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Comparison of ascites production for monoclonal antibodies in BALB/c and BALB/c-derived cross-bred mice

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BALB/c male mice were mated with either Swiss-Webster or MF1 females to produce first generation cross-bred offspring. Hybridoma cell lines, from the fusion of P3-NS1-Ag4/1 myeloma cells with spleen cells sensitised to the porcine coronavirus causing transmissible gastroenteritis, were injected intraperitoneally into these mice to produce ascitic fluid containing monoclonal antibodies. Mice of 11 weeks of age weighing between 26 and 34 g were used. The volume of ascites produced by mice injected with four of the five hybrid cell lines tested was greater in the cross-bred offspring than in the BALB/c parent. The fifth cell line gave comparable volumes in the MF1 cross-breed and BALB/c parent but a lesser volume in the Swiss-Webster cross-breed. The antibody titres of the ascites as determined by virus neutralisation, radioimmune and indirect immune fluorescence assays, did not differ significantly between mouse types. The ability to use all offspring from a litter of cross-bred mice, irrespective of sex, and the increased volume of ascitic fluid formed in each mouse, permits fewer animals to be used for the production of ascites in these strains, thereby offering considerable economic and ethical advantages over the use of BALB/c mice.

Key words: Monoclonal antibody; Ascitic fluid; (BALB/c × Swiss-Webster)F1; (BALB/c × MF1)F1; (Mouse)

Introduction

The increasing usage of monoclonal antibodies (MAb) as immunological reagents and for research requires the production of high titre preparations. It has been shown that mouse hybridoma cell lines will rapidly produce ascites tumours in histocompatible mice, yielding milligram quantities of antibody relatively uncontaminated by non-mouse proteins. This represents a simpler method of production compared to normal cell culture techniques, but is expensive in terms of the number of mice needed and their management.

The study of Brodeur and Tsang (1986), using a hybrid mouse line to produce large volumes of high titre MAbs, suggested a cheaper and more efficient method of producing the reagents required for our studies on the antigenicity of the porcine transmissible gastroenteritis virus (TGEV). We have extended their study and this report describes the production of first generation crosses
derived from the BALB/c inbred mouse line and two outbred mouse strains, their use in raising ascites tumours and analysis of the immunoglobulin yield from five hybridoma cell lines.

Materials and methods

Production of cross-bred mice

BALB/c mice were purchased from Charles River (U.K.) (Margate, Kent), Swiss-Webster mice and MF1 mice were purchased from Harlan Olac (Bicester, Oxon). The greatest litter sizes were achieved by mating BALB/c males with either Swiss-Webster or MF1 females, the resulting pups being weaned at 3 weeks of age. Male and female pairs were housed together and repeat matings usually occurred post-partum. Optimum breeding conditions permitted six litters per pair before litter size and fecundity decreased.

Production of ascites

6-week-old BALB/c male mice are preferentially used for ascites production because they develop demonstrably more fluid than females (Brodeur et al., 1984). Mixed groups of randomly selected male and female cross-bred mice of approximately 11 weeks of age, as recommended in the study of Brodeur et al. (1986), were compared to an equivalent number of BALB/c male mice of 6 weeks of age, for their ability to produce ascitic fluid. Mice, at the appropriate age, were treated with 0.5 ml pristane (4,6,10,14-tetramethylpentadecane; Koch-Light) 7 days before intraperitoneal injection of 1 × 10^6 hybridoma cells, harvested from actively growing cultures and suspended in 1 ml of isotonic saline.

The hybridomas used produce IgG1 antibodies. Two of them (3C156 and 6D442) produce antibody capable of neutralising TGEV, the remaining three (DA3, 4B115 and 9D298) have no detectable neutralising capability. DA3 recognises an epitope on the viral nucleoprotein while the remainder are specific to the viral surface projection (peplomer) (Garwes et al., 1987, 1988). Competition binding assays have shown that each of these antibodies recognises a different epitope on the peplomer and they have been classified as belonging to the following competition groups: A (3C156), D (9D298), F (6D442) and G (4B115).

Ascites were allowed to develop until the mice were visibly distended, at which time they were killed and the ascitic fluid collected. The sex of the mouse, the volume of fluid removed from it and the presence of solid tumours was noted. After clarification of the fluid by centrifugation at 10,000 × g, the final volume was measured and the fluids stored at −20°C until required.

