under-developed nations where it infects 0.1 to 10% of the livestock (e.g. cattle, swine, sheep, goats, dogs and poultry), wild life (e.g. bison, caribou, wolves, dolphins) and people.

Currently, there are no vaccines for human use to protect against brucellosis. In the past researchers have vaccinated people at high risk (e.g. veterinarians, abattoir workers) with an attenuated vaccine strain, *B. abortus* S19, but this appears to be attenuated for cattle and can be pathogenic or cause brucellosis in humans. There was a French vaccine (PI, or phenol insoluble) that removed the toxic lipopolysaccharide (LPS) component with phenol, but the phenol insoluble residue gave a high rate of reactogenicity (at least 53%) and led to hyper-sensitivity (vaccinates exposed to *Brucella* antigens were susceptible to anaphylactic shock). This latter vaccine has been discontinued and hence there are no human vaccines for brucellosis presently available.

The vaccines presently used for livestock also have their inadequacies. The one used for cattle, an attenuated *B. abortus* S19 vaccine strain, does not give absolute protection from disease and is about 80% protective, occasionally reverts to a pathogenic form that can cause abortions, the vaccinates cause confusion in serological tests (i.e. in some cases the positive serology can be caused by vaccination, infection, or vaccination and subsequent infection), it is virulent for animals other than cattle and it can be pathogenic for people.

In the development of a vaccine against brucellosis, the view of the scientific community was exceptionally discouraging. Below are the key points they raised:

1) *Brucella* was recognized over 100 years ago and for over a century researchers around the world have tried to raise a vaccine against brucellosis without success. Given the time, number of investigators and talent involved, the evidence was obvious that a vaccine could not be developed.

2) *Brucella* was a facultative parasite that could sequester inside tissues. Not only was it protected from antibiotics and vaccine-induced antibodies of humoral immunity, but it also had mechanisms for controlling its host phagocyte (i.e. it secretes thymidine and cyclic GMP which inactivate the host cell) and hence cellular immunity is ineffective.

3) Polysaccharides and bacterial glucans are very poor immunogens. The evidence is that these are the least likely candidates for vaccines.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a safe and effective vaccine against brucellosis.

Specifically, the invention provides a vaccine, for stimulating protection against brucellosis, comprising as the active component an immunoprotective and non-toxic quantity of outer-polysaccharide (OPS) extracted from *Brucella abortus* or any bacteria cross reactive thereto.

Further, the vaccine can be used for protection against infection from a variety of bacteria.

In addition, the vaccine can be used as a brucellosis treatment after infection.

Brief Description of the Drawings

These and other features of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings wherein:

Figures 1 to 8 illustrate the humoral response to *Brucella abortus* antigen in mice tests.

Detailed Description of the Preferred Embodiment

Despite the views of world renowned *Brucella* experts and polysaccharide chemists, there were a few observations that gave indications that a vaccine was possible:

1) Protection does occur in the field. *Brucella* is only about 70% infectious (either to animals or people) which suggests that there is something occurring to protect the 30% which do not come down with brucellosis. Also, once a cow aborts due to *Brucella*, it has a natural immunity to this disease.

2) There was an unexplained but well accepted observation: although the outer polysaccharide (O-polysaccharide or OPS), which gives a bacterium its serological identity, does not induce an immunological response, the immuno-dominant antigen of *Brucella* (about 80% of the antibodies are to this) is this same OPS when it forms part of the bacterial LPS or smooth lipopolysaccharide.

3) It has been determined that *Brucella* infected animals did produce antibodies which could precipitate OPS only when it was part of LPS (Bundle et al., Canadian Patent No. 1,212,051, issued September 30, 1986). It was evident that the OPS was somehow involved with immunity but that this immunity was different from antibody activity. As investigators have never reported the use of OPS as a vaccine, there appeared to be an exceptional opportunity ignored by everyone else.

4) Further, OPS was on hand for vaccine trials due to new methods in its purification (Cherwonogrodzky et al., "Antigens of *Brucella*", Animal Brucellosis (1990), 19-64, K. Nielsen and J.R. Duncan (ed.)).

The other concept advanced by the present inventors was the ability of one vaccine to protect against other cross-reactive diseases. Table I shows that several bacteria have similar OPS structures. As will be noted later, proof for this claim is the finding that the *B. abortus* OPS is a very effective vaccine for protecting pigs from *B. suis* infections. Also, the *Yersinia enterocolitica* O:9 OPS can replace the OPS of *B. abortus* in general immunity experiments in mice.

Therefore, the present study examined the use of OPS as a vaccine to protect Balb/c mice from brucellosis.

Materials and Methods

Brucella abortus 30, 413 and 2308 were acquired from Agriculture Canada, Animal Diseases Research Institute (ADRI-Nepean), Nepean, Ontario, Canada. The bacteria were grown either in Brucella broth (Difco/BDH Inc., Edmonton, Alberta) or on Brucella agar plates (supplemented with 1 ppm crystal violet) and incubated with 5% CO_2 at 37°C for 2 days. To make an inoculum for mice, it was observed that a suspension of *B. abortus* that gave an OD_{620} of 0.2 on a Spectronic 20™ spectrophotometer (Milton Roy Co., Fisher Scientific Co., Ottawa, Ontario) corresponded to 1.1×10^9 colony forming units (cfu). Bacterial cultures were either diluted or suspended in sterile 1% saline to approximate this value, diluted further to yield about 2.5×10^5 cfu/ml (0.2 ml of this suspension was the inoculum) then part of this was placed on Brucella agar and incubated to confirm these estimates.

The OPS and LPS used as vaccines were purified by methods already reported (Cherwonogrodzky et al., 1990) from *B. abortus* 413 cells killed with 2% phenol. Briefly, for OPS, the killed cells were suspended in 2% acetic acid, 1% saline solution (the suspension was 20% cells, v/v), placed in a boiling water bath for 2 hours, centrifuged to remove the cells, trichloroacetic acid (final concentration 0.2M) was added to remove proteins, centrifuged and the supernatant was extracted at room temperature with an equal volume of phenol. The OPS was precipitated from the phenol layer with 3 washes of 5 volumes of methanol with 1% sodium acetate (w/v), dialysed then purified on a G-50 Sephadex™ with 0.4% acetic acid and 0.4% pyridine as the buffer, then lyophilized. For LPS, the killed cells were suspended in 1% saline (cells were 20% v/v) and

extracted with an equal volume of phenol, the mixture being constantly stirred at 70°C for 30 minutes. The crude LPS was washed 3 times with 5 volumes of methanol-acetate, dialysed against 0.01M TRIS-HCL buffer (pH 7.%) with 1% saline and 0.04% sodium azide, digested with lysozyme, RNAse, DNAse (all 25 μg/ml, 6 hours at room temperature) and proteinase K (50 μg/ml, another 48 hours incubation at room temperature). The mixture was ultra-centrifuged, then the final LPS pellet was re-suspended in water and lyophilized. Samples of OPS and LPS dissolved in water did not absorb at $A_{260,280}$ and contained less than 1% protein.

