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Biocompatibility of Wound Management Products: Standardization of and Determination of Cell Growth Rate in L929 Fibroblast Cultures

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Abstract—To facilitate the development of a bioassay procedure by which the biocompatibilities of materials used in wound management may be assessed and compared, those environmental factors affecting cell growth in mouse L929 fibroblast cultures have been identified. Standardization of the initial cell number and frequency of change of medium resulted in the virtual elimination of variation of growth curves of L929 cells cultured in flasks of specified surface area. In addition, three methods for assessing fibroblast growth rate in the presence of alginate products used in wound management were evaluated. These were the haemacytometer counting chamber method, the Coulter counting method, and a liquid scintillation counting method. The first two methods determine the number of cells in a given volume of a cell suspension, whereas the third method determines the rate of synthesis of deoxyribonucleic acid (DNA), and hence cell growth, by measuring the incorporation of [^{3}H]thymidine. The haemacytometer method had significant advantages over the other two procedures in providing both qualitative and quantitative data on culture morphology and cell growth response.

In their transition from passive to interactive to active materials, 'surgical dressings' have evolved into 'wound management products'. This evolution has been facilitated by a better understanding of both wound physiology and the physico-chemical characteristics of the new materials. In recent years this has led to the development of a wide range of products based on synthetic, semi-synthetic and natural polymers, and perhaps most importantly to the so-called hydrogel and hydrocolloid dressings (Turner et al 1986). The efficacy of these wound management products is currently determined either by clinical assessment, which remains guided by empirical observations and anecdotal reports, or through the use of wound models. Both procedures give only minimal information on the local cellular response to the presence of intrusive materials such as hydrogels and hydrocolloids.

The further development of these materials requires that a better understanding be gained of the qualitative and quantitative aspects of the cellular response to their presence. Our objective has been to design an in-vitro bioassay system that will provide such information and hence information on their biocompatibility and, indirectly, on their suitability for use in wound management. This could allow the optimization of existing product use and facilitate the development of new materials with intrinsic biological activity.

Animal cell culture methods used to determine the biocompatibility of medical materials and devices (Wilsnack 1973; Fehn & Schottler 1983; Johnson & Northup 1983; BSI 5736: part 10: 1988) generally rely upon the microscopical assessment of a developing cell monolayer in the presence of the material or a prepared extract. We require a quantitative determination of cell growth to allow comparison of different products. If bioassay procedures are to be developed by which the biocompatibilities of cell types found in healing wounds with wound management products may be assessed and compared, those factors affecting growth, and hence yield of cells in control cultures of appropriate cell lines at any specific time during culture, have to be identified and standardized. The mouse L929 fibroblast was selected as an appropriate cell line for this developmental work. As suitable culture media for this cell line are in established use, environmental sources of variation, other than those associated with the deliberate alteration of culture medium composition, needed to be investigated. These included the frequency of change of medium, the initial cell number, and the surface area available for growth. In addition, three standard counting procedures (Freshney 1983) were selected for comparison: the Coulter and haemacytometer cell counting methods, and the liquid scintillation method for determination of DNA synthesis, and hence cell growth. These methods were evaluated using mouse L929 fibroblast cultures in the presence of two similar alginate dressings which have previously been found to produce contrasting cell responses (Schmidt et al 1986; Fry 1986).

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Materials and Methods

Cell lines

L929 mouse fibroblasts were obtained from Flow Laboratories, Rickmansworth, UK.

Culture media

Unless otherwise stated, all materials were purchased from Flow Laboratories, Rickmansworth, UK.

Complete growth medium for the L929 cells consisted of Minimum Essential Medium Eagle (Modified) with Earle's salts (EMEM) containing 20 mM *N*-2-hydroxyethylpipera-

zine-*N'*-2-ethanesulphonic acid (HEPES) supplemented with non-essential amino-acids (1% dilution of MEM concentrate), 2 mM L-glutamine, and 10% foetal bovine serum (FBS).

Dulbecco's phosphate buffered balanced salt solution (BSS) without Ca^{2+} or Mg^{2+} containing trypsin (0·25% w/v) was used in cell trypsinisation procedures. Antibiotics (penicillin 100 units mL^{-1} and streptomycin 100 μg mL^{-1}) were used in the stock cultures but not in the test dishes.

