Immunology, 1968, **14**, 649.

Formation of C'_6 by Rabbit Liver Tissue *In vitro*

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(*Received* 29*th August* 1967)

Summary. Various rabbit tissues such as liver, spleen, peritoneal macrophages and kidney, were cultured *in vitro* at 37° for periods of up to 72 hours. The combined content of C'_6 in tissue and culture medium was determined at the end of the incubation period. Production of C'_6 activity was found in liver cultures and possibly in spleen and macrophage cultures. The rate of inactivation of serum C'_6 at 37° in culture medium was also determined.

INTRODUCTION

Studies on the formation of serum proteins associated with complement (C′) activity were made possible by the isolation and characterization of β_{1C} as C'_3 (Müller-Eberhard, Nilsson and Aronsson, 1960; Müller-Eberhard and Nilsson, 1960) of β_{1E} as C'_4 (Müller-Eberhard and Biro, 1963), and of 11S protein as C'_{1q} (Müller-Eberhard and Kunkel, 1961; Morse and Christian, 1964). Incorporation of radioactive amino acids into these serum proteins by various tissues and cell types *in vitro* has been studied in different species (Thorbecke, Hochwald, van Furth, Müller-Eberhard and Jacobson, 1965) and it has been shown that both liver and lymphoid tissues produce these three proteins (Stecher, Morse and Thorbecke, 1967). The liver, however, was active only after exposure to agents known to enhance the production of a variety of serum proteins in this organ (Williams, Asofsky and Thorbecke 1963; Hurlimann, Thorbecke and Hochwald, 1966). A few epithelial tissues were also active in the production of one or more of these proteins.

Among the various isolated cell types studied, the only specific cell type demonstrating incorporation of ^{14}C-amino acids into these proteins was the peritoneal and lung macrophage (Stecher and Thorbecke, 1967b). A recent report of Olitzki and Gershon (1967) supports the proposal that lung macrophages may indeed synthesize several complement components since cells were found in lung washings which produced plaques when incubated in an agar containing sensitized sheep erythrocytes.

Studies on the site of formation of haemolytically active complement factors, however, have been hampered by the lability of many complement components at 37°. The lack of knowledge regarding possible inhibitors present in tissue extracts, which would effect results of component titrations as used for serum C′ activity, has been an additional discouraging factor.

According to Colton, Borsos and Rapp (1966), the only guinea-pig tissue capable of producing C'_{1a} activity at 4° and at 30° *in vitro* is the ileum. Liver was found to contain

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and produce insignificant amounts of C'_1 activity. Siboo and Vas (1965) found an increase in C'_2 and C'_4 activities both in liver and spleen cultures during a 24–48-hour incubation period.

The present studies were undertaken to determine which rabbit tissues or cells might be active in the production of C'_6 activity. This component was chosen because of the availability of serum specifically lacking in C'_6 activity from complement deficient rabbits (Rother, Rother, Müller-Eberhard and Nilsson, 1966), which allows detection and titration of C'_6 activity with high sensitivity. In addition, since C'_6 is a relatively heat stable component, studies with tissues incubated at 37° could be carried out without an overwhelming loss of activity during the incubation period. It is the purpose of this paper to show that the rabbit liver is a site of C'_6 production.

MATERIALS AND METHODS

Animals

Adult and foetal rabbits of the New Zealand type were used. Some of the adult animals were injected intramuscularly with 2 ml paratyphoid–typhoid vaccine (Wyeth Laboratory, Marietta, Philadelphia) 24 hours before killing; others were bled 60 ml from the ear 24 hours before use.

Tissue culture procedures

Tissues were removed aseptically, and were minced and distributed over the walls of several roller tubes. The tissues (approximately 100 mg wet weight/tube) were allowed to drain off excess fluid for about 15 minutes, and then 2 ml of culture medium was added to each tube. The method of collecting and isolating peritoneal macrophages has been described previously (Stecher and Thorbecke, 1967b). Several media were used, some of which were found to be more effective than others. Eagle's minimal essential medium (MEM) with penicillin (200 U/ml) and supplemented with 20 per cent C'_6 defective rabbit serum or with 0·5 per cent bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Illinois) was found to give better results than MEM with 0·5 per cent rabbit serum albumin (Pentex Inc., Kankakee, Illinois). In most experiments, Phenol Red was omitted from the medium since its presence interfered with C'_6 titrations. Culture fluids containing Phenol Red were dialysed before titrations were performed. Hydrocortisone (0·1 μg/ml) and insulin (50 μg/ml) were usually added to the media (see Stecher and Thorbecke, 1967b).

