PROTECTIVE IMMUNITY INDUCED BY 67 K OUTER MEMBRANE PROTEIN OF PHASE I *COXIELLA BURNETII* IN MICE AND GUINEA PIGS

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Received November 4, 1994

Summary. – A 67 K outer membrane protein (OMP) isolated from phase I *Coxiella burnetii* QiYi strain was purified with monoclonal antibodies (MoAb) coupled to CNBr-Sepharose 4B. Chemical analyses of the 67 K protein showed that it contained seventeen kinds of amino acids and no LPS. The immunogenicity and protectivity of the 67 K protein against *C. burnetii* was evaluated in mice and guinea pigs by *in vitro* lymphocyte proliferation assay, delayed-type skin test, antibody conversion rate, and immunization and challenge tests. Intraperitoneal injection of the 67 K protein resulted in antibody production against phase I and II whole cell antigens. The anti-67 K antibody conversion rate was found to be 100% in mice and guinea pigs as well. Lymphocytes were responses *in vitro* to specific antigen. In addition, delayed-type hypersensitivity appeared two weeks after immunization with the 67 K protein. Moreover, 100% of mice and guinea pigs inoculated with the 67 K protein were protected against a challenge with 10^3 ID_{50} virulent *C. burnetii*. In conclusion, these results demonstrate that the 67 K OMP elicits *in vivo* and *in vitro* both B cell-mediated and T cell-mediated immunity in mice and guinea pigs. Thus the 67 K protein is a candidate for an effective subunit vaccine against Q fever.

Key words: *Coxiella burnetii;* outer membrane protein; protective immunity; vaccine

Introduction

Infection of individuals with phase I *C. burnetii*, the etiological agent of Q fever results in the development of serum antibodies and of cell-mediated immunity (Heggers *et al.*, 1974; Hinrichs and Jerrels, 1976; Kishimoto and Burger, 1977). Protection against natural or experimentally induced infection with *C. burnetii* is obtained by vaccination with suspension of killed phase I whole cell vaccines (Damrow *et al.*, 1981). The whole cell vaccines have many disadvantages, such as the induction of low immunogenicity and immunopathological reactions in humans and animals (Baca and Paretsky, 1983; Fries *et al.*, 1993), especially in individuals which contacted specific antigens. The major obstacle in developing a suitable vaccine is the poor understanding of the nature of immunogenic moieties associated with proteins. Recent studies have focused on OMPs as a possible candidate for vaccine development. Hendrix *et al.* (1991) reported that mice, guinea pigs and cattle inoculated with 27 K *C. burnetii* OMP were protected against a lethal challenge with live rickettsiae. In previous studies, we purified two OMP of 67 K and 17.1 K from *C. burnetii* with MoAbs coupled to CNBr-Sepharose 4B. Mice immunized with these proteins were protected against an infectious challenge with the same rickettsial strain (Zhi *et al.*, 1992). Chemical analyses of the 67 K protein showed that it contained 17 kinds of amino acids and no LPS (Zhi *et al.*, 1993). In order to determine the immunogenicity and protection of the 67 K protein, experiments were designed to evalute the effects of the 67 K protein on the induction of humoral immunity and cell-mediated immunity *in vivo* and *in vitro*, and to study the possibility of use of the 67 K protein as a subunit vaccine.

Materials and Methods

Animals. BALB/c mice and male guinea pigs (7 – 10 animals per group) were supplied by the Animal Production Centre, Third Military Medical College, Chongqing, PRC. Mice weighed 18 – 22 g and guinea pigs 300 – 400 g at the time of inoculation.

C. burnetii. The *C. burnetii* strain QIYI(CBQY) was originally isolated from a patient with chronic Q fever in 1962 (Yu *et al.*,

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1964). The red plaque purified phase I cells ($CBQYIC_3$) were used in this study (Cheng *et al.*, 1989). $CBQYIC_3$ were propagated, suspended, purified as previously described (Zhi *et al.*, 1992).

Preparation of OMPs from *C. burnetii* was carried out the method of Cai and Zhang (1988). Purified rickettsial suspension was diluted to 0.06 –0.22 mg protein/ml in PBS and sonicated for 5 mins. The sediment from centrifugation at 14,000 × g for 20 mins was resuspended in PBS and sonicated again. The supernatant containing OMPs was collected by centrifugation at 143,000 × g for 2 hrs and analyzed by SDS-PAGE.

Protein purification. MoAbs directed against phase I and II *C. burnetii* were produced by Yu *et al.* (1986). The titers of the MoAbs used in this study were 10^{-9} for phase I(E_7) and 10^{-3} for phase II(A_5) as detected by ELISA. The MoAbs were coupled to CNBr-Sepharose 4B according to the manufacture's instructions. The OMPs were purified by column chromatography according to Zhi *et al.* (1992). The antigenic activity of the eluted fractions was determinal by ELISA.

