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Construction of a Bovine–Murine Heteromyeloma Cell Line; Production of Bovine Monoclonal Antibodies against Rotavirus and Pregnant Mare Serum Gonadotrophin

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ABSTRACT

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Bovine–murine heteromyeloma cell lines were prepared by fusing lymphoid cells from a bovine leukemia virus (BLV)-infected cow with mouse myeloma cells. Selection of hybrid cell colonies was based on the ratio of bovine and murine chromosomes, the presence of cell-surface immunoglobulins and growth characteristics. First-generation fusion partners were compared for fusion efficiency and the number of antigen-specific antibody-producing clones generated. Hybrid cell colonies that initially secreted antibodies were selected from first-generation heteromyelomas to function as second-generation fusion partners. Although fusion efficiencies for both generations did not differ, the second-generation heteromyelomas yielded a higher number of specific antibody-producing clones. Fusion of heteromyelomas with either lymph node cells or splenocytes indicated that fusion with lymph node cells results in a higher number of specific antibody-producing clones, whereas fusion efficiency was found to be higher with splenocytes. The optimal time intervals between the final booster injection and fusion were found to be 4 days for splenocytes and 7 days for lymph node cells. Finally, the characterization of bovine monoclonal antibodies against bovine rotavirus and pregnant mare serum gonadotrophin and their neutralizing capacities in vitro are described.

INTRODUCTION

In animal production monoclonal antibodies have become important tools in diagnostic and fundamental research. In addition, monoclonal antibodies
can be used to optimize physiological processes such as growth and reproduction, and are important in the treatment of certain animal diseases (Booman, 1988). Their murine or rat origin may limit the use of monoclonal antibodies in passive immunization of other species. In human clinical trials with murine monoclonal antibodies, almost all non-immunocompromised patients developed anti-mouse immunoglobulin antibodies which inhibited the therapeutic effect (Cole et al., 1985). In pigs, repeated administration of murine antibodies generated a significant antibody response to mouse immunoglobulins (Arriëns and Booman, 1989). In addition, immunoglobulins have species-specific effector determinants, which allow co-operative cellular effects not possible with heterologous antibodies (Nose and Wigzell, 1983).

The production of antibodies other than those of murine or rat origin has been greatly hampered, however, by the lack of suitable myeloma fusion partners. Nevertheless, some success has already been achieved in the development of monoclonal antibodies from livestock species. Srikumaran et al. (1984) demonstrated the potential of interspecies hybridomas produced by fusing mouse myeloma cells with bovine lymphocytes. Such interspecies fusions resulted in bovine antibodies against bovine enteric coronavirus (Raybould et al., 1985) as well as in antigen-specific ovine monoclonal antibodies (Beh et al., 1986; Groves et al., 1987b). A severe limitation to interspecies hybridomas, however, is their genetic instability, due to the selective elimination of the non-murine chromosomes. In another approach, followed by Tucker et al. (1984; 1987), Groves et al. (1987a; 1988) and by us, bovine–murine hybrid myelomas (heteromyelomas) have been constructed in an attempt to obtain a better fusion partner for the production of bovine monoclonal antibodies. It was expected that a heteromyeloma would retain the superior fusion characteristics of the mouse myeloma cells and be better able to support stable bovine antibody production because of the presence of bovine chromosomes.

The present paper reports the construction and selection of a bovine–murine heteromyeloma cell line. The optimal interval between boosting and harvesting of the lymphocytes and the source of lymphocytes was also studied to a limited extent. In this study bovine monoclonal antibodies were produced against rotavirus and pregnant mare serum gonadotrophin (PMSG).

