SIMPLE AND RAPID STAINING FOR DETECTION OF ENTAMOEBA CYSTS AND OTHER PROTOZOA WITH FLUOROCHROMES

Fumihiko KAWAMOTO, Sahoko MIZUNO, Hisashi FUJIOKA, Nobuo KUMADA, Etsuro SUGIYAMA¹, Tsutomu TAKEUCHI², Seiki KOBAYASHI², Motohiro ISEKI³, Minoru YAMADA⁴, Yoshitsugu MATSUMOTO⁴, Tatsuya TEGOSHI⁴ and Yukio YOSHIDA⁴

Department of Medical Zoology, Nagoya University School of Medicine, Tsurumacho, Showa-ku, Nagoya 466, ¹Department of Medical Zoology, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113, ²Department of Parasitology, Keio University School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo 160, ³Department of Medical Zoology, Osaka City University School of Medicine, Asahimachi, Abeno-ku, Osaka 545, and ⁴Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kawaramachi, Kamikyo-ku, Kyoto 602

(Received December 24, 1986. Accepted April 1, 1987)

SUMMARY: Three fluorochromes were applied to stain various parasitic protozoans. By double staining with 4',6-diamidino-2-phenylindole and propidium iodide, differentiation of the nuclei from the cytoplasm can easily be achieved within several seconds. The chromatoid bodies in Entamoeba cysts were stained bright red. Plasmodium yoelii at all stages except late trophozoites and young gametocytes was easily identified. In the oocysts of Cryptosporidium sp., the nuclei and cytoplasm of the sporozoites fluoresced bluish white and red, respectively, whereas the residual body appeared blue or green. The third fluorochrome, Calcofluor white M2R, was suitable for detecting the cysts of Entamoeba spp. and Chilomastix mesnili.
INTRODUCTION

Fluorescence microscopy combined with fluorochrome stains has become a valuable means for rapid examination of various cell components. Recently, many fluorochromes have widely been used for this purpose or for epifluorometric assay for DNA contents of various cells and protozoans. Arroyo-Begovich et al. (1) have reported that Calcofluor white M2R (M2R) stains chitin in the fixed cyst wall of *E. invadens*. No fluorochrome stain, however, has enabled one to observe the nuclei or chromatoid bodies in *Entamoeba* cysts.

It is well known that 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) bind specifically to double-stranded DNA and nucleic acids (DNA and RNA), respectively (2-5). During epifluorometric studies on DNA content of *E. invadens* cysts with DAPI and PI (unpublished), we found that these fluorochromes stained the nuclei bluish white and the chromatoid bodies red. In this paper, we report application of these fluorochromes for detection of protozoan parasites in such genera as *Entamoeba, Giardia, Balantidium, Chilomastix, Cryptosporidium, Plasmodium, Trypanosoma, Pneumocystis*, and *Toxoplasma*. We further investigated to find whether M2R stains cyst or oocyst walls of these parasites.

MATERIALS AND METHODS

*Parasites: a) Entamoeba.* A xenic strain of a reptilian amoeba, *E. invadens* originally isolated at London School of Hygiene and Tropical Medicine was offered by Prof. A. Ishii, Okayama University, Okayama. The trophozoites were cultured at 28°C in TYI-S-33 medium (6) without elimination of symbiotic bacteria. To induce encystation, the trophozoites were cultured at 25°C for 2-3 days in the encystation medium reported by Rengpien and Bailey (7). The cysts yielded were harvested by centrifugation at 500 ×g for 5 min and fixed with absolute methanol, ethanol (70-100%), acetone (50-100%), or Carnoy’s fixative (ethanol : glacial acetic acid, 3:1).

Cysts of *E. coli* and *E. histolytica* were collected from fresh stools of human patients and naturally-infected Japanese macaques, *Macaca fuscata fuscata*, by centrifuging stool suspensions in saline at 500 ×g for 5 min and stored in various fixatives. Some *E. histolytica* cysts were collected by the formalin-ether floatation method (MGL method) and washed with water several times before staining.

