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MICROCALORIMETRIC STUDY OF VIRUS INFECTION
The effects of hyperthermia and α 1b recombinant homo interferon on the infection process of BHK-21 cells by foot and mouth disease virus

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The metabolic process of BHK-21 cell line infected by foot and mouth disease virus (FMDV) was determined by using LKB-2277 Bioactivity Monitor. The aim of the present study is to investigate the metabolic thermal power of the virus infection process of BHK-21 cells, the effects of combinational treatments of hyperthermia and α 1b recombinant homo interferon on this process. In contrast to the metabolic thermal power of uninfected BHK-21 cells, the thermogenetic curves show that the energy metabolism mechanisms of BHK-21 cells were significantly changed by the virus infection process. The maximum thermal power decreased and the time needed to reach the maximal thermal power increased with the increasing concentration of interferon. The results also show that the infection process was thermosensitive. But no apparent synergetic effect of the combinational treatments of hyperthermia and interferon was observed. The present microcalorimetric results are in accordance with the cytomorphology observations.

Keywords: *BHK-21 cell line, FMD virus, hyperthermia, interferon, metabolism, microcalorimetry*

Introduction

Foot and mouth disease virus (FMDV) is the prototype member of the *Aphthovirus* genus of the family *Picornaviridae*. FMDV is the causative agent of the economically most important animal viral disease world-wide that has been responsible for large economic losses in different parts of the world [1].

In vitro studies of energy metabolism in isolated cells infected by virus or uninfected contribute to improved knowledge of energy metabolism under normal and pathological conditions. In principle, the growth processes are not different from conventional chemical processes [2]. The metabolism of cells from substrates is a chemical reaction, albeit a complex one, is accompanied by a change in enthalpy [3]. The amount of heat is related to the sum of process taking place in cells. The maximal thermal power and the total heat production in the calorimetric experiments can be used to evaluate the metabolic activity of cell, therefore, it can be the basis of studying the antiviral effect of combination of hyperthermia and drugs.

In the past decades, microcalorimetry has been used extensively in the study of the life science [4–6]. But in the field of animal or human cells, many studies are focused on the metabolism of cell [7–9] and on the action of drugs and related substances on cells [10, 11]. Only a few papers [12, 13] are concerned with the important physiological process of virus infection.

This paper describes the application of microcalorimetry in pharmacology and drug effect evaluation. It is a quantitative, inexpensive and versatile method for determining the therapeutic effectiveness of certain treatment. In this study, the BHK-21 cell line and FMDV are used to study the metabolic process of infected cells by microcalorimetry. The effects of interferon, temperature and their combinational treatment on the infected BHK-21 cells are also reported.

Materials and methods

Instrument

LKB-2277 Bioactivity Monitor (Thermometric AB, Sweden), a type of heat-flow microcalorimeter, was used. The detection limit of this system is 0.15 μW and its baseline stability (over a period of 24 h) is 0.2 μW. The performance of this instrument and the details of its construction have been previously described [14]. The amplifier of the monitor was set at the scale of 100 μW and the signal was recorded by a self-made data acquisition system.

LH50A Microscope was from Olympus Inc., Japan.

Materials

BHK-21 cells, and FMDV (serotype O) were provided by China Center for Type Culture Collection (CCTCC), Wuhan University, Wuhan 430072, P. R. China.

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BHK-21 cells were cultured in a T-25 plastic flask (Costar Co. USA). The cells were grown in Minimum Essential Medium (MEM), pH=7.2–7.4, with 10% heat-inactivated fetal calf serum at 37°C in 5% CO_2. The MEM and trypsin (1:250) were obtained from GIBCO Co. USA. The same medium was used for the calorimetric experiments.

α 1b recombinant homo interferon (10 μg/10^6 Unit) was obtained from Tri-Prime Gene Co. Ltd, Beijing, P. R. China.

Experimental

Microcalorimetry assay

The ampoule method was used in the microcalorimetry assay. Each ampoule contained 1 mL sample or reference and 2 mL air. Cells in exponential growth phase were trypsinized and counted using a Thoma's hemocytometer. The concentration of the cell suspension was adjusted to 10^5 cells mL^{-1} by adding appropriate culture medium. The 1 mL cell suspension was added into a 3 mL glass ampoule. After the cells being cultured in the ampoule for 24 h at 37°C in 5% CO_2, the cells reached above 80% confluency. For the FMDV infection assay, the used media was aspirated from the ampoule and then 50 μL of FMDV (10^6 $TCID_{50}$ 0.1 mL^{-1}) was added to the ampoule. After the cells were infected for 1 h at 37°C, the inoculum was then replaced with 1 mL fresh culture medium. Following these procedures, the ampoule was sealed and put into the microcalorimeter to determine the thermal power of the infected BHK-21 cells.

