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Authors
Lackie, JM
Armstrong, PB

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STUDIES ON INTERCELLULAR INVASION *IN VITRO* USING RABBIT PERITONEAL NEUTROPHIL GRANULOCYTES. II. ADHESIVE INTERACTION BETWEEN CELLS

J. M. LACKIE AND P. B. ARMSTRONG*
Strangeways Research Laboratory, Wort's Causeway, Cambridge CB1 4RN, U.K.

SUMMARY

A possible mechanism for intercellular invasion is that the strength of adhesion between host and invading cells is greater than the average of the strengths of homotypic adhesions. This hypothesis has been examined by a study of the kinetics of aggregation of dispersed populations of an invasive cell type (the rabbit peritoneal neutrophil granulocyte) and a host cell type (the chick embryo heart fibroblast) in shaken suspension culture. Since aggregation in mixed populations of the 2 cell types demonstrated tissue specificity, the hypothesis is not supported by these studies, heterotypic adhesions seem in fact to be weaker than homotypic adhesions.

INTRODUCTION

Intercellular invasion is the migration of invasive cells into the interiors of dissimilar host tissues. A possible mechanism for invasion based on analogy with the differential adhesion hypothesis of Steinberg (Steinberg, 1963, 1964, 1970) is that the heterotypic adhesions established between invasive and host tissue cells are favoured over homotypic adhesions. Invasion could then be treated as a converse phenomenon to the 'sorting out' of heterotypic populations of non-invasive cells (Moscona & Moscona, 1952; Armstrong, 1970, 1971; Steinberg, 1970).

In the present study, we report a test of this hypothesis using the rabbit peritoneal neutrophil granulocyte-chick embryo fibroblast invasion system described in a previous report (Armstrong & Lackie, 1975). The neutrophil granulocyte (PMN) is highly invasive *in vivo* and has been shown to be invasive to aggregates of chick embryo fibroblasts *in vitro* (Armstrong & Lackie, 1975). Study was made of the aggregation kinetics of mixed populations of fibroblasts and granulocytes. Contrary to our expectations, heterotypic aggregation was not favoured over homotypic aggregation. These observations do not support the hypothesis outlined above.

* Permanent address: Department of Zoology, University of California, Davis, California 95616, U.S.A.

MATERIALS AND METHODS

Neutrophil granulocytes (PMNs) were obtained and prepared for aggregation experiments as described previously (Armstrong & Lackie, 1975; Lackie, 1974). Chick heart fibroblasts (CHFs) were grown in 90-mm tissue culture grade plastic Petri dishes (Armstrong & Lackie, 1975) and suspended when sub-confluent by mild trypsinization. Cells were treated at room temperature with 0·05 % trypsin (Difco 1:250) in divalent cation free (CMF) Hanks' containing 0·5 mM Na_2EDTA. After 3 min, the trypsin solution was replaced with CMF Hanks'-Hepes at 4 °C and cells were released from the dish by pipetting. The cell suspension was washed 3 times in cold CMF Hanks'-Hepes and the cells were suspended at the appropriate concentration in Hanks'-Hepes or Medium 199 with 10 % calf serum.

Aggregation experiments

Aggregation was carried out in plastic scintillation vials containing 2 ml of cell suspension on a reciprocating waterbath shaker (4 m^{-2} stroke; 100 strokes per min) at 37 °C. Aliquots of 0·2 ml were removed from the vials at various times using an 'Oxford' micropipette with an enlarged (2-mm) pipette aperture. The samples were diluted to 10 ml with filtered 150 mM NaCl and counted electronically on a Model 401 Celloscope (Ljunberg; Sweden) fitted with a 100-μm aperture tube and a 545-μl metering unit.

Within an experiment the aggregation vials contained equal numbers of cells (except when deliberately diluted) and comparisons within an experiment present no problems. The validity of comparisons between experiments is doubtful, since preparations of PMNs differ markedly in their adhesiveness (Lackie, 1974), and paired-sample statistics have been used where appropriate.

