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CYTOPLASMIC SPREADING OF HUMAN SYNOVIAL CELLS IN CULTURE*

I. NOTES ON EXPERIMENTAL METHOD AND THE EFFECTS OF HEAT-INACTIVATED SERUM

BY

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When taken into suspension by trypsin or maintained in suspension by agitation, synovial cells retract the cytoplasmic processes characteristic of their growth on surfaces and adopt a rounded form. These processes spread again when the cells are allowed to settle and attach once more to a surface in a suitable medium. This behaviour of cells in culture is well known, and has been designated alternatively as flattening, stretching, or spreading. It forms a distinct phase in the propagation of cells by serial passage. Our interest in cytoplasmic spreading was aroused by the observation (Fraser and Catt, unpublished) that toxic influences could be recognized by their inhibition of the phenomenon. A deliberate study was therefore undertaken, partly to develop a quantitative method for assessing cytotoxic agents and also to determine optimal conditions for maintenance of synovial cell cultures

Methods

Stock cultures of synovial cells were isolated and propagated as previously reported (Fraser and McCall, 1965). The selected cultures were rinsed with calcium- and magnesium-free Hanks's balanced salt solution, and the cells suspended by incubation and agitation in trypsin 0·25 per cent. in phosphate-saline buffer at 37° C. This was done as quickly and gently as possible, but dispersal was verified by microscopic examination. The cell density of an aliquot of the trypsin suspension was determined in a Coulter electronic cell counter, and a volume of suspension sufficient to give a final cell density of 40,000 to 50,000 cells per ml. was spun down at 1,000 r.p.m. The cells were resuspended in Medium 199 containing 3 per cent. heat-inactivated human serum, which was included to maintain dispersion of the cells. This suspension was divided into parts of 4 ml. each. To each of these was added 4 ml. serum diluted in Medium 199 to give the desired final concentration. Each preparation was gently mixed and inoculated into two polystyrene dishes with recessed covers* (Cooper, 1961). 3·8 ml. neatly formed a meniscus between the dish and cover. The dishes were incubated in a humidified atmosphere of 5 per cent. carbon dioxide in air at 37° C. At intervals of 5, 24, and 48 hours, 400 cells were examined in each preparation by negative high phase contrast, and the proportion of cells showing cytoplasmic spreading was recorded. Fields were selected randomly and the coded dishes identified at the end of the experiment. Human serum was prepared aseptically from volunteers, strictly fasted overnight and not taking drugs. Canine serum was similarly prepared. Visible lipaemia or haemolysis was avoided. Other serum and media were supplied by the Commonwealth Serum Laboratories. All serum was heated at 56° C. for 30 minutes before the experiments.

Results

Human Serum

The degree of spreading in human serum depended strikingly on the concentration. Table I (opposite) records fourteen comparisons of individual sera in concentrations of 50 and 10 per cent. after 5, 24, and 48 hours. Spreading was on the whole much greater in 50 per cent. serum. This superiority was just

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* Falcon Plastics

TABLE I

COMPARISON OF HEAT-INACTIVATED HUMAN SERA, 10 PER CENT. AND 50 PER CENT., IN FOURTEEN EXPERIMENTS

Experiment No.	Serum Concentration (per cent.)	Percentage of Cells showing Cytoplasmic Spreading			Age of Culture (days)
		5 hrs	24 hrs	48 hrs	
1	50 10	42 31	87 80	96 84	21
2	50 10	68 14	78 34	82 18	26
3	50 10	3 7	36 5	37 12	48
4	50 10	33 15	49 22	73 11	49
5	50 10	6 4	25 13	65 15	56
6	50 10	7 4	55 8	78 8	61
7	50 10	58 38	66 69	90 45	39
8	50 10	3 2	39 9	86 17	63
9	50 10	7 12	87 44	91 35	70
10	50 10	2 3	21 5	67 3	76
11	50 10	37 28	52 22	53 6	34
12	50 10	15 3	72 14	92 41	69
13	50 10	40 14	88 35	76 15	113
14	50 10	60 55	76 48	77 25	34

INTERACTION TABLE, TOTALS OF TRANSFORMED VALUES*

Time (hrs)	Serum Concentration (per cent.)		Total
	50	10	
5	403·9[a]	305·2[a]	709·1[c]
24	713·8[a]	435·9[a]	1149·7[c]
48	864·6[a]	390·0[a]	1254·6[c]
Total	1982·3	1131·1	3113·4

Standard Errors:
Treatment Totals[a] (of 14) = 34·6
Marginal Totals[b] (of 42) = 59·8
Marginal Totals[c] (of 28) = 48·9
To compare two treatment totals, significant differences
at 5 per cent. level = 95·8, and at
1 per cent. level = 126·2

* Derived from analysis of variance after angular transformation (Fisher and Yates, 1953).

significant at the 5 per cent. level after 5 hours, but highly significant after 24 hours, when spreading was greater in all but one observation. There was a progressive increase of spreading in 50 per cent. serum throughout, but in 10 per cent. serum there was a slight regression after 24 hours.

