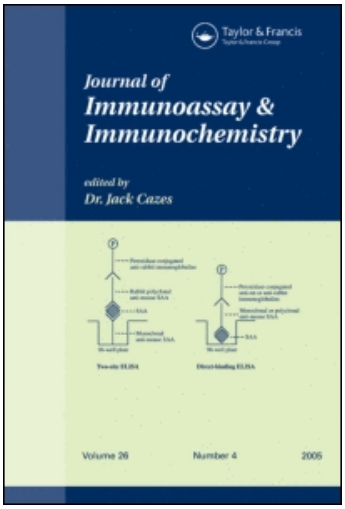

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Cartledge, Christine , McLean, Charles and Landon, John(1992) 'Production of Polyclonal Antibodies in Ascitic Fluid of Mice: Time and Dose Relationships', Journal of Immunoassay and Immunochemistry, 13: 3, 339 — 353
To link to this Article: DOI: 10.1080/15321819208021237
URL: http://dx.doi.org/10.1080/15321819208021237

JOURNAL OF IMMUNOASSAY, 13(3), 339-353 (1992)

PRODUCTION OF POLYCLONAL ANTIBODIES IN ASCITIC FLUID OF MICE: TIME AND DOSE RELATIONSHIPS

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ABSTRACT

Further studies of the humoral immune response have been undertaken using ovine immunoglobulin G as the immunogen and BALB/C mice as the recipients. Mice immunized by the intraperitoneal route weekly for five weeks with an emulsion of the immunogen in Freund's complete adjuvant and given an intraperitoneal injection of pristane on day 14 produce relatively large volumes of ascitic fluid. A simple fluoroimmunoassay is used to determine specific antibody titres. The mice do not lose condition, remain active and eat normally throughout, although all show extensive granuloma formation within the peritoneal cavity. Tapping is performed before ascitic fluid production is excessive and each study is complete within 49 days, with a maximum of 5 taps. Only a single immunization site is required, since no higher antibody titres are obtained using combined intraperitoneal plus intramuscular plus subcutaneous immunization routes.

A dose response study has shown that relatively large amounts (100 μg) of ovine immunoglobulin G must be injected to evoke a maximum response. Studies are in progress of various adjuvants and potential modulators of the immune response and of ways in which the immunogencity of macromolecules can be changed. This approach can also be used to obtain antibodies for diagnostic purposes more rapidly than in rabbits and sheep.

(KEY WORDS: Ovine immunoglobulin G, polyclonal antibodies, murine model, ascitic fluid)

INTRODUCTION

Antibody production has long remained an art rather than a science (1). This reflects the difficulty and cost of immunizing statistically relevant numbers of rabbits, goats or sheep and the time taken (at least 12 weeks) for specific antibodies to attain their maximum titre in these species. Mice are relatively inexpensive to purchase and house, making possible the use of statistically significant numbers, and their immune response is relatively rapid. However, they provide insufficient serum to enable detailed assessment and use of the resulting antibodies. One solution is to obtain the antibodies from their ascitic fluid, which has a similar protein distribution to serum albeit at only 50% to 60% of the concentrations found in serum. Munoz (2) first suggested this possibility and Tung and others undertook the pioneering work (3) which now makes this a reliable approach. Thus it is known that an appropriate strain of mice must be used (4); female mice produce larger volumes of ascitic fluid than males (5); and 10 to 12 week old mice are best suited for this purpose (3).

In a previous study from our laboratory (6) polyclonal antibodies were raised in murine ascitic fluid to insulin, human growth hormone and ovine immunoglobulin G (oIgG) and the intraperitoneal (ip) route of immunization was found to be superior to the subcutaneous route in terms of the maximum titres of specific antibodies attained. It was decided that oIgG was the preferred immunogen for use in further studies because of its freedom from side effects, low cost and wide availability. This

paper summarizes the optimization of the procedure with regard to the adjuvant used, the time of the pristane injection and the dose and route of administration of the oIgG. The time course of ascitic fluid and antibody production were then studied.

