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Production of Milligram Quantities of H-2K and Thy-1 Alloantigens by Large-Scale Mammalian Cell Culture (39793)

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The investigation of the structure and function of mammalian cell-surface antigens has become one of the most intriguing and intensive fields of modern biological research. These antigens allow a cell to communicate with its environment and may well provide the key to the detection of foreign or transformed cells by the immune system. The murine T lymphocyte provides an excellent model for the study of these molecules. In particular murine T lymphocytes express two antigens of special interest to immunologists, Thy-1 or θ and H-2 (1, 2). The Thy-1 alloantigen is found on murine and rat brain and thymus tissues where it reflects the state of differentiation. This antigen exists as one of two allelic forms, Thy-1.1 or Thy-1.2, in the various strains of inbred mice. Recent work on the rat analog indicates that Thy-1.1 from the thymus is a glycoprotein of 26,000 molecular weight (3), but its function remains a matter of speculation. The H-2 locus codes for the major histocompatability antigens in the mouse which consist of two polypeptide chains of 45,000 and 12,000 molecular weight (4) and are expressed in varying amounts on most of the tissues of the body. While some progress has been made in determining the partial structure of these molecules (5–9), little has been learned concerning their biological function due to the paucity of each component available. In order to gain a thorough understanding of these cell-surface antigens, each component must be purified away from other cellular materials in sufficient quantities to conduct reliable experiments. Such a procedure requires vast amounts of material since purification of 10,000-fold or more may be required. In order to provide this large amount of material we have employed large-scale mammalian cell culture to provide kilograms of murine thymic-derived leukemia cells each week. Our initial studies have centered on two murine T-cell lymphoblastoid cell lines, BW 5147 and ASL1-W. BW 5147 was derived from an AKR/J lymphocytic leukemia and expresses the antigens Thy-1.1 and H-2K^k, D^k (i.e., is H-2^k). ASL1-W was derived from an A-strain mouse lymphoma and has Thy-1.2 and H-2K^k D^d (H-2^a) on its surface. Thus, kilogram quantities of these cells provide milligram quantities of H-2K^k and Thy-1 alloantigens for study of their structure and function.

Methods and materials. Cell lines. BW 5147 was obtained from the Salk Institute, San Diego, California. ASL1-W was obtained from Dr. Mike Wolcott at the University of Alabama in Birmingham. Cells were grown in UAB-1 (RPMI-1640 plus extra glucose (2.5 g/liter) and asparagine (0.2 g/liter)) containing 10% fetal calf or horse serum.

Spinner cultures were started in 50-ml Bellco flasks and transferred to larger vessels up to 4 liters as the rate of growth permitted. Cells were maintained between 8 $\times$ 10^5 and 3 $\times$ 10^6 cells/ml during this stage by adding new media every 24 hr. Dissolved oxygen (D.O.) and pH and were not monitored at these levels. Large-scale culture was begun in a modified 12-liter bacterial fermentor (New Brunswick Scientific Co. Model 19, New Brunswick, N. J.) where pH was controlled at 6.9 $\pm$ 0.1 by sparging either CO_2 or air through the solution (10). At this stage cells frequently exceeded 3 $\times$ 10^6/ml. The full 12-liter vessel was then used to inoculate the 70-liter stainless-steel vessel, which in turn provided the inoculum for the 200-liter vessel. In both large vessels pH was maintained at 6.9 $\pm$ 0.1, temperature at 37°, agitation rate at 100 rpm, and the dissolved oxygen level was monitored. We found it necessary to continuously sparge air through vessels larger than 4 liters

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to maintain viability, and had little success in growing our cell lines in conventional 8-liter spinner flasks without sparging. Using this scale-up technique we have taken cells from 30-ml T-flasks up to the 70-liter culture vessel in 1 week.

Mice. AKR mice were obtained from Cumberland View Farms, (AKR/Cum) Clinton, Tennessee, and the Jackson Laboratory (AKR/Jax), Bar Harbor, Maine.

Quantitation of alloantigens. Thy-1 and H-2K^k antigens were monitored by quantitative adsorption of cytotoxic activity (11, 12). H-2K^k antisera (D-3b) was obtained from Dr. John Ray of the National Institutes of Health and was used at a dilution of 1:300. This antiserum was made in a (C3H · H-2^o × 129)F1 against C3H lymphocytes and recognized specificities 11, 25, 45, and 23 on the H-2K^k molecule. Thy-1.1 antiserum was AKR/Cum anti-AKR/Jax thymocytes and was used at a dilution of 1:600. Thy-1.2 was quantitated with AKR/Jax anti-AKR/Cum thymocytes at a 1:48 dilution. Adsorptions and cytotoxicity assays were performed in microtiter plates. Five hundred thousand ^{51}Cr-labeled AKR spleen (H-2K) or thymus cells (Thy-1) per well were used.

