

100 ml. conical flask containing 25 ml. of culture solution consisting of Linsmaier and Skoog's⁵ nutrient medium without the optional constituents and with the indoleacetic acid concentration at 8 mg/l. and kinetin at 2.56 mg/l. The flasks were fixed horizontally onto an illuminated vertical wheel which was rotated at 8 r.p.m. Within 3-4 weeks each tip developed into a mass of callus bearing several terminal leaf initials (Fig. 1). After a further 3-4 weeks, protrusions of the callus developed below these initials, each bearing several small green leaves. The protrusions became detached and "budded" profusely into a ball of callus units (Fig. 2), many of which also became detached and continued to proliferate. This process was accelerated if the cultures were grown on a horizontal shaker operating at approximately 80 cycles/min.

The proliferation into callus units continued if portions of the culture were transferred to new flasks and the shaking was maintained. If, however, the callus units

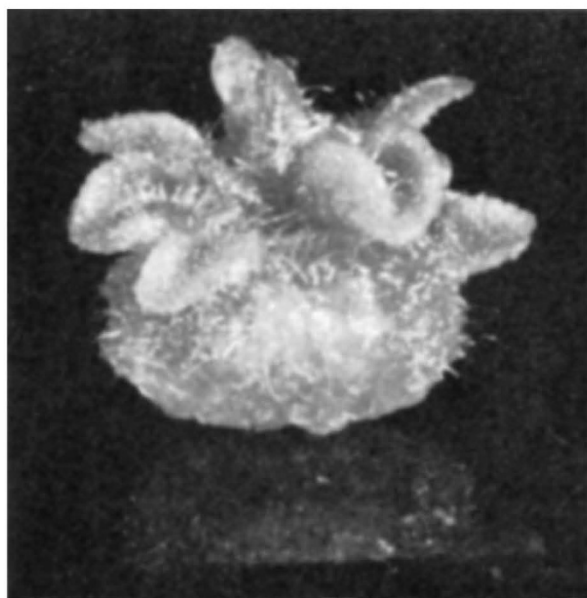


Fig. 1. 3-4 week old culture showing callus mass and terminal leaf initials. ($\times 15$.)

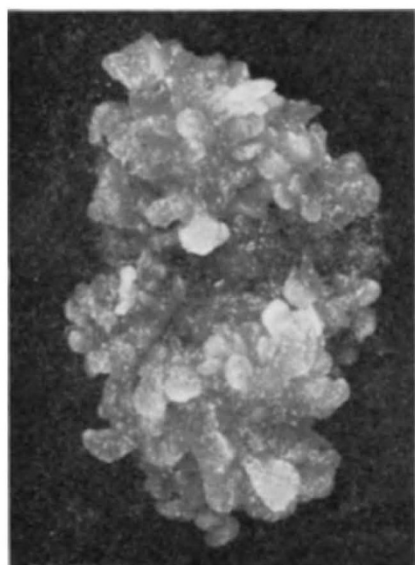


Fig. 2. 5-8 week old culture showing "ball" of proliferating callus protrusions. ($\times 3$.)



Fig. 3. Young *N. rustica* plant produced after transfer to still culture. ($\times 2/3$.)

were removed and placed on filter paper bridges in still culture⁶, each developed roots and shoots and grew into a normal plant (Fig. 3).

This method of culture enabled very large numbers of clonal plants to be raised from one apical tip. It seems to be the first occasion on which propagation of a dicotyledonous plant from a meristem by this particular means has been demonstrated, although somewhat similar cultures have been made from single cell and callus tissue of carrot and other plants⁷⁻¹⁰. It has also been shown¹¹ that carnation meristems can be induced to form multiple apices in culture from which numerous explants can be grown.

The possible application of the shake culture method for the multiplication of other dicotyledonous plants is being investigated.

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Rubella Haemagglutinin prepared in BS-C-1 Cells

STEWART *et al.*¹ described procedures for the preparation of rubella virus haemagglutinin in monolayers of BHK21 cells. Others expanded these studies to include extraction of haemagglutinin with high pH buffers from BHK21 suspension cultures infected with rubella virus². Produc-

tion of rubella virus haemagglutinin in an established line of African green monkey kidney cells (VERO) was recently reported³. We describe the production of haemagglutinin in the BS-C-1 line of African green monkey cells by two different strains of rubella virus and subsequent establishment of a chronically infected culture which continues to shed virus and haemagglutinin after serial passage.

