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THE EFFECT *IN VITRO* OF ESTRONE AND BRADYKININ ON A NUCLEAR ABNORMALITY IN A HAMSTER ASCITES TUMOR*

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Abstract—Bradykinin caused a decrease in the number of polynucleated cells only in the presence of estrogens. Bradykinin may influence cell permeability to estrogens, and estrogens may be the active factor instead of bradykinin. However, when bradykinin, estrone, and washed polynucleated cells were incubated *in vitro*, decrease in polynucleated cell numbers did not occur until after 2 hr. Incubation of bradykinin and estrone for 4 hr before addition of washed polynucleated cells resulted in decreased numbers of polynucleated cells in less than 2 hr. This suggests that bradykinin and estrone form a complex or third product which is the active agent and that the phenomenon may not be explained entirely on the basis of altered cell permeability to estrogens.

Effect of agents upon polynucleated tumor cells has been determined by microscopic counts of polynucleated cells before and after incubation. Evidence indicated that reduction in the number of polynucleated cells was the result of cytokinesis occurring around each nucleus in a polynucleated cell. This resulted in an increased number of small tumor cells in the culture. Use of the Coulter particle-size distribution plotter to determine cell size shifts and increased numbers of smaller tumor cells after incubation with bradykinin and estrone offered a simple objective method to replace the laborious and subjective microscopic determination of polynucleated cell numbers.

A HAMSTER ascites tumor has been described which had a large number of polynucleated tumor cells.[1] Polynucleated cells are usually found in cancerous tissue but are normally present in limited numbers.[2] In the hamster ascites tumor, however, as many as 25 % of the tumor cells in some hamsters were polynucleated. This nuclear abnormality probably resulted from fragmentation and lobation[3] or amitosis.[4]

Early experiments on transplantation of hamster ascites tumor were made with female hosts. Chance transplantation of the tumor into male hamsters resulted in a significant increase in the number of polynucleated tumor cells.[5] The ascites tumor was originally produced from a methylcholantrhene-induced sarcoma[6] and was not gonadal in origin. The difference in the number of polynucleated cells appeared to be dependent on the sex of the host hamster. A study of the effects of estrogens and androgens on abnormal polynucleated tumor cells in hamsters was undertaken by using the number of polynucleated cells as a parameter against which to measure effect.

Experiments *in vivo* and *in vitro* demonstrated that estrogens caused a decrease in the number of polynucleated cells.[5] The nature of that effect was elucidated by using a

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defined culture medium to replace the ascites fluid used in earlier experiments *in vitro*. Evidence from experiments using washed polynucleated tumor cells in defined culture medium showed that estrogens alone did not decrease the number of polynucleated cells.[7] Subsequently it was shown that estrogens and small amounts of ascites fluid or serum from cancer-carrying hamsters had to be added to the defined culture medium before a decrease in the number of polynucleated cells occurred.[7] Serum from normal hamsters plus estrogen did not decrease the number of polynucleated cells.[7] On the basis of these results it appeared that a factor occurred in hamster ascites fluid or cancer serum which acted with estrogens to cause a decrease in the number of polynucleated cells.

Further experiments have demonstrated that normal serum plus estrone caused a decrease in the number of polynucleated cells if normal blood were allowed to remain at 24° for 1 hr before the serum was removed.[8] It was subsequently shown that platelets caused normal serum to develop activity and to decrease the number of polynucleated cells as effectively as cancer serum.[8] The hypothesis was advanced that the factor which caused a decrease in the frequency of the nuclear abnormality was present in both cancer and noncancer serum. It appeared to be in a bound state so that normally its presence was not detected in noncancer serum. Platelets in some manner released the factor under the influence of cancer or *in vitro* at 24° for 1 hr.[8]

Attempts to isolate the active factor from cancer serum are in progress and thus far results indicate that it occurs in the α_2-globulin fraction,[9] and might be a polypeptide. Several biologically active peptides were tested *in vitro* on the polynucleated tumour cells to determine whether they significantly decreased the number of polynucleated cells. The results of those experiments are presented in this report.

METHODS

Ascites tumor in 0·5 ml dosages was injected intraperitoneally into golden hamsters (*Mesocricetus auratus*)* ovariectomized 14 days earlier.