*b) Giardia lamblia and Chilomastix mesnili.* Cysts of *G. lamblia* and *C. mesnili* were obtained from fresh human stools and stored in 70% ethanol. Trophozoites of *G. lamblia* (Portland-I strain) were cultivated in the medium reported by Keister (8).
c) Balantidium coli. Trophozoites of B. coli were obtained from a naturally-infected Japanese macaque, M. fuscata fuscata, and stored in absolute methanol.

d) Cryptosporidium sp. Oocysts of Cryptosporidium sp. were obtained from naturally-infected Norway rats, Rattus norvegicus, and smears were fixed with absolute methanol.

e) Plasmodium yoelii nigeriensis and Trypanosoma brucei gambiense. P. yoelii n. and T. brucei g. (Wellcome strain) were maintained in ICR mice by serial passages of the infected blood. Thin blood smears fixed with absolute methanol were used for staining.

f) Pneumocystis carinii. To induce the disease, prednisolone acetate (3.5 mg/ml saline) was administered subcutaneously to Wister rats twice a week for 14 weeks. Impression smears of the rats' lung tissue were air-dried and fixed with absolute methanol.

g) Toxoplasma gondii. RH strain of T. gondii was maintained by serial passages of the peritoneal fluid in dd mice. Smears of the peritoneal fluid from infected mice were fixed with absolute methanol.

Chemicals: 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), Calcofluor white M2R (M2R) and RNase A were purchased from Sigma Chemicals Co. DAPI (10 µg/ml) and M2R (100 µg/ml) were dissolved in 0.05 M Tris-HCl buffer, pH 7.4, and PI (10 µg/ml) was in 0.01% sodium citrate. These staining solutions were stored in dark at 4°C. RNase was dissolved in 0.05 M Tris-HCl buffer (pH 7.4) at a concentration of 500 µg/ml and boiled for 10 min to destroy contaminated DNase. It was stored at -20°C in adequate volumes until use.

Staining methods: Stainings with DAPI and PI were done by putting a drop of each staining solution on the fixed smear. For double-staining with DAPI and PI, a fixed smear was first stained with a drop of DAPI, and several seconds later, a drop of PI was overlaid without washing DAPI out.

Staining was performed by adding a drop of M2R solution on a live or a fixed material of Entamoeba spp. and fixed ones of G. lamblia, C. mesnili, Cryptosporidium sp. and P. carinii. A drop of 0.01% gluconate chlorohexidine (Hibiten soln.) was added to prevent decay of fluorescence.

RNase treatment of E. invadens cysts: RNase was applied to E. invadens cysts according to the methods of Kuroiwa et al. (9) and Uno et al (10). A drop each of RNase and PI solutions was added to DAPI-stained samples, which were incubated for 30 min at 37°C.

Fluorescence microscopy: A cover glass was put on the stained preparation with the staining solutions as mounting media. The preparation was then sealed with nail polish. Observations were made with an Olympus microscope (BH-2) with a combination of an ultraviolet excitation filter (UG-1, 334 nm) and a L-435 (435 nm) filter for barrier.
RESULTS

Staining Entamoeba spp. with DAPI and/or PI

When methanol-fixed E. invadens cysts were stained with DAPI, only the nuclei appeared bright bluish white. When stained with PI, the chromatoid bodies as well as nuclei became visible, exhibiting bright red fluorescence. The chromatoid bodies fluoresced stronger than did the nuclei (data not shown), but it was difficult in most E. invadens cysts to distinguish the nuclei from the chromatoid bodies.

Due to the highly specific affinity of DAPI for DNA and PI for DNA and RNA, it was expected that the DAPI-DNA complex would not react to additional staining with PI, and that RNA remaining in the cytoplasm would do with PI. This prediction on the differential staining was confirmed when methanol-fixed cysts of E. invadens were stained with DAPI-PI (Fig. 1A): The chromatoid bodies were displayed as bright red-colored rods and easily distinguished from the bluish white-colored nuclei. The nuclei of trophozoites also appeared bluish white, whereas their cytoplasm looked blue with scattered red spots, which were indicative of ribosomes (Fig. 1B). Frequently, however, some nuclei in the trophozoites and cysts changed from bluish white to pink by an unknown reason.

