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OPTIMIZATION OF CULTURE CONDITIONS FOR FELINE × MURINE HETEROHYBRIDOMAS

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Abstract—Feline splenocytes were fused to the murine myeloma lines NSO or Ag8. Autologous serum and taurine were used as media supplements for the cat × mouse heterohybridomas. The best results were obtained by the use of NSO as fusion line with taurine-supported media.

Key words: Heterohybridomas, felines, taurine, cat serum.

Résumé—Des cellules spléniques de chat ont été fusionnées avec des cellules de myélome de souris NSO ou Ag8. Du serum autologue et de la taurine ont été utilisés pour supplémenter le milieu de croissance pour les hétérohybrides chat × souris. Les meilleurs résultats ont été obtenus en fusionnant des cellules NSO avec dans milieu supplémenté avec de la taurine.

Mots-clefs: Hétérohybridomes, félinés, taurine, serum de chat.

INTRODUCTION

Feline Infectious Peritonitis (FIP) is a severe disease in cats caused by a coronavirus (FIPV) and characterized by the formation of FIP-associated immune complexes. Immunoglobulin G (IgG) is considered to be a major component of these complexes [1]. Unlike many other mammalian species, in cats only two IgG subclasses, designated IgG_1 and IgG_2, have been postulated so far [2, 3]. For a better understanding of the involvement of these complexes in the immunopathology of FIP we were interested in the two proposed IgG-subclasses.

For the subclass determination of human immunoglobulins the use of paraproteins, secreted by myelomas (plasmacytomas) *in vivo* or by cell lines adapted to *in vitro* growth, proved to be extremely useful in the past [4]. Compared to other species, the occurrence of feline myelomas is a very rare event. Most feline paraproteins which have been more closely characterized until now, have been determined as IgG without further discrimination of IgG-subclasses [5, 6].

Another possibility to obtain antibodies belonging to one distinct subclass is offered by the hybridoma technology developed by Köhler and Milstein [7] for the production of monoclonal antibodies (mAb). This approach is not directly applicable in cats, because no

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Abbreviations used: Complete culture medium, CCM; dimethylsulfoxide, DMSO; feline infectious peritonitis, FIP; feline infectious peritonitis virus, FIPV; hypoxanthine, aminopterin, thymidine, HAT; hypoxanthine, thymidine, HT; immunoglobulin G, IgG; immunoglobulin M, IgM; monoclonal antibody, monoclonal antibodies, mAb; *o*-phenylenediamine, OPD; phosphate buffered saline, PBS.

feline myeloma line has been adapted as fusion partner. Instead, feline lymphocytes can be fused to HAT-sensitive murine myeloma lines, used in conventional hybridization experiments. The development of a panel of cat × mouse heterohybridoma lines, secreting feline monoclonal antibodies should be very useful as reference standards for distinct subclasses. A similar approach was chosen by Srikumaran *et al.* [8] for the characterization of bovine IgG subclasses by bovine × murine heterohybridomas, and by Galakhar *et al.* [9] for IgG subclass studies in minks using mink × mouse heterohybridomas.

In this preliminary study we tried to optimize the outgrowth efficiency of feline × murine mAb by heterohybridomas.

MATERIALS AND METHODS

Fusion lines

NSO were obtained from European Culture Collection (ECACC No. 85011432), P3X63-Ag8.653 (Ag8) were from ATCC (ATCC CRL 1580). The cells were propagated in a culture medium consisting of RPMI-1640 supplemented by 7% heat-inactivated foetal bovine serum (Boehringer), 2 mM L-glutamine, 1 mM of sodium pyruvate, 10 IU/ml penicillin, 10 μg/ml streptomycin and 5×10^{-2} mM 2-mercaptoethanol. Both cell lines do not synthesize antibodies and were checked for HAT-sensitivity regularly.