Ten to twelve days after inoculation, the ascites tumor from six or more animals was pooled and heparinized (7·5 mg sodium heparin/5 ml tumor). The tumor was centrifuged at 3,700 rev/min for 5 min. The cells were suspended in cold Hank's saline, recentrifuged for 5 min, and resuspended in a medium consisting of 45% Hank's saline, 40% TC medium 199, and 15% horse serum at pH 7·2; 0·1 μg of estrone and the test substances were added to each ml of culture medium containing 1·5 to 2·0 × 10^7 tumor cells. Aliquots of 2·5 ml in 25-ml Erlenmeyer flasks were incubated at 37° in a Dubnoff metabolic apparatus and shaken at 60 rev/min in an atmosphere of 95% O_2, 5% CO_2 for 6 hr. The cells were smeared, fixed, and stained by the Feulgen reaction.

Results were determined by counting 100 cells in each of four corners and in the center of each slide and calculating the percentage of polynucleated and mononucleated tumor cells. Any tumor cell with two or more nuclei was considered to be polynucleated. Each test material was coded and incubated in duplicate. Slides were coded, selected at random, and read independently by two individuals. The results obtained by two investigators for each duplicate experiment were pooled, averaged, and statistically analyzed. Each experiment was repeated a minimum of two times and all experimental data are presented.

* Dennen Animal Industries, Gloucester, Mass.

A reduction in the number of polynucleated cells by 30% or more is considered a positive effect.[7]

A model B Coulter counter* was used to determine the number of tumor cells and their volume. The instrument was calibrated with ragweed pollen to determine the volume in cubic microns per threshold division. A threshold division was found to be the equivalent of 140 cubic microns when the aperture current was 2 and amplification 4. Stepwise increases in the threshold and total cell counts at a given threshold setting were used to determine tumor cell-size distribution curves. Aliquots of *in-vitro* tumor cell incubations were diluted with isotonic saline to obtain 10 to 20 thousand cells per ml, and their distribution was determined from a stepwise series of increasing threshold levels.

RESULTS

Table 1 presents the results obtained when synthetic bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and estrone were incubated with polynucleated cells.

TABLE 1. THE DECREASE IN NUMBER OF POLYNUCLEATED CELLS AFTER TREATMENT WITH BRADYKININ AND ESTRONE

Component material	Culture medium (μg/ml)	Estrone culture medium (μg/ml)	Polynucleated cells* (%)
Control			18·13 ± 1·41
Estrone		0·10	18·61 ± 1·70
Bradykinin-1†	0·10		17·41 ± 1·75
	0·01	0·10	10·37 ± 1·47
	0·05	0·10	9·89‡ ± 1·36
	0·25	0·10	17·90 ± 1·41
Control			22·15 ± 1·65
Bradykinin-2	0·10		19·35 ± 1·81
	0·20	0·10	12·75‡ ± 1·30
	0·10	0·10	13·35‡ ± 1·67
	0·05	0·10	20·80 ± 1·47

* Average value ± S.D.

† Bradykinin-1 kindly supplied by Dr. Joseph Fried, Squibb Institute; Bradykinin-2 obtained from Sandoz Inc.

‡ Decrease is significant ($P < 0·01$).

Two synthetic bradykinins obtained from different sources were effective in decreasing the number of polynucleated cells. Bradykinin-1 had a lower minimal dose level than bradykinin-2. The difference can probably be attributed to greater purification of bradykinin-1.

Two glycine-substituted bradykinins, 6-glycine bradykinin[10, 11] (Arg-Pro-Pro-Gly-Phe-Gly-Pro-Phe-Arg) and 7-glycine bradykinin[10, 11] (Arg-Pro-Pro-Gly-Phe-Ser-Gly-Phe-Arg), as well as retrobradykinin[10, 12] (Arg-Phe-Pro-Ser-Phe-Gly-Pro-Pro-Arg), were also tested for ability to decrease the number of polynucleated cells. As shown

* Coulter Electronics, Hialeah, Fla.

in Table 2, 6- and 7-glycine bradykinin had no observable effect on polynucleated cells even at dose levels twenty times greater than bradykinin-1. Retrobradykinin was effective in decreasing the number of polynucleated cells. The minimal dose level was twice that of bradykinin-1 which was obtained from the same source.

Several readily available biologically active peptides, histamine, and serotonin were tested on the polynucleated cell system. The results are presented in Table 3. None of the peptides tested caused a decrease in the number of polynucleated cells. Histamine and serotonin had no observable effect on the polynucleated cells.

