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The effect of a mutagen (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine) on cultured cells from adult *Schistosoma japonicum*

Abstract The transforming effect of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) on cultured cells from *Schistosoma japonicum* (*S. japonicum*) was studied using mono-factor and orthogonal tests. Under the influence of MNNG, cultured cells grew well, and cell survival time was more than 246 days in low-serum medium. When treated with 3 μg/ml MNNG for 48 h, the number of dividing cells increased significantly as determined by bright-field and scanning electron microscopy (SEM). Under these conditions, abundant microvilli, ruffles, microridges, papillae and blebs were observed on the surface of the induced cells. Treatment with MNNG may overcome existing limitations to get continually proliferating schistosome cells and open the possibility to immortalize isolated cells.

Introduction

Schistosomiasis is a disease of worldwide significance affecting millions of people (Ross et al. 2002). Although chemotherapeutic treatment is possible using praziquantel, problems emerged due to the observed resistance towards this drug (Doenhoff et al. 2002). Despite considerable efforts for vaccine development, no effective vaccine is available today (Bergquist 2002). Godal (1993) already pointed out that, to promote the development of a vaccine or new drugs, the establishment of a cultured cell line of schistosomes would be one of the important objectives in the future (*TDR towards the year 2000: strategic considera-*

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tions). A cell line would be a useful source of parasite material for immunological, biochemical, pharmacological, developmental biology, and enzymatic studies. Despite the immense value such a cell line would have in various studies of schistosomes, only few attempts have been made towards this end.

Hobbs et al. (1993) reported the successful isolation of cells from *Schistosoma mansoni* (*S. mansoni*), however, proliferation has not been achieved although various external substances such as hemolymph, tissue extracts, purified attachment factors, hormones, or peptide growth factors have been used. Outgrowth in some tissues and a large number of round cells were observed in *S. mansoni* after the worms were treated with the mutagen ethyl methane sulphonate (EMS), but cultured cells from *S. mansoni* did not proliferate (Coles and Fitzgerald 1986).

Recently, a protocol for the successful isolation of cells from *Schistosomajaponicum* (*S. japonicum*) was reported (Dong et al. 1995a,b, 2002). To induce cell divisions, the cells were treated with phytohaemagglutinin (PHA). Although many new round cells emerged, PHA treatment did not lead to a continuous cell line (Dong et al. 1998). In the present study, we tested whether *N*-methyl-*N*-nitro-nitrosoguanidine (MNNG) may be able to induce a stronger proliferation of isolated *S. japonicum* cells. By acting directly on nucleic acids, MNNG is a strong mutagen that is able to induce cell transformation leading to immortalized cell lines (Gichner and Veleminsky 1982; Liu 1998). Results will be presented that show the feasibility of this approach for schistosome cells.

Materials and methods

Worm collection and cell culture

A Chinese strain of *S. japonicum* was maintained in *Oncomelania hupensis* and rabbits as final hosts as described before (Dong et al. 2002). Twenty-eight-day-old worms were collected by perfusion with 0.85% saline containing antibiotics (1,000 μg/ml penicillin and 1,000 μg/ml

streptomycin) and 10 μg/ml heparin. After thorough washing, the harvested worms were minced into small fragments, which were digested for 6–10 h at 4°C by a trypsin and EDTA (1:5) solution. The obtained cell pellets were collected by centrifugation (1,200 rpm, 10 min) and washed two times (1,200 rpm, 4 min). Then, they were resuspended in medium to be inoculated onto T-25 flasks or cover slips. The culture temperature was 36–37°C, and pH was about 7.2–7.4. Twice a week, half of the culture medium was renewed.

MNNG treatments of cells

After inoculation, cells were first incubated in general medium [RPMI-1640 (GIBCO, USA)] with 20% calf serum (YaFa Biotechnology, Wuhan) and antibiotics (100 μg/ml penicillin G and 100 μg/ml streptomycin) for 3 days. After that, MNNG treatment was done for either varying time periods (0–48 h, each using a concentration of 3 μg/ml MNNG), or with different concentrations (0–9 μg/ml, each for 48 h), or at different time points (at days 3–8, respectively). Each test was done in triplicate (Tables 1 and 2). After incubation, the cells were thoroughly washed three times with basic medium (RPMI-1640) and cultured afterwards in general medium for up to 3 weeks. Then, the medium was replaced with low-serum medium (basic medium containing 5% calf serum and antibiotics).