MATERIALS AND METHODS

Groups of 10 BALB/c female mice, aged 8 to 10 weeks and weighing from 15 to 22 g at the time of their first ip immunization, were used for each study.

Freund's complete (FCA) and incomplete (FIA) adjuvants were purchased from Difco Laboratories, Detroit, USA; normal sheep serum from ILS, London, UK; donkey anti-sheep serum (batch number 0962K) from SAPU, Carluke, Lanarkshire, UK; polyethylene glycol 6000 (PEG) from BDH, Poole, Dorset, UK; fluorescein isothiocyanate isomer 1 (FITC), bovine serum albumin (batch number A4503) and pristane from Sigma, Poole, Dorset, UK; and DEAE Affi-Gel Blue from Bio-Rad, Hertfordshire, UK. Reagents were of analytical grade quality whenever possible.

Production and Labelling of Ovine Immunoglobulin G

Normal sheep serum was precipitated with solid sodium sulphate (18% w/v) and the precipitate washed with an equal volume of aqueous sodium sulphate (18% w/v), spun in a bench centrifuge and the supernate decanted. The precipitate was then resolubilized in the original volume of 0.15 M sodium chloride before reprecipitation, as described above. Final purification

was by ion exchange chromatography using DEAE Affi-Gel Blue to remove contaminating albumin and proteases.

FITC and oIgG were reacted in a molar ratio of 4:1 in sodium bicarbonate buffer (0.1 M, pH 9.0) and incubated overnight at room temperature. The reaction mixture was applied to a column of Sephadex G-25 fine grade and eluted with sodium bicarbonate buffer. The labelled protein peak, identified by its colour, was well separated from minimal amounts of unreacted FITC and was collected, aliquoted and stored at -20 °C until required.

Production of Polyclonal Antibodies in Murine Ascitic Fluid and Serum

The immunogen used in the final protocol comprised 1 mL of the purified oIgG in saline (5 g/L) added to 9 mL of FCA as a water-in-oil emulsion, prepared by vigorous mixing after addition of 3 to 5 drops of Brij 20. Each mouse received 200 μL (containing 100 μg of oIgG) of this emulsion by the ip route on days 0, 7, 14, 21 and 28, together with 500 μL of pristane on day 14. Thereafter the mice were weighed twice weekly and ascitic fluid was collected as described previously (6) when they had gained 10 g or more over their original weight, giving a maximum of twice weekly tapping from days 31 to 63. On day 63 the mice were sacrificed by cervical dislocation, exsanguinated and the serum separated and stored at -20 °C until assayed. Subsequently, the protocol was shortened to 49 days, with a maximum of 5 taps, and granuloma formation within the peritoneal cavity recorded.

Monitoring Animal Welfare during Polyclonal Antibody Production

The mice were observed daily after day 28 and a record kept of unexpected findings. Abdominal distension was relieved by tapping of ascitic fluid and, if they became ill, the study was terminated.

Assessment of Polyclonal Antibodies to Ovine Immunoglobulin G in Murine Ascitic Fluid and Serum

To 100 μL of doubling dilutions of mouse anti-oIgG ascitic fluid or serum in sodium phosphate buffer (50 mM, pH 7.4, containing 0.5% bovine serum albumin and 0.1% sodium azide) is added 250 μL (5 μg protein) of FITC:oIgG. After 1 hour at room temperature, 1 mL of 4% PEG in sodium phosphate buffer (50 mM, pH 7.4) is added and the tubes are vortexed vigorously and spun at 4 ^{o}C for 1 hour at 2,500 rpm to separate the bound and free fractions. The background signal for each tube is established by replacing the FITC:oIgG with the same volume of assay buffer. Fluorescence intensities of the supernates (free fraction) are measured using a Perkin-Elmer model 1000 fluorimeter. The percentage total intensity measured at each dilution of test material is corrected for background, and titres calculated at 50% binding. The precision of the assay was determined using donkey anti-sheep serum; the within-assay precision was 4.5% and between-assay precison 9.8%, both calculated on 50 measurements.