Feline lymphocytes

Lymphocytes were obtained from seven cats which had to be sacrificed due to severe non-infectious diseases. The spleens were removed immediately after euthanasia and immersed in 70% ethanol. The splenocytes were prepared as single cell suspensions from small pieces of the spleen, washed three times with cold PBS containing antibiotics and counted in a haemocytometer (Bürker–Türk ruling). The percentage of viable cells was estimated by 0.5% trypan blue dye exclusion test.

An aliquot of the isolated lymphocytes was frozen for further experiments in a medium containing 20% foetal bovine serum and 10% DMSO (Sigma), at a density of 1×10^7 cells/ml according to Schreier *et al.* [10] and stored in liquid nitrogen.

Media supplements

Cat serum was collected by venous puncture from healthy donors, incubated for 25 min at 56°C for complement-inactivation, filter sterilized twice and frozen in small aliquots at −30°C and used at a final concentration of 5% (v/v). Taurine (2-aminoethanesulphonic acid, Sigma) was used at a final concentration of 0.8 mM (0.1 mg/ml culture medium).

Production of cat × mouse hybridomas

Fusions were done following standard protocols [11, 12] with 50% (v/v) polyethylene glycol 1500 (Boehringer), using the myeloma lines Ag8 or NSO, respectively, at the logarithmical phase of growth. Fused cells were seeded at a concentration of

Table 1. Setup of experiments

Experiment	Fusion lines	Culture medium
1	Ag8, NSO	CCM
2	Ag8, NSO	CCM + 5% cat serum
3	Ag8, NSO	CCM + 0.8 mM taurine

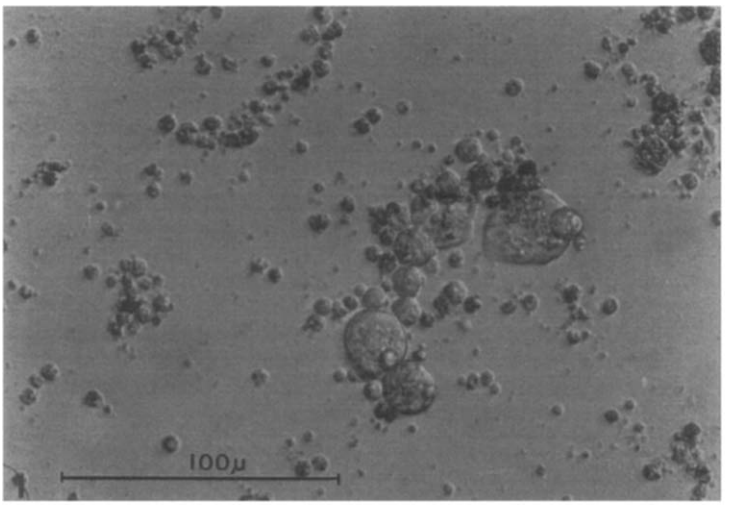

Fig. 1. (×400) Clone of cells on day 10 after the fusion.