The experimental design is similar to that described by Curtis (Curtis, 1969, 1970*a*, *b*; Curtis & van de Vyver, 1971), except that, since the Couette viscometer aggregation system was not used, collision efficiency could not be calculated. In his analysis of aggregation data, Curtis utilized the formula

$$\log P = -\mathrm{k}Et$$

where P (the particle ratio) is the number of particles present at time t divided by the number at time zero, k is a constant for a given aggregation system, and E is the collision efficiency (the fraction of collisions that result in adhesions).

In binary cell mixtures the particle ratio may be calculated

$$\log P_{AB} = n_1 \log P_A + n_2 \log P_B,$$

where P_A, P_B and P_{AB} are the particle ratios, at time t, for pure suspensions of type A or type B cells and for mixtures of the two in which n_1, n_2 are the fractions of the 2 cell types present. The value of P_{AB} so calculated assumes that there is no specificity and that collisions between cells of type A and type B are likely to be effective in proportion to the adhesiveness of the two cell types.

To calculate P_{AB} on the expectation of complete specificity (P') it is possible to use Curtis formula

$$E_{AB} = n_1^2 E_A + n_2^2 E_B$$

but since we cannot derive E (collision efficiency) from our system we have calculated P'_{AB} by combining the particle ratios for appropriately diluted suspensions of the two cell types. Thus if $P_{0.5A}$, $P_{0.5B}$ are the particle ratios for 50 % dilutions of A and B then for the 50:50 mixture,

$$\log (P'_{AB}) = 0{\cdot}5 (\log P_{0.5A}) + 0{\cdot}5 (\log P_{0.5B}).$$

This assumes that the 2 cell types aggregate independently and that for either cell type the sole consequence of the mixture is one of dilution.

Membrane filter assay

The composition of the aggregates present in mixed suspensions of CHFs and PMNs following 2 h of agitation in the reciprocating shaker at 37 °C was determined by direct micro-

scopic examination. The cell suspension to be examined was filtered on to a cellulose acetate membrane filter (1·2-μm pore size: Millipore Filter Corp., New Bedford, Mass.) and fixed with neutral-buffered formalin. The filter was then stained with Giemsa, dehydrated in isopropyl alcohol, cleared in toluene and mounted in Canada balsam as described previously (Armstrong, 1966; Steinberg, Armstrong & Granger, 1973). Under these conditions, loss of cells from the filter was negligible, and fibroblasts and PMNs could readily be distinguished from one another on the basis of size, nuclear morphology and cytoplasmic staining. In excess of 1000 aggregates were examined on each filter by scanning consecutive fields with a Zeiss microscope fitted with a Neofluar 40/0·65 objective. The number of cells of each type was recorded from every aggregate encountered.

