# BAI Strain A Avian (Myeloblastosis) Leukosis Virus from Myeloblast Tissue Culture.* (31468)

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Studies on the BAI strain A avian tumor (myeloblastosis) virus(1,2) have been hampered by restrictions on the amount of virus available from the blood plasma of birds with leukemia induced by the agent. A possible alternate virus source was suggested by the capacity of myeloblasts from leukemic chickens to grow and elaborate virus *in vitro*(3). Under suitable conditions, about $2.5 \times 10^7$ cells/ml liberate 20-60 virus particles/cell/hour, and the virus content of the culture fluid reaches levels of $3\text{-}5 \times 10^{10}$ particles/ml before medium change is necessary. In a recently devised culture system virus concentrations attained levels in the medium comparable with those in many plasmas from leukemic birds(4). This has been accomplished by use of high culture content of myeloblasts and short-term control of nutritional and pH levels by dialysis against an appropriate external medium. This report describes the system and some aspects of the agent derived from the culture.

*Materials and methods.* Fig. 1 illustrates the apparatus:‡ (A) culture and dialysis chambers; (B) reservoir for feeder fluid; and (C) the pump for fluid circulation. A 3-liter pyrex flask (A) was fitted with 2 ports, the bottom was removed slightly below the greater curvature, and the cut surface was smoothed and squared in the flame. The base, in 2 parts, was milled from 2 nylon blocks 1″ × 10″ × 10″ (Almac Plastics, Inc., New York). The upper, (F) (inset of Fig. 1), was machined, 22.3 mm OD, with a square inner shoulder, (E), 5 mm deep and 3 mm wide to receive a rubber gasket and the flask edge and shaped further, 15 cm final open diameter, with a curvature simulating that of the removed part of the flask. A well 0.7 cm deep and 15.3 cm diameter was cut in the lower block, (I), and 2 holes were bored from opposite sides with an upward slant to open at the upper edge of the well. A sheet of teflon (Engineered Plastics, Inc., Gibsonville, N. C.) 1 mm thick was cut to a disc to fit between the blocks and milled with oblong fenestrations, (H), to support the dialysis membrane tubing 5¾″ flat width (Arthur H. Thomas Co., Philadelphia, Pa.) as indicated at (G). Joints between lower base and teflon support; teflon and dialysis membrane; and membrane and upper base were sealed with stopcock grease. The parts of the base were held together with 8 ¼″ × 2.5″ stainless steel bolts. A clamp to hold the flask to the base after autoclaving was a lucite disc ¾″ thick fitted over the flask as shown and attached to the base by 4 bolts.

The reservoir, (B), was a 4-liter pyrex aspirator bottle, and the pump, (C), a rubber tube with ball valves within a glass chamber and activated by air pressure delivered

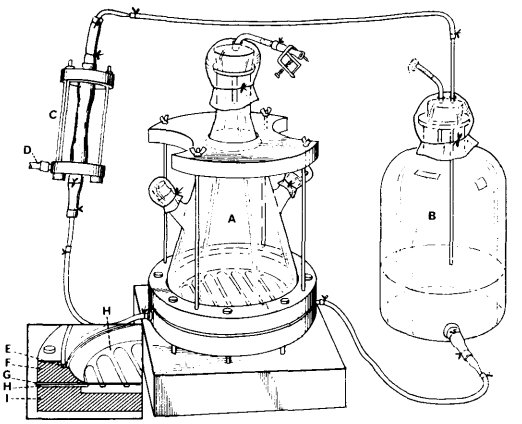


FIG. 1. Culture chamber.

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‡ Constructed by the Surgical Instrument Shop, Duke University Med. Center.

at (D) and regulated by a windshield wiper motor (not shown).

For sterilization, the teflon support, dialysis membrane and bases were assembled, and the base bolts were tightened lightly. The glass flask with cotton-stoppered mouth and ports was pressed (not clamped) onto the rubber gasket, and 100 ml of distilled water were introduced onto the membrane. The rubber stopper of the reservoir, (B), was fitted loosely. After 1 hour at 15 pounds pressure, the flask clamp (not autoclaved) was installed; the bases were tightened firmly; and, in the sterile room, the cotton plugs were removed; the water was poured out through the port; and white rubber stoppers with paper shields closed the openings.