At the outset of the incubation period one or two tubes which served as controls, were frozen to −20° immediately after receiving the tissue and the medium. The other tubes were incubated in a roller drum at 37° for 24–72 hours. At the end of the incubation period all tubes were stored at −20°. The contents of each tube were then subjected to sonic oscillation (Raytheon magnetostriction oscillator, 9 k-cycles, 10 minutes, 0°) and centrifuged at 15,000 ***g*** for 20 minutes. The clear supernatants were titrated for C'_6 and C'_3 activities.

The tissue of a parallel culture was exposed to a medium containing [^{14}C]lysine and [^{14}C]isoleucine of 100–200 mc/mM, 1 μc of each per millilitre. The technique for demonstrating incorporation of labelled amino acids into serum proteins by means of autoradiography (AR) of immunoelectrophoretic (IE) patterns has been described (Hochwald, Thorbecke and Asofsky, 1961; Hurlimann *et al.*, 1966). Concentrated culture fluids were added to IE slides after addition of a carrier serum to the antigen well. IE patterns were

developed with a sheep antiserum to whole rabbit serum (Mrs M. B. McGinness of the Department of Health of the City of New York Bureau of Laboratories, Otisville Branch, New York, was kind enough to immunize the sheep for us) and a duck antiserum to β_{1C} (Mardiney and Müller-Eberhard, 1965). This antiserum recognized the active and the inactivated form of C'$_3$ which, in the rabbit, have similar immunoelectrophoretic mobilities (Linscott and Cochrane, 1964).

C'$_3$ activity

C'$_3$ activity was determined by the immune adherence (IA) assay described by Nishioka and Linscott (1963). The method was not used in the present study for exact quantification, but rather as an indicator for relative differences in C'$_3$ activity.

C'$_6$ activity

C'$_6$ activity was assayed as by Rother *et al.* (1966), with minor modifications. Chicken erythrocytes (E) were used in this assay since they are known to be more sensitive in titrations of rabbit C' than are sheep erythrocytes (Linscott, 1967). The cells were sensitized with a rabbit anti-Forssman serum (Baltimore Biological Laboratories, Baltimore, Maryland). The standardized suspension of sensitized cells (EA) was incubated with rabbit serum lacking C'$_6$ activity (C'$_{def}$) to result in cells with the activity of EAC'$_{1a\ 42a\ 3}$. (C'$_{1a}$: the activated first component of complement, etc.). The cells were washed and resuspended in EDTA-buffer and an excess amount of C'$_{def}$ furnishing the post-C'$_3$ factors with the exception of C'$_6$, plus the test sample, were added for incubation for 60 minutes at 32°. A calibration curve with pooled normal rabbit serum was prepared for each titration and the amount of C'$_6$ present in the test sample was calculated (graphically) and expressed in terms of microlitre serum equivalent.

C'$_6$ inhibition tests

Tests were performed to confirm the functional specificity of the lytic activity found in the C'$_6$ assay. Rabbit antiserum against rabbit C'$_6$ was prepared in C'$_6$ defective rabbits and the C'$_6$ inhibition test was carried out under the criteria described by Rother *et al.* (1966).

Diluents

All dilutions in the C' determinations were made in veronal buffer, pH 7·4 (Mayer, 1961). Gelatin was added to 0·1 per cent for the IA determinations. EDTA-buffer is veronal buffer plus Na_3 EDTA added to a final molarity of 0·01.

RESULTS

STABILITY OF RABBIT C'$_3$ AND C'$_6$

In view of the known instability of the activity of C' components, information was sought on the rate of loss of rabbit C'$_3$ and C'$_6$ activity in culture medium to facilitate the estimation of their production in the cultures. A 1:10 dilution of normal rabbit serum in MEM was incubated at 37° and samples were withdrawn at various time intervals. Whereas approximately 50 per cent of C'$_6$ activity still persisted after 2 days, the C'$_3$ titre in 0·02 ml serum declined from a strong ++ IA reaction to + after 24 hours, and was barely discernible after 48 hours.