Test results are expressed as arithmetic means. Comparison between groups was performed using the Mann Whitney U test and p

values of less than 0.05 were taken to be statistically significant.

RESULTS

Comparison of Freund's Complete and Incomplete Adjuvants

One group of 10 mice was subjected to the protocol summarized earlier and a second group to an identical protocol except that FIA was substituted for FCA. No ascitic fluid was formed in the group receiving the incomplete adjuvant, whereas those receiving FCA produced a mean volume of 16.2 mL per mouse.

Timing of Pristane Injection

A total of 40 mice were divided equally into 4 groups and received the standard protocol except for the timing of the pristane, which was injected ip on either days 0, 7, 14 or 21. The data are summarized in Table 1. The largest volume of ascitic fluid was formed when pristane was given on day 14 and this time was chosen for all subsequent studies.

Different Routes of Administration

A total of 30 mice were divided into groups of 10. The standard protocol was observed except that one group received immunogen by a combined ip plus subcutaneous (sc) plus intramuscular (im) route and another by an im plus sc route.

The data are summarized in Table 1. There were very significant differences ($p = <0.005$) between individual titres when

TABLE 1

Effects of Variations in Protocol on Ascitic Fluid Production

Timing of Pristane Injection

Day of Pristane Injection	Percentage of Responders	Mean Total Volume (mL) Ascites	Mean Titre (Reciprocal)
0	60%	8.2	109
7	70%	11.5	67
14	70%	17.2	85
21	90%	13.4	63

Different Routes of Administration of the Adjuvant/Sheep IgG Emulsion

Route of Immunization	Percentage of Responders	Mean Total Volume (mL) Ascites	Mean Titre (Reciprocal)
ip + im + sc	70%	9.2	105
im + sc	70%	8.2	8
ip	90%	10.1	68

Dose Response to Sheep IgG

Dose of Sheep IgG (μg)	Percentage of Responders	Mean Total Volume (mL) Ascites	Mean Titre (Reciprocal)
1	80%	10.6	0
5	100%	8.6	12
25	100%	14.9	17
50	100%	9.6	41
100	80%	17.7	69
200	100%	9.5	69
400	80%	10.9	73

comparing the ip plus sc plus im route with the im plus sc route and the ip route with the im plus sc route. No significant differences were obtained on comparing the ip plus sc plus im route with the ip route and so the ip route was chosen for subsequent studies because it was less time-consuming to perform and less stressful to the mice.

Dose Response to Sheep Immunoglobulin G

A total of 80 mice were divided into batches of 10 and studied in 2 experiments. In the first, groups of mice received either 1, 5, 25 or 100 μg oIgG and in the second, either 50, 100, 200 or 400 μg.

The data are summarized in Table 1 and the individual titres for each mouse are shown in Figure 1. There were significant differences between individual titres when the 5 μg dose was compared with the 50, 100, 200 and 400 μg doses ($p = <0.005$) and when the 25 μg dose was compared with the larger doses ($p = <0.005$). The greatest volume of ascites was produced using 100 μg and this dose was chosen for use in the mouse model.

Control Data

A total of 100 mice were subjected to the standard protocol outlined earlier which involves the use of FCA and 100 μg of oIgG and the ip injection of pristane on day 14. The 89 mice which had ascitic fluid produced a mean total volume of 13.0 mL per mouse, containing a mean antibody titre of 1:54 (Table 2a). Ascitic