Myeloblasts were from Line 15 White Leghorn chicks(1) with advanced leukemia induced with BAI strain A virus. Cultures, 2-4, of 50-ml volume in 500-ml Erlenmeyer flasks were prepared with myeloblasts ($2.5 \times 10^7$ cells/ml) from a single bird. Medium was changed as necessary, and the cultures were divided until the total cell number was about $100 \times 10^8$. The cells were then sedimented and resuspended in 100 ml of 50% chicken serum in medium 199 containing antibiotics, and added glucose and folic acid(3,5) and introduced into the culture flask. The reservoir received 1 liter of the same medium but with only 25% chicken serum. Serum in contact with the cells was heated at 56°C for 30 minutes and centrifuged for 2 hours at 105,500 *g* before use.

The cultures were incubated at 37°C with the culture chamber on a gyratory oscillator (70-80 RPM) (Gyrotory Shaker, S3, New Brunswick Scientific Co., New Brunswick, N. J.)(6). Virus particle number was estimated by measurement of adenosinetriphosphatase (ATPase) activity(5,7) or electron microscopic count(8), and cell counts were made in Neubauer counting chambers with trypan blue(6) to distinguish nonviable cells. Virus infectivity was assayed in 3-day-old chicks by the latent period procedure(9).

*Results.* BAI strain A virus concentration attainable in myeloblast cultures is dependent on cell density in the medium and length of culture period. From the rate of virus production, it was evident that the cell content should be $10^8$/ml or greater to yield virus in desirable concentrations. Preliminary experiments showed that such cultures could not be maintained for the necessary periods without a feeder system for control of pH and supply of nutrients. The efficacy of various feeder fluids was tested by varying the concentrations of chicken serum and glucose or by addition of pancreatic substance(10). Serum concentrated 4-fold by pervaporation was also added to the culture medium in some experiments. In 2- to 8-day cultures, virus reached concentrations of $4.2\text{-}7.8 \times 10^{11}$ particles/ml. Nevertheless, virus infectivity was low, less than 10% of the control on a virus-particle basis in 72-hour cultures, for example, and the proportion of nonviable cells increased to levels of 25% in the same period. Moreover, fresh cells were required for each culture.

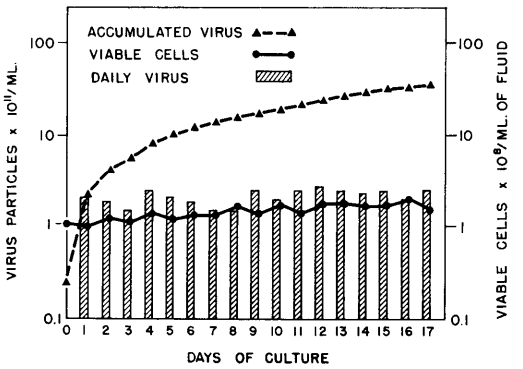


FIG. 2. Daily cell and virus concentrations and accumulated virus/ml in a culture carried 17 days.

Further experiments resulted in development of the procedure yielding the typical data described in Fig. 2. Myeloblasts, $1.1 \times 10^8$ cells/ml, were established in culture as described. The cells were removed each day, counted, sedimented and resuspended in fresh medium. Between 0 and 5 days, the cells increased to $1.6 \times 10^8$/ml. On the 5th day, the cells in 20 ml of the culture fluid were discarded, and the remainder were put into fresh medium yielding a cell density of $1.3 \times 10^8$/ml. This increased to $1.9 \times 10^8$/ml by the 13th day, and 10 ml of suspended cells were discarded. On the 17th day, cell concentration was $1.7 \times 10^8$/ml. On alter-

nate days, the culture was transferred to a fresh chamber to eliminate a fatty precipitate collecting at the air-fluid junction, and the feeder fluid was replaced at this time.

An average of 88.1 ml of culture fluid containing an average of $2.1 \times 10^{11}$ virus particles/ml were harvested daily. The total yield in 1499 ml was $316.6 \times 10^{12}$ particles which represented about 237 mg dry weight of virus ($7.5 \times 10^{-16}$ g dry weight/particle).

This yield was compared with that from 113 chicks inoculated with the agent at 3 days of age. Of these, 31 developed a plasma virus content of $1 \times 10^{11}$ or more particles/ml. The total plasma yield was 61 ml with a virus content of $7.39 \times 10^{11}$ particles/ml or $45.1 \times 10^{12}$ particles. Virus from the culture was thus approximately 7-fold that from the chickens in about the same time.

Infectivity of culture virus relative to that of agent in plasma from leukemic chicks is shown in Table I. Culture fluids harvested at various 24-hour intervals in an experiment like that of Fig. 2 were inoculated along with the control plasma virus in dose groups of 30 chicks each. Potency ratios calculated on the basis of virus particle number in relation to latent period of disease onset(11) varied from about 2 to 0.63 indicating that culture virus infectivity was closely similar to the infectious activity of plasma virus.