For liposomal encapsulation of OPS and LPS, briefly, negatively charged liposomes were prepared using phosphatidylcholine:cholesterol:phosphatidylserine in a molar ration of 7:2:1. The lipids were dissolved in a small volume of chloroform:methanol (2:1 v/v), dried to a thin film on a RotaVap™ (under vacuum, flask was immersed in 37°C water bath), then further dried in a vacuum chamber to remove residual solvent (Note: the lipids are sensitive to oxygen). Either OPS or LPS in 1% saline (the saline was autoclaved and cooled to remove dissolved oxygen) was added to the lipid film and a thick emulsion was made on the RotaVap™. The emulsion was transferred to centrifuge tubes, purged with nitrogen gas, left for an hour to reconstitute, then re-suspended in 100mM HEPES buffer (pH 6.7) in normal saline. The liposomes were washed (centrifuged 125,000 x g/4°C/30 min., supernatant discarded, pellet re-suspended in HEPES-saline), the preparation was purged with nitrogen gas and the tubes sealed with Parafilm™ until required.

Balb/c mice were 15-16 grams (29-35 days old) females purchased from Charles River (Quebec) and were cared for in accordance with the guidelines set by the Canadian Council for Animal Care. All procedures were reviewed and approved by the Animal Care Committee

(members consist of a veterinarian, scientists and lay people) at the Defence Research Establishment Suffield (DRES). Immunization (on weeks 0, 1 and 5) was done by suspending the vaccines in sterile saline and delivering a total of 0.2 ml in 2 subcutaneous and 2 intra-muscular injections. Blood samples were drawn from, and infectious inocula (on week 6) were given by, the intra-venous route using the tail vein which had been mildly warmed under a heat lamp. Spleen counts were assessed by sacrificing each animal (on week 7), aseptically removing the spleen, homogenizing this in 2 aliquots of 1 ml sterile saline, serially diluting the preparation, plating each dilution on Brucella agar (5% CO_2, 37°C, 1 week for incubation) and counting the resulting colonies. Protection was identified when the total spleen count was 100-fold (i.e. 2 $\log_{10}$) less than the inoculum given.

Specific IgG and IgM levels against LPS and OPS in serum samples from the weekly bleedings were assayed by an indirect FELISA, as known in the art (Fulton et al., *J. Virol. Methods*, 22, 1988, 149-164). Due to the large number of samples, equal volumes of the sera from the mice (sets of 3-4 mice given the same vaccine concentration) were pooled. Briefly, the wells of the microtitre plates were coated with 50 µl of *B. abortus* LPS (20 µg/ml in 0.05M carbonate-bicarbonate buffer, pH 9.6). This antigen was used to detect the antibody response to OPS, liposome encapsulated OPS (LIP-OPS), LPS and liposome encapsulated LPS (LIP-LPS). After blocking steps of 2% bovine serum albumin, 0.1% Tween 20, 0.14% sodium phosphate, 1% NaCl pH 7 (BT-PBS), serially diluted serum samples were added to the wells. The specific IgG and IgM levels were detected by alkaline phosphatase-labelled anti-mouse IgG or IgM conjugates.

Results

1) Mouse Studies at DRES

Balb/c mice were immunized with purified OPS from *Brucella abortus* 1119-3 and initially the results were discouraging. As expected, the IgG or IgM antibody titres (reflective of humoral immunity) were very low with OPS, whether given as a single dose or as multiple (3) doses. The antibody titres were more pronounced when LPS was given as the antigen. The antibody titres could be enhanced when these antigens were liposomal encapsulated, but again the titres were still low for OPS (see Figures 1 to 8).

When these mice were challenged with a virulent strain of *B. abortus* 2308, however, OPS did appear to protect the mice from infection. Indeed, the poorer the antibody response to a given antigen, the better appeared to be the protection as shown in Table II which presents date on mice immunized three times with the noted concentration of purified antigens then challenged with *B. abortus* 2308.

Table III compares mice immunized either once or three times with the noted concentrations of purified antigens then challenged with *B. abortus* 30 (another infectious strain that was isolated from an aborted bovine fetus by Agriculture Canada several years ago). Other studies such as those with mice in Chile and with guinea pigs in Colombia appear to suggest that there is a low element of randomness in protection studies, likely due to individual susceptibility or resistance to brucellosis.

2) Guinea Pig Studies (Colombia)

Dr. Olga Marino of the Instituto Colombiano Agropecuario (ICA), Bogota, Colombia, did

an independent investigation on the protective properties of the OPS vaccine. Guinea pigs were used as these are perhaps the most sensitive animal species to *Brucella* infections (Garcia-Carillo, "Laboratory Animal Models for Brucellosis Studies", Animal Brucellosis (1990), 423-442, K. Nielsen and J.R. Duncan (ed.)). A vaccine that is protective to these susceptible animals is likely to be protective for humans.

Results for the first set of experiments are not available, although it was reported that 1 mg of OPS was able to protect a 400g guinea pig from a challenge of 5×10^4 cells of *B. abortus* 2308. At the United Nations University Brucellosis Researchers Network meeting in Valdivia, Chile (April, 1995), another study was presented as noted in Table IV. Results were said to be similar to that of before, except that previously 1000 μg was 100% protective while three injections to 1000 μg was only partially effective.

The similarities between the two studies suggest that OPS is protective for guinea pigs against *Brucella* infection, that single doses are more protective than multiple doses, and that protection appears to be inversely related to antibody production.

3) Swine Study in Venezuela

In Venezuela, swine are infected not with *Brucella abortus* but with *Brucella suis*, a more infectious species of *Brucella* than the former. The disease is sexually transmitted, passed from an infected boar to a susceptible sow at breeding.

In the presented studies, sows were either left as controls or were vaccinated with different doses of potential vaccines. The swine were cared for six months then both the vaccinates and the controls were mated with the same four infected boars to ensure insemination and infection. The

animals were housed in the same general area on a farm and could be identified by ear tags.

Table V gives a brief summary of the results. From the results it was found that:

a) A single dose of 100 μg of OPS (from *B. abortus*) was 100% effective in protecting the sows from *B. suis* infection. Protected swine did not have significant serum titres to *Brucella*. Not only did the pregnancies come to a successful term, but the litter size averaged 11 to 12 robust piglets. There is good evidence, therefore, that the DRES OPS vaccine, made from *B. abortus* cells, can protect against infections from cross-reactive bacteria (e.g. *B. suis*).

b) For the controls, 68% sero-converted with high titres to *Brucella*, and of these 45% aborted. For control sows that did come to term, 5% had still-born piglets in their litters. For the remainder, although the litter appeared healthy, the average size was 5 to 6 piglets.

