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RAPID COMMUNICATION

ELIMINATION OF MYCOPLASMA CONTAMINANTS FROM CELL CULTURES WITH ANIMAL SERUM

C. N. NAIR

Department of Cell and Molecular Biology*
Medical College of Georgia
Augusta, GA 30912

Repeated treatment with guinea pig or rabbit serum, but not with human serum, was found to eliminate mycoplasma contaminants from mammalian cell cultures as judged by staining with the fluorescent dye Hoechst 33258. Following treatment with rabbit serum and several passages, M. hyorhinis could not be detected by staining, isolation on agar, or specific immunofluorescence in a human prostate carcinoma cell line heavily contaminated with this organism. There was no evidence for the involvement of antimycoplasma antibodies in the bactericidal activity of rabbit serum. Mycoplasmacidal activity of rabbit serum was associated with a heat-labile component(s) which could be inactivated by incubation of the serum with goat antirabbit complement component C3.

Introduction. Mycoplasmas frequently contaminate cell cultures (1) and profoundly alter their functional and metabolic characteristics (2) rendering them unsuitable for many types of studies. Since an entirely satisfactory procedure for eliminating mycoplasmas from contaminated cell cultures is not available, valuable cell cultures have to be often discarded. Among the procedures that have been reported for the elimination of mycoplasmas from contaminated cultures are: treatment with 5-bromouracil and the Hoechst compound 33258 followed by exposure to visible light (3), passage in nude mice (4) in vitro cultivation with macrophages (5) and growth in the presence of tetracycline and kanamycin (6) of mycoplasma contaminated cells. I have found that repeated treatment with guinea pig or rabbit serum may eliminate mycoplasmas from monolayer cultures of several types of cells. The details of this procedure and the results obtained with it so far are summarized in this paper.

Materials and Methods. Mycoplasma contaminated DU-145 cells, a cell line derived from human prostate carcinoma, and BHK-21 hamster kidney cells were kindly provided by Robert Raynor and George Schuster, respectively. Mycoplasma contamination of HeLa cells, CV-1 monkey kidney cells, and mouse L-929 cells were detected during routine screening of these cell cultures in this laboratory. Monolayer cultures of each cell line were grown in minimal essential medium (MEM) containing 10% heat-inactivated calf serum (HICS), 100 units/ml each of penicillin and streptomycin and .25 μg/ml of fungizone. Mycoplasma species M. hominis, M. orale, M. pneumoniae and M. salivarium and specific antiserum against M. hyorhinis were obtained from the Research Resources Branch, National Institutes of Health. The DNA specific fluorescent dye Hoechst 33258 was purchased from American Hoechst Corporation. Lyophilized guinea pig complement was purchased from Grand Island Biological Company or Cappel Laboratories and goat antirabbit complement C3 and fluorescein-conjugated pooled rabbit IgG from Cappel Laboratories.

Serum from guinea pigs or rabbits was obtained as follows. Blood was

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withdrawn into a sterile glass tube and allowed to clot at room temp. for 45 min. Serum was separated by low speed centrifugation and used in experiments either immediately or after storage at -80°C up to several weeks. Mycoplasma-contaminated monolayer cultures were dispersed by treatment with 0.1% trypsin-.02% EDTA in phosphate buffered saline, and the cells were suspended in MEM containing 10% HICS. In addition, guinea pig serum or rabbit serum prepared as described above was added to the cell suspension to a final concentration of 10% by volume. After mixing, the cells were incubated in 35 mm plastic culture dishes (Corning) at 37°C in a humidified CO_2 atmosphere. The above steps were repeated at the next few (2 to 4) passages. To monitor mycoplasma contamination, serum-treated and control cultures were trypsinized and suspended in MEM containing 10% HICS. An aliquot of each suspension was planted in a slide chamber (Lab-Tek) and incubated until the cultures attained moderate confluence. The cultures were stained with the DNA-specific fluorescent dye Hoechst 33258 and examined under a fluorescence microscope as previously reported (7). The presence of M. hyorhinis in serum treated and untreated DU-145 cells was monitored by the above technique and by immunofluorescence using fluorescein-conjugated antiserum against M. hyorhinis. Serum treated DU-145 cell cultures were also screened for the presence of mycoplasma at the mycoplasma testing service laboratory of Microbiological Associates by Hoechst stain (catalogue #81-141) and by isolation on agar using a large volume (50 ml) of the culture fluid (catalogue #81-139). The above laboratory also identified the mycoplasma contaminant in DU-145 cells as M. hyorhinis by specific immunofluorescence (catalogue #81-140). The identity and the treatment history of the cultures were unknown to the testing laboratory. Between serum treatment and testing, the cultures were passed several times in antibiotic-free MEM containing 10% HICS.

