Virus resistance transferred from human rheumatoid cells to rabbit synovial cells

II. Cell fusion

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Cultured human rheumatoid synovial cells (RSC) appear resistant to lytic infection with rubella virus (RV) in contrast to the susceptibility of nonrheumatoid synovial cells (NSC) (Grayzel and Beck, 1970). In the previous paper we reported transfer of RV resistance from RSC to rabbit synovial cells by means of intra-articular injection of RSC into normal rabbit joints (Smith, Hamerman, Janis, and Habermann, 1974). In parallel with these studies, we attempted to transfer RV resistance from RSC to other cells by the technique of cell fusion. These efforts were successful when normal rabbit synovial cells were used as the 'susceptible cell'.

Material and methods

CULTURE MEDIUM

Dulbecco-modified Eagle's medium with 10 per cent. calf serum was used at all times unless otherwise noted.

CELLS AND SOURCES

Human RSC and NSC were obtained as described earlier (Smith and others, 1974). Normal rabbit synovial cells were derived in a similar manner from joints of New Zealand female rabbits killed at 3 to 6 months of age. Other cells used for fusion and passages are listed in Table I.

CELL FUSION TECHNIQUE

Synovial cells were derived by trypsinization from culture plates five to fifteen cell generations after complete primary explant outgrowth. RSC and NSC used in each fusion experiment had a comparable duration of culture life. Cells were dispersed in medium containing 2 per cent. calf serum, counted by haemocytometer, and 3 to 5 million human cells (RSC or NSC) were mixed in equal number with the test cells. The procedure used was adapted from Coon and Weiss (1969). The fusion factor was β -propiolactone-inactivated Sendai virus, obtained from Dr. Barbara Knowles, Wistar Institute, and added at a titre of 2 to 8,000 HAU per 10^7 cells. The mixtures were shaken in an ice bath for 20 min., and then in a 37°C. water

Table I *Types and sources of cells used for fusion and passages*

Cells	Sources
CHO (Chinese hamster ovary)	Dr. H. Koprowski
CV-1 (Monkey kidney line)	Dr. H. Koprowski
HEK (Human embryo kidney)	Flow Labs
DF (Human dermal fibroblasts)	Our lab
NSC, RSC (Human synovial cells)	Our lab
HuEF (Human embryo fibroblasts)	Dr. H. Klinger
BHK-21 (Baby hamster kidney)	Flow Labs
PtK2 (Rat kangaroo)	Dr. D. Axelrod
3T3 (Mouse embryo line)	Dr. H. Green
HeLa (Human cervical carcinoma)	Dr. M. Scharff
ME (Mouse embryo)	Dr. H. Green
SIRC (Rabbit cornea)	Dr. I. Spigland
WI-38 (Human lung)	Dr. I. Spigland
AGMK (African green monkey kidney)	Dr. I. Spigland
VERO (African green monkey kidney line)	Flow Labs

bath for 30 to 60 min. The mixture was then sedimented for 1 hr through a 12.5 cm. column of calf serum-enriched (30 per cent.) culture medium, which permitted cells to fall to the bottom and unattached Sendai virus to be retarded at the top of the column. The cells were then plated as desired for further study. In all cases, the 'test cells' (Table I) were pre-labelled for 72 hrs with 0.2 μ curies/ml. of thymidine methyl- 3 H. After fusion, the cells were subjected to radioautography to determine the percentage of multinucleated cells containing both labelled and unlabelled nuclei (heterokaryons).

CO-CULTIVATION

In two cases, co-cultivation of rabbit synovial cells with RSC was carried out using the same fusion procedures except that inactivated Sendai virus was omitted.

USE OF SUBCULTURES OF THE CELL FUSIONS

Chromosomal analysis was carried out as previously described on the rabbit fusions after six to ten cell generations (Coon and Weiss, 1969).

Accepted for publication June 29, 1973.

