cell within each layer, while a heat flux from below produces a smaller scale turbulent motion.

The interfacial phenomena described here are also somewhat similar to the "interfacial turbulence" observed by chemical engineers during the process of solvent extraction (Sherwood and Wei[4]), but the mechanisms are quite different. All their observations relate to the boundary between *immiscible* solvents, and the motion near such interfaces has been explained in terms of the variations of surface tension with concentration or temperature.

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*INFECTION OF HUMAN AND SIMIAN TISSUE CULTURES WITH ROUS SARCOMA VIRUS**

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Several investigators have described the induction of tumors in rats,[1] hamsters,[2] mice,[3] guinea pigs,[4] rabbits,[5] and newborn monkeys[6] with Rous sarcoma virus (RSV). Svoboda and Chyle[7] have also reported the "malignization" of rat fibroblasts in tissue culture exposed to a strain of RSV, and in 1962, Bergman and Jonsson[8] described the appearance of a "few foci of granulated cells" in cultures of rats, guinea pigs, and mouse tissues exposed 9 days previously to RSV. The latter authors were unable, however, to show similar changes in rabbit and human fibroblast cultures.

Since RSV produced tumors in primates,[6] it was decided to expose normal human fibroblasts and normal monkey cells to RSV and to study the effect of the infection.

Materials and Methods.—Strains of virus: One preparation of the "Schmidt-Ruppin" strain of RSV was obtained from Dr. W. Nichols of the South Jersey Medical Foundation, who had in turn received it from Professor Ahlström of Lund, Sweden. It was received in the form of a frozen fragment of tumor excised from a chicken of a leukosis-free flock which had been injected intramuscularly with the virus. After thawing, the tumor tissue was finely minced, suspended in phosphate-buffered saline at five times the volume of its net weight, and the suspension rapidly frozen and thawed three times. The suspension was then centrifuged for 20 min at 2000 rpm, and the supernatant used as inoculum for the tissue cultures. This pool is referred to as SR-1. Its titer in chick fibroblasts was 10^6 focus-forming units (FFU) per ml; 0.3 ml of undiluted material was used as inoculum for one milk dilution bottle of tissue culture cells.

TABLE 1

RESULTS OF EXPOSURE OF HUMAN AND GREEN MONKEY KIDNEY CULTURES TO ROUS SARCOMA VIRUS

Origin of Cultures			Designation of Culture Passage				Virus		Observed time after exposure
Species	Organ	Cell types	Strain	Substrain	No.	Growth phase	strain	Lesions—type	(days)
Human	Fetal lung	Fibroblasts	WI-38	XVI	15	II	SR-1	Plaques of vacuolated cells	5–7 days after subculture
				"	19	"	"		
				XIX	18	"	"		
				XXI	16	"	SR-2		
				IV	42	III	SR-1		4–7 days
				XVII	43	"	"		
	Adult buccal mucosa	Fibroblasts	W-5 CM	D-285*	15	Slow proliferation	SR-1	Vacuolated cells, detachment	4–7 days
				D-333†	15	Slow proliferation			
				D-90*	25	III	SR-1	Vacuolated cells and foci	4–14 days
	Embryonic kidney	Fibroblasts and epithelial	—	—	Primary	—	Bryan-1	Vacuolated fibroblasts	4–7 days after subculture
Monkey	Adult kidney	Epithelial	WGM	1	25	II	SR-1	Foci	2 days after 1st subculture
			"	1	17		SR-2		
			"	1	17		Bryan-2		
			CV	1	2		SR-2		
			"	1	2		Bryan-2		

* Described in detail in ref. 10.
† Substrain D-333 incubated for 333 days at the 12th culture passage without medium changes.

Another preparation of the SR strain (SR-2), obtained from Dr. Rauscher of the National Cancer Institute as partially purified material (T_2 preparation), had a titer of 10^6 FFU/ml.

The Bryan strain of RSV was kindly supplied by Dr. R. Bryan of the National Cancer Institute in the form of a frozen preparation of partially purified virus. The undiluted material, containing 10^7 FFU/ml, was used as inoculum. Two pools, lots CT 935 and TV 11-21, were employed and are referred to as Bryan-1 and Bryan-2, respectively.

