Rous Sarcoma Virus Infection of Chicken Buffy Coat Macrophage.* (32155)

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It has been shown that extensive fibroblastic elements developed in some chicken buffy coat macrophages in tissue culture(1). These developed from connective tissue contamination in the buffy coat which gained entrance depending on the method of obtaining the blood. The development of the connective tissue elements in the contaminated macrophage cultures was greatly stimulated by chick embryonic materials such as filtered chorioallantoic membrane extract or Rous sarcoma virus (RSV) prepared from virus induced chorioallantoic membrane tumors. Macrophage cultures which developed fibroblastic elements after treatment with RSV preparations invariably supported extensive multiplication of the virus with subsequent cell changes in the fibroblasts.

The *in vitro* susceptibility of chicken macrophages to the RSV has remained in dispute. The claims of the earlier investigations (2,3) that the buffy coat macrophage cultures can be infected by RSV were questioned(4,5), on the grounds that fibroblastic contamination of the monocyte cultures could occur since unfiltered embryonic extracts were used for making the culture. This report indicates that pure chicken buffy coat macrophage cultures, in the complete absence of fibroblastic elements, can also harbour the sarcoma virus for as much as 4 months after virus treatment.

Chicken macrophages in tissue culture. A brief resume of certain pertinent *in vitro* characteristics of chicken macrophages from the chapter on 'Macrophages'(6) helps to explain the paucity of reports dealing with virological studies utilising chicken macrophages(7,8,9). "Whilst it is relatively easy to set up a great number of buffy coat cultures in flasks and even to establish pure macrophage populations, to maintain and carry on such culture sets for long periods is very difficult. Even when a set of cultures has been successfully established, sooner or later greater variation in morphology from flask to flask are encountered than in the case with other cell lines. Continuous propagation of the macrophage cultures by transplantation of the cells either by detaching them mechanically or by means of trypsin or versene have not been successful and as yet cloned cultures of macrophages do not seem to have been established." Our experiments agree with this and therefore detailed quantitative analysis on the susceptibility of chicken macrophages to RSV have not been feasible.

Material and methods. Detailed procedures for obtaining buffy coat macrophage cultures from blood obtained by both cardiac and venous routes have been described previously (1). In the venous route technique 7 to 10 drops were allowed to flow out before blood was collected in a syringe in order to prevent fibroblastic contamination of the blood(1). Cultures were maintained in a medium containing 50% chicken serum. Neutralizing antibodies to RSV in these sera were determined according to the method described by Bang and Foard(10). The sera used were found to possess a neutralization index of less than 2 for the sarcoma virus, which is considered negative.

Viruses. CVA strain. In the majority of the experiments the CVA strain(10) of RSV was used for infection. This strain was derived from and has antigenic properties identical to the Bryan strain. Stock virus preparations consisted of a 20% tumor extract of a pool of large second passage chorioallantoic membrane tumors initiated by the virus, and had a titre of 1×10^6 vascular

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lesions forming units (v.f.u.) in chick embryos.

High titre Bryan strain: Stocks of this strain have been shown to contain a Rous associated agent and it is also believed that the sarcoma virus itself is defective(11). Stock samples of this strain were kindly provided in 1 ml quantities by Dr. M. K. Kaighn, Department of Embryology, Carnegie Institution of Washington, Baltimore. These preparations had a titre of 1.5 to 2 $\times$ 10^7 tissue culture focus forming units (f.f.u.) per ml of wing web tumor extracts.

Schmidt-Ruppin strain (S-R): Stock virus of S-R strain titred 1.6 $\times$ 10^5 f.f.u. per ml of chicken breast tumor extract. The original tissue culture stock was obtained from Dr. R. M. Dougherty, State University of New York, Upstate Medical Center, Syracuse.

Stocks of the CVA and S-R strains were prepared according to methods described earlier(1) involving high speed homogenization in 0.05 M citrate buffer at pH 6.8, rapid freezing and thawing, and 2 cycles of centrifugation at 2500 rpm at 4°C.

Assays. Presence and quantity of RSV was determined by the method of intravenous inoculation of chick embryos(12). In experiments for the assay of S-R strain of RSV the tissue culture assay method(13) was used.