Aided by grants from the U.S. Public Health Service (AM-05082 and AM-08729), and the Research Corporation, Providence, Rhode Island.

* Senior Investigator, Arthritis Foundation. Presented in part at the annual meeting of the American Society for Clinical Investigation, April 30, 1972.

Lysis and passage 5 days after fusion 2×10^6 cells were lysed by two cycles of freeze-thawing, and serially passaged in an attempt to recover infectious virus produced in the fused cultures. Cells used for passage studies are listed in Table II. Serial passages in each case were carried out weekly for 1 to 6 months.

Virus challenge Cells were subcultured for 7 to 14 days after fusion, and one additional subculture was carried out before initial challenge with RV and simultaneous chromosome counts. This schedule meant evolution of six to ten cell generations over the course of 3 to 4 weeks post-fusion. Challenges with NDV were carried out as described in the accompanying paper. NDV challenge was repeated at least once on a later subculture.

Mycoplasma testing was performed on fused cells and virus preparations using Hayflick's medium, prepared by us, containing: 3 per cent. PPLO agar, 20 per cent. horse serum, 10 per cent. yeast extract, 0.02 per cent. thallium acetate, fungizone 5 µg./ml., and penicillin 50 units/ml.

Results

Effects of serial passage

When cell lysates derived from fusion of the human and test cells were serially passaged upon the cell types shown in Table II, there were no cytopathic effects, cell lysis, or formation of inclusions.

Table II Cells used for fusion with human nonrheumatoid (NSC) or rheumatoid (RSC) synovial cells and subsequent serial passage

Fusion*	Passaged on*
CHO	DF, NSC, HEK, BHK, PtK2
CV-1	DF, NSC, HEK
HEK	HEK
HuEF	HuEF
3T3	3T3
PtK2	PtK2
HeLa	HeLa
ME	ME
BHK-21	BHK-21
Rabbit synovial	Rabbit synovial, NSC, HeLa, SIRC, WI-38, HEK, VERO, AGMK

* For abbreviations see Table I.

Only the results of fusion between human synovial cells and normal rabbit synovial cells will now be described in detail; the findings with a variety of other test cells revealed no differences between cells fused with NSC or RSC. Clinical sources of the human NSC and RSC used in rabbit cell fusions are shown in Table III.

Morphological appearance of fused cells

Autoradiographic studies 24 hrs after fusion revealed that both the NSC and RSC cell cultures contained 3 to 4 per cent. true heterokaryon formation with rabbit synovial cells.

Table III Clinical sources of human non-rheumatoid (NSC) and rheumatoid (RSC) synovial cells fused with rabbit synovial cells

Cell strain	Patient's sex and age (yrs)	Diagnosis and surgery
NSC-13	F-62	Idiopathic carpal tunnel syndrome
NSC-19	F-59	Osteoarthritis (hip replacement)
NSC-42	M-32	Traumatic synovitis (Meniscectomy)
NSC-57	M-57	Traumatic synovitis (Meniscectomy)
RSC-7	F-60	RA—Knee synovectomy
RSC-8	F-59	RA—Hip replacement
RSC-14	F-29	RA—Knee synovectomy
RSC-30	F-55	RA—Proximal interphalangeal joint replacements
RSC-31	F-63	RA—Knee synovectomy

After 3 to 4 weeks and two subcultures, the fused cultures appeared to be composed entirely of the rabbit synovial cells, which had rapidly overgrown their slower-growing human counterparts. Chromosome counts of 100 cells from each fused culture showed that this was indeed the case. This finding was of particular importance, since it appeared by morphology and by chromosome counts that no human RSC remained to contribute to the RV resistance demonstrated in these rabbit cultures subsequent to fusion.