Tissue culture preparations: Table 1 shows the origin of the 15 tissue cultures used in the present study. The W-5 CM D-90 and WI-38 IV and XVII cultures were at the end of their *in vitro* lifetime (phase III) when exposed to RSV.[9] The W-5 CM D-285 and D-333 cultures were in a stage of semiarrested growth following "prolonged" storage at 37°C.[10] Other substrains of WI-38, XVI, XIX, and XXI, and the green monkey kidney cultures, WGM-1[11] and CV-1, were infected during the stage of rapidly proliferating growth (phase II).[9] Modified Eagle's basal medium supplemented with 2× concentration of amino acids and vitamins, with Earle's balanced salt solution and 10% calf serum (Millipore filtered and inactivated at 56°C for 30 min) was used in all cultures. General handling of normal and infected cultures and techniques used for morphological examinations were similar to those reported before.[12]

Assays for infectious RSV: Assays were performed by the standard *in vitro* method, described by Temin and Rubin,[13] employing cultures of RIF-free chick embryos, and also by inoculation of 3–4-day-old chicks via the wing-web route.

Complement-fixation tests: Tests for Rous tumor antigen were performed, as described by Sarma and Huebner,[14] against sera from hamsters bearing nonvirus-shedding Rous tumors. Cells from RSV-exposed human and green monkey kidney cultures were trypsinized and made into a 10% suspension by volume in phosphate-buffered saline. The crude suspension was used as such, or as a supernatant fluid collected after centrifugation at 3000 rpm for 10 min.

Results.—Exposure of rapidly proliferating WI-38 substrains (phase II in Table 1) to the SR tumor extracts did not cause immediate morphological changes. However, following the first cell transfer, cells with vacuolated cytoplasm appeared either singly or in groups on the fifth to seventh day after the original exposure (Figs. 1 and 4).

With each subsequent cell transfer, the RSV-exposed cultures showed, in contrast to noninfected WI-38 controls, an abnormally high number of mitotic figures resulting in a disorganized multilayer growth pattern. The vacuolated cells (Fig. 4), showing enlarged nuclei with a single, abnormally large nucleolus, could not be detected immediately in the new monolayer, but they appeared in the culture within 3–4 days of the transfer. Up to now these cultures have been maintained *in vitro* for 5 months of continuous cultivation, and the presence of the characteristic vacuolated cells has been observed at each culture passage.

If, instead of serial cell transfer, the cultures were maintained after only one subcultivation, vacuolated cells accumulated in clearly defined areas of the monolayer, forming large plaques, as shown in Figures 2 and 3.

In contrast to these results, exposure of slowly proliferating cultures, such as the W-5 CM and WI-38 in phase III (Table 1), to SR-1 resulted in the appearance of vacuolated cells as early as the fourth day after exposure before the cell transfer was made. These cells piled up in foci in one culture (W-5 CM-D90) 7–14 days after infection, when pronounced degenerative changes (CPE) were also noted. The cytopathic effect was also observed in the four other cultures which did not show piling up of cells. Transfer of cells from the infected W-5 CM cultures resulted in complete lysis after the first passage, whereas the infected WI-38 (phase III) cultures lysed after several passages *in vitro*.

When primary human embryonic kidney culture was exposed to the Bryan strain of RSV (Table 1), there were degenerative changes of the fibroblastic elements