Microscopical analyses

For bright-field microscopy, cultured cells were analyzed daily using an inverted microscope (Olympus) at a magnification of ×400, and survival rates were recorded. It was regarded as degeneration when cells became flat and translucent, and had more granules in their cytoplasm. The time interval from inoculation until 95% of the cultures had degenerated was recorded as their survival times. For scanning electron microscopy (SEM), cultured cells were collected once a week from week 1 to 11 after being treated with MNNG. The collected cells were washed three times (1,200 rpm, 3 min) in phosphate-buffered saline (PBS) and then fixed using cacodylate-buffered glutaraldehyde (2.5%) for 2 h at 4°C. After washing in PBS (1,200 rpm, 3 min, three times), the cells were post-fixed in 1% osmium tetroxide for 1 h, then dehydrated through a graded ethanol

Table 2 The $L_9(3^4)$ orthogonal design and its results of the inducement of cultured cells from *S. japonicum* with MNNG

Test number	1 (A)	2 (B)	3 (E)	4 (C)	Survival time (days)
1	1	1	1	1	60
2	1	2	2	2	165
3	1	3	3	3	246
4	2	1	2	3	16
5	2	2	3	1	165
6	2	3	1	2	231
7	3	1	3	2	35
8	3	2	1	3	180
9	3	3	2	1	200
K_1	471	111	471	425	1298
K_2	412	510	381	431	
K_3	415	677	446	442	
R	59	566	90	17	

series, critical point dried, and sprayed with gold by a vacuum sprayer. Finally, cells were observed and photographed under SEM (Hitachi-570).

Results

Effects of different concentrations of MNNG on cultured cells

Early after inoculation, cultures not treated with MNNG showed a predominance of small round cells (diameter 8 μm or less), which adhered individually or in groups of 5–200 cells to the surface of the flasks, similar to "fried eggs" described by Hobbs et al. (1993). The small round cells grew well, were homogeneously bright, and had high nuclear-to-cytoplasm ratios with few organelles. No dividing cells were observed in the test and control groups. To overcome this problem, we made use of MNNG, a mutagen that had been shown before to be able to induce cell division (Du et al. 1984; E 1995; Su et al. 1995). First, we tested the effect of different concentrations of MNNG, and analyzed the cells by bright-field and electron microscopy as discussed later. When MNNG was applied for 48 h, at day 3 after inducement most cells grew well with few

Table 1 Factors and levels of the $L_9(3^4)$ orthogonal test

Factors/ levels	Basic medium (A)	Induction concentration of MNNG (μg/ml) (B)	Induction time of MNNG (h) (C)
1	RPMI-1640	6	24
2	TC-199	2	36
3	DMEM/F-12	3	48

Table 3 Variance analysis of the results in Table 2

Factors	Square sum of mean square deviation (SS)	Degree of freedom (ν)	Mean square (MS)	F	P
A[a]	736.22	2	368.1	0.51	>0.05
B[b]	56382.89	2	28191.45	39.18	<0.05
C[c]	49.56	2	24.8	0.03	>0.05
E[d]	1438.89	2	719.5	–	–

[a]Basic medium
[b]Inducing concentration of MNNG
[c]Inducing time of MNNG
[d]Errors

Figs. 1–5 Cultured cells with smooth surfaces and degenerative cells of *S. japonicum*
Fig. 1 Cells of the control groups with smooth surface or some papillae, cultured for 49 days
Figs. 2–3 Degenerative cells of the control groups with some holes on their surfaces, cultured for 70 or 89 days, respectively
Fig. 4 Cells of the test groups with smooth surfaces, cultured for 3 weeks after treatment with MNNG on day 6
Fig. 5 Degenerative cells of test groups, cultured for 4 weeks after treatment on day 6
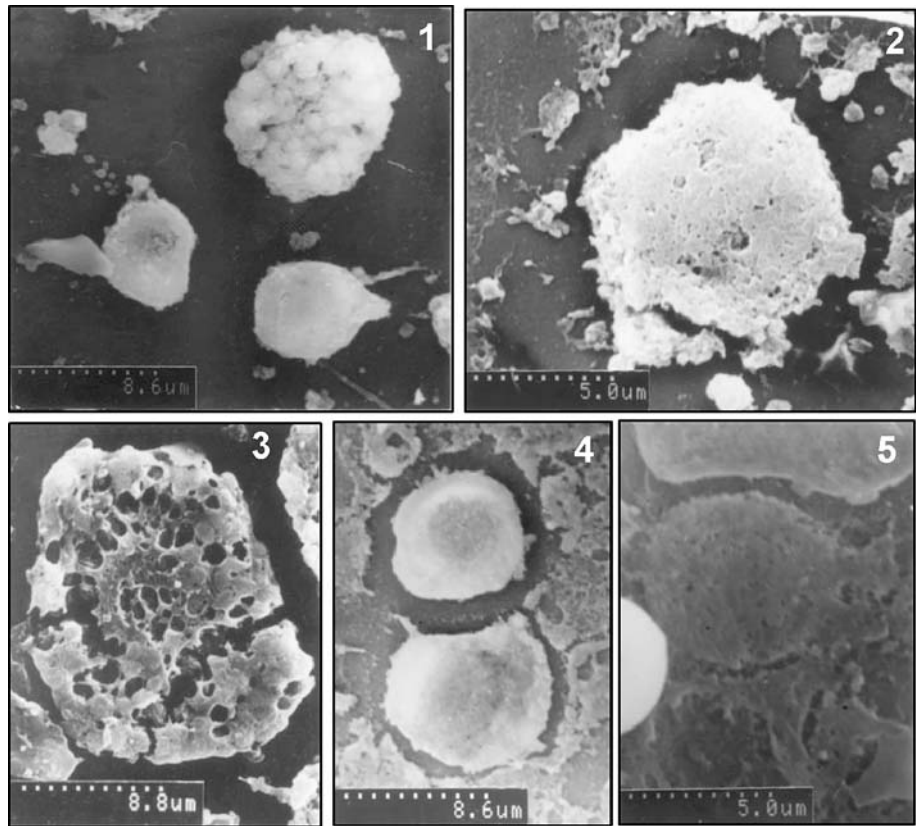
cells detaching from the flasks in the groups treated with 1 or 2 µg/ml MNNG. In the groups treated with 3 and 4 µg/ml MNNG, although a few cells detached and degenerated, most cells survived well. In the groups treated with 6 and 9 µg/ml MNNG, however, most cells detached and degenerated with few cells surviving. At day 35, small round cells still predominated in the 1- to 2-µg/ml-MNNG-treated groups, but no dividing cells were observed. In the groups treated with 3 and 4 µg/ml MNNG, respectively, surviving cells remained healthy and some grew bigger in volume, mostly being polygonal or round. Bi- or multi-nuclear cells, whose cytoplasms were connected by thin cytoplasmic bridges, could be occasionally observed. Most cells of the groups treated with 6 and 9 µg/ml MNNG, respectively, degenerated and died. At day 150, most cells of the 1- to 2-µg/ml-MNNG-treated groups degenerated, while small round cells still survived. Most of these cells grew well, but they did not divide. In the groups treated with 3 or 4 µg/ml MNNG, especially in the 3-µg/ml-treated groups, the number of the cells increased, and the volume of some cells enlarged (>10 µm in diameter), as did the nuclear-to-cytoplasm volume ratio. Dividing cells were constantly observed, and the survival time significantly increased up to 246 days.

