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Conditioned medium from gerbil—mouse T cell heterohybridomas improved antibody secretion

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Abstract: Mongolian gerbils (*Meriones unguiculatus*) are widely used as animal models for a variety of infectious diseases. However, immunological reagents such as cytokines have not been characterized. Two heterohybridomas, D9(E6)C2B3 and D9(E4), obtained by fusion of gerbil splenocytes with mouse myeloma cells (P3-X63-Ag8.653), expressed gerbil *CD3G* mRNA. These cells were suggested to be T cell heterohybridomas. They also expressed gerbil *IL6* [D9(E6)C2B3] and *TGFB* [D9(E4) and D9(E6)C2B3] mRNAs. The addition of conditioned medium (CM) obtained from the culture of D9(E6)C2B3 significantly enhanced antibody secretion and expression of gerbil Cγ1 and Cε *IGHC* mRNAs in the B11D2(C2) heterohybridoma, which secretes gerbil IgG1. However, the addition of CM from both heterohybridomas did not improve in proliferation of B11D2(C2) cells. These results indicate that CM from D9(E6)C2B3 improved the culture of gerbil–mouse heterohybridomas, possibly by secreting gerbil IL6.

Key words: Conditioned medium, Heterohybridoma, Mongolian gerbil

Introduction

Mongolian gerbils (*Meriones unguiculatus*) are used as animal models in studies of cancer and some infectious diseases. However, studies are limited by a lack of gerbil-specific immunological reagents such as cytokines and monoclonal antibodies (mAbs). Cytokines are low-molecular-weight proteins that regulate immune responses and inflammatory reactions [20]. They elicit different biological activities in different target cells and have overlapping actions. The cDNA sequences of the gerbil cytokines IL2 [14], IL12 [5], and IL18 [6] have been reported and their biological activities have been estimated, but there are far fewer reports of gerbil cytokines than of mouse or rat cytokines.

To generate large amounts of cytokines, which are present in minute amounts *in vivo*, the T cell hybridoma technique is used. Howard *et al.* (1979) [8] fused HAT-sensitive AKR thymoma (BW5147) cells with mouse spleen cells and reported the secretion of hemopoietic colony-stimulating factors. Hybridomas obtained from CEM-1 leukemia cells expressed low levels of lymphocyte-derived CD3 antigens [7]. CD3 is a T-cell-specific marker comprising three distinct polypeptides: γ (25 kDa), δ (20 kDa), and ε (20 kDa). The binding of peptide–MHC (major histocompatibility complex) complexes to the T cell receptor transmits a signal via the tightly associated CD3 and ζ molecules into the interior of the T cell [4]. In our previous research, we reported the fusion of gerbil spleen cells with mouse myeloma cells to create gerbil–mouse heterohybridomas that secrete gerbil immunoglobulins (Igs) [22]. In the fusion experiments, some cell colonies did not produce Igs (IgM and IgG), but produce low molecular proteins in protein assay. The results suggested that some of those cells might be fused mouse myeloma cell and gerbil T cell, but not gerbil B cell, and assumed to be gerbil T cell heterohybridoma. In case the products from those cells

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are gerbil cytokines, they would be useful reagents for the culture of gerbil cells. In this study, to characterize the two lines, D9(E4) and D9(E6)C2B3, which did not show Igs secretion, we evaluated their effect on the cell proliferation of and antibody secretion by another heterohybridoma that secretes gerbil IgG1 [21]. This report describes the production of stable T cell heterohybridomas and the characteristics of the cells secreting a factor capable of stimulating the production of Ig.

Materials and Methods

Animals

Mongolian gerbils bred at this laboratory [10] were maintained at 22 ± 3°C with lighting from 0500 to 1900 h (14L:10D). They were given food pellets (Labo MR Stock, Nihon Nosan Kogyo, Yokohama, Japan) and water *ad libitum*. All experimental procedures were conducted in accordance with the guidelines for animal experiments of the College of Bioresource Sciences, Nihon University.