MATERIALS AND METHODS

Construction and selection of 1st-generation heteromyeloma cell lines

Bovine–murine heteromyeloma cell lines were constructed by fusing lymphoid cells from a bovine leukemia virus (BLV)-infected cow with cells of the X63-Ag8.653 mouse myeloma cell line (Kearney et al., 1979). Seven years previously, this cow had been experimentally infected with BLV strain J 5 (Ressang et al., 1974). The supposedly transformed lymphoid cells were selected
from spots in the popliteal lymph node with a hyalin appearance. After fusion, growth characteristics such as doubling time, viability after freezing and subsequent thawing, and homogeneity of cell type of the colonies of hybrid cells were determined. In addition, the ratios of numbers of bovine and murine chromosomes and of bovine and mouse cell-surface Ig, and the secretion of bovine Ig, were assessed. Selected hybridoma colonies were cloned twice by limiting dilution and the subclones checked for chromosomal content, cell-surface Ig and growth characteristics. Subclones chosen to be fusion partners were made hypoxanthine, aminopterin, thymidine (HAT)-sensitive by growing them for at least 2 months in medium supplemented with 30 μg/ml 8-azaguanine (Sigma). Cells were tested at intervals for HAT sensitivity by culturing them in HAT medium. The fusion partners were screened for the presence of BLV by the procedures described below.

Next, heteromyeloma cell lines were each fused in two consecutive experiments with popliteal lymph-node cells from calves immunized against rotavirus. Heteromyeloma cell lines were selected on the basis of fusion efficiency, number and stability of specific anti-rotavirus bovine Ig-producing clones 6 weeks after fusion, as well as the parameters mentioned earlier. Fusion efficiency was defined as the number of colonies per 1 × 10^7 lymphocytes 3 weeks after fusion. Colonies were counted using an inverted microscope.

**Comparison of 1st- and 2nd-generation heteromyeloma cell lines**

From the hybrid cell colonies obtained by fusing 1st-generation heteromyelomas with lymphoid cells from calves immunized against rotavirus, colonies that initially secreted antibodies were selected to function as 2nd-generation bovine-(bovine-murine) heteromyelomas. Cells of these colonies were cloned twice and rendered HAT-sensitive. The 2nd generation of heteromyelomas produced in this way were compared with 1st-generation heteromyelomas in fusion experiments with cells from the popliteal lymph nodes of calves immunized against rotavirus.

**Timing of fusion and source of lymphocytes**

The finally selected heteromyelomas were used for fusion experiments with splenocytes and popliteal lymph-node cells on day 7 or day 4 after the last booster injection, respectively. For these experiments calves were immunized with PMSG.

**Immunization of calves**

Male Jersey calves aged between 6 and 12 months were injected intramuscularly (i.m.) with 300 μg density gradient-purified bovine rotavirus (Dutch
field isolate 26B) in complete Freund's adjuvant (CFA), followed by an injection of a similar dose (i.m.) in incomplete Freund's adjuvant (IFA) 3 weeks later. About 6 weeks later, i.e. 4 days before fusion, calves received 300 µg rotavirus in 10 ml phosphate buffered saline (PBS) intravenously. The purification procedure for rotavirus was as follows. Rotavirus was grown on MA 104 cells. After one cycle of freezing and thawing, cell debris was removed by centrifugation for 10 min at 1500 rpm. The supernatant was loaded on 10 ml of 45% w/v sucrose/PBS. After centrifugation for 3 h at 27 000 rpm, the pellet was suspended in 5 ml of PBS and centrifuged once more for 15 min at 1500 rpm to remove insoluble material. The supernatant was used for immunization.

For immunization against PMSG, calves were injected five times i.m. with 3000 IU PMSG (Intervet) and adjuvant at intervals of 4 weeks. CFA was used in the first injection, and IFA thereafter. Calves were boosted intravenously with 3000 IU PMSG in 10 ml PBS about 6 weeks after the last injection, i.e. 7 or 4 days before fusion.

Fusion

Heteromyeloma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin, streptomycin and glutamine. Fusions between spleen or lymph-node cells and myeloma cells were performed as previously described (Booman et al., 1984) with minor modifications. If necessary at a later stage assays for bovine Ig and determination of Ig-isotype were performed, using hybrid cells grown in DMEM with 10% horse serum.