Effects of different treatment periods of MNNG on cultured cells

When the concentration of MNNG was 3 µg/ml, both the cell survival time and the number of dividing cells increased along with the treatment period. At day 35 after MNNG addition, the cells of the 24-h-treatment groups were similar to those of the controls (not treated with MNNG). Both of them were composed of healthy small round cells. In the 36-h-treatment groups, the cultured cells grew well, and dividing cells were occasionally observed. However, in the 48-h-treatment groups, some cells grew larger in volume, and dividing cells could be observed with a higher frequency compared to the other groups. At day 75, the 24-h-treatment groups and the controls were still mainly made up of small round cells, and still no dividing cells were observed. At the same time point, the 36-h-treatment groups had a mix of small and large round cells, with a few dividing cells. In the 48-h-treatment groups, however, there were mostly large round cells, and dividing cells were constantly observed. After being treated with MNNG, the cells of this group still grew well for more than 246 days, whereas cells of the other groups degenerated during this time period.

The optimal concentration and time of MNNG was tested by $L_9(3^4)$ orthogonal test (Table 1). The statistical analyses shown in the Tables 2 and 3 revealed that a treatment of cells with 3 µg/ml of MNNG for 48 h with RPMI-1640 as basic medium was most beneficial for the growth and division of the cultured cells. Under these conditions, cells survived longer (more than 246 days), grew better, and divided with a higher frequency compared to the other groups. As seen in Table 3, the working concentration of MNNG had the most significant effect on the cell culture parameters measured in this study.

Bright-field and electron microscopy studies
of *S. japonicum* cells treated with MNNG

Bright-field microscopy showed that cells of all the test groups grew well. Dividing cells were observed at day 35 following treatment with 3 µg/ml MNNG for 48 h, and big, round cells predominated in this mixed cell population. Cell division processes were even observed during analyses (data not shown). However, in the control groups, most cells were small and round, and degenerated grad-

ually after day 35 in culture. Here, no cell divisions were observed.

Results of SEM analyses of control cells showed that the surface topography of these cells was smooth except that some had papillae (Fig. 1). Along with the extending time of culture, the phenotype of the cells changed gradually, the diameter of the cells decreased, the color of the cells turned from white to gray, and even the papillae on cell surface disappeared gradually. Eventually, the cells became flat and degenerated (Figs. 2 and 3). Again, no dividing cells were observed.