4) Production of 150,000 "Human Equivalent Doses" of Vaccine

If 100 μg of the OPS vaccine can protect a 25 kg sow from a highly virulent strain of *B. suis*, it is likely that 300 μg of the same vaccine will protect a 75 kg person. The initial plan was to produce enough *B. abortus* cells and from this enough OPS vaccine to supply the amounts required for collaborative studies with our allies. However, as DRES has had its Level 3 Contaminant suites under renovations during the term of this task, killed *B. abortus* cells were acquired from external sources. The two sources were:

1) VECOL, Empresa Colombiana de Productos Veterinarios
D.C. Calle 26 (Av. El Dorado), No. 82-93
Bogota, Colombia

2) United States Department of Agriculture

National Veterinary Services Laboratory

1800 Bayton Road

Ames, Iowa, USA, 50010

The OPS vaccine was then extracted from the above cells using the "Rapid Method" reported by Cherwonogrodzky et al. (1990) which was summarized above. It should be noted that Lot #1 differs from Lot #2 in that the former used autoclaving as a source of heat for the hydrolytic release of polysaccharide while the latter used a boiling water bath. For Lot #1, the cells were first washed and re-suspended in 1% NaCl, 2% acetic acid. The cells were then autoclaved but due to a malfunction the conditions were 140°C instead of 121°C, the pressure was about 23 psi instead of 15 psi, and the time was about 1 hour instead of 30 minutes. Charing and yellowing of the OPS was observed, although the Colombian study with guinea pigs suggests that this did not seriously affect the potency of the vaccine. For the Lot #2 extractions, the cells were washed and re-suspended as before in 1% NaCl, 2% acetic acid, but instead were heated in a boiling water bath at 99°C for 2 hours. The yield of OPS was less (8g instead of 30g per kg) but there was less charing and less yellowing of the vaccine. A total of 45g (150,000 human equivalent doses) has been purified for research and experimental purposes.

The following is evidence that the *Brucella abortus* OPS vaccine is protective against bacteria other than *B. abortus*:

1) One of the most encouraging results is the absolute protection that the OPS vaccine gave swine against *B. suis* infection. Not only did it offer protection but it is likely that this protection also extends against cross-reactive bacteria as evidenced by the fact that the

polysaccharide vaccine used was from *B. abortus* yet protected swine from *B. suis*.

2) The hope was that the vaccine would protect swine from brucellosis. Not only were small amounts (i.e. 100 μg) of vaccine protective, but a single injection protected swine that were exposed to disease six months later. Also, a year after these studies were done, these same swine were protected from any incidence of infection (i.e. the vaccine is long lasting). Curiously, the farm where these swine were kept had an epidemic of *Haemophilus pleuropneumonia*. Unlike the rest of the swine, those immunized with the OPS vaccine remained healthy. Recently, similar studies were done in Venezuela except that the vaccine was given orally to swine rather than by injection. The same concentration of vaccine (except given orally) gave the same effective level of protection against brucellosis. Oral vaccination raises the possibility that food pellets with vaccine may be able to vaccinate wildlife.

3) Related work at DRES has found that the OPS vaccine may be a powerful immuno-modulator, enhancing general immunity against disease. In this work it was found that the OPS from *Yersinia enterocolitica* O:9 can be used to replace the OPS from *B. abortus*. This is the first evidence that the OPS vaccine made from any of the cross-reactive bacteria, such as those in Table I, may be of complete or partial protection against the other noted bacteria.

As mentioned above, Figures 1 to 8 give a representation of the humoral response of the immunized mice to different potential vaccines of different concentrations. General trends in these responses are summarized below:

a) OPS appeared to be a poor immunogen. Anti-*Brucella* IgG and IgM levels after a single dose were either undetectable or at the lower limits of detection, even when OPS was

liposome encapsulated. When multiple injections of OPS were given, anti-*Brucella* IgM levels were still at the lower limit of detection and then only detectable for the higher concentrations of OPS given. Anti-*Brucella* IgG levels were higher for multiple injections than for a single dose of OPS, but even then the response was at the limit of detection for the low concentrations of OPS given. Liposomal encapsulation of OPS did enhance anti-*Brucella* IgG titres about 4 fold.

b) LPS appeared to be a better immunogen than OPS. The anti-*Brucella* IgG and IgM levels were higher with greater concentrations of antigens given, were higher for multiple rather than single dose injections, and were higher when the LPS was liposomal encapsulated. For a single dose of 0.1 or 1 μg LPS or LIP-LPS, no anti-*Brucella* IgG levels were detected. Either the levels were below the limits of detection or the concentrations were below a required threshold for an IgG response.

c) Despite the above trends, when the same mice were challenged with *B. abortus* (similar results were observed for strains 30 and 2308 and hence have been combined), protection did not appear to be correlated with anti-*Brucella* IgG or IgM levels (see Table VI). Indeed, the results suggest an inverse relationship whereby the best protection was observed for mice injected with antigens that gave the lowest anti-*Brucella* antibody titres (i.e. single doses, OPS).

Discussion

In the presented study, it has been found that purified OPS is a poor immunogen for anti-*Brucella* IgG or IgM titres in the mouse. These titres can be enhanced if multiple rather than a single dose is given, if OPS is associated with lipids (either in the LPS form or liposomal

encapsulated) and if high concentrations are used. It was also observed that these titres had little to do with protection, and indeed there appeared to be a general trend that greater protection was correlated with poorer anti-*Brucella* responses. This lack of correlation is understandable given that the *Brucella* species are facultative parasitic bacteria that can invade white blood cells, organs and bone marrow (F.M. Enright, Animal Brucellosis (1990), 301-320, K. Neilsen and J.R. Duncan (ed.); P. Nicoletti and A.J. Winter, *ibid*, 97-126), sequestering themselves away from the bactericidal effects of antibodies. Although antibodies are unlikely to have an influence on established intra-cellular infections, these still have a significant effect on reducing bacterial counts circulating in the blood after an initial inoculation or in humoral bacteraemia (L.B. Corbeil et al., *Infect. Immun.* (1988) 3251-3261).

As cattle immunized with *B. abortus* S19 are resistant to brucellosis, it is likely that some antigens can induce a cell-mediated immunity (Nicoletti and Winter, 1990). The present study indicates that purified OPS can induce such an immunity and this has subsequently been supported in other studies using mice (Rojas et al., not published).

As the OPS of *B. abortus* was an effective vaccine against brucellosis, other sources for this component may also be possible. Possibilities are *Escherichia coli* recombinants, OG6 and OG8, carrying *Brucella* genes, cross-reactive bacteria such as *Yersinia enterocolitica* O:9 and *Escherichia hermannii*, and defective strains of *Brucella*. For the latter, colonies of *B. melitensis* B115 are rough in appearance because, even though they do produce OPS (R. Diaz et al., *J. Clin. Microbiol.* (1979), 10, 37-41), this strain is defective on combining it to its LPS and hence either store or secrete the OPS. Similar defective strains may not only be a source of OPS *in vitro*, but may be a

potential vaccine candidate as a result of their secretion of the OPS *in vivo*.

Conclusions

As the O-polysaccharide, or OPS, is an integral part of the smooth-lipopolysaccharide, or LPS, an immunosorbant assay (the indirect FELISA) was used to quantify antibodies in mice immunized with either *B. abortus* OPS or LPS. The present results confirmed the view that LPS was more immunogenic than OPS, that multiple injections gave better response than a single injection, and that liposome encapsulation of antigens raised anti-*Brucella* IgG or IgM titres. The humoral response appeared to have little correlation with protection against brucellosis, and indeed the most effective vaccine appeared to have been purified OPS, and even then one injection of this novel vaccine appeared to have been more effective than multiple injections.

This suggests that recombinants capable of expressing OPS, cross-reactive bacteria that express similar polysaccharides, or defective strains of *Brucella* that synthesize but do not couple the OPS to LPS, may be novel vaccine candidates.

It has been found in other studies that *Brucella* vaccines or filtrates of *Brucella* cultures gave therapeutic relief for brucellosis patients. In view of this finding and the results presented above it can be concluded that the OPS vaccine of the present invention can be used as a treatment after infection.

Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the appended claims.

Table I: OPS of Cross-Reactive Bacteria

Bacterium	O-Polysaccharide on their LPS[a]
Brucella abortus	(1,2-linked) perosamine
Yersinia enterocolitica O:9	(1,2-linked) perosamine
Brucella melitensis	(1,3-linked) perosamine
Escherichia hermanii	(1,3-linked) perosamine
Brucella suis	(1,2/1,3-linked) perosamine
Vibrio cholerae	glycero-tetronic perosamine
Salmonella landau	glu,fu,acetyl-gal,acetyl-perosamine
Salmonella godesburg	glu,fu,acetyl-gal,acetyl-perosamine
Escherichia coli O157:H7	glu,fu,acetyl-gal,acetyl-perosamine
Pseudomonas maltophilia 555	rham,acetyl-gal,acetyl-perosamine
Francisella tularensis	dideoxy sugar-perosamine
Yersinia pestis	?-perosamine[b]

[a] perosamine, 4-formamido-4,6-dideoxy-D-mannose; glu, D-glucose; fu, L-fructose; gal, D-galactose; rham, D-rhamnose

[b] some strains of plague cross-react with *Brucella*, the mechanism is unknown (personal communication, Dr. M. Corbel, 1994)

Table II: Balb/c Mice Vaccinated and then Challenged with *B. abortus* 2308

Antigen		Antibody titre [1]		Spleen Count ($log_{10}CFU$)[2]	Protection	
		IGg	IgM		(no./total)	%
None		<6.6	<6.6	6.1, 6.5, 6.5, 6.6 (avg. 6.4)	0/4	0
LPS	1 μg	8.6	7.6	3.7, 4.8, 4.8, 5.8	1/4	25
	100 μg	11.6	8.6	2.6, 2.6, 3.7, 6.0	3/4	75
LIP-LPS	1 μg	11.6	7.6	3.0, 3.2, 4.6, 5.9	2/4	50
	100 μg	13.6	10.6	4.3, 4.7, 4.7, 6.3	1/4	25
OPS	1 μg	7.6	<6.6	3.7, 5.0, 5.5, 5.6	1/4	25
	100 μg	8.6	6.6	0, 0, 3.2, 3.4	4/4	100
LIP-OPS	1 μg	7.6	<6.6	0, 0, 3.3, 3.8	4/4	100
	100 μg	11.6	7.6	3.3, 3.4, 3.7, 4.5	3/4	75

[1] Log_2 of average reciprocal antibody titres at 6 weeks of immunization.

[2] Initial inoculum was 5×10^4 or 4.7 log_{10} CFU. Each number is the spleen count for a single

mouse.

Table III: Single vs. Multiple Injections of Antigens as Vaccines in the Protection of Balb/c Mice Against *B. abortus* 30

		Single Injection of Antigen		Multiple Injections of Antigens	
Antigen		**Spleen Counts (log_{10} CFU)**	**Protection (no./total) (%)**	**Spleen Counts (log_{10} CFU)**	**Protection (no./total) (%)**
Control (none)		4.78, 5.20, 5.69, 4.59, 4.00	0/5 (0%)	see previous column	see previous column
LPS	100 μg	3.48, 0, 3.48	1/3 (33%)	0, 5.20	1/2 (50%)
	10 μg	3.84, 4.30, 0	1/3 (33%)	4.81, 3.60, 3.30	0/3 (0%)
	1 μg	0, 0, 8.0	2/3 (66%)	0, 4.61	1/2 (50%)
	0.1 μg	4.15, 5.08, 5.78		0, 5.34	1/2 (50%)
LIP-LPS	100 μg	0, 0	2/2 (100%)	0, 0, 4.82	2/3 (66%)
	10 μg	0, 3.60, 0	2/3 (66%)	0, 0, 0	3/3 (100%)
	1 μg	0, 5.11	1/2 (50%)	0, 5.36	1/2 (50%)
	0.1 μg	0, 5.25, 3.0	1/3 (33%)	0, 0	2/2 (100%)
OPS	100 μg	0, 0, 0	3/3 (100%)	0, 4.40	1/2 (50%)
	10 μg	0, 0	2/2 (100%)	3.60, 3.30, 0	1/3 (33%)
	1 μg	0, 0	2/2 (100%)	0, 0	2/2 (100%)
	0.1 μg	0, 0, 0	3/3 (100%)	3.00, 5.23	0/2 (0%)
LIP-OPS	100 μg	8.18, 0	1/2 (50%)	0, 0, 0	3/3 (100%)
	10 μg	3.84, 4.20, 0	1/3 (33%)	0, 0, 0	3/3 (100%)
	1 μg	3.85, 3.48, 0	1/3 (33%)	5.62, 5.11, 4.28	0/3 (0%)
	0.1 μg	0, 0	2/2 (100%)	0, 0, 5.04	2/3 (66%)

Mice were immunized on week 1 for single injection, weeks 1, 2 and 5 (intra-muscular) for multiple injections. On week 7 mice were challenged with 5×10^4 (log_{10} of 4.70) of *B. abortus* 30,

on week 8 the mice were sacrificed and their spleens assayed for bacteria.

Table IV: Guinea Pigs Immunized with OPS and Challenged with *B. abortus* 2308

Antigen		Infected Animals/Total	% Protection
None (controls)		6/6	0%
Single dose of OPS	10 μg	1/4	75%
	100 μg	1/4	75%
	1000 μg	3/4	25%
Three doses of OPS	3 x 10 μg	3/4	25%
	3 x 100 μg	3/4	25%
	3 x 1000 μg	0/4	100%

Table V: Venezuelan Swine Study for Vaccinated and Control Sows Challenged with *Brucella suis*

No.	Vaccine	Amount	Dose	Result	1 year later
10	Bab-OPS	100 μg	1	no abort., sero-	protected
10	Bab-OPS	500 μg	1	no abort., sero+/-	protected
10	Bab-OPS	100 μg	3	no abort., sero-	protected
10	Bab-OPS	500 μg	3	no abort., sero+/-	protected
10	Bsu-OPS	100 μg	1	no abort., sero-	protected
10	Bsu-OPS	500 μg	1	no abort., sero+/-	protected
10	Bsu-OPS	100 μg	3	no abort., sero+/-	protected
10	Bsu-OPS	500 μg	3	no abort., sero+/-	protected
10	Bsu-cell	100 μg	1	no abort., sero+	protected
10	Bsu-cell	500 μg	1	no abort., sero+	protected
10	Bsu-cell	100 μg	3	no abort., sero+	protected
10	Bsu-cell	500 μg	3	no abort., sero+/+	protected
10	RB51	10^6	1	no abort., sero-	protected
10	RB51	10^7	1	no abort., sero-	protected
10	RB51	10^8	1	no abort., sero-	protected
10	RB51	10^9	1	no abort., sero-	protected
30	Controls			31% abort., 68% sero+	25% abortions

Bab-OPS is *Brucella abortus* 1119-3 O-polysaccharide vaccine, **Bsu-OPS** is an O-polysaccharide vaccine produced in Venzuela from *B.suis*, **Bsu-cell** is *B. suis* cells killed with 2% phenol, **RB51** is an attenuated live vaccine strain of *B. abortus* from Dr. G. Shurig, Blacksburg, West Virginia.

