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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Zoologia. — *Further research on* in vitro *production of* C 4 *component by rat macrophages*(*). Nota(**) di GIANNI A. AMIRANTE, LIA BELLONE e GABRIELLA POZZI, presentata dal Socio S. RANZI(***).

RIASSUNTO. — Si è voluto verificare la produzione di una frazione del complemento (C 4) da parte di macrofagi di mammifero. La presenza di questa frazione del complemento, in macrofagi di ratto in coltura, era già stata constata in ricerche precedenti, ma per dimostrare la sintesi attiva da parte di tali cellule, si sono sottoposte le colture pure allestite da essudato peritoneale di ratto, a trattamento con un inibitore della sintesi proteica (Actinomicina D). Dopo il trattamento con tale sostanza le colture venivano trattate con siero anti–C 4, coniugato con isotiocianato di fluoresceina, che conferisce al sito cellulare cui si lega, una spiccata fluorescenza. Insieme alle colture trattate con actinomicina, venivano fatte reagire con l'antisiero fluoresceinato anche colture di controllo non trattate. Si è rilevata una nettissima differenza nel numero di cellule fluorescenti nelle colture trattate rispetto ai controlli. Da ciò si può concludere che i macrofagi peritoneali di ratto sono sicuramente implicati nella sintesi attiva della frazione C 4 del complemento.

To date not many studies have been carried out on cells involved in complement synthesis. The earliest research dates back to Rice (1953) who first noticed a lowering of the complement rate in the blood of animals whose liver was injured with carbon tetrachloride. Later on, *in vitro* incorporation of ^{14}C marked amino acids was studied in the liver, spleen and endothelioreticular system. Moreover, in these organs the presence of $C\,1_q$, C 3 and C 4 fractions was also observed (Thorbecke *et al.*, 1965; Stecker V. J. *et al.*, 1967). In connection with $C\,1_q$, Lai A. Fat and Furth (1975) have recently suggested that macrophages or lymphnode cells may be responsible for their synthesis. Observations on C 4 were carried out by Littleton *et al.*, (1970), who used a modified Jerne lysis plaque technique on guinea pig pulmonary and peritoneal macrophage cultures. Furthermore, a production of C 6 in rat liver cultures and, probably, also of C 5 in cells of medullar origin, was observed (Rother *et al.*, 1966). Colten *et al.* (1968) observed C 1 synthesis in epithelial cells of the small intestine. C 2 production was noticed in guinea pig spleen cultures and in lung, in lymphonodes and in peritoneal exudate (Rubin J. *et al.*, 1971). C 9 synthesis was observed in cultures of rat hepatoma cells of the MH_1C_1 group (Rommel *et al.*, 1970). Nothing is known about C 7 synthesis, whereas the only works on C 8, by Geiger *et al.* (1972), concern swine fetal tissues. Amirante and Lombardi (1972) studied the presence of C 4 in white rat peritoneal macrophages by means of the lysis plaques technique. Since this work gave some interesting results, it seemed advisable to pursue studies using more refined techniques with culture celles in order to directly observe its synthesis.

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(***) Lavoro dedicato al prof. Silvio Ranzi in occasione del suo 75° compleanno.

Rat peritoneal macrophages and monocytes were cultured in a liquid medium. The presence of C 4 was shown up by treating the cultures with fluorescein isothiocyanate conjugated immunoglobulins (FITC-Ig). Finally, to evidence that the presence of the C 4 fraction in these cells was due to an active synthesis, cultures were treated with Actinomycin D which, as is well known, inhibits protein synthesis.

Culture preparation: 4 ml of 4% peptone in saline solution were injected intraperitoneally into adult white rats (Sprague-Dowley). Five days later the rats were killed and the peritoneal exudate was drawn off washing the cavity with sterile Hanks' solutions. After centrifugation cells were suspendend again in a liquid culture medium. The culture medium consisted of NCTC 135 or NCTC 109 (Difco Lab.) 9.7 g/l complemented with 30% calf serum lacking in C 4 fraction (R 4). As regards R 4 preparation, we followed the above method (Amirante and Lombardi, 1972), dialyzing the serum against a 0.01 M ammonia solution. Cultures were prepared in leighton tubes, containing about 3×10^6 cells each, and then stored at 37 °C. The culture medium was changed every 48 hours. Every 25 hours test samples of the culture were stained according to the method of Pappenheim, to ascertain when cell populations could be considered " pure ". Only when pure monocyte and macrophage cultures were obtained, treatments with Actinomycin D and with antibodies were performed.