Cell lines

Three gerbil–mouse heterohybridomas—B11D2(C2) (which secrete gerbil IgG1 specific to keyhole limpet hemocyanin; KLH) [21, 22], D9(E4), and D9(E6)C2B3, which were generated by fusing gerbil splenocytes with mouse myeloma cells (P3-X63-Ag8.653, provided by the RIKEN BioResource Center)—and mouse myeloma cells (P3-X63-Ag8.653) were cultured in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Tokyo, Japan), 100 U/ml penicillin (Meiji, Tokyo, Japan), 100 μg/ml streptomycin (Meiji), MEM nonessential amino acids (Invitrogen Gibco, Tokyo, Japan), 5×10^{-2} M 2-mercaptoethanol (Wako, Tokyo, Japan), and 2 μg/ml $NaHCO_3$ (Nacalai Tesque, Tokyo, Japan). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Media were changed three times a week.

Simultaneous GISH of heterohybridomas

For genomic *in situ* hybridization (GISH), chromosomal preparations were made from D9(E4) and D9(E6)C2B3 by conventional methods [22]. Total genomic DNA for use as probes was extracted from gerbil splenocytes and mouse myeloma cells with a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The probes were labeled with biotin-16-dUTP by using a Biotin-High Prime Kit (Roche, Tokyo, Japan; for mouse DNA) and with DIG-11-dUTP by using a Dig-High Prime kit (Roche; for gerbil DNA) according to the manufacturer's protocol. Signals were detected with Alexa Fluor 488-streptavidin (Invitrogen, Tokyo, Japan; green fluorescence for mouse DNA) and anti-digoxigenin-rhodamine (Roche; red fluorescence for gerbil DNA). GISH was conducted as reported previously [23].

RT-PCR for gerbil cytokine mRNA expression

Total RNA was isolated from splenocytes of 18-week-old female gerbil, mouse myeloma cell line P3-X63-Ag8.653, and heterohybridomas with a Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RT-PCR was performed with an RNA PCR Kit (AMV) v. 2.1 (Takara, Shiga, Japan) according to the manufacturer's instructions. Primers for gerbil cytokines (IL4, IL6, IL10, IL12p35, TNFA, IFNG, and TGFB), gerbil Ig heavy-chain constant region (*IGHC*) genes (Cμ, Cγ1, Cγ2, Cε, and Cα) [24, 25], and gerbil CD3G were designed from the cDNA sequences (Table 1). The PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide.

Preparation of conditioned medium (CM)

D9(E6)C2B3 and D9(E4) were acclimatized in serum-free medium (SFM; PFHM-II protein-free hybridoma medium 1×; Invitrogen) at 37°C in a 5% CO_2 incubator for a month. Cells (1×10^6/ml) were cultured with SFM for 5 days and the culture supernatants were harvested. Cells and debris were removed by centrifugation at 180× *g* for 5 min. The supernatant was filtered through a 0.22-μm membrane filter (Millipore Corporation, Billerica, MA, USA). The supernatants (named D9(E6)C2B3-CM and D9(E4)-CM) were stored at −20°C until use.

Cell proliferation assay

B11D2(C2) cells were used in the cell proliferation assay. Cells were acclimatized in SFM at 37°C in a 5% CO_2 incubator for a month. The cells were diluted in SFM, 20% D9(E6)C2B3-CM, or 20% D9(E4)-CM, seeded in triplicate into 96-well microtiter plates at 1×10^4 cells/well, and cultured for 7 days. The cells were counted on days 1, 3, 5, and 7 in a hemocytometer. Half the volume (100 μl) of medium was replaced with fresh medium on day 5 to prevent a decrease in cell proliferation due to deterioration of the medium.

Table 1. Primer used for the expression analysis of gerbil *CD3G* and cytokine mRNAs