Routine maintenance of the cultures

Stock L929 cells were stored in liquid nitrogen. New cultures were initiated from frozen stock; cells were grown in sterile 75 cm^2 tissue culture flasks in the complete EMEM medium at 37°C. Cultures were passaged weekly and media changed every two days.

Reagents, etc.

[Methyl-^{3}H] Thymidine 2 Ci $mmol^{-1}$ (74 GBq) and an LKB Internal Standard Kit containing [^{3}H]cholesterol 0·09 μCi were obtained from Amersham International UK. Glass microfibre discs grade GF/A 1·5 cm diameter, trichloroacetic acid (TCA), perchloric acid 70% Analar, and chloroform were obtained from BDH Chemicals UK. Scintillation fluid (Scintillator 299) was obtained from Packard.

Alginate 1 and Alginate 2 were obtained from N.I. Medical and Brit-Cair Ltd as pre-marketing samples for evaluation. They do not represent currently available proprietary products (Sorbsan and Kaltostat).

Standardization of culture variables

Growth curves of L929 cells were produced for periods of up to 8 days from cells in different passages (572–577); each experiment was conducted with cells from the same passage. Cells were grown in a humidified incubator at 37°C in a 5% CO_2 in air atmosphere. The use of sera with different batch numbers was unavoidable, but otherwise the composition of the culture medium was not varied between experiments. The effect of frequency of change of medium, initial cell number, and substrate area on the growth of the L929 cells was investigated:

(a) *Change of media.* To examine the effect of frequency of change of medium on the growth rate of L929 cells, four groups of replicate cultures containing the same initial cell number (1×10^5 cells mL^{-1}) in 5 mL medium were prepared in identical 25 cm^2 flasks. Medium was changed either on days 2 and 6, on days 4 and 6, or on days 1 and 6. The fourth group comprised cultures in which a change of medium was made on days 2, 4, and 6. This group was regarded as the control. Three cultures from each group on days 2, 4, 6, and 8, were trypsinized using 5 mL trypsin solution in BSS, and the cells counted in three samples from each culture using the haemacytometer method described below. For each sample, the average of 15 field counts was calculated and the standard deviation computed.

(b) *Initial cell number.* The effect of the initial cell number of the cultures on the final yield of cells was investigated by preparing replicate cultures in 25 cm^2 flasks with 5 mL of cell suspension containing cell concentrations varying from 80×10^3 to 320×10^3 cells mL^{-1}. Media changes were carried out on days 2, 4, and 6. Three cultures were taken from each replicate group on days 2, 4, 6, and 8, and the cell monolayers trypsinized using 5 mL trypsin solution. The cells in three samples from each culture were counted using the haemacytometer method as in (a) above. For each sample, the average of 15 field counts was calculated and standard deviation computed.

(c) *Growth surface area.* To investigate the effect of the growth surface area on the growth rate of the cells, 2×10^5 L929 cells mL^{-1} were inoculated into three types of culture vessels: 25 cm^2 flasks, 21 cm^2 dishes, and multi(24)-well dishes (surface area of each well: 2·25 cm^2). Volumes of 5 mL and 1 mL of the cell suspension were used in the flasks/dishes and multi-well dishes, respectively. Media changes were made every two days. Three cultures were removed on days 2, 4, 6, and 8, trypsinized, and the cells counted as in (a) and (b) above.

Preparation of the alginate samples

Samples of alginate products having dimensions 3×3 cm were removed aseptically from the manufacturer's packaging and placed in complete EMEM medium until saturated. The saturation volumes were found to be $0{\cdot}90 \pm 0{\cdot}1$ mL for Alginate 1 and $0{\cdot}75 \pm 0{\cdot}1$ mL for Alginate 2.