Most cells of the test groups also had smooth surfaces (Fig. 4), and degenerated gradually just like the cells of the control groups (Fig. 5). Some cells, however, changed greatly during the course of culture, revealing particular features on the cell surface such as microvilli, ruffles, blebs, papillae, pseudopodia, cilia, or microridges. These features appeared individually or together after the cells were treated with MNNG, and they were never observed on control cells. The microvilli on cells treated with MNNG varied greatly in length, number, diameter, shape and distribution. On some cells, the microvilli were distributed sparsely, and were short and wide (Figs. 6 and 7), or long
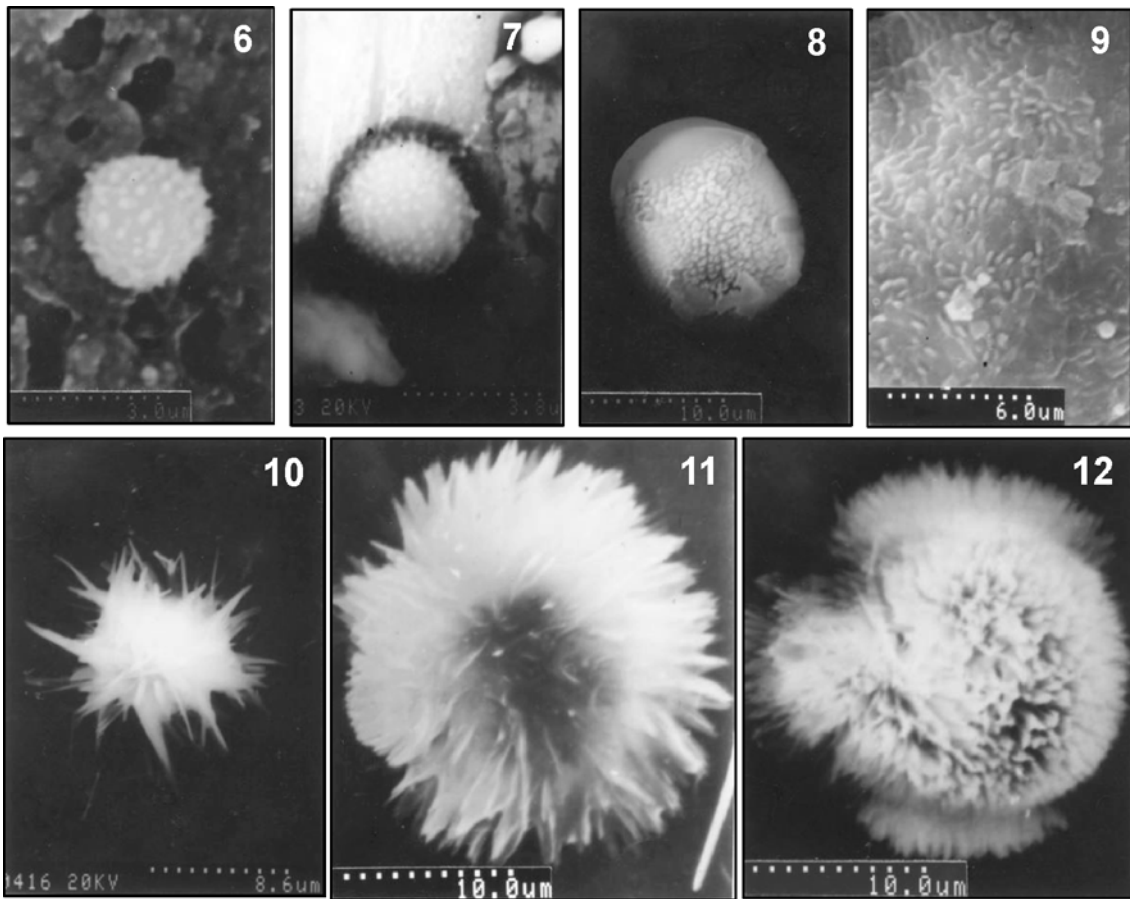
Figs. 6–12 Microvilli on the cells of *S. japonicum* treated with MNNG
Figs. 6–9 Cells with short microvilli distributed sparsely or densely, cultured for 3, 4, 8, and 3 weeks after stimulation on days 8, 7, 5, and 5, respectively

Figs. 10–12 Cells with crowded microvilli in different lengths, cultured for 5, 4, and 4 weeks, respectively, after treatment on day 5

