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A Quantitative Assay for Avian Myeloblastosis Virus.* (32316)

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Various methods for the assay of avian myeloblastosis virus (AMV) have been reported in the literature. Direct counts of virus particles with the electron microscope and assays of virus-associated adenosine triphosphatase have been used by Mommaerts *et al* (1) and by Beaudreau and Becker(2). Tissue culture techniques for the assay of infectious AMV include the interference test with Rous sarcoma virus and the fluorescent focus test described by Vogt and Rubin(3). The neoplastic potential of AMV has so far been assayed only with endpoint procedures performed *in vivo* or *in vitro*(4,5,6). Since endpoint techniques lack precision and economy, it was desirable to design an assay based on enumeration of individual lesions caused by the oncogenic action of the virus *in vitro*. Such an assay is described here.

Materials and method. The stock of AMV used to infect chicken yolk sac and bone marrow cultures consisted of plasma obtained from leukemic birds. The details of the preparation of the virus stock have been published (7). The modified Eagle's medium and serum supplements were those recommended by Baluda and Goetz(6). Chicken serum added to the tissue culture medium was always pretested for the presence of antibody against AMV and for cell toxicity. Primary yolk sac cultures were prepared from individual fertile chicken eggs on the 14th day of incubation according to a procedure reported in the literature(6). From the same eggs, embryos were excised and used for fibroblast cultures in order to determine genetic susceptibility to avian tumor viruses by challenging with Rous sarcoma virus pseudotypes of the A and B subgroups(8). By this technique most of the embryos as well as the corresponding yolk sac cultures were susceptible to all serotypes of subgroups A and B but some embryos and their respective yolk sac cultures were resistant to the subgroup B serotypes of avian leukosis viruses. In accordance with published work, fully susceptible embryos were designated as C/O and embryos showing selective genetic resistance to subgroup B were termed C/B(8). The source of chick embryos was

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line 934F, a hybrid white leghorn line of Hyline Farms, Des Moines, Iowa. Fertile eggs of this line were obtained from a local hatchery. The eggs used had an incidence of congenital infection with avian leukosis viruses that was about 1% (Vogt, private communication). For the focus assay of AMV, 8 $\times 10^6$ cells were seeded in 2 ml of complete medium on a 35 mm Falcon plastic dish. The plates were fed with fresh nutrient medium once 6 days after seeding. On the next day, the cell monolayers were infected with 0.1 ml of the appropriate virus dilution. Three dishes were used for each virus dilution. No difference in number of foci was found when virus adsorption was allowed to occur for 4-5 hours. For practical reasons therefore the infected cultures were incubated overnight. The fluid medium was then replaced with 2 ml of nutrient agar. This overlay consisted of 5% heat inactivated (30 min 56°C) chicken serum, 5% heat inactivated calf serum, 10% tryptose phosphate, 0.5% Difco purified agar in a modified Eagle's medium(6). The cells were incubated under this primary overlay for 6 days. At that time 2 ml of a second overlay were spread on the dishes, and the cultures were incubated for an additional 6 days. At around 12-14 days after infection foci of transformed cells were large enough to be counted at 25× magnification with the inverted microscope.

Results. The morphology of a transformed focus after infection of chick yolk sac cells appears as clear area 2-5 mm in diameter where packed myeloblasts can be distinctly seen (Fig. 1). To determine whether the number of foci obtained by this method was proportional to the virus concentration, serial dilutions of virus were assayed. As shown in Fig. 2, there was a linear relationship between the number of foci and the concentration of AMV in the inoculum. Variation of the focus counts occurred primarily between different embryos and may have been due to the physiological condition of the cells. The virus stock represented on the graph had a titer of 1.5×10^5 FFU/ml (focus forming units) on susceptible C/O cells. In contrast, when the same virus stock was used to infect C/B cells an at least 30-fold reduction in transforming

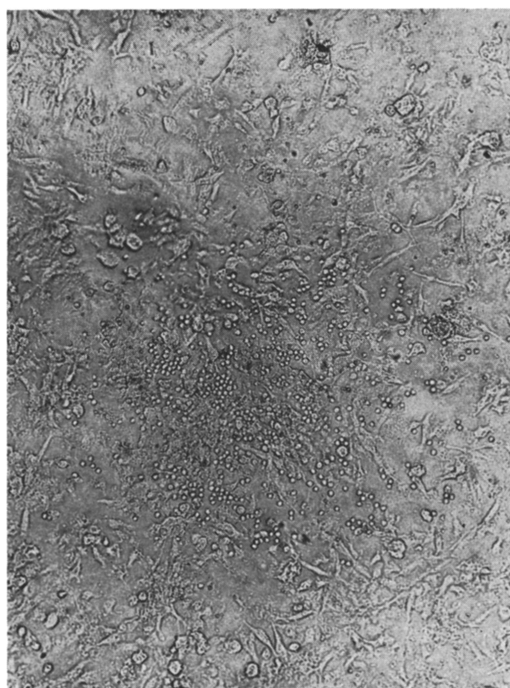

FIG. 1. AMV transformed focus 12 days after inoculation of chicken yolk sac cells (×47).