Primer		Accession number	Sequence 5'→3'	Product size (bp)	Annealing temperature (°C)	Cycle
Gerbil	*IL4*	L37779	F: CACATCCCTGACGGTAGAATTC R: CTGAGGATCCCGGAGTTGTTCT	421	62	40
	IL6	AB164706	F: CACCCCCAACAGACCAGTAT R: AGCCATTCCGTCTGTGACTC	441	62	40
	IL10	L37781	F: GCCCCAGTCATAAACAGAGC R: GAATCCCTCTGAGCTGTTGC	490	62	40
	IL12p35	AF288849	F: GAAGACGGCCAGACAAAAGC R: GGATGCTGAAAGCATGGAGT	446	62	40
	TNFA	AB177841	F: ACTGGAATTCGGGGTGTTTGGTC R: GAGAACCTGGGAATTCACGAGG	254	62	40
	IFNG	L37782	F: TCACCCTAAGGAAGCGGAAA R: GACATGTTCTCCAGGCACCA	572	62	40
	TGFB	AF161218	F: ACCTGGGCTGGAAGTGGATC R: ATGAATTCTTGCGGCCCACGTAGTAGA	194	62	40
	CD3G	AB429404	F: GGCCCAGTCAAAACAAGAAA R: CTGGTCATTTTGCAACAGAGTC	412	62	40
	GAPDH	AY066007	F: GCAAGTTCAACGGCACAGTC R: TTTCCAGAGGGACCATCCAC	452	62	40
Mouse	*β-actin*	X03672	F: CCCATCTACGAGGGCTAT R: AAGAAGGAAGGCTGGAAA	310	62	40

Sources: *IL4* [27], *TNFA* [16], mouse *β-actin* [3]. Each cycle consisted of denaturation at 94°C for 2 min, annealing at the given temperature for 30 s, and extension at 72°C for 1 min.

Measurement of gerbil IgG1 secretion

The level of gerbil IgG1 specific to KLH in the culture supernatants secreted by B11D2(C2) in SFM was measured by indirect ELISA using a commercial kit (ELISA-mate, Kirkegaard & Perry, Gaithersburg, MD, USA) as previously described [22]. The cells were diluted in SFM, 20% D9(E6)C2B3-CM, or 20% D9(E4)-CM, seeded in triplicate into 96-well microtiter plates at 1×10^4 cells/well, and cultured for 7 days. Culture supernatants were collected on days 1, 3, 5, and 7 for measurement in 96-well microtiter ELISA plates (Nalge Nunc Int., Rochester, NY, USA), which had been coated with 100 μg/ml KLH in coating buffer for 1 h at 25°C in an incubator. Horseradish peroxidase–conjugated rabbit anti-gerbil IgG (1/1,000, Immunology Consultants Laboratory, Newberg, OR, USA) was used as the secondary antibody. The absorbance at 405 nm was measured in a 96-well plate reader (Bio-Rad, Hercules, CA, USA).

RT-PCR for gerbil IGHC mRNAs in heterohybridoma

B11D2(C2) cells were used in the analysis of the expression of *IGHC* mRNAs. Cells acclimatized in SFM were incubated at 1×10^6 cells/ml in 20% D9(E6)C2B3-CM in SFM. After 0, 4, and 12 h, total RNA was isolated from the cell with a Trizol Reagent kit (Invitrogen) according to the manufacturer's protocol. RT-PCR was conducted as above. The PCR conditions and product sizes for each *IGHC* primer are listed in Table 2.

Statistical analysis

Data are presented as means ± SEM. Differences between means were tested by Student's *t*-test. A probability level of $P<0.05$ was considered to be significant.

Results

Identification of gerbil chromosomes in heterohybridomas

To provide the evidence of heterohybridoma comprising both gerbil and mouse chromosomes, the chromosome analysis of two cell lines was performed by simultaneous GISH. GISH revealed gerbil chromosomes (violet) and mouse chromosomes (aqua) in gerbil–mouse heterohybridomas D9(E4) (Fig. 1a) and D9(E6)C2B3 (Fig. 1b).

Expression of cytokine mRNAs in heterohybridomas

The characteristics and differences between two heterobybridomas were evaluated by mRNA expression of several cytokines. RT-PCR analysis of mRNAs for gerbil cytokines (IL4, IL6, IL10, IL12p35, TNFA, IFNG and TGFB) and CD3G revealed the expression of *CD3G* and *TGFB* in D9(E4), D9(E6)C2B3, and gerbil spleen

Table 2. Primers used for the expression analysis of gerbil *IGHC* mRNAs

Primer	Accession number	Sequence 5'→3'	Product size (bp)	Annealing temperature (°C)	Cycle
Cμ *IGHC*	AB663133	F:AGGTCCGCCATGGCAACAAT R:TGTCGTATGATGCCAGGTTT	498	60	28
Cγ1 *IGHC*	AB663132	F:AGAATACCACTGCAAGCCTT R:GGGTGCTCTTGAAGTTCGGC	558	62	17
Cγ2 *IGHC*	AB597231	F:CGGTGGACCATCTGTCTTCA R:TTCTCTCCCATTCCACGTCA	446	64	23
Cε *IGHC*	AB663134	F:GACAGGACATACCTAATGCG R:TTGGTATCCACTGGCAGAAT	410	60	28
Cα *IGHC*	AB663135	F:AGCTATGAACGTGACCTGGG R:TGAGTCACCGTGCAGGTGAA	495	62	24
GAPDH	AY066007	F:GCAAGTTCAACGGCACAGTC R:TTTCCAGAGGGACCATCCAC	421	60	28