BS-C-1 cells in the forty-ninth passage and rubella virus, strain Putnam, were obtained from the Division of Biologics Standards, US National Institutes of Health, and rubella virus, strain Baylor, was received from Dr W. Rawls, Baylor University. Virus titrations were carried out by the ECHO 11 interference test⁴ and are expressed as tissue culture interference dose-50 per cent/0.1 ml. Titres and passage histories of virus pools used were as follows: Putnam, $10^{6.5}$, RK13/12, MA104/1, BHK21/2; and Baylor, $10^{4.7}$, BHK21/6. Haemagglutination and haemagglutination inhibition tests were carried out with erythrocytes from 1 day old chicks by methods previously described⁵.

BS-C-1 monolayers were prepared in 4 ounce glass prescription bottles and inoculated with rubella virus at a multiplicity of 5. After incubation for 2 h at 36° C, the cells were rinsed three times with saline and fed a measured volume (15 ml.) of medium 199 containing 2 per cent kaolin-treated foetal bovine serum. Infected and uninoculated control cultures were incubated at 36° C and observed daily for cytopathic effects (CPE). Maintenance medium was replaced daily and harvests tested for the presence of haemagglutinins without prior treatment.

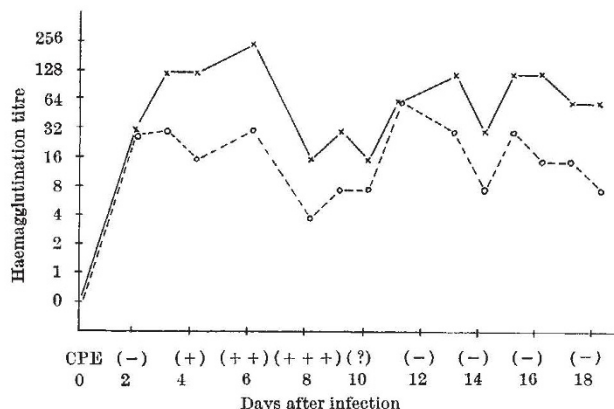


Fig. 1. Production of haemagglutinins in BS-C-1 cells by the Putnam (x) and Baylor (o) strains of rubella virus.

Results from a typical experiment are summarized in Fig. 1. Haemagglutination antigen was detected 2 days after infection and reached maximum titre within 3 days. Between 6 and 8 days the haemagglutination titre dropped 8-16-fold in cultures infected with both Putnam and Baylor and then gradually increased to previous levels. The sharp drop in titre coincided with extensive cell destruction. Haemagglutinin was present in all harvests throughout 18 days. Harvests from Putnam infected cells usually contained 4-fold higher titres of haemagglutinin than cells infected with Baylor. CPE was first observed in cultures on the fourth day after infection and progressed to involve most of the cells by the eighth day. Cells appeared granular, pyknotic and loosely attached giving a "peppered" appearance to the culture. CPE did not progress completely to destroy the cells and by 10-12 days it became increasingly difficult to distinguish infected from uninoculated control cultures. By 18 days a light monolayer had once again formed which was indistinguishable from the control culture.

It was of interest to determine whether BS-C-1 cells that had been infected by rubella virus and showed extensive CPE and then sheeted out again could be passaged. We therefore chose a culture infected 18 days with Putnam and dispersed the cells with 0.25 per cent trypsin and subcultured at a 2:1 ratio in medium 199-20 per cent foetal bovine serum. Cultures were split 2:1 every 5-7 days thereafter. When cultures became confluent they were rinsed twice with saline and refed medium 199-20 per cent kaolin-treated foetal bovine serum. The following day the medium was harvested and titrated for virus and haemagglutinin and the cells subcultured. Results after five serial passages are presented in Table 1. The cells are chronically infected as shown by the continued shedding of low levels of virus ($0.7-2.3 \log_{10}$) and moderate amounts of haemagglutinin throughout each of five passages.