Results. Photomicrographs of mycoplasma-contaminated DU-145 cells stained with the Hoechst compound before and after treatment with rabbit serum are shown in Figure 1. When the cells were stained before serum treatment, fluorescence covered the entire surface of virtually every cell (Fig. 1A) indicating heavy contamination with mycoplasma. On the other hand, when staining was carried out after four cycles of serum treatment, only the ovoid nuclei appeared fluorescent (Fig. 1B) suggesting the elimination of mycoplasma contaminants. A drastic reduction in mycoplasmal DNA-specific fluorescence could be observed even after a single treatment with serum, whereas two cycles of treatment were frequently effective in the total elimination of such fluorescence. The absence of mycoplasma in two cultures of DU-145 cells separately treated with rabbit serum, was confirmed by another laboratory by Hoechst stain and large volume isolation techniques (see Methods).

Treatment with guinea pig serum eliminated nonnuclear fluorescence due to unidentified mycoplasmas from HeLa, CV-1, L-929, and BHK-21 cell lines. In HeLa and CV-1 cells that were examined by Hoechst stain after several passages, nonnuclear fluorescence did not reappear. Treatment with guinea pig serum also eliminated nonnuclear fluorescence due to M. orale, M. hominis, M. salivarium, or M. pneumoniae from HeLa cells that were deliberately contaminated with these organisms.

Mycoplasmacidal activity was regularly present in guinea pig serum and rabbit serum. It was present only in some batches of commercially obtained guinea pig complement and absent in sera obtained from two human volunteers. The activity was heat labile and in terms of filterability, associated with a molecule(s) of $\geqslant$50,000 molecular weight.

To test whether the activity might be due to the activation of the indirect complement pathway, 0.1 ml of rabbit serum was mixed with 10 µl of goat anti-rabbit complement component C3. The mixture was incubated for 10 min at room temperature and added to one-half of a suspension of contaminated DU-145 cells. As control, a mixture of 0.1 ml of rabbit serum and 10 µl of normal goat serum was incubated in parallel and added to the other half of the cell suspension. Following incubation to form monolayers, trypsinization and serum treatment were repeated. After three cycles of serum treatment, staining with the Hoechst compound revealed