Determination of the effect of the alginate products on the growth of L929 cells

(a) *Haemacytometer method.* L929 cells in their 575th passage were used. The cultured monolayer was trypsinized to produce a suspension containing 1×10^5 cells mL^{-1} of EMEM medium. Five mL aliquots were dispensed into 21 cm^2 tissue culture dishes and the cultures maintained in a humidified incubator at 37°C in a 5% CO_2 in air atmosphere. Twenty four hours later media were removed and the dishes were divided into three groups. The control dishes received 5 mL of medium. The second group was supplemented with the saturated Alginate 1 samples plus 4·1 mL of medium. The third group received the Alginate 2 samples plus 4·25 mL of medium. Every 24 h, three dishes from each group were removed and counted using an improved Neubauer haemacytometer chamber. Each dish was counted in triplicate and the average of five field counts was calculated.

(b) *Coulter counter method.* A 100 μm tube suitable for particles within a size range 2–40 μm was used. A Coulter counter Model ZB was calibrated using divinylbenzene spheres of 9·7 μm diameter; the calibration factor (k) was found to be 2·1. The L929 cells had a diameter range of 3–22 μm.

To count cells, monolayers were trypsinized and suspended in BSS. Two counts were performed on each L929 sample to obtain concordant readings.

Liquid scintillation counting method for measuring uptake of [^{3}H]thymidine in-vitro

A quench curve was prepared using the internal standard method. The amount of [^{3}H]thymidine incorporated into the cells over 48 h was used to measure the growth of cells in the

cultures supplemented with Alginate 1 and Alginate 2 and in the control cultures. Cultures were established at a density of $1{\cdot}2 \times 10^5$ cells mL^{-1}. Five mL of cell suspension was dispensed into each tissue culture dish to produce replicate cultures. After 24 h incubation at 37°C in a humidified incubator, the saturated samples of the alginate products were placed onto the formed monolayers. Twenty four dishes were treated with Alginate 1, 24 dishes with Alginate 2, and the remaining 24 dishes were used as a control. At the same time, the medium was replaced by a medium containing [^{3}H]thymidine at a concentration of 1 μCi mL^{-1}. Quadruplicate cultures were removed at 6, 12, 24, 30, 36 and 48 h from the time of application of the samples and the amount of [^{3}H]thymidine residue in the culture media determined. The amount of [^{3}H]thymidine activity incorporated into the cells was measured using a modification of the filter disc technique of Cope & Double (1972).

The culture dishes were washed with BSS (5 mL), trypsinized, and the cell suspension treated with cooled 5% TCA (5 mL). The precipitate was collected on Whatman glass fibre discs, which were extracted with 5 mL of 0·5 M perchloric acid for 30 min at 70°C. Duplicate samples of 0·1 mL of the extracts of each dish were mixed with 10 mL of scintillation fluid and counted in a Liquid Scintillation Counter model 1217 Rackbeta LKB Wallac.

Statistical method

Duncan's test for multiple comparisons (Duncan 1955) was used to evaluate the difference between the control cultures and those treated with the alginate products.

Results and Discussion

In our development of a bioassay method for the comparative evaluation of biocompatibility of materials used in wound management, we have identified and standardized those factors (other than composition of medium) which affect L929 cell growth in the cultures. These include the frequency of change of medium, the relationship between initial cell number and volume of culture medium, and the surface area of the growth vessel.

From the growth curves shown in Fig. 1, it can be seen that the rate of increase in cell number was affected by the frequency of medium change. Representative growth curves derived from cultures having an initial cell number of 1×10^5 cells mL^{-1} and grown in 25 cm^2 flasks are shown in Fig. 1.

Our results are consistent with the view that rate of growth slows as nutrient levels in the medium become limiting. It is clear that a 48 h interval between media changes encourages steady growth of the L929 cells.

