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EXPERIMENTS ON THE SENSITIVITY OF STRAINS OF HUMAN FIBROBLASTS TO INFECTION WITH RHINOVIRUSES.

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HUMAN embryo fibroblasts have been observed and studied for many years in the outgrowths from explant cultures of human tissues. Recently Hayflick and Moorhead (1961) have described a technique for subculturing such cells in a reproducible manner. The cells retain the morphology of fibroblasts, they eventually die out after 30 to 50 passages, but they retain the typical diploid chromosome number almost to the end. They have accordingly been called human diploid cell strains (HDCS).

The rhinoviruses form a group of viruses related to enteroviruses (Tyrrell and Chanock, 1963) and can be recovered from cases of the common cold. Strains which have been isolated in monkey or human kidney cell cultures will grow in and cause degeneration of HDCS (Taylor-Robinson, Hucker and Tyrrell, 1962). Other strains have been isolated from clinical specimens by inoculation into HDCS (Hamparian, Ketler and Hilleman, 1961 ; Johnson, Bloom, Chanock, Mufson and Knight, 1962), although the isolation rates are apparently lower than those obtained in optimally sensitive cultures of human embryo kidney. We encountered one strain of H rhinovirus which would not multiply freely when infected nasal washings were inoculated into human embryo kidney cultures ; it did grow in the WI–26 strain of Hayflick and Moorhead (Tyrrell, Bynoe, Buckland and Hayflick, 1962). Washings were tested in 11 strains of diploid cells, each derived from the lungs of a different human embryo, and the virus grew in only 4 strains. Also, although the H.G.P. virus produced marked and rapid cytopathic effect in W1–38 cells it produced none or only occasional slight effects in primary cultures of human lung fibroblasts (Tyrrell and Parsons, 1960).

In view of these facts we decided to study further the sensitivity of a number of cell strains to rhinoviruses in order to find cells of maximal sensitivity for the isolation and titration of rhinoviruses. We studied the effect of the virus used in the test, modification in the medium, the length of cultivation *in vitro* and the origin of the cells.

MATERIALS AND METHODS

Cultures

Cell strains were initiated by trypsin dispersal of the lungs of normal human embryos (obtained at hysterotomy in most cases). The cells were passed in bottle cultures by repeated trypsinization, as described by Hayflick and Moorhead (1961). They were subcultured into test tubes. The cells were usually grown in a medium consisting of 10 per cent calf serum, 2 per cent of a stock solution containing 10 per cent of peptone and 88 per cent of Eagle's medium with antibiotics; they were maintained at 33° in 2 per cent calf serum and 98 per cent Eagle's medium, often remaining in excellent condition for 3 or 4 weeks without a medium change. The diploid chromosome number was not established for any of our strains, but their general behaviour and appearance on cultivation were very like that of well-characterised cell strains such as WI–38. We therefore call them HDCS for convenience.

Virus

We used viruses which had not been passed in HDCS in case in addition to becoming adapted to diploid fibroblasts they had also become adapted to a particular type of fibroblast. In general, viruses were used either as nasal washings obtained from volunteers inoculated with virus passed only in the human respiratory tract, or as fluids from tissue cultures which had been inoculated with such washings.

H.G.P. and P.K. were M rhinoviruses serologically indistinguishable from each other.

B816 was an H rhinovirus used only as a washing collected from infected volunteers. (Kendall, Bynoe and Tyrrell, 1962).

D.C. was an H rhinovirus used after a total of 6 passages in HDCS and 1 in HEK or as a nasal washing (Tyrrell *et al.*, 1962).

Coxsackie virus A21 derived originally from a virus recovered from an airman in the R.A.F. (see Parsons, Bynoe, Pereira and Tyrrell, 1960); it was used after 1 passage in human amnion cells, 1 in human embryo kidney, and 2 in HeLa cells.

Pools of virus were divided between numerous small tubes and stored at −60°. One tube was thawed at each test.

Conduct of sensitivity tests

Serial dilutions of tissue culture fluids or undiluted nasal washings were added in 0·2 ml. volumes to groups of 3 to 5 confluent or almost-confluent tissue cultures. These were observed for up to 2 weeks. Sometimes the amount of cytopathic effect was measured by counting microplaques (Parsons and Tyrrell, 1961), but usually by a simple arbitrary scoring system. Serial 10-fold dilutions were usually used and the results were, on the whole, reproducible. The results given in the table represent single titrations or the mean of 2 titrations and we have regarded tenfold differences (i.e. 1 log. unit) as significant.

