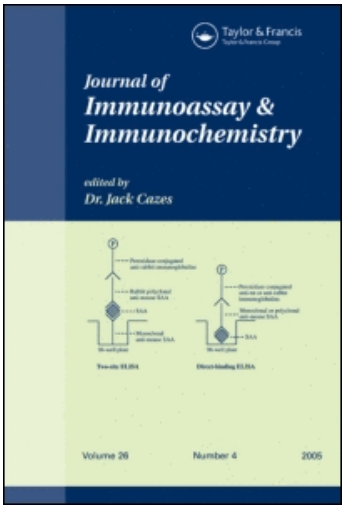

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PRODUCTION OF POLYCLONAL ANTIBODIES IN ASCITIC FLUID OF MICE: TECHNIQUE AND APPLICATIONS

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ABSTRACT

Balb/c mice immunised intraperitoneally with insulin developed significantly higher antibody concentrations in their serum than mice immunised subcutaneously. The antibody response was dose related, 50 ug amounts being more effective than either 20 ug or 5 ug amounts. In contrast, the antibody titres in the ascitic fluid of mice immunised with growth hormone were significantly higher after 5 ug when compared to 25 and 100 ug amounts.

Repeated intraperitoneal injections of an emulsion of sheep IgG or human growth hormone in complete Freund's adjuvant, together with a single intraperitoneal injection of Pristane, induced ascites formation in most mice within 5 weeks. Up to 90 mL could be obtained from a single mouse by abdominal tapping, and the antibodies derived from the ascitic fluid proved suitable for application in a radioimmunoassay.

(KEY WORDS: ascitic fluid, murine polyclonal antibodies)

INTRODUCTION

Antibodies are the most important reactants in all immunoassays since it is they which largely determine both specificity and the ultimate sensitivity that can be achieved. A guinea-pig antiserum formed the basis of the first radioimmunoassay (1), to determine

circulating levels of insulin, and guinea-pigs remain the species of choice for the production of anti-insulin sera. However, the need for cardiac puncture and the small volumes of blood that can be obtained has mitigated against their widespread use and most polyclonal antibodies are currently raised in rabbits, sheep or goats. Not all immunoassayists have access to such animals and the costs involved in their purchase, housing, and maintenance are considerable. Furthermore, the expense prevents the immunisation of large enough numbers of these animals to produce statistically meaningful results, which may explain why antibody production, despite its central role in immunoassay development, remains more of an art than a science (2).

It would be both easy and relatively inexpensive to immunise large groups of mice but, although central to monoclonal antibody production, mice have not been employed extensively to raise polyclonal antibodies. This reflects the difficulty and time required to bleed mice from their tail veins or by cardiac puncture and, even if sacrificed, each mouse provides only about 0.5 ml of serum, thus making this an extravagant method of obtaining antibodies.

An alternative method of obtaining murine antibodies is to induce formation of ascitic fluid after primary immunisation. Thus, in 1957, Munoz reported that repeated intraperitoneal injections (IPI) of egg or ovine albumin emulsified with Freund's complete adjuvant, often caused formation of ascitic fluid containing specific antibodies (3) in concentrations approximating those in serum (4). Lieberman and her colleagues extended these studies (5,6) by introducing different bacterial antigens. Further modifications of the method including the timing of the immunisation schedule, selection of appropriate strain of mouse (7), use of a high ratio of adjuvant to immunogen, and the IPI of Pristane (8,9) now make this a reliable procedure.

Antibodies produced by this technique have been employed for many purposes, such as studies on the structure of murine immunoglobulins (10,11) and the immune response to infectious agents (12,13). Most antibodies have been raised against cell surface antigens on red blood cells, bacteria or viruses, and the technique does not appear to have been used to study factors influencing the immune response (such as improved adjuvants) or to provide antibodies and carrier proteins for immunoassay purposes.

In an attempt to optimise the method further with regard to the quantity of antibody produced, we dealt with some of the issues involved separately. First, we compared antibody production following the administration of a small soluble antigen (insulin) via the subcutaneous or intraperitoneal route. Second, we investigated the influence of the dose of two peptide antigens differing in molecular weight (insulin and human growth hormone) on the antibody titre either in serum or ascites and, third, we studied the effect of different adjuvant combinations containing sheep immunoglobulin G (IgG) as immunogen on the volume of ascitic fluid produced.

MATERIALS AND METHODS

Female Balb/c mice aged 8 to 9 weeks at the beginning of the immunisation and ranging in weight from 15 to 22 g were used for all studies.

