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SYNTHESIS AND SECRETION OF IgE BY AN ESTABLISHED HUMAN MYELOMA CELL LINE

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SUMMARY

The rate of IgE production *in vitro* and the cell population proliferation rate by the established human myeloma line 266 Bl have been studied quantitatively under various tissue culture conditions. The rate of extracellular IgE accumulation depended on the type of medium used, the cell density and the period of time elapsed after explantation. The maximum production rate of $8{\cdot}1 \times 10^{-12}$ g IgE/cell/48 hr was noticed at cell densities $< 10^{6}$/30 ml and with the presence of feeder human skin fibroblasts or glia-like cells or with the use of conditioned media harvested from such cells. The rate of cell proliferation and secretion of IgE to the medium ran parallel suggesting that the IgE production is highest when cells are in the best physiological condition. During more than one year the rate of synthesis of IgE remained stable. This functionally stable human myeloma line is suitable for further studies on immunoglobulin biosynthesis at the cellular and subcellular level under the tissue culture conditions found optimal in this study.

INTRODUCTION

Investigations with myeloma cells of human origin were for long hampered by the very short life span *in vitro* of these cells. In recent years, however, when more adequate tissue culture methods were used a few myeloma lines were established *in vitro*—one from a patient with IgGL myeloma (Matsuoka, Moore, Yagi & Pressman, 1967) and two from an IgEL myeloma (Nilsson, Bennich, Johansson & Pontén, 1970).

The line of Matsuoka *et al.* (RPMI 8226) produced dimers of λ-chains, antigenically identical to those found in the patient's urine (Matsuoka, Yagi, Moore & Pressman, 1969). The cell lines derived from the E myeloma patient (266Bl, 268Bm) secreted complete molecules of IgEL indistinguishable from the myeloma protein synthezised *in vivo*.

Human myeloma cells are generally accepted as malignant counterparts to plasma cells and are known, in most cases, to be stable producers of intact immunoglobulin *in vivo*.

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Complete myeloma proteins should probably not be considered abnormal 'paraproteins' but normal immunoglobulins since some M-proteins have been shown to have antibody activity (Waldenström, Winblad, Hallen, & Livingman, 1964; Beaumont, Jocotot, Vilain, & Beaumont, 1965; Eisen, Little, Osterland & Simms, 1967; Stone & Metzger, 1967; Terry, Boyd, Rea & Stein, 1970). The biosynthesis of immunoglobulin therefore remains essentially unaltered after the malignant transformation. This would make the human myeloma cells suitable for controlled *in vitro* studies on the biosynthesis and secretion of immunoglobulins.

The production of immunoglobulin by human myeloma cells has been studied quantitatively in short term cultures by Salmon & Smith (1970). Quantitative study of the lambda light chain secretion by line RPMI 8226 was reported by Matsuoka *et al.* (1968). Some data on the secretion rate of IgEL by the same 266Bl line as was used in this study were given recently (Nilsson *et al.*, 1970)

The growth rate and viability of the 266Bl myeloma line have been found to vary a great deal depending on the tissue culture conditions (Nilsson, 1971). For the further studies with this cell line on the mechanisms involved in synthesis and secretion of IgE it is therefore of importance to define the type of tissue culture to be used.

This communication describes the production of IgE by asynchronous myeloma cells 266Bl in relation to their proliferation under various tissue culture conditions.

MATERIALS AND METHODS

Maintenance of cell line 266Bl

The establishment the maintenance and tissue culture characteristics have been described before (Nilsson *et al.*, 1970; Nilsson, 1971).

Essentially, the line is maintained in Erlenmeyer flasks containing monolayers of allogeneic skin fibroblasts or glia-like cells (Pontén & Macintyre, 1968). Cultures are incubated in a humidified CO_2-in-air atmosphere at 37°C. The medium, Nutrient medium F-10 or RPMI 1640 (Grand Island Biol. Comp., New York, U.S.A.) supplemented with 10% post-natal calf serum and antibiotics (100 IE/ml penicillin, 50 μg/ml stretomycin, 1·25 μg/ml amphotericin B) is changed twice a week.

Cell counts

A celloscope (Celloscope 502, Linson Instruments, Stockholm) was used for counting cells. Viability was estimated by the trypan blue exclusion method after incubation of cells in standard medium containing 0·2% trypan blue for 15 min at 37°C.

Quantitation of IgE secreted in the medium.

