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ROUTINE HEAT INACTIVATION OF SERUM REDUCES ITS CAPACITY TO PROMOTE CELL ATTACHMENT

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SUMMARY

Heat inactivation (56° C for 40 min) of bovine calf serum was shown to diminish its capacity to promote the attachment of cells to plastic or glass surfaces. This effect was not observed in stationary cultures (culture dishes) but became manifest under conditions in which the cells were subjected to a small amount of liquid shear force, i.e. by growing cells in roller bottles or culture tubes. Of four cell lines tested on bovine calf serum (SV-BHK, BALB-3T3, CV-1, and FS-4) SV-BHK and CV-1 cells showed the greatest sensitivity to loss of attachment-promoting activity. Fetal bovine serum also seemed to be affected by heat inactivation but to a lesser degree than bovine calf serum. Treatment of vessel surfaces with either unheated calf serum or specific attachment factors (gelatin, poly-D-lysine, and fibronectin) greatly increased cell attachment in the presence of heat inactivated serum. Heat inactivation did not seem to affect the ability of cells to grow after attachment. Of the four cell lines tested, the normal human fibroblast line (FS-4) was shown to be most effective at conditioning medium and restoring its capacity to promote the attachment of all four cell lines.

Key words: heat inactivation; attachment; serum; cells; conditioned medium.

INTRODUCTION

Heating serum has become an established means of inactivating complement for various immunological assays (19). Heat inactivation usually involves heating serum to approximately 56° C for periods from 30 to 60 min. Although this procedure is known to produce a complicated series of effects on hemolytic and anticomplementary activity, which can cause problems in some immunologic assays (3,16,19), it has generally been assumed to have little or no effect on the attachment and growth of animal cells in culture. It has therefore become common practice to heat inactivated serum for the purpose of destroying viruses and other microorganisms (23).

In our laboratory we do not routinely heat inactivate serum. On the few occasions when we have, it was noted that cells grown in roller bottles (or other vessels in which a small amount of liquid shear force was applied) were slow to attach, although cells grew well once attachment had taken place. In the present study, a variety of cell types were evaluated under various conditions, to determine to what extent heat inactivation of serum affects the attachment of cells to commonly used substrates.

MATERIALS AND METHODS

Cells used in these studies were FS-4, a normal human fibroblast line obtained from Jan Vilček, New York University School of Medicine, NY; SV-BHK, an SV40-transformed BHK line obtained from David Evans, Wayne State University, Detroit, MI; CV-1, an established line of monkey kidney cells obtained from Mel DePamphilis, Harvard Medical School, Boston, MA; and Balb3T3 cells obtained from George Todaro, Oncogen Inc., Seattle, WA. Stock cultures of cells were maintained in large (850 cm^2) plastic roller bottles (Corning Glass Works, Corning, NY). Growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) purchased from K. C. Biologicals, Lenexa, KS, supplemented with 10% bovine calf serum (Whittaker M. A. Bioproducts, Walkersville, MD). Serum samples for testing were obtained from Whittaker M. A. Bioproducts; GIBCO, Grand Island, NY; K. C. Biologicals; and Hazleton Dutchland, Inc., Denver, PA. Tryspin, for cell dispersion, was obtained from Worthington, Freehold, NJ; EDTA, used in conjunction with trypsin was purchased from Sigma Chemical Co., St. Louis, MO.

Attachment assay. Stock SV-BHK cultures used for attachment assays were washed with phosphate buffered saline (PBS) containing no Mg or Ca and dispersed with a solution of trypsin (0.008%) and EDTA (0.02%). After detachment, trypsin was inactivated by addition of DMEM + 10% heat inactivated serum (on a 1:1 volume basis). The suspension was pipetted to ensure that cells were evenly dispersed, then centrifuged at 1000 rpm for 5 min to pellet cells. Cells were then resuspended in DMEM (no serum) and counted in a hemacytometer using trypan blue to determine viability. Cells were then seeded in the appropriate test medium into either small (490 cm^2)