Each cycle consisted of denaturation at 94°C for 2 min, annealing at the given temperature for 30 s, and extension at 72°C for 1 min.

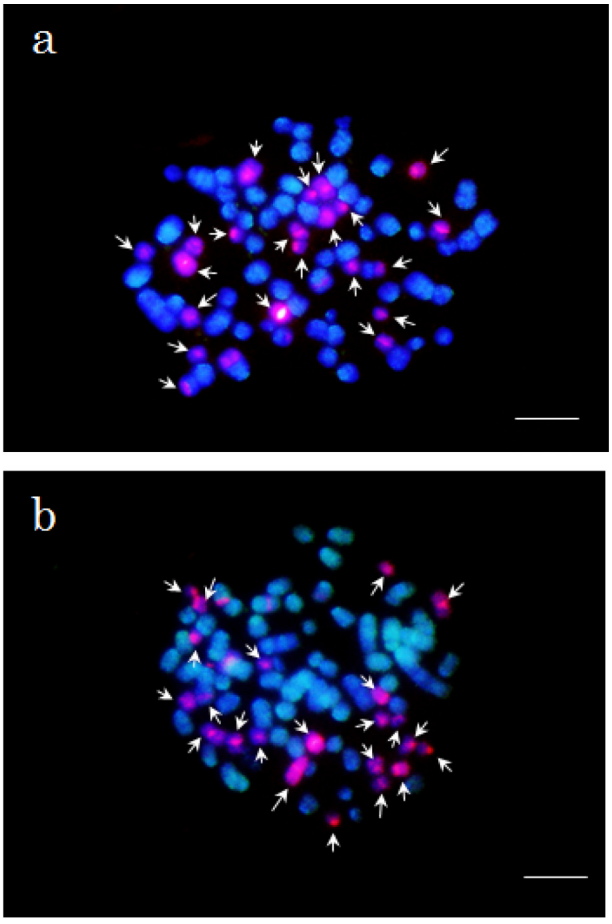

Fig. 1. Simultaneous genomic *in situ* hybridization (GISH) with total genomic DNA of gerbil (violet) and mouse (aqua) as probes. (a) Gerbil–mouse heterohybridoma D9(E4); (b) D9(E6)C2B3. Arrows indicate gerbil chromosomes. Bar=10 μm.

cells (Fig. 2), and of *IL6* in D9(E6)C2B3. In contrast, none were detected in mouse myeloma.

Effects of CM on cell proliferation and antibody production

Effects of the conditioned medium from two heterohybridomas on cell proliferation and antibody production of the heterohybridoma B11D2(C2) secreting gerbil IgG1 were evaluated. D9(E4)-CM and D9(E6)C2B3-CM did not improve the proliferation of B11D2(C2) cells; significantly less number of the cells than the control on days 5 and 7 (Fig. 3). On the other hand, D9(E6)C2B3-CM significantly increased the production of antibody specific to KLH (P<0.05) compared with the control from days 3 to 7 (Fig. 4).

Expression of gerbil IGHC mRNAs in heterohybridoma

Effects of the conditioned medium from two heterohybridomas on the expression of gerbil *IGHC* mRNA of the heterohybridoma B11D2(C2) secreting gerbil IgG1 were evaluated. D9(E6)C2B3-CM increased the expression of Cγ1 and Cε *IGHC* mRNAs by B11D2(C2) cells (Fig. 5). The expression of Cγ1 *IGHC* mRNA increased from 0 h to 4 h and remained high at 12 h. That of Cε *IGHC* mRNA increased from 0 h to 4 h and further to 12 h. The expression of Cμ, Cγ2, and Cα *IGHC* mRNAs was not detected.