RESULTS

Nasal washings were inoculated to cultures of 11 strains of cells and it was noted that there were apparent differences in sensitivity. The numbers that were inoculated were limited by shortage of washings, which were used undiluted. It can be seen from the representative results shown in Table I that virus was isolated from cells grown in 3 different media, and that a resistant and sensitive cell strain were found, both of which had been grown up in the same medium, namely Eagle's. The numbers of positive cultures were rather small, but it seemed likely that the strain BWHEL 1 was highly resistant to all strains, and subsequent experiments have shown that it is at least 100-fold less sensitive to H.G.P. virus than a number of other strains. Altogether, 2/10 strains failed to detect H.G.P., 2/10 failed to detect B816 and 4/6 failed to detect D.C.—however, apart from BWHEL 1, the resistant cells were different for different viruses—this might have been due to random variation or to specific differences in the cells and the two

TABLE I.—*The Recovery of Virus from Nasal Washings in Human Diploid Cell Strains (HDCS).*

Cell strain	Medium	Number of positive cultures in those inoculated with: M rhinoviruses H.G.P.	H rhinoviruses B816	H rhinoviruses D.C.
HEL 126	199	1/3*	1/3	3/3
S 44	Eagle's	3/3	3/3	0/3
HEL 117	Lactalbumin hydrolysate	3/3	1/3	1/2
BWHEL 1	Eagle's	0/4	0/4	0/4

* Numerator = number infected and denominator = number inoculated.

possibilities could not be distinguished without more quantitative experiments. However, it was concluded that sensitive strains of cells could be grown in several different media and that resistant or sensitive strains might be grown out in the same medium.

Titration of viruses in different cell strains

We next attempted to study the differences in sensitivity more quantitatively and some of our results are shown in Table II. The first 3 rows are of interest

TABLE II.—*Effect of Various Factors on the Sensitivity of HDCS to Rhinoviruses.*

Cell strain and medium	Titre (negative $\log_{10}$ per 0·2 ml.) of 4 viruses obtained from human embryo kidney cultures† and assayed in indicated cell strain: M rhinovirus H.G.P.	H rhinoviruses B816	H rhinoviruses D.C.	Coxsackie A21
WI–26 (Eagle's) 28.11	3·2	0·5	0·2	3·5
21.11	2·5	neg.*	neg.	3
10.10	1·5	neg.	neg.	4
WI–38 (Eagle's)	2·3	1·1	0·5	3·3
MEH (199)	2·7	0·2	0·5	3·3
HEL 130 (199)	1·7	neg.	neg.	5
MRC 1 (Eagle's)	⩾2	neg.	neg.	4·5
Human embryo kidney A	⩾2	0·2	1·0	>4
Human embryo kidney B	<1	neg.	neg.	—

* No virus CPE in three cultures receiving undiluted inoculum.

† The pools were all obtained from the same batch of HEK cultures inoculated with nasal washings, except for D.C. which had been passed 6 times in HDCS and once in HEK to adapt it to HEK cells.

because they show that the same cell strain may vary in sensitivity on different occasions, depending on how " healthy " the cultures are. Those prepared on the 10.10 appeared less healthy than the others and those on 21.11 although they looked normal had grown out more slowly than others. WI–38 was at least as sensitive as WI–26. In subsequent experiments it was shown that cultures which grew out well might be kept at 30° for many days before inoculation and still retain their sensitivity. A healthy cell strain grown in medium 199, namely MEH, was as sensitive as the above strain while HEL 130 and MRC 1 were relatively less sensitive ; nevertheless the latter seemed to be as sensitive as the others to cox-

sackievirus A21, if not more so. Finally, two batches of primary human embryo kidney cells were shown to vary over tenfold in sensitivity, and over at least as wide a range as the diploid cells. It was thought possible that virus might be multiplying in the apparently resistant cells without producing a cytopathic effect, and the medium from negative cultures found at and just beyond the end-point of a titration of P.K. was therefore inoculated into sensitive cells (monkey kidney cells). No virus was recovered from the normal looking cultures although it was readily recovered from cultures showing a cytopathic effect. It was concluded that the difference in sensitivity really represented a difference in the susceptibility to virus infection rather than a difference in the ability to show a cytopathic effect. It was also concluded that strains were on the whole sensitive or resistant to all the rhinoviruses used.