Table II records three further experiments simultaneously comparing spreading in several titres. The same direct relationship between spreading and serum concentration was apparent.

Examination of the individual results further illustrates the analysis of the grouped observations. The 5-hour readings did not show the consistency of discrimination revealed by the later observations. The lower level and later regression of spreading were also very pronounced in low titres of some sera.

Heterologous Serum

Bovine and human sera were studied together in concentrations of 5 and 20 per cent. (Table III).

TABLE II

COMPARISON OF HEAT-INACTIVATED HUMAN SERA IN SEVERAL TITRES IN THREE EXPERIMENTS

Experiment No.	Serum Concentration (per cent.)	Percentage of Cells showing Cytoplasmic Spreading			Age of Culture (days)
		5 hrs	24 hrs	48 hrs	
1	5	19	7	10	107
	10	62	45	50	
	15	22	48	52	
	25	21	61	94	
2	10	14	35	15	113
	20	46	74	39	
	30	41	72	56	
	50	40	88	76	
3	10	55	48	25	34
	30	44	65	46	
	50	60	76	77	

TABLE III

COMPARISON OF HEAT-INACTIVATED HUMAN AND BOVINE SERA IN THREE EXPERIMENTS

Experiment No.	Serum	Concentration (per cent.)	Percentage of Cells showing Cytoplasmic Spreading		Age of Culture (days)
			24 hrs	48 hrs	
1	Human	20	74	81	43
	Bovine		87	87	
	Human	5	62	25	43
	Bovine		93	85	
2	Human	20	26	43	50
	Bovine		93	97	
	Human	5	10	8	50
	Bovine		74	96	
3	Human	20	10	25	92
	Bovine		53	47	
	Human	5	4	7	92
	Bovine		21	21	

INTERACTION TABLE, TOTALS OF TRANSFORMED VALUES*

Serum	Serum Concentration (per cent.)		Total
	20	5	
Human	243·6[a]	143·5[a]	387·1[b]
Bovine	382·5[a]	334·3[a]	716·8[b]
Total	626·1[b]	477·8[b]	1103·9

Standard Errors:
Treatment Totals[a] (of 6) = 27·3
Marginal Totals[b] (of 12) = 38·6
To compare two treatment totals, significant differences
at 5 per cent. level = 75·7, and at
1 per cent. level = 96·6
* Derived from analysis of variance after angular transformation (Fisher and Yates, 1953).

The spreading activity of bovine serum proved superior in both titres. Furthermore, it did not show a significant reduction in 5 per cent. concentration, by contrast with that of the human sera.

The activity of foetal calf serum also surpassed that of human serum (Table IV). The remarkable persistence of this potency in low dilutions is further illustrated in Table V.

Studies of canine serum were limited by difficulties in preparing sterile serum from fasting animals. Of the two studies, one showed a low level of spreading in both titres, but in neither study was there any loss of activity in the lower concentration (Table VI, overleaf).

Discussion

Method of Study.—The method described here was gradually devised from our earlier observations. Certain points need special emphasis. The significant variation between experiments was anticipated from empirical experience of culture maintenance. Some of this variation was no doubt due to the samples of serum. However, the cell strain, previous cultural history, and cell preparation were also

TABLE IV

COMPARISON OF HEAT-INACTIVATED HUMAN AND FOETAL CALF SERA IN THREE EXPERIMENTS

Experiment No.	Serum	Concentration (per cent.)	Percentage of Cells showing Cytoplasmic Spreading		Age of Culture (days)
			24 hrs	48 hrs	
1	Human	20	88	82	86
	Foetal Calf		79	86	
	Human	5	59	35	86
	Foetal Calf		78	76	
2	Human	20	9	7	93
	Foetal Calf		47	53	
	Human	5	3	9	93
	Foetal Calf		54	54	
3	Human	20	59	45	100
	Foetal Calf		88	72	
	Human	5	14	7	100
	Foetal Calf		88	76	

INTERACTION TABLE, TOTALS OF TRANSFORMED VALUES*

Serum	Serum Concentration (per cent.)		Total
	20	5	
Human	259·7[a]	151·3[a]	411·0[b]
Foetal Calf	348·5[a]	347·7[a]	696·2[b]
Total	608·2[b]	499·0[b]	1107·2

Standard Errors:
Treatment Totals[a] (of 6) = 24·2
Marginal Totals[b] (of 12) = 34·2
To compare two treatment totals, significant differences
at 5 per cent. level = 67·0, and at
1 per cent. level = 88·2
* Derived from analysis of variance after angular transformation (Fisher and Yates, 1953).