Fig. 3 shows the comparative sedimentation characteristics of virus preparations(12) from tissue culture and from plasma centrifuged in a potassium tartrate density gradient(13). The respective virus boundaries were at the same level, but the boundary of the tissue culture virus was somewhat more diffuse upward (lower density) than that of the comparable amount of plasma-derived agent. Electron micrographs of the preparations sedimented and dried on an agar surface(8) showed typical virus particles and also a small population of amorphous nonviral particles generally smaller than the agent. This disclosed a contaminant which was also visible in sedimented pellets as a trace of yellowish material layered in the clear virus component. On disc gel electrophoresis(14), components of virus after treatment with sodium deoxycholate migrated in a pattern essentially indistinguishable, Fig. 4, from that of constituents of the virus from plasma treated in the same way.

TABLE I. Relative Infectivity (Potency) of Tissue Culture Fluids in Comparison with Standard Control Virus-Containing Blood Plasma.

| Day of culture | Relative potency | Day of culture | Relative potency |
|---|---|---|---|
| Control | 1 | 8 | .63 |
| 1 | 2.02 | 16 | .78 |
| 4 | 1.84 | 17 | 1.75 |
| 5 | 1.8 | | |

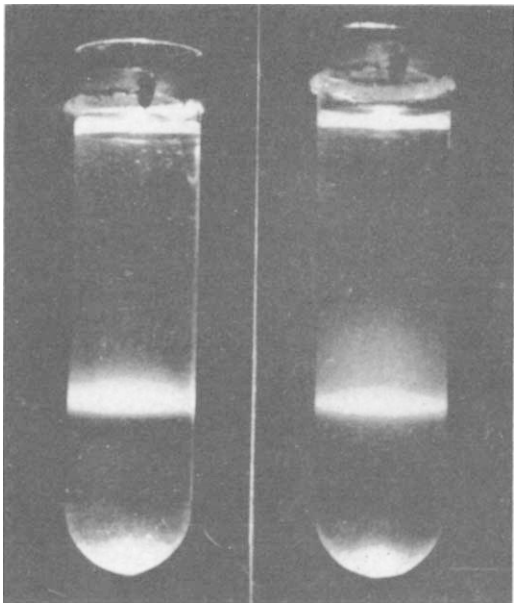

FIG. 3. 0.1 ml of virus concentrated from tissue culture fluid (right) and myeloblastosis plasma (left) by one cycle of sedimentation, resuspension and clarification(12) was layered on a preformed gradient of 2%-40% potassium tartrate in 0.05% bovine albumin and centrifuged 90 min at 40,000 rpm in a Spinco SW50 rotor.

The culture fluid has been used thus far primarily as a routine source of virus for isolation of ribonucleic acid (RNA)(15). Yields of high molecular weight viral RNA (about 60s) ranged from about 30-60% of the total RNA extracted. Other consistent RNA components sedimented at about 16s and 26s with a larger peak at about 5s. These yields were comparable to those from virus isolated from plasma, and variations appeared to depend more on manipulation of the virus and the particular preparation than on the source of the agent.

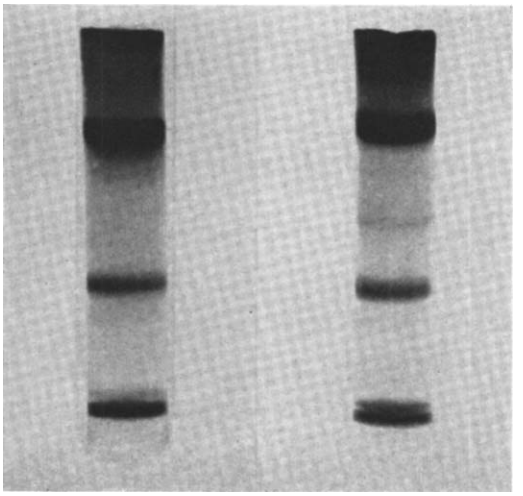

FIG. 4. Disc gel electrophoretic pattern of proteins of virus from plasma (left) and from culture fluid (right) purified by 4 cycles of alternate high (30,000 *g*) and low (2500 *g*) centrifugation(12). Virus pellets were treated with 1% sodium deoxycholate, and .05 ml samples (.56 mg virus dry wt.) were applied to the columns. Electrophoesis was similar to that described(14) using 7% acrylamide and a running pH of 9.5. The current was 5 mAmp. for each column, and duration of the run was 30 min. Gels were stained with Amido Schwartz and destained with acetic acid. The lowest band is the detergent moving with the tracking dye of bromphenol blue.