The relationship between the initial cell number and the

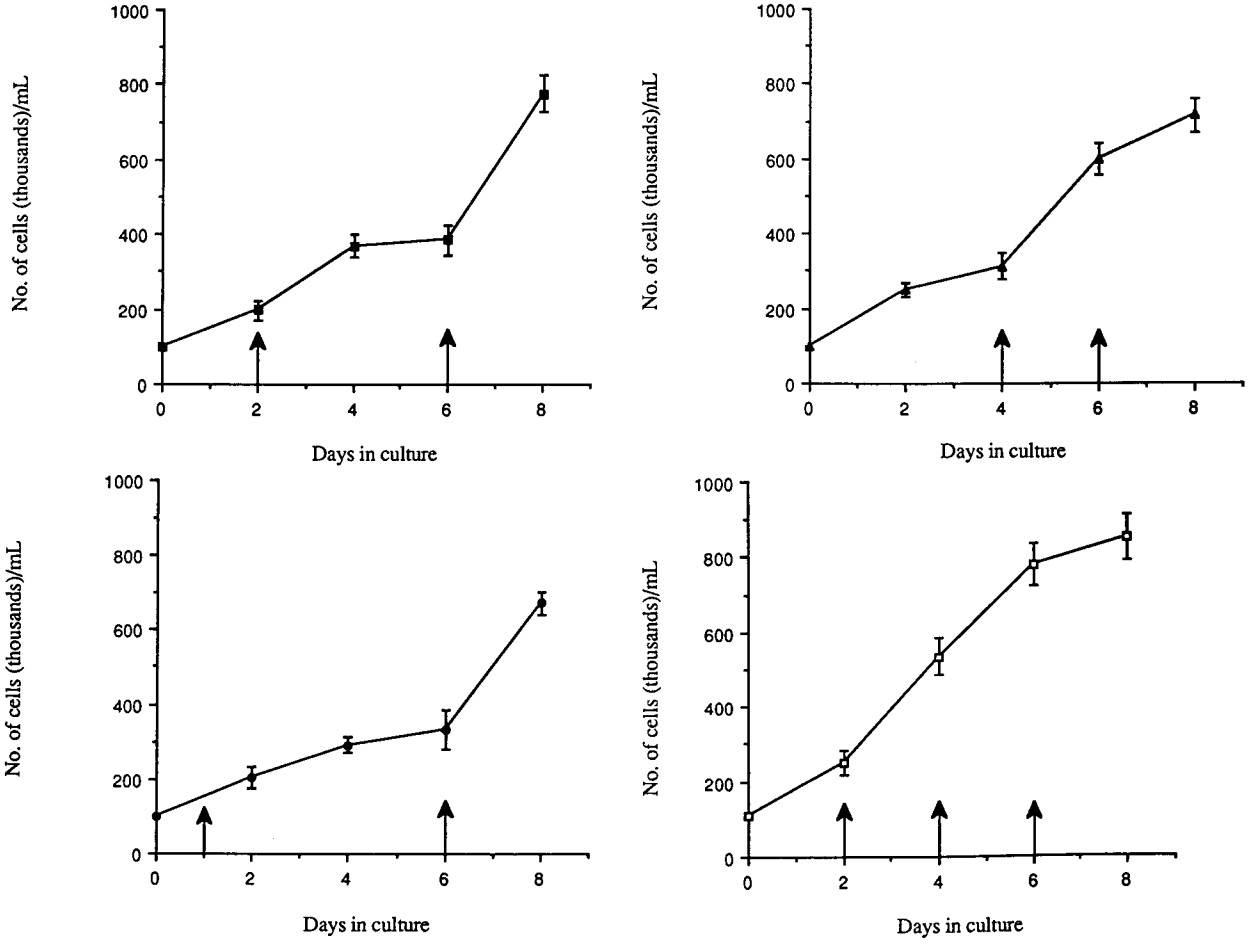

FIG. 1. Growth curves of L929 cultures, showing standard deviations of mean counts, with medium change on: days 2 and 6 (top left); days 4 and 6 (top right); days 1 and 6 (bottom left); and days 2, 4, and 6 (bottom right).

final percentage increase in number was determined from 20 independent groups of replicate cultures and is presented in Fig. 2. The initial cell number was found to be related to the final percentage increase by the equation $y = 283 \cdot 10^7 \cdot x^{-1.33}$. This equation enables the prediction of the final percentage increase (y) in culture when the initial cell number (x) is known, and when the cells are grown in 25 cm^2 flasks in standard nutrient medium of known compatibility with the cell line, when nutrient depletion does not limit growth. The optimum initial cell number which produced the greatest yield of cells (approx. 600×10^3) was in the range $75–150 \times 10^3$ cells mL^{-1}.