titer was observed. Table I shows results obtained with 9 individual C/B yolk sac cultures, as compared to the average titer of 8 C/O cultures. The reason for this resistance of C/B cultures to AMV-induced transformation is being investigated. The same virus suspension had a titer of $1.0 \times 10^{6.0}$ transforming units per milliliter when assayed on C/O yolk sac cultures by endpoint procedures. This means that about 15% of the total particles which appear as transforming units by the endpoint dilution method are able to form foci by the focus assay procedure. A similar ratio of focus formers to infectious units has

TABLE I. Transforming Titers of AMV in C/O and in C/B Yolk Sac Cultures.

Cell phenotype	Egg No.	Titer, FFU/ml
C/O	Avg of 8 eggs	1.5×10^5
C/B	1	1.2×10^3
	2	9.0×10^2
	3	$<4.5 \times 10^2$
	4	3.1×10^3
	5	4.9×10^3
	6	$<4.5 \times 10^2$
	7	4.5×10^2
	8	2.3×10^4
	9	$<4.5 \times 10^2$

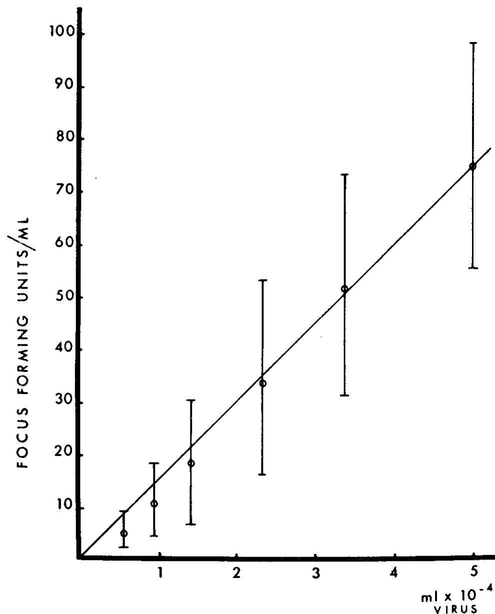

FIG. 2. Relationship between concentration of AMV and number of transformed foci in C/O yolk sac cultures. Each point represents the mean of counts from 8 experiments. Bars represent standard deviation for the same experiments.

been reported in RSV focus assay(9). A linear relationship between virus concentration and number of foci was also observed in cultures of bone marrows obtained from individual one-day-old chicks. In these cases genetic resistance could not be tested for and the average transforming titer of the AMV stock on 6 sets of bone marrow cultures was 1.6×10^4 FFU/ml.

Discussion. The data obtained in this study show the linear dependence of the number of foci on virus concentration. This proves that one AMV infectious unit is sufficient to initiate a focus. This focus assay will permit more experiments in the analysis of viral oncogenicity. For instance, cloning of viral populations derived from single infectious units will elucidate the question of the multipotency of this virus.

There are no clear data on the growth curve analysis of AMV in chicken yolk sac cultures. Since viral multiplication and cell transformation seem to be two separate events in AMV infection, the focus assay will be of help in clarifying this point.

One aspect of this study is concerned with the difference in the transforming activity between susceptible (C/O) and resistant (C/B) chick embryo cells. Data to be published soon, demonstrate that susceptibility and resistance to AMV transformation are based on the genetic properties of certain chicken strains, and on the heterogeneity of AMV. As has been shown, avian myeloblastosis virus strain BAI-A contains at least 2 viral agents which have been termed AMV-1 and AMV-2(10). The latter seems to be the only viral component for inducing leukemia and cellular transformation. Our studies clearly indicate that C/B cells do not allow penetration and multiplication of AMV-2 unless it is mixed together with AMV-1 component. This is due to phenotypic mixing of the two viruses and explains the lower efficiency of transforming activity in C/B cells reported here. The role of AMV-1 with regard to the leukosis virus complex is currently being studied.

Summary. A quantitative assay for a leukosis virus has been developed. Chick embryo yolk sac and bone marrow cultures were infected with serial dilutions of avian myeloblastosis virus and were overlayed with purified agar dissolved in nutrient medium. Under these conditions well defined foci of transformed cells developed. There was a linear relationship between the number of these foci and the concentration of virus.

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