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Modification of RPMI 1640 for Use in In Vitro Immunological Studies of Host-Parasite Interactions in Giardiasis

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Incubation of trophozoites for 6 h in RPMI 1640 affected the viability of the parasite; however, RPMI 1640 supplemented with L-cysteine did not affect trophozoite viability, ability to grow when transferred to fresh TYI-S-33, or ability to infect gerbils. Similarly, incubation of murine spleen cells in modified medium did not affect the viability of the cells or proliferative responses to mitogens. RPMI 1640 supplemented with 11.4 mM L-cysteine is a suitable maintenance medium for in vitro studies in immunoparasitology because it maintains viability as well as some of the physiological functions of both trophozoites and lymphocytes.

Giardia lamblia is an anaerobic parasitic protozoan which colonizes the small intestine of its host (8). Gillin and Diamond (5) and Gillin and Reiner (6) have established cultures of *G. lamblia* in a medium (TYI-S-33) developed for the culturing of *Entamoeba histolytica* (3). For trophozoites of *G. lamblia* to grow in this medium, it has to be supplemented with L-cysteine HCl, a reducing agent, and the culture tubes must be filled to further reduce oxygen tension. Since the availability of axenic cultures, workers have shown interest in performing immunological studies of host-*G. lamblia* relationships (1, 7, 10, 12). RPMI 1640 has been used frequently in these studies. This medium was designed by Moore and colleagues (9) for the culturing of human lymphocytes, and it is unlikely that it will meet the conditions that are necessary to keep *G. lamblia* trophozoites alive. In this study, we have modified RPMI 1640 by adding L-cysteine to accommodate parasitic cells. We report here on the ability of modified RPMI 1640 to maintain both the viability and the attachment of *G. lamblia* trophozoites as well as to maintain the viability and mitogen-induced proliferative responses of murine spleen cells.

RPMI 1640 with L-glutamine (GIBCO) and supplemented with 10% fetal bovine serum (Flow Laboratories), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma), and 100,000 U of penicillin-streptomycin (Sigma) was used as standard RPMI 1640. L-Cysteine HCl (Fisher) was added to RPMI 1640 at concentrations of 2.8 (RPMI-2.8), 5.7 (RPMI-5.7), and 11.4 (RPMI-11.4) mM. *G. lamblia* WB used in this study was originally isolated by Smith et al. (11). The trophozoites attached to the vessel wall were used to initiate new cultures. Cells were washed in cold phosphate-buffered saline (0.15 M, pH 7.2), resuspended in the different experimental media, and sampled for enumeration in 1% Formalin with a hemacytometer.

To obtain the lymphocytes, we sacrificed CD-1 Swiss mice by cervical dislocation and removed their spleens. Cell suspensions were prepared by gently seiving the spleens through 80 mesh stainless steel screens into 10 ml of RPMI 1640. Cell viability was determined by staining with 0.2% trypan blue. The experiments were repeated three times, and triplicate samples were taken. Data were analyzed by Student's *t* test. We determined the effects of RPMI 1640 supplemented with different concentrations of cysteine on trophozoite attachment and viability. Attached trophozoites to inoculate culture plates were obtained by gently removing the medium and the free trophozoites from each well. The medium was replaced with 2 ml of ice-cold phosphate-buffered saline, and the plates were put on ice for 10 min. Trophozoite numbers were determined with a hemacytometer and viability was determined on the basis of flagellar movement and morphology.

Figure 1 shows the effect of cysteine on the attachment and survival of *G. lamblia* trophozoites in culture plates. As the cysteine added to RPMI 1640 was increased from 0 to 11.4 mM, both the attached and the total numbers of live trophozoites recovered increased. Trophozoites incubated in RPMI 1640 with no cysteine did not exhibit flagellar movement and were morphologically distorted. Trophozoites preincubated in RPMI-5.7 or RPMI-11.4 reproduced as well as did those preincubated in TYI-S-33. They were also able to infect gerbils (2, 4; data not shown).