Virus neutralisation test (VNT)

Ascites-3C156 and 6D442 were tested for TGEV neutralising ability. Ascitic fluids from daily harvests were diluted 1/10 followed by serial three-fold dilutions in duplicate in medium (Eagle's MEM buffered with 0.14% sodium bicarbonate), 50 mM HEPes, 2.5% calf serum (Gibco) and containing penicillin, streptomycin and mycostatin (at 100 U, 100 µg and 250 U·ml⁻¹ respectively). An equal volume of the same medium containing 200 TCID₅₀ of the FS772/70 strain of TGEV was added and incubated at 37°C for 1 h before 150 µl of each sample was transferred to confluent monolayer cultures of secondary adult pig thyroid (APT/2) cells in 96 well plates. After a further 3 days incubation at 37°C, end points were determined by scoring for cytopathic changes. The titre of the antibody was expressed as the reciprocal of the highest dilution that protected a cell sheet against infection.

Fluorescent antibody test (FAT)

LLC-PK1 cell monolayers (Garwes et al., 1984) in 96 well plates were infected with approximately 10 p.f.u. of TGEV/cell for 10 h at 37°C then fixed with cold 80% acetone for 10 min at 20°C. Ascites were diluted as for the VNT but in PBSa (Dulbecco and Vogt, 1954); 100 µl aliquots were introduced onto the fixed infected plates, incubated at 37°C for 1 h and then washed three times with PBSa. FITC-rabbit anti-mouse immunoglobulin (Nordic) was diluted 1/40 in PBSa and 25 µl was added to each well. After incubation at room temperature for 30 min the plates were washed as before then examined with a Leitz Ortholux microscope fitted with a fluorescence vertical illuminator. The titre was expressed as the
reciprocal of the highest antibody dilution showing fluorescence.

**Radioimmunoassay (RIA)**

TGEV was pelleted from tissue culture supernatant fluid through 30% sucrose at 30,000 × g for 2 h. After resuspension in distilled deionised water (DDW), to 1/100 of the original volume, the virus was treated with 1% Nonidet NP-40 for 10 min at room temperature then diluted 1/10,000 in DDW. Aliquots of 100 µl were air-dried onto polyvinyl 96 well plates (Falcon, Becton & Dickinson) at 37°C and then fixed with 80% acetone and blocked with PBSa containing 0.1% Tween 20, 1% gelatin (PTG) for 30 min at 37°C.

Serial three-fold dilutions of ascites were made in PTG on the antigen-coated plates, incubated at 37°C for 1 h and then washed three times in PTG. Biotinylated sheep anti-mouse Ig (Amersham International, U.K.) was diluted 1/1000 in PTG and 100 µl added to each well. The plates were then incubated and washed as before. To each well was added 100 µl of 125I-streptavidin (Amersham International U.K.) diluted 1/750 (130 nCi/ml) in PTG. After incubation at 37°C for 15 min the plates were washed extensively and individual wells counted.

The data were subjected to linear regression analysis and the highest dilution giving a count greater than mean + three times the standard deviation (\(\bar{x} + 3\sigma\)) of the negative control was taken as the end point.

**Results**

Average litter sizes were determined from 60 to 100 litters of both the parental and F1 strains and the average weight for each type and sex of mouse at 6 and 11 weeks of age was determined from approximately 100 mice in each group. Table I shows a summary of these findings. Statistical analysis showed that both types of cross were significantly heavier than the BALB/c parent (\(P < 0.0001\)) and that the BALB/c × MF1 (MF1/×) cross are significantly heavier than the BALB/c × Swiss-Webster (SWR/×) cross (\(P < 0.0001\)).

The volume of ascitic fluid harvested from individual mice was compared, firstly to the volumes taken from other members of the same group,
### TABLE II
MEAN AND STANDARD DEVIATION OF VOLUME OF ASCITIC FLUID HARVESTED FROM EACH MOUSE TYPE

| Hybridoma cell type | Group total | Mean Harvested | Producers |
|---------------------|-------------|----------------|-----------|
|                     | Mice in group | ml (± SD)       | mice a ml (± SD) |
| Mouse line: BALB/c |
| 3C156               | 15          | 2.27 (1.36)     | 14 | 2.43 (1.25) |
| 6D442               | 15          | 2.57 (0.73)     | 15 | 2.57 (0.73) |
| DA3                 | 15          | 0.95 (0.91)     | 12 | 1.19 (0.91) |
| 4B115               | 15          | 2.10 (1.20)     | 14 | 2.25 (0.90) |
| 9D298               | 15          | 1.43 (0.65)     | 14 | 1.54 (0.54) |
| Mean volume/mouse b | 1.86 (0.66) | 2.00 (0.60)     | 0.10 (0.05) |
| Mean volume/g body weight c | | |