Final cell yield was also related to the surface area available to the cells when the number of cells per unit volume and frequency of media change were kept constant. Fig. 3 shows that the final yield of L929 cells increased as the initial number of cells per unit area decreased. The values for the number of cells per unit area are calculated from the initial numbers of cells per mL, the areas of the flasks into which they were inoculated, and the volumes of the inoculates. Thus, when initial cell concentrations are held constant, final cell concentrations and hence also final percentage changes in cell concentrations, increase as the area available for cell attachment increases. The growth curves of L929 cells obtained in the three types of culture vessels, which show the relationship between cell yield and cell density, are presented in Fig. 4.

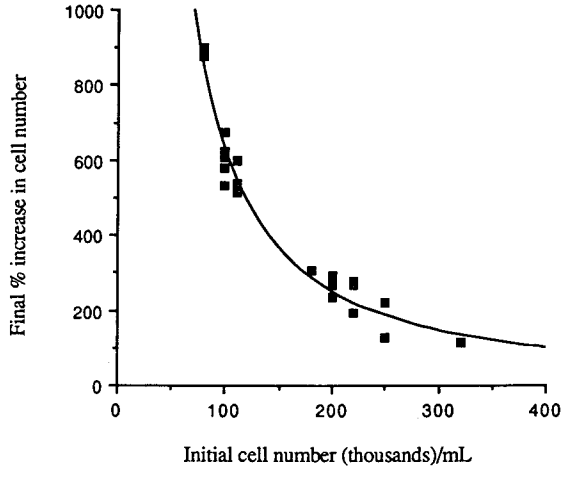

FIG. 2. Relationship between initial cell number and final percentage increase in L929 cultures growing in 25 cm^2 flasks.

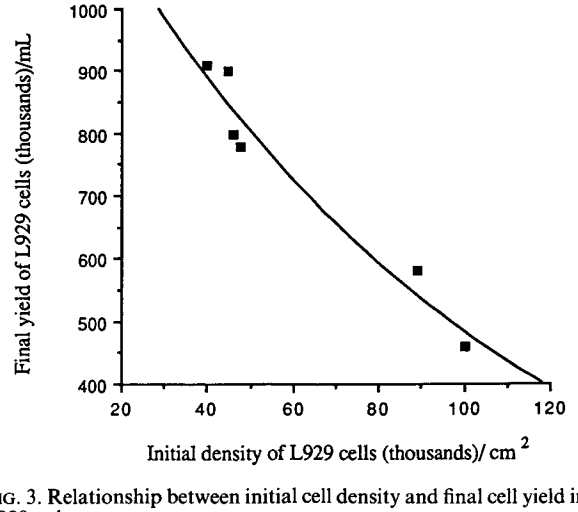

FIG. 3. Relationship between initial cell density and final cell yield in L929 cultures.

When all experimental conditions, as well as the above identified factors affecting cell growth, were maintained constant, the growth pattern of the cells was standardized, and variation of the growth curves of the L929 fibroblasts was virtually eliminated. Remaining sources of variability over and above those derived from sampling and counting errors and from normal biological variation, are probably ascribable to batch-to-batch variation in the serum used. In practice, extrinsic sources of error appeared to be less significant than intrinsic biological variability.

Several ways of determining the rate of growth of cultures are in current use. Biocompatibility assays, by their nature, involve the addition to cultures of exogenous materials that are likely to interfere with the counting procedure. We therefore evaluated three methods for determining cell growth against two alginate wound management products. All three methods were found to be suitable for assessing cell growth in the absence of added materials.

The two alginate samples were chosen as test materials since pilot studies had shown them to have profoundly different effects on cultured fibroblasts, notwithstanding their apparent chemical similarity (Schmidt et al 1986). It has since been reported that the cause of the inhibition in cell growth seen with Alginate 2 was a trace residue of a quaternary ammonium surfactant remaining from the manufacturing process (Keys 1986). It should be stressed that Alginates 1 and 2 are not representative of currently available proprietary alginate products.