plastic Corning roller bottles (5×10^6 cells/bottle), 16×125-mm borosilicate glass culture tubes (KIMAX brand) at 2.5×10^5 cells/tube, Corning plastic culture dishes (60×15 mm) at 2.5×10^5 cells/dish, or KIMAX glass culture dishes (60×15 mm) at 2.5×10^5 cells/dish. Roller bottles were incubated in a 37° C warm room on a roller rack (Bellco Glass Co., Vineland, NJ) using a rolling rate of 1 rpm. Tubes were incubated on a Bellco roller drum in a CO_2, humidified incubator also at a rate of 1 rpm. Culture dishes were also incubated in a CO_2 incubator. Initially, the pH of the medium was adjusted to approximately 7.4. At the appropriate times the number of attached cells was determined as follows: cells were washed with PBS, detached with trypsin-EDTA, diluted 1:2 with growth medium and counted by hemacytometer or Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Production and testing of conditioned media. For production of conditioned media (CM) cell lines were seeded into small roller bottles containing DMEM × 10% heat inactivated bovine calf serum, at 5×10^6 cells/roller bottle. When cells were approximately 50% confluent (3 to 4 d), spent medium was discarded, and fresh medium containing heat inactivated serum was added. Twenty-four hours later, the CM was removed, centrifuged at 1500 rpm to remove cell debris, and frozen at −20° C in glass bottles until tested. To test CM, each cell line was seeded in small roller bottles or glass culture tubes in either a) CM; b) DMEM + 10% heat inactivated serum, or c) DMEM + 10% unheated serum. Duplicate cell counts were made at the appropriate times by hemacytometer or Coulter counter as previously described.

Heat inactivation of sera. Serum was heat inactivated using the following procedure: a) Serum from a 500-ml bottle stored at −20° C was thawed and aliquoted into 100-ml borosilicate glass bottles at 50 ml/bottle. Some samples were left untreated and stored at −20° C. b) Samples to be heat inactivated were first placed in a 37° C waterbath for 20 min along with a bottle containing water (with a thermometer inserted) to be used for calibration purposes. c) Bottles were then placed in a 56° C waterbath and left for 40 min after the temperature of the serum had reached 56° C (as indicated by the calibration bottle). d) Heat inactivated samples were then stored along with unheated samples at −20° C. All samples were handled in exactly the same way with regard to freezing and thawing and no precipitates were observed.

Pre-treatment of culture vessels. Culture tubes were exposed to serum or solutions of attachment factors (2 ml/tube) for 4 h at 37° C. Attachment factors were used at a concentration of 5 $\mu g/cm^2$. Serum treatment utilized a concentration of 10%. Tubes were then washed twice with sterile, distilled water, after which 2 ml of the appropriate attachment medium containing 2.5×10^5 SV-BHK cells were added to each tube. Cultures were incubated for 4 h at 37° C while rotating at 1 rpm. The number of attached cells was determined by trypsin-EDTA dispersion followed by counting in a Coulter counter.

Quality control. All cell lines were tested for mycoplasma by a DNA staining technique (18) and by cultivation on artificial media, and found to be free of contamination.

Results

Measurement of Cell Attachment on Plastic and Glass Surfaces

Our initial observations on the effect of serum heat inactivation on cell attachment were made with SV-BHK cells grown in plastic roller bottles. Later, for economic reasons as well as convenience, we conducted our attachment studies in glass roller tubes. To determine whether the phenomenon of reduced attachment of cells in heat inactivated serum under conditions which included shear was applicable to glass as well as to plastic surfaces, we measured attachment of SV-BHK cells in plastic and glass vessels in stationary (culture dishes) and nonstationary (roller tubes and bottles) culture using both heat inactivated and unheated serum. The results, shown in Table 1, indicate that with plastic and glass culture dishes, almost identical amounts of attachment occur in either unheated or heat inactivated serum. Under conditions of mild shear, cell attachment in heat inactivated serum was greatly diminished and to approximately the same degree on both plastic and glass surfaces. The extent of attachment in stationary culture was significantly greater than in nonstationary culture even in unheated serum.

Effect of Heat Inactivation on Cell Growth

An experiment was set up to determine whether heat inactivation of bovine calf serum affects growth as well as attachment of SV-BHK cells. Cells were first seeded in roller bottles (5×10^6 cells/roller bottle) in medium containing unheated serum and incubated to allow attachment over a 4-h period. Medium containing unattached cells was then removed, cells were washed with PBS, and fresh medium added. One half of the bottles received medium with heat inactivated serum, the

TABLE 1

ATTACHMENT OF SV-BHK CELLS ON PLASTIC AND GLASS SURFACES IN UNHEATED AND HEAT-INACTIVATED CALF SERUM[a]

Vessel	Percent Attachment	
	Unheated Serum	Heat inactivated Serum
Culture dish, plastic	54.4	60.7
Culture dish, glass	60.1	57.7
Roller bottle, plastic	19.1	0.4
Roller tube, glass	12.2	0.5

[a]Cells were seeded at a concentration of 5×10^6 cells/roller bottle ($\sim 1 \times 10^4$ cells/cm^2) and 2.5×10^5 cells/tube or dish ($\sim 1 \times 10^4$ cells/cm^2). After 4 h of incubation at 37° C, the number of attached cells was determined by trypsin-EDTA dispersion and counting with a Coulter counter.