| Mouse line: SWR /x |
|---------------------|-------------|----------------|-----------|
| 3C156               | 10          | 1.80 (1.72)     | 6 | 3.00 (0.63) |
| 6D442               | 10          | 2.90 (1.24)     | 10 | 2.90 (1.24) |
| DA3                 | 15          | 1.91 (2.05)     | 9 | 3.33 (1.17) |
| 4B115               | 15          | 2.80 (2.20)     | 11 | 3.86 (1.60) |
| 9D298               | 15          | 3.30 (2.23)     | 11 | 4.50 (1.00) |
| Mean volume/mouse b | 2.54 (0.66) | 3.52 (0.66)     | 0.12 (0.06) |
| Mean volume/g body weight c | | |

| Mouse line: MFI /x |
|---------------------|-------------|----------------|-----------|
| 3C156               | 15          | 2.67 (2.71)     | 9 | 4.44 (1.99) |
| 6D442               | 15          | 4.27 (1.91)     | 13 | 4.92 (0.86) |
| DA3                 | 15          | 3.00 (2.43)     | 11 | 4.09 (1.83) |
| 4B115               | 15          | 2.97 (2.16)     | 12 | 3.70 (1.48) |
| 9D298               | 15          | 3.60 (1.73)     | 14 | 3.86 (1.47) |
| Mean volume/mouse b | 3.30 (0.64) | 4.20 (0.49)     | 0.13 (0.04) |
| Mean volume/g body weight c | | |

* Mice that produced ascitic fluid.
  * Mean volume and standard deviation of fluid produced by the mouse line irrespective of hybridoma cell type.
  * Mean volume and standard deviation of fluid produced per g body weight.

Secondly to mice of the same sex in the other groups (where possible) and thirdly to the mean volume taken from the BALB/c male mice. This determined any factors affecting the production of fluid such as the hybridoma cell type, the mouse type or the sex of the mouse used. It has been observed (Brodeur et al., 1984) that male mice produce more fluid than the females. In this study we could find no correlation between sex and yield of fluid from the cross-bred strains (results not shown). After clarification by centrifugation the volume of fluid recovered represented approximately 80% of the original volume for all cell types and mouse strains. The remaining 20% comprised blood cells and other cellular debris.

The number of mice producing ascites differed with the type of hybridoma cell used. In one case (3C1) the cells induced the formation of hard packed masses of cells associated with the injection site and the mesentery, rather than the more normal plasmacytomas. In other cases (DA3, 4B1 and 9D2) there were areas of cellular aggregation throughout the mesentery, but these did not appear to interfere with the production of the fluid. Some mice died before the ascites could develop and this occurred more frequently with the 3C156 and DA3 groups in both the cross-bred mouse strains. The data presented for yield of ascites therefore reflect premature death and hard tumour production and the individual volumes of ascitic fluid.
TABLE III

TITRES OF ASCITIC FLUIDS AS DETERMINED BY VIRUS NEUTRALISATION TEST (VNT), FLUORESCENT ANTIBODY TEST (FAT) AND RADIOIMMUNOASSAY (RIA)

| Cell line | Mouse type | Mean titre a |
|-----------|-----------|--------------|
|           |           | VNT | FAT | RIA |
| 3C156     | BALB/c    | 3.5×10^5 | 7.3×10^3 | 7.2×10^4 |
|           | SWR/×     | 2.0×10^5 | 7.3×10^3 | 2.5×10^5 |
|           | MF1/×     | 2.0×10^5 | 2.2×10^4 | 8.0×10^4 |
| Previous stock | BALB/c | 2.0×10^5 | 2.2×10^4 | 7.5×10^4 |
| 6D442     | BALB/c    | 8.1×10^2 | 2.7×10^2 | 2.0×10^4 |
|           | SWR/×     | 8.1×10^2 | 2.4×10^3 | 5.0×10^4 |
|           | MF1/×     | 8.1×10^2 | 4.1×10^3 | 2.0×10^4 |
| Previous stock | BALB/c | 2.7×10^2 | 2.7×10^2 | 2.0×10^3 |
| DA3       | BALB/c    | NA b     | 2.2×10^4 | 2.0×10^3 |
|           | SWR/×     | NA b     | 2.2×10^4 | 2.1×10^3 |
|           | MF1/×     | NA b     | 2.2×10^4 | 2.1×10^3 |
| Previous stock | BALB/c | NA b     | 2.2×10^4 | 1.5×10^3 |
| 4B115     | BALB/c    | NA       | 7.3×10^3 | 5.0×10^4 |
|           | SWR/×     | NA       | 2.4×10^3 | 7.0×10^4 |
|           | MF1/×     | NA       | 7.3×10^3 | 8.0×10^4 |
| Previous stock | BALB/c | NA       | 7.3×10^3 | 3.0×10^4 |
| 9D298     | BALB/c    | NA       | 7.3×10^3 | 1.8×10^5 |
|           | SWR/×     | NA       | 7.3×10^3 | 1.8×10^5 |
|           | MF1/×     | NA       | 7.3×10^3 | 2.6×10^5 |
| Previous stock | BALB/c | NA       | 7.3×10^3 | 1.5×10^5 |

a Ascitic fluids were collected over a span of several days. Each day's sample was tested individually to assess variation. Since no variation with time was found the mean titre is shown above.
b Not applicable.