It has been reported that the chromatoid bodies are composed of crystalline-arrayed ribosomes (11). To find whether the red-colored materials are really chromatoid bodies, DAPI-stained samples of E. invadens cysts were treated with RNase. As shown in Fig. 1C, the red-colored materials disappeared by the RNase treatment and the cytoplasm appeared blue, indicating that the red-colored materials are composed of RNA. However, all the nuclei in the cysts fluoresced pink in color after the RNase treatment.

Similarly, cysts of E. histolytica and E. coli were well observed by double-staining with DAPI and PI as shown in Figs. 1D-F and G-H, respectively. In both species, the cytoplasm of uninuclear cysts was stained entirely red. As the cysts matured, typically shaped chromatoid bodies appeared red with PI (Figs. 1E-G). Then, the red-colored chromatoid bodies disappeared by the time of full maturation of the cysts, and the cytoplasm displayed entirely red (Fig. 1H).

The double staining with DAPI and PI, when applied to cysts of three Entamoeba species fixed with ethanol, acetone or Carnoy's fixative, also gave good coloration. The materials fixed with formalin or collected by the MGL method were unsuitable for the staining. Nevertheless, removal of formalin from the freshly-fixed material by several washings was effective to revive good stainability.
Fig. 1.
Fig. 2.
Explanation for figures

Fig. 1. *Entamoeba* spp. stained with DAPI and PI (A-H) or M2R (I-N). Bars represent 10 μm in A-H and K-N, and 50 μm in I-J.
A-C, *E. invadens* cysts (A, C) and trophozoites (B, arrowheads).
D-F, *E. histolytica* cysts with one (D), two (E) and four nuclei (F). Three of four nuclei are seen in F, and bar-shaped chromatoid bodies are recognized in E-F.
G-H, *E. coli* cysts with two (G, large arrowheads) and eight nuclei (H). Note that typical chromatoid bodies are seen in G (small arrowheads).
I-J, Live cysts of *E. invadens* displaying the ring structure (small arrowheads). The same field photographed by light (I) and fluorescence microscopies (J). Note that live trophozoites (large arrowheads) do not emit fluorescence as shown in J.
K-L, Live (K) and methanol-fixed cysts (L) of *E. histolytica*.
M-N, living (M) and methanol-fixed cysts (N) of *E. coli*.

Fig. 2. Various protozoans stained with DAPI and PI (A-B, D-E, and G-N) or M2R (C and F). Bars represent 10 μm in A-C, and E-N, and 50 μm in D.
A-C, A trophozoite (A) and cysts (B-C) of *Giardia lamblia*. Note that the cyst wall is not stained with M2R (C).
D, A *Balantidium coli* trophozoite. An arrowhead indicates a micronucleus.
E-F, *Chilomastix mesnili* cysts. An arrowhead in E indicates a cytostome-like structure in a cyst. Note that the cyst wall stained with M2R emits strong fluorescence (F, arrowhead).
G-H, Oocysts of *Cryptosporidium* sp. Note that residual bodies (small arrowheads) exhibit blue to green fluorescence, differing from that of nuclei (large arrowheads) and cytoplasm.
I-J, *Plasmodium yoelii*. Note that each developing stage parasite, or free merozoite (m), ring form (r), young schizont (y), mature schizont (s), macrogametocyte (ma), and microgametocyte (mi) can be identified.
K, *Trypanosoma brucei*. Large and small arrowheads indicate the reticulocytes and kinetoplasts, respectively.
M-N, *Pneumocystis carinii*. Seven of the eight intracystic bodies are seen in M. Trophozoites (small arrowheads) of various sizes and reticulocytes (large arrowheads) are seen in N.
Staining Entamoeba spp. with M2R

M2R was applied to live and fixed cysts of *E. invadens*, *E. coli*, and *E. histolytica*. Under ultraviolet excitation, all cysts of these species displayed bright blue rings that correspond to the cyst walls (Figs. 1J-M), whereas live trophozoites were not stained (compare Fig. 1I with 1J). As M2R penetrates the walls of the fixed cysts (1), the inside chromatoid bodies were frequently observed (Figs. 1L, N). Trophozoites became stainable after fixation (data not shown).