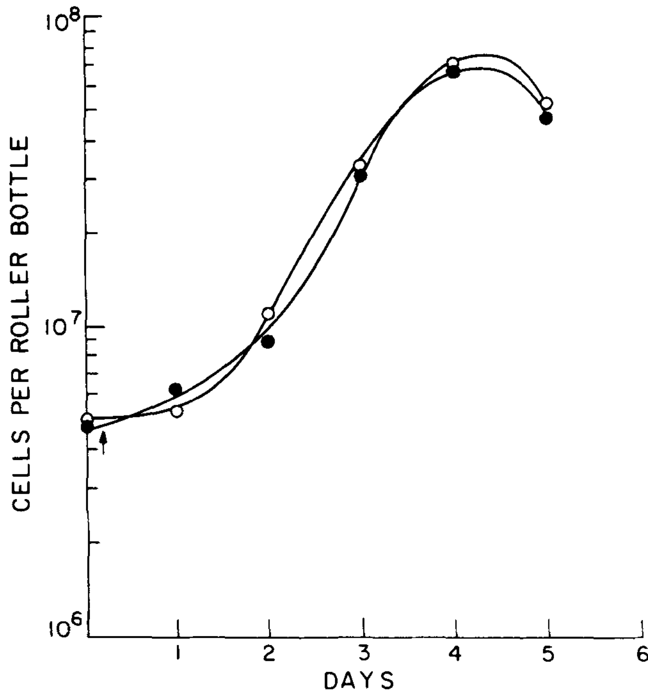

FIG. 1. Growth of SV-BHK cells in medium supplemented with either heat inactivated (O) or unheated (●) bovine calf serum (10%) after attachment in medium with unheated serum. Cells were seeded in roller bottles (5×10^6 cells/roller bottle) in DMEM + 10% unheated serum. After 4 h of incubation (*arrow*) medium containing unattached cells was discarded, attached cells were washed twice with PBS, and fresh medium supplemented with either heat inactivated or unheated serum was added to cultures in duplicate. Beginning on Day 1 (24 h) attached cells were dispersed with trypsin-EDTA and counted using a hemacytometer. The initial cell density (5×10^6 cells/roller bottle) refers to cells inoculated rather than to cells attached.

other half, unheated serum. All bottles were then incubated and daily cell counts were made to compare growth rates in the two different media. Figure 1 shows resulting growth curves, and indicates that for this particular cell line, essentially the same growth rate and maximum density are achieved in either medium. Other cell lines (CV-1, Balb-3T3 and FS-4) have shown similar results (data not shown).

Comparative Effect of Heat Inactivation on Bovine Calf and Fetal Bovine Sera

A comparison was made between bovine calf serum and fetal bovine serum with regard to the effect of heat inactivation on cell attachment and growth. SV-BHK cells were inoculated into roller bottles containing medium supplemented with each type of serum under heat inactivated and unheated conditions. Figure 2 shows a comparison of the growth curves obtained. With bovine calf serum (Fig. 2 *A*), under unheated conditions, there is essentially no lag phase. Attached cells exceeded 10^7/roller bottle on Day 1 (obviously due in part to cell growth) and maximum density was achieved by Day 4. By contrast, in heat inactivated serum, the number of attached cells on Day 1 is approximately 20-fold less than the unheated control. Excellent growth is then seen, reaching a density almost equal to the unheated culture by Day 5. The effect of heat inactivation on fetal bovine serum (Fig. 2 *B*) was much less marked. The combination of poor attachment with unheated serum (five-fold less than bovine calf) and better attachment in heat inactivated serum (two-fold greater than bovine calf) resulted in a relatively small difference between treated and untreated fetal bovine serum cultures on Day 1. Cells then grew at the same rate and achieved approximately the same density in heat inactivated and unheated serum.