Treatment with Actinomycin D: The concentration of Actynomycin D was determined by several attempts, treating cultures with concentrations that varied from 20 to 0.01 micrograms/ml while time of treatment varied from 16 to 24 hours. Dilution of Actinomycin was made with the culture medium. At the end of every treatment, cultures were stained in order to control cell viability and conditions, and consequently to be able to find the highest concentration which allowed the cells to be kept in culture. We found that the best values of concentration and the time that allowed good cellular conditions and a good synthesis inhibition were 0.01-0.015 micrograms/ml for 24 hours. Treatment with Actinomycin was effected on three, four, five and seven day old cultures. At the end of Actinomycin treatment, cells were in good condition even if less numerous than in the control (Plate I, *a* and *b*). Thus after having fixed time and concentrations, the cultures so treated were exposed to anti-C 4 serum, marked with FITC, and observed under the fluorescence microscope (Leitz U. V. mod. SM, filters UG 1, BG 38). As a control, the same treatment was applied to cultures that had not been treated with Actinomycin.

Treatment of cultures with marked antiserum: After a short methanol fixation and washings in PBS pH 7.2, controls and Actinomycin treated cultures were exposed for 30 minutes at 37 °C to anti-C 4 serum (Behring) [1], marked with fluorescein isothiocyanate. After further PBS washings the cultures were buffered and imbedded in glycerine in order to be observed under the fluorescence microscope.

Cells were drawn 5 days after the intraperitoneal peptone injection, because we noticed that at this moment cell reaction against inflammation was at a maximum. Cultures were stained daily according to the method of Pappenheim and observed under the microscope; cells were in good condition. Cell composition was rather homogeneous: in fact monocytes, macrophages and mast cells could be found. It must be noticed that cells cultivated in both NCTC 135 and NCTC 109 did not show significant variations. Cell percentage

(1) We used this antiserum because, according to preliminary tests, it showed a highly specific cross reaction against the rat C 4 fraction we purified.

TABLE I

Average percent values of the various cell types in control in vitro *cultures from 2 to 8 days old.*

Day	Macrophages	Monocytes	Mast cells
2	73.8	25.1	1.1
3	97.6	0.2	2.2
4	96.5	3.1	0.4
5	98.4	1.6	—
6	99.3	0.7	—
7	99.5	0.5	—
8	99.8	0.2	—

varied as time passed. The third day, nearly pure macrophage and monocyte cultures were present (Plate I, *a*); furthermore, we could notice an increase in the percentage of macrophages (Table I). Therefore, it was decided to start treatments the third day of culture. Pure cultures were treated for half an hour with fluoresceined antiserum to display the presence of C 4. Using this conjugation time, fluorescence in the inside of the cells was clear and the percentage of marked cells amounted to about 52 % (Plate I, *c*). To demonstrate that the presence of C 4 fraction in the cells was due to an active synthesis, protein synthesis of cultivated cells was inhibited by treatment with Actinomycin D. These cultures and some controls were treated with anti-C 4 FITC serum; in some cases fluorescence was completely lacking; generally a considerable lowering of fluorescence, in comparison with the controls, was observed (Table II and fig. 1).

TABLE II

Average percent values of fluorescent cells in control and in Actinomycin treated cultures from 3 rd *to* 7 th in vitro *culture day.*

Day	Controls	Actin. D .010 μg/ml	Actin. D .015 μg/ml
3	37.5	0.0	0.0
4	55.0	8.9	10.4
5	69.2	17.0	7.6
7	47.0	5.6	0.0

From the data presented above, it can be inferred that the presence of C 4 in peritoneal macrophages and monocytes is certainly due to an active protein synthesis because in the presence of Actinomycin the percentage of fluorescent cells is very low or absent as compared with control cultures.

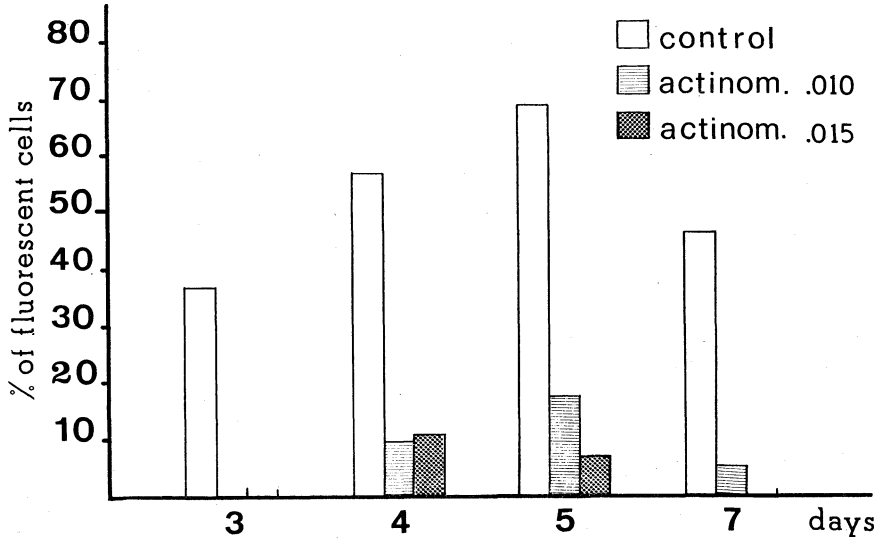

Fig. 1. - Average percent values of fluorescent cells in control and Actinomycin treated cultures.