*Discussion.* With heightened interest in viruses causing tumors, there is an increasing need for such agents in quantities sufficient for comprehensive physical and chemical studies. Usefulness of the BAI strain A virus in this respect has already been demonstrated (1,16). Although the virus may occasionally reach levels of $2 \times 10^{12}$ particles/ml of plasma from birds with myeloblastosis, the concentrations are usually considerably lower, and use of the chick as a source of agent is tedious and very costly. The present results demonstrate the feasibility of producing the virus with a simple and inexpensive *in vitro* system in amounts far exceeding those obtainable from the bird under practical conditions.

The shape of the chamber, swirling motion imparted by the oscillator and the ratio of culture volume to that of the chamber were of much importance as previously seen(3,6). Substitution of other types of chambers and stirring with various devices and air bubbling resulted in damage to the cells. Daily virus harvest and medium change were essential for preventing excessive virus inactivation and supporting cell growth which permitted repeated use of the same cells. Maintenance of cell density at or less than $2 \times 10^8$/ml was critical for continued cell growth and viability.

Virus preparations obtained from culture fluid by centrifugation in alternate high and low gravitational fields were not of the same homogeneity as those yielded by similar treatment of virus-containing leukemic plasma. The contaminant of unknown origin could be largely but not completely eliminated by repeated cycles of sedimentation. Nevertheless, the culture virus showed essentially the same infectious activity on a virus-particle basis as that from leukemic plasma and has proved entirely satisfactory as a source of virus RNA, showing the same components and component distribution as plasma virus RNA(15). Further work is necessary to find the means for eliminating the contaminant.

*Summary.* A combined culture-dialysis chamber was devised for producing BAI strain A virus *in vitro* in amounts greatly increasing the scope of physical and chemical studies of the agent. The yield of virus was approximately 15 mg dry wt/day/chamber. Infectivity of the culture virus was the same on a particle basis as that of virus from leukemic chicks. Properties of the culture virus were indicated by gradient centrifugation, gel electrophoresis and by the character of RNA extracted from the agent.

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## Relationships of V1030, V1880 and Pett Viruses to the Prototype A24 Coxsackievirus.* (31469)

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V1030 and V1880 viruses were isolated in 1961 from fecal specimens of 2 children vaccinated with inactivated polio-diphtheria-pertussis-tetanus vaccine. The two viruses possessed properties of picornaviruses, but no definite relationship between them and the known enteroviruses tested could be established(1). Subsequently they were submitted to this laboratory as potentially new enterovirus prototypes. Our preliminary study of these 2 viruses and reference antisera indicated a relationship between V1030 and coxsackievirus A24. Further study showed that V1030 and V1880 viruses were related to each other and that both were related to the prototype strain of coxsackievirus A24. Another unassigned virus, named Pett, was included in this study because of some evidence of antigenic relationship between it and coxsackievirus A24(2). The Pett virus was isolated during the summer of 1956; it is the prototype of 8 agents obtained from fecal specimens of 10 children during an outbreak of respiratory illness(3).

*Materials and methods. Viruses.* V1030 and V1880 were received from Dr. Wolf Henigst of Munich, Germany, as infected fluid overlays of the sixth passage in primary human amnion cultures. Coxsackievirus A24 was the National Institutes of Health (NIH) reference virus prepared at this laboratory. The Pett virus was received from Dr. Julius A. Kasel of the NIH as infected HeLa cell culture fluid. All 4 viruses were passed in primary human amnion cells and purified by 3 terminal dilution passages. For the immunization of monkeys and performance of serologic tests a stock of each virus was prepared in Roux bottles containing human amnion cells.

*Antisera.* Three monkeys were inoculated using the schedule described for enteroviruses (4). One or two additional booster doses were given in order to obtain satisfactory homologous antisera.

*Cell cultures.* Human amnion cell cultures were used for (a) propagation of all 4 viruses, (b) performance of serum neutralization tests and (c) preparation of both hemagglutinating and complement fixing antigens. For determining spectra of cell susceptibilities of V1030 and V1880 viruses, cell cultures of WI-38, rhesus and green monkey kidney and BHK-21 were used. Growth and maintenance of these cells were according to the procedures described by Schmidt(5).

*Newborn mice.* One-day-old white Swiss mice were inoculated either intracerebrally and intraperitoneally or intracerebrally and subcutaneously. When no symptoms appeared, mice were sacrificed after 14 days and a 20%

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