SDS-PAGE and immunoblot analysis of purified proteins was performed as previously described by Zhi *et al.* (1991).

Chemical analyses of the 67 K protein. Amino acids, total sugar, heptose and 3-deoxy-D-mannooctulosonic acid (KDO) were analyzed as described by Amano and Williams (1984).

Immunization and challenge. Mice were immunized sc with 100 μg of the 67 K protein in incomplete Freund's adjuvant twice at 15-day intervals and challenged ip with 10^3 ID_{50} of $CBQYIC_3$ 10 days after the last injection. Guinea pigs were immunized 300 μg and challenged in the same way. The control mice and guinea pigs were injected with saline by using the same protocol. Seven to ten animals were used in each group.

Lymphocyte proliferation assay. Uptake of [^{3}H] thymidine ([^{3}H] TdR) by spleen cells from mice injected with $CBQYIC_3$, OMP was determined by the procedure as follows. Spleen cells were isolated by using Ficoll-Hpaque an incubated with RPMI 1640 medium at 1×10^6 cells/ml for 120 hrs at 37 °C in 5% CO_2 in moist air. A total of 1 μCi of [^{3}H] TdR (Science Nuclear Corp, PRC) was added to each culture tube. The cultures were incubated for another 18 hrs. Cells were collected and the extent of the uptake of [^{3}H] TdR was determined by standard scintillation technique with the LS-9000 (Beckman Instruments, Inc., Fullerton, Ca, USA). For each experiment, results from triplicate samples from five mice in each group were expressed as SI (cpm for antigen-stimulated cells/cpm for unstimulated cells). The procedure of uptake of [^{3}H] TdR by periphery blood lymphocytes from guinea pigs was the same as the procedure by spleen cells from mice except that periphery blood lymphocytes were incubated for 48 hrs.

The dealyed-type hypersensitivity (DTH) test was performed on the footpads of mice by the method of Collins *et al.* (1968). Fifty μg of the 67 K protein in a volume of 0.05 ml were injected sc into the left hind footpads of mice. An equal volume of saline containing 0.02% Triton X-100 was injected into the opposite footpads. The footpad thickness was measured with calipers at 24 hrs and 72 hrs post elicitation. DTH was expressed as the absolute increase in footpad thickness. The footpads in mice immunized with the 67 K protein were thickest at 72 hrs, when they were collected and fixed with formaldehyde. For routine pathology, tissue was dehydrated, embedded, stained with haematoxylin eosin, and observed under light microscope.

Detection of specific antibody. Antigens including phase I and phase II CBQY in whole cells and purified 67 K protein (5 mg/ml), respectively, were coated on plastic plates. Antibodies were detected by ELISA. The titers of the positive control serum samples were determined by the mean indices ± SD of the mean in the negative control group.

Results

Purification and identification of the 67 K protein

$CBQYIC_3$ OMP was purified by MoAbs coupled to CNBr-Sepharose 4B and detected by ELISA. The antigen (40 μg per track) was analyzed by SDS-PAGE and Coomassie Briliant Blue staining. A band of apparent M_r of 67 K was observed in the gel. The 67 K band reacted with MoAb E_7 in immunoblot analysis (Fig. 1).

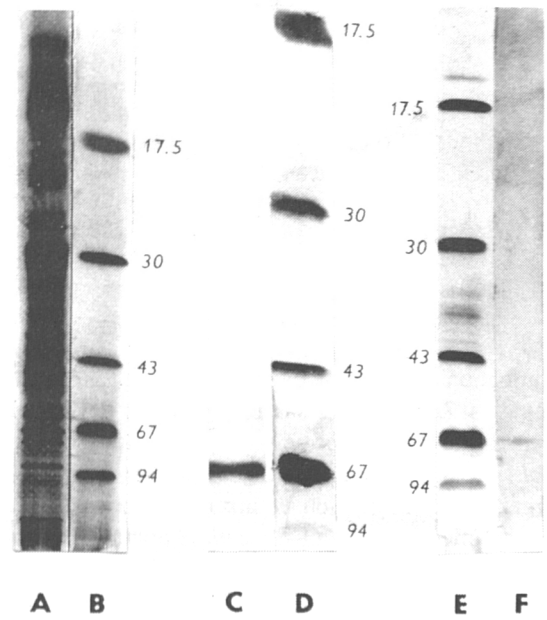

Fig. 1
SDS-PAGE and immunoblot analysis
a,b,c,d,e: SDS-PAGE; f: immunoblot (MoAb E_7); a: $CBQYIC_3$ OMP; c,f: 67 K protein; b,d,e: site markers.