A. *Incubation of cells.* 266Bl cells were harvested from optimally growing asynchronous stock cultures by low speed centrifugation, washed once or twice in phosphate buffered saline (PBS) and resuspended in a small, volume of fresh medium. Total and viable cells were counted and, in routine experiments approximately 10^6 viable cells were transferred to each Erlenmeyer flask with or without a monolayer of feeder cells on its bottom. Fresh medium was added to a final volume of 30 or 40 ml. During the course of the experiments, lasting for 1–14 days, no fresh medium was added.

TABLE 1. Influence by different culture conditions on the accumulation of IgE in 30 ml cultures. 7 days experiments

Exp. No.	Medium	No viable cells/culture ($\times 10^6$)		Accumulation of IgE (μg/culture)		IgE(μg) produced by 10^6 initially inoculated cells		Special characteristics	Date
		Initial	Final	Days 0–4	Days 0–7	0–4 days	0–7 days		
1	F-10+10% calf serum	5·0	5·9	16·5(±2·5)	25·7(±3·9)	3·3	5·1	Initial cell number high	August 1969
2	F-10+10% calf serum	0·7	0·8	4·1(±0·6)	6·8(±1·0)	5·6	8·5	—	March 1970
3	F-10+10% calf serum	4·7	6·6	12·5(±1·9)	36·5(±5·5)	2·7	5·5	Initial cell number high	April 1970
4	F-10+20% calf serum	0·9	1·1	4·1(±0·6)	8·1(±1·2)	4·6	9·0	Calf serum concentration higher than routine	April 1970
5	Conditioned F-10+5% fresh calf serum	0·7	2·8	7·6(±1·1)	21·6(±3·2)	10·8	30·9	F-10+10% serum harvested after one day's incubation on 403CG glia-like cells	April 1970
6	Conditioned F-10+5% fresh calf serum	0·7	1·7	5·0(±0·8)	8·5(±1·3)	7·1	12·1	F-10+10% serum harvested after one day's incubation on 389CG glia-like cells	April 1970
7	Conditioned F-10 No serum	1·0	0·8	2·4(±0·4)	4·0(±0·6)	2·4	4·0	Medium harvested from lymphoblastoid line 61M grown under serum free conditions	April 1970
8	F-10+10% calf serum	0·8	1·1	16·5(±2·5)	31·0(±4·7)	20·6	39·0	403CG glia-like cells present in the culture. Final cell number too low since myeloma cells became attached to glia cells.	March 1970

TABLE 2. Influence by different culture conditions on the accumulation of IgE in 30 ml cultures. 14 days experiments (Fig. 1) August 1970

Exp. No.	Medium	No viable cells/ culture ($\times 10^6$)		Accumulation of IgE (μg/culture)				Special characteristics
		Initial	Final	Days 0–2	Days 0–4	Days 0–7	Days 0–14	
9	F-10+10% calf serum	1·1	0·9	2·9(±0·4)	5·4(±0·8)	10·8±(1·6)	25·6(±3·8)	—
10	RPMI 1640+ 10% calf serum	1·1	1·0	Not done	5·5(±0·8)	10·8(±1·6)	19·6(±2·9)	—
11	Conditioned F-10 +5% fresh calf serum	1·1	7·2	6·0(±0·9)	18·0(±2·7)	28·3(±4·2)	129·1(±19·4)	F-10+10% calf serum harvested after one day's incubation on skin fibroblasts (393S)
12	Conditioned F-10 +5% fresh calf serum	1·1	5·1	8·1(±1·2)	10·0(+1·5)	21·8(±3·3)	93·7(±14·1)	F-10±10% calf serum harvested after one day's incubation on glia-like cells (403CG)
13	F-10+10% calf serum+ feeder cells	1·1	4·9	5·5(±0·9)	14·3(±2·2)	21·5(±3·2)	120·3(±18·0)	Feeder cells (393S fibroblasts) present as a confluent monolayer on the bottom of the Erlenmeyer flask
14	F-10+10% calf serum+feeder cells	1·1	3·7	5·7(±0·9)	13·0(±2·0)	22·3(±3·4)	121·0(±18·2)	Feeder cells (403CG glia-like cells) present as a confluent monolayer.

In other experiments conditioned media were used. Such media, F-10 or RPMI 1640 containing 10% post-natal calf serum, were harvested from confluent monolayers of human adult skin fibroblasts or glia-like cells after 1 day's incubation, filtered sterile and supplemented with 5% fresh post-natal calf serum. The different tissue culture conditions studied are detailed in Tables 1 and 2.