Discussion

Simultaneous GISH analysis confirmed that D9(E6) C2B3 and D9(E4) are heterohybridomas comprising both

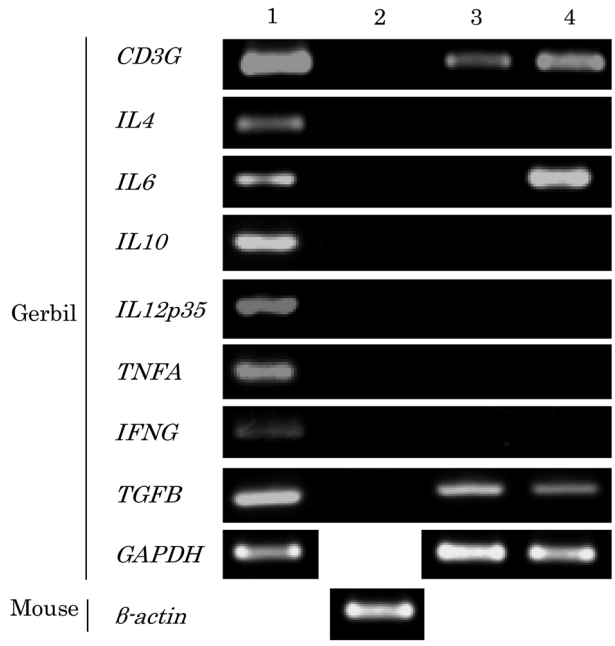

Fig. 2. Expression of gerbil *CD3G* and cytokine mRNAs in gerbil–mouse heterohybridomas D9(E4) and D9(E6)C2B3. Total RNA was extracted from gerbil spleen (lane 1), mouse myeloma P3-X63-Ag8.653 (lane 2), D9(E4) (lane 3), and D9(E6)C2B3 (lane 4). RT-PCR products were electrophoresed and stained with ethidium bromide.

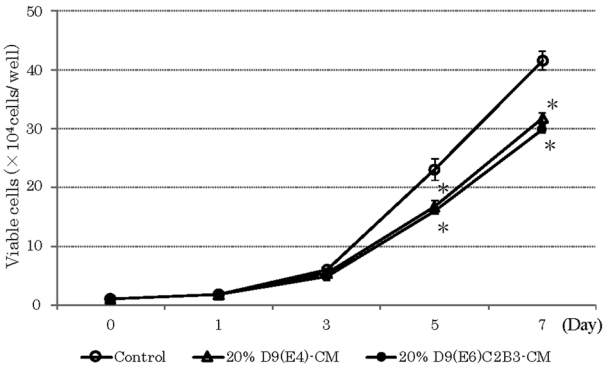

Fig. 3. Proliferative effects of each CM on heterohybridoma B11D2(C2). Cells were cultured in SFM as control (○), 20% D9(E4)-CM (Δ), or 20% D9(E6)C2B3-CM (●) for 7 days. Values are the average of three independent experiments.

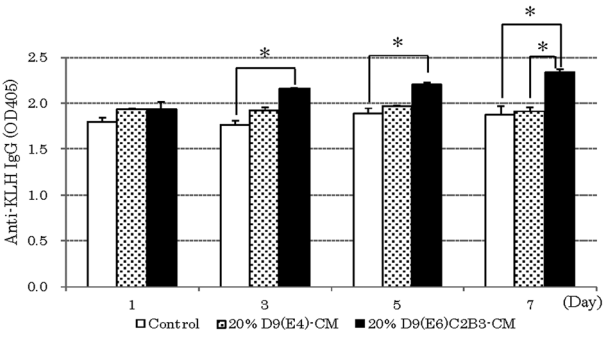

Fig. 4. Antibody production by B11D2 (C2) cells cultured in SFM (□), 20% D9(E4)-CM (▫), or 20% D9(E6)C2B3-CM (■) for 7 days. Values are the average of three independent experiments.

gerbil and mouse chromosomes (Fig. 1). These cells expressed gerbil *CD3G* mRNA (Fig. 2). Since human T cell hybridomas produce low levels of lymphocyte-derived CD3 antigens [7], D9(E6)C2B3 and D9(E4) might be T cell heterohybridomas formed by the fusion of gerbil T lymphocytes with mouse myeloma cells. Although B cell heterohybridomas formed by the fusion of B lymphocytes of many species with mouse myeloma cells have been established [2, 12], the fusion of heterogeneous T lymphocytes with mouse myeloma cells has not been reported before. Our results suggest that mouse myeloma cells can fuse with both gerbil B and gerbil T lymphocytes.