Chemical composition of the 67 K protein

Analysis of amino acid composition of the 67 K protein indicated that it contained seventeen kinds of amino acids. The relative content of amino acids was highest for glycine and lowest for alanine, cysteine and valine. The concentra-

Table 1. Amino acid composition of the 67 K protein

Amino acid	Relative content (moles/His moles)
ASP	3.51
THR	2.34
SER	3.59
GLU	5.73
PRO	2.01
GLY	14.02
MET	0.26
ILE	3.71
LEU	1.79
TYR	0.36
PHE	2.01
HIS	1.00
LYS	2.70
ARG	1.92
ALA	trace
CYS	trace
VAL	trace

tion of total sugar was 0.06% KDO and heptose were not present in the 67 K protein (Table 1).

Protectivity of the 67 K protein

Mice and guinea pigs were immunized with the 67 K protein and challenged with CBQYIC$_3$. Results showed that 100% of mice and guinea pigs were protected against the infectious challenge (Table 2). All immunized animals showed no overt signs of illness. There was no fever in guinea pigs during 3 weeks after the challenge, and no microorganism was demonstrated in spleen of mice at the sixth day by microscopic examination.

Effect of the 67 K protein on lymphocyte responsiveness

The induction of cell-mediated immunity was measured by the incorporation of [^{3}H] TdR by lymphocytes in response specific antigens. Responses of spleen lymphocytes from immunized mice and responses of periphery blood lymphocytes from immunized guinea pigs to the 67 K protein were tested in tube culture and expressed as SI. Mice receiving 100 μg of the 67 K protein showed an enhanced antigenic activity of lymphocytes with SI of 5.76 for specific recall antigen on the sixth day. Similarly, periphery blood lymphocytes from guinea pigs receiving 300 μg of the 67 K protein gave SI of 4.88 on the third day. This indicated that the 67 K protein produced an increased activity and proliferation response of lymphocytes (Fig. 2, Table 3).

Table 2. The protectivity of the 67 K protein against challenge of mice and guinea pigs with 10^3 ID_{50} of CBQYIC$_3$

Animals	Immunized animals		Control animals	
	Results (A/B)	Protection (%)	Results (A/B)	Protection (%)
Mice	0/10	100%	10/10	0
Guinea pigs	0/7	100%	7/7	0

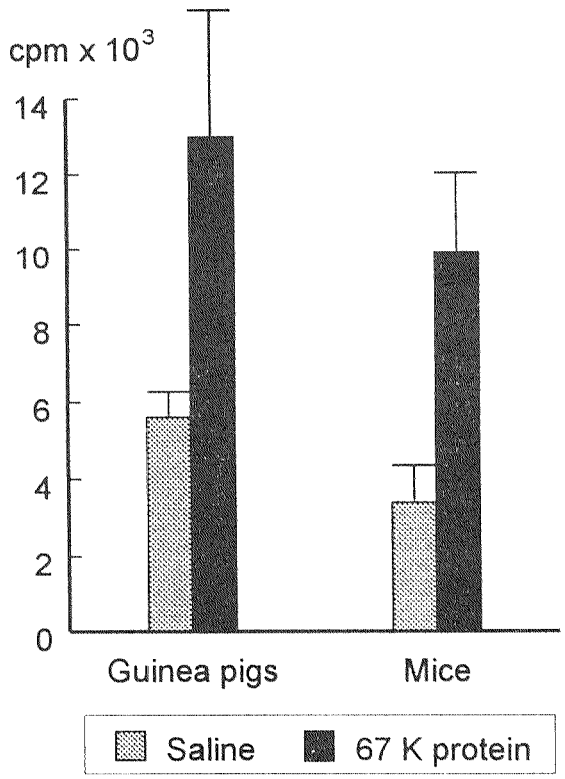

Fig. 2
Incorporation of [^{3}H] TdR into lymphocytes

DTH in the 67 K protein immunized mice

The development of cell-mediated immunity is known to be important for the control of *C. burnetii* infection. DTH is an *in vivo* manifestation of the cell-mediated immunity and has been shown to parallel the development of protective immunity in mice. Hence, the cell-mediated immunity in the 67 K protein immunity of mice was studied by using DTH as a parameter.