TABLE 2. THE EFFECT OF 6- AND 7-GLYCINE BRADYKININ AND RETROBRADYKININ ON POLYNUCLEATED CELLS

Component material	Culture medium (μg/ml)	Estrone Culture medium (μg/ml)	Polynucleated cells* (%)
Control			18·13 ± 1·33
6-Glycine bradykinin†	0·1	0·1	17·80 ± 1·52
	0·2	0·1	17·20 ± 1·33
	1·0	0·1	16·55 ± 1·18
7-Glycine bradykinin	0·1	0·1	16·53 ± 2·33
	0·2	0·1	18·35 ± 1·18
	1·0	0·1	16·75 ± 0·92
Retrobradykinin	0·1		16·76 ± 1·22
	0·1	0·1	9·55‡ ± 1·15
	0·05	0·1	15·73 ± 1·31

* Average value ± S.D.
† Glycine substituted and retrobradykinin kindly supplied by Dr. Joseph Fried, Squibb Institute.
‡ Decrease is significant ($P < 0·01$).

TABLE 3. THE EFFECT OF HISTAMINE, SEROTONIN, AND SOME PEPTIDES ON POLYNUCLEATED CELLS

Component materials*	Polynucleated cells† (%)	Component materials	Polynucleated cells (%)
Control	18·51 ± 1·26	Polylysine	19·40 ± 2·94
Histamine	16·25 ± 1·19	MO 149	16·15 ± 1·44
Serotonin	16·05 ± 1·00	FSH	18·07 ± 1·37
Oxytocin	19·60 ± 0·87	ACTH	17·17 ± 1·96
Vasopressin	18·00 ± 0·65	LH	21·35 ± 3·88

* One μg of test substance and 0·1 μg of estrone per ml of culture medium. Dose levels ranging from 0·1 μg to 10 μg of test substance gave the same results as 1 μg.
MO 149 = histidyl-phenylalanyl-citrullyl-tryptophyl-glycine; kindly supplied by Dr. Joseph Fried, Squibb Institute.
† Average values ± S.D.

Several steroids other than estrogens were tested on the polynucleated cell system with bradykinin. The results are presented in Table 4. Classes of steroids tested, other than estrogens, caused no decrease in the number of polynucleated cells.

Bradykinin-1 and estrone were incubated with polynucleated cells for 6 hr. At 2-hr intervals the incubation was interrupted and the percentage of polynucleated cells

determined on a small aliquot of the incubate. The results are presented in Fig. 1. All values are averages of three separate experiments; the standard deviation for each set of values was 1·50 or less.

The results in Fig. 1A demonstrate that bradykinin and estrone did not lower the number of polynucleated cells after 2 hr incubation time. However, by 4 hr the

TABLE 4. THE EFFECT OF SOME STEROIDS OTHER THAN ESTRONE ON POLYNUCLEATED CELLS

Steroid	Polynucleated cells* (%)	Steroid	Polynucleated cells (%)
Control	16·65 ± 1·49	Cortisone	17·40 ± 1·35
Testosterone	16·87 ± 0·78	Progesterone	16·35 ± 1·17
Cortisol	15·90 ± 1·65	Aldosterone	16·30 ± 1·18

* Average values ± S.D.; 0·1 μg of bradykinin-1 and 0·1 μg of steroid per ml of culture medium.

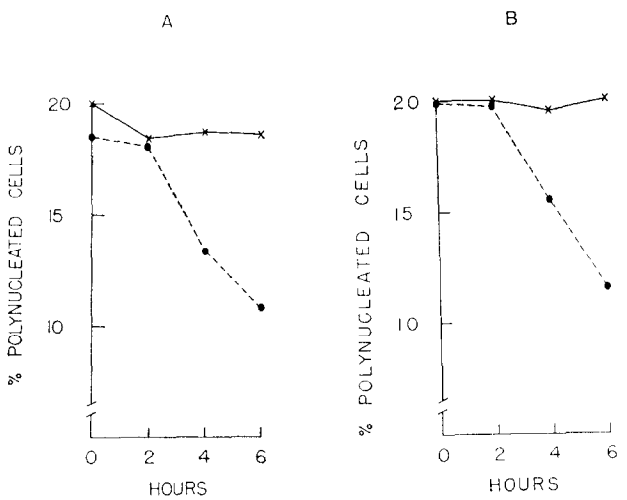

FIG. 1. A. The rate of decrease in the number of polynucleated tumor cells treated with 0·1 μg of bradykinin and 0·1 μg of estrone per ml. ×—×, Control. . ●--●, Experimental.
B. Same as A except that 17 β-estradiol (0·1 μg/ml) was substituted for estrone.

decrease was evident. In the next series of experiments estrone and bradykinin were incubated without polynucleated cells. Washed polynucleated cells in culture medium were added after the estrone–bradykinin incubation had proceeded for either 2 or 4 hr. The incubation was then continued to a total of 6 hr. The results are presented in Fig. 2A.