Cell associated virus was tested for by subcutaneous inoculation in the inguinal region of two-day-old chicks. Cells from infected cultures were removed mechanically with a rubber policeman and suspended in about 0.5 ml of fresh medium. Due to the extreme fragility of the cells, only an approximate estimate of the cells inoculated was possible. The cell counts ranged from 7 $\times$ 10^3 to over 8 $\times$ 10^5 in the inoculum. The inoculated chicks were observed for over 30 days for tumor appearance at the site of inoculation. Virus presence in the tumor was inferred when red vascular lesions developed in the tumor bearing animals or was tested by assaying an extract of tumor itself.

Method of infection. Five-day-old cultures were treated with 0.2 ml of a 50% virus preparation in balanced salt solution at room temperature (26°C) for 40 minutes. The cultures were rocked gently at intervals and after absorption the treatment fluid was drained and replaced with culture medium.

TABLE I. Tumor Induction by Sarcoma Virus Treated Macrophage Culture Cell Inoculations.

Exp	Animal	Source of blood	No. positive/No. tested: Infected	No. positive/No. tested: Uninfected controls
1	5	C	2/2	—
2 & 3	24	C	2/7	0/1
4	362	C	2/2	—
5	6	C	8/8	0/2
6 & 7	132	V	1/3	0/2
8 & 9	155	V	2/6	0/2
10–12	156	V	5/6	0/2
13–16	129, 197 218, 347	V	1/11	0/9
			23/45	0/18

C—cardiac route; V—venous route.

Fluorescent antibody technique. Hyperimmune sera to the CVA strain of RSV were prepared in commercially obtained chickens following the development of regressing tumors. These chickens were challenged at least twice after tumor regression with 10^5 to 5 $\times$ 10^5 v.f.u. of virus at intervals of 10 days, and bled a week after the last challenge.

Fluorescent antibody conjugation followed the procedure described by Coons(14). The protein to dye ratio was 1:50 and the conjugate was absorbed twice with beef liver acetone powder at 100 mg per ml for 1 hour. Each ml of conjugate was further absorbed with 10^7 chick embryo fibroblasts for 1 hour twice.

Cultures grown on coverslips were fixed in cold acetone for 15 to 20 minutes. The coverslips were stained for ½ hour at 37°C, washed with phosphate buffered saline and mounted in buffered glycerol.

Results. Table I shows the results of 16 experiments in which RSV treated chicken buffy coat macrophage cultures were tested by subcutaneous inoculation in 2-day-old chicks. Some of the cultures were also tested for presence of free virus in their supernatant fluid.

Nearly 51% of the virus treated cultures induced tumors when the cells of these cultures were tested 14 to 116 days after virus treatment. In 9 tumor bearing animals red vascular lesions were observed all over their bodies indicating the liberation of virus from

TABLE II. Tumor Induction by Macrophage Cultures Treated with Different Strains of Rous Sarcoma Virus.

Virus strain	No. positive/No. tested
CVA I	18/37
Bryan high titre I and II	3/4
Schmidt-Ruppin	2/4
	23/45

the tumors. Also, 3 other tumor extracts had a virus titre of 5.5×10^4 to 3.5×10^7 v.f.u. per gram of tumor tissue. In some experiments all the virus treated cultures were positive for tumor induction while in others only a few induced tumors.

Cell inoculations from macrophage cultures treated with all of the 3 different strains of RSV induced tumors in 2-day-old chicks as shown in Table II. None of the untreated control cultures induced tumors when tested in 2-day-old chicks (Table I). All of these macrophage cultures lacked fibroblastic colonies during the entire period of observation.

Of 16 cultures which were tested and found positive for tumor production only 6 (<50%) contained free virus in their supernatant fluid when their fluids were repeatedly tested by intravenous inoculation in chick embryos (Table III). The amount of virus was always less than 10^2 v.f.u./ml of fluid. In 3 cultures (6,1, 6,2 and 6,8 Table III) free virus was absent at first but was detected by chick embryo inoculation of the supernatant fluid many days after the cells themselves had been shown to induce tumors in 2-day-old chicks. The supernatant fluid of

TABLE III. Virus in the Supernatant Fluid of Macrophage Cultures Positive for Tumor Induction when Inoculated as Cell Suspension. All cultures here included were positive.