The footpad thickness (1.7375 ± 0.7981) at 72 hrs in mice immunized with 67 K protein was significantly

Table 3. Incorporation of [^{3}H] TdR into lymphocytes

Immunized animals	Incorporation of [^{3}H] TdR		
	Nonstimulated cultures (cpm)	Culture stimulated with the 67 K protein	
		cpm[a]	Stimulation index
Mice	1738.8 ± 324.4	10315.2 ± 2056.3	5.76
Guinea pigs	2380.4 ± 393.5	13036.6 ± 2938.0	4.88

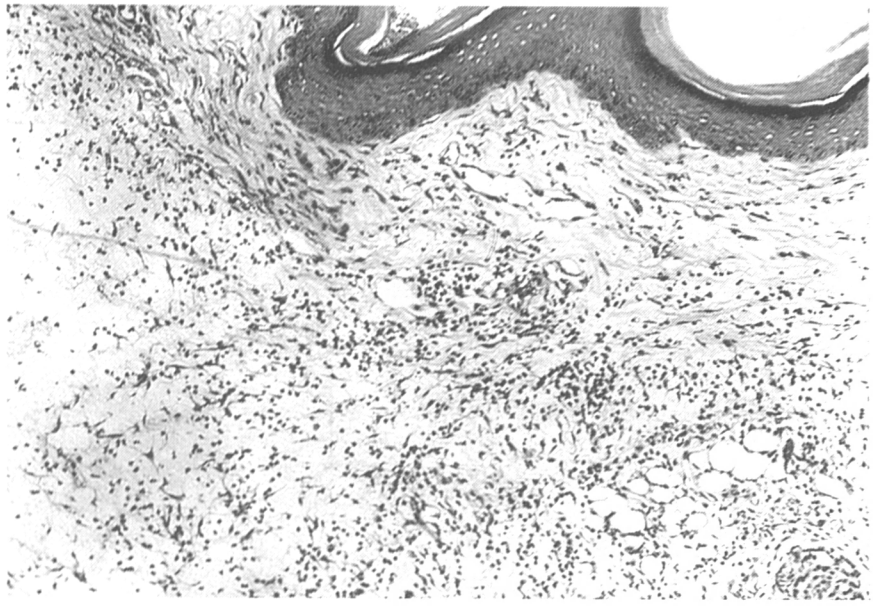

Fig. 3
Mononuclear cell in the skin of mouse footpad (light microscopy)

($P < 0.001$) greater than that (0.6625 ± 0.3512) in mice which received saline. Light photomicrograph showed that mononuclear cells accumulated in the skin of footpad (Fig. 3).

Specific antibody level

Serum titers of antibodies were measured in immunized (67 K) mice and guinea pigs. The anti-67 K antibody conversion rate was found by ELISA to be 100% in mice as well as in guinea pigs. Titers of anti-67 K antibodies were 1:4200 for guinea pigs. Titers of antibodies against phase I whole cells were 1:2700 and against phase II cells were 1:16800 in guinea pigs immunized with the 67 K OMP.

Discussion

Infection of individuals with phase I *Coxiella burnetii,* the etiological agent of Q fever, results in the development of serum antibodies and of cell-mediated immunity, like with other obligatory intracellular bacteria. Previous studies on humans and experimental animals infected with Q fever demonstrated the presence of cell-mediated immunity which was proved in macrophage migration inhibition test, delayed-type skin test (Ascher *et al.,* 1983; Lackman *et al.,* 1962), or *in vitro* lymphocyte proliferation assay (Jerrells *et al.,* 1975). The cell-immunity was also demonstrated *in vivo* by spleen cells of immunized mice. However, the role of antibodies in the immunity of Q fever was not understood. Phase I antibodies were considered to be in relation to protection against *C. burnetii* infection. Phase II antibodies were found not to correlate with protection although the titers of anti-phase II antibodies were very high. Mice injected with antiphase I IgM mixed with *C. burnetii* showed a suppression of the proliferation of *C. burnetii* in spleen cells (Williams *et al.,* 1990). Similar findings were observed by neutralization test and passive protection assay with MoAbs against *C. burnetii* in our laboratory (Yu *et al.,* 1989).

In this study the 67 K protein from $CBQYIC_3$, was purified with MoAbs coupled to CNBr-Sepharose 4B and determined to be a major structural protein of *C. burnetii* by SDS-PAGE and immunoblots. Studies on the immunogenicity of the 67 K protein were based on the antibody

conversion rate, the detection of cell-mediated immunity with DTH test or the lymphocyte proliferation assay *in vitro*. The immunization of mice and guinea pigs with the 67 K protein induced high level of antibodies and lymphocyte proliferation. The anti-67 K antibody conversion rate was detected to be 100% in mice as well as in guinea pigs. DTH *in vivo* appeared two weeks after immunization with the 67 K protein. The latter elicited both B cell- and T cell-mediated immunity *in vivo* and *in vitro*. These results demonstrated that the 67 K protein was immunogenic in animals. Moreover, 100% of mice and guinea pigs inoculated with the 67 K protein were protected against an infectious challenge with 10^3 ID_{50} CBQYI. We assume that the protective immunity of the 67 K protein is based on humoral and cell-mediated immunity in immunized animals.