Incubation of bradykinin and estrone for 2 hr before polynucleated cells were added resulted in a 2-hr delay before a decrease in the number of polynucleated cells occurred. Incubation for 4 hr, however, resulted in an immediate decrease in the number of polynucleated cells upon their addition to the estrone–bradykinin incubate. It is

interesting to note that the same type of curve and time sequence was obtained when a similar experiment was performed with cancer serum and estrone.[7] The time sequence in the decrease of polynucleated cells (Fig. 2A) suggests that estrone and bradykinin formed a complex or a third product which was the effective agent in lowering the number of these cells.

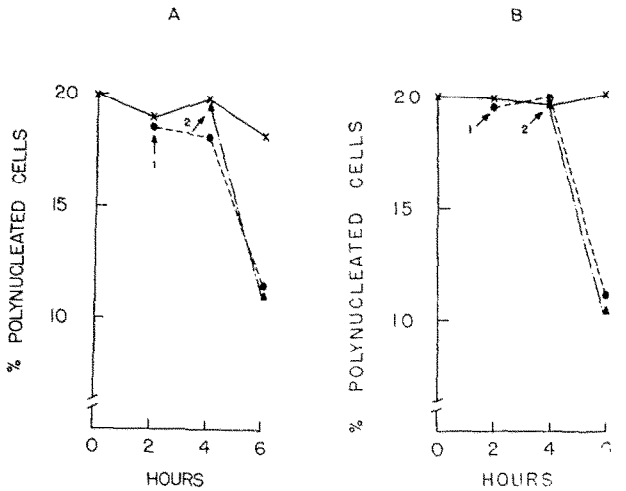

FIG. 2. A. Effect of incubation of bradykinin (0·1 μg/ml.) and estrone (0·1 μg/ml) for 2 or 4 hr before addition of washed polynucleated cells. ×—×, Control. ●– –●, Washed tumor cells added at 1. ▲— —▲, Washed tumor cells added at 2.
B. Same as A except that 17 β-estradiol (0·1 μg/ml) was substituted for estrone.

The experiments presented in Fig. 1A and 2A were repeated with 17β-estradiol instead of estrone (see Fig. 1B and 2B). The time sequences in decrease of the number of polynucleated cells treated with bradykinin and estradiol were identical with those obtained with bradykinin and estrone. This ruled out a possibility that the time delay was the result of conversion of estrone to estradiol, with estradiol being the active hormone.

The decrease in the number of polynucleated cells after treatment with bradykinin and estrone appeared to result from cytokinesis around each nucleus in a polynucleated cell without chromosome formation. Several experimental approaches were used to determine this effect. (1) Estrone and bradykinin were incubated for 4 hr. Washed polynucleated cells in culture medium were added and a few drops of the culture sealed under a coverslip on a microscope slide. The preparation was placed in a stage incubator at 37° and observed by phase microscopy. Cytokinesis occurred around each nucleus in some polynucleated cells without chromosome formation. (2) Total tumor cell numbers were determined before and after incubation with bradykinin and estrone (see Table 5). An increase in total tumor cell numbers occurred. (3) Mitotic figure counts were made before and after incubation (Table 5); no statistically significant change occurred in the mitotic rate, the apparent increase in the experimental culture having a P value of 0·9 .This indicated that increased cell numbers were not due to increased mitotic division. (4) Cytokinesis in a polynucleated cell

would result in mononucleated cells whose volume would be less than that of the parent polynucleated cell. Cell size distribution plots were obtained before and after 6 hr incubation with the Coulter cell-size distribution apparatus. An increase in the number of smaller tumor cells occurred after incubation with bradykinin and estrone (Fig. 3).