The titres of fluids collected on different days after injection did not differ significantly within each experimental group and the results were pooled. Table III shows the pooled results for each assay with each hybridoma cell line raised in the three types of mice.

Discussion

The cross-bred mice were used at 11 weeks of age as suggested by Brodeur and Tsang (1986). However, further work at this laboratory has shown that the MF1 × mice are ready for use at 6 weeks of age. Although they have a slightly lower body weight at this age they produce as much ascitic fluid as their 11-week-old counterparts. Although BALB/c, SWR and MF1 mice are reasonably docile and easy to handle, there is a marked difference in the temperaments of both
types of cross-breed. Even at 11 weeks of age the cross-bred mice are much more agitated and aggressive when handled.

After injection with the hybridoma cells ascitic fluid can be harvested at least 2–4 days earlier from BALB/c mice than from both types of cross. The number of days required for all the mice in each group to be completely harvested is also markedly different. The BALB/c mice are usually ready for fluid harvest 16–21 days (mean 17.508 days ± 2.3823) following injection so that the fluid is collected within a short time span. However, the SWR/× mice require 16–26 days (mean 20.29 days ± 3.6964) and the MF1/× mice require 18–24 days (20.70 days ± 1.7822) before the ascites are ready. This may only be of importance if ascites production is required in minimum time.

Statistical analysis of the volumes of fluid removed from the three mouse strains showed that there was a significant difference between the MF1/× and BALB/c yields (P < 0.01) whereas there was only a slight difference between the BALB/c and SWR/× yields (P = 0.14) and between the SWR/× and MF1/× yields (P = 0.10). That these differences were not due solely to the larger size of the MF1/× mice is suggested by analysis of the yields of fluid/g body weight. There was a significant difference between the values from BALB/c and MF1/× (P < 0.01) while the differences between BALB/c and SWR/× (P = 0.06) and SWR/× and MF1/× (P = 0.41) were not significant.

Analysis of the data from the various tests showed no significant differences between the titres of the antibodies found in the ascites from the different strains of mouse, irrespective of the length of time between injection of the cells and removal of the ascitic fluid. The cross-bred mice, especially the MF1/×, showed greater consistency with regard to the volume of fluid that can be collected from a given number of mice. At this laboratory there have been some problems with BALB/c mice producing an increased proportion of hard tumours, so reducing the volume of fluid that can be collected. We are now using MF1/× mice for the routine production of ascitic fluids where the longer time taken for the fluids to form is of no importance. The choice of these mice in preference to the SWR/× was based on

the larger litter sizes and the greater body weight. In addition to this, unlike the BALB/c parents, when the hybridoma cells induced hard tumours there was a continued, if reduced, production of fluid in these mice.

There appears to be some correlation between the body weight of the mice used and the amount of fluid produced in them. In many laboratories fluid is removed from the mouse by the repeated insertion of a sterile hypodermic needle into the abdominal cavity to permit drainage of the fluid. In this laboratory the mice are allowed to produce fluid until they are visibly distended and then killed. In larger mice the peritoneal cavity will provide a greater area for the fluid to accumulate before the mice start swelling. It may also take a longer time for full distension to occur.

It has been observed that male BALB/c mice produce greater quantities of ascitic fluid compared to the females (Brodeur et al., 1984). In the cross-bred mice no such correlation between sex and yield was seen, indicating the possibility of using both male and female mice. This implies a more economical use of all animals from a litter of MF1/× mice, rather than just the males as is normal with BALB/c mice. The mean volume of ascitic fluid harvested from the three strains also shows that the MF1/× produce up to twice as much fluid as the BALB/c strain. This suggests that only half the number of mice is needed to generate the same volume of fluid as the BALB/c strain. This represents not only a more ethical usage of mice since fewer mice are required but is also advantageous in that less time, media and culture vessels are needed to grow the necessary hybridoma cells. Alternatively, one might use the same number of cross-bred mice as the BALB/c mice resulting in a larger volume of fluid. The result of this would be a long-lasting reserve of that particular ascites obviating the necessity for repeated use of more mice to generate further fluid. The advantage of this latter course is that one could be relatively certain that the antibody one is using remains constant in terms of titre and specificity.

This study indicates the value of first generation cross-bred offspring from the parental BALB/c strain and an outbred mouse strain for the production of large volumes of ascitic fluid
containing high titre monoclonal antibodies. The first generation crosses are easy to produce, moderately easy to handle, and give consistently large volumes of high titre ascitic fluid compared to the BALB/c parent.

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