Williams *et al.* (1990) reported a major surface of roughly 29.5 K from the phase I Nine Mile strain (Williams *et al.*, 1990). No phase I lipopolysaccharide (LPSI) was detected in the 29.5 K protein preparation by three different LPSI MoAbs. Mice immunized with two 25 μg injections of LPSI induced antibodies against LPSI and phase I whole cells. No antibody was detected against phase II whole cells. However, immunization with the 29.5 K protein induced antibody against the LPSI fraction and phase I and II whole cells. The 29.5 K protein was more protective than LPSI in the challenge assay. In this study, analyses of the 67 K protein indicated that it contained only 0.06% of sugar and no KDO and heptose. Therefore it is considered that the 67 K protein did not contain LPS because KDO and heptose are principal components of LPS of Gram-negative bacteria. 0.06% of sugar in the 67 K protein might be remnants of LPS. Immunization with the 67 K protein induced the production of high level of antibodies against specific antigen, phase I and II whole cell antigens. This also suggested that the 67 K protein was effective immunogenic and might be of great value in the development of vaccine. Analyses of protein by amino acid composition demonstrated that the 67 K protein was qualitatively different from the bacterial 62 K heat shock protein (HSP). The 67 K protein contained cysteine which was absent in the 62 K HSP. While the 67 K protein was devoid of asparaginate and glutamine these amino acid were confirmed to be present in the HSP.

At present, for specific prophylaxis of rickettsial infection by vaccination, only Q fever vaccines are available for common use. The best practical progress has been achieved in the preparation and evaluation of vaccines against Q fever (Kazár *et al.*, 1991). The soluble chemovaccine obtained by trichloroacetic acid extraction of a LPS-protein complex from phase I *C. burnetii* cells was recommended for Q fever vaccination by sc route (Brezina *et al.*, 1974) and successfully used in several hundreds persons professionally exposed to Q fever in Czechoslovakia (Kazár *et al.*, 1982). The residue of chloroform-methanol extraction of cells (CMR) had replaced phase I *C. burnetii* cells in the use for human vaccination, since CMR was more immunogenic than the killed whole cells and was nontoxic. But the CMR provided at least one of the determinants which induced immunosuppresion. The induction of negative regulation might be initiated by a complex composition of the antigen, especially in relation to lipid A of LPS (Williams *et al.*, 1982,1986). Based on this opinion, excellent vaccines emphasize the need for specific antigenic determinant isolated from *C. burnetii* that possess protective antigens without producing undesirable biological and immunological tissue reactions. A highly purified 67 K surface protein containing seventeen kinds of amino acids might be used as effective and harmless subunit and recombinant vaccine.

Acknowledgement. The project was supported by the National Science Foundation of the People's Republic of China.

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thrice and the yields of virus (PFU) were assayed (Dulbecco and Vogt, 1954). The experiment was repeated thrice and the data were analyzed in completely randomized design (Cochran and Cow, 1967).

Virus growth studies. (a) At high multiplicity of infection. HS and control BHK-21 cells were inoculated with 10 PFU/cell. The progeny virus was assayed at 0.2, 4, 6, 10, 16, 20, and 24 hrs p.i. The results were compared using t-test. (b) At low multiplicity of infection. HS and control PS and BHK-21 cells were inoculated with 0.1 PFU/cell. These cultures were assayed for progeny virus on days 1 – 5 p.i. The results were compared using t-test. (c) Virus growth at different passage level. Monolayers of PS and BHK-21 cells at different passages levels (15 – 47) were subjected to heat shock and then inoculated with 20 PFU of the virus. These cultures were assayed for virus yield after 48 hrs p.i. (d) Growth of different strains of JEV. Monolayers were inoculated with different dilutions of 821564 (Kolar), 785730 (Gorakhapur) and 7812474 (Dibrugarh) strains of virus. Virus was grown and the yield was assayed on day 2 p.i.

Virus inactivation studies. Virus stocks were prepared without stabilizer by inoculating control and HS-BHK-21 cell monolayers. The stocks were diluted in PBS, incubated at 37 °C and samples were taken at various time intervals (0 – 24 hrs) for virus titration. The results were compared using t-test.

Results

Effect of heat shock on cell viability

Monolayers of PS and BHK-21 cell lines in Bellco tubes were exposed to 43 or 41 °C for different time intervals. Viable cell count and pH of cultures were recorded. PS cells retained 100 – 95% viability when exposed to 43 °C from 2 to 24 hrs. On the other hand, BHK-21 cells retained only 84 – 20% viability when exposed to 43 °C from 2 to 16 hrs and by 24 hrs the viability was lost completely. When BHK-21 cells were exposed to the lower temperature (41 °C), 95 – 98.7% cells retained viability up to 24 hrs. BHK-21 cells were, thus, more thermosensitive than PS cells. All the cultures showed slight alkalinity when exposed to higher temperature. In conclusion, the heat treatments given to PS (43 °C for 4 hrs) and BHK-21 cells (41 °C for 4 hrs) did not adversely affect the cell viability (Table 1).