The effects of adding L-cysteine to RPMI 1640 on the viability and mitogen-induced proliferative responses of splenic lymphocytes were determined. Cells grown in the five media were transferred to 24-well plates (10^6 live cells per ml per well) and incubated at 37°C in 5% CO_2. The viability of splenic lymphocytes was not affected when cells were incubated in the experimental media for 6 h (Fig. 2). After 24 h, cultures incubated in RPMI-11.4 and TYI-S-33 had, respectively, 20 and 60% less live cells than did cultures incubated in RPMI 1640 without cysteine. The viability of lymphocytes incubated in RPMI-2.8 did not differ from that of lymphocytes incubated in RPMI 1640 without cysteine after 48 h.

We studied the effects of modified RPMI 1640 on the ability of splenic lymphocytes to respond to T- and B-cell mitogens. Live cells (5×10^6) suspended in the five media were added to 24-well plates and incubated. After 6 h, the cells were collected, pooled in each medium, and washed twice by centrifugation in RPMI 1640 without cysteine. The cells were transferred to 96-well plates at a concentration of 5×10^5 live cells per well. Concanavalin A (type IV-S; 5 μg/ml; Sigma) or lipopolysaccharide (*Salmonella typhosa*; 35 μg/ml; trichloroacetic acid extract; Sigma) was added to each well, and the plates were incubated for 48 h. All

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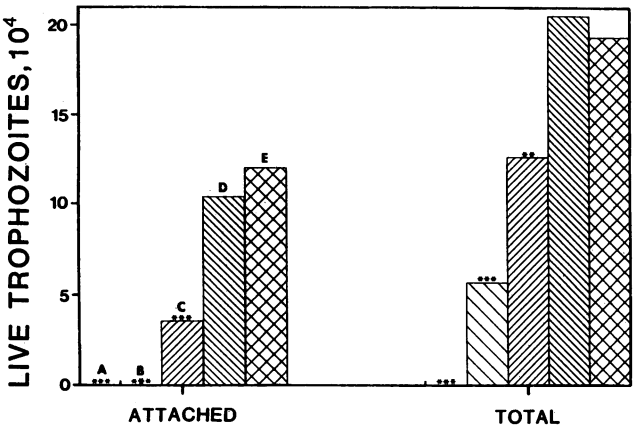

FIG. 1. Effects of incubation in standard RPMI 1640, RPMI-2.8, RPMI-5.7, and RPMI-11.4 upon the recovery of *G. lamblia* trophozoites. Trophozoites (2×10^5/ml) were incubated in 24-well plates with standard RPMI 1640 (A), RPMI-2.8 (B), RPMI-5.7 (C), RPMI-11.4 (D), or TYI-S-33 (E). Asterisks indicate that results differed significantly at $P < 0.01$ (**) or $P < 0.001$ (***).

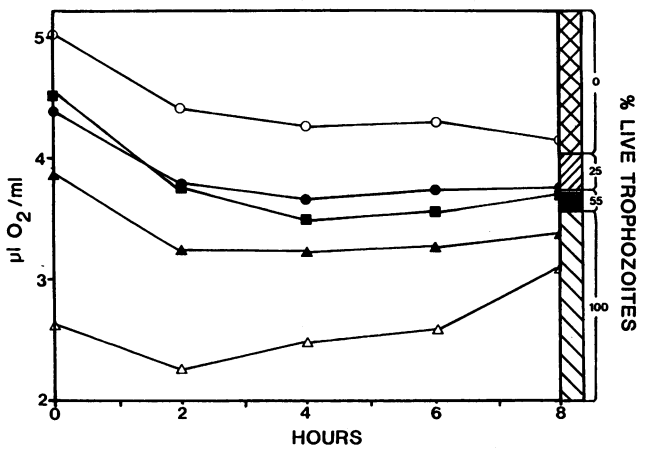

FIG. 3. Concentrations of oxygen measured by an oxygen electrode in five different media dispensed in 24-well plates. On the right axis is shown the proportion of *G. lamblia* trophozoites which were alive in the different media. Symbols are as in Fig. 2.

incubations were done at 37°C in 5% CO_2. Tritiated thymidine (specific activity, 6.7 Ci/mmol; ICN Radiochemicals) was added to each well. The cells were incubated for a further 18 h and harvested onto glass fiber filters (Whatman GF/C). Filters were air dried, dissolved in Cytoscint (ICN Radiochemicals), and counted in a liquid scintillation counter (Rackbeta Spectral; LKB). T- and B-cell-induced proliferation of lymphocytes incubated in RPMI 1640 containing up to 11.4 mM cysteine was not significantly different from that of lymphocytes incubated in RPMI 1640 without cysteine after 6 h.