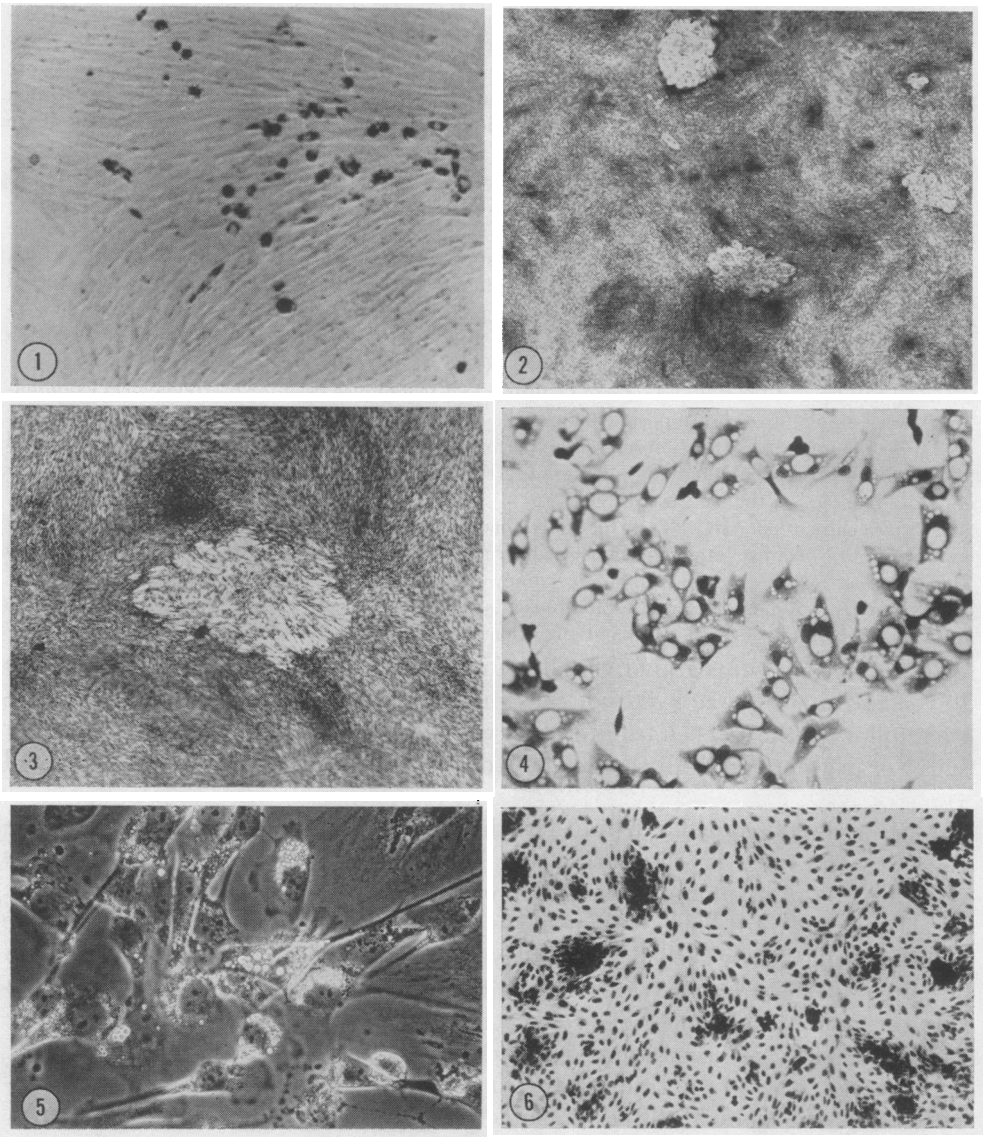

FIG. 1.—WI-38 XVI 14 days after exposure to RSV SR-1 and 7 days after the first subculture. Note the cytopathic effect of cells with granulated cytoplasm. Live, unstained preparation. FIG. 2.—WI-38 XVI; plaques in cell sheet. Fixed and stained with May-Greenwald Giemsa. FIG. 3.—Higher magnification of one plaque shown in Fig. 2. FIG. 4.—Cells from center of plaque illustrated in Fig. 3. Note vacuolated cytoplasm. The nucleus is displaced in each cell by a single large vacuole. FIG. 5.—Human embryonic kidney cells infected with the Bryan strain of RSV 14 days previously and subcultured 7 days after infection. Typical cytopathic effect. Live, unstained cells—phase contrast. FIG. 6.—WGM-1 culture exposed to RSV SR-1, showing typical foci of transformed cells. Foci are in different stages of development. Fixed and stained with May-Greenwald Giemsa.

of the culture, as shown in Figure 5, without any apparent effect on the epithelial elements of the culture. Since human epithelial cells usually cannot be maintained in serial subcultivation beyond the second or third passage, the infected cultures

could not be propagated because of the apparent destruction of the fibroblast elements by the RSV at the first passage level.