TABLE 5. TOTAL TUMOR CELL COUNT AND PERCENTAGE MITOTIC FIGURES FOR BRADYKININ AND ESTRONE-TREATED INCUBATIONS

Component materials	Cells per ml 0 hr	Cells per ml 6 hr	Per cent mitotic figures* 0 hr	Per cent mitotic figures* 6 hr
Control	$1{\cdot}87 \times 10^7$	$1{\cdot}76 \times 10^7$	$1{\cdot}66 \pm 0{\cdot}67$	$1{\cdot}71 \pm 0{\cdot}26$
Bradykinin + estrone, 0·1 μg/ml	$1{\cdot}66 \times 10^7$	$1{\cdot}78 \times 10^7$	$1{\cdot}46 \pm 0{\cdot}61$	$1{\cdot}71 \pm 0{\cdot}20$

* Percentage mitotic figures ± S.D. Percentage mitotic figures determined by counting 20,000 tumor cells each for bradykinin-treated and control experiments.

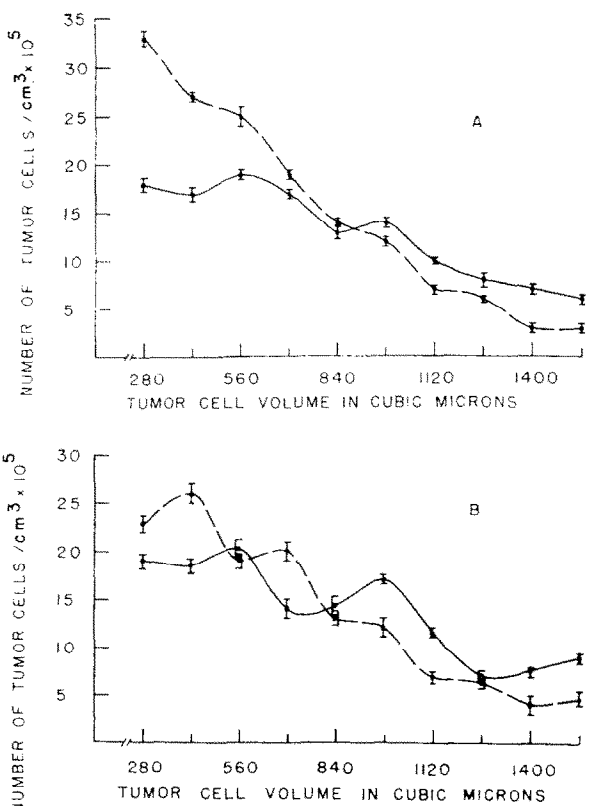

FIG. 3. A. Number and size distribution of tumor cells before and after incubation with 0·1 μg of bradykinin/ml and 0·1 μg of estrone/ml using model B Coulter counter with 200-μ orifice and aperture current of 2; ●—●—●, 0 hr; ● — — ●, 6 hr. Each point is the average of 10 separate experiments. The bars indicate the standard deviation.

B. Control experiment. Same as A except that bradykinin was not added to the culture medium.

DISCUSSION

The pharmacological properties of bradykinin have been extensively studied by Rocha e Silva.[13] The response of smooth muscle and the peripheral vascular system to bradykinin have been well established.[14] Otherwise, the physiological and pathological significance of bradykinin is largely unknown.[15] The evidence presented in this report indicates that bradykinin and estrone decreased the number of tumor polynucleated cells in hamster ascites *in vitro* as effectively as cancer serum and estrone. Bradykinin, retrobradykinin, or cancer serum and estrone appear to be specific in their action on polynucleated cells in that no other agents tested produced that effect. This indicates that the active factor in cancer serum which decreased the number of polynucleated cells may be bradykinin or a closely related polypeptide.

It should be noted that Rubin *et al.*[16] found retrobradykinin to be less than 1/300 as potent as bradykinin in serveral vascular and smooth muscle preparations. These authors also found 6- and7-glycine bradykinin to be at least as potent as, and 1/100 as potent as, bradykinin, respectively. However, 6- and 7-glycine bradykinin in high concentration with estrone failed to decrease the number of polynucleated cells.

Brachet has suggested that study of nuclear abnormalities may provide information about the mitotic process.[17] Bradykinin reduced the number of polynucleated cells, a nuclear abnormality, by causing cytokinesis to occur around each nucleus in a polynucleated cell without chromosome formation. This raised the possibility that bradykinin or a closely related polypeptide may be involved in cell division.

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