For the control assay, all the procedures were just like the above except that no infection process was included.

Interferon was added into the ampoule with the fresh culture medium after the infection process to determine its antiviral effect. Three final concentrations of 0.2, 0.5, 1.0 μg mL^{-1} were used.

The measurements were carried out at 37°C, which was corresponding to the physiological body temperature, and 40, 42°C i.e. hyperthermia, respectively.

The final interferon concentration of 1.0 μg mL^{-1} (at 37°C) was used to see the effect of interferon on the uninfected BHK-21 cells.

BHK-21 Cell line morphology observation

The cells were cultured and infected in 3 mL ampoule just the same as the microcalorimetry assay. Cells were observed on LH50A Microscope (Olympus Inc., Japan) 24 h post infection. The amplification was set at 150×.

Results

The metabolic thermogenetic curves of uninfected BHK-21 cells and FMDV infected BHK-21 cells are shown in Fig. 1. There is a significant difference between these two curves that can only contribute to the result of FMDV infection. The thermogenetic curve of uninfected BHK-21 cells formed a broad peak while for that of FMDV infected BHK-21 cells, there was a peak that consisted of an exponential increasing phase and an exponential decreasing phase.

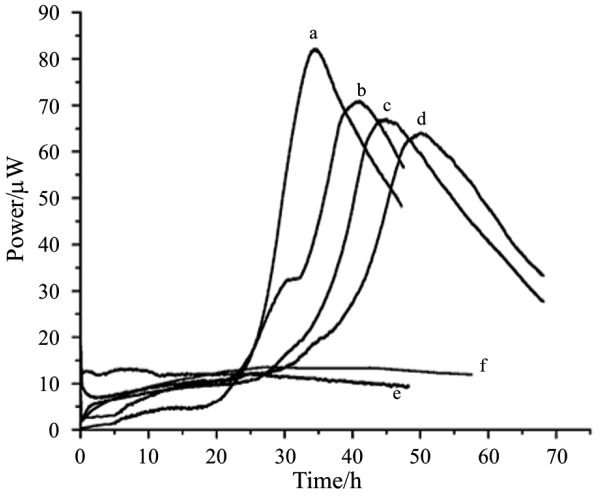

Fig. 1 Thermogenetic curves of BHK-21 cells under different conditions at 37°C. a – FMDV infected BHK-21 cells without interferon, b –, c –, d – FMDV infected BHK-21 cells with 0.2, 0.5, 1.0 μg mL^{-1} interferon, e – uninfected BHK-21 cells with 1.0 μg mL^{-1} interferon, f – uninfected BHK-21 cells without interferon

The thermogenetic curves of FMDV infected BHK-21 cells under the action of interferon are shown in Fig. 1. It is clear that with the increasing of interferon's concentration, the maximal thermal power became smaller and the time it took to reach the maximal thermal power became longer. For the uninfected BHK-21 cells, there was no significant difference between the thermogenetic curves whether the interferon was present or not (Fig. 1).

The inhibitory ratio of interferon can be defined as:

$$I\% = (P_{max,0} - P_{max})/P_{max,0} \times 100\% \quad (1)$$

in which P_{max} is the maximal thermal power with the present of interferon at 37°C, $P_{max,0}$ is the maximal thermal power without interferon at 37°C. Table 1 gives the values of the inhibitory ratio (in I%) and t_{max}, in which t_{max} is the time (in hour) to reach the maximal thermal power. The I% and t_{max} become larger with the increasing of the concentration of interferon. From Table 1 we can obtain the inhibitory

Table 1 The values of maximal thermal power (P_{max}), the time to reach the maximal thermal power (t_{max}) and the inhibitory ratio (I%) *vs*. various concentrations of interferon (c) at 37°C and at 40°C

c/μg mL^{-1}	T/°C	I%	P_{max}/μW	t_{max}/h
0.0	37	0.0	82.20	34.3
0.2	37	13.7	70.96	41.1
0.5	37	18.4	67.05	44.8
1.0	37	22.0	64.08	50.1
0.0	40	43.9	46.09	42.0
0.2	40	45.1	45.09	42.5

ratio (I%) *vs*. the concentration of interferon (c) obey the following equation:

$$I = 1.55 + 22.2\sqrt{c} \quad (c\text{: from 0 to 1.0 } \mu g\ mL^{-1}, R = 0.98) \tag{2}$$