Optimization of heat treatment

When PS and BHK-21 cells were exposed to 43 °C and 41 °C, respectively, for various time intervals (0 to 24 hrs) and studied for their ability to support the growth of the virus, they were found to yield significantly more virus than the unexposed cells ($P < 0.01$), The highest yields (7.9 log PFU/ml and 8.2 log PFU/ml) were obtained in PS and BHK-21 cells, respectively, when heat shock was given for 4 hrs (data not shown).

Table 1. Viability of PS and BHK-21 cells after heat shock

Period of exposure (hrs)*	Viability (% of control)		
	PS cells	BHK-21 cells	
	43 °C	43 °C	41 °C
2	100	84	99
4	100	60	99
8	99	50	98
16	97	20	95
24	95	0	95

*Control cells showed 100 – 98.5% viability and pH of medium in the range 6.8 –7.0.

After studying the optimal conditions for heat treatment, it was decided to expose the monolayers of PS and BHK-21 cell cultures to 43 and 41 °C, respectively, for 4 hrs before inoculation. Henceforth these cultures will be designated as heat shocked (HS) cultures. HS and control cultures were used to study (1) their sensitivity to virus, (2) efficiency of plating of the virus and (3) viral growth. Progeny virus obtained from HS cultures was studied for its thermostability at 37 °C.

CPE and virus yield

HS and control monolayers of BHK-21 and PS cells were infected with various dilutions of virus stocks. Four tube cultures were used for each dilution. These cultures were observed for CPE for up to 10 days and virus yields were assayed. All HS cultures showed profound CPE one day earlier than controls. Similarly complete cell degeneration was also observed one day earlier in HS cultures. Virus yield was increased by 1.7 and 2 logs/0.1 ml in HS-PS and HS-BHK-21 cells, respectively (Table 2).

Table 2. CPE and virus yield

Cells	CPE observed (day p.i.)	Complete cell destruction observed (day p.i.)	Virus yield ($\log TCID_{50}$/0.1ml) after complete cell destruction
PS (control)	2	5	6.0
HS-PS	1	4	7.7
BHK-21	2	4	6.5
HS-BHK-21	1	3	8.5

Efficiency of plating of virus in HS cells

Monolayers of HS and control PS cell cultures were inoculated with 50, 100 and 150 PFU of the virus per culture, adsorbed for half an hour at 37 °C. Unadsorbed virus (supernatant) was collected and quantitiated. The infected cultures were overlaid with agar medium and incubated at 37 °C. On the 4th day p.i. the cultures were fixed, stained with crystal violet and plaques were counted. The experiments were repeated four times.

When equal quantum of JEV was plated (50, 100 and 150 PFU/culture) in HS and control PS cultures, the HS cultures yielded an average of two times more plaques than the control cultures indicating an enhancement of plaque production (data not shown).

Virus growth

From the one step growth curve (Fig. 1) it can be seen that there was no substantial difference in viral yields in HS and control cells up to 10 hrs p.i. However, significant increase in the yield was obtained in HS cells after 16 hrs p.i. ($P < 0{,}01$). Growth of virus at low multiplicity of infection in HS-PS and HS-BHK-21 cells is shown in Fig. 2 and 3, respectively. The heat treated cells showed higher yields during the whole observation period.

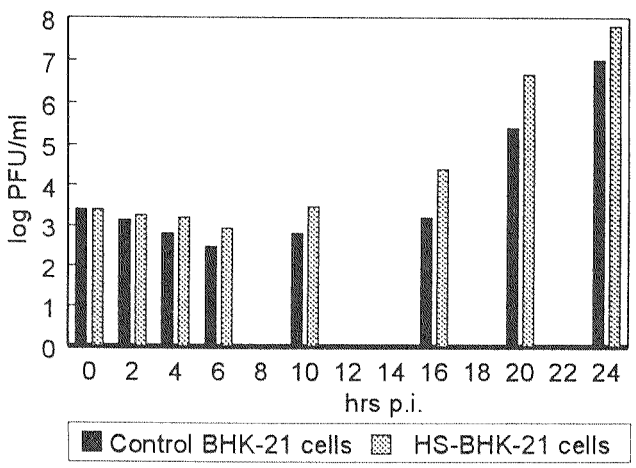

Fig. 1
One-step growth curve of JEV in HS and control BHK-21 cells
Multiplicity of infection 10 PFU/cell.

Heat treated cells at different passage levels (15 – 47) persistently produced significantly higher yields of virus by 0.6 to 1.4 log PFU/ml units (data not shown).