STUDIES ON THE PRODUCTION OF C'_6 ACTIVITY

Cultures of nine out of thirteen different rabbit liver specimens showed increasing concentrations of C'_6 activity upon incubation for 24 or 48 hours. The gain in activity was equivalent to the amounts of C'_6 present in 1·1–31·7 μl of serum (Table 1). Four cultures did not show such an increase. However, the decrease in activity even in these samples was distinctly less than the expected rates of natural inactivation of the initial content of C'_6 in the cultures (Fig. 1). The foetal liver specimens had been removed from foetuses at 24 days gestation. One produced a moderate amount of C'_6; in the other culture, the C'_6 titre paralleled the expected rate of inactivation.

TABLE 1

C'_6 ACTIVITY IN CULTURE MEDIUM OF RABBIT TISSUES* AFTER DIFFERENT PERIODS OF INCUBATION AT 37°

Tissue cultured	C'_6 activity (μl serum equivalent) at:				C'_6 activity maximal gain
	0 hours	24 hours	48 hours	72 hours	
Liver	9·3	9·2	8·3	8·3	
	6·6	6·0	5·9	6·3	
	6·1	6·1	5·7	5·7	
	7·2	7·2		7·0	
	6·1	7·2	7·1	6·6	1·1
	6·8	8·2	8·1	8·3	1·5
	8·5	9·3		10·3	1·8
	7·8	9·7	8·2	8·0	1·9
	12·0	15·2	16·7		4·7
	13·3	17·5	18·4		5·1
	7·2	16·6			9·4
	15·2	24·0	31·5		16·3
	13·3	13·3	45·0	18·7	31·7
Foetal liver	8·0	5·9			
	9·3	10·6			1·3
Peritoneal macrophages	5·3	4·0			
	5·2			4·1	
	3·6		2·5		
	4·4			5·2	0·8
Spleen	11·0	6·6			
	6·1	5·6	5·7		
	6·1		6·1		
	6·1	6·1		6·1	
	8·6	8·3	8·8	8·0	0·2
Kidney	8·4	6·4	6·2		

* At the end of the incubation period, tissues were frozen and sonicated to release C'_6 content into the medium.

In one of four peritoneal macrophage cultures a slight increase was seen in C'_6 activity; in another culture the titre remained fairly constant, and in two others there was a decrease in activity similar to the expected natural loss. One spleen culture showed a decrease similar to the natural instability of C'_6, but the remaining four spleens maintained almost constant levels of activity. In the one kidney culture studied, the decrease in activity was not significantly different from the rate of inactivation observed in serum containing medium alone (Table 1).

All samples of the three most active liver cultures were subjected to the C_6' inhibition test. Lytic activity was abolished by anti-C'_6 serum in all samples, confirming the identity of the produced lytic activity as C'_6.

STUDIES ON THE PRODUCTION OF β_{1C} AND OF C'_3 ACTIVITY

Both liver and spleen incorporated [^{14}C]amino acids into β_{1C}-globulin. The degree of labelling of β_{1C} by liver varied between weak and very strong (graded w+ to +++). Some of the livers with high C'_6 production were also among the best producers of other serum proteins, as judged by β_{1C} and albumin labelling—but this was not always the case. Labelling of β_{1C} by the spleen or by peritoneal macrophages varied between + to ++. Kidney did not label β_{1C}. Foetal liver, taken approximately 1 week before birth, showed significant labelling of β_{1C}, and was very active in the production of other serum proteins, such as albumin and transferrin (Stecher and Thorbecke, 1967a).