At the same time we can obtain the t_{max} *vs*. the concentration of interferon (c) obey the following equation:

$$t_{max} = 34.2 + 15.6\sqrt{c} \quad (c\text{: from 0 to 1.0 } \mu g\ mL^{-1}, R = 0.99) \tag{3}$$

Temperature's effect on the infection process are shown in Fig. 2, from which we can see that at 40°C the maximal thermal power is markedly smaller (only 56% of that at 37°C) and the time to reach the maximal thermal power is almost 8 h longer than those at 37°C. While at 42°C, the peak was sharply reduced in the thermogenetic curve of infected BHK-21 cells.

The temperature's effect to the infection was evaluated by the inhibitory ratio too. The inhibitory ratio caused by hyperthermia is defined as following:

$$I\% = [P_{max,0} - P_{max,0}(T)]/P_{max,0} \times 100\% \tag{4}$$

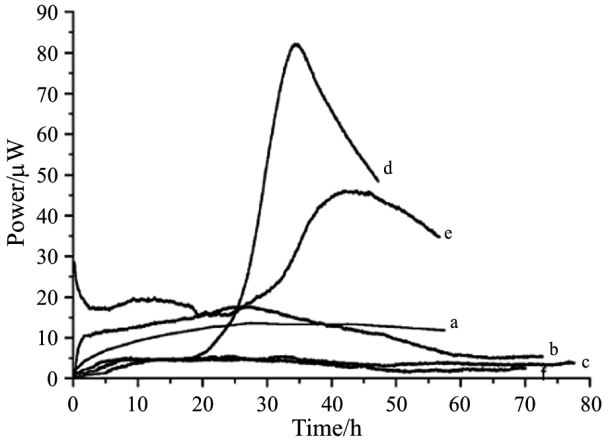

Fig. 2 Thermogenetic curves of FMDV infected BHK-21 cells at 37, 40 and 42°C. a – , b – , c – uninfected BHK-21 cells at 37, 40, 42°C; d –, e – , f – FMDV infected BHK-21 cells at 37, 40, 42°C

in which $P_{max,0}(T)$ is the maximal thermal power without interferon at temperature T, $P_{max,0}$ is the maximal thermal power without interferon at 37°C. The analysis of Fig. 2 is listed in Table 1.

When the measurements of FMDV infected BHK-21 cells were performed at 40°C under the action of 0.2 μg mL^{-1} interferon, the maximal thermal power is slightly smaller than that at 40°C without interferon (Fig. 3). The inhibitory ratio caused by the combination of hyperthermia and interferon is defined as:

$$I\% = [P_{max,0} - P_{max}(T)]/P_{max,0} \times 100\% \tag{5}$$

in which $P_{max}(T)$ is the maximal thermal power with interferon at temperature T, $P_{max,0}$ is the maximal thermal power without interferon at 37°C. The analysis results are listed in Table 1.

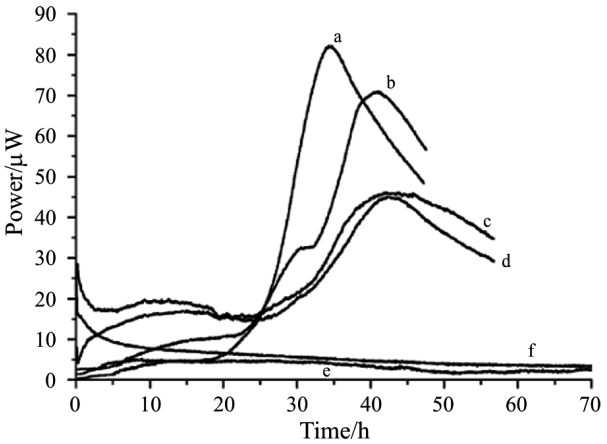

Fig. 3 Thermogenetic curves of the combination effects of interferon and temperature to FMDV infected BHK-21 cells. a – 37°C without interferon, b – 37°C+0.2 μg mL^{-1} interferon, c – 40°C without interferon, d – 40°C+0.2 μg mL^{-1} interferon, e – 42°C without interferon, f – 42°C+0.2 μg mL^{-1} interferon

Since there was not distinctly discernable peak on the thermogenetic curve at 42°C, the inhibitory ratio was not available.