Effect of Heat Inactivation on Attachment of Different Cell Lines

The effect of heat inactivation of bovine calf serum on the attachment of various cell lines is shown in Table 2. The data showed a striking contrast between stationary culture (plastic culture dishes) and nonstationary culture (glass roller tubes and plastic roller bottles) with regard to the extent of attachment in heat inactivated serum. In culture dishes, the extent of attachment in heat inactivated serum was approximately equal to that in unheated serum. By contrast, in roller tubes and roller bottles, the degree of attachment in heat inactivated serum was significantly less than in unheated serum for all four cell lines. Thus, the introduction of a small amount of liquid shear (tubes were rotated at 1 rpm), seems to make the cell lines tested more dependent on serum attachment activity which is lost due to heat treatment. Of the four cell lines tested, SV-BHK and CV-1 cells showed the greatest decreases in attachment in heat inactivated serum (21-

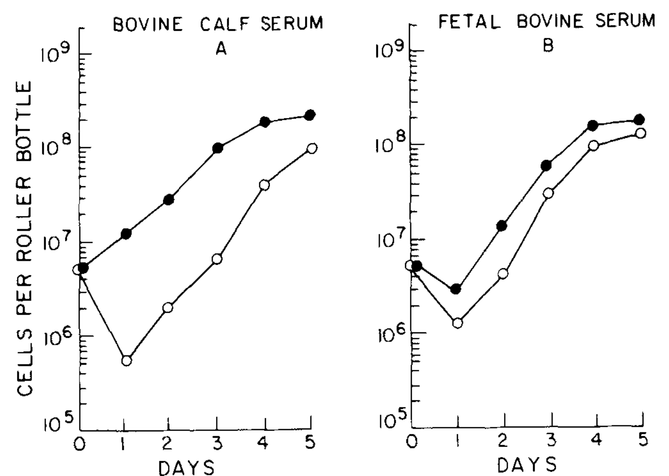

FIG. 2. Attachment and growth of SV-BHK cells in bovine calf serum and fetal bovine serum. Cells were seeded in roller bottles in medium supplemented with either heat inactivated (O) or unheated (●) serum (10%). After 24 h and on a daily basis thereafter, duplicate cultures were dispersed with trypsin-EDTA and cells were counted using a hemacytometer. The initial density (5×10^6 cells/roller bottle) refers to cells inoculated rather than to cells attached.

TABLE 2

EFFECT OF HEAT INACTIVATION OF BOVINE CALF SERUM ON THE ATTACHMENT OF VARIOUS CELL LINES[a]

Type of Vessel	Serum	Percent Attachment			
		SV-BHK	BALB-3T3	CV-1	FS-4
Roller tube, glass	Unheated	35.8	29.5	41.1	36.6
	Heat-inact.	1.7	8.6	2.5	15.7
Roller bottle, plastic	Unheated	17.2	32.0	76.0	48.0
	Heat-inact.	0.2	16.2	3.0	22.0
Culture dish, plastic	Unheated	56.4	39.4	79.3	83.4
	Heat-inact.	59.0	47.4	73.7	77.0

[a]Vessels were seeded in duplicate with the indicated cell lines suspended in DMEM + 10% serum (either untreated or heat inactivated). Tubes and dishes received a total of 2.0 ml of medium containing 2.5×10^5 cells total. Roller bottles received 50 ml containing a total of 5×10^6 cells. After 4 h of incubation at 37° C (tubes and roller bottles rotated at 1 rpm), the number of attached cells was determined by trypsin-EDTA dispersion and counting with a Coulter counter. The roller bottle data were acquired from a separate experiment and incorporated into this table.

and 16-fold decreases, respectively, in roller tubes and 86- and 25-fold decreases, respectively, in roller bottles).

Pretreatment of Vessel Surfaces

Effect of serum pretreatment on attachment of SV-BHK cells. The decrease in attachment-promoting capacity observed in heat inactivated serum could be due to either the destruction of attachment activity or to the formation of some toxic or inhibitory material in the serum. In an effort to distinguish between these alternatives, glass culture tubes were treated with either unheated serum or heat inactivated serum, after which cell attachment was measured in serum-supplemented media and under serum-free conditions. Results (Table 3) showed a decrease in the capacity of heat inactivated serum to pretreat (or coat) glass surfaces so as to promote cell attachment (0.9% compared to 24.4% for unheated serum when heat inactivated serum was used in the attachment medium). Although the extent of attachment observed in heat inactivated serum (24.4%) was not as great as that obtained in any of the cultures using unheated serum in the attachment medium (28.5 to 40.6%), these differences may not be significant. Pretreatment of tubes with heat inactivated serum did not appear to substantially decrease cell attachment compared to other pretreatments when unheated serum was used in the attachment medium. Attachment in DMEM was approximately the same as in heat inactivated serum, following pretreatment with all three of the pre-treatment solutions.