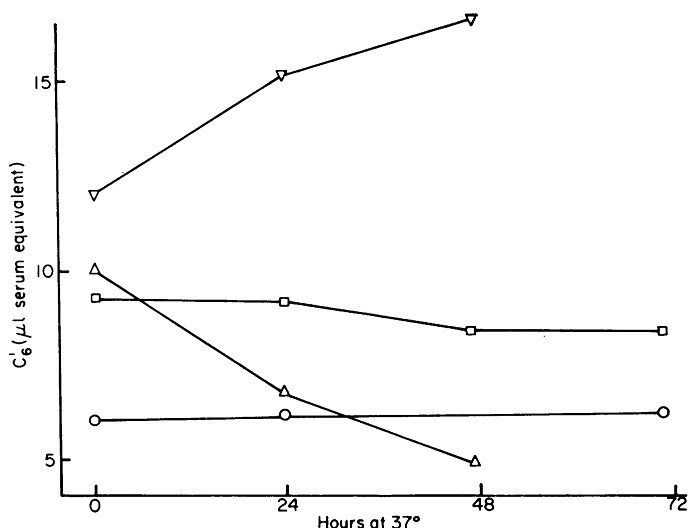

FIG. 1. Approximately 50 per cent of C'_6 activity is lost when serum is incubated with medium for 2 days at 37° (△), as contrasted with a fairly constant level in a representative spleen culture (○). The majority of the liver cultures showed a significant increase, some exceeding the example depicted above (▽). A few liver cultures developed a slight decrease in C'_6 content as illustrated by □.

It was not possible to associate the labelling of β_{1C} with production of C'_3 activity. C'_3 apparently undergoes rather rapid inactivation upon incubation at 37° since the activity equivalent to 0·02 ml of fresh rabbit serum—giving a strong IA reaction—was only barely detectable by this test after 48 hours of incubation. In view of this loss of C'_3 activity, and considering that the highest C'_6 production per culture was equivalent to the activity present in 0·03 ml of serum, it is not surprising that production of C'_3 activity was not detectable. It should be emphasized that in the rabbit there is no clearcut shift from the β_{1C} position as C'_3 undergoes inactivation (Linscott and Cochrane, 1964) and therefore the C′ active form of the protein is not distinguishable, by immunoelectrophoresis, from the inactive form. The β_{1C}-globulin which was labelled by liver grown in the presence of [^{14}C]amino acids could thus represent either form of this protein.

DISCUSSION

The present observations strongly suggest that the rabbit liver is a site of formation of C'_6. The interpretation as production rather than release of preformed material is based on the continuous increase in C'_6 activity during the first 48 hours, which assumes even more significance when contrasted with the demonstrated rate of decay by temperature (Fig. 1). Further, since all cultures were subjected to disintegration by sonication prior to the collection of the test sample, any accumulation of preformed intracellular material should not have escaped detection. The rate of production varied within a wide range. While in the most active culture an increase in C'_6 activity equivalent to 31·7 μl serum was noted, some other cultures barely compensated for the rate of inactivation.

The observations on rabbit foetal liver suggest some C'_6 production in the later part of the gestation period but no firm conclusion can be reached on the basis of the limited data collected in this investigation. Similarly, no conclusions on production of C'_6 in the foetus can be drawn from the presence of C'_6 in the initial (zero time) tubes of the foetal liver cultures since no information is available on the possible supply of C′ factors by the mother to the foetal rabbit. The C'_6 activity which could be demonstrated at zero time with the sonicated tissues does not necessarily imply intracellular material. It may indeed predominantly represent serum C'_6 trapped in the extracellular compartment. Since removal of free extracellular fluid was achieved only by washing and draining of the cultured tissue fragments prior to the addition of medium, enough C'_6 could have been left so as to be recognized by the titration method.

Only a few rabbit organs were selected for study in the present investigation. No attempt was made to identify further the producing cell type in the liver, or to investigate the presence of productive cells in organs other than those in Table 1. C'_6 production has not been previously studied. However, it is of interest to compare the present findings with observations on the site of formation of other factors functional in the chain of C′ reactions. The exclusive production in the small intestine of C'_1 described by Colten *et al.* (1966) and the production of C'_2, C'_3 and C'_4 in liver, spleen and bone marrow reported by Siboo and Vas (1965) suggest that these functionally related proteins may not all be produced by the same type of cell. On the other hand, the results obtained on incorporation of [^{14}C]amino acids into three serum proteins associated with the complement system, as well as the lysis of sensitized erythrocytes following incubation in agar with lung macrophages (Olitzki and Gershon, 1967), suggest that many C′ factors are produced in a single type of cell: the omnipresent macrophage.

ACKNOWLEDGMENTS

This work was supported by United States Public Health Service Grants AI-03076 and RM-08986, and was performed while J.H. was a Fellow of the Swiss Academy of Medicine.

One of us (G.J.T.) is a recipient of Research Career Development Award GM-K3-15,522 from the United States Public Health Service. V.J.S.-L. is a Fellow of the United States Public Health Service, National Institute of General Medical Sciences (5-F1-GM-20,043).

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