B. *Assay of IgE in the medium.* During each experiment 1 ml samples of supernatant were harvested at intervals. The IgE concentration as kindly determined by Dr S. G. O. Johansson in the unconcentrated supernatants by the radioimmunosorbent test (RIST) as described by him and co-workers (1968). A substandard of the British Research Standard (68/341) designated to contain 10,000 units of IgE (Rowe *et al.*, unpublished results) was employed. When E myeloma protein ND (Johansson & Bennich, 1967) was tested it was found that one unit of the substandard was approximately equal to 1 ng of protein ND. Further studies, however, indicate that the levels obtained by the RIST and given in this report are roughly 20% below the true value (Rowe & Johansson, personal communication). The tabulated error of estimation, calculated as the standard deviation from the mean value at duplicate analyses, was 10–15%.

Autoradiographic studies

Myeloma cells in suspension cultures were exposed to 1 μC tritiated thymidine (^{3}H-Tdr) (Spec. act. 2 Ci/mmol, Schwarz Bio Research Inc., Orangeburg, N.Y., U.S.A.) per ml medium (F-10 or RPMI 1640+10% calf serum) for 60 min. Autoradiographs were prepared as follows: cells were centrifuged onto microscope slides by a Cytocentrifuge (Shandon Scientific Comp. Ltd., London) dried, washed twice in PBS and fixed in methanol-acetic acid (3:1) for 1 hr. The slides were coated by Kodak AR 10 stripping film. After exposure for 10 days at 4°C films were developed in Kodak D-19B. The coated microscope slides were finally stained in 0·05% (v/v) tolouidine blue for 5 min. The incorporation of ^{3}H-Tdr was estimated by counting 500 cells on each autoradiogram. All values were based on duplicate or triplicate cultures.

Control experiments

To establish whether, during the course of the experiments, there was a break down or other alterations of IgE molecules making them non-reactive in the RIST, known amounts of IgE were incubated in F-10 medium+10% calf serum without cells during the same maximum time (14 days) as for the other experiments. Since the death rate of 266B1 cells has been found to be high IgE was also added to medium containing freeze-thawed myeloma cells to investigate if the content of dead myeloma cells could affect the IgE molecules. In no such experiments the initial concentration of IgE changed indicating that IgE is unaffected under the tissue culture conditions used in the experiments.

RESULTS

The influence of various tissue culture conditions on the production of IgE and the proliferation of the myeloma cells

Three sets of experiments (August 1969, March 1970, April 1970) of 7 days length were performed (Table 1). Myeloma cells from stock cultures of a viability varying between

60–65 % were used. The following parameters influencing the rate of IgE production by the myeloma cells were studied.

1. *Concentration of post-natal calf serum in the medium* (Table 1)

Under ordinary *serum free* conditions the myeloma cells died rapidly in 1–3 days. When, however, cells were incubated in medium harvested from human lymphoblastoid cell cultures (grown in serum free F-10 medium for 14 months) and supplemented by 0·5% bovine serum albumin (BSA) 2·4 μg IgE/10^6 cells was accumulated in the medium during days 0–4 (Exp. 7). In contrast to the cell death under serum free conditions the initial cell number only decreased by 20% during the 7 days. During the second hour of this experiment 27 % of the cells were labelled by ^{3}H-Tdr. The addition of 0·5 % BSA to non-conditioned, serum free, F-10 medium did not support growth and IgE production better than serum free F-10. The percentage of labelled cells during the second hour in these experiments was 16–18 %. This indicates that human lymphoblasts produce a conditioning factor(s).

In experiments using the standard serum concentration of 10% the accumulation of IgE was more rapid, 5·6 μg/10^6 cells/0–4 days (Exp. 2). When F-10 containing 20% serum was employed 4·6 μg IgE/10^6 cells/0–4 days was produced (Exp. 4). The rate of IgE secretion and the proliferation of the myeloma cells was thus not higher at this serum concentration, than under standard conditions. Because of this finding no further experiments with higher serum concentrations were performed.

2. *Cell density* (Table 1)

As a routine, cultures were initiated with 10^6 viable cells/30 ml medium. At initial cell concentrations of 5×10^6 (Exp. 1) and $4{\cdot}7 \times 10^6$ (Exp. 3) the rate of IgE production, calculated for 10^6 viable cells during day 0–4, was 3·3 μg, and 2·7 μg, respectively. These figures, considerably lower than those obtained in routine experiments (i.e. Exp. 2) indicate that initial cell concentrations of the order 5×10^6 cells/culture ($1{\cdot}7 \times 10^5$/ml medium) are too high, when immunoglobulin synthesis is to be studied with cells secreting the globulin at highest possible rate. The myeloma cells do not seem to be dependent on a minimal cell density since the rate of IgE synthesis and the proliferation of the cells remained unaltered at cell concentrations lower than 10^6/cultures.