Further comparative titrations

We prepared a fresh set of virus seeds, and, because the storage of some cell strains had been unsuccessful, a fresh set of cell strains, and titrated the former in the latter. The objects were to search for highly sensitive cell strains in which to try and grow hitherto uncultivatable cold viruses and also to see whether the differences in sensitivity found with rhinoviruses were also observed with a typical enterovirus, namely poliovirus, and with a rhinovirus passed in different cells and to different extents in other cultures, such as monkey kidney or human embryo kidney. The results obtained are shown in Fig. 1. It may be seen that the infectious dose of poliovirus was the same in all the cell strains whereas with all the other virus seeds the strain HEL 148 was least susceptible, the strain HEL 149 more so, while HEL 150 and 151 were almost as sensitive as WI–38. Several other similar strains have been recovered since. They appear to be highly sensitive to H rhinoviruses and have been used successfully for detecting and quantitating small doses of coxsackievirus A21 in clinical specimens or in aerosols. The more resistant cells seemed to grow less well, to be finely granular in appearance and more likely to degenerate in maintenance medium than were sensitive cells.

Factors affecting the sensitivity of cell strains

We wondered whether sensitivity or resistance of a cell strain was present on first cultivation or gradually acquired and how the sensitivity of HDCS compared with that of primary HEK. In a preliminary experiment we prepared tube cultures of human embryo lung and inoculated these with serial dilutions of virus. Later bottle cultures were similarly subcultured and parallel cultures of WI–38, a sensitive HDCS, were likewise tested. The results are given in Table III, which

TABLE III.—*Relative Insensitivity of Early Passages of a Resistant Cell Strain.*

Test virus	Titre (negative $\log_{10}$ per 0·2 ml.) of M rhinovirus H.G.P. assayed in indicated cells			
	HEL 146		WI–38	
	Pass	Titre	Pass	Titre
First pass in HEK (pool A)	0	neg.	19	3
First pass in HEK (pool A)	1	<0·5	—	—
Nasal washing	2	neg.	20	1·5
First pass in HEK (pool B)	3	neg.	20	>2·5

shows that the resistance of the cell strain HEL 146 was present from the first culture and was manifested against several different pools of virus. Rather similar experiments in which one pool of virus was used are shown in Table IV and Fig. 2. These show that the lungs yielded primary cultures which were always much more resistant to the M rhinovirus than were the kidney cells, but that with subculture the lung cells gradually become more sensitive, although they usually "levelled

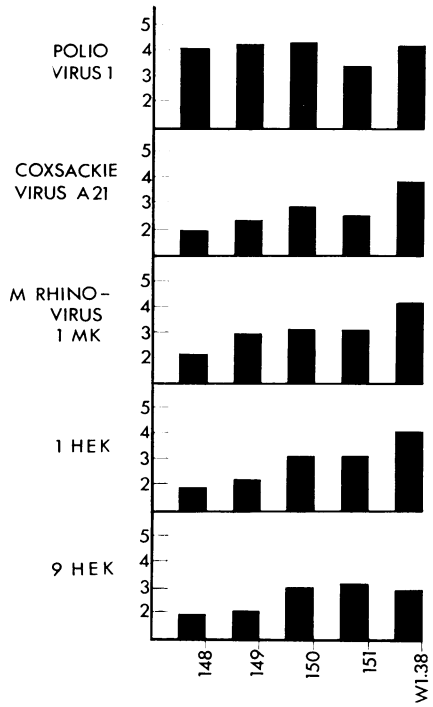


FIG. 1.—Five pools of virus were titrated in the five HDCS, 148, 149, 150, 151 and WI38. The titres are shown by the height of the columns and are expressed as $\log_{10}$ TCD_{50} per ml. of inoculum. The titres of the poliovirus were the same in all the cell strains, but varied with the other viruses, strain 148 being least readily infected.

TABLE IV.—*Relative Sensitivity of Cultured Kidney and Lung Cells of Seven Consecutive Human Embryos.*

Embryo	Titre of M rhinovirus assayed in: Primary kidney	Lung: 1st pass	Lung: Later pass
1	3·2	1·2	—
2	2·5	0·7	—
3	⩾4·2	—	⩾2·2 (8)*
4	4	0·7	3·7 (9)
5	3·2	1·2	3 (9)
6	3·4	1·2	2 (7)
7	3	neg.	3·2 (5)

* Number of serial passages.

out " at a degree of sensitivity which was less than that of the kidney cultures. The resistant cultures obtained on first passage were almost entirely composed of fibroblast-like cells (Fig. 3), but occasionally groups of epithelium-like cells were seen. It is possible that those embryos which yielded the more sensitive kidney cells also yielded the more sensitive HDCS, but this cannot be regarded as proved from our results.