The photographs of BHK-21 cells (control, infected cells, infected cells with interferon) are shown in Fig. 4. BHK-21 cells are fibroblast-like cells. When BHK-21 cells was infected by FMDV, they show cytopathic effects, i.e. cells rounded up, became less adherent, lysed. From Fig. 4, the control cells are spindle shaped and formed a monolayer. For the infected cells, part of them had detached from the substrate and lysed, some of them rounded up and only a small part attached to the substrate. In the presence of interferon, fewer cells rounded up and more cells attached to the substrate.

There was not distinct morphological difference between the control cells at 37 and 40°C. But fewer

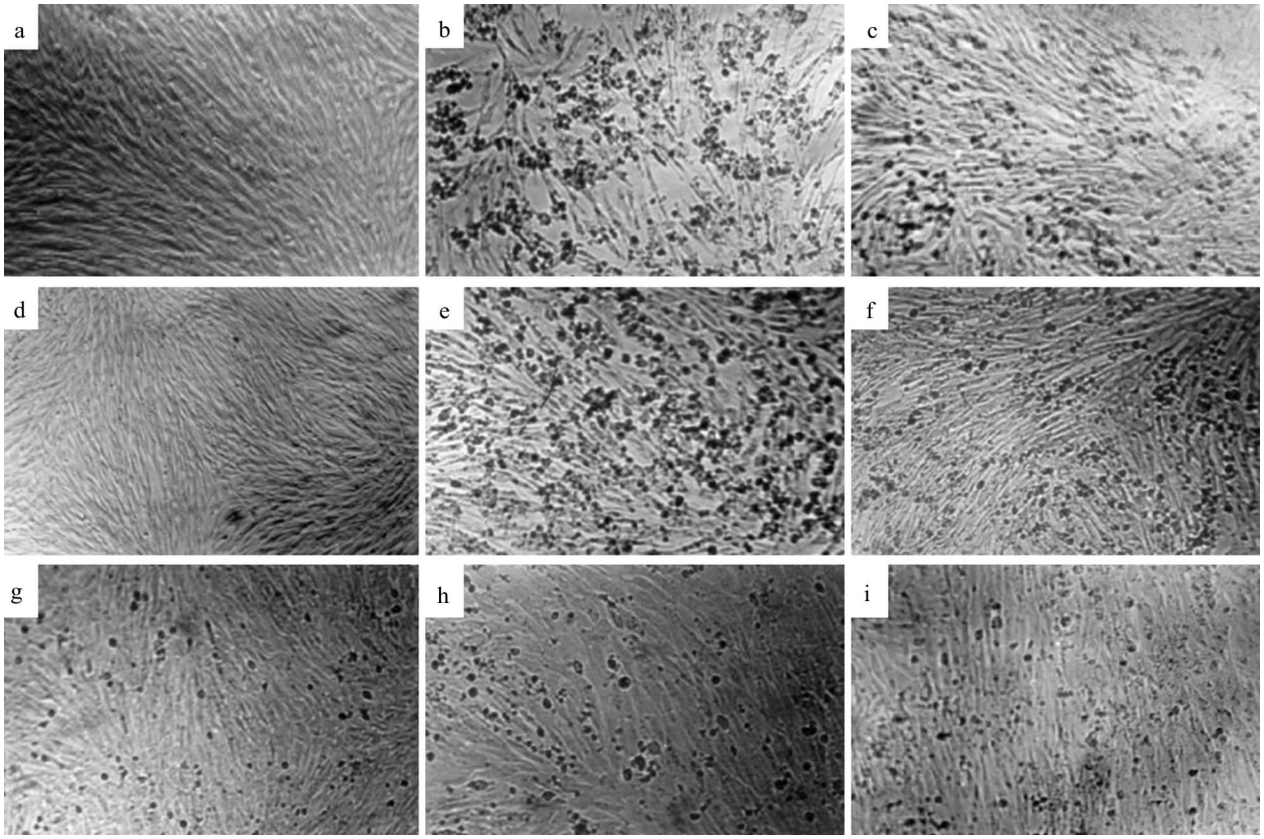

Fig. 4 The morphology of different BHK-21 cells was examined with an Olympus LH50A microscope. The magnification was set at 150× and all the photos were taken after 24 h. a – control at 37°C, b – FMDV infected at 37°C, c – FMDV infected and with 0.2 μg mL^{-1} interferon at 37°C, d – control at 40°C, e – FMDV infected at 40°C, f – FMDV infected and with 0.2 μg mL^{-1} interferon at 40°C, g – control at 42°C, h – FMDV infected at 42°C, i – FMDV infected and with 0.2 μg mL^{-1} interferon at 42°C

infected cells detached from the substrate than those at 37°C. When interferon was added to the infected cells, fewer cells rounded up and more cells remained attached than those without interferon. But the morphological difference between the infected cells in the presence or absence of interferon at 40°C was not as distinct as that at 37°C.