Table VI: Protection Against *Brucella abortus* for Balb/c Mice Given Different Antigen Vaccines

Injection[a]	Vaccine[b]	Spleen Count[c]	Protection[d]	Unifected Mice[e]
	None (control)	5.48 ± 0.24	0/12 (0%)	0/12 (0%)
Single Dose (wk 0)	OPS: 1 μg	1.53 ± 0.69	6/6 (100%)	3/6 (50%)
	100 μg	2.24 ± 0.85	4/7 (57%)	3/7 (43%)
	LIP-OPS: 1 μg	3.26 ± 0.55	1/7 (14%)	1/7 (14%)
	100 μg	4.17 ± 1.06	1/7 (14%)	1/7 (14%)
	LPS: 1 μg	4.01 ± 1.12	2/7 (29%)	2/7 (29%)
	100 μg	3.54 ± 0.70	1/7 (14%)	1/7 (14%)
	LIP-LPS: 1 μg	3.12 ± 0.71	3/7 (42%)	1/7 (14%)
	100 μg	1.43 ± 0.64	6/6 (100%)	3/6 (50%)
Multiple Doses (wks 0, 1, 5)	OPS: 1 μg	3.85 ± 0.66	2/10 (20%)	2/10 (20%)
	100 μg	3.07 ± 0.70	3/10 (30%)	3/10 (30%)
	LIP-OPS: 1 μg	3.38 ± 0.55	3/11 (27%)	2/11 (18%)
	100 μg	2.56 ± 0.51	3/11 (27%)	3/11 (27%)
	LPS: 1 μg	3.39 ± 0.54	5/10 (50%)	1/10 (10%)
	100 μg	2.88 ± 0.60	5/10 (50%)	2/10 (20%)
	LIP-LPS: 1 μg	3.38 ± 0.52	6/10 (60%)	1/10 (10%)
	100 μg	3.46 ± 0.61	5/11 (45%)	2/11 (18%)

[a] dose given in 2 subcutaneous and 2 intra-muscular injections.

[b] total amount for each dose.

[c] average (with standard error about the mean) *B. abortus* counts (log_{10} colony forming units) for spleens.

[d] number of mice with 2 log_{10} less *B. abortus* c.f.u. in spleens/total group number.

[e] number of mice with no detectable *B. abortus* in spleens/total group number.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A vaccine for stimulating protection against brucellosis consisting of as the active component an immunoprotective and non-toxic quantity of outer-polysaccharide extracted from *Brucella abortus* or any bacteria having antigens cross-reactive to the *Brucella abortus* outer-polysaccharide.

2. The vaccine of claim 1 wherein the outer-polysaccharide is extracted from the group consisting of: *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*, *Pseudomonas maltophila* 555, *Escherichia coli* O:157, H:7, *Escherichia hermannii*, *Yersinia enterocolitica* O:9, *Salmonella landau*, and *Salmonella godesberg*.

3. A vaccine for stimulating protection against infection from *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*, *Pseudomonas maltophila* 555, *Escherichia coli* O:157, H:7, *Escherichia hermannii*, *Yersinia enterocolitica* O:9, *Salmonella landau*, or *Salmonella godesberg*, consisting of as the active component an immunoprotective and non-toxic quantity of outer-polysaccharide extracted from any of said bacteria.

4. A vaccine for protection in a mammal against infection from *Brucella* or any bacteria having antigens cross reactive to *Brucella* outer-polysaccharide consisting of as the active component an immunoprotective and non-toxic quantity of *Brucella* outer-polysaccharide.

5. The vaccine of claim 4 wherein said vaccine offers protection against infection from Bruc*ella abortus*, *Brucella suis*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*, *Pseudomonas maltophila* 555, *Escherichia coli* O:157, H:7, *Escherichia hermannii*, *Yersinia*

enterocolitica O:9, *Salmonella landau*, *Salmonella godesberg*, or *Haemophilus pleuropneumoniae*.

6. A pharmaceutical composition consisting of outer-polysaccharide extracted from Brucella or any bacteria having antigens cross-reative to the *Brucella* outer-polysaccharide, and a pharmaceutically acceptable carrier or excipient.

7. The composition of claim 6 wherein said outer-polysaccharide is extracted from *Brucella abortus*.

8. A pharmaceutical composition consisting of an active component for stimulating protection against brucellosis, said active component consisting of outer-polysaccharide extracted from a microorganism chosen from the group consisting of *Brucella abortus*, *Brucella melitensis*, *Francisella tularensis*, *Vibrio cholerae*. *Pseudomonas maltophila* 555, *Escherichia coli* O:157, H:7, *Escherichia hermannii*, *Yersinia enterocolitica* O:9, *Salmonella landau,* and *Salmonella godesberg*, and a pharmaceutically acceptable carrier or excipient.

9. A use of the composition as claimed in any one of claims 6 to 8 as a stimulant of cell-mediated immunity against brucellosis in a mammal.

