SHORT COMMUNICATION

Long-Term Cultivation and Productive Infection of Primary Thymocyte Cultures by a Thymocytopathic Murine Retrovirus

P. K. Y. WONG, KUNAL SAHA, Y. C. LIN, W. S. LYNN, and P. H. YUEN

The University of Texas, M. D. Anderson Cancer Center, Science Park-Research Division, P.O. Box 389, Smithville, Texas 78957

Received June 26, 1995; accepted November 14, 1995

ts1, a mutant of MoMuLV, selectively kills T cells and neurons in the infected host resulting in neuroimmunodegeneration. In the infected thymus there is an early increase in mitosis of thymocytes followed by rapid death, suggesting that thymocyte death may be induced by viral mitogenic activation. Studies on thymocytes obtained from ts1-infected mice indicated that the ts1-induced depletion of thymocytes is mediated through activation-induced death by apoptosis. To further investigate the interaction between ts1 and thymocytes, we have established long-term primary murine thymocyte cultures by placing the thymocytes together with thymic remnants in culture medium containing IL-2 and IL-7. These thymocytes retained their immature phenotype and were susceptible to infection by ts1 and its parental wild-type MoMuLV. ts1-infected thymocytes proliferated initially at accelerated rate but subsequently produced more infectious virus and died much faster than control or MoMuLV-infected thymocytes. These in vitro studies to some extent reflect our in vivo studies reported previously.

A major goal of our research has been to understand the pathogenic mechanisms of immunosuppression and neurodegeneration induced by an neuroimmunopathogenic mutant of Moloney murine leukemia virus (Mo-MuLV), designated ts1 (1-4). As in human immunodeficiency virus infection (5-9), ts1-infection typically exhibits the selective depletion of CD4+ T cells and thymocytes as well as neurons (2, 10-13). When injected intraperitoneally, ts1 replicates in the thymus and spleen, particularly in thymocytes and splenic CD4+ T cells, before spreading to the central nervous system. In the thymus, during the early phase of ts1 infection there is an increase in thymocytic mitosis, which is followed by progressive and rapid thymocytic cell death particularly in the cortex region (10). An ex vivo study of thymocytes from ts1-infected mice at 20-25 days postinfection (dpi) indicated that the ts1-induced T-cell death is the result of apoptosis (14). We also demonstrated that the killing of thymocytes by ts1 is a direct result of virus infection of the thymus (15).

To further investigate the specific events leading to T-cell depletion by ts1, we have established a long-term primary culture system using dissociated thymocytes. This system provides an environment that to some extent simulates the microenvironment of the thymus. We found that the thymocytes in culture retained their double-positive (CD4+ CD8+) immature phenotype. We also found that these thymocytes could be readily infected by ts1 or wild-type (WT) MoMuLV. To our knowledge, this is the first time that primary neonatal murine thymocytes have been productively infected with murine retrovirus in vitro and maintained for any length of time.

To establish primary thymocyte cultures, single-thymocyte suspensions were prepared from thymus lobes taken from 1- to 2-day-old FVB/N mice. Thymocytes were prepared mechanically by mincing thymus and teasing apart the cells with a rubber-head tissue homogenizer into cold RPMI-1640 medium supplemented with 2% FCS and penicillin and streptomycin. Most of the thymocytes were released from the thymus in this way. The cells were washed once with medium and plated on a plastic tissue-culture dish for 2 hr at 37°C. The nonadherent cells were then transferred to another culture dish at a density of 2 × 10^6 cells/ml of medium containing 20% FCS, human recombinant IL-2 (50 U/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN) and human recombinant IL-7 (30 ng/ml) (a generous gift from Sterling Winthrop, Inc., Malvern, PA). To each of the culture dishes the remnant from two thymuses after the mincing and teasing procedure to obtain thymocytes described above were added. In a separate experiment, equal number of thymic remnants alone (i.e., from two thymuses) was placed in each culture dish with medium only to determine the number of cells which might release from the
We found that by culturing thymocytes obtained from 1- to 2-day-old FVB/N mice in various concentrations of IL-2 and IL-7 along with thymic remnants, we have established a condition that can maintain these thymocytes in cultures for over 3 weeks (Fig. 1A). During the first 3 days of culture in this environment, there was a 50% increase in the number of viable cells (determined by trypan blue exclusion assay). Although this viable cell number did not increase exponentially, the fact that we also observed a high number of dead cells (40%) by Day 3 of culture (data not shown) indicates that a rapid turnover of the thymocytes occurred during this period and that the rate of cell growth is faster than the rate of cell death at least up to 3 days after culture. More importantly the number of viable cells in culture with IL-2 and IL-7 and thymic remnants remained high (about $2 \times 10^6$ viable cells/ml) up to 18 days of culture. It is possible that some thymocytes may have been released from the remnants after we put the remnants back in the cultures and this may have influenced our results. This possibility, however, is not likely because in duplicate culture plates in which only the thymic remnants were placed in medium supplemented with IL-2 and IL-7 and cultured identically to the thymocyte culture mentioned above, the number of cells that were released from these remnants were less than 10% of the total cell population (data not shown). Therefore, the cell growth observed in the thymocyte suspension cultures apparently resulted from proliferation of these cells. However, we cannot completely rule out the possibility that some thymocytes might be released from the thymic remnants under this condition. By Day 3 of culture, the viable cell number in thymocyte cultures with IL-7, IL-2, and the thymic remnants, was 10-fold higher than the viable cell number in thymocyte cultures that were not supplemented with these cytokines or thymic remnants and twofold higher than in cultures that contained IL-2 and IL-7 but no thymic remnant. By 10 days of culture, the cell viability was more than 10-fold higher in the presence of IL-7, IL-2, and the thymic remnants, than thymocyte cultures with IL-2 and IL-7 alone, and several orders of magnitude higher than thymocyte cultures without any lymphokines and thymic remnants. These results indicate that a combination of IL-2, IL-7, and thymic remnant provides a good growth condition for primary thymocytes in culture. It is well known that thymic stromal cells produce many factors that play important roles in the growth of thymocytes (16). Several cytokines have been previously shown to augment the growth of T cells (17–20). IL-2 was initially shown to be a major primary T-cell growth factor (21), but over the past several years, IL-7, which was initially isolated as a B-cell growth factor, has also been shown to be a potent T-cell growth factor (18, 22, 23) and in the ab-