Results. As shown in Fig. 1, approximately 25 liters of BW 5147 cells at 9 × 10^5 cells/ml were used to initiate the culture in the 70-liter vessel on Day 0. The cells grew rapidly during the first 24 h and fresh medium was added twice during that period. On Day 1, 50 liters from the 70-liter vessel was used to inoculate the 200-liter vessel and both vessels were then operated simultaneously for 4 days. On Day 5 the entire 200-liter-vessel volume was harvested and, following replacement of an air-outlet filter, the 200-liter vessel was resterilized and reinoculated with the total content of the 70-liter vessel. The 200-liter vessel was operated for an additional 4 days, after which the growth period was terminated. Throughout the entire 9-day growth period cell viability exceeded 95%, the cells doubled every 10 to 16 hr, and densities frequently surpassed 4 × 10^6 cells/ml in both the 70- and 200-liter vessels. During this period, approximately 3.1 × 10^{12} BW 5147

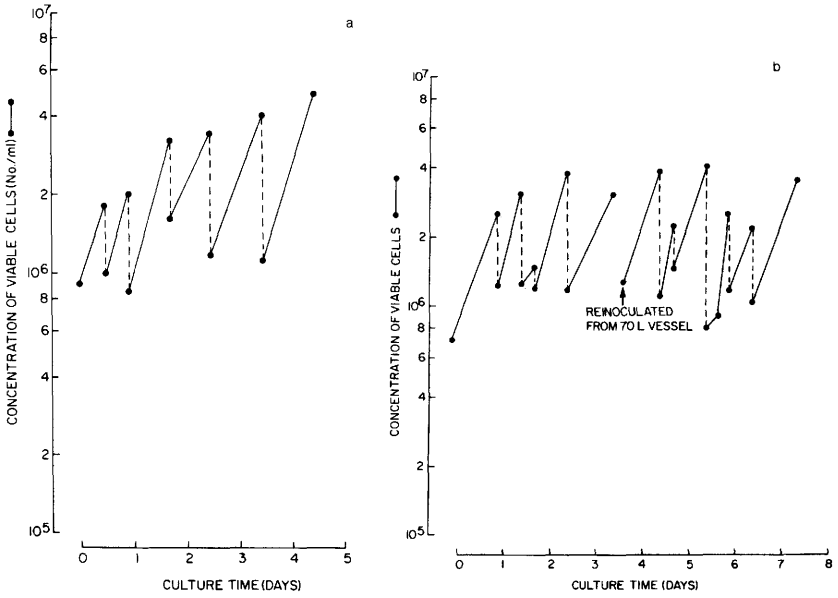

FIG. 1. (a) Growth of BW 5147 cells in the 70-liter vessel. The viable cell density in cells per milliliter, as determined by counting in a hemocytometer using trypan blue exclusion, is represented by ●——●. Dashed lines represent cell harvests and addition of new media to provide additional nutrients. Cells were grown in UAB-1 containing 10% fetal calf serum. (b) Growth of BW 5147 in the 200-liter vessel. On Day 4 the entire vessel was harvested and following resterilization the vessel was reinoculated from the 70-liter vessel. Dashed lines and ●——● are as in (a). Day 0 on this vessel corresponds to Day 1 on the 70-liter vessel.

cells, or over 3 kg (wet weight), were harvested. Similar growth characteristics were observed for the ASL1-W cell line in the 70-liter vessel except that the maximum cell density was slightly over 2×10^6 cells/ml.

Figure 2 displays the alloantigen expression on ASL1-W in the 70-liter culture vessel. Thy-1.2 expression is displayed as thymocyte equivalents per cell. As can be seen, ASL1-W expresses four- to sixfold more Thy-1 on its membrane than does the normal thymocyte. H-2K^k on ASL1-W is compared to the amount on normal spleen cells since that tissue is the highest known source of H-2 molecules in the mouse. Approximately equal amounts of H-2K^k were detected on ASL1-W and AKR spleen cells. Similar results were obtained for BW 5147 cells in both the 70- and 200-liter vessels except that Thy-1.1 expression averaged only two times that of normal thymocytes and H-2K^k was approximately one-half the amount on spleen cells.