Serial No.	Animal No.	Culture No.	Titre of virus*	Tumor induction by supernatant fluid†	Days culture tested‡
1	5	1	0 (7); 0 (13)	— (17)	17
		2	0 (7); 0 (13)	— (17)	17
2	132	1	0 (11); 0 (18); 0 (33)	— (33)	33
3	24	1	0 (16); 0 (32); 0 (44)	— (44)	44
		2	0 (16); 2.8×10^1 (32); 0 (44)	— (44)	44
4	6	1	0 (17); 0 (34); 9.6×10^1 (61); 6×10^0 (72)	+ (116)	21; 51; 61
		2	0 (17); 0 (34); 0 (52); 8×10^0 (61); 4.4×10^1 (72)	+ (116)	21; 61; 116
		3	0 (9); 0 (17); 0 (63)	— (91)	91
		4	0 (9); 0 (17)	nt	21; 116
		5	0 (17); 0 (34)	+ (91)	21; 91
		6	0 (17); 0 (34)	— (91)	21; 91
		7	0 (17); 2.8×10^1 (34); 0 (61)	+ (91)	61; 91
		8	0 (17); 0 (34); 3×10^1 (61)	+ (21); + (61)	21; 61; 116
5	156	1	0 (8); 0 (14); 0 (18)	nt	18
		2	nt	nt	19
6	362	1	1×10^0 (7); 1×10^1 (14); 5×10^0 (19)	nt	19
		2	0 (7); 0 (14); 5×10^0 (18)	nt	18
7	156	1	0 (30)	— (30)	30
		2	nt	— (15)	15
8	156	1	nt	+ (17)	17
9	155	1	0 (30)	— (30)	30
		2	nt	— (15)	15
10	195	1	0 (25)	— (28)	28

* Intravenous inoculation of supernatant fluid in chick embryos. Titre expressed as v.f.u. Number within parentheses indicates days supernatant fluid tested after virus treatment.

† Subcutaneous inoculation in 2-day-old chicks. Number in parentheses indicates days supernatant fluids tested after virus treatment.

‡ Subcutaneous inoculation of cell suspensions in 2-day-old chicks. All tests were positive for tumor induction.

+, tumor induction; —, no tumor induction; nt, not tested.

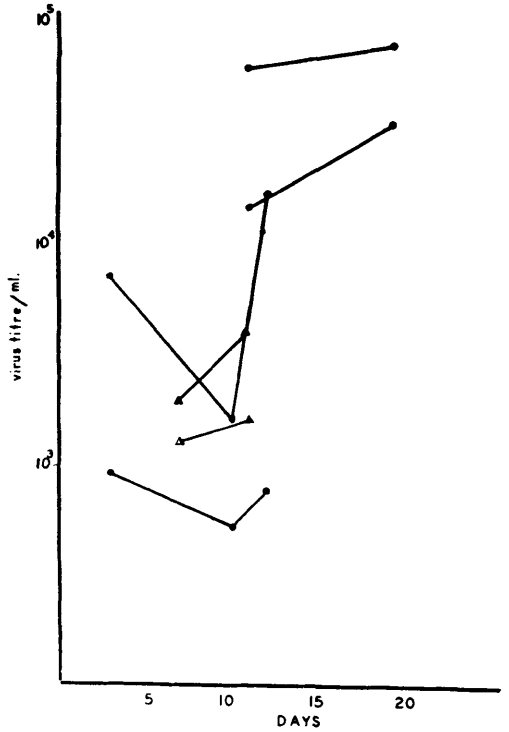

FIG. 1. Free virus in the supernatant fluid of sarcoma virus infected chicken fibroblast cultures in 3 different experiments.

one culture (6.5) induced tumor in 2-day-old chick, though earlier tests of the supernatant fluid were negative for free virus.