Freund's complete (FCA) and incomplete (FIA) adjuvants were purchased from Difco Laboratories, Detroit, USA; normal sheep serum and sheep anti-mouse IgG from IIS, London, UK; charcoal, (Norit GSX)

and polyethyleneglycol 6000 (PEG) from BDH, Poole, Dorset, UK; Dextran T 70 from Pharmacia, Hounslow, Middlesex, UK; DEAE Affi-gel Blue from Bio-Rad, Watford, Hertfordshire, UK; and Pristane (2, 6, 10, 14-tetramethylpentadecane) from Sigma, Poole, Dorset, UK. All reagents were, whenever possible, of analytical grade quality.

Insulin Study

Semisynthetic human insulin of porcine origin was kindly donated by Novo, Copenhagen, Denmark, and dissolved in normal saline at the required concentrations. These aqueous solutions were then emulsified with FCA for the first immunisation and with FIA for reimmunisations at a ratio of 1:10, together with a few drops of Brij 20%.

125 I- labeled human insulin was purchased from Amersham International, Amersham, UK.

A total of 60 mice were divided into groups of 10 and the mice in each group received either 5 ug, 20 ug, or 50 ug of insulin in 200 uL volumes of emulsion by either the subcutaneous route (SCI) or IPI on days 0, 14, 28, and 44. Twenty mice given 100 ug of insulin either SC or IP were immunised only once. Blood samples were collected (on days 14, 28, 44, and 64) from a tail vein into calibrated tubes so that the volume obtained could be estimated. A mean volume of 50 uL was collected and this was diluted 1:10 in assay buffer, centrifuged to remove red blood cells and the diluted sera stored at -20°C until their assessment.

Antibody titres were assessed by incubating 200 uL of a 1:100 dilution of each antiserum with 100 uL (containing 20 pg) of 125 I - labeled human insulin and 100 uL of assay buffer (0.04 M phosphate, pH

7.4, containing 0.15 M NaCl, 0.01 M EDTA, 0.5% BSA, and 0.1% Na-azide) for 24 h at 4°C . Separation of the bound and free fraction was achieved by adding 400 uL of dextran coated charcoal (2.5 g charcoal + 0.25 g dextran T70/100 ml assay buffer), centrifugation at 2500 rpm for 30 min at 4°C, aspirating the supernates to waste and counting the charcoal pellets (containing the free fraction) in a multi-well scintillation counter. Nonspecific binding was estimated by including tubes containing no added antiserum, was generally below 5% and was not accounted for. Percentage bound was calculated by substracting the counts of tubes containing antisera from total counts and subsequent division by total counts.

Growth Hormone Study

Human growth hormone was purified from frozen human pituitary glands as described previously (14) and dissolved in normal saline at the required concentrations. These aqueous solutions were emulsified with FCA and 200 uL volumes injected by the IP route into mice using a 23 gauge needle on days 0, 14, 21, and 28. On day 14, each mouse also received an IPI of 500 uL of Pristane.

The mice were tapped as soon as visible ascites had formed (earliest day 21) and this was continued for up to 12 weeks.

125 I-labeled hGH was prepared using iodogen, as described previously (15). The antibody titre was estimated by incubating 100 uL (200 pg) of tracer with 100 uL of doubling dilutions of ascitic fluid for 16h at room temperature. Separation was achieved by addition of 50 uL of a 1:100 dilution of normal mouse serum, followed by 50 uL of a 1:10 dilution of sheep anti-mouse IgG and, after 15 min,

1 ml of PEG 6000 (to give a final concentration of 4%). The tubes were then centrifuged for 30 min at 4°C and 2500 rpm and the precipitates, containing the bound fraction, counted in a multi-well scintillation counter. The antibody titre is given as the final dilution that bound 50% of the labelled hormone.

Thirty mice were split into groups of ten and given either 5 ug, 25 ug or 100 ug amounts of the immunogen per immunisation.

Volume Production Study

Normal sheep serum was precipitated with solid sodium sulphate (18% w/v). Each precipitate was washed with an equal volume of aqueous sodium sulphate (18%), spun in a bench centrifuge and the supernate decanted. The precipitate was then resolubilised in the original volume of 0.15 M NaCl before reprecipitation as described above. After resolubilisation further purification was by ion exchange and affinity chromatography using DEAE Affi-gel Blue to remove contaminating albumin and proteases.