ELISAs

Detection of bovine Ig. A double antibody sandwich assay was used for the detection of bovine Ig (Van Zaane and IJzerman, 1984). Briefly, microtiter plates were coated with a monoclonal antibody recognizing a common determinant of bovine IgG1, IgG2, IgA and IgM. After incubation with the culture supernatants, bound antibody was detected using a mixture of rabbit antisera against bovine IgG, IgM and IgA conjugated with horseradish peroxidase (HRP).

Determination and quantitation of bovine Ig isotypes. In a similar type of assay, plates coated with monoclonal bovine Ig isotype-specific antibodies were used (Van Zaane and IJzerman, 1984) for the determination and quantitation of bovine Ig isotypes. Two-fold dilutions of bovine Ig isotype standards and culture supernatants were added and the assay developed as described before. The concentration of bovine Ig in culture supernatants was estimated from titration curves.
Detection of anti-rotavirus bovine antibodies. An antibody capture assay was used for detection of specific anti-rotavirus antibody-secreting clones (Van Zaane and IJzerman, 1984). Microtiter plates were coated with the anti-bovine Ig monoclonal antibody. After incubation with the culture supernatants, rotavirus was added and subsequently bound antibody was detected using swine anti-rotavirus antibodies conjugated with HRP.

Detection of anti-PMSG bovine antibodies. Screening for monoclonal antibodies directed against PMSG was performed by an indirect double antibody sandwich assay. Microtiter plates were coated with rabbit anti-PMSG antiserum (10 μg Ig/ml, 100 μl/well). After blocking with 1% BSA, a standard solution of PMSG (6 IU/ml) was incubated for 75 min at 37°C. Culture supernatants were then incubated for 1.5 h at RT or overnight at 4°C, and rabbit anti-bovine Ig(G,M,A) conjugated with HRP (Nordic Immunological Laboratories) was used as a conjugate. Detailed procedures were similar to those described earlier for the ELISA (Booman et al., 1984).

BLV screening. Cell suspensions of the 1st-generation heteromyeloma lines were screened for the presence of BLV by a double antibody sandwich assay using monoclonal antibodies directed against two BLV-p24 antigenic determinants (De Boer et al., 1987). A semi-purified BLV preparation was used as positive control. The heteromyelomas were also tested for the presence of BLV by intraperitoneal inoculation of 5 × 10^8 cells in four 6-month-old male Fle-volander lambs, from which serum samples were collected at 4-week intervals over a 6-month period. The serum samples were tested for anti-BLV antibodies by an agar gel precipitation test and a complex-trapping-blocking ELISA-p24 (De Boer et al., 1987).

Immunofluorescent staining of cell-surface immunoglobulins

The presence of bovine and murine surface immunoglobulins was detected by direct immunofluorescence tests. Cells were incubated (30 min on melting ice) with fluorescein-conjugated rabbit anti-bovine Ig F(αb')_2 fragments for the detection of cell-surface bovine Ig, and with a rabbit anti-mouse Ig conjugate to detect murine Ig. After washing the cells, fluorescence was examined on a microscope slide under a phase-contrast UV microscope.

Karyotyping

Chromosome preparations were made following standard procedures. The chromosomes were examined after GTG banding, as described by Hageltorn and Gustavsson (1973). For each cell line to be karyotyped, the total chromosome number and the number of bovine chromosomes were determined for
five individual cells. Bovine chromosomes were differentiated from mouse chromosomes on the basis of their negatively stained centromeric regions (Lin et al., 1977).

Characterization of anti-rotavirus and anti-PMSG bovine monoclonal antibodies

To study the reactivity of anti-rotavirus monoclonal antibodies against double-shelled rotavirus of bovine, porcine and/or human origin, one monoclonal antibody was further characterized by immunogold electron microscopy (Vreeswijk et al., 1988). Briefly, rotavirus was absorbed onto Ni-collodion carbon-coated grids and incubated with monoclonal antibody in PBS for 2 h at 37°C. After washing, bound monoclonal antibody was detected following incubation with 1 μg Protein A/ml PBS (30 min at RT) and subsequently rabbit anti-Protein A Ig (Sigma), conjugated with colloidal gold (30 min at RT). Some rotavirus-coated grids were floated on a drop of 0.5% glutaraldehyde in PBS for 1 min before incubation with the monoclonal antibody was begun. The strains used were bovine rotavirus RA174, purified from pooled faeces from calves on different farms, the Pensaert porcine rotavirus strain 277 (probably subtype 1, serotype 4), and a human rotavirus from a child with severe diarrhoea.