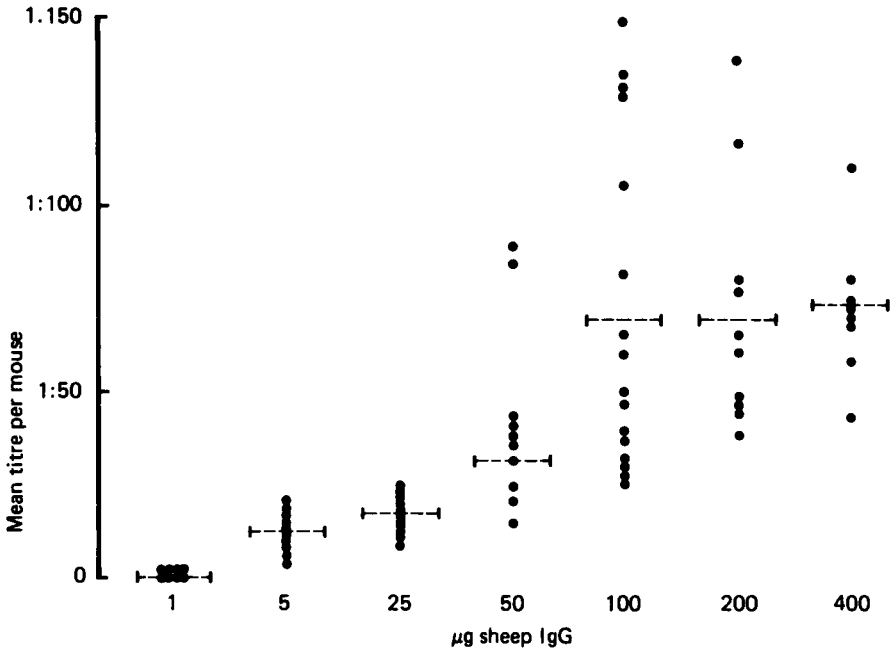

FIGURE 1 Mean antibody titres per mouse to sheep IgG over the period of ascitic fluid production for different doses of sheep IgG.
——— denotes the group mean titre

fluid production was maximal during the seventh week (Figure 2 and Table 2b), with mean volume per mouse (4.2 mL) being highest on day 49. The highest antibody titre (1:78) was found on day 42 (Table 2b), 14 days after the last immunization. Thereafter the titre fell to approximately half this value (1:41) on day 63 when serum titres were measured. The titre for the serum pool collected on day 63 was 1:65, which is 1.6 times that of the ascites pool on that day. This is in agreement with total protein and SDS PAGE analysis of the serum and ascites (data not shown).

TABLE 2

Cumulative Data for the Murine Model

(a) Ascites Data per Mouse over the Period of Ascitic Fluid Production

Percentage of Responders	Mean Total Volume (mL) Ascites	Mean Titre (Reciprocal)
89%	13.0	54

(b) Ascites Data per Mouse for Different Days of Tapping

Day of Tapping	Mean Total Volume (mL) Ascites	Mean Titre (Reciprocal)
31	2.2	31
35	3.7	76
38	3.1	71
42	3.2	78
45	3.0	68
49	4.2	64
52	2.8	52
56	3.3	50
59	2.5	47
63	2.6	41

DISCUSSION

Several of our findings are in accord with those of previous workers. Thus Freund's incomplete adjuvant is unsuitable since no ascitic fluid was produced; the ip route of administration is to be preferred; and the volume of ascitic fluid is increased substantially if pristane is given on day 14. Sommerville (7) produced antibodies to both human and rabbit IgG in the ascitic fluid of mice in titres ranging from 1:32 to 1:64, with each mouse providing between 10 and 15 mL of ascites. However, he did not

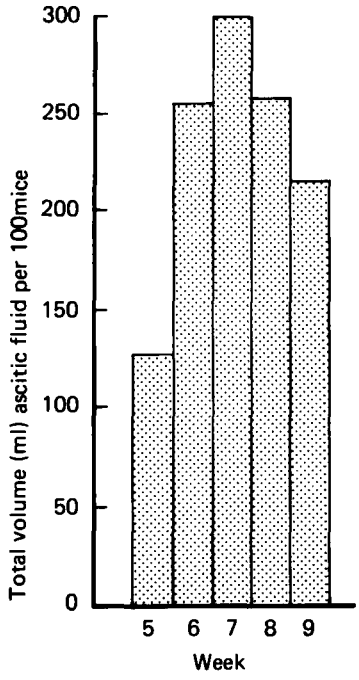

FIGURE 2 Total volume (mL) of ascitic fluid collected per 100 mice on different weeks for the control group. Week 5 = ascitic fluid collected on day 31 + 35; week 6 = ascitic fluid collected on day 38 + 42; week 7 = ascitic fluid collected on day 45 + 49; week 8 = ascitic fluid collected on day 52 + 56; and week 9 = ascitic fluid collected on day 59 + 63

study the time course of the response or undertake a detailed dose response experiment.