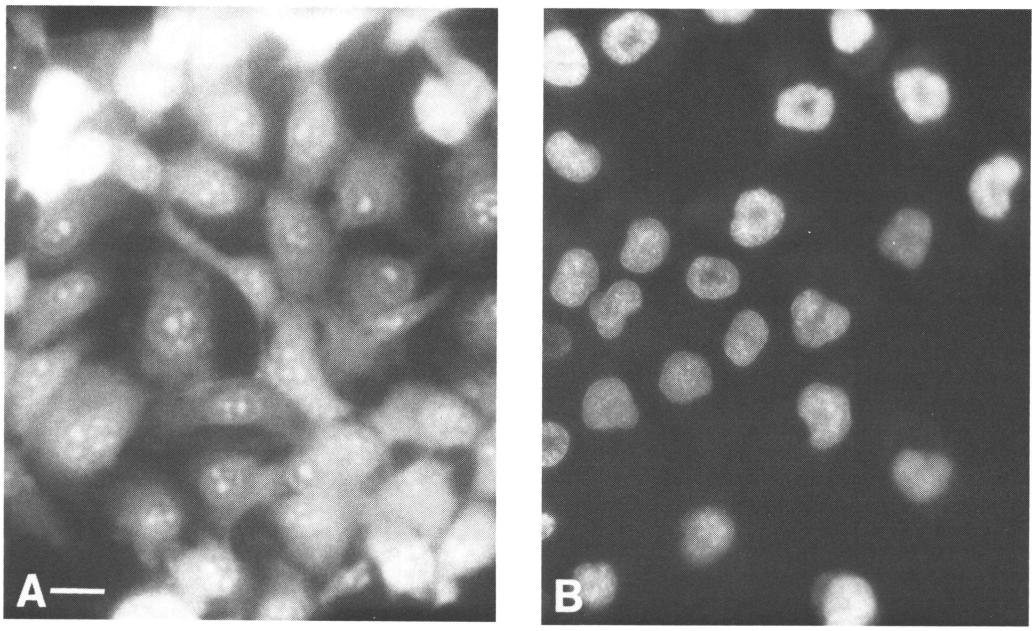

Fig. 1. DNA-specific fluorescence of mycoplasma contaminated DU-145 cells before and after treatment with rabbit serum. Monolayer cultures were subjected to four cycles of treatment with rabbit serum as described in the text and passed repeatedly in medium lacking antibiotics. Serum treated and untreated cultures were grown in slide chambers, stained with the Hoechst compound and viewed under a Zeiss fluorescence microscope. Panel A: untreated control culture showing diffuse fluorescence. Panel B: serum-treated culture showing fluorescence restricted to the cell nuclei. Bar = 20 μm.

no reduction in nonnuclear fluorescence in the test culture as opposed to the control culture which appeared to be 'mycoplasma-free'.

To examine the possibility that the mycoplasmacidal activity of rabbit serum was due to the presence in the serum of antimycoplasma antibodies, slide chamber cultures of rabbit serum-treated or untreated DU-145 cells were stained with fluorescein-conjugated a) pooled rabbit IgG and b) anti M. hyorhinis serum. Intense fluorescence was observed in the untreated culture (Fig. 2A) stained with anti M. hyorhinis serum whereas no fluorescence over background could be detected in either culture stained with rabbit IgG (results not shown) or in the rabbit serum-treated culture stained with anti M. hyorhinis serum (Fig. 2C).

Discussion. The results of this study indicated that simple treatment with guinea pig serum or rabbit serum might eradicate mycoplasma contaminants from cell cultures. When staining with the Hoechst compound was used as the sole monitoring technique, serum treatment

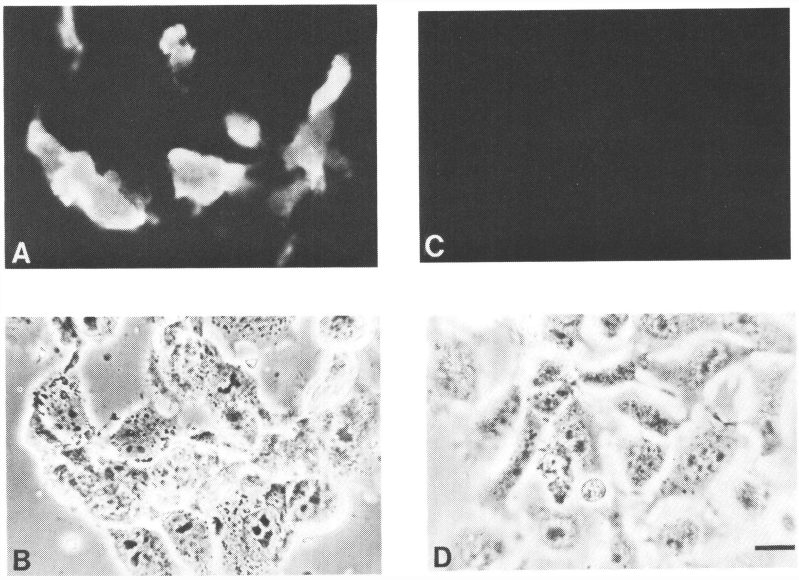

Fig. 2. Immunofluorescent staining of mycoplasma contaminated DU-145 cells before and anfter treatment with rabbit serum. Monolayer cultures of DU-145 cells were grown in slide chambers, stained with anti M. hyorhinis serum by incubation for 30 min at 37°C and after extensive washing with phosphate buffered saline, examined under a Zeiss fluorescence microscope. Panels A and B: untreated control culture. Panels C and D: Culture subjected to four cycles of treatment with rabbit serum. Panels A and C show fluorescence micrographs of the same fields as seen in panels B and D respectively. Bar = 20 μm.