Figs. 13–21 Ruffles, blebs and papillae on the cells of *S. japonicum* treated with MNNG
Figs. 13–15 Ruffles on the surfaces of cells cultured for 5, 1, or 11 weeks, respectively, after treatment on day 5
Figs. 16–17 Blebs on the surfaces of cells, cultured for 3 or 7 weeks after treatment on days 5 or 6, respectively
Figs. 18–20 Papillae on the surface of cells, cultured for 3 weeks after treatment on day 5
Fig. 21 A cell showing uropod formation, cultured for 5 weeks after treatment on day 8
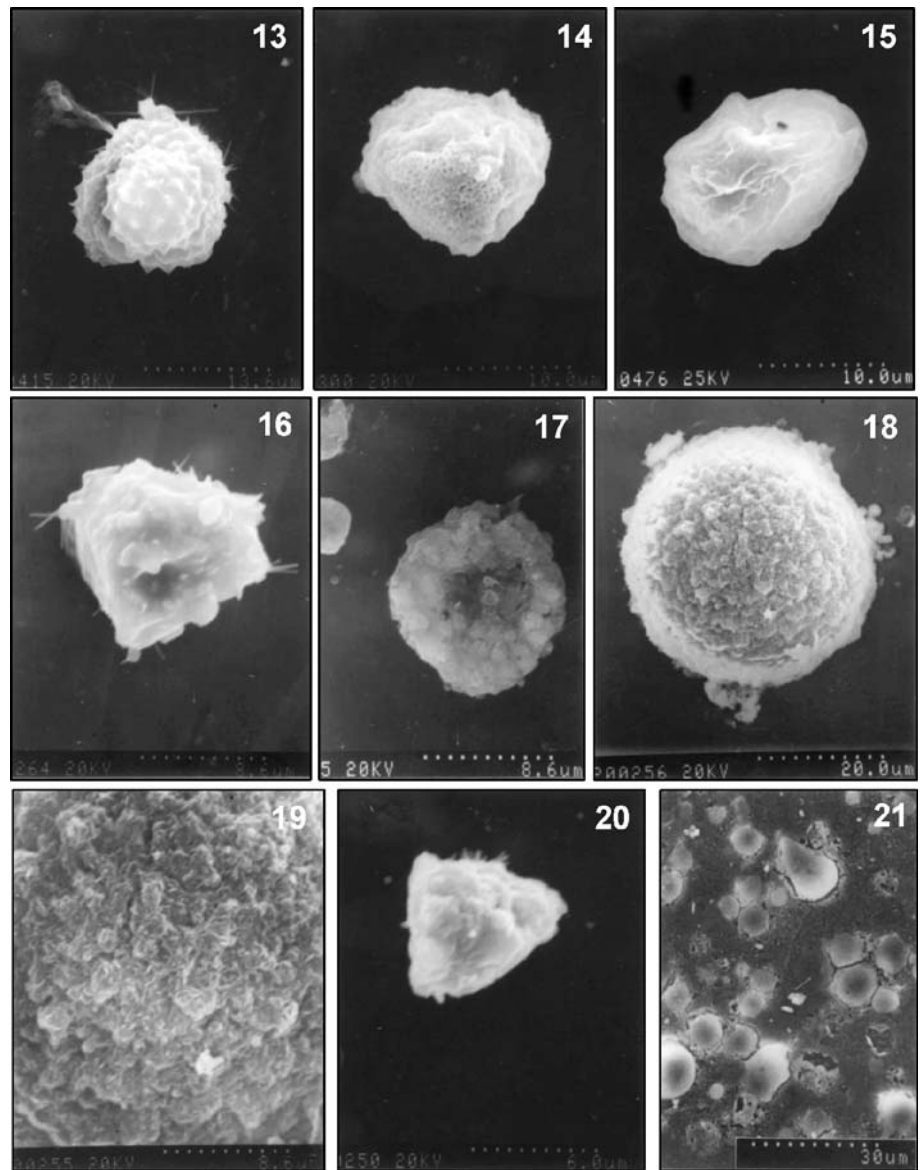
and thin (Figs. 13, 16, and 20). On other cells, the microvilli were uniform in length and diameter, and distributed densely (Figs. 8 and 9). Some cells also had disorderly, crowded microvilli on their surfaces of different lengths and diameters (Figs. 10, 11, and 12). Ruffles were usually observed on the margin (Figs. 13 and 14) or upper surface (Fig. 15) of the cells, and were undulating or stub-like folds. The ruffles usually co-existed with other features, for example, blebs (Fig. 16) or microvilli (Figs. 13 and 16). Some cells also showed a few blebs, which are small, spherical projections of the surface. The blebs had smooth surfaces, and were attached to the cell with a pedicle (Figs. 16 and 17). The papillae were distributed sparsely or densely on some cell surfaces and attached to it with wide base, and usually had many short microvilli on their surfaces (Figs. 18, 19, and 20). Other surface features were seen also. Some cells had wide pseudopodia (Fig. 21), or long cilia (Fig. 22); some had many microridges that were distributed evenly on cells (Figs. 23, 24, 25, and 26).

Some had concavities and small holes of different sizes among microvilli, ruffles or microridges (Figs. 16 and 26).

Related proliferation changes were also observed under SEM. Active cell division was observed at 5 weeks after treatment, especially in those cells that were treated with MNNG at day 4 of inoculation. Apart from the ordinary binary division of cells, tripolar or multipolar divisions were also observed in the cultures. After binary division, the daughter cells were identical to each other, and they usually had smooth surfaces (Figs. 27 and 28). In the case of multipolar cells, two forms were observed. One is a direct form with cells dividing into three or more similar cells simultaneously (Figs. 29, 30, and 31), while the other was an indirect form, in which a cell first divided into two daughter cells, and then one of these daughter cells divided again (Figs. 28, 32, 33, and 34). The three daughter cells were distributed in a bead-like fashion (Figs. 28 and 29), or triangle-like shape (Figs. 32, 33, and 34), and they were