TABLE V

COMPARISON OF HEAT-INACTIVATED FOETAL CALF SERA IN SEVERAL TITRES IN TWO EXPERIMENTS

Experiment No.	Serum Concentration (per cent.)	Percentage of Cells showing Cytoplasmic Spreading		Age of Culture (days)
		24 hrs	48 hrs	
1	1	89	83	34
	2	91	87	
	3	87	74	
	5	89	84	
2	1	94	91	35
	2	90	93	
	3	94	94	
	5	90	95	

TABLE VI
COMPARISON OF HEAT-INACTIVATED CANINE SERA, 10 PER CENT. AND 50 PER CENT. IN TWO EXPERIMENTS

Experiment No.	Serum Concentration (per cent.)	Percentage of Cells showing Cytoplasmic Spreading			Age of Culture (days)
		5 hrs	24 hrs	48 hrs	
1	50	7	21	10	83
	10	7	33	39	
2	50	48	70	76	50
	10	36	68	77	

suspected causes of variation though difficult to measure. The chosen cultures were therefore carefully maintained with regular changes of medium and controlled *p*H. The most important feature of the method was the preparation of a common cell suspension in Medium 199 rather than separate suspensions of spun-down aliquots of cells in each complete test medium. The final mixing and inoculation of each test preparation could thus be done in virtually the same way. The number of comparisons in each experiment was arbitrarily limited to reduce the time between completion of each test mixture.

Many methods have been described to assess cell attachment or spreading in culture, but not all are designed for precise measurement. Lieberman and Ove (1958) defined a unit of activity as the smallest amount causing attachment and flattening of approximately half the number of cells. Plating efficiency or cloning has been used, in which the cell inoculum is sparse enough to enable individual cell colonies to be counted after a period of weeks, and to be regarded as the progeny of single cells. Zaroff, Sato, and Mills (1961), in the study of cells from marrow, kidney, thymus, liver, and spleen, found that plating efficiency was constant over a wide range of inoculum density. However, the proportion of cells which formed clones was extremely low, and evidence was cited to suggest that the surviving cells were antigenically related to connective tissue. The highly-specialized cells of these organs clearly did not survive culture, in contrast with our experience of synovial intima. Moreover, cells do not always establish with maximum efficiency in low density (Pace and Aftonomos, 1957; Puck, Cieciura, and Fisher, 1957). Fisher, Puck, and Sato (1958) measured cell attachment "by microscopic counting on the glass surface either of viable cells or fixed and stained preparations", each dish being inoculated with a known number of cells. Weiss (1959-1960) has used elegant means of applying shearing stresses to assess attachment of cells. However, these methods destroy the preparation after one measurement. In the present study, the use of Cooper dishes allowed serial observations of each culture with excellent optical conditions. Moreover, spreading was measured as a proportion, which avoided the necessity for absolute cell counts with the attendant sources of error. Rounded but unattached cells which would be lost in stained preparations would settle on the culture surface and could also be counted.

Significance of Spreading.—The phenomenon of cell spreading is undoubtedly complex. In the case of synovial cells, attachment to a surface is an essential first step. Though we have been able to keep such cells viable in suspension for many days, they have always remained rounded until allowed to settle in a stationary culture. Cell attachment and spreading seem to involve two distinct phenomena. The early phase has been extensively studied by Taylor (1961), who found that cells would attach very rapidly and spread, even when treated with formalin. He observed that this immediate type of spreading was retarded by a variety of sera and proteins, and depended little on cell-type or vessel-surface; on the other hand, cells spread in the absence of protein tended later to regress, detach, and degenerate. He contrasted this phase of spreading with that observed in the presence of serum which started after a delay of some hours, but was sustained. These findings are consistent with the variability of our five-hour observations which suggest the interplay of these phases (Exp. 11, Table I; Exp. 3, Table IV). The later observations in our studies proved to be a more significant index of the sustained spreading of viable cells. Nevertheless, this stage also involves a number of factors. 24-hour readings in the "lag" phase before multiplication presumably provide the best estimate of spreading alone. In the later stages, dissolution of dead cells and clumping of damaged cells in unfavourable media would also tend to increase the apparent proportion of spread cells. Nevertheless, these changes are usually slow enough to allow recognition of cells in a discrete rounded state when undergoing regression (see

Table I). After 24 hours, cell multiplication in favourable media may increase the apparent degree of spreading. However, there is strong evidence that adhesion to surfaces and to other cells depends on metabolic activity as well as the passive physicochemical role of the medium (Moscona, 1961). The system therefore seems appropriate for the quantitative study of cytotoxic effects, provided that the cells are not rapidly destroyed.