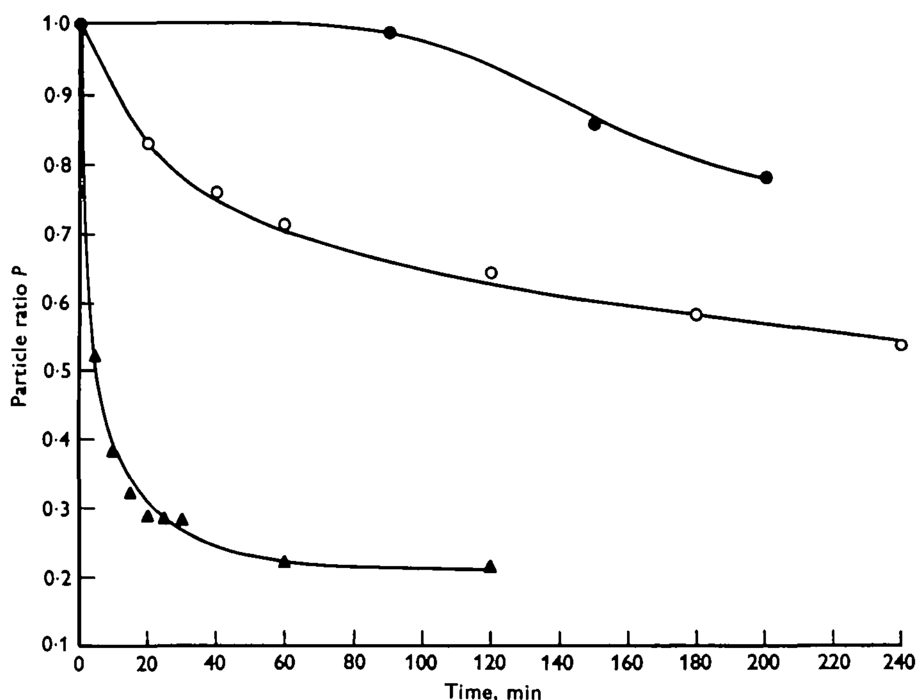

Fig. 1. The rates of decline of the particle ratios (*P*) (see Table 1) with time for: chick heart fibroblasts (CHFs) suspended using 0·1 % trypsin for 5 min (●—●); CHFs suspended using 0·05 % trypsin for 3 min (○—○); and rabbit PMNs (▲—▲). Note the lag in aggregation for the more heavily trypsinized fibroblasts. A similar effect has been reported for chick embryo neural retina cells; harsh trypsinization produces an aggregation lag whereas mild trypsinization does not (Steinberg *et al.* 1973). Each point represents the mean value from at least 3 separate flasks.

RESULTS

Aggregation experiments

Both PMNs and chick heart fibroblasts will spontaneously aggregate, though at rather different rates, when monodisperse cell suspensions are shaken at 37 °C (Fig. 1; see also Lackie, 1974). The extent of aggregation and its rate can be quantitated by following the decline in total particle number in the suspension, since aggregates count only as single particles. In mixed cell suspensions, a proportion of the collisions,

which may or may not lead to cell-cell adhesion and hence aggregation, are between dissimilar cells, and it is these collisions which are of particular interest. If heterotypic collisions are ineffective then the rate of aggregation of the individual cell types will be reduced. If there is no tissue specificity during aggregation, then the log of the rate of aggregation of the mixture should simply be related to the proportion of each cell type present, and the logs of their respective monotypic aggregation rates (Curtis, 1970*b*; Curtis & van de Vyver, 1971). Were heterotypic adhesion preferred, then the rate of aggregation in the heterotypic suspension should be greater than would be predicted on the basis of this simple linear relationship.

Table 1. *The difference between observed values for the particle ratio of* 50:50 *mixtures of PMN and CHF* ($P_{50/50}$) *at* 3 *different times and values calculated from the particle ratios of* 100% *pure PMN* (P_{PMN}) *and CHF* (P_{CHF}) *on the expectation of no specificity of adhesion*

Time, min	P_{PMN}	P_{CHF}	$P_{50/50}$ Observed	$P_{50/50}$ Calculated	Mean difference ± 5% C.I.	No. of pairs of flasks	't'
30	0·428	0·834	0·761	0·583	0·179 ± 0·050	14	7·664
60	0·368	0·715	0·646	0·499	0·147 ± 0·046	14	6·857
180	0·386	0·608	0·608	0·447	0·161 ± 0·087	8	4·356

The calculations are described in the Methods section. In order to combine the data from 5 separate experiments, the mean difference between the observed and calculated values (± 5% confidence interval estimate) was obtained using paired samples. If no specificity were shown then the mean difference should not be significantly greater or less than zero.