Effect of pretreatment with various attachment factors. Cell attachment in heat inactivated serum was measured in untreated culture tubes and compared to attachment obtained in tubes pretreated with various attachment factors. The data in Table 4 indicate that all three attachment factors (gelatin, poly-D-lysine, and fibronectin) showed a capacity to enhance cell attachment in medium with heat inactivated serum. However, not all factors were able to promote the attachment of all four cell lines tested. For example, gelatin promoted the attachment of SV-BHK and FS-4 cells, but not CV-1 and Balb 3T3 cells. Fibronectin enhanced attachment to a significant degree in only SV-BHK and CV-1 cells. Only poly-D-lysine promoted the attachment of all four cell lines. However, it should be pointed out that only one concentration of attachment factor (5 μg/ml) was used in these experiments and that other factor concentrations could give different results. In spite of the overall increases in attachment due to pretreatment with attachment factors, in only one instance (Balb 3T3 on poly-D-lysine) was the attachment rate (19.6%) as great as in control cultures containing unheated serum (19.1%).

Effect of Conditioned Medium on Cell Attachment Rates

Conditioned medium was produced by growing each cell line in medium with heat inactivated bovine calf serum, then tested by comparing it to media with either unheated serum or heat inactivated serum, for ability to

TABLE 3

EFFECT OF SERUM PRETREATMENT OF SUBSTRATE ON ATTACHMENT OF SV-BHK CELLS[a]

Pretreatment	Percent Attachment in Various Attachment Media		
	10% Heat inact. Serum	10% Unheated Serum	DMEM
10% Heat inactivated serum	0.9	28.5	1.4
10% Unheated serum	24.4	35.1	17.5
DMEM (no serum)	1.7	40.6	2.3

[a]Culture tubes were exposed to the indicated pretreatment for 4 h at 37° C (2 ml/tube). Tubes containing the above attachment media were then inoculated with 2.5×10^5 cells each (10^4 cells/cm^2). Cultures were incubated for 4 h at 37° C while rotating at 1 rpm. The number of attached cells was determined by trypsin-EDTA dispersion followed by counting in a Coulter counter.

TABLE 4

EFFECT ON CELL ATTACHMENT OF PRETREATMENT OF SUBSTRATE WITH ATTACHMENT FACTORS[a]

Attachment Medium[b]	Vessel Pretreatment	Percent of Attachment			
		SV-BHK	FS-4	CV-1	Balb-3T3
Unheated serum	none	54.1	57.4	77.3	19.1
Heat inactivated serum	none	4.0	12.6	4.9	5.6
Heat inactivated serum	gelatin	16.3	24.6	2.6	6.9
Heat inactivated serum	poly-D-lysine	28.3	34.1	29.6	19.6
Heat inactivated serum	fibronectin	39.5	19.1	19.2	7.2

[a]Glass culture tubes were either left untreated or exposed for 4 h to the various attachment factors, diluted in PBS (no Mg or Ca) to give a concentration of 5 $\mu g/cm^2$ (62.5 $\mu g/ml$). Duplicate tubes were then inoculated with 2.5 $\times$ 10^5 cells/tube in the medium indicated (2 ml/tube) and incubated on a roller drum (1 rpm) for 4 h at 37° C. The number of attached cells was determined by trypsin-EDTA dispersion, followed by counting in a Coulter counter.

[b]Attachment medium consisted of DMEM plus the indicated serum at a concentration of 10%.

promote the attachment of cells in roller bottle cultures over 24-h. The results are given in Table 5. Results after 4 h indicate that with all cell lines, less attachment occurs in the heat inactivated media compared to unheated controls. These data are in good agreement with results obtained in other experiments (Tables 2 and 4). As in previous experiments, SV-BHK and CV-1 cells were affected to a greater degree by heat inactivation than other cell lines (40- and 25-fold less attachment, respectively). FS-4 cells and Balb 3T3 cells showed reasonably good attachment (approximately 50% of controls). The differences between heat inactivated and unheated cultures were less pronounced after 24 h than after 4 h except in the case of SV-BHK cells. Conditioning the medium was shown to promote early attachment of all cell types except SV-BHK.