appeared to 'eliminate' unidentified mycoplasmas from various cell lines and four known species of mycoplasmas from HeLa cells. Strong evidence for the effectiveness of this procedure was provided by the experiments with DU-145 cells heavily contaminated with the common mycoplasma contaminant M. hyorhinis. After treatment with rabbit serum, the presence of mycoplasma could not be detected in these cells by the staining technique, specific immunofluorescence or isolation on agar. These are the three most commonly used procedures for mycoplasma detection (9). The fact that between serum treatment and testing, the cultures had been passed several times in the presence of antibiotic-free medium further encourages one to believe that total elimination of mycoplasma was accomplished.

The observation that staining with fluorescein-conjugated pooled rabbit IgG failed to cause fluorescence in mycoplasma contaminated DU-145 cells under conditions in which fluorescein-conjugated specific antiserum produced pronounced fluorescence in these cells suggests that the mycoplasmacidal activity of rabbit serum was not due to the presence in the serum of antimycoplasma antibodies. This finding together with the observation that anticomplement C3 antiserum neutralized mycoplasmacidal activity of rabbit serum suggests the involvement of complement C3 in this activity. It will be of interest to determine whether complement C3 is

involved also in the killing of mycoplasmas during passage of contaminated cells in nude mice (4).

Disappearance of mycoplasma-specific fluorescence from serum treated cultures was associated with smoother appearance of cells and greater definition of cellular morphology. There was no indication that serum treatment had any adverse effect on the growth rate of surviving cells. In fact, serum-treated DU-145 cells appeared to reach confluence faster than their untreated counterparts. This must be contrasted with the reported reduction in the cloning efficiency and growth rate of certain cells exposed to the combined action of the Hoechst compound and 5-BrU (3). It must be pointed out that cloning of serum-treated cells was not necessary to obtain a mycoplasma-free culture. The extreme simplicity of serum treatment described in this study and its apparent effectiveness against different known species of mycoplasmas including M. hyorhinis and unidentified mycoplasmas contaminating various cell-lines suggest that further evaluation of the use of animal serum for eliminating mycoplasmas from cell cultures will be rewarding.

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References

1. Stanbridge EJ. Mycoplasmas and cell cultures. Bacteriol Rev 35: 206-227, 1971.
2. Barile MF. Mycoplasmal contamination of cell cultures: mycoplasma-virus-cell culture interactions. in: Fogh, J., ed. "Contamination in tissue culture", New York, Academic Press, pp. 131-172, 1973.
3. Rottem S, Marcus M. Elimination of mycoplasmas from contaminated cell cultures. Isr J Med Sci 17: 569-571, 1981.
4. van Diggelen O P, Shin S, Phillips DM. Reduction in tumorigenicity after mycoplasma infection and elimination of mycoplasma from infected cultures by passage in nude mice. Cancer Res 37: 2680-2687, 1977.
5. Schimmelpfeng L, Langenberg U, Peters J. Macrophages overcome mycoplasma infection of cells in vitro. Nature 285: 661-662, 1980.
6. Gurney T, Woolf MJ, Abplanalp LJ, McKittrick NH, Dietz JN, Cole BC. Elimination of mycoplasma hyorhinis infections from four cell lines. In Vitro 17: 993-996, 1981.
7. Chen TR. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp Cell Res 104: 255-262, 1977.
8. McGarrity GJ. Cell cultures: detection of contamination. In: Jacoby WJ and Pastan IH, eds. Methods in Enzymology. New York: Academic Press Vol. 58: pp. 18-29, 1979.

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