Fluorescent antibody studies. The fluorescent antibody method for detecting virus antigens was applied to the virus treated macrophage cultures. Cultures from 2 chickens were used. One of these was animal 156 whose virus treated macrophage cultures induced tumors on cell inoculation in 3 different experiments (Serial Nos. 5, 7 and 8, Table III). Cultures parallel to those of experiment No. 8 (Table III) from this bird were utilised for fluorescent antibody studies. These cultures were examined from 4 to 28 days after virus treatment at periodic intervals. In addition to the above cultures, cell samples from macrophage cultures 6.1, 6.2 and 6.7 (Serial No. 4, Table III) proved to be infected by many prior tests, were also examined. These cells were incubated briefly at 37°C in medium to allow them to attach and spread on coverslips. RSV antigen was not detected in any of the 20 different cultures examined; nor did the cells from cultures positive for tumor induction show virus antigen. In these latter studies cells for fluorescent antibody staining as well as those for tumor induction studies came from the same batch of cells detached mechanically from the culture tubes. RSV infected chick embryo fibroblast cells set up as positive controls on the other hand showed bright fluorescence everytime tested.

Infection of fibroblast cultures from adult chickens. For comparative purposes, the susceptibility of fibroblast cultures obtained from the knee joint and subcutaneous tissue of adult chickens and grown in the same medium was studied in a few experiments. Results of these are shown in Fig. 1. Free virus was found as early as the third day after infection and the concentration of virus in the supernatant fluids was 10^3 to 10^5 v.f.u. per ml. This indicated that RSV grew well in susceptible cells when grown in this medium, and may again indicate that fibroblastic cells were absent from the macrophage cultures.

Discussion. In this study RSV treated chicken buffy coat macrophage cultures were tested for their ability to induce tumors in 2-day-old chicks. A number of such tests proved positive for tumor induction (Table I); however, free virus in the supernatant fluids was absent in more than 50% of the tumor positive cultures. Of the 23 cultures found to induce tumors on cell inoculations only 3 were tested for tumor induction for as long as 4 months after virus treatment (Table III). These were positive. Many other cultures induced tumors when tested at periods exceeding a month. Thus an "intimate" association of the sarcoma virus and macrophage cells occurred.

The virus detected in these cultures might be a residue of the original virus inoculum but this seems improbable for the following reasons: (i) the half life of RSV at 37°C is only 155 to 24 minutes(15,16) and in the experiments of Sanford *et al*(5) the virus survived only for 48 hours in the absence of living cells; (ii) in the present study the maintenance medium of the virus treated cultures was regularly changed every fourth day,

but virus was detected for as long as 4 months after infection.

Sanford *et al*(5) observed tumor induction by 7 of 124 (5.6%) virus treated macrophage cultures when tested 8 to 35 days after virus treatment. In their experiments none of the cultures tested after the 14th day were positive for tumor induction. The higher percentage (51%) of tumor induction by virus treated macrophage cultures observed in the present study may be due to the use of 2-day-old chickens. In the early studies of Rous(17) it was recognized that young chickens were more susceptible to the virus than older ones and Harris(18) showed that the minimal infective dose for one-day-old chicks was on the average 1/10 to 1/50 of that needed for 6-week-old chickens of the same strain.

Due to the technical difficulties experienced in separating the macrophages, as mentioned earlier, it has not been possible to establish the actual number of cells that were infected. The fact that tumors were obtained following inoculation of groups of cells taken from the same culture at intervals separated by a month or more (Table III) suggests that the virus is distributed in more than a single cell or cell group. Failure to detect virus antigens by the fluorescent antibody method in any of the cells examined together with the ability to induce tumor by cell clumps obtained from the same infected cultures which provided cells for fluorescent staining may be due to a low concentration of virus antigen in the infected cells. The ease with which virus antigens were detected in infected chick embryo fibroblast cultures indicates that the serum used for fluorescent technique was satisfactory.