Table 1. PRODUCTION OF RUBELLA VIRUS AND HAEMAGGLUTININ IN CHRONICALLY INFECTED BS-C-1 CULTURE

Passage No.	Interval after infection (days)	Infectivity titre* ($TCID_{50}/0.1$ ml.)	Haemagglutination titre*
1	28	1.7	16
2	33	0.7	8
3	40	1.5	64
4	45	1.7	16
5	50	2.3	32

* Titre of overlay fluid on day of split.

Table 2. RUBELLA HAEMAGGLUTINATION INHIBITION TITRES IN RANDOM HUMAN SERA TESTED WITH THREE DIFFERENT HAEMAGGLUTINATION ANTIGEN PREPARATIONS

Sera identification	Haemagglutination antigen source		
	Putnam prepared in BS-C-1	Baylor prepared in BS-C-1	Commercial prepared in BHK21
6	<8	<8	<8
65	<8	<8	<8
66	<8	<8	<8
73	<8	<8	<8
25	16	32	32
163	64	64	64
60	64	32	128
59	128	128	128
68	128	256	256
76	256	256	512
160	256	1,024	512
158	1,024	1,024	2,048
159	1,024	4,096	4,096

Rubella virus haemagglutinins produced in BS-C-1 by the Putnam and Baylor strains and a commercial antigen prepared in BHK21 cells were used to measure haemagglutination inhibition antibody in thirteen human sera (Table 2). A titre of less than 8 was obtained in four sera with each of the three antigens. Rubella virus haemagglutinins prepared in BS-C-1, similar to haemagglutination antigen prepared in VERO cells, were as sensitive in measuring haemagglutination inhibition antibody as antigen prepared in BHK21 cells.

These results further establish that production of rubella haemagglutination antigen is not unique to a particular virus-host cell system but rather a common occurrence requiring only infection of cells at a high input multiplicity and initial replication of virus to relatively high titres. Continued proliferation at high titre does not seem necessary because haemagglutination antigen was shed from chronically infected cells producing relatively small quantities of virus. It has been reported^{6,7} that low levels of infectious rubella virus were obtained from chronically infected human foetal tissue and LLC-MK₂ cells, but no data were available on haemagglutination production.

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Effect of Preliminary Irradiation of Mice on Radioresistance of Immunologically Competent Spleen Cells

RESISTANCE to radiation is increased in animals repeatedly exposed to ionizing radiation¹⁻⁶. We know of no quantitative studies of radiosensitivity of haematopoietic or immunocompetent cell populations from previously irradiated animals.

Our aim has been to compare the radioresistance of immunologically competent cells from intact and previously irradiated animals by counting the affected cells. The plaque technique⁷ has made possible the determination of a number of single antibody-forming cells⁷.

The animals were (C57B × CBA)_F₁ female mice which weighed 18–20 g, and were 3–4 weeks old. The principle of the method used to obtain antibody-forming cells has been described before^{8,9}. Splens of intact and previously irradiated (500 r.) donors were removed and suspensions of spleen cells were prepared in TC 199 medium 1 (ref. 10) 7–21 days after irradiation. Samples of the cell suspension were irradiated *in vitro* with doses varying from 0 to 1,000 r. The cells were then washed two or three times with TC 199 medium and, after washing, sheep red blood cells were added to each sample. The treated cells were injected into lethally irradiated (850 r.) syngeneic recipients. Each recipient received 0.5 ml. of mixture, consisting of 25×10^6 spleen cells and 2×10^8 sheep red blood cells. Because preliminary experiments had shown that maximal accumulation of cells producing antibody is reached in the spleen of the recipients on the sixth day after the transfer of the cells the recipients were killed 6 days after injection. Their splens were removed and suspensions of spleen cells were prepared. Antibody-forming cells were counted by the Jerne plaque technique⁷. Mice and suspensions of cells were irradiated with ⁶⁰Co X-rays in an EGO-2 apparatus, at a dose rate of 641–587 r./min.