The cultures resulting from NSC- and RSC-rabbit fusions were observed for morphology and growth characteristics throughout their culture lives, which ranged from fifteen generations (about 7 weeks) to more than 100 generations (2 years). Only one pair (NSC-rabbit and RSC-rabbit) of fused cultures has survived for this exceptionally long period, and from the fourth subculture, or roughly the 10th to 12th generation after fusion, this pair of cultures exhibited a marked variance in behaviour. This is particularly interesting because the normal rabbit cells used for this NSC and RSC fusion came from the same rabbit cell culture. The culture resulting from the RSC-rabbit fusion began to show a slower growth rate, larger cell size, and lower confluent density than its NSC-rabbit control. These differences have persisted, and were quite comparable to those observed in two of the cultures earlier derived from RSC-injected rabbit joints; they have not yet been observed in any of the NSC-rabbit control fusions, or cultures derived from NSC-injected rabbit joints.

RV challenge

This was carried out on five of the RSC-rabbit fusions and four NSC-rabbit fusions (Table IV, overleaf). All four NSC fusions were susceptible to RV, showing severe cytopathic effects and cell death and lysis 14 to 21 days after infection. Four of the five RSC rabbit fusions were RV resistant, retaining both their normal appearance at 21 days, and their ability to be

Table IV *Virus challenge of cells after fusion*

Fused population	RV		NDV	
	Susceptible	Resistant	Susceptible	Resistant
Human NSC × Rabbit synovial	4	0	2	0
Human RSC × Rabbit synovial	1	4	0	3

subcultured. One of the RSC rabbit fusions proved to be sensitive to RV.

NDV challenge

Three RSC-rabbit fusions and two NSC-rabbit fusions were available for testing with NDV by the hemadsorption test. These three were NDV resistant as compared to the two control fusions (Table IV).

Titration of RV production in fused cells

One NSC-rabbit and one RSC-rabbit fusion were infected with RV and the virus from the culture lysates was titred after 48 hrs. The NSC-rabbit fusion produced virus which assayed at 10^8 TCID₅₀/ml., while the RSC-fusion produced only 10^2 TCID₅₀/ml. These titres were strikingly comparable to those of the one assayed pair of NSC- and RSC-injected rabbit joint cultures, as described in the previous paper. It was evident that there was a much more limited type of productive infection in the RSC-rabbit fusion, which was unable to produce obvious cytopathic effects in this culture when followed for 21 days.

Co-cultivation

On two occasions normal rabbit synovial cells and RSC were co-cultivated without addition of inactivated Sendai virus. The subsequent population of rabbit cells remained susceptible to RV as judged by cytopathic effect after challenge. All RSC cultures used for cell fusions and co-cultivation were previously determined to be RV resistant.

Sterility testing

No mycoplasma was identified in the cell fusions or virus preparations. This, of course, does not rule out the presence of fastidious mycoplasma requiring other factors for growth and identification.

Discussion

Cell fusion has been used to 'rescue' virus from cells harboring a virus genome but not releasing infectious progeny. In some cases, if these cells are fused with a susceptible cell using inactivated Sendai virus as fusion factor, virus can be recovered. Perhaps most thoroughly studied are cells transformed by SV 40 virus; when these cells are fused with susceptible cells, virus progeny are released from heterokaryons (Koprowski, 1971; Watkins and Dulbecco, 1967) (Fig. 1).

FIG. 1 *Diagram of 'rescue' of SV 40 from transformed mouse fibroblasts*

We fused RSC with a variety of test cells, but it was not until normal rabbit synovial cells were used as a 'susceptible' cell that significant results were obtained. Our purpose is shown schematically in Fig. 2. After cell fusion and subsequent serial cell culture, the remaining cells were rabbit cells, as verified by chromosome count. Rabbit cells remaining after fusion with either NSC or RSC showed no cytopathic

FIG. 2 *Diagram of our purpose in using cell fusion in an attempt either to 'rescue' an agent in rheumatoid synovial cells, or to transfer rubella virus resistance to normal rabbit synovial cells*

effects, and passage of the lysates onto a variety of other cell strains produced no lytic or other changes. However, when the rabbit cells resulting from RSC-rabbit fusions were challenged with RV, no cytopathic effect was observed in four out of five cases. In contrast, lysis and cell death developed in all four rabbit cell cultures resulting from NSC-rabbit fusions.