10. The vaccine of claim 3 wherein said vaccine offers protection from infection against *Haemophilus pleuropneumoniae*.

FIGURE 1. Titres of IgG for Balb/c mice given *Brucella abortus* OPS

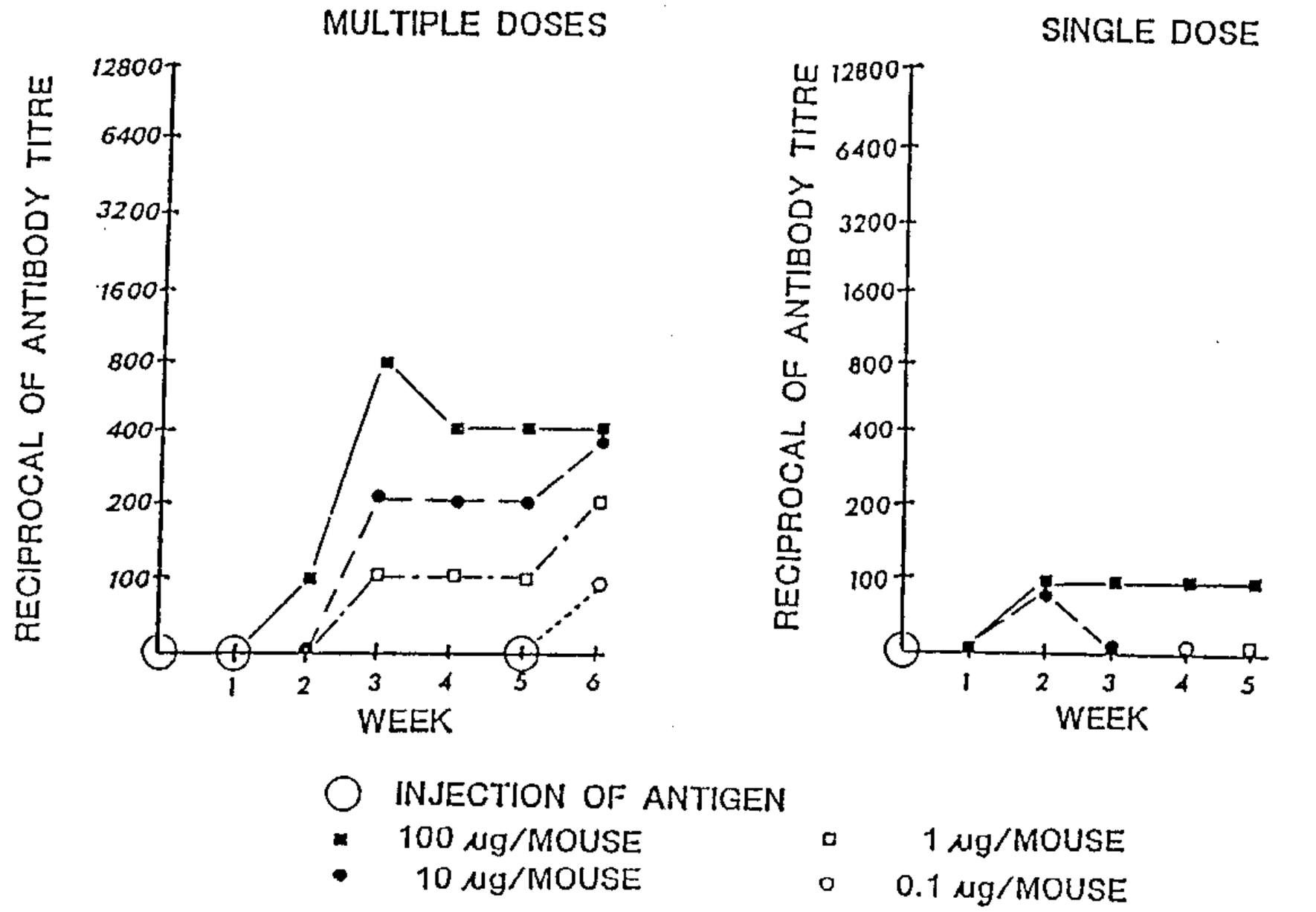

FIGURE 2. Titres of IgG for Balb/c mice given *Brucella abortus* LIP-OPS