Since the production of cytokines by immunocompetent cells such as T cells is extremely small, their purification is very difficult. T cell hybridomas can be developed to obtain large amounts *in vitro* [8]. D9(E6)C2B3 expressed gerbil *IL6* mRNA, and D9(E6)C2B3 and D9 (E4) expressed gerbil *TGFB* mRNA. Culture in D9(E6)C2B3-CM significantly enhanced antibody production and expression of gerbil Cγ1 and Cε *IGHC* mRNAs by B11D2(C2). IL6, a multipotential cytokine that plays roles in regulating immune responses, acute-phase reactions, and hematopoiesis, induces proliferation of and antibody production by hybridoma cells [18]. Mouse IL6 stimulates cell proliferation [1, 17, 26] and antibody production [15, 19] by some mouse hybridoma cell lines. Recombinant human IL6 enhanced Ig secretion by rabbit–rabbit hybridoma cells [13]. In contrast, culture in D9(E6)C2B3-CM and D9(E4)-CM did not improve the proliferation of B11D2(C2) cells. TGFB is produced by T cells, is required for cell survival, and activates monocytes, astrocytes, and microglia [9]. It also inhibits proliferation of T and B cells, maturation of cytotoxic lymphocytes and natural killer cells, and activation of macrophages [11]. These results suggest that gerbil TGFB in D9(E6)C2B3-CM and D9(E4)-CM suppressed the proliferation of B11D2(C2) cells.

D9(E6)C2B3-CM significantly elevated the production of antibody by B11D2(C2) compared with the con-

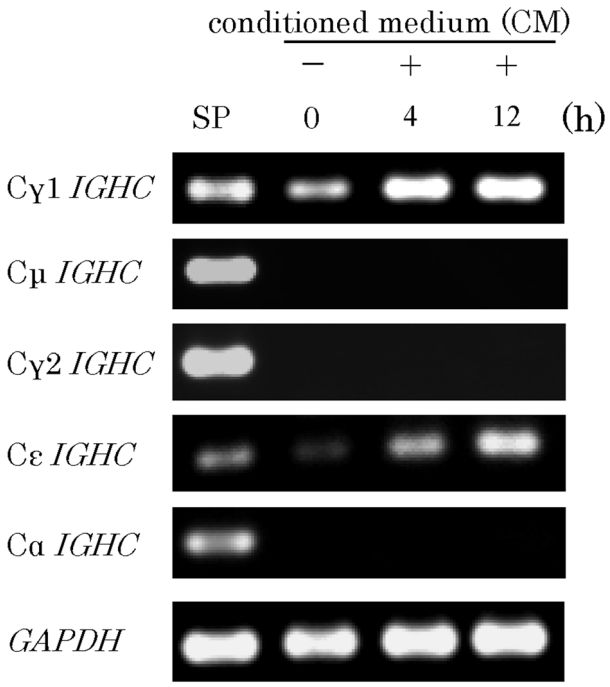

Fig. 5. Expression of gerbil *IGHC* mRNAs by B11D2(C2) cells. Total RNA was extracted from gerbil spleen (SP; lane 1), B11D2(C2) with no stimulation (lane 2), B11D2(C2) after 4 h culture in 20% D9(E6)C2B3-CM (lane 3), or after 12 h in 20% D9(E6)C2B3-CM (lane 4). RT-PCR products were electrophoresed and stained with ethidium bromide.

trol, but D9(E4)-CM showed no effect. Both D9(E6)C2B3 and D9(E4) are derived from the D9 heterohybridoma. Expression analysis of gerbil cytokines shows that the lines differed in the expression of gerbil *IL6*. This difference suggests that D9(E6)C2B3-CM contained a factor that elicits antibody production, and gerbil IL6 was one candidate.

Immunological reagents specific to gerbil cytokines or surface markers have not been available. Gerbil cytokines produced by gerbil–mouse heterohybridomas will be valuable for the culture of gerbil cells *in vitro*, the establishment of mAbs specific to gerbil cytokines, and a better understanding of innate and acquired immunity in gerbil.

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