Figs. 22–26 Other features of cells of *S. japonicum* treated with MNNG
Fig. 22 Long ciliate-like convexity on the cell surface of cells cultured for 6 weeks after treatment on day 6
Figs. 23–25 Microridges on the surfaces of cells cultured for 4, 9, or 10 weeks after treatment on days 7, 7, or 5, respectively
Fig. 26 Concavity on the surface of cells cultured for 8 weeks after treatment on day 5
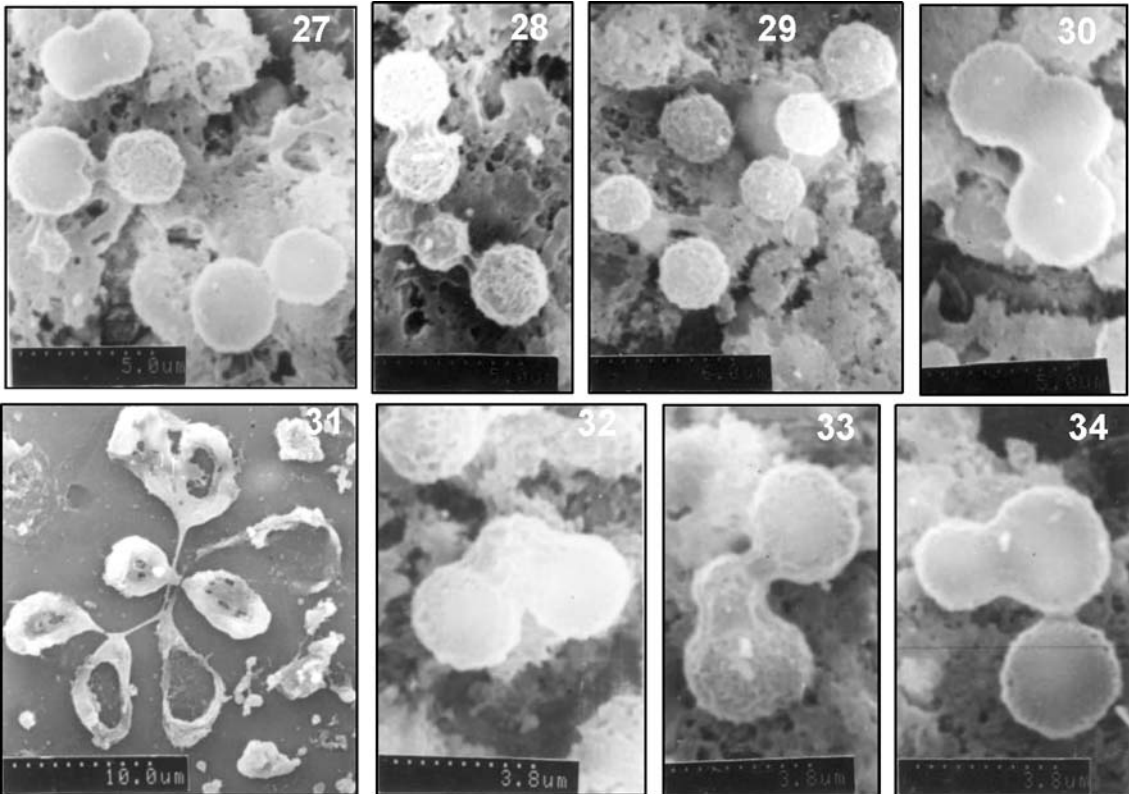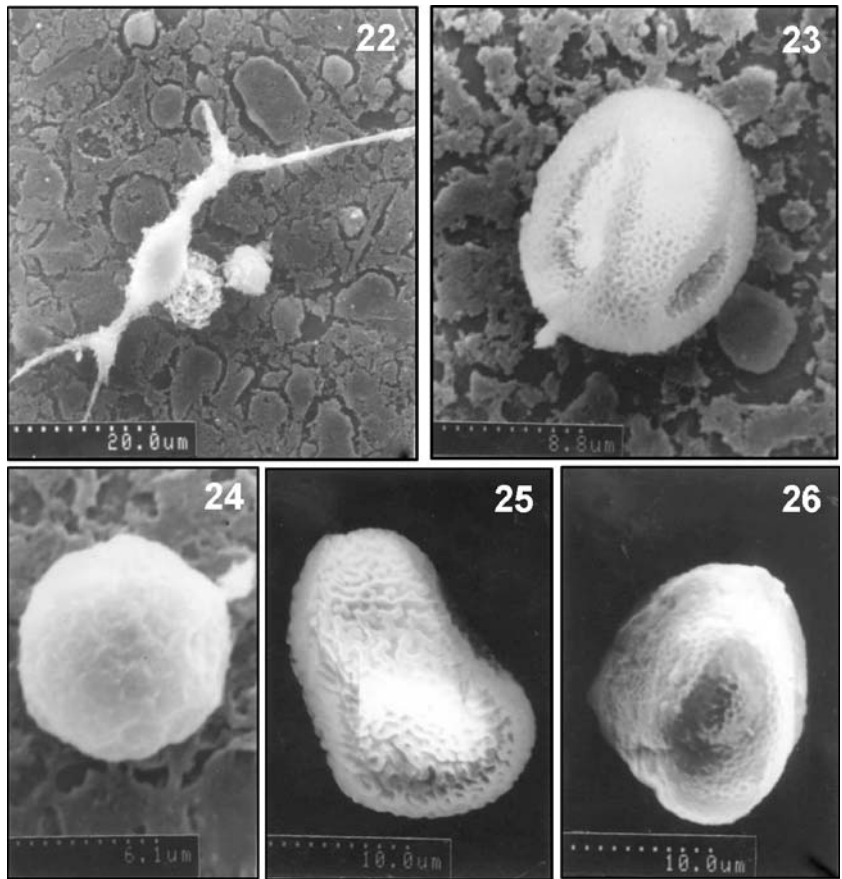
Figs. 27–34 Dividing cells of *S. japonicum* treated with MNNG
Figs. 27–28 Two dividing cells with similar size and shape, cultured for 5 weeks after treatment on day 4