For preparation of the immunogen mixture, aliquots of 1 ml containing 2.5 g/L of IgG were added to 9 ml of complete or incomplete Freund's adjuvant and emulsified by vigorous vortex mixing after addition of 3 - 5 drops of Brij 20%.

Four groups each of 10 mice were given IPIs of 200 uL of adjuvant/immunogen (containing 50 ug IgG) on days 0, 7, 14, 21, and 28.

Groups 1 and 2 were given complete Freund's adjuvant throughout, Groups 3 and 4 received incomplete Freund's adjuvant throughout.

In addition to adjuvant/immungen, groups 1 and 3 received a single intraperitoneal injection of 500 uL of Pristane on day 14.

Statistics

Test results are expressed as means +/- SEM if not indicated otherwise. Comparison between means was performed using the Mann-Whitney test. When more than two means were compared, the difference between the multiple groups was first established by the Kruskal-Wallis test. p-values (2-tailed) of the Mann-Whitney test performed afterwards were corrected for the number of groups compared and values less than 0.05 were taken to be statistically significant and denoted by *.

RESULTS

Insulin Study

Five out of ten mice given 100 ug of insulin and two of the ten injected with 50 ug of insulin by the IP route died within two days, whereas only one mouse given these amounts subcutaneously failed to survive.

The mice given 100 ug amounts were followed without further injections of immunogen. There was a statistically significant

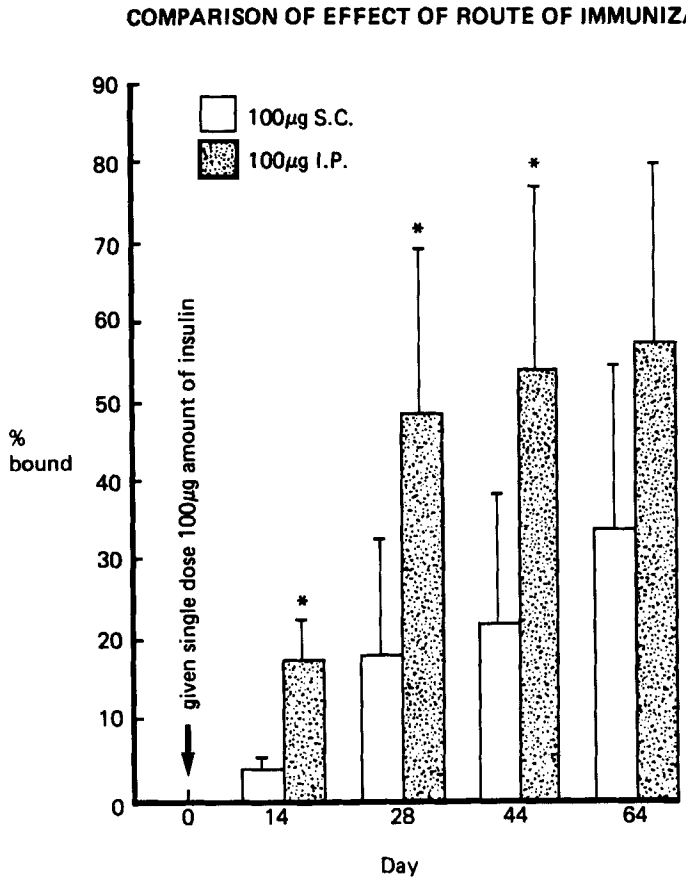

FIGURE 1

increase in tracer binding between day 14 and 64 after the single SCI, but no significant increase after day 14 following the IPI. Intraperitoneal immunisation caused significantly higher serum antibody titres than subcutaneous inoculation on days 14 to 44 (Figure 1).