The evidence for the susceptibility of chicken macrophages in the complete absence of fibroblastic elements is supported by the following points: i) At no time during the entire period of observation were groups of spindle cells or spindle cell sheets observed in these macrophage cultures; ii) in those experiments where buffy coat was obtained from venous blood, precautions, as described under *Methods,* were taken to eliminate connective tissue contamination of the buffy coat cells; iii) whenever connective tissue elements were present in macrophage cultures they overgrew the macrophages especially after treatment of the cultures with chick embryonic materials such as RSV prepared in chorioallantoic membrane(1); iv) in a few instances where subcultures were made from macrophage cultures with a potential to induce tumors, the cellular migration pattern showed only isolated pleomorphic cells migrating from the clumps similar to macrophage cultures. (Fig. 2): these subcultures themselves induced tumor when implanted into 2-day-old chicks; v) lastly, failure to detect virus antigens in any of the infected macrophage cultures by the fluorescent antibody technique argues against the presence of fibroblastic cells in the cultures.

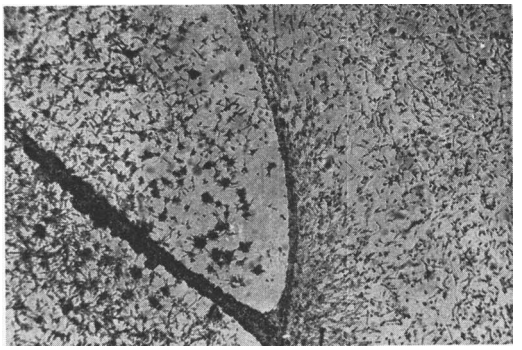

FIG. 2. Subculture on collagen coated coverslip of accumulated clump of cells from sarcoma virus infected macrophage culture. Migration pattern of pleomorphic isolated cells and small clumps. Lysis and retraction of collagen film noticed on left half of picture. Implantations from this culture induced tumors in 2-day-old chicks on 2 different occasions.

The results strongly support the establishment of a low grade persistent infection of the chicken macrophages by the sarcoma virus. The mechanism by which the virus persists in these cultures is not clear though an "intimate" association of the virus with the macrophage cell occurs, and the nature of this association needs to be investigated.

Summary. Over 50% of RSV treated chicken buffy coat macrophage cultures induced tumors when the cells were tested in 2-day-old chicks. Some of these cultures were positive when tested as late as 4 months after infection. Sarcoma virus from the

tumors and red vascular lesions were observed in many of the tumor bearing animals. The macrophage cultures lacked detectable fibroblastic elements. Free virus was absent in the supernatant fluid of most of the virus treated macrophage cultures. More than one-half of the cultures whose cells induced tumors did not contain free virus at any time when their supernatant fluids were tested. Whenever detected, the titre of free virus was less than 10^2 v.f.u./ml of supernatant fluid. This value was about 1/1000 that of the free virus in infected fibroblast cultures which had a titre of 10^4 to 10^5 v.f.u. per ml of supernatant fluid. Sarcoma virus antigens were not observed in the virus treated macrophage cultures when tested by the fluorescent antibody staining technique including cell samples from macrophage cultures positive for tumor induction.

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Toxicity of L-Asparaginase to Resistant and Susceptible Lymphoma Cells *in vitro*.* (32156)

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It has previously been shown that cells of 6C3HED lymphoma are susceptible to guinea pig serum L-asparaginase *in vitro*, correlating with their susceptibility *in vivo*(1).

It has previously been shown that the blood lymphocytes of patients with chronic lymphocytic leukemia are more susceptible to death *in vitro* than are lymphocytes from normal volunteers when incubated with L-asparaginase from *E. coli*(2).

The present report describes the relative susceptibility to a wide range of concentrations of *E. coli* L-asparaginase *in vitro* of normal murine spleen and thymus lymphocytes and tumor cells from a strain of L-asparaginase-susceptible lymphoma (6C3HED) and from a strain of a derived *E. coli* L-asparaginase-resistant tumor (6C3HED-ECLAR1).

Materials and methods. Enzyme. E. coli L-asparaginase was obtained from Worthington Biochemical Corp. The preparations were purified by salt fractionation and chromatography and had a specific activity of 5.0 to 6.7 international units per mg protein. The lyophilized enzyme preparations were reconstituted in normal saline and sterilized by filtration through a 220 mμ Millipore® filter. Protein was determined by the method

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