The Coulter counter method offers a quick way of counting (2–3 min per count), but does not allow any observation of the cells to be made and does not distinguish between viable and non-viable cells, single cells and clusters, and foreign particles. When the alginate products were present in the cultures, the Coulter counter method proved to be unsuitable because alginate fibres remaining in the

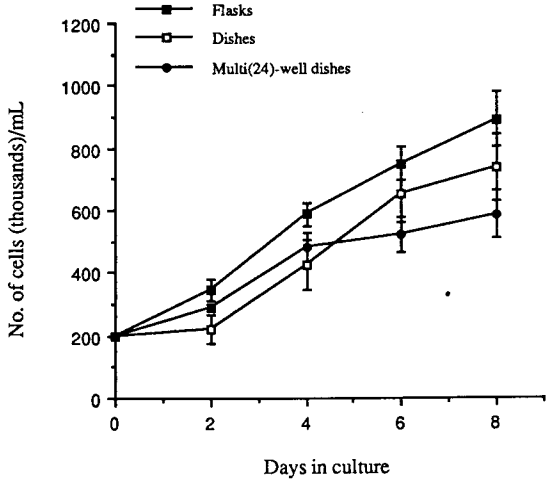

FIG. 4. Growth curves for L929 cultures, with standard deviations of mean counts, showing the relationship between initial cell density and final cell yield for three types of culture vessel.

cultures obstructed the flow of cells through the counting orifice. Upon microscopic examination of the cultures, some clustering of cells was also evident, a factor that would contribute to counting errors.

The advantages of the haemacytometer method over the other two methods are that direct observation of the morphology of the cells can be made, as can the simultaneous determination of viability using trypan blue as a viability stain. In addition, a single cell suspension can be confirmed—a primary requirement for accurate cell counts. The disadvantages are the possible error due to sampling and transfer to the chamber, and the necessity of counting a large number of cells to produce accurate counts. It is also a time-consuming technique requiring approximately 20 min per sample. The presence of test materials in the culture samples did not interfere with the counting procedure. Results obtained from the haemacytometer method are shown in Fig. 5 (top). Cell numbers recorded in the cultures supplemented with the Alginate 1 were higher than the control, but the differences were not significant at day 7 ($P=0{\cdot}651$). The reduction in the cell numbers in the Alginate 2 cultures was highly significant ($P=0{\cdot}001$).

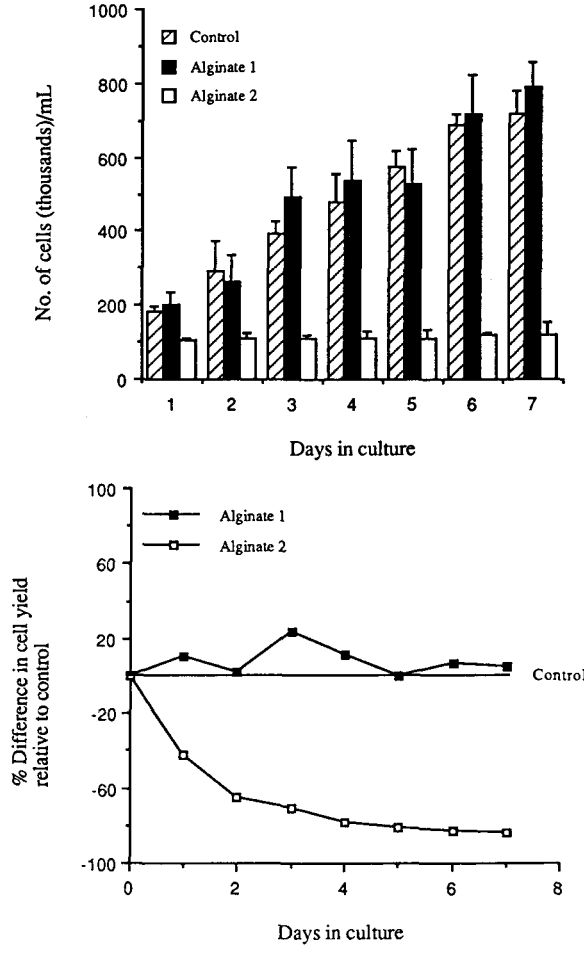

FIG. 5. Results obtained from the Haemacytometer method. Effect of calcium alginate dressings on L929 cells in culture. Each bar represents the mean of five field counts of triplicate cultures (bottom). Percentage difference in cell yield between the control cultures and those treated with the two alginate products (bottom).