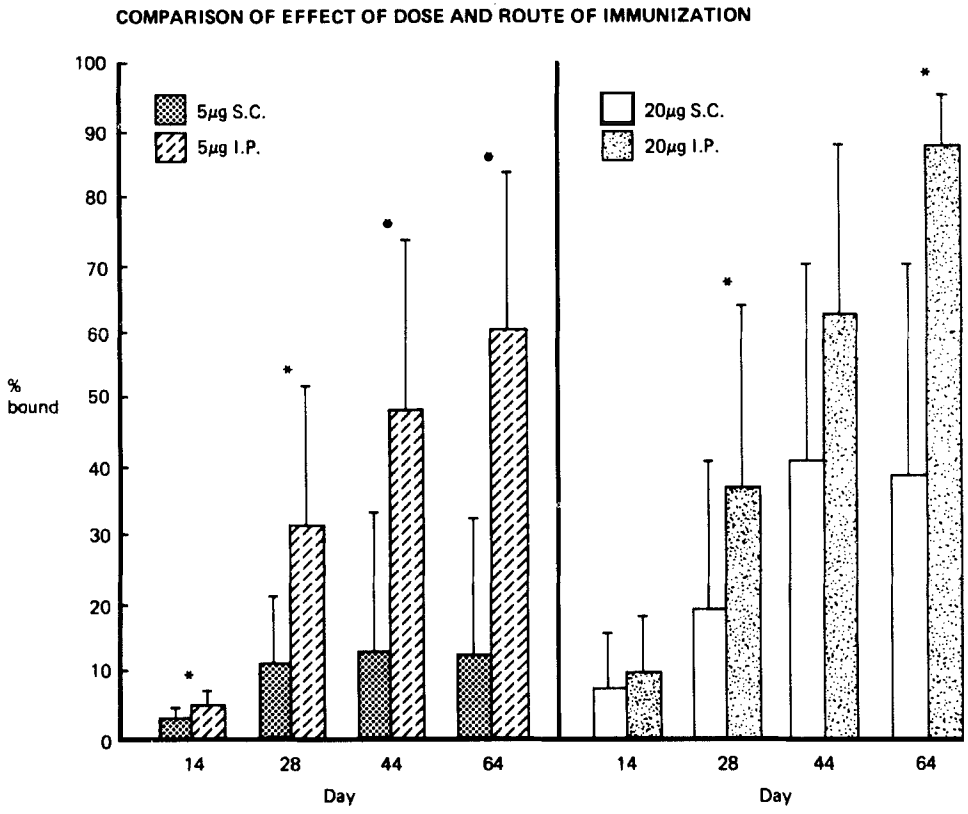

FIGURE 2

Repeated IPIs of 5 or 20 ug amounts also caused significantly higher (approximately double) serum antibody titres than SCIs of the same dose (Figure 2).

Following repeated IPIs, 50 ug amounts caused significantly higher antibody titres than 5 ug amounts on day 28 and 44. 20 ug injected repeatedly gave rise to significantly more binding than 5 ug on day 64 (Table 1).

TABLE 1

Percentage Binding After Graded Intraperitoneal Injections of Human Insulin

Day of Study	Dose/IPI (ug) Mean (SD) 5	20	50	100
14	5 (2)	9 (8)	10 (8)	18 (19)
28	31 (21)	37 (27)	66 (17)	--
44	48 (25)	62 (25)	89 (5)	--
64	60 (23)	87 (6)	--	--

Growth hormone study

Results for the 30 Balb/c mice given three different doses of hGH by IPI are summarised in Table 2. Two died and a further five did not produce sufficient ascitic fluid to tap. Antibody titres in the group receiving only 5 ug amounts of hGH were significantly greater than those in the ascitic fluid from the groups injected with 25 ug ($p<0.05$) or 100 ug ($p<0.01$) amounts. The mean total volumes of ascitic fluid produced did not differ between the three groups.

Volume Production

No ascites could be removed from any of the animals immunised with FIA (group III and IV), whether they had received additional Pristane or not. That this is due to a failure to produce ascites rather than to difficulties in removing it is evident from the finding

TABLE 2

Ascites Volume and Antibody Titre after Graded Intraperitoneal Injections of Human Growth Hormone

Animal No	Total Volume of Ascitic Fluid (ml)	Antibody Titre (Reciprocal)
Given 5 ug hGH/Immunisation		
1	2,5	34.000
2	49,0	72.000
3	90,0	48.000
4	,0	--
5	,0	--
6	13,0	40.000
7	20,0	52.000
8	65,5	56.000
9	22,0	120.000
10	19,0	60.000
Mean (SEM)	28,1 (9,6)	60.250 (9.490)
Given 25 ug hGH/Immunisation		
11	Died	--
12	,0	--
13	8,5	34.000
14	13,0	6.000
15	82,0	13.000
16	33,0	48.000
17	18,5	11.000
18	24,0	9.000
19	37,5	32.000
20	Died	--
Mean (SEM)	27,1 (9,0)	21.857 (6.069)
Given 100 ug hGH/Immunisation		
21	40,5	5.000
22	20,5	4.000
23	,0	--
24	22,5	4.000
25	48,0	26.000
26	,0	--
27	51,0	5.000
28	35,0	18.000
29	81,0	16.000
30	76,0	9.000
Mean (SEM)	37,5 (8,8)	10.875 (2.906)

TABLE 3

Volume of Ascitic Fluid after Intraperitoneal Injections of Complete Freund's Adjuvant/IgG without (Group I) or with (Group II) Addition of Pristane on Day 14.