Application of DAPI, PI, and M2R to Other Protozoans

Good coloration was obtained by the DAPI-PI staining method on every parasite mentioned below, but the nuclei were sometimes stained pink to pinkish white as was the case with *Entamoeba*.

Figs. 2A-B show the results of DAPI-PI staining applied to the trophozoites and cysts of *Giardia lamblia*. Their nuclei displayed blue to white fluorescence, whereas the cytoplasm appeared red with PI. When the fixed cysts were stained with M2R (Fig. 2C), the dye penetrated into the cytoplasm, exhibiting only entire dim blue fluorescence.

Fig 2D shows a trophozoite of *Balantidium coli* stained with DAPI and PI. The macro- and micro-nuclei and the cytoplasm were well observed by this staining.

When the double staining method was applied to a freshly-fixed stool specimen from a patient who complained of diarrhea, *Chilomastix mesnili* cysts were found in it (Fig. 2E). The nucleus exhibited bluish white color in the red-stained cytoplasm. The cytostome-like structure that could not be stained with PI was also distinguished in the stained cytoplasm. The cyst wall stained with M2R fluoresced in strong blue color tone (Fig. 2F). Care should be taken in discrimination of *C. mesnili* cysts from yeasts that may have similar size or M2R-positive cell walls.

Oocysts of *Cryptosporidium* sp. stained with DAPI and PI are shown in Figs. 2G-H. Besides the four nuclei stained with DAPI and the cytoplasm stained with PI, a residual body was easily identified as a blue or greenish structure. M2R did not stain the oocyst wall (data not shown).

DAPI-PI staining was applied also to blood and tissue protozoans such as *Plasmodium yoelii* (Figs. 2I-J), *Trypanosoma brucei* (Fig. 2K), *Toxoplasma gondii* (Fig. 2L) and *Pneumocystis carinii* (Figs. 2M-N).

*P. yoelii* (Figs. 2I-J) all stages including free merozoites was well stained with these dyes, whereas normal red blood cells were not. Due to red coloration of
P. yoelii cytoplasm, the double staining made it easier to identify each of these developmental stages from others except late trophozoites and young gametocytes. The cytoplasm of mature macrogametocytes was stained bright red, while that of mature microgametocytes appeared blue to reddish blue.

The nuclei of trypomastigotes of T. brucei displayed reddish or bluish white fluorescence, while their kinetoplasts and cytoplasm appeared blue and red, respectively (Fig. 2K). Some of non-infected erythrocytes were stained red with PI. They were considered to be reticulocytes which contain ribosomal RNA.

Tachyzoites of T. gondii stained with DAPI-PI are shown in Fig. 2L. The nucleus, cytoplasm, and mitochondrion of the parasite emitted bluish white, red, and blue fluorescence, respectively.

Figs. 2M-N show a cyst and trophozoites of P. carinii stained with DAPI and PI. The nuclei of the intracystic bodies (Fig. 2M) and trophozoites (Fig. 2N) were stained bluish white, and their cytoplasm emitted bright red fluorescence. Reticulocytes were also well observed. M2R did not stain the cyst wall (data not shown).

**DISCUSSION**

We demonstrated a simple and rapid technique for staining parasitic protozoans with DAPI and PI by which differential coloration of the nucleus from the cytoplasm could easily be achieved within several seconds. Compared with the staining with DAPI alone, the coloration of cytoplasmic RNA with a subsequent PI-stain made the outline of the parasites clearer and increased the brightness of the field of vision. Furthermore, differential staining of nuclei with DAPI from cytoplasm with PI enabled one easier discrimination of the parasites than in single staining with any of the known fluorescent dyes such as DAPI (3-5), mithramycin (12), acridine orange (13,14), rhodamine (15,16), ethidium bromide (17) and auramine (18,19). It should be noted that double staining with DAPI-PI was inadequate to observe mitochondrial DNA except that in T. brucei and T. gondii, and that pigment granules or some organelles such as flagella and undulating membrane were not visualized. Further, the nuclei of protozoan parasites were frequently stained pink to pinkish white. We cannot explain why this color change happens, particularly in E. invadens cysts treated with RNase.