The aggregation data for 50:50 mixtures of PMNs and CHFs are presented in Table 1. The degree of aggregation is expressed as the particle ratio (P):

$$P = \frac{\text{number of particles at time } t}{\text{number of particles at time zero}}$$

High values of the particle ratio mean that little aggregation has occurred. As can be seen by inspection, the observed degree of aggregation of mixed cell suspensions is significantly less than the degree of aggregation that would be predicted if no specificity existed. If complete specificity of aggregation is assumed (i.e. PMNs aggregate only with PMNs; CHFs only with CHFs) then, in the early stages of the aggregation process, only a fraction of collisions (i.e. the homotypic collisions) are likely to lead to adhesion and the aggregation rate of each cell type will be reduced. Thus in a 50:50 mixture, the aggregation rate of the PMNs is likely to be comparable with the rate observed in 50% dilutions of the PMNs, and similarly for the CHFs. If the predicted aggregation rate for the 50:50 mixture is calculated on this basis, then a better approximation to the observed rate is obtained (Fig. 2, Table 2).

It has been suggested that cells may release substances into the culture medium which inhibit the adhesion of other cell types (Curtis & van de Vyver, 1971; Curtis & De Sousa, 1973). For this reason, the effect of CHF-conditioned medium on the

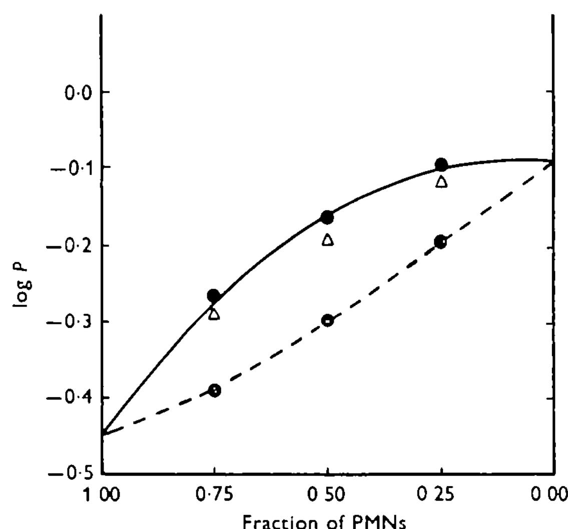

Fig. 2. The log of the degree of aggregation at 60 min for mixed suspensions of PMNs and CHFs as a function of the ratio of PMNs in the cell suspension. The broken line (– – –) indicates the expected result if no adhesive specificity is exhibited (calculated as $(n_{PMN} \log P_{PMN} + n_{CHF} \log P_{CHF})$). The triangles are the values calculated on the basis of complete specificity from the formula:

$$\tfrac{1}{2}(\log P_{n_{PMN}} + \log P_{n_{CHF}}),\ \text{where } P_{n_{PMN}} \text{ and } P_{n_{CHF}}$$

are the particle ratios at 60 min for suspensions of PMNs and CHFs diluted to a cell concentration equal to the fractional concentrations of each cell type in the mixtures.

Table 2. *The observed and calculated values of the particle ratio (P) at 60 min for various mixtures on the basis of either no specificity or complete specificity, and the mean difference between the observed and calculated values (± 5 % confidence interval estimate)*

		No specificity			Complete specificity	
		P				
Fraction of PMN	Fraction of CHF	Observed	Calculated	Mean difference ± 5 % C.I.	P calculated	Mean difference ± 5 % C.I.
1·00	0·00	0·358	—	—	—	—
0·75	0·25	0·543	0·400	0·114 ± 0·052*	0·517	0·027 ± 0·038
0·50	0·50	0·689	0·506	0·166 ± 0·092*	0·601	0·087 ± 0·058*
0·25	0·75	0·805	0·640	0·159 ± 0·107*	0·722	0·083 ± 0·020*
0·00	1·00	0·811	—	—	—	—

This table is based on the results from 5 paired sets of flasks drawn from 2 experiments in which the whole range of mixtures and dilutions were done. Note that the aggregation of mixtures is lower even than expected on the basis of complete specificity, but that the difference between the observed values and the values calculated on this basis is less than that calculated on the expectation of no specificity.