	GROUP I		GROUP II	
	MEAN VOLUME (ml/mouse)	TOTAL VOLUME (ml/group)	MEAN VOLUME (ml/mouse)	TOTAL VOLUME (ml/group)
DAY 35	0	0	1,9	18,9
DAY 38	1,3	13,2	1,8	17,6
DAY 42	2,0	18,3	3,0	29,7
DAY 45	1,2	10,6	1,8	18,1
DAY 49	,5	4,6	1,5	13,5
DAY 52	,5	4,8	1,1	10,3
DAY 56	,4	3,5	1,0	9,4
DAY 63	0	0	,8	6,9
TOTALS	6,0	55,0	12,9	124,4

that these animals gained only an average of 2 g in weight over the study period (data not shown).

Every animal administered FCA/IgG produced some ascites, though not all on the same day. One animal out of each ascites forming group died during the observation period (after day 38 in group I, after day 45 in group II). The total volume produced ranged from 0.7 to 12.2 ml per mouse in group I and from 2.3 to 23.2 ml in group II. Mice in group II began to form ascites three days earlier and for longer than those of group I.

The total volume produced by group I over the entire study period was 55 ml, as compared to 124.4 ml by group II (Table 3). This difference is statistically significant.

Clinical Uses

The ascitic fluid pool from the mouse (No 25) with the highest avidity was substituted for the rabbit anti-hGH serum in the routine hospital service assay at a final dilution of 1:25,000. The results obtained with the two different antibodies for clinical samples correlated closely (r=0.98) and the equation of the regression line was : y = 1.2 * X - 0.1 . The antibody supply from this one mouse would be sufficient for about 5 million tubes.

Finally, it was shown that the IgG in ascitic fluid from mice given FCA alone by IPI served equally well as normal mouse serum IgG as the carrier in second antibody separation, although the ascitic fluid had to be used at a lesser dilution as expected from its lower total protein content.

DISCUSSION

The present study emphasises the usefulness of mouse ascitic fluid as a source of specific antibodies and nonspecific murine proteins.

Using this method, the advantages of mice as laboratory animals (small costs of purchase and maintenance, availability of inbred strains, possibility to study large numbers) may be fully exploited while at the same time the restrictions caused by their size (very limited volume of serum) are partly overcome.

In this study we first presented evidence that intraperitoneal immunisation is superior to the subcutaneous route in terms of

antibody titre. The higher mortality of mice given large amounts of insulin by IPI than that of those receiving the same dose by SCI suggests that insulin is more rapidly absorbed from the peritoneum and may be taken up by other parts of the reticulo-endothelial system. Following such injections, the peritoneum becomes lined with small nodular granulomata containing proliferating plasma cells, lymphocytes and histiocytes, and the abdominal and cervical lymph nodes enlarge (6).

In the volume production study we demonstrate the critical importance of using complete Freund's adjuvant with a soluble immunogen to induce ascites formation, which contrasts with the previous findings of Lieberman and her colleagues (6). As recommended by Tung (8), we used a high volume ratio of adjuvant to immunogen for preparation of the immunogen emulsion. In addition, we formally tested the effect of a single intraperitoneal injection of Pristane and found a significant increase in volume production.

The total ascitic fluid protein content is about 50% that of serum and the antibodies are predominantly of the IgG class (6) and the IgG1 and IgG2 subclasses (16). Both ascitic fluid and serum have a similar protein distribution, as evidenced by electrophoresis (17) and immunoelectrophoresis (18,19).

The study of the influence of the dose on the immune response after repeated IPIs shows conflicting results for insulin and hGH. Whereas higher doses also gave higher antibody titres following insulin injections, the reverse was true for hGH. This may reflect differences in the immunogenicity of the two compounds.

TABLE 4

Day	Immunogen/Adjuvant (1 part/ 9 parts)	Pristane
0	200 uL	--
14	200 uL	500 uL
21	200 uL	--
28	200 uL	--

After day 28 wait for ascites to form, which normally occurs by day 35. Tap mice and check weekly for fluid formation. Further injections of Immunogen/Adjuvant only when fluid production begins to decrease or antibody titre not satisfactory.

* Complete Freund's Adjuvant

According to the experience gained so far, we would recommend an immunisation scheme as outlined in Table 4.