We had hoped that the small size of the mice would ensure that they required minimal amounts of immunogen since this could be of considerable importance when studying macromolecules of limited availability. The fact that it required repeated injections of 100 μg of oIgG to obtain maximum titres was a

surprise since we (unpublished observations) and others (8) have obtained high circulating levels of specific antibodies to oIgG in donkeys immunized with similar amounts. That such large quantities of immunoglobulin are required, as compared with 50 μg for insulin and only 5 μg for human growth hormone (6), probably reflects differences in the relative immunogenicity in mice of these three soluble antigens and emphasizes the need to undertake a detailed dose response study for each immunogen. Fortunately, highly purified ovine IgG is easy and inexpensive to prepare.

Monoclonal antibodies are commonly produced in ascitic fluid using BALB/c mice (9) but such treatment leads to cachexia and severe abdominal distension, partly due to the tumour mass which develops. The use of FCA as adjuvant in the present studies causes granuloma formation in the peritoneum but overall there is probably no severe stress to the mice since steroid levels are not elevated (10). Our standard protocol (Table 3) has been modified to terminate on day 49, rather than day 63, with a maximum number of 5 taps. Even by this time the 89 mice which responded had produced over 650 mL of ascitic fluid. Since titres in the ascitic fluid fell progressively after day 42, shortening the procedure did not reduce the quality of the polyclonal antibodies.

Antibodies to atrial natriuretic peptide (ANP) have been produced by the present technique, using 50 μg ANP per immunization and sensitive RIA and IRMA's developed using the ascites as the 'link' antibody in the latter (11). In addition, mice immunized with ANP developed greater volumes of ascites than

TABLE 3

Standard Protocol for the Murine Model

Day	Immunogen Sheep IgG/FCA* Volume (μL)	Pristane (μL)	Collect Ascitic Fluid	Blood
0	200	-		
7	200	-		
14	200	500		
21	200	-		
28	200	-		
31			+	
35			+	
38			+	
42			+	
45			+	
49#			+	+

* 1 part/9 parts
\# sacrifice mice before tapping to ensure a maximum of only 5 taps

All injections to be given via the intraperitoneal route. After day 28 wait for ascites to form, which normally occurs by day 35. Ascitic fluid formation is indicated by abdominal distension and a gain in weight of more than 10 g above the initial weight. Check the mice daily for well being, culling any that fail to thrive. Useful indicators of cachectic mice include no gain in weight, a lack of mobility and poor condition of the coat.

controls immunized with a similar immunogen which did not contain ANP (Tattersall and Dawnay, personal communication). Similarly, antibodies to the Brugia malayi adult antigens raised in mice were useful diagnostically in detecting circulating filarial antigen in Bancroftian filariasis (12) using a sandwich ELISA technique.

We are using this approach to study ways to modify immunogenicity. For example, there is evidence that covalently coupling polyethylene glycol to bovine serum albumin markedly decreases the immunogenicity of the latter (13) and we are currently investigating whether this approach can prevent the immune recognition of oIgG by B and T cells (14). We are also determining the relative immunogenicity of IgG of various species and especially their $F(ab)_2$ and Fab fragments. Our preliminary findings show that, although equine antisera remain the usual treatment for envenomation, ovine IgG is less immunogenic than equine IgG in mice. However, this cannot be taken as final evidence that similar findings occur in man.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help and advice given by Mr K Mansfield and his colleagues in the Biological Services Unit

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