As reported by Kuroiwa et al. (9) and Uno et al. (10), PI-nucleic acid complexes should be excited by green light to avoid possible quenching of
fluorescence that may be caused by ultraviolet ray. In fact, some degree of quenching occurred during our observation with DAPI-PI under ultraviolet light. To suppress such decay of fluorescence, we recommend addition of gluconate chlorohexidine or 2-mercaptoethylamine (5) to the staining solution, or dissolving fluorochromes in "NS" buffer (9) containing 2-mercaptoethanol.

The chromatoid bodies in *Entamoeba* cysts were well stained with DAPI-PI, emitting strong red fluorescence. Since these bodies are not generally seen in young or maturing cysts of *E. histolytica* or *E. coli*, staining with M2R may help rapid detection. For reliable identification of *Entamoeba* cysts, it is necessary to discriminate the internal structures such as chromatin granules, karyosomes, and glycogen granules, which are not visualized with DAPI-PI or M2R. In such cases, M2R staining combined with conventional one may be efficient and reliable to screen and identify the species.

Having applied M2R staining to the formalin-fixed cysts of *E. invadens*, Arroyo-Begovich et al. (1) observed bright yellow-green fluorescence on the cyst wall under a violet excitation system. In our observations, M2R stained also the cyst wall of living *Entamoeba* under ultraviolet excitation. The trophozoite and the inner part of the cyst became stainable only after fixation. These results may suggest that M2R can be used as a rapid vital stain for *Entamoeba*.

It has been reported that M2R reacts with hexopyranose polymers with $\beta$-configuration that compose chitin, cellulose, etc. (20). The results obtained by M2R staining may indicate that the cyst wall of *C. mesnili* is composed of such polymer(s).

For detection of cryptosporidial oocysts, several stains, e.g. modified Ziehl-Neelsen stain, Kinyoun acid-fast stain, etc., have been used, enabling us to distinguish the oocysts from various yeasts (18,19,21). Except for a modified acid-fast stain (19,21), however, it is difficult to observe the internal structure of the oocyst. By DAPI-PI staining, we can easily differentiate the nuclei, the crescent structure of sporozoites, and the residual body in three different colors. It is worthy to note that rapid scanning as well as reliable identification of *Cryptosporidium* sp. were feasible by observing both the oocyst wall and the inside fluorescence with combined light and fluorescence microscopy.

*P. yoelii* at all developmental stages except late trophozoites and young gametocytes was easily identified by DAPI-PI staining. The mature macro- and microgametocytes differed from each other in color of their cytoplasm. This might be caused by the difference in RNA contents between the two. In fact, an increase of cytoplasmic RNA during macrogametocyte-genesis (22,23) was reported. The
nature of the blue fluorescence observed in the micro-gametocytes remains to be investigated.

From the results described above, it is concluded that the DAPI-PI and M2R staining methods would be useful for rapid detection of the parasitic protozoans responsible for human and animal diseases. Since no rinse is necessary in the DAPI-PI or M2R staining procedures, even a small number of pathogens, if any, in test specimens can be detected without any loss from the slide preparations. The specimens stained with fluorochromes, if air-dried, can be re-stained with conventional dyes for permanent recording.

ACKNOWLEDGEMENTS

We wish to express our sincere thanks to Prof. A. Ishii, Okayama University, Prof. M. Suzuki and Dr. S. Waki, Gunma University, Dr. S. Shinonaga, Tokyo Medical and Dental University and Drs. T. Takayanagi and Y. Yabu, Nagoya City University for providing parasite materials and for their valuable advice. We thank also Prof. K. Tanaka and Dr. K. Yamashita, Nagoya University, for their valuable suggestions on this study, and Mrs. H. Chigusa for her technical assistance.

REFERENCES