Effects of Heat-inactivated Serum.—In sustained synovial cell culture, we have sought to achieve two main objects; first, to maintain a cell strain with excess for experiment, and secondly to minimize changes in the nature of the cell population. A high degree of cell attachment and spreading after subculture is important for both purposes. As serum appears to be one of the main factors influencing attachment and spreading, the present studies were undertaken as it was not felt justified to apply results from other studies. Many of these have concerned the "permanent" established lines long-adapted *in vitro* rather than recently isolated strains. Furthermore, "fibroblastic" cells from different organs may differ in their cultural behaviour (Puck and others, 1957). Nevertheless, the effects of sera reported here agree generally with other studies. The three types of heterologous sera showed a high degree of heat-stable spreading activity sustained in dilutions of 10 per cent. and less, and foetal calf serum was remarkably effective in very low dilutions. The overall benefit of foetal calf serum in the culture of euploid cells has been noted by Puck, Cieciura, and Robinson (1958), and its specific effect on the spreading of HeLa cells by Fisher and others (1958). The latter group of authors related its spreading activity to an α-globulin identical with fetuin which comprises 40 per cent. of foetal calf serum. On the other hand, Weiss (1959) found the attachment of several human cell types in 10 per cent. heat-inactivated human serum superior to that in heterologous sera, This difference could be due to the cells, the sera, and other factors mentioned earlier, as well as the techniques. The chemical nature of the glass or plastic used for the culture vessel might be expected to affect cell attachment and spreading, and it is surprising that there is such a measure of agreement in the several studies mentioned.

It is well known that some individual sera are toxic in cell culture. It might be thought that the use of individual human sera in the present study could be responsible for the differences from pooled foetal calf and bovine sera. However, if this were so, spreading should have been less in the higher titres, as in the first experiment with canine serum. It can be concluded that in respect to human synovial cells, heat-inactivated human serum is relatively deficient in spreading activity in the technique used. This fact is clearly relevant in experiments requiring the use of human serum alone, and in the efficient maintenance of stock cultures by serial passage. In routine culture, cell dispersal does not need to be as vigorous, and complete spreading is more closely approached but the tendency to regression of cell spreading which we have noted in lower titres of human serum would impair the efficient maintenance of the numbers and nature of a cell strain.

Summary

A method is described for measuring the rate of cytoplasmic spreading of synovial cells on attachment to culture vessels after dispersal by trypsin. The effect of heat-inactivated serum on the spreading of human synovial cells is described. The spreading activity of certain heterologous sera (bovine, foetal calf, and canine) is maintained at a high level in concentrations of 10 per cent. and less. The spreading activity of human serum is relatively low in concentrations of 5-10 per cent. and maximal only at much higher concentrations. These findings are relevant to the successful maintenance of synovial cell strains and the design of experiments.

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Expansion cytoplasmique des cellules synoviales humaines en culture

I. Notes sur un procédé expérimental et sur les effets du sérum inactivé par la chaleur

RÉSUMÉ

On décrit un procédé pour mesurer la vitesse de l'expansion cytoplasmique des cellules synoviales en culture, après dispersion par la trypsine. On décrit l'effet du sérum inactivé par la chaleur sur l'expansion des cellules synoviales humaines. Le pouvoir de certains sérums hétérologues (bovin, foetal du veau et canin) de favoriser l'expansion est bien maintenu en concentrations de dix pour cent et même moins. A cet égard le sérum humain est plus faible aux concentrations de 5 à 10 pour cent et n'atteint son pouvoir maximum qu'à des taux beaucoup plus élevés. Ces résultats sont importants pour le maintien des souches des cellules synoviales et pour préparer des projets d'expériences.

Expansión citoplásmica de las células sinoviales humanas de cultivo

I. Notas sobre el método experimental y sobre los efectos del suero inactivado por el calor

SUMARIO

Se describe un método para medir la velocidad de la expansión citoplásmica de las células sinoviales de cultivo después de la dispersión por la tripsina. Se describe el efecto del suero inactivado por el calor sobre la expansión de las células sinoviales humanas. El poder de ciertos sueros heterólogos (bovino, ternero fetal y canino) de favorecer la expansión se mantiene bien en concentraciones de 10 por ciento y de menos. En este respecto el suero humano es menos fuerte en concentraciones de 5 a 10 por ciento y alcanza su mayor poder en concentraciones mucho más altas. Estos resultados importan mucho para cultivar cepas de células sinoviales y para preparar proyectos de experimentos.