The neutralizing capacity of the anti-rotavirus monoclonal antibody was determined by Dr. P.P. Pastoret, Université de Liège, Bruxelles (Thiriart et al., 1987), using bovine rotavirus strain 81/36F (Castrucci et al., 1983).

The cross-reactivity of a bovine anti-PMSG monoclonal antibody with bovine LH and FSH was determined by indirect double antibody sandwich ELISAs as described (Booman et al., 1990). The in vitro neutralizing capacity of the monoclonal antibody was determined in a validated assay (M.J.H. Hoeijmakers, Intervet Int. B.V., Boxmeer). Standard solution PMSG was preincubated with the antibody and the mixture tested for residual PMSG by a double antibody sandwich ELISA. In this assay a mouse monoclonal antibody against PMSG was used as the coating and rabbit anti-PMSG-HRP as the conjugate.

RESULTS

Construction and selection of 1st-generation heteromyeloma cell lines

Fusion of lymphoid cells from a BLV-infected cow with cells of X63-Ag8.653 resulted in 32 growing hybrid colonies after 6–8 weeks of culture. The hybrid cells contained variable numbers of chromosomes, ranging between 53 and 112 with a mean of 67.6 ± 12.6. The percentage of bovine chromosomes ranged between 0 and 29 with a mean of 14.8 ± 8.4. Only two hybridoma colonies were
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positive for bovine cell-surface Ig (1 II and 3 V), and were secreting bovine IgM. Mouse cell-surface Ig was not detected.

On the basis of their Ig production, 1 II and 3 V were selected for use as the 1st-generation heteromyeloma fusion partners together with two hybrids (3 XIV and 1 XXIII) with a high content of bovine chromosomes and favorable growth characteristics. 3 V also contained a high number of bovine chromosomes. No BLV was detected in cell suspensions of 1st-generation fusion partners, and no BLV-specific antibody was detected in serum samples from inoculated lambs.

Fusion of the 1st-generation fusion partners with lymph-node cells from calves immunized with rotavirus resulted in large differences in numbers of hybrid colonies generated by 1 II and 3 V on the one hand and 3 XIV and 1 XXIII on the other. The two latter cell lines showed negligible fusion efficiencies, and therefore appeared not to be useful as fusion partners. Although the fusion efficiencies of 1 II and 3 V were relatively high (42 and 70, respectively), only two hybridomas per fusion partner produced anti-rotavirus antibodies for more than 6 weeks. Three of these four anti-rotavirus antibody-producing cell lines secreted a mixture of IgM and IgG1, and one cell line secreted only IgG1. Of the total number of hybrid colonies generated after fusion with 1 II and 3 V, about 20 and 50%, respectively, produced bovine Ig. About 70% of these bovine Ig-producing hybridomas secreted a mixture of IgM and IgG1, and one cell line secreted only IgG1.

When karyotyping a group of bovine Ig-producing hybridomas (n = 15), the total number of chromosomes ranged between 68 and 154 with a mean of 111.2 ± 24.9. The percentage of bovine chromosomes varied between 11.6 and 36.5 with a mean of 26.2 ± 4.4.

Comparison 1st- and 2nd-generation heteromyeloma cell lines

The hybridoma colonies 13 XIII and 33 VI, generated in fusion experiments with 1 II and 3 V, respectively, were chosen as 2nd-generation fusion partners. Both hybridomas secreted anti-rotavirus antibodies for the first 3–4 weeks after fusion, but subsequently lost this activity. They did not secrete unrelated bovine Ig either. In Table 1 characteristics of 13 XIII and 33 VI are compared with those of the 1st-generation fusion partners 1 II and 3 V.