An attempt was made to increase the sensitivity of 3 cell strains by adding the virus to freshly trypsinized cells rather than to cultures which had grown out; the cells were observed for cytopathic effects as usual. The sensitivity of this new

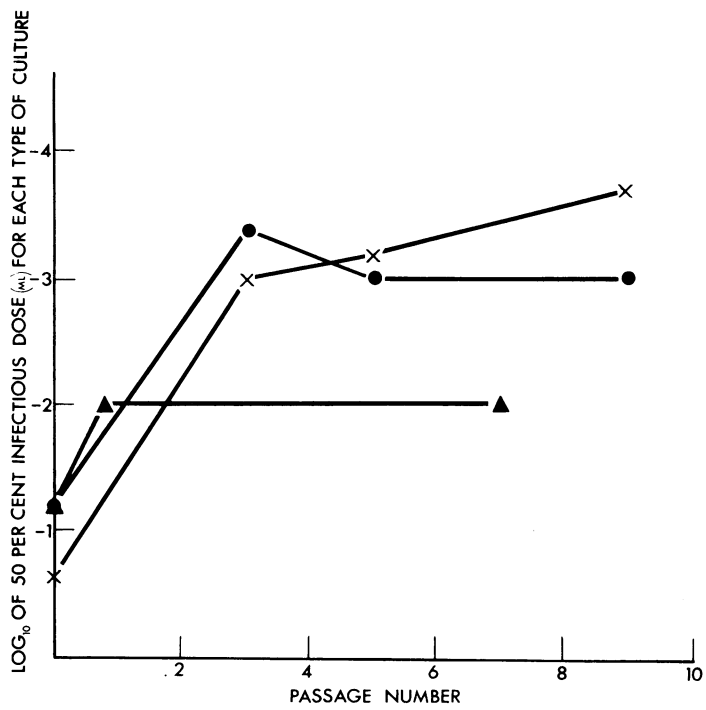


FIG. 2.—One pool of M rhinovirus strain P.K. was passed once in primary human embryo kidney cells and titrated in tube cultures of diploid cells prepared at various passage levels. The titres are expressed as in Fig. 1. The sensitivity of the cell strains to the virus increased rapidly after the first passage. ×—× Embryo 4. ●—● Embryo 5. ▲—▲ Embryo 6.

EXPLANATION OF PLATE.

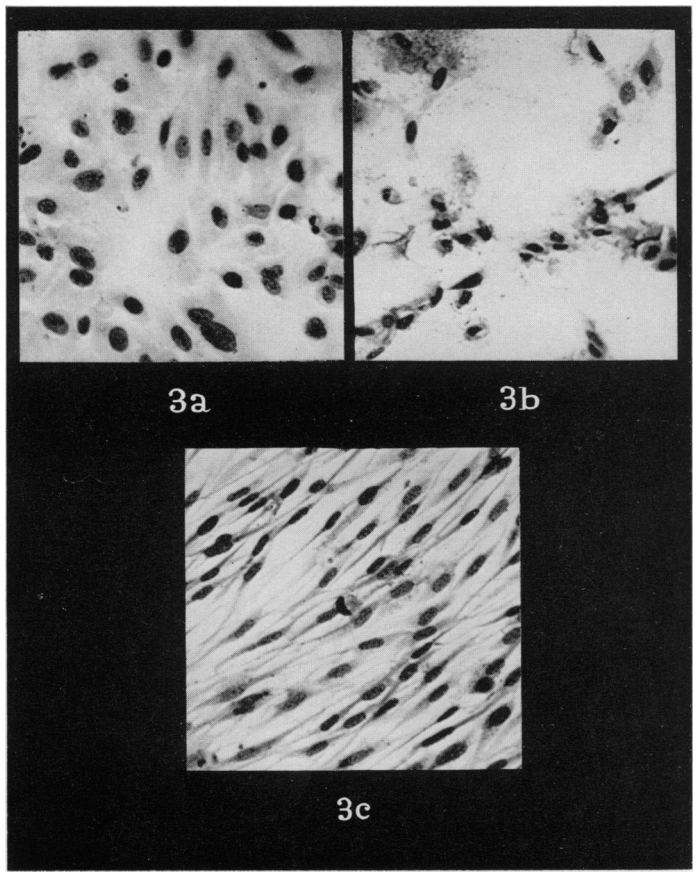
FIG. 3.—Fixed and stained preparations from embryo.