Mouse ascitic fluid may serve as an alternative source of antibodies for use in immunoassays, if success is not being achieved using the conventional types of animal. Furthermore, mice can be used to assess, whether a new immunogen is suitable prior to its injection in large quantities into larger animals, with a result in 35 days. This approach may also be used to address such questions as the optimal ratio of carrier to hapten or the advantage of new adjuvants employing large enough numbers of animals to produce statistically meaningful results.

Apart from being a convenient source of antibodies, mouse ascites can also be used as a source of nonspecific proteins ,for example for use as carriers in second antibody separation systems.

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REFERENCES

1 Yalow, R A and Berson, S A. Immunoassay of endogenous plasma insulin in man. J Clin Invest 1960, 39, 1157-1164.

2 Hurn, B A L and Landon, J. Antisera for radioimmunoassay. In: Kirkham, K E and Hunter, W M (Eds.). Radioimmunoassay Methods. London & Edinburgh, Churchill Livingstone, 1971:121-130.

3 Munoz, J: Production in mice of large volumes of ascites fluid containing antibodies. Proc Soc Exp Biol Med 1957; 95: 757- 759.

4 Anacker, R L and Munoz, J. Mouse antibody. I. Characterization and properties of antibody in mouse peritoneal fluid. J Immunol 1961; 87:426-432.

5 Lieberman, R, Douglas J O A and Humphrey, W Jr. Ascites induced in mice by staphylococcus. Science 1959; 129: 775.

6 Lieberman, R, Douglas J O A and Mantel, N. Production in mice of ascitic fluid containing antibodies induced by staphylococcus- or salmonella-adjuvant mixtures. J Immunol 1960; 84: 514-529.

7 Lieberman, R, Mantel, N and Humphrey, W. Ascites production in 17 mouse strains. Proc Soc Exp Biol Med 1961; 107: 163-165.

8 Tung, A S, Ju, S-T, Sato, S and Nisonoff, A: Production of large amounts of antibodies in individual mice.
J Immunol 1976; 116 (3): 676-681.

9 Tung, A S. Production of large amounts of antibodies, nonspecific immunoglobulins, and other serum proteins in ascitic fluids of individual mice and guineapigs.
Methods in Enzymology 1983; 93: 12-22.

10 Merryman, C and Benacerraf, B. Studies on the structure of mouse antibodies. Proc Soc Exp Biol Med 1963; 114: 372-375.

11 Friedenson, B, Tung, A S and Nisonoff, A. Constancy of amino-terminal amino-acid sequences of antibodies of defined specificity and shared idiotype from individual inbred mice. Proc Natl Acad Sci 1975; 72: 3673-3679.

12 Brandt, W E, Buescher, E L and Hetrick , F M. Production and characterization of arbovirus antibody in mouse ascitic fluid.
Am J Trop Med and Hyg 1967; 16 (3): 339-347.

13 Millman, I, Ziegenfuss, J F Jr, Raunio, V, Lonodn, W T, Sutnick, A I and Blumberg, B S. The production of antibodies to Australia antigen in mouse ascites fluid. Proc Soc Exp Biol Med 1970; 133: 1426-1431.

14 Jones, R L, Benker, G, Salacinski, P R, Lloyd, T J and Lowry, P J. Large-scale preparations of highly purified pyrogen-free human growth hormone for clinical use. J Endocrinol 1979; 82: 77-86.

15 Salacinsky, PRP, Mclean, C, Sykes, J E C, Clement-Jones, V V, and Lowry, P J. Iodination of proteins, glycoproteins and peptides using a solid phase oxidising agent, 1,3,4,6-tetra-chloro-3,6-diphenyl glycouril (Iodogen). Anal Biochem 1981; 117:136-146.

16 Mattioli, C A, Yagi, Y and Pressman, D. Production and properties of mouse antihapten antibodies. J Immunol 1968; 101 (5): 939- 948.

17 Jordan, R T, Banovitz, J, Trapani, I L and Campbell, D H. Electrophoretic analysis of serum and ascitic fluid proteins from immunized and nonimmunized mice. J Immunol 1962; 88: 314-319.

18 Ishii, H. Studies on mouse hemolysin. I. Immune ascites as a source of hemolysin. Jap J Exp Med 1967; 37: 515-524.

19 Wagner, O and Raesaenen, J A. Mouse immune ascites in immunoelectrophoresis. Ann Med exp Fenn 1967;45: 170-173.