At 42°C some of the control cells died and rounded up due to the high temperature. To the infected cells in the presence or absence of interferon, there were not significant cytopathic effects but the cell necrosis ascribed to the high temperature increased.

Discussion

For the uninfected BHK-21 cells (Fig. 1), the thermal power kept rising slowly until reached its peak and then decreased slowly too, which may be attributed to the exhaustion of the oxygen or nutrient material in the medium and the decrease in pH in closed ampoule [15]. For the metabolic process of FMDV infected BHK-21 cells, in the beginning the thermal power increased slowly, then the thermal power increased quickly and reached the maximum, ended with the thermal power decreasing rapidly too. The metabolic thermal power of FMDV infected cells increased more distinctly than of uninfected cells. The significant difference between the metabolic thermal power can only be ascribed to the alteration of the BHK-21 cells' metabolism mechanism after infection. There is not any heat producing structure in virus. Virus is incapable of replication outside cells [16]. When virus penetrates into the host cell, the nucleic acid of virus is released, and then the metabolism of the infected cell is changed with the replication of the genome of virus, production of virus-encoded protein, and the assembly of the virus. The results showed that the biochemical processes in infected BHK-21 cells went on fast and more metabolic heat was released than uninfected cells.

Interferon was discovered as a substance that was secreted from cells and induced other cells to become resistant to subsequent virus infection [17]. Interferon, in particular, has been reported to induce antiviral activity *vs*. many viruses in vitro [18]. Once exposed to interferon, responsive cells initiate a signaling cascade that culminates in the specific induction

of 30 or more interferon-inducible genes [19]. So when interferon was added, the replication of the virus was inhibited and the metabolism of the virus-infected cells must be altered. According to Fig. 1 we can see that there was not significant difference in the thermal power with the uninfected cells under the action of 1.0 mg mL^{-1} interferon but, with the infected cells, the maximal thermal power decreased and the time to reach the maximal thermal power became longer with the addition of interferon. From Fig. 4, it is clear that fewer cells show cytopathic effect under the action of interferon. The results show that interferon can inhibit the replication of FMDV. From this, we also assumed that the maximal thermal power and the time to reach the maximal thermal power could be used as a criterion to evaluate the extent of infection.

At 37°C with the concentration of interferon increasing, *I*% increased simultaneously (Table 1), but the increasing rate of *I*% is slower than the increasing rate of the concentration of interferon (*c*). When the *c* increased from 0.2 to 1.0 μg mL^{-1}, the *I*% only increased from 13.7 to 22.0%. This may be interpreted as the following reasons. Interferon induced gene products which function at multiple levels is paramount to the cellular antiviral response [19]. Cells themselves play an important role in the antiviral process. Under the experiment condition, the cell number was fixed, if cells were 'saturated' by the interferon, the antiviral protein produced by the cells was limited, and so the additional interferon would not enhance its effect largely. This was partly proved by the Eqs (2) and (3) that the antiviral effect of interferon (*I*%, t_{max}) was correlated with the extraction of interferon's concentration rather than the interferon's concentration itself.

Physiological process is sensitive to temperature. Growing cells at too high temperatures is more detrimental than at too low temperatures [20]. Some studies have been concerned the thermosensitivity of cell [21, 22]. For the infection process, it was restrained severely at temperature above 37°C (Fig. 2, Table 1). The photographs (Fig. 4) of infected cells illustrated that the higher the temperature, the fewer the cells showed cytopathic effect. The results indicated that virus infection was sensitive to high temperature. Viruses are unable to replicate on their own but must enter a host cell and use the host cell macromolecular machinery and energy supplies to replicate. At 40°C the enzyme, membrane, etc. of the host cell was affected to some extent. So at a higher temperature, the infection process was inhibited partly. Photographs illustrated that a huge cell injury occurred and some cells died at 42°C, which is 5°C higher than the normal culture temperature. We think that the main reason why there was not distinctly discernable peak on the thermogenetic curve may lie on this fact.

