SYNTHESIS OF THE FIRST COMPONENT OF HUMAN COMPLEMENT IN VITRO*

By HARVEY R. COLTEN, M.D., JAMES M. GORDON, M.D., TIBOR BORSOS, Sc.D., AND HERBERT J. RAPP, Sc.D.

(From the Biology Branch and the Immunology Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

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In previous publications we have presented evidence that the first component of guinea pig complement (C'1) is synthesized by the small intestine (1, 2). This conclusion was based on the observations that, in vitro, small intestine was the only tissue capable of production of hemolytically active C'1, that this production was reversibly inhibited by puromycin and actinomycin D and that ^{14}C-lysine was incorporated into molecules which had the properties of C'1 (C'1-like molecules) (1). Furthermore, by use of a hemolytic plaque assay technique (3) adapted to the study of C'1 synthesis by individual cells (2), it was found that columnar epithelial cells of the small intestine were the only cells capable of producing hemolytic plaques.

We now report that human colon as well as ileum are sites of synthesis of C'1. There was no detectable synthesis of C'1 in any of the other tissues examined. The cell type producing hemolytically active human C'1 has not yet been identified.

Materials and Methods

The preparation of partially purified guinea pig C'2 (the second component of C') (4), the cell intermediate EAC'4 (5) consisting of sensitized sheep erythrocytes (E) (4), rabbit antibody to boiled stromata of E (A) (4), and fourth component of C', and C'EDTA (guinea pig serum diluted 1/50 in 0.01 M *Na*-ethylenediaminetetraacetate, pH 7.5) (6) have been described. Fresh frozen guinea pig serum was obtained from Suburban Serum Laboratories, Silver Spring, Md. Isotonic Veronal-buffered saline (VBS), $\mu = 0.15$, and isotonic Veronal-buffered sucrose, $\mu = 0.0090$, were prepared as previously described (7).

Assay of C'1.—The assay of C'1 activity is based on the ability of C'1 to convert EAC'4 to EAC'1a,4 (8). Tissue fragments (average wet weight, 40 mg) were incubated with 0.5 ml of EAC'4 (1.5×10^8 cell/ml) in VBS, for 10 min at 30°C. The second component of C' (C'2) and C'EDTA (to supply the late acting C' components) were added, and the number of C'1 molecules transferred from the tissue to EAC'4 was calculated from the degree of hemolysis (9). Where the C'1 content of a tissue was too high to permit its estimation in the one step assay described above, a two step assay for C'1 content was performed as follows: Tissue fragments were incubated with 0.5 ml of EA (1.5×10^8 cells/ml) for 10 min at 30°C to permit transfer of C'1 from the tissue to the EA; at this time, suitable dilutions of the EAC'1

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were incubated for 10 min at 30°C with EAC'4 at $\mu = 0.15$ to permit transfer of the C'1 from EAC'1 to EAC'4. After addition of the remaining complement components, the amount of C'1 transferred was calculated from the degree of lysis.

Preparation of Organs.—Isolated organs were obtained either at postmortem examinations within 3 to 4 hr of time of death or at surgery. Tissues were placed immediately into Ringer's lactate solution at 0°C. Segments of the alimentary tract, liver, spleen, kidney, lymph node, lungs, and thymus were washed at 0°C in Ringer's lactate solution with 5% glucose (Abbott Laboratories, North Chicago, Ill.) containing 100 units of aqueous crystalline penicillin and 50 μg streptomycin/ml. At this stage of preparation, tissues contained C'1 activity, probably due to the presence of serum (1). This initial C'1 activity was eliminated by incubating each of the tissues in 0.01 M EDTA buffer for 40 min at 37°C. The tissues were washed four times at 0°C in the Ringer's lactate solution to remove EDTA and then tested for residual C'1 activity.

Incubation Media.—Tissues were incubated in medium 199 containing 100 units of penicillin and 50 μg streptomycin/ml. In experiments to test the effect of inhibitors of protein synthesis on the production of C'1, tissues were incubated in medium 199 containing puromycin (10–15 μg/ml) or actinomycin D[1](10–15 μg/ml), and penicillin (100 units/ml), and streptomycin (50 μg/ml).