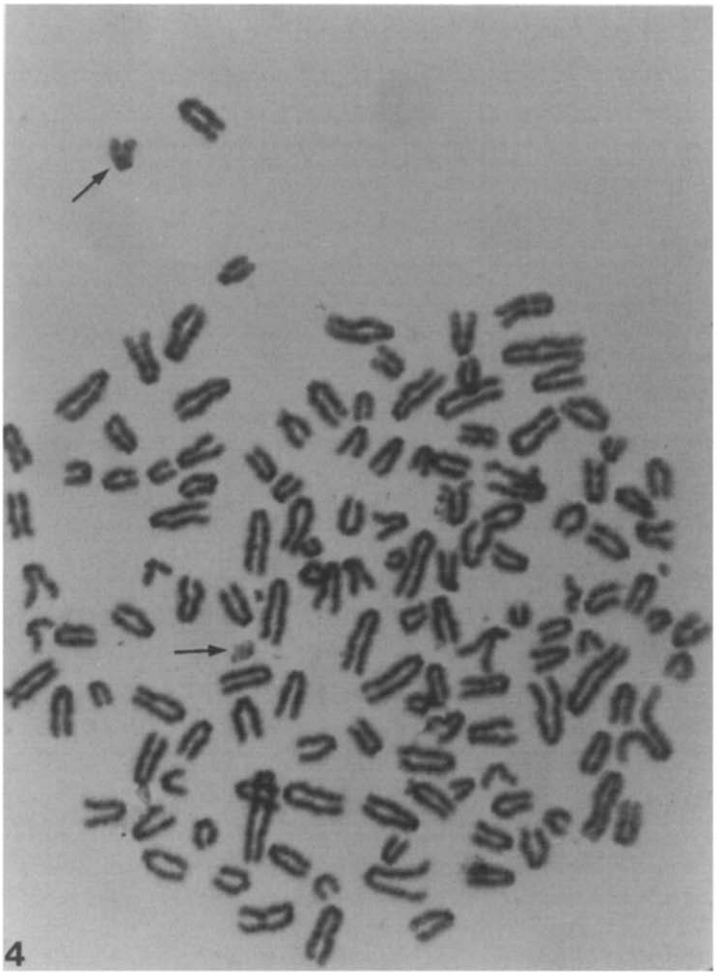

Fig. 4. (× 1000) G-banded metaphase spread of a cat × NSO hybridoma clone. Arrows indicate two feline chromosomes; total number of chromosomes is 136.