(*a*) Normal primary human kidney cells stained haemotoxylin and eosin. × 140.

(*b*) As above, inoculated 5 days previously with M rhinovirus P.K., passed once in human embryo kidney cells and diluted 1/100.

(*c*) Primary lung fibroblast cells inoculated and fixed as in (*b*) except that the virus was undiluted.

There is a definite cytopathic effect in (*b*) and none in (*c*). Although parallel cultures to (*c*) showed a cytopathic effect 5 days later, no cytopathic effect was seen in cultures inoculated with virus diluted 1/10.


3a
3b
3c

method was apparently at least 10-fold less than that of inoculating grown-out cultures.

We wondered whether the resistance or sensitivity of these cultures might be due to a transmissible factor, such as a latent virus and we attempted to make resistant cultures sensitive and vice versa by growing up cell sheets in the medium from cultures of the opposite type and then titrating virus in the confluent tubes in the usual way. No striking effects were observed, but in some control experiments it was noted that if cells were grown up in a mixture of one part of medium which had been used for growing similar cells and one part of fresh medium, then the cultures were as much as 3 or 4-fold more sensitive than cultures which had been fed only with fresh medium. The whole matter required further study.

DISCUSSION

The studies described in this paper seem to us to have raised more questions than they have settled. It seems clear that our cell strains vary in their sensitivity to rhinoviruses although they seem roughly equally sensitive to poliovirus, a finding which conflicts with the report of Hayflick and Moorhead (1961) that their strains varied in sensitivity to poliovirus. It is obvious that cells may be resistant because of some fault in the medium or culture conditions and may become sensitive due to an ill-understood change taking place during the early passages of the cells *in vitro*. It is also very likely that there is an intrinsic difference in the susceptibility of the cells in the first place, just as there are differences between the kidney cultures obtained from different embryos—a fact which we noted some years ago in attempts to isolate rhinoviruses and which has also been reported by Johnson *et al.* (1962).

We have, nevertheless, no idea what the nature of the resistance or sensitivity may be. The foetuses were not known to come from infected mothers and were apparently normal, but it is possible that they were infected with a virus, or that a virus was acquired from the culture medium. Medium from the uninoculated sensitive strain HEL 110 apparently produced colds in volunteers (Tyrrell *et al.*, 1962) and we therefore thought that the sensitive strains might be contaminated with a virus. However, as neither sensitivity nor resistance could be transmitted for certain in a number of experiments we could find no evidence to support these conjectures.

It is possible that cells are resistant because of differences in their chemical constitution which may in turn reflect the genetic constitution of the host from which they came. It is possible, for example, that our standard medium is slightly suboptimal for cells derived from a small proportion of embryos. Monocytes obtained from certain strains of mice have been found to be resistant or sensitive to certain viruses like their donor animal ; also the factor confering this property can be extracted from them and partly identified chemically (Kantoch and Bang, 1962). Further experiments on these lines are needed in our system, and we also plan to find out at what stage of the growth cycle virus multiplication is unable to progress.

The resistance of the first passage cells is also not understood. It might be due to the absence of a receptor substance, analogous to the receptors for enteroviruses which monkey kidney and human amnion cells acquire during the first few days of cultivation *in vitro* (McLaren, Holland and Syverton, 1960). The first passage

cells were found in one of our experiments to be of reduced sensitivity to both poliovirus and an M rhinovirus. On the other hand, resistance might be due to the presence of a small number of non-fibroblast cells which in some way confer resistance on a whole culture.

It may be that resistance is in all cases due to one factor, but we think it is equally possible that resistance will be found to be due to the operation of one or other of several different mechanisms.

SUMMARY

Fibroblast cell strains have been derived from the lungs of a number of human embryos. Some cell strains are more readily infected with M and H rhinoviruses than are others. The sensitivity may vary a hundredfold or more and is probably as great as that between different kidney cells obtained from different human embryos. Poor cultural conditions will reduce the sensitivity of a cell strain, but sensitive cell strains have been obtained in several different media. The resistance of one cell strain was detected on first passage and persisted ; several sensitive cell strains were found to be relatively resistant on first isolation, but acquired sensitivity after a few passages.

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