Figure 5 (bottom) shows the percentage difference in the growth of the cells in the presence of the two alginate products relative to the control.

The results obtained from the liquid scintillation method are presented in Figs 6, 7. Liquid scintillation counting provided results that closely mirrored those obtained by the haemacytometer method. Fig. 6 shows that the activity incorporated into the Alginate 1 cultures was less than the control after 48 h, but this was not significant ($P=0{\cdot}102$). The difference between the activity incorporated into the control and the activity incorporated into the Alginate 2 cultures was highly significant ($P=0{\cdot}001$).

Figure 7 shows the amount of [^{3}H]thymidine remaining in the media after incubation of the L929 cultures with the two alginate products. The amount of [^{3}H]thymidine remaining in the Alginate 1 cultures was markedly decreased and not significantly different ($P=0{\cdot}706$) from that in the control. In contrast, the cells in the Alginate 2 cultures failed to incorporate any thymidine.

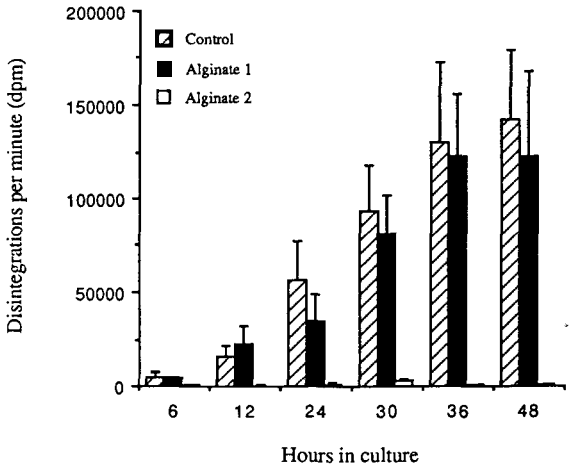

FIG. 6. Results obtained from the liquid scintillation method. L929 cultures were incubated with [^{3}H]thymidine for 48 h in the presence of alginate dressings. Each bar represents the mean of eight counts (d min^{-1}).

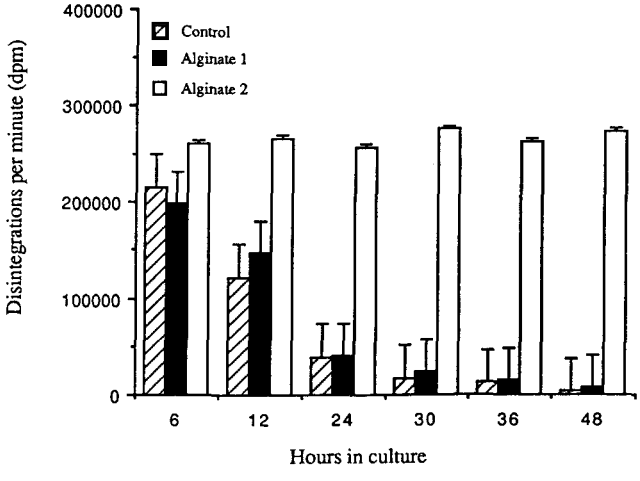

FIG. 7. [^{3}H]thymidine remaining in the culture media after 48 h incubation in the presence of the two alginate dressings. Each bar represents the mean of eight counts.

In conclusion, the Coulter method proved to be unacceptable for the determination of cell numbers in cultures containing residual fibres from the added alginate products. The liquid scintillation and haemacytometer methods both provided similar results even though the former measured DNA synthesis rather than actual cell numbers. The haemacytometer method had the advantage of providing both qualitative (direct cell observation) and quantitative (cell numbers) data in the determination of cell compatibility of the test materials.

These results have enabled us to optimize culture conditions and to identify a suitable counting procedure for our biocompatibility assays. Because the growth of cells in control cultures has been standardized, the cell yield in control cultures can now be used as a baseline against which the response of cell cultures to culture additives may be compared and hence quantified.

Acknowledgement

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