In Table 2 results of the fusion experiments of the selected 1st- and 2nd-generation fusion partners with lymph-node cells from calves immunized against rotavirus are summarized. No difference in fusion efficiency was observed between the 1st-generation heteromyelomas and their 2nd-generation derivatives. However, the number of specific anti-rotavirus antibody-producing hybridomas was higher with the 2nd-generation fusion partners. For this reason, and since 13 XIII and 33 VI are non-secretors, these cell lines were preferable to 1 II and 3 V as fusion partners. In the case of the 2nd-generation heteromyelomas, fusion efficiency of 33 VI was higher than that of 13 XIII,
TABLE 1

Characteristics of 1st- and 2nd-generation fusion partners

| Fusion partner | Number of chromosomes¹ | Cell-surface bovine Ig | (Sub)class of secreted bovine Ig | Doubling time (h)² |
|----------------|------------------------|-----------------------|---------------------------------|-------------------|
|                | Before cloning and aza treatment | After cloning and aza treatment |                                |                   |
|                | Total | Bovine (%) | Total | Bovine (%) | Total | Bovine (%) | Total | Bovine (%) |                   |
| 1st-generation |       |           |       |           |       |           |       |           |                   |
| 1 II           | 58.8  | 3.8 (6.5) | 52.3  | 2.0 (3.8) | +     | IgM       | 15    |           |                   |
| 3 V            | 112.3 | 27.3 (24.3)| 98.5  | 25.5 (25.9)| +     | IgM       | 19    |           |                   |
| 2nd generation |       |           |       |           |       |           |       |           |                   |
| 13 XIII        | 121.2 | 31.2 (25.7)| 116.7 | 21.7 (18.6)| -     | -         | 18    |           |                   |
| 33 VI          | 110.3 | 30.7 (27.8)| 113.4 | 29.4 (25.9)| -     | -         | 21    |           |                   |

¹Mean of 5 cells per fusion partner.
²2 × 10⁶ cells/10% FCS.

TABLE 2

Fusion results of 1st- and 2nd-generation fusion partners

| Fusion partner | Fusion efficiency¹ | Spec. anti-rotavirus clones² | Ig (sub)class of spec. clones |
|----------------|-------------------|------------------------------|-------------------------------|
| 1st generation|                   |                              |                               |
| 1 II          | 151               | 2                            | IgM + IgG₃ (2)                |
| 3 V           | 332               | 3                            | IgM + IgG₃ (2), IgG₃ (1)      |
| 2nd generation|                   |                              |                               |
| 13 XIII       | 163               | 7                            | IgG₃ (5), IgM (2)             |
| 33 VI         | 302               | 6                            | IgG₃ (3), IgM (3)             |

¹Mean number of colonies per 1 × 10⁷ lymphocytes for two fusion experiments 3 weeks after fusion.
²Total number of specific clones from two fusion experiments 6 weeks after fusion.

but the number of specific antibody-producing hybridomas was about the same. Consequently, subsequent experiments were performed with the two 2nd-generation heteromyeloma cell lines.

For five antigen-specific antibody-producing hybrid colonies generated from 13 XIII and 33 VI, the total number of chromosomes ranged between 113 and 162 with a mean of 137.8 ± 17.4. The percentage of bovine chromosomes varied between 26 and 31 with a mean of 27.9 ± 1.9. Table 3 summarizes the karyotyping results for hybrid cells obtained by fusion of X63-Ag8.653 with lymphoid cells of the BLV-infected cow and by fusion of bovine lymphocytes with the 1st- and 2nd-generation fusion partners.
| Chromosomes of bovine-murine hybrid colonies | Bovine X 1st-gen. fusion partners (bovine-murine hybrids) | Bovine X 2nd-gen. fusion partners (bovine-murine hybrids) |
|---------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| Number of chromosomes                       | Number of hybrid colonies karyotyped.                     |
| 1X63-Ag8, 653 + BLV-lymph.                  | 111 (n=10)                                                 | 137.8 ± 17.4                                             |
| (n=32)                                      |                                                           | 111.2 ± 24.9                                             |
|                                              | 23.3 ± 8.9                                                | 38.2 ± 3.4                                               |
|                                              | 29.5 ± 3.8                                                | 27.9 ± 1.9                                               |
| Total                                       | 67.6 ± 12.6                                               | 104.4 ± 27.6                                             |
| Bovine                                      | 10.8 ± 7.0                                                | 23.3 ± 8.9                                               |
| % Bovine                                    | 14.8 ± 8.4                                                | 24.6 ± 3.8                                               |