Capacity of Conditioned Media from One Cell Type to Promote the Attachment of Other Cell Types

Conditioned media, which had demonstrated the capacity to enhance the attachment of the cells that produced it (Table 5), were tested for their ability to promote the attachment of other cell lines (Table 6). FS-4 conditioned medium showed the greatest capacity to promote attachment, achieving attachment rates comparable to unheated serum controls with all cell lines tested. CV-1 cells also demonstrated the ability to enhance attachment of other cell types by conditioning media. By comparison, conditioned medium from Balb 3T3 cells demonstrated little, if any, capacity to promote attachment of other cell types.

TABLE 5

EFFECT OF CONDITIONED MEDIUM ON CELL ATTACHMENT[a]

Cell Line	Type of Medium	Number of Attached cells per Roller Bottle ($\times 10^6$)[c]	
		4 h	24 h
FS-4	10% unheated serum	2.4 ± 0.5	3.1 ± 0.2
	10% heat inactivated serum	1.1 ± 0.2	2.4 ± 0.3
	conditioned medium	2.5 ± 0.47	3.4 ± 0.14
SV-BHK	10% unheated serum	0.8 ± 0.20	2.0 ± 0.58
	10% heat inactivated serum	<0.02[b]	<0.02[b]
	conditioned medium	<0.02[b]	<0.02[b]
CV-1	10% unheated serum	3.8 ± 0.1	4.5 ± 0.39
	10% heat inactivated serum	0.15 ± 0.07	3.1 ± 0.32
	conditioned medium	0.37 ± 0.01	3.1 ± 0.49
BALB 3T3	10% unheated serum	1.6 ± 0.46	1.8 ± 0.28
	10% heat inactivated serum	0.81 ± 0.05	2.2 ± 0.1
	conditioned medium	1.5 ± 0.18	2.3 ± 0.3

[a]Roller bottles (490 cm^2) of each cell line were seeded in duplicate in each type of medium and incubated for the time indicated. Conditioned medium was prepared as described in Materials and Methods. The number of attached cells was determined by counting in a hemacytometer.

[b]Insufficient number of cells to count using standard hemacytometer.

[c]Values represent the means ± SD of duplicate cultures.

TABLE 6

CAPACITY OF CONDITIONED MEDIA TO PROMOTE THE ATTACHMENT OF OTHER CELL TYPES[a]

Attachment Medium	Percent Attachment			
	SV-BHK	FS-4	CV-1	Balb-3T3
DMEM + unheated serum	26.7	19.1	35.3	27.3
DMEM + heat inactivated serum	1.6	4.3	1.3	2.8
Conditioned medium (FS-4)	29.6	13.2	30.9	20.4
Conditioned medium (CV-1)	14.2	11.6	14.8	17.8
Conditioned medium (Balb-3T3)	3.4	6.2	1.4	5.1
DMEM (no serum)	2.2	5.4	5.1	6.4

[a]Cells were suspended in the media indicated and duplicate culture tubes were inoculated with 2.5×10^5 cells (2 ml/tube). Cultures were incubated at 37° C for 4 h and rotated at 1 rpm. The number of attached cells was determined by trypsin-EDTA dispersion, followed by counting in a Coulter counter. Conditioned medium was prepared as described in Materials and Methods.

Discussion

In this study, it was shown that heat inactivation of bovine calf serum diminished its capacity to promote the attachment of animal cells to glass and plastic surfaces under conditions of mild shear. Although this phenomenon could result from either the destruction of attachment factor(s) or the production of toxic or inhibitory substances in the serum, our data suggest that the former possibility plays the greater role. First, the fact that heat inactivation had no apparent effect on cell attachment in stationary culture suggests that toxic or inhibitory substances, if present, are at low concentrations. Furthermore, our experiments on serum pretreatment of culture surfaces showed clearly that heat inactivation reduces the capacity of serum to treat (coat) surfaces in such a way as to enhance attachment. However, because pretreatment of vessels with either unheated serum or attachment factors did not produce the amount of attachment seen in control cultures with unheated serum in the attachment medium, the possibility of toxic or inhibitory material being present in heat-inactivated serum cannot at this point be ruled out.