3. *Conditioned medium* (Table 1)

With feeder cells present (Exp. 8) a rapid production of IgE was noted. Calculated per 10^6 cells, 20·6 μg was produced during day 0–4.

The very presence of feeder cell was not a prerequisite for a high rate of IgE secretion. As shown by Exp. 5 media harvested from glia-like cells were as efficient to induce and maintain a high secretion rate of IgE as was the presence of feeder cells. The amount of IgE secreted per day was of the same order as in Exp. 8. The feeding effect of different cell types varied. Adult skin fibroblasts and glia-like cells had the most potent conditioning capacity but even among these cell types individual lines seemed to differ in that respect. 389CG glia-like cells were for instance less conditioning than 403CG (Exp. 6).

Comparison of the influence on cell proliferation and IgE secretion rate by non-conditioned and conditioned media.

A separate set of experiments, summarized in Table 2 and Fig. 1 (a–f) was performed in August 1970 to confirm earlier results (Table 1) and to compare the differences between

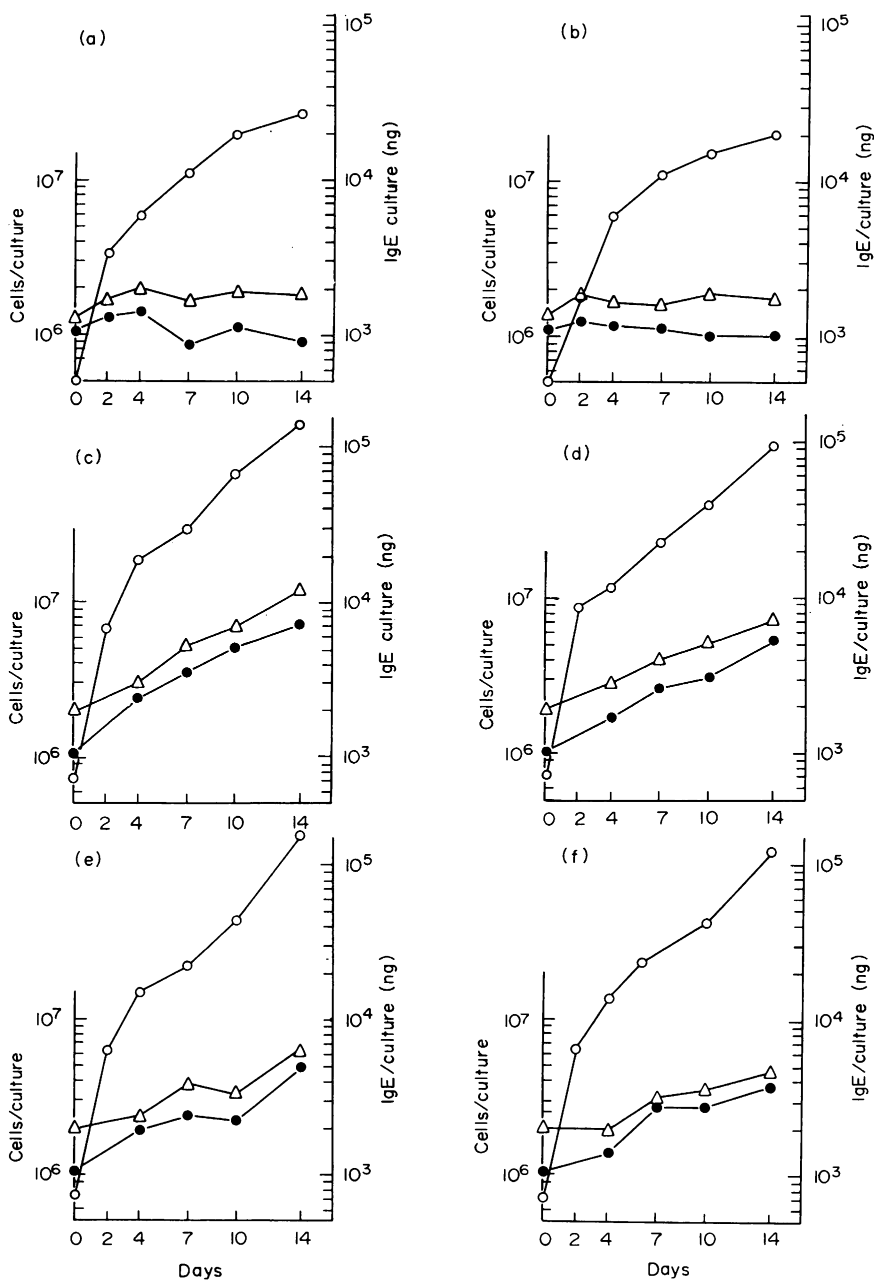

FIG. 1. IgE secretion and cell proliferation in different media (Exp. 9–14). (a) F-10+10% calf serum; (b) RPMI 1640+10% calf serum; (c) F-10+15% calf serum conditioned by adult skin fibroblasts (393S); (d) F-10+15% calf serum conditioned by adult glia-like cells (403CG); (e) F-10+10% calf serum, Adult skin fibroblasts (393S) present in the culture: (f) F-10+10% calf serum. Adult glia-like cells (403CG) present in the culture. The symbols, ○, △, ●, indicate the amount of IgE per culture, total cell number and viable cell number, respectively.

conditioned and non-conditioned medium. Cells were harvested from the same optimally growing stock culture and washed twice in cold PBS. Aliquots of $1{\cdot}1 \times 10^6$ viable cells were incubated for 14 days under different conditions. The IgE concentration in the medium, total and viable cell number were determined on Day 0, 2, 4, 7, 10 and 14.

In F-10 medium (Exp. 9) or RPMI 1640 (Exp. 10) the production of IgE during Days 0–4 was $4{\cdot}9\ \mu g/10^6$ and $5\ \mu g/10^6$ cells, respectively. The viability declined considerably in both cultures from day 4 and onwards. Finally, at 14 days the viable cell numbers were slightly lower than at day 0.

As in the previous 7 days experiments (Table 1) growth was faster and the IgE secretion rate was higher in cultures supplied with media harvested from feeder cells or with such cells present. No significant differences was found in 'feeding' capacity between adult skin fibroblasts, adult glia-like cells and media harvested from such cells. Cells multiplied with a population doubling time ranging from 4 (Exp. 11) to 6 (Exp. 14) days. Viability remained at the same level throughout these experiments.