The incorporation of ^{14}C-labeled amino acids into C'1-like molecules was studied by incubating EDTA-treated tissues in medium 199 in which ^{14}C-labeled leucine (>130 mc/mmole), lysine (>180 mc/mmole), arginine (>130 mc/mmole), and valine (>120 mc/mmole) (^{14}C-protein-labeling mixture, Schwarz BioResearch Inc., Orangeburg, N.Y. lot No. 6801) at a final concentration of 2 μc/ml were substituted for the corresponding unlabeled amino acids. At timed intervals tissue fragments were removed from this mixture, washed once in medium 199 lacking radioactive amino acids, and then incubated for 15 min at 30°C with 1.0 ml of EA (1×10^9 cell/ml). At this time the tissue fragment was removed and the red cells were washed once in VBS sucrose buffer ($\mu = 0.065$), then collected by centrifugation and resuspended in 5.0 ml of VBS-sucrose buffer. The cell suspension was divided into 2.4 ml portions one of which received 10.0 ml VBS-sucrose and was incubated for 10 min at 37°C followed by one wash with VBS-sucrose buffer. The other 2.4 ml portion received 0.24 ml of 0.1 M EDTA and 10.0 ml of 0.01 M EDTA buffer and was also incubated for 10 min at 37°C followed by one wash with 0.01 M EDTA buffer. Both cell suspensions were then washed three times in VBS-sucrose buffer, transferred to Corex centrifuge tubes, and centrifuged. Each cell button was lysed with 10.0 ml of distilled water and the resulting stromata were centrifuged at 0°C in a Sorvall centrifuge type SS 34 rotor for 30 min at 39,100 *g*. The sedimented stromata were dissolved in NCS reagent (Nuclear-Chicago Corp., Des Plaines, Ill.) and transferred quantitatively in a final volume of 1.0 ml to counting vials. 10 ml of scintillation fluid[2] were added, and the radioactivity was counted for 10 min in a Packard Tri-Carb scintillation spectrometer (model 4312). Control determinations were performed exactly in the above manner except that E were substituted for EA. In some instances the amount of hemolytically active C'1 on EA and E was also determined by the C'1 transfer test (9).

RESULTS

Site of C'1 Synthesis—EDTA-treated portions of transverse colon, ileum, stomach, liver, spleen, and kidney obtained at autopsy were incubated in

[1] We are indebted to the Drug Development Branch of the Cancer Chemotherapy National Service Center, NCI, for supplying puromycin (NSC-3055) and actinomycin D (NSC-3053).

[2] Solution of 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis 2-(5-phenyloxazolyl) benzene in 1 liter of toluene.

medium 199 at 10°C and their C'1 content was determined at timed intervals. The segments of colon and ileum were the only tissues capable of in vitro production of hemolytically active C'1 (Fig. 1). It was a consistent finding

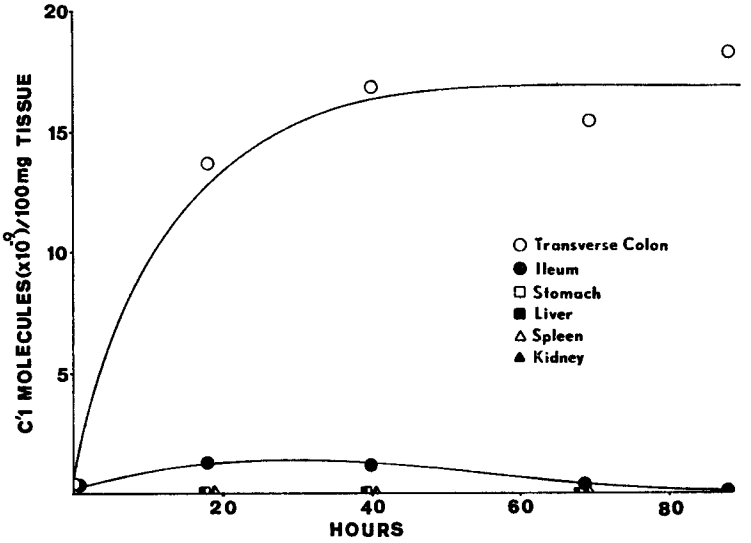

FIG. 1. In vitro production of hemolytically active C'1 by isolated human tissue. Tissues were incubated in medium 199 at 7°C.

TABLE I

In Vitro Production of Hemolytically Active C'1 by Intestinal Tissues Obtained at Postmortem Examination

	C'1 molecules ($\times 10^{-8}$)/100 mg of tissue			
	Time of incubation, *hr*			
	0	17	42	66
Ileum*	0.07	