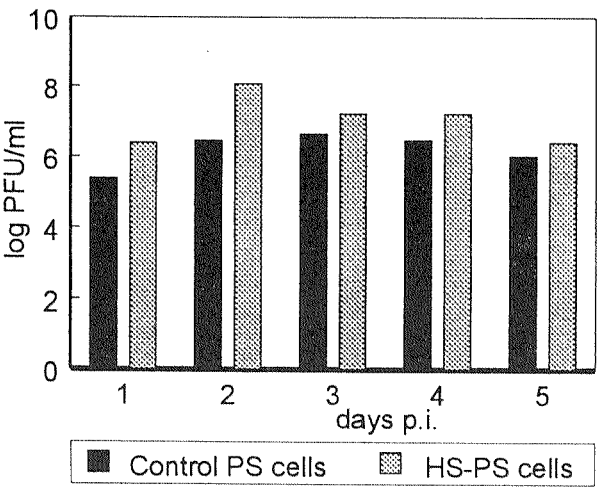

Fig. 2
Growth of JEV in HS and control PS cells infected at low multiplicity of infection
Multiplicity of infection 0.1 PFU/cell.

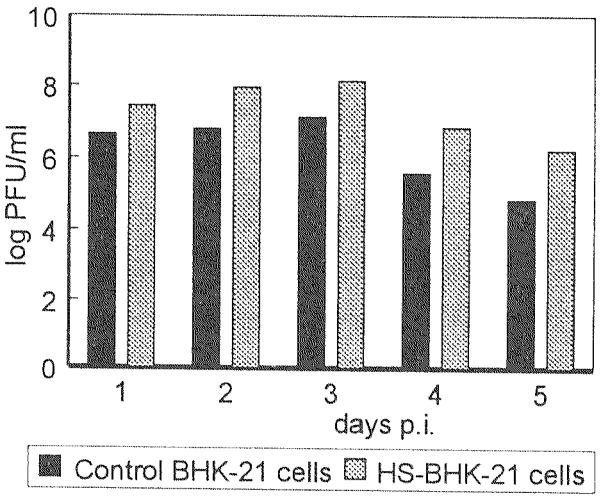

Fig. 3
Growth of JEV in HS and control BHK-21 cells infected at low multiplicity of infection
For legend see Fig. 2.

Virus inactivation

The rates of inactivation of virus determined as slopes of linear regressions were 3 and 2.5% per hr at 37 °C in control and HS-BHK-21 cells, respectively. The difference in the residual virus titer was apparent at 8 hrs ($P < 0.05$) and highly significant ($P < 0.01$) at 24 hrs (Fig. 4). The progeny virus obtained from HS-BHK-21 cells showed comparatively higher thermostability as evidenced by slower inactivation.

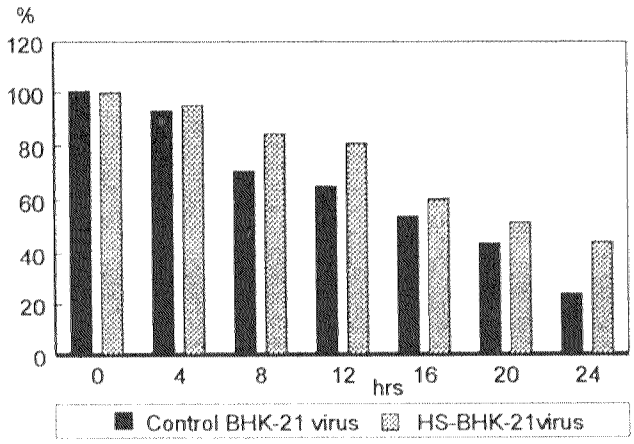

Fig. 4
Inactivation at 37 °C of JEV grown in HS and control BHK-21 cells
Ordinate: % of residual virus.

Growth of different strains of JEV

Increased yield by 0.5 to 2.0 log PFU/ml units was obtained on day 2 p.i. by inoculating different strains of JEV (Kolar, Gorakhpur and Dibrugarh) in HS cells (data not shown).

Discussion

Viability of PS and BHK-21 cells was not adversely affected by the heat shock treatment. It may be noted that PS and BHK-21 cells exposed to 43 and 41 °C, respectively, for 4 hrs and shifted down to 37 °C for 12 hrs could be successfully subcultured. A strain of PS cell line has been maintained at 40 °C for more than 50 passages (results not shown).