In parallel experiments the percentage of ^{3}H-Tdr labelled cells was determined on day 0, 4 and 7 in routine cultures supplemented with conditioned and non-conditioned RPMI 1640 plus 10% calf serum. On day zero 35% of the cells in both types of media incorporated ^{3}H-Tdr. On Day 4 a significantly higher percentage of labelled cells (38·3) was found in conditioned than in non-conditioned cultures (29). Finally, on Day 7 the difference in the number of ^{3}H-Tdr incorporating cells was even greater, 38·5% versus 26%, indicating the superiority of conditioned medium to non-conditioned to support proliferation of IgE myeloma cells.

Experiments 9–14 show that, in unsynchronized cultures, IgE synthesis is optimal when cells are in the best physiological condition as measured by proliferation rate and that proliferation rate correlates with production rate of IgE.

DISCUSSION

The main conclusion of this and previous reports (Nilsson *et al.*, 1970; Nilsson, 1971) is that myeloma cells are unusually exacting in their metabolic requirements. Only if special media, rich in vitamins, amino acids etc. (for instance F-10, RPMI 1640), are conditioned by the presence of growing cells will the IgE production become optimal. It will then approach the secretion rate obtained in fresh explants of human myeloma cells which presumably reflect the synthetic rate *in vivo* (Salmon & Smith, 1970; Salmon *et al.*, 1971). Measured over a 48-hr period the optimal rate was $1{\cdot}7 \times 10^{-13}$ g of IgE per cell per hour.

Maximal rate of synthesis was correlated with rapid cell growth. This is consistent with the proposal by Buell & Fahey (1969) and Byars & Kidson (1970) that immunoglobulin synthesis is restricted to a particular part of the cell cycle. It is, however, also possible that the correlation is non-specific in the sense that IgE production and a high growth rate are both indicators of high viability.

The present cell line 266 B1 is the only one of human myeloma origin which continuously produces complete immunoglobulin molecules *in vitro* identical to those manufactured *in vivo*. The elaboration of a technique which permits a high rate of IgE synthesis over long periods of time should make it possible to study the regulation of immunoglobulin synthesis and secretion by myeloma cells.

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