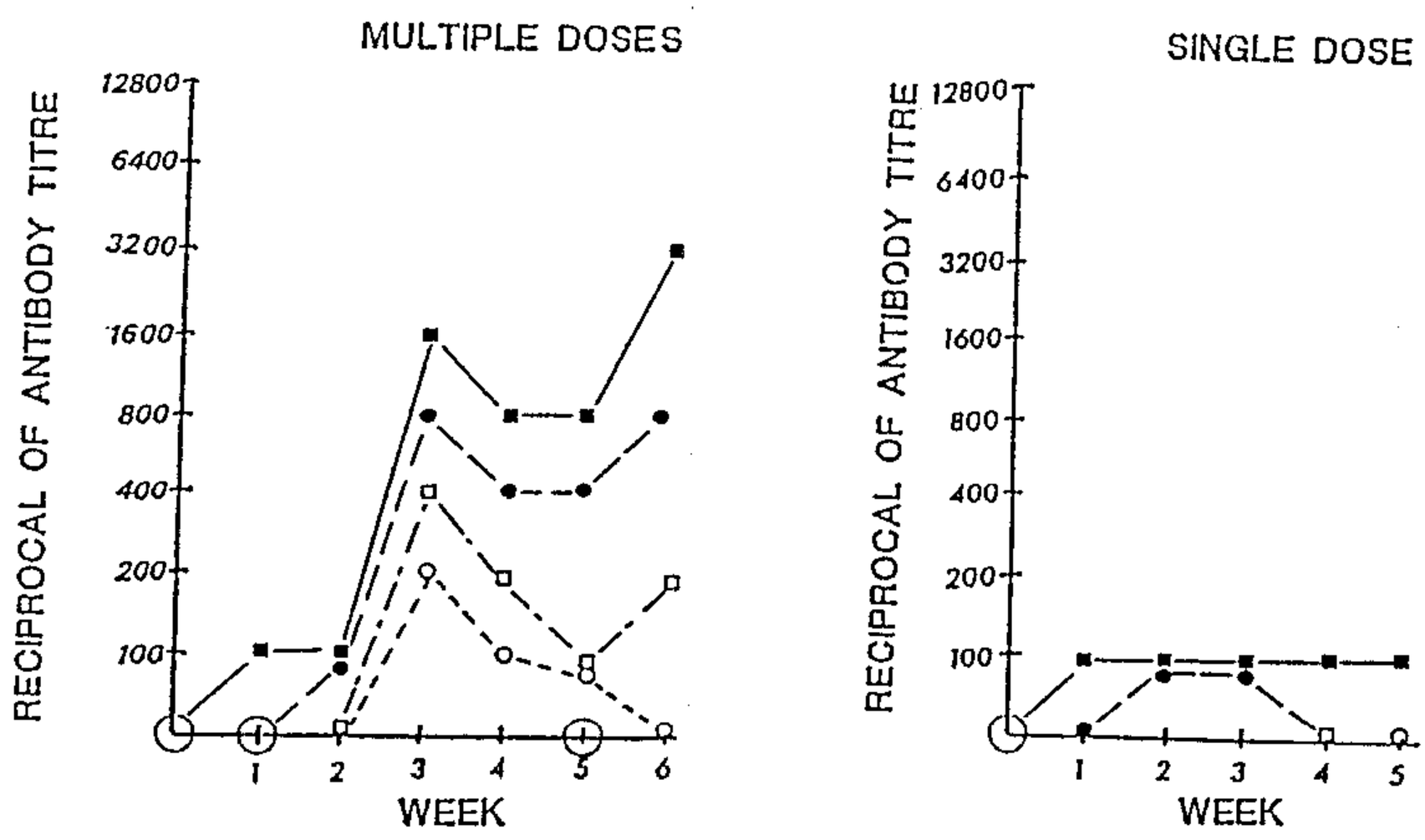

FIGURE 3. Titres of IgM for Balb/c mice given *Brucella abortus* OPS

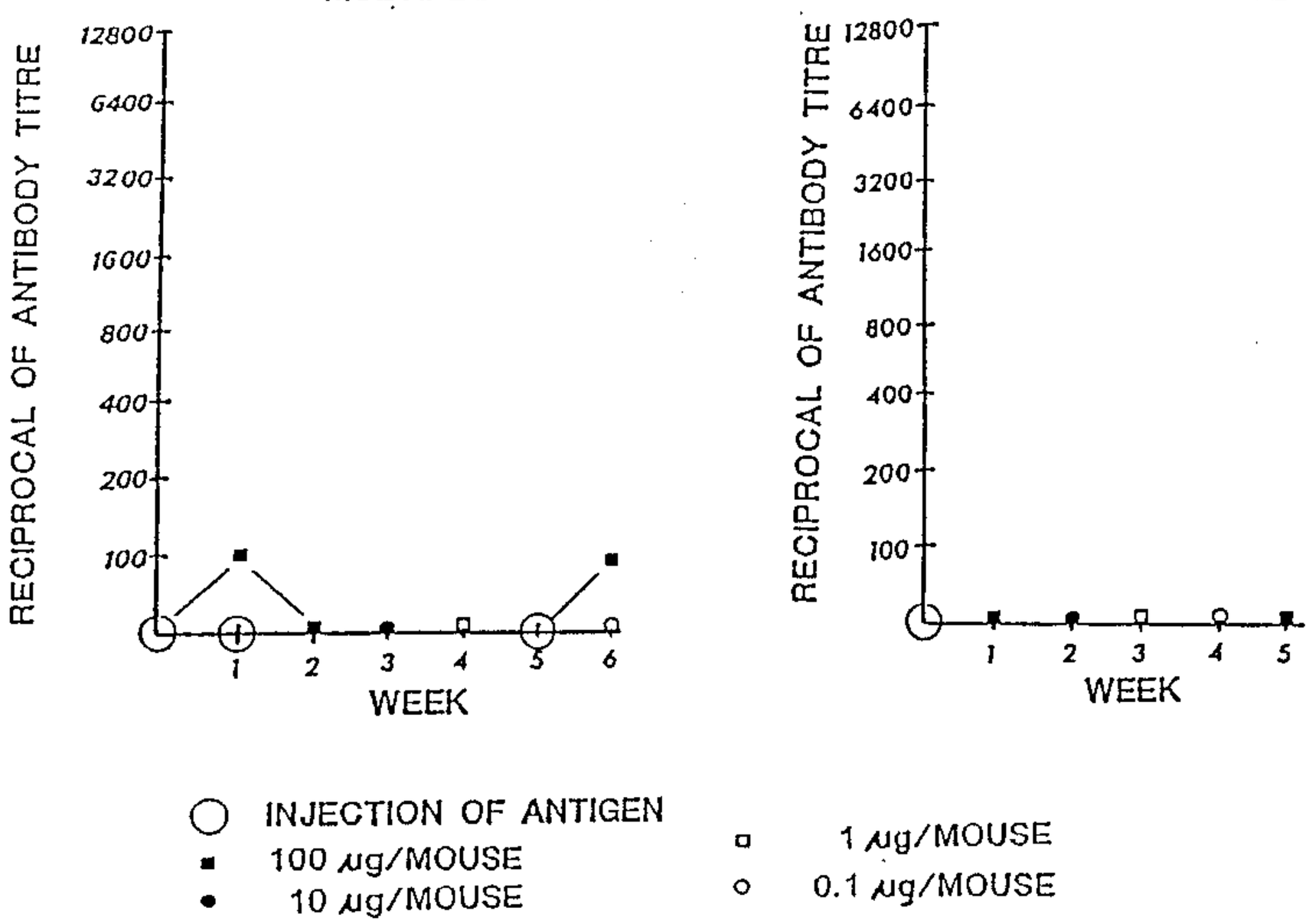

FIGURE 4. Titres of IgM for Balb/c mice given *Brucella abortus* LIP-OPS

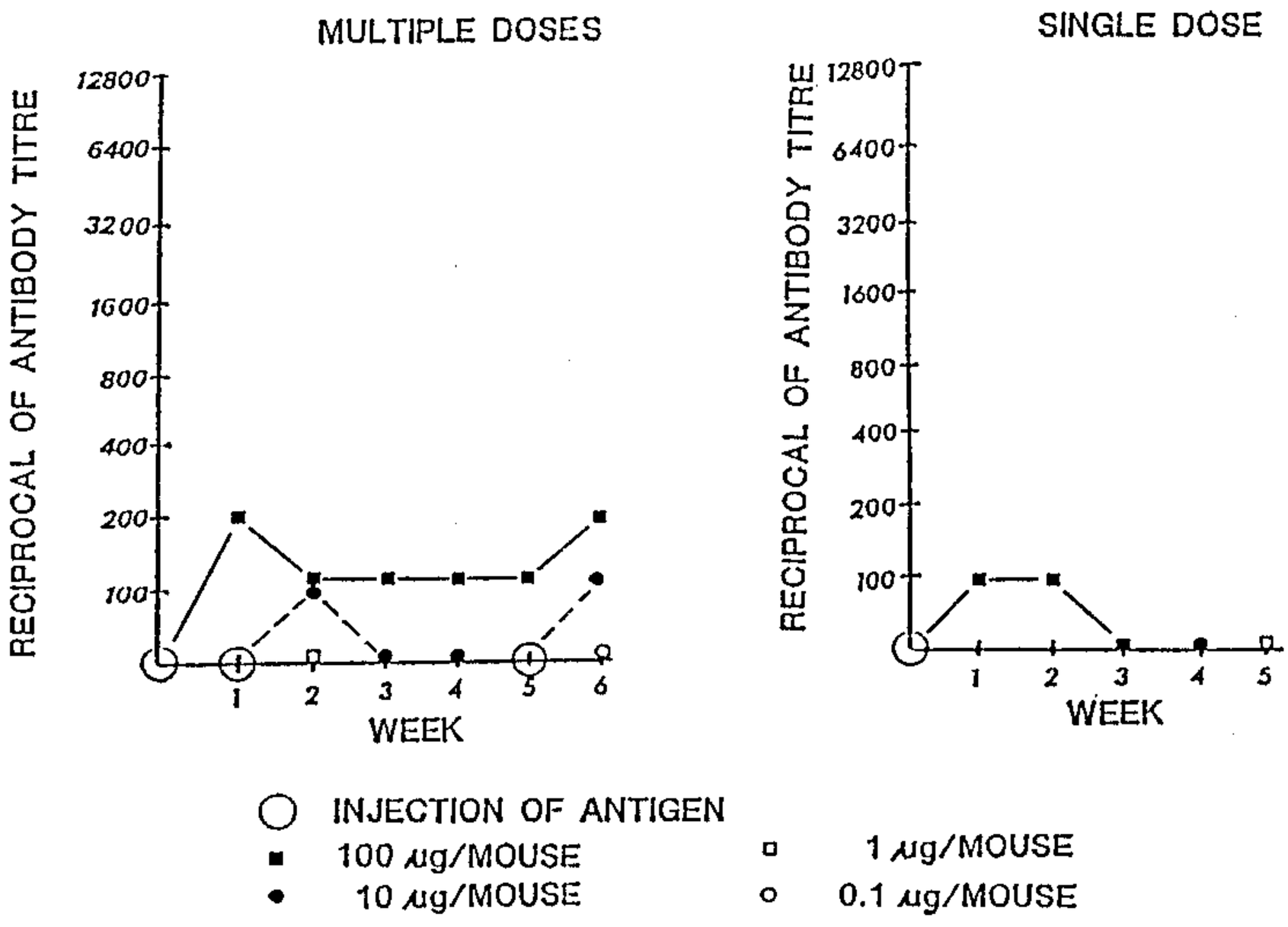

FIGURE 5. Titres of IgG for Balb/c mice given *Brucella abortus* LPS