* Significantly different from zero at the 5 % level.

aggregation of PMNs was investigated. No significant difference could be detected in the 8 pairs of flasks used (P (control) $\pm$ 1 standard deviation = 0·392 $\pm$ 0·025; P (fibroblast conditioned medium) = 0·420 $\pm$ 0·032) but the data in Table 2 suggest that there may be some effect in the present system.

The data from the aggregation experiments suggest that the mixed cell suspensions behave as nearly independent populations, and that little or no heterotypic aggregation occurs.

Table 3. *Distribution of cells in aggregates produced during 2 h of aggregation of a mixed cell suspension containing equal numbers of chick heart fibroblasts (CHF) and rabbit neutrophil granulocytes (PMN)*

Aggregate class	Composition	Number of aggregates in each class: Observed	Expected
2-cell	CHF	141	45
	Both	22	90
	PMN	17	45
3-cell	CHF	58	14
	Both	15	84
	PMN	39	14
4-cell	CHF	31	5
	Both	12	70
	PMN	37	5
5-cell	CHF	12	1·2
	Both	6	36
	PMN	20	1·2
> 5-cell	CHF	18	< 3*
	Both	114	> 160*
	PMN	43	< 3*

The figures for the number of aggregates in the homotypic classes have had subtracted from them the numbers of homotypic aggregates of each class that were present in the fibroblast and PMN suspensions at the beginning of the experiment. The figures in this table represent only those aggregates formed during the 2-h aggregation period, and are drawn from 2 replicate flasks. The expected number of aggregates has been calculated on the assumption of no adhesive specificity, i.e. that heterotypic aggregates are as likely to form as homotypic aggregates. It can be seen from the table that PMN aggregates tend to be larger than CHF aggregates.

* These values are calculated for 6-cell aggregates and therefore represent the limits of the expected values.

Membrane filter assay

The suggestion that specificity of aggregation occurs in mixed suspensions of CHFs and PMNs is supported by direct examination of the aggregates formed after 2 h in a 50:50 mixture of the 2 cell types (Table 3). Few heterotypic aggregates were present; the majority of aggregates were homotypic. If there had been no specificity of aggregation, there would be many more heterotypic aggregates than homotypic aggregates,

and had there been preferential heterotypic adhesion then heterotypic aggregates would have been expected to constitute the predominant class.

DISCUSSION

The intermingling of cells that occurs during intercellular invasion may depend upon a variety of factors, among them the ability of invasive cells to move over the surfaces of normal cells. We have shown (Armstrong & Lackie, 1975) that PMNs will move on chick heart fibroblasts, and have argued that this is an important attribute for an invasive cell. It could, however, be argued that PMNs tend to move so as to maximize their adhesive interactions with the surrounding cells, or indeed that the preferential formation of heterotypic adhesions between invasive cells and host-tissue cells would be sufficient in itself to cause invasion. In the present study we have examined the hypothesis that invasion is a consequence of preferential heterotypic adhesion, and that it may be considered the converse of sorting out. Our results, which show that both chick heart fibroblasts and PMNs show tissue-specific aggregation *in vitro*, indicate that this hypothesis is untenable.

One major problem that should be emphasized is the difficulty involved in relating data derived from short-term aggregation studies to situations in which a complex equilibrium of adhesive forces may be involved (Steinberg, 1964, 1970; Niederman & Armstrong, 1972). Nevertheless, since migration by PMNs in the acute inflammatory response occurs rapidly (McGovern, 1957; Florey & Grant, 1961), and with a time course similar to that used in these experiments, we feel justified in considering that short-term adhesive interactions are more likely to be important than long-term adhesive equilibria. Three pieces of evidence indicate that PMNs do not preferentially adhere to fibroblasts rather than to one another: the aggregation kinetics of mixed suspensions, the composition of the aggregates so formed, and the low collecting efficiency of fibroblast lawns for PMNs (Armstrong & Lackie, 1975). Thus invasion does not seem to be the converse of sorting out, and must presumably involve some other mechanism which overcomes the homotypic adhesive preference.

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