The phenomenon of reduced attachment-promoting capacity in bovine calf serum due to heat inactivation is not an isolated instance of a defective batch of serum. We have tested numerous batches of serum (data not shown) from different companies and have found all lots to be affected to a similar degree by heat treatment. It seems likely that other types of sera will be similarly affected, perhaps to differing degrees. A limited amount of the data presented in this study suggests that heat treatment may not affect fetal bovine serum to the extent that bovine calf serum is affected. If this proves to be true, it would be of interest to determine whether the differences are quantitative or qualitative in nature.

Experiments are currently under way to determine which protein factor(s) might be lost during heat inactivation. To date, the best characterized serum attachment factors are fibronectin (8,25) and plasma-spreading factor (1,4,6,12,22,24). Although cell surface fibronectin has been shown to mediate the cell attachment and spreading process (4,7,11,20), its role in commercial sera has been reported to be minimal (9,12,13). Two principle reasons for this are: a) the serum preparation procedure at 4° C can effectively remove most of the fibronectin (9,12); and b) at serum concentrations >3%, extracellular fibronectin has been reported to be ineffective, presumably due to competition with serum albumins for adsorption sites (5,13). A serum concentration of 10% was used in all our studies. Plasma-spreading factor, on the other hand, has been reported to be effective over a wide range of serum concentrations (4,12) and presumably is not removed during serum processing. Although either or both of these factors could be affected by heat inactivation, it does not seem likely that fibronectin would be affected because it undergoes denaturation at higher temperatures than used in our studies (10). It is also possible that serum contains proteins other than those reported to date, which are important for cell attachment only when cells are subjected to shear forces.

The decrease observed in cell attachment in heat inactivated serum seems to reflect an increased cellular dependency on serum attachment activity under conditions of mild shear. Wide differences were observed among the cell lines tested with regard to their sensitivity to the loss of attachment activity. FS-4 and Balb 3T3 cells, both of fibroblast origin, were less affected by heat inactivation than SV-BHK and CV-1 lines, one of which is transformed (SV-BHK) and both of which are of epithelial origin. Differences observed between various cell types could be due in part to differences in cell membrane composition. For example, the membranes of cultured fibroblast strains such as FS-4 have been reported to contain significant amounts of fibronectin, whereas transformed cells such as SV-BHK have been shown to contain little or no fibronectin in their membranes (15,17,21).

It is also possible that the capacity of different cell types to condition media by releasing factors into it could play a role in the eventual attachment of cells in medium with heat inactivated serum. With the exception of SV-BHK cells, cell lines used in this study demonstrated the ability to condition medium and thereby either fully or partially restore attachment-promoting activity to the medium (Table 5). In addition, conditioned medium from two of the cell lines (FS-4 and CV-1) were shown to significantly enhance attachment of all four cell lines used in these studies (Table 6). It is of interest that Balb-3T3 cells, although capable of utilizing attachment factors produced by other cell types, seemed less capable of producing attachment factors than either FS-4 or CV-1 cells. It is therefore possible that these cells, as well as SV-BHK cells, are defective in the synthesis of adhesion proteins.

Both fibroblast cells and epithelial cells have been shown to be capable of producing proteins that enhance both cell attachment and spreading. Dickey and Seals (2) isolated a protein from the spent medium of rat hepatoma cells capable of promoting cell attachment to collagen-

coated petri dishes. Yamada et al. (26) reported that rat embryo fibroblasts were capable of conditioning medium to promote cell adhesion and spreading, particularly to collagen-coated surfaces. Studies with human diploid fibroblasts (5,14) have shown that cell surface components active in cell attachment and spreading are released in an active form into the culture media. In view of these and other reports, as well as results we have presented, it seems likely that medium conditioning could account, at least in part, for the eventual attachment of cells in heat inactivated serum.

Results of the studies presented here have practical value for investigators involved in cell attachment studies as well as those involved in the production of anchorage-dependent cells in roller bottles and other vessels in which shear forces are introduced. It seems obvious that for certain applications, heat inactivation of serum is contraindicated although we have shown that the effect of heat inactivation can be overcome to a significant degree by pretreatment of vessel surfaces with a variety of factors. In addition, the wide differences among cell types with regard to dependency on serum-attachment activity may provide the basis for a method of isolating or separating different cell types, e.g., normal and transformed cells. Finally, the observed increase in serum dependency under conditions of shear may provide the basis for a unique approach to the study of cell attachment mechanisms.

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