Figs. 29–34 Multi-mitoses of cells, cultured for 5 weeks after treatment on day 4 (except in Fig. 31). Cells were cultured for 8 weeks after treatment on day 5

different from each other. The daughter cells mentioned above were all connected via cytoplasmic bridges.

Discussion

MNNG is a monofunctional alkylating agent that causes chromosomal DNA damage, which in turn provides a signal, triggering the DNA damage response that involves the coordinate control of many signal transduction pathways (Gichner and Veleminsky 1982; Liu 1998; Lindahl and Wood 1999; Schar 2001). MNNG has been successfully used in the transformation of several cell lines in vitro (Du et al. 1984; E 1995; Su et al. 1995). Therefore, it was used in our study to induce the division and transformation of cultured cells from adult *S. japonicum*. The normal working concentration of MNNG is 0.5–1 µg/ml, and working time is 12–16 h (E 1995). Using similar conditions (1 or 2 µg/ml, 12–20 h), we detected only few differences between the test groups and the control groups in first experiments. At these concentrations, MNNG was not capable to induce the transformation of cultured cells. When the concentration of MNNG was 6 µg/ml or higher, most of the cells detached and degenerated at day 3 after treatment, showing that inevitable damage was caused by the toxic effect of high concentrations of MNNG on cells (E 1995). Divisions occurred in some cells even in low-serum medium only when the concentrations of MNNG were 3 or 4 µg/ml. Especially in the 3-µg/ml-treated groups, the number of dividing cells constantly increased during the culture period. When the treatment of MNNG was done for 48 h, cell division occurred most frequently, and cells survived longer than 246 days. It was obvious that both the appropriate concentration and the time of MNNG treatment were important factors determining the proliferation and survival of cultured cells. Orthogonal tests indicated that, compared to treatment time, the concentration of MNNG was the more critical parameter for cell proliferation and survival. Although the passage of dividing cells was tried, it failed, probably because the numbers of mutant cells induced by MNNG was not high enough to predominate in the mixed cell culture.

In the past, some approaches were undertaken to cultivate cells of schistosomes (Bayne et al. 1994). Although cells of several schistosome strains could be isolated and kept for some time in vitro (Bayne et al. 1994; Coles and Fitzgerald 1986), continuous cell divisions were not observed in these primary cell cultures, although various biological substances were tested for their inducing capacity. Results from our study indicate that it may be necessary to stimulate the division of schistosome cells chemically using mutagens. In addition, the time point of stimulation seems to be important. The most appropriate time point for chemical treatment in our study was day 4 after isolation.

When cells transform, their size, shape, metabolism, and movement changes accordingly, as do their surface features, such as the number, shape, and distribution of ruffles and microvilli (Du et al. 1984). It was reported that soon after human peripheral blood lymphocytes had been treated with phytohaemagglutinin (PHA), a T-cell-specific mitogen, their fairly smooth surfaces were covered by a mixture of stub-like or long conical villi, blebs or ruffles. But these surface features decreased or disappeared eventually when PHA was cleared away (Hoffman et al. 1977; Newell and Roath 1978). The surface topography of the cell can reflect its function, being related to cell movement, growth, proliferation, aging, metabolism, or other functions (Ferenczy 1980; Newell 1980). In the control experiments of this study, most cell surfaces were smooth except for a few papilla, ruffles or microvilli on them. This finding is in accordance with the data of Dong et al. (2001) showing that chemically unstimulated cells were weak in proliferation and metabolism in culture. The surface topography of some cells in the chemically stimulated test groups of the present study, however, changed dramatically. During the low-serum culture phase, significant morphological features such as villiae, microridges, ruffles, blebs, concavities and microvilli emerged on the cell surfaces. Especially, microvilli varied in number, shape, and distribution. These features are characteristic for the transformation and proliferation of cells, and also for metabolic activity (Kessel 1974; Du et al. 1984). Furthermore, many binary and multipolar divisions could be observed in cells that were chemically stimulated at day 4 after inoculation, indicating an enhancement of DNA synthesis and cell proliferation.