1Mean number of chromosomes ± s.d. of hybrid colonies.
2Number of hybrid colonies karyotyped.
Timing of fusion and source of lymphocytes

Results of fusion experiments of 2nd-generation fusion partners 13 XIII and 33 VI with spleen and popliteal lymph-node cells on day 4 or day 7 after the final intravenous booster injection are summarized in Table 4. It can be concluded that the fusion efficiency of the heteromyeloma cell lines was considerably lower with lymph-node cells than with spleen cells; the number of specific antibody-producing hybridomas, however, was much higher with the lymph-node cells.

Fusion with lymph-node cells on day 4 resulted in a higher fusion efficiency than fusion on day 7. For antigen-specific antibody-producing hybridomas, however, fusion on day 7 was preferable. In contrast, with spleen cells only fusions on day 4 resulted in specific antibodies, while fusion efficiencies were about equal on days 4 and 7.

Comparing the fusion partners, 33 VI showed a higher fusion efficiency with lymph-node cells than 13 XIII. The number of specific antibody-producing hybridomas generated from 33 VI was, however, lower both with lymph-node and spleen cells. The best combination appeared to be fusion of 13 XIII with lymph-node cells on day 7.

Characterization of specific bovine monoclonal antibodies

Anti-rotavirus antibody

One of the anti-rotavirus antibody-producing hybrid colonies (12 XX), generated by fusion of 1 II with stimulated lymph-node cells, was further chara-

| 2nd-generation fusion partner | Fusion efficiency | Lymph node cells spec. anti-PMSG clones | Number of fusion exp. | Fusion efficiency | Splenocytes spec. anti-PMSG clones | Number of fusion exp. |
|------------------------------|-------------------|----------------------------------------|----------------------|------------------|-----------------------------------|----------------------|
| 13 XIII + 33VI               |                   |                                        |                      |                  |                                   |                      |
| day 4 + 7                    | 54                | 45                                     | 6                    | 99               | 9                                 | 6                    |
| day 4                        | 62                | 22                                     | 4                    | 104              | 9                                 | 4                    |
| day 7                        | 37                | 23                                     | 2                    | 90               | 0                                 | 2                    |
| 13 XIII                      |                   |                                        |                      |                  |                                   |                      |
| day 4 + 7                    | 38                | 29                                     | 3                    | 103              | 7                                 | 3                    |
| 33 VI                        |                   |                                        |                      |                  |                                   |                      |
| day 4 + 7                    | 69                | 16                                     | 3                    | 95               | 2                                 | 3                    |

1Mean number of colonies per $1 \times 10^7$ lymphocytes for the number of fusion experiments indicated, 3 weeks after fusion.

2Total number of specific clones for the number of fusion experiments indicated, 6 weeks after fusion.
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Fig. 1. Colloidal gold immuno-electron microscopy carried out directly (A, B) or after fixation (C) on virus particles of bovine rotavirus. Single-shelled virus particles did not show a reaction with the monoclonal antibody (B, ss). When fixation was carried out before immunoreactions, the labelled particles could easily be identified as double-shelled particles (C, ds; arrow, outer shell). Similar results were obtained with swine and human rotaviruses. Bar in (A) indicates 50 nm.