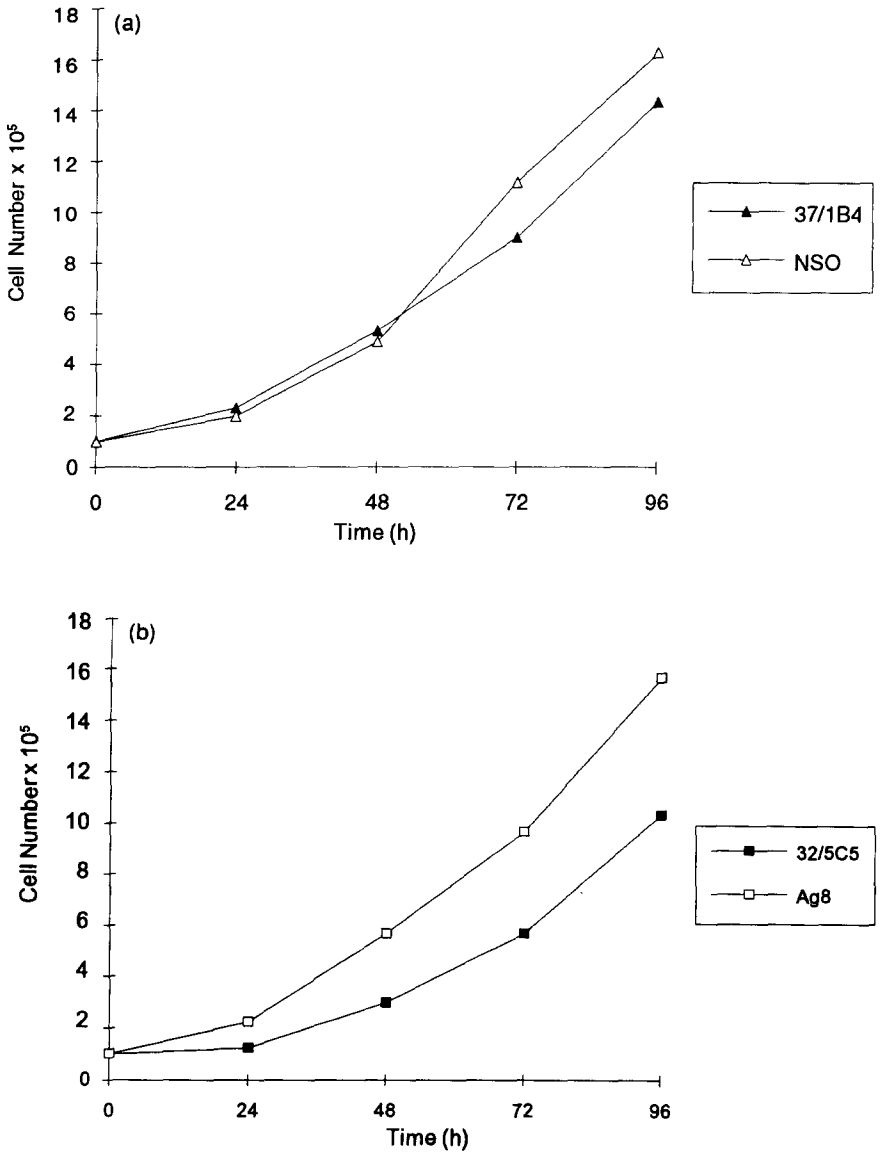

Fig. 2(a) and (b). Comparison of the growth of two fused cell clones to their parental lines.

5×10^6 lymphocytes/ml in 48-well culture plates (Costar). The culture medium described above was used, additionally supplemented by 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine (HAT, Boehringer) and 25% (v/v) macrophage-conditioned medium [13] instead of feeder cells (=complete culture medium, CCM). In the initial phase of growth the heterohybridomas were cultured as described in Table 1.

HAT was replaced by HT without macrophage-conditioned medium 12–15 days and

Table 2. Outgrowth efficiency of NSO-derived hybridomas using frozen and native splenocytes

Treatment of lymphocytes	Number of wells seeded	Number of wells showing growth	E (%)
Native	502	41	8.17
Frozen	288	11	3.82

again 21–25 days after fusion. Control wells containing native feline lymphocytes or the myeloma fusion partners respectively in HAT-medium, were set up together with the fused cells.

Outgrowth efficiency

The outgrowth efficiency (E) was used as a parameter for the comparison of fusion lines and media supplements. Culture plates were screened for clonal growth of the hybridomas 10–20 days after fusion and then about once a week. Wells which contained colonies consisting of at least four to eight large, contiguous cells were counted positive. The outgrowth efficiency was calculated by the formula:

$$E(\%) = \frac{\text{number of wells containing clones}}{\text{total number of wells}} \times 100.$$