Control cultures treated with marked antiserum showed a remarkable percentage of fluorescent cells that increased until the fifth day, and then slowly decreased. The small percentage of cells treated the fourth day showing fluorescence lets us assume a specific messenger RNA to be present and preformed before Actinomycin treatment. In fact, this substance inhibits transcription but does not affect translation.

In future we propose both to further investigate C 4 production, treating cultures with substances that operate on translation, like cycloheximide, and to study production of other complement factors in these cells, using other labelling agents.

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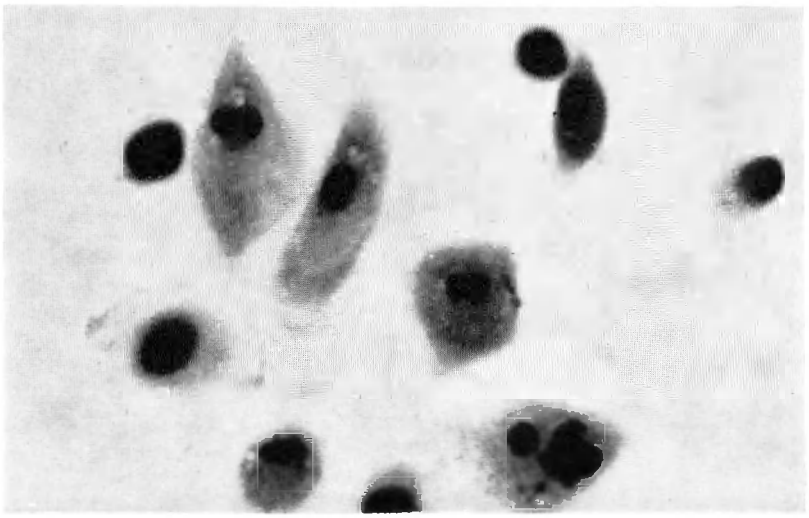
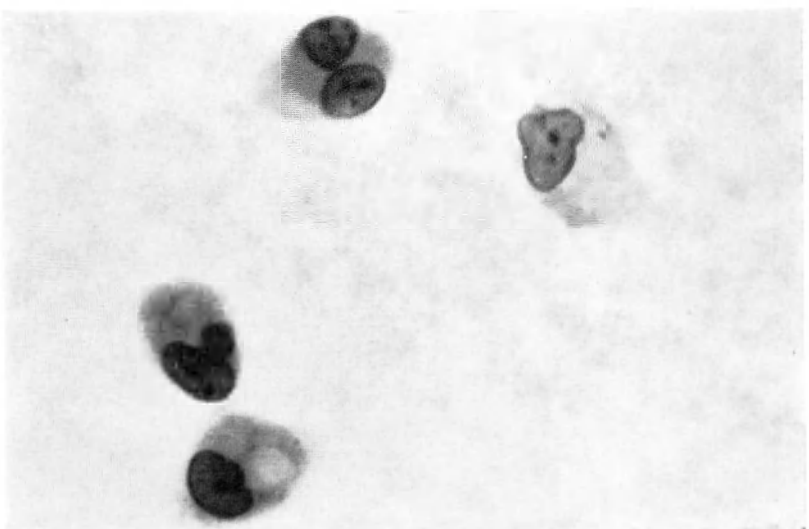
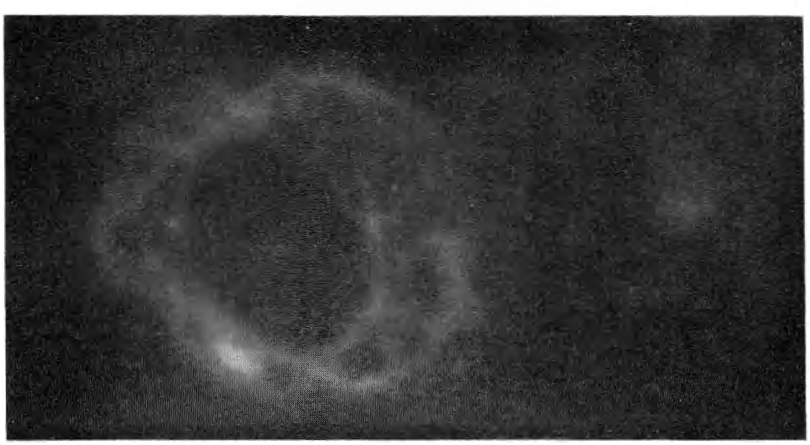


a) Control macrophages at 3rd day of culture (Pappenheim staining; magnification ×640).



b) Actinomycin D treated macrophages at the 3rd day of culture (Pappenheim staining; magnification ×640).



c) Immunofluorescence of C 4 in control macrophage treated with FITC anti-C 4 serum (Magnification ×1,500).

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