Characterized. The total number of chromosomes before cloning was 120, of which 22 (18%) were of bovine origin. After cloning, the cells contained 109 chromosomes including 17 (16%) of bovine origin. Before cloning the 12 XX hybrid cells secreted immunoglobulins of class IgM + IgG1; after cloning only IgG1 was secreted. The concentration of bovine IgG1 in the supernatant was about 10 μg/ml per 2.5 × 10^5 cells/48 h. The hybridoma cell line has been actively secreting in culture for more than 6 months. The doubling time (2 × 10^5 cells, 10% FCS) was about 26 h. In immuno-electron microscope preparations of untreated and glutaraldehyde-treated bovine rotavirus, two distinct types of virus particles could be recognized: labelled (Fig. 1A, C) and unlabelled (Fig. 1B). The unlabelled particles were easily identified as single-shelled rotaviruses. Labelled particles in unfixed preparations showed a fuzzy outline (Fig. 1A), while in some cases gold particles appeared to have drifted away from the characteristic rotavirus backbone (Fig. 1A, arrowheads). After glutaraldehyde fixation we found gold particles bound to the intact outer shell of double-shelled particles (Fig. 1C, ds; arrow, outer shell). Similar results were obtained with swine and human rotavirus (not shown). In the in vitro neutralization assay of Thiriart et al. (1987) the antibody did not neutralize bovine rotavirus (strain 81/36 F).

Anti-PMSG antibody. 4 XII is a hybrid cell line generated by fusion of 33 VI with stimulated lymph-node cells. The total number of chromosomes after cloning was 156, of which 42 (27%) were of bovine origin (no data are available on the chromosome content before cloning). The cells of hybridoma cell line 4 XII secreted antibodies of the IgG1 subclass at a level of 10–15 μg per 2.5 × 10^5 cells/48 h. This cell line has been actively secreting in culture for more than six months. Doubling time (2 × 10^5 cells, 10% FCS) was about 29 h. The an-
tibodies did not show cross-reactivity with bovine FSH and LH. The in vitro neutralizing capacity of this antibody in 10× concentrated serum-free supernatant (2.5×10^6 cells/48 h) was 550 IU PMSG/ml. Cells were easily adapted to growth in serum-free medium (HB 102; HANA Media).

DISCUSSION

Fusion of lymph-node cells from a BLV-infected cow with murine myeloma cells resulted in hybrid colonies with a highly variable number of bovine chromosomes. From the observation that two hybrid cell lines retained cell-surface Ig from the parent BLV-cells and secreted bovine IgM, it was reasoned that they would be able to support stable bovine antibody production after fusion with stimulated bovine B lymphocytes. Although the number of cell lines secreting antigen-specific antibodies was low, the expectation has been confirmed. Whether two additional non-secreting hybrid cell lines that had been selected as fusion partners based only on a relatively high number of bovine chromosomes and favorable growth characteristics would also sustain secretion of immunoglobulins could be evaluated, as the fusion efficiency of both of these heteromyelomas was negligible. The results of the karyotype analysis of the four 1st-generation heteromyelomas suggest that there is no direct relationship between the number of bovine chromosomes and characteristics important for the selection of the 1st-generation fusion partners. This was likewise reported by Murphy et al. (1986) in human monoclonal antibody production. The few bovine chromosomes present in the cells of heteromyeloma 1 II are apparently sufficient to support endogenous Ig production as well as the generation of stable hybridomas that secrete relatively high yields of bovine Ig. Yarmush et al. (1980) reported that they could not detect any rabbit chromosomes in rabbit-mouse interspecies hybridomas secreting rabbit immunoglobulins, and presented evidence for translocation of the relevant rabbit genes to a mouse chromosome. On the other hand, there seems to be a correlation between bovine chromosome content and bovine Ig production, since the higher number of bovine chromosomes present in 3 V compared to 1 II corresponds to the percentages of hybrid Ig-secreting cell lines (50 and 20 percent, respectively). Hybrid cell colonies generated by fusion of the 1st-generation fusion partners retained much more bovine chromosomes than the cell colonies produced by fusion of the mouse myeloma cells with the lymph-node cells of the BLV-positive cow.