As a result of infection of the WGM-1 and CV-1 green monkey kidney cultures with either the SR-1, SR-2, or Bryan-2 strains of RSV, rounded cells with granulated cytoplasm which piled up in characteristic clusters (Fig. 6) could be observed on the seventh day after exposure and on the second day after the first cell transfer. These clusters, resembling foci of Rous-infected chick embryo fibroblasts, could be separated from the remaining portion of the culture by trypsinization and transferred separately to a new culture vessel. Following incubation, a monolayer was formed consisting of cells containing vacuoles and elongated processes in the cytoplasm which were not capable of further division and ultimately underwent complete degeneration. The infected monkey kidney cultures, however, could be maintained *in vitro* for an unlimited number of passages if the entire culture was subcultivated; new clusters then appeared at each fresh cell transfer (Fig. 6). The same findings were described for rat fibroblast cultures exposed to RSV.[7]

Tests for infectious virus: Cultures of chick embryo fibroblasts obtained from leukosis-free birds were exposed to culture medium and extracts from frozen and thawed cells of RSV-exposed human and green monkey kidney cultures at various subculture levels. In addition, viable cells from these cultures were seeded directly onto the chick embryo fibroblast monolayers. In no case has infectious virus been recovered from human and green monkey cultures, although the chick embryo fibroblasts were fully susceptible to infection with the partially purified Bryan strain of RSV. Inoculation of the RSV human and monkey kidney cell extracts and intact cells into the wing web of 3–4-day-old chicks failed to produce tumors during 2 months of observation. However, when the WGM-1 SR-1 cells were grown together with chicken embryo fibroblasts and the cell mixtures implanted into chickens, tumors developed.

Presence of complement-fixation antigens: Suspensions of various human and green monkey cultures exposed to RSV and harvested at different passage levels were used in the complement-fixation test against the serum of a hamster bearing RSV tumor, known to fix complement in the presence of antigen common to the Rous-avian leukosis complex. The results, summarized in Table 2, indicate that

TABLE 2

RSV COMPLEMENT-FIXATION ANTIGEN TITRATIONS

Preparation	CF titer (vs. 4 units serum)	AC titer
WI-38XIX-SR-1 (10% susp.)	1:8	<Undiluted
" "	1:8	Undiluted
" "	1:8	Trace
" "	1:8	<Undiluted
WGM1-SR-1	Undiluted	<Undiluted
" "	1:2	<Undiluted
" "	1:2	<Undiluted
" "	1:2	<Undiluted
Rous hamster tumor (20% susp.)	1:32	1:4
WI-38 control A	<Undiluted	<Undiluted
" " B	Trace	Undiluted
WGM1 control	<Undiluted	<Undiluted
HeLa	<Undiluted	<Undiluted
WI-26	Undiluted	Undiluted
WI-26-SV40	Undiluted	Undiluted
W18-SV40	<Undiluted	<Undiluted

CF = complement-fixation; AC = anticomplementary.

antigen prepared from the cells of WI-38 substrain XIX (Table 1), collected at various passage levels after exposure to RSV, fixed complement at a 1:8 dilution, as compared with the 1:32 titer of an antigen prepared from a 20 per cent suspension of an RSV-induced hamster tumor. The concentration of C-F antige nin the SR-1-infected WGM-1 monkey kidney line is lower than that observed in the infected WI-38 line. Rous antigen was *not* detected in noninfected human fibroblasts, green monkey kidney (WGM-1), and HeLa cells. Moreover, two cell lines transformed by SV40 and containing the specific SV40 C-F tumor antigen, WI-26 VA 4 and W-18 VA 2, did not fix complement in the presence of anti-Rous serum.