When hyperthermia and interferon act at the same time, no distinct synergistic effect of hyperthermia with interferon can be seen (Table 1, Fig. 3). The photographs in Fig. 4 were in accordance with the microcalorimetric results. We believe that this may be explained by the mechanism of interferon. Interferon acts *vs*. viruses indirectly and other cellular genes need to be expressed for the antiviral effect to be apparent [23]. So it can be concluded that the interferon's effect would be reduced at higher temperatures, since the enzyme system of the host cell is inhibited to some extent.

Our experiments indicate that microcalorimetry can be used as a promising tool in the study of the virus infection. The maximum thermal power and the time to reach the maximal thermal power may be used as a criterion to evaluate the extent of virus infection. With increasing the concentration of interferon, the shape of the thermogenetic curves of the FMDV infected BHK-21 cells illustrated regular changes. Based on this point, we may establish a model to evaluate the effect of antiviral drugs, vaccine by virtue of microcalorimetry. Hyperthermia as a useful tool has been employed in the therapy of carcinoma [24, 25]. Under the condition of 40 and 42°C, the results displayed that the process of virus infection was thermosensitive. It showed that hyperthermia might be a promising method for the therapy of virus disease.

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References

1 D. Esteban, B. Eric, E. Cristina and S. Francisco, Comp. Immunol. Microb., 25 (2002) 297.
2 E. H. Battley, J. Therm. Anal. Cal., 74 (2003) 709.
3 R. B. Kemp, Handbook of Thermal Analysis and Calorimetry, Vol. 4, From Macromolecules to Man, Elsevier, Amsterdam 1999.
4 X. Y. Xie, C. X. Wang and Z. Y. Wang, J. Therm. Anal. Cal., 76 (2004) 275.
5 H. Aki, J. Therm. Anal. Cal., 68 (2002) 553.
6 B. Chardin, P. Gallice, J. C. Sari and M. Bruschi, J. Therm. Anal. Cal., 70 (2002) 475.
7 R. B. Kemp, Thermochim. Acta, 193 (1991) 253.
8 J. Nittinger and P. Fürst, Thermochim. Acta, 251 (1995) 155.
9 R. B. Kemp and Y. Guan, Thermochim. Acta, 332 (1999) 203.
10 Y. Xie, H. K. Joseph, W. Depierre and L. Nassberger, J. Pharmacol. Toxicol. Methods, 40 (1998) 137.

11 A. M. Tan, B. Xu, S. Q. Huang and S. S. Qu, Thermochim. Acta, 333 (1999) 99.
12 K. Ijungholm, I. Wadsö and L. Kjellen, Acta Path. Microbial. Scand. Sect. B, 86 (1978) 121.
13 A. M. Tan and J. H. Lu, J. Biochem. Biophys. Methods, 38 (1999) 225.
14 J. Suurkuusk and I. Wadsö, Chem. Scr., 20 (1982) 155.
15 P. Backman, T. Kimura, A. Schon and I. Wadsö, J. Cell Physiol., 150 (1992) 99.
16 E. K. Wagner and M. J. Hewlett, Basic Virology, Blackwell Science Inc., Berlin 1999.
17 A. Isaacs and J. Lindenmann, Proc. R. Soc. Lond. B., 147 (1957) 258.
18 I. R. Tizard, The biology of veterinary cytokines interferons. In: M. J. Myers and M. P. Murtablgh (Eds), Cytokines in Animal Health and Disease. Marcel Dekker Inc, New York 1995.
19 G. J. Michael and G. K. Michael, Pharmacol. Therapeut., 78 (1998) 29.
20 J. P. Mather and P. E. Roberts, Introduction to Cell and Tissue Culture: Theory and Technique, Plenum Press, New York 1998.
21 P. Lönnbro and A. Schön, Thermochim. Acta, 172 (1990) 75.
22 Y. Feng, Z. F. Luo, S. S. Qu, C. Y. Zheng and H. Xu, Thermochim. Acta, 303 (1997) 203.
23 L. S. Geoffrey, A. S. Julian and A. Antonio, Sem. Virol., 8 (1998) 409.
24 H. Robins, W. L. I. Longo, R. A. Steeves, Lagoni, K. Rhonda, A. J. H. Anders, M. Ch. B. Neville, S. O'Keefe, W. Giese and C. L. Schmitt, Int. J. Radiation Oncology Biol. Phys., 15 (1988) 427.
25 J. van der Zee, D. G. Gonzalez, G. C. van Rhoon, J. D. P. van Dijk, W. L. J. van Putten and A. A. M. Hart, The Lancet, 355 (2000) 1119.

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