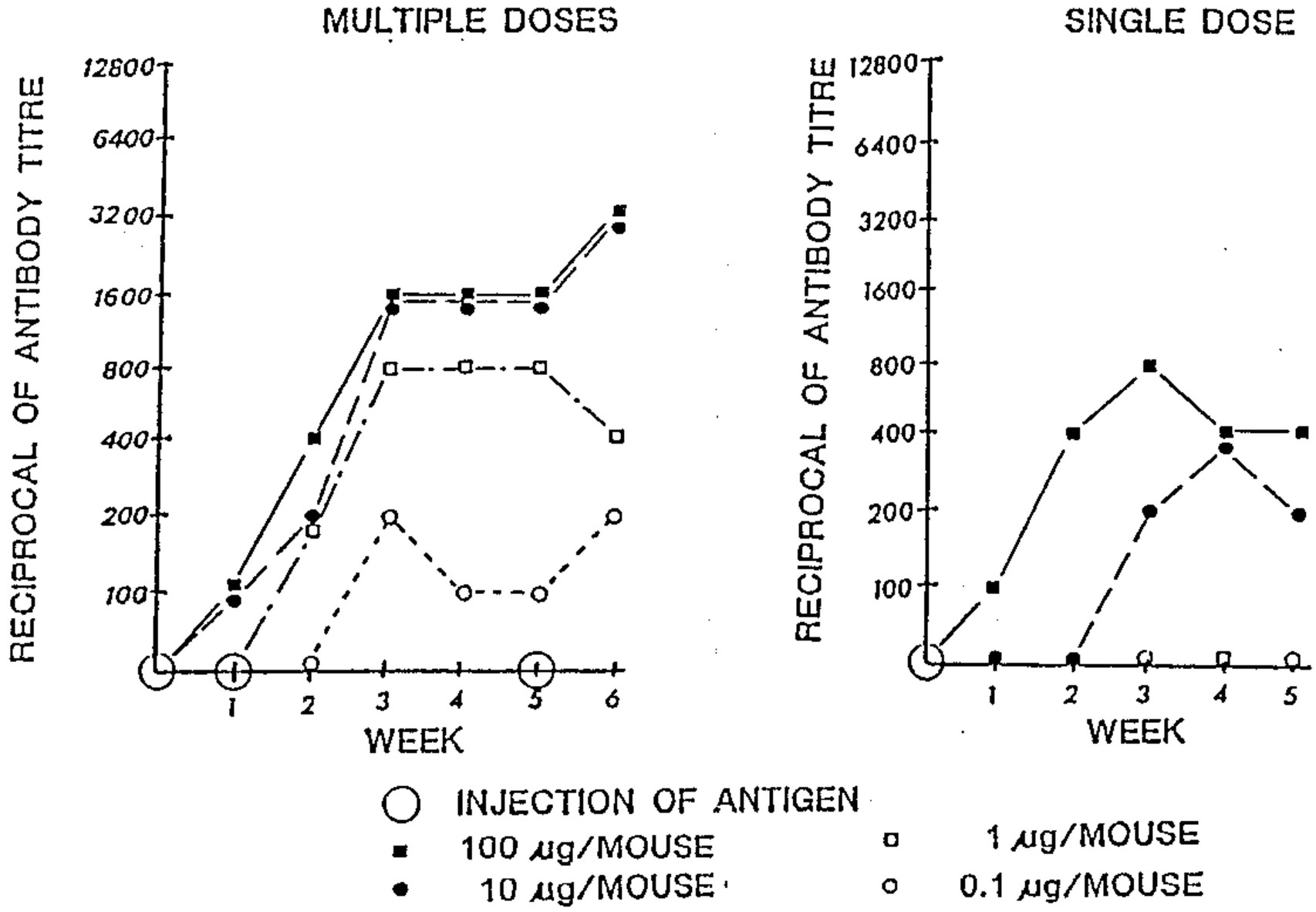

FIGURE 6. Titres of IgG for Balb/c mice given *Brucella abortus* LIP-LPS

FIGURE 7. Titres of IgM for Balb/c mice given *Brucella abortus* LPS

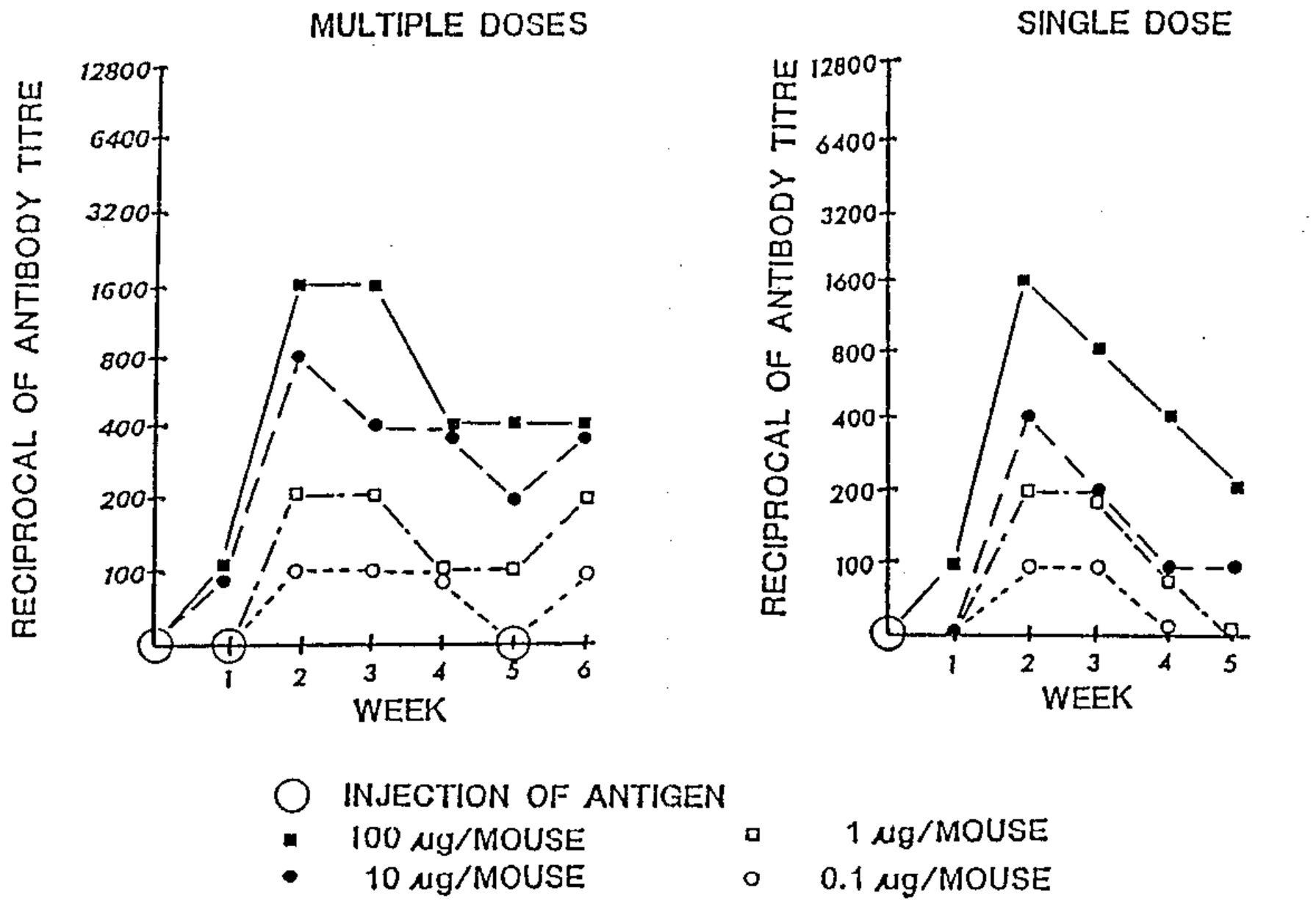

FIGURE 8. Titres of IgM for Balb/c mice given *Brucella abortus* LIP-LPS