It was expected that the 2nd-generation non-secreting heteromyelomas would still have the apparatus for support of immunoglobulin secretion and would generate a higher yield of antibody-producing cell lines. Indeed, substantially more antigen-specific antibody-producing lines were obtained with the 2nd-generation than with the 1st-generation fusion partners. This cannot simply be ascribed to differences in bovine chromosome numbers (see 3 V and 33 VI
and their fusion products). The number of bovine chromosomes in 111 and 13 XIII was indeed different, but in the fusion products of both heteromyelomas this difference had largely disappeared. For that matter, Tucker et al. (1987), using the same approach, found an increase in the retention of bovine chromosomes in 2nd-generation fusion partners compared with 1st-generation fusion partners, and an increase in number of antigen-specific clones. On average, fusion of the 2nd-generation fusion partners with bovine B lymphocytes resulted in an increase in the absolute number of bovine chromosomes, while the relative number tended to rise to a certain limit. There was considerably less variability in chromosome number. Apparently, the number of bovine chromosomes has an upper limit. In human–mouse heterohybrids a similar threshold seems to be observed. Hybrids usually have at most five times more mouse than human chromosomes (Raison et al., 1982; Teng et al., 1985). Whether a 3rd-generation bovine–murine heteromyeloma cell line will improve the levels of antibody secretion and ensure that high levels of secretion are sustained is currently being explored.

For each of the selected 1st- and 2nd-generation fusion partners, many more antigen-specific immunoglobulin-producing clones could be detected in the first weeks after fusion (data not shown). However, in general their secretion level was very low, and within 6 weeks they lost their activity. The numbers of this type of colony were about twice as high as in the mouse–mouse system. A similar limitation occurs in human monoclonal antibody production, generally attributed to either overgrowth by non-secreting lymphocyte ‘contaminants’ or to the loss of structural genes coding for immunoglobulin. As suggested by James and Bell (1987), other factors include deficits in growth and differentiation factors and their receptors, structural and regulatory gene defects and imperfections in the synthetic and secretory machinery of the cells.

Our experiments indicate that the fusion efficiency is higher with splenocytes than with lymph-node cells. However, the number of antigen-specific antibody-secreting clones was lower in the case of splenocytes, notwithstanding the intravenous route for the final booster which preferentially stimulates splenocytes. Apart from our work no comparative studies of this nature have been published in the area of bovine monoclonal antibody production. In contrast to human monoclonal antibody technology, where no differences between spleen and lymph-node fusions were found (Jahn et al., 1988), in mice fusion of poplileal and inguinal lymph-node lymphocytes also induced a higher fusion efficiency as well as a higher frequency of hybridomas secreting specific antibody than fusion with splenocytes (Mirza et al., 1987).

As in murine and human antibody production (for references, see James and Bell, 1987), we found that in cattle the preferred interval between the final booster injection and fusion of splenocytes is 3–4 days. The optimal time for lymph-node cells seems somewhat longer. Additional immunocytochemical data from our laboratory (Wissink et al., 1989) show that the appearance of plasma
cells in medullary cords of peripheral lymph nodes takes place 4–5½ days after the final booster injection.

Further studies on one of the anti-rotavirus and anti-PMSG monoclonal antibodies proved the usefulness of the heteromyelomas for the production of bovine monoclonal antibodies. Both antibody-producing cell lines showed good cloning efficiency (about 30%), cells can grow in a minimal medium even under serum-free conditions, and secretion levels obtained are relatively high in comparison with other reports in bovine (Tucker et al., 1984; Groves et al., 1987a) as well as in human monoclonal antibody production (for references, see Kozbor and Croce, 1985). In addition, preliminary results from our laboratory indicate that it is possible to obtain bovine monoclonal antibodies via ascites production or high serum titers in irradiated (300 rad), pristane-treated calves by inoculation of at least $1 \times 10^8$ hybrid cells (data not shown). Although the anti-rotavirus antibody was directed against an outer capsid glycoprotein which might theoretically carry antigenic sites for virus neutralization, the antibody did not neutralize bovine rotavirus in vitro. The in vitro neutralizing capacity of the anti-PMSG monoclonal antibody was very potent. Further studies on the in vivo neutralizing capacity of this antibody are currently in progress. Since a murine anti-PMSG monoclonal antibody has recently become available commercially (Neutra-PMSG, Intervet), a comparative study of the inhibitory capacity and the efficacy of the bovine and murine antibodies after repeated treatment is now possible.

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