![Graphs showing survival and production of virus in cultured thymocytes under different conditions.](image-url)
sence of other cytokines to induce IL-2 and IL-4 secretion (20, 24, 25). Watson et al. (20) have reported long-term T-cell growth using intact fetal thymus lobes in the presence of IL-2 and IL-7. However, long term growth of thymocytes from neonatal or adult mice has not been reported.

Fluorescence-activated cell sorter analysis of these thymocyte cultures at 2 to 3 days after culture with labeled antibodies against various T-cell antigens (Thy 1.1, CD4, and CD8) showed that these thymocyte cultures contained largely CD4\(^+\)/CD8\(^-\) double-positive T cells (Fig. 2), thus retaining their phenotype in vivo (14). Although we did notice a slight increase in the percentage of single-positive cells (CD4\(^+\) or CD8\(^+\)) as the cultures grew older (data not shown), the vast majority (>75%) of the cells retained their double-positive phenotype for at least up to 10 days after culture.

To assess whether these primary cultures of thymocytes are susceptible to ts1 and WT infection, freshly isolated neonatal thymocyte suspension cultures as described above were diluted to a density of 2 x 10^6 cells/ml in RPMI 1640 medium supplemented with 5% FCS. The cells were washed several times and shaken in a sterile disposable plastic tube for 4 hr at 37\(^\circ\)C in the presence of polybrene (2 \mu g/ml). These thymocytes were then infected with ts1 or WT virus at an m.o.i. of 1 and agitated at 37\(^\circ\)C for 1 hr. The cell suspensions were washed twice and transferred to 24-well culture plates. One milliliter of RPMI medium containing 20% FCS, IL-2 (50 U/ml), and IL-7 (30 ng/ml) and supplemented with thymic remnants was then added to each culture plate and incubated at 37\(^\circ\)C. One half milliliter of the supernatants were taken every 2-3 days from each sample and cells were spun down. The supernatants were collected and the cells were resuspended with 0.5 ml of complete medium (with IL-2 and IL-7) and added back to the wells. The supernatants were assayed for virus infectivity by 15F cell assay (26). As shown in Fig. 1B, the primary cultures of thymocytes were infected by both ts1 and WT. The virus titers (1 x 10^2 - 2 x 10^4 IU/ml) obtained from these cultures were considerably lower than those (1 x 10^7 IU/ml) obtained from primary culture of endothelial cells or astrocytes (27). The overall virus production in ts1-infected thymocytes, however, was consistently higher than that of WT-infected thymocytes. By 9 dpi, viral production in ts1-infected thymocytes reached 2 x 10^4 IU/ml and exceeded that of WT-infected thymocytes by about 10-fold.

To assess whether ts1 and WT exert any cytopathic effects on the thymocytes in vitro and whether they are comparable, the number of viable cells was determined every 2-3 days and assayed by trypan blue exclusion. As shown in Fig. 1C, by the first 3 dpi, ts1-infected thymocytes grew faster than WT-infected or uninfected thymocytes. However, the number of viable thymocytes infected with ts1 declined much faster after the initial activation. By 15 dpi, ts1-infected thymocyte number was about sixfold less than that of the uninfected control cells, and about fourfold less than that of WT-infected (Fig. 1C). These results indicate that both ts1 and WT induced thymocytic cell death, that ts1 after the initial activation induced T cell killing at a much faster rate than did WT virus. This data is in agreement with the results of our previous ex vivo studies of thymocytes isolated from ts1- or WT-infected mice (15).

Our system of long-term cultures of thymocytes from postnatal mice reported here not only reflect the capacity of culture primary murine thymocytes to function in vitro but also correlate to what happens to these cells in vivo during ts1 infection. This system may also serve as a tool to elucidate the mechanism(s) underlying ts1-MoMuLV-mediated thymocyte death. Furthermore, these primary thymocyte cultures will also be useful in studies on the mechanisms involved in controlling the fates of T cells during development.

ACKNOWLEDGMENTS

We thank D. Hollowell for her technical assistance, D. Walker for his help with the FACS analysis, and C. McKinley for her excellent assistance in the preparation of this manuscript. This work was supported by Public Health Service Grants AI 28283 and CA45124 and also by National Cancer Institute Core Grant CA16672.

REFERENCES