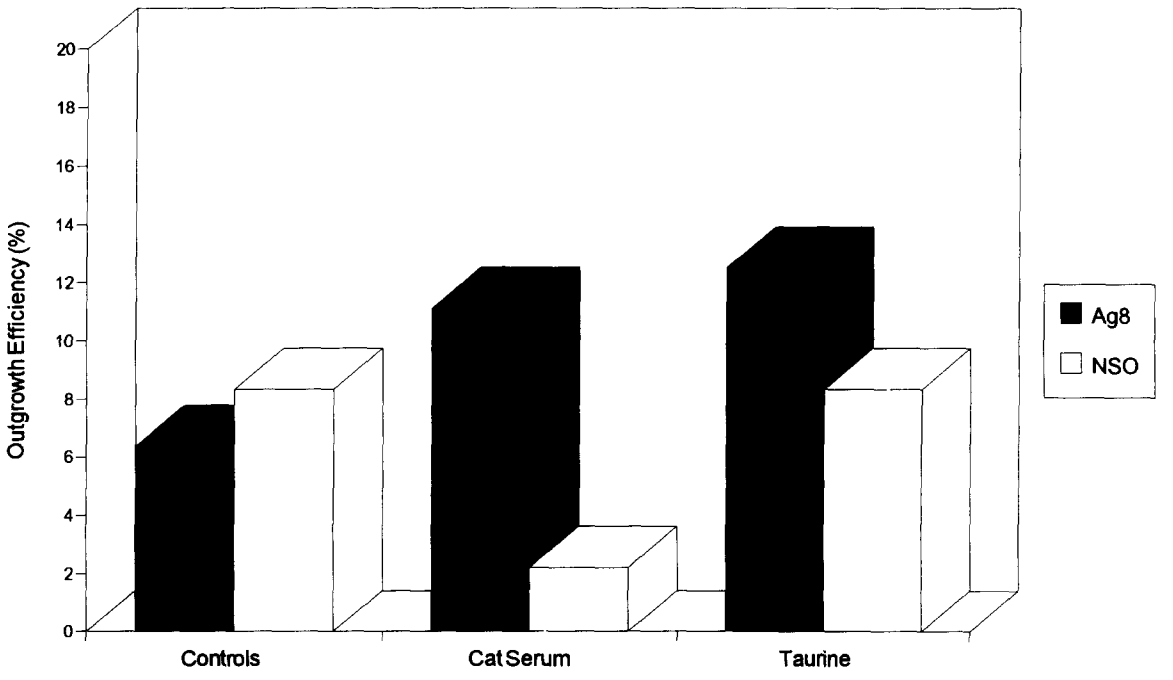

Fig. 3. Comparison of the outgrowth efficiency of different experiments 15 days after the fusion.

Table 3. Mean numbers of chromosomes of two hybridomas and their parental lines

Cell clone	x	s	Parental line	x	s
34/4B3	66.6	3.4	NSO	61.8	1.4
30/2C6	106.6	2.9	Ag8	52.3	6.2

Growth rates and size of heterohybridomas and parental lines

Numbers of parental lines and heterohybridomas were adjusted to 5×10^5 cells/ml and 1 ml of cell suspension was added in triplicate in a 24-well culture plate. Cell numbers were counted at 24, 48, 72 and 92 h by a haemocytometer and an automated counter (Cobas Minos Vet, Roche); the results were expressed as means of the three wells at each time point. The cell size was determined by microscope from native monolayers.

ELISA screening

Culture supernatants were screened by ELISA for feline IgM and IgG production 1 week after the second medium replacement. High affinity 96-well assay plates (Greiner) were coated overnight at 4°C with 50 μl culture supernatant diluted with the same volume of a carbonate–bicarbonate buffer (pH 8.9). Supernatants from control wells (NSO or Ag8) were taken as blanks, CCM containing 5% cat serum was used as positive control. Goat anti-cat-IgM (H- and L-chain specific, 1:10,000, Accurate Chemicals) or goat anti-cat-IgG (H-chain specific, 1:50,000, Bethyl) was used as first antibody. After each step the plates were washed three times with PBS containing 0.01% Tween 20 using an automatic ELISA-washer (SLT). The assay was developed with an affinity purified rabbit anti-goat-IgG (H-chain specific, 1:20,000, Accurate Chemicals) conjugated to horseradish peroxidase with OPD as substrate.

Karyotyping

Chromosome preparations were made following standard procedures. The metaphase spreads were examined after G-banding [14]. For each cell line to be karyotyped, the total number of chromosomes and the number of cat chromosomes were determined for 15 individual cells. Cat chromosomes were differentiated from mouse chromosomes on the basis of banding pattern and the position of the centromere [15].

RESULTS

Twelve experiments resulted in 223 colonies (7.4% of wells seeded) which survived at least 15 days, 23 (0.8%) of them survived for several months and were stored in liquid nitrogen after propagation in HAT-medium for 2 weeks. Under the conditions described above, the fusion experiments did not result in more than one colony per well, i.e. there was one developing colony in 5×10^6 cells seeded. No heterohybridomas secreting feline IgM or IgG could be detected by ELISA.

The heterohybridoma cells were larger than their parental lines. Typically, one clone (37/5C5) was 15.13 ± 1.14 μm dia, compared to 12.71 ± 0.69 μm for its parental line Ag8 (Fig. 1). Depending on the parental line heterohybridomas generally multiplied slower [Fig. 2(a), (b)] and had a lower maximum cell density compared to NSO or Ag8.