The effect of heat shock on the course of JEV replication was studied in the HS cultures. They showed greater or earlier CPE than the control cells. Also increased yields in $TCID_{50}$ were obtained in HS-PS and HS-BHK-21 cells infected with the virus. Our results are in agreement with those reported earlier on an increased sensitivity to virus and virus yield in heat treated cells infected with VSV, EMC and influenza WSN viruses (Kognovitskaya *et al.*, 1987), CMV and EBV (Zerbini *et al.*, 1986), rabies virus (Kawai *et al.*, 1992), VSV and Rous sarcoma virus (Bromley and Voellmy, 1983) and some bacteriophages (Wiberg *et al.*, 1988).

The increase in cellular sensitivity for CPE and higher virus yields in the HS cultures could be attributed to various factors. Zerbini *et al.* (1986) observed 2-fold increase in early antigen production and 2.5-fold increase in virus capsid proteins in human lymphoblastoma cells exposed at 44 °C for 10 mins and infected with EBV. Increased synthesis of rabies viral mRNA and capsid proteins in BHK-21 cells exposed to 44 °C for 4 hrs was reported by Kawai *et al.* (1992). Sun *et al.* (1993) reported 2-fold increase in p24 viral antigen and 20-fold increase in the release of reverse transcriptase in HTLV-1-infected MT-2 cells exposed to 42 °C for 5 hrs.

Li *et al.* (1980) observed increased fluidity of lipid bilayer of cell membrane at higher temperature which otherwise is gel-like. Functions of membrane enzymes and fatty-acid composition of membrane phospholipid were found altered due to temperature-linked transition (Fergusson *et al.*, 1975). Such changes may be responsible for higher intake of virus. One of the phospholipid groups, polyphosphoinositides is known as the receptor substance for arboviruses.

Kognovitskaya *et al.* (1987) reported switching off of interferon production in heat shocked cells as a possible cause of higher yield of virus. PS cell cultures, which we have used in these studies are considered to be non-producers of interferon as there are no reports available on induction of interferon in these cells. Our own observations also confirm this assumption (unpublished results). Yamazak (1965) reported that two strains of JEV (CEC-adapted Hotta and attenuated V42 J strain) were unable to induce interferon in hamster kidney cells. Moreover, it required exposure for 18 hrs at 41 – 44 °C for interferon inhibition. We have exposed our cells to higher temperature for only 4 hrs before infection. Therefore, the increased viral yields in the HS cultures may not be due to the inhibition of interferon production in these cells.

Increased virus yields were obtained in HS cultures by inoculation of both low and high virus doses. Our observation is in agreement with reports of Zerbini *et al.* (1986). They claimed that 10 PFU and 1 PFU virus inoculum yielded increased recovery of human CMV in human embryonic fibroblasts.

The enchanced virus yield may be due to changes in DNA and protein synthesis in host cells during heat shock. Rapp and Turner (1979) observed enhanced plaque production by CMV in the presence of diethylstibestrol which is able to induce a subset of HS proteins (Busin and Bournias-Vardiabis, 1984). Hence, enhanced virus replication in these conditions is mediated by a mechanism which is similar to heat shock response. It is possible that sufficient levels of HS/stress proteins are essential for productive viral infection (Young and Elliot, 1989).

The one-step growth studies in our investigation do not indicate any major differences in the early phase of the virus growth. Thus no differences in the adsorption and eclipse (and therefore penetration and uncoating of the virus) were found in HS and control cells. It is only after 16 hrs p.i. that

HS cultures show significantly higher yields of virus. The assembly of viral components and their maturation into complete infectious virions appear to take place much more efficiently in the late phase of the virus growth in HS cultures. Zerbini *et al.* (1986), however, observed enhanced CMV replication in human fibroblast cells due to shortened eclipse phase by exposure of cells to 44 °C for 10 mins.

We have also observed higher efficiency of plating of virus in HS cells which may be responsible for obtaining higher virus yield. Thus the thermostimulation of these cultures has resulted in an increase in the cellular sensitivity for CPE production and the enhancement of both plaque and virus production.

The viral progeny obtained in HS cells was found to be more thermostable which might be contributing to higher viral yields observed in these studies. JEV is an extremely thermolabile virus. Acquisition of a considerable degree of thermostability by the progeny virus obtained after replication in the HS cultures is an interesting finding, which needs to be analyzed further.

The fact that both PS and BHK-21 cultures at different passage levels gave increased yields of various strains of JEV after heat shock indicates that it is a general phenomenon.

From the present studies, it appears that the heat shock technique may be a more sensitive tool for (1) early detection of JEV (2) obtaining higher virus yields and (3) increasing the stability of the virus.

Acknowledgement. The authors are indebted to Dr. K. Banerjee, Director of the National Institute of Virology for his continuous encouragement and valuable suggestions. The authors gratefully acknowledge the kind co-operation from all the staff members of Tissue Culture and Cell Biology Division and the assistance of Ms C.A. D'Mello, Sh. S.S. Bedekar and Sh. V. Simha in the typing of the script and data analysis.

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