Provided that cell division and proliferation could be strengthened and sustained, the presented approach may be a suitable way to establish a continuous cell line of *S. japonicum* as well as other schistosome species.

References

Bayne CJ, Menino JS, Hobbs DJ, Barnes DW (1994) In vitro cultivation of cells from larval *Schistosoma mansoni*. J Parasitol 80:29–35

Bergquist NR (2002) Schistosomiasis: from risk assessment to control. Trends Parasitol 18:309–414

Chen XB, Dong HF, Jiang MS (1997) Orthogonal test studies on the cultured conditions of cells from *Schistosomulum*. Chin J Zoonoses 13:41–42

Coles GC, Fitzgerald J (1986) Effect of mutagen on cultured *Schistosoma mansoni*. J Helminthol 60:135–142

Doenhoff MJ, Kusel JR, Coles GC, Cioli D (2002) Resistance of *Schistosoma mansoni* to praziquantel: is there a problem? Trans R Soc Trop Med Hyg 96:465–469

Dong HF, Jiang MS, Li Y, Yang MY, Zhou SL (1995a) Preliminary studies on the culture methods of cells from *Schistosoma japonicum*. Acta Hydrobiol Sin 19:382–383

Dong HF, Jiang MS, Li Y, Ni YH, Chen JQ (1995b) Preliminary studies on the cultured conditions of cells from adult *Schistosoma japonicum*. Chin J Schisto Control 7:257–261

Dong HF, Jiang MS, Chen XB, Yang MY, Zhang PX, Zhou SL (1998) Function of PHA on proliferation of cultured cells of adult *Schistosoma japonicum*. Chin J Zoonoses 14:41–44

Dong HF, Chen XB, Ming ZP, Zhong QP, Jiang MS (2002) Ultrastructure of cultured cells from *Schistosoma japonicum*. Acta Trop 82:225–234

Du YX, Chen JK, Wu ZL, Feng JW, Chen XW, Chen GQ (1984) Observation of some biological characteristics on transformed cells induced by MNNG in vitro. Chin J Ind Hyg Occup Dis 2:155–161

E Z (1995) Tissue culture and molecular cytology technology. Beijing Publishing House, Beijing, China, pp 220–226

Ferenczy A (1980) The female reproductive system. In: Hodges GM, Hallowes RC (eds) Biomedical research applications of scanning electron microscopy, Academic, London, pp 127–166

Gichner T, Veleminsky J (1982) Genetic effects of *N*-methyl-N'-nitro-*N*-nitrosoguanidine and its homologs. Mutat Res 99:129–242

Godal T (1993) TDR towards the year 2000: strategic considerations (working document for discussion at JCB-16, June 1993; document No. TDR/PTR-SCI/92.3 Rev. 4). TDR, Geneva, pp 19–23

Hobbs DJ, Fryer SE, Duimstra JR, Hedstrom OR, Brodie AE, Collodi PA, Menino JS, Bayne CJ, Barnes DW (1993) Culture of cells from juvenile worms of *Schistosoma mansoni*. J Parasitol 79:913–921

Hoffman CC, Moore KC, Shih CY, Blakley RL (1977) Scanning electron microscopy of human lymphocytes during transformation and subsequent treatment and methotrexate. J Cell Sci 28:151–165

Kessel RG (1974) Scanning electron microscopy. Springer, Berlin Heidelberg New York, pp 66

Lindahl T, Wood RD (1999) Quality control by DNA repair. Science 286:1897–1905

Liu YG (1998) Foundations of health and toxicology. People's Medical, Beijing, China, pp 97–100

Newell DG (1980) The white cell system. In: Hodges GM, Hallowes RC (Eds) Biomedical research applications of scanning electron microscopy. Academic, London, pp 219–305

Newell DG, Roath S (1978) The surface morphology of mitogen stimulated human peripheral blood lymphocytes. Br J Haematol 39:615–623

Porter KR, Fonte V, Weiss G (1974) A scanning microscope study of the topography of hela cells. Cancer Res 34:1385–1394

Ross AGP, Bartley PB, Sleigh AC, Olds GR, Li Y, Williams GM, McManus DP (2002) Schistosomiasis. N Engl J Med 346:1212–1220

Schar P (2001) Spontaneous DNA damage, genome instability, and cancer—when DNA replication escapes control. Cell 104:329–332

Su XL, Ning T, Ke Y (1995) Study of cytoskeletons in human gastric epitheilial cell treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. Acta Anatomica Sinica 26:3391–3393

Weller TH, Wheeldon SK (1982) The cultivation in vitro of cells derived from adult *Schistosoma mansoni*. I. Methodology, criteria for evaluation of cultures, and development of media. Am J Trop Med Hyg 31:335–348