Effects of freezing splenocytes

Native lymphocytes showed >95% viability, in frozen splenocytes viability was reduced to *c.* 40% as determined by 0.5% trypan blue dye exclusion test. The outgrowth efficiency of native and previously frozen splenocytes was compared for NSO-controls until day 15 after hybridization (Table 2).

Effect of media supplements

Compared to the controls, autologous serum as well as taurine influenced the growth of some of the hybridomas during the first 12–15 days after fusion (Fig. 3).

Chromosomal analysis

The fusion of feline splenocytes with NSO or Ag8 caused an increase in the number of chromosomes of the resulting hybridomas, depending on the parental line (Table 3). A variable number of feline chromosomes was found in different heterohybridoma clones (Fig. 4).

DISCUSSION

Little is known about the specific needs of feline lymphoid cells in culture. Cat leukocytes and neutrophils, like human ones, contain a high intracellular concentration of taurine (22 and 26 mM respectively) [16]. Unlike other species, taurine is an essential amino acid in cats and the effects of feline taurine deficiency have been investigated during the last years [17]. It was shown, that a taurine-free diet has an impact on the development and function of the feline immune system *in vivo* [16]. Added to a lymphocyte transformation assay *in vitro*, taurine causes an increase in proliferation to Con A [18]. Human lymphoblastoid lymphocyte-derived cells are also stimulated and proliferate in taurine-supplemented media [19]. In our experiments we found that the addition of taurine to culture media significantly improved the outgrowth efficiency for Ag8-derived hybridomas (Fig. 3), but no marked effect was noticed on NSO-derived hybridomas.

Autologous serum was successfully used by Raybould and Takahashi [20] for the production and improvement of stability for rabbit × mouse heterohybridomas. Compared to controls, cat serum had a clear growth-supporting effect in Ag8-derived heterohybridomas, but when NSO was used as parental line, cat serum obviously had a rather growth-suppressive effect. The reasons for the different modes of action of cat serum and taurine on the two fusion lines are unknown so far, but the choice of the appropriate fusion line seems to be important for the production of interspecific hybridomas. Galakhar *et al.* [9] found the fusion lines Ag8 and NSO to be superior to Sp2/0-Ag14 in the yield of mink heterohybridomas. Other possible influences of Ag8 or NSO on heterohybridomas, such as antibody production could not be studied in the present experiments.

A number of HAT-resistant clones could be isolated from either fusion partners which contained cat chromosomes and were larger than their parental lines, proving that a fusion of feline and murine cells had taken place.

No clones secreting feline IgG (or IgM) could be detected for two possible reasons. (1) Splenocytes of unstimulated cats were used for our fusions experiments. According to literature some experiments using lymphocytes of unimmunized animals resulted in antibody-secreting heterohybridomas [21, 22], others did not and showed reduced outgrowth efficiency [23]. (2) Early screening followed by rapid cloning is frequently

recommended to stabilize secreting clones [22, 24], but due to slow cellular growth ELISA screening could not be done earlier than 4 weeks after the fusion.

Experiments with *in vivo* stimulated (originated from FIP-infected animals) or *in vitro* stimulated cat splenocytes are in progress.

Somatic hybridization experiments (cat × mouse and cat × Chinese hamster) were reported by O'Brien and Nash [15] for the study of chromosomal linkage groups in cats, but no information about culture conditions was given. Therefore the chosen approach was useful in order to optimize the culture system and experiments to adapt some of the heterohybridoma clones described above for growth in 8-azaguanine are currently in progress. They should be valuable as HAT-sensitive fusion partners for the production of 2° heterohybridomas (hybridhybridomas). Such cell lines tend to be more stable than 1° heterohybridomas in the production of monoclonal antibodies [25].

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