Discussion.—Although the development of tumors in mammals after exposure to RSV has been described on many occasions in the past,[1-5] transformation of mammalian cells in tissue culture by RSV has been reported only recently by Svoboda and Chyle.[7] These workers described the acquisition of neoplastic properties by rat embryonic fibroblasts grown together with RSV-infected chicken fibroblasts. When, on the other hand, the rat cells were exposed to a purified preparation of RSV in the absence of chick embryo fibroblasts, they became transformed only if maintained in culture for a prolonged time both before and after exposure to the virus.[7]

In contrast, in the present study with human and monkey kidney cultures it was possible to produce the characteristic lesions without cultivating the cells for a long time prior to infection with the Schmidt-Ruppin or Bryan strains of RSV. Similarly, as early as 9 days after exposure to the virus there was already an accumulation of vacuolated cells in the human cultures. The lesions appeared sooner in cultures in which growth was arrested or "slowed down" than in those which were exposed to the virus during their phase of rapid proliferation. This somewhat parallels the observation of a more rapid transformation of cultures of human fibroblasts nearing the end of their *in vitro* lifetime by another tumor virus, the SV40.[10] Transfer of cells by splitting the originally infected cultures also seemed to accelerate the appearance of lesions in the RSV-infected human and monkey kidney cultures.

The vacuolated cells observed in RSV-infected human cultures seemed to represent "transformed" fibroblasts characteristic for this particular tissue culture system. Their accumulation in the form of small or large plaques (Fig. 2) also seemed to represent a unique feature of the RSV infection of human fibroblasts. Conversely, foci similar to those appearing on green monkey kidney cell monolayers infected with RSV have been observed in connection with the transformation of other mammalian tissue culture systems, such as rat, mouse, guinea pig, and hamster,[4, 7, 8] and also resemble foci seen in RSV-infected cultures of chick embryo fibroblasts. However, tumors developed when transformed rat cells were grown together with chicken fibroblasts and the mixture of the cells implanted into chickens.[15] Similarly, in the present work, tumors developed in chickens after they were inoculated with WGM-1 SR-1 cells, which had been grown with chicken fibroblasts.

Although vacuolated human fibroblasts and the focus-forming monkey kidney cells could not be maintained in cultures consisting entirely of these cells, in cultures propagated without separation of the focus- or plaque-forming elements, new lesions were observed at each passage level 3–4 days after cell transfer. It seems, therefore, that a "transforming" agent was carried in this culture system which could

infect fresh cells at each subculture. The presence of this agent could not be demonstrated either in chick embryo fibroblast cultures exposed to material from human and monkey kidney cultures or in chickens implanted with the RSV-exposed human and monkey kidney cells. Other workers were also unable to isolate infectious virus from either medium or cell extracts of RSV-exposed mammalian cultures[7] and also failed to induce tumors in chickens implanted directly with rat fibroblasts transformed in tissue culture by RSV.[7]

However, the only imprint left directly by the virus infection in human and monkey cells is the complement-fixation antigen which could be detected in monkey kidney cells maintained for as long as 8 months after exposure. At present, it is impossible to say whether the presence of this antigen is indicative of continuous infection of the cells with a "defective" [16] virus, and since only one of the two RSV strains used in this study was considered to persist in "defective" form requiring a helper virus to complete its maturation.[16]

If the RSV in human and monkey cultures is in need of a helper virus, the search for the latter may encompass more than the use of avian leukosis viruses which act as helpers for RSV in chick embryo fibroblasts.[16] Should a defective-helper virus system be species-specific, only exposure of RSV-infected human and monkey kidney cultures to human leukemic material may result in the appearance of infectious RSV. This, at the same time, would serve as demonstration for the presence of a "helper" virus in human leukemia. Experiments bearing on this problem are currently in progress.

Note: At the time this manuscript was being prepared, Professor L. A. Zilber, then visiting in the United States, told the authors that he and Shevlagin had obtained similar results with human embryonic cells exposed to chicken sarcoma virus at the Gamaleya Institute in Moscow.

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