The reason for using rabbit synovial cells was the ability to transfer RV resistance to such cells by intra-articular injection of RSC into rabbit joints, as described in the previous paper. Little is known about rabbit synovial cells in culture, but their fine structure appears similar to human synovial cells (Ghadially and Roy, 1966; Mitchell and Blackwell, 1968). It is not certain whether other *synovial* cells would acquire RV resistance after fusion with RSC. This possibility was pursued by fusion of human NSC with human RSC but the resulting cultures could not be distinguished morphologically as nonrheumatoid or rheumatoid, and this approach could not be pursued. Other cells from animal joints, such as guinea pig synovial cells or rabbit chondrocytes are in the process of being used for fusion to see if synovial cells alone among various cell types derived from joints acquire RV resistance from RSC after fusion.

How RV resistance is transferred from RSC, presumably, by way of heterokaryons, to rabbit cells in the culture is not known, but fusion with inactivated Sendai virus is necessary since mere co-cultivation failed to reproduce this finding. Nor is it known whether all the rabbit cells resulting from RSC-rabbit fusion are RV resistant or only some of the cells. Cloning of the rabbit cells might resolve this question. If only a few of the rabbit cells proved to be RV resistant, it might be assumed that the whole cultures were protected by some interferon-like substance released from the few RV resistant cells.

Despite the acquisition of RV resistance, the rabbit cells resulting from RSC-rabbit cell fusion do not appear to release detectable infectious virus, as judged by failure to observe cytopathic effects in the cultures or in the lysates passaged on to a variety of cells. One possibility is that very few of the rabbit cells are releasing virus, and the titre is too low to produce detectable cytopathic effects. Other possibilities exist:

- (a) The acquisition of RV resistance is unrelated to a virus genome;
- (b) An incomplete virus genome has been transferred

to rabbit cells, such that the portion coding for RV resistance is present, but the genome is unable to make a complete infectious virus;

- (c) The affected rabbit cell may be susceptible or 'permissive' for the expression of RV resistance, but 'non-permissive' for the complete virus particle.

We have attempted to approach and study some of these alternatives. For example, if any one RSC used for fusion contained deficient virus genetic information to express infectivity, multiple strains (Knowles, Jensen, Stepkowski, and Koprowski, 1968) of RSC in culture could be used for fusion with the rabbit cells. In one such experiment, no infectious virus was produced. An attempt was also made to fuse RV-resistant rabbit cells and normal rabbit cells as a means of augmenting possible virus release, but none was detected. Finally, with Dr. A. Grayzel, an attempt was made to induce virus production by incubating RV-resistant rabbit cells with 5-bromo-deoxyuridine (Lowy, Rowe, Teich, and Hartley, 1971; Aaronson, Todaro, and Scolnick, 1971), but this was also unsuccessful.

The central question in this work is whether or not RV resistance in rabbit cells represents the partial expression of virus genetic material. The only unequivocal answer will be the demonstration of infectious virus progeny in these rabbit cultures. Some techniques currently available have been tried without success; it is not certain that any known methods will lead to a successful outcome even assuming the answer to the question is positive. New directions of work needed here parallel those current in virus oncogenesis.

Summary

Inactivated Sendai virus was used to fuse human rheumatoid synovial cells with normal rabbit synovial cells. Heterokaryon formation was 2 to 4 per cent. Successive subcultures revealed only rabbit cells, as shown by chromosome count. These rabbit cells had acquired the property of resistance to rubella virus infection, a trait that mere co-cultivation failed to transfer. Thus, two independent means are available to transfer rubella virus resistance: intra-articular injection of rheumatoid cells into rabbit joints, described in the preceding paper; and cell fusion, discussed in this paper. The relationship of this finding to a possible latent virus agent in the rheumatoid cells is discussed.

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