The injection of spleen cells stimulated by antigen from normal mice into lethally irradiated recipients resulted in the accumulation in their splens of $\sim 2,500 \times 10^{-8}$ antibody-forming cells. Use of the same quantity of transplanted cells from previously irradiated donors (500 r.) led to a significantly smaller accumulation of cells producing antibody. There were about 150×10^{-8} cells if the donors were irradiated 7 days before the splens were removed, and $\sim 170 \times 10^{-8}$ if splens were removed 21 days after irradiation.

Cell suspensions from intact and previously irradiated donors were radiated *in vitro*. The results (dose response) for three groups of the cell populations are summarized in Fig. 1. In each case inactivation of antibody-forming cells was calculated as a percentage with respect to the value obtained in each experiment with a non-irradiated *in vitro* cell suspension. Curve 1 characterizes the dose response of spleen cells from intact donors. Using the exponential part of this curve, the D_{37} was calculated to be 125 r. and the extrapolation number (n) is 1.0 ($LD_{50} = 95$ r.). The dose response curve of mouse spleen cells

irradiated 7 days before the beginning of radioresistance studies was characterized as $D_{37} = 325$ r. and $n = 1.0$ ($LD_{50} = 235$ r.). The greatest difference in accumulation of antibody-forming cells, in comparison with the normal cell suspension, occurred when the spleen cells were isolated from the donors 21 days after the preliminary irradiation. The inactivation curve of antibody-producing cells then gave $D_{37} = 180$ r. and $n = 23$ ($LD_{50} = 680$ r.). These results indicate that the population of immunocompetent spleen cells in the body of previously irradiated mice is significantly more radioresistant than that in intact animals.

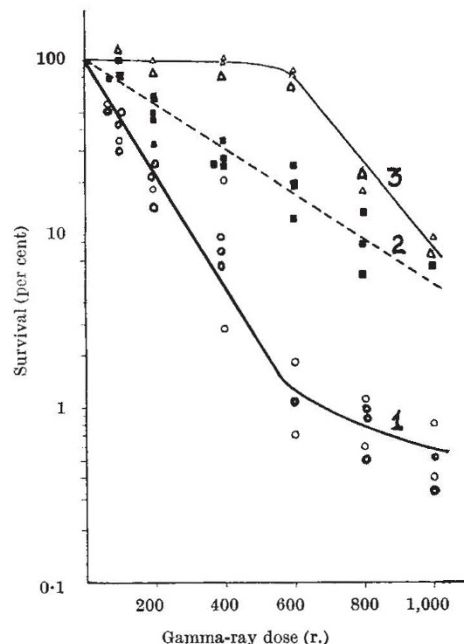


Fig. 1. Effect of irradiation of spleen cell suspensions on the accumulation of antibody-producing cells after antigenic stimulation. ○, Inactivation curve for spleen cells from intact mice; ■, inactivation curve for spleen cells from mice irradiated (500 r.) 7 days and, △, 21 days before irradiation *in vitro*. Every point represents data obtained from at least five mice. Curve 1, $D_{37} = 125$ r., $n = 1$; curve 2, $D_{37} = 325$ r., $n = 1$; curve 3, $D_{37} = 180$ r., $n = 23$.

One possible explanation is that there are a few radioresistant stem cells or immunocompetent cell precursors in the body of a normal animal. Preliminary irradiation damages a radiosensitive cell population and could be a selecting factor for the pre-existing cell population of radioresistant cells, so that the ratio of the two cell populations changes in favour of radioresistant cells. Theoretical analysis shows that curve 1 agrees most closely with two possibilities: (1) there is 1 per cent of radioresistant cells which are six times more radioresistant than the remaining 99 per cent of cells, and (2) there is 5 per cent of radioresistant cells, which are four times more radioresistant than the remaining 95 per cent.

This may be correct¹¹, but there is another possible explanation for our results in another way. The radioresistance of immunocompetent cells which has been demonstrated in previously irradiated mice is probably only a relative phenomenon. The spleen cell population is very heterogeneous. The most typical form of cell inactivation occurs when cells are able to divide one, two or more times before death^{12,13}. The generation time of such cells is longer than usual. If the number of cells capable of limited division after exposure to ionizing irradiation is the same in both normal and previously irradiated populations, it is simple to calculate that the percentage of such cells in an irradiated normal spleen cell population