Discussion. As we began this approach to producing large quantities of mammalian

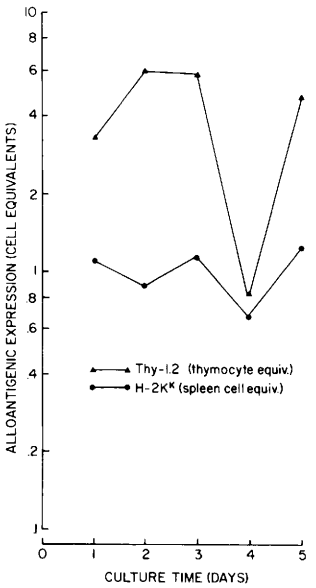

FIG. 2. Alloantigen expression of ASL1-W in the 70-liter culture vessel. Antigen expression on a per-cell basis was compared to normal AKR/Cum spleen (H-2K) or thymus (Thy-1) cells by quantitative absorption of cytotoxic activity, and the ratios are plotted. Cells were grown in UAB-1 supplemented with 10% horse serum.

cell-surface antigens, two potential problems confronted us: (i) Possibly cells would not grow in large-scale culture vessels as in the smaller conventional spinner flasks, and (ii) cells might cease to express surface antigens in large-scale culture. As shown in Figs. 1 and 2, neither of these possibilities has materialized. Indeed, cells appear to grow better in our large vessels where pH and dissolved oxygen can be maintained at optimal values. While BW 5147 cells seldom exceed densities of 3×10^6 cells/ml in smaller flasks, a density of 4×10^6 cells/ml is easily obtained in our large-scale facility. Moreover, doubling times averaged 10 to 16 hr in the large vessels as compared with a doubling time approaching 24 hr in spinner flasks. This allows a harvest of $\frac{3}{4}$ the volume of each vessel, or approximately 200 liters, daily from the 70- and 200-liter vessels.

The increased antigen expression on our transformed cell lines compared to that on normal cells is most likely attributed to their increased surface area. These cell lines are much larger than normal cells and, given the same density of antigen per surface area, would be expected to exhibit more of these antigens per cell. Normal thymocytes express approximately 25% as much H-2 as do spleen cells (13). Thus, ASL1-W which express four times the amount of Thy-1 as normal thymocytes also express four times the amount of H-2K^k.

As can be seen in Fig. 2 antigen expression on the cells did vary somewhat in our system throughout the culture period. Cikes (14) reported decreased H-2 expression on cells in rapidly growing cultures. We have investigated Thy-1 and TL expression as a function of the stage of growth of cell lines grown in a 14-liter vessel at constant pH (15). Under these conditions Thy-1 was found to be expressed in greatest quantities on cells in the logarithmic phase of growth. Thus, the variation in antigen expression cannot be explained by differing percentages of cells in the various stage of growth since any increase in H-2 should be accompanied by a decrease in Thy-1, and, as can be seen in Fig. 2, this is not observed. In our study TL did not vary greatly during the various phases of growth (15). This is consistent with the data reported for HL-A

expression by Pellegrino *et al.* (16). The factors affecting antigen expression in our large vessels are unknown at present but studies to determine these parameters are currently underway. All sera utilized in this study were from a single lot and would therefore not contribute to variation. It should be noted, however, that we have never observed a net decrease in antigen expression during prolonged culture in our large-scale facility.

Since our cell lines express more antigen per cell than normal thymocytes, the 3.1×10^{12} BW cells represent approximately 60,000 thymus equivalents of Thy-1 and over 15,000 spleen equivalents of H-2, produced at a fraction of the cost of 15,000 mice. The number of H-2 molecules on spleen cells and Thy-1 on thymocytes is approximately 500,000 per cell (17, 18). Thus, the 3.1×10^{12} cells represent more than 3 μmole of Thy-1 antigen and 500 nmole of H-2K^k. This amount of material should greatly facilitate the complete structural and functional determination of these molecules.

Large-scale mammalian cell culture is now an economical, practical approach to answering many of the questions in molecular biology. Recent work indicates that mammalian cells may be grown for prolonged periods in simple serum-free media (19). Indeed, preliminary work in our laboratory has shown that the BW 5147 cell line grows as well in 2% horse serum as in 10% fetal calf with the same Thy-1 and H-2 expression, reducing serum costs by an order of magnitude. In addition to the two membrane components, H-2K and Thy-1, under investigation in our laboratory, fractionation of the cellular components by differential and gradient centrifugation provides nuclear and cytoplasmic material in previously unattainable quantities. Investigators can now design experiments to utilize this vast source of material for unraveling many mysteries of the mammalian cell.

Summary. A system for the production of milligram quantities of cell-surface alloantigens is presented. This system consists of 70- and 200-liter mammalian cell culture vessels capable of growing murine T-cell lines in suspension culture. The two cell lines examined exhibited superior growth properties in these vessels than in small spinner flasks. Expression of H-2K^k and Thy-1 alloantigens were higher on these cell lines than normal thymocytes. Approximately 3 μmole of Thy-1.1 and 500 nmole of H-2K^k antigens were produced in an 8-day period. The potential of the large-scale mammalian cell culture approach is briefly discussed.

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