The relative oxygen concentrations of the five media were determined at 2-h intervals for 8 h. Cysteine was added fresh to RPMI 1640 and TYI-S-33 media, and the oxygen contents were determined. One-milliliter aliquots of each medium were dispensed into 24-well plates and incubated at 37°C in 5% CO_2. Media were sampled from the wells and the relative oxygen concentrations were measured with a YSI model 5300 biological oxygen monitor (model 5331 standard oxygen probe) (Scientific Division, Yellow Springs Instrument Co. Inc.). The oxygen concentration was at its highest at time zero (Fig. 3). However, after 2 h of contact with cysteine, the oxygen concentration decreased and remained constant for up to 8 h. The oxygen concentration of RPMI-11.4 was significantly higher than that of TYI-S-33 containing the same amount of cysteine. When the oxygen concentration of the media decreased, the percentage of live trophozoites recovered increased (Fig. 3, right-hand axis).

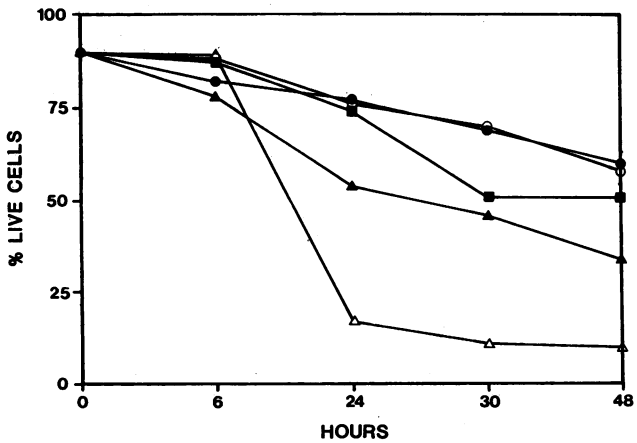

FIG. 2. Percentage of live murine spleen cells (200 cells were counted) after incubation in RPMI 1640 containing different concentration of cysteine or in TYI-S-33. Symbols: ○, RPMI 1640; ●, RPMI-2.8; ■, RPMI-5.7; ▲, RPMI-11.4; △, TYI-S-33. Each point is the mean of three wells.

The maintenance medium designed for in vitro studies should maintain the viability and the normal physiological functions of both the parasites and the host cells. Studies on the immunological aspects of host-*G. lamblia* interactions (1, 7, 10, 12) have been performed with defined medium developed for the culturing of lymphoid cells. Smith et al. (10) used RPMI 1640 to determine the cytotoxicity of human mononuclear cells against *G. lamblia* trophozoites (strain WB). They concluded that peripheral blood monocytes-macrophages were spontaneously cytotoxic for *G. lamblia* trophozoites. Aggarwal and Nash (1), in an attempt to repeat the experiments of Smith and colleagues (10), reported on the failure of human mononuclear cells to kill trophozoites. Instead, these authors attributed the death of trophozoites to the inability of the parasites alone to survive under the assay conditions. The results indicated that the medium used was not adequate to support the physiological functions of both the parasites and the mammalian cells.

We report here that the addition of 11.4 mM L-cysteine to standard RPMI 1640 maintains the viability as well as some of the physiological functions of both *G. lamblia* trophozoites and murine spleen cells. The modified medium does not affect trophozoite attachment to culture vessel walls, ability to reproduce when transferred to fresh TYI-S-33 culture medium, or infectivity for gerbils. The experiments involving splenic lymphocytes demonstrated that the addition of up to 11.4 mM cysteine to RPMI 1640 does not alter the viability or the proliferation of these cells after 6 h of incubation.

The oxygen concentrations of the media correlated negatively with the recovery of live *G. lamblia* trophozoites. It appears that trophozoites of the WB strain can withstand

oxygen concentrations of 3.3 μl/ml, since 100% of trophozoites were recovered after incubation in RPMI-11.4. Both RPMI-11.4 and TYI-S-33 contained 11.4 mM cysteine, but the oxygen concentration of TYI-S-33 was significantly lower than that of RPMI-11.4. This result suggests that other components present in TYI-S-33 must contribute in decreasing the oxygen concentration. To allow for the reduction of the oxygen concentration, cysteine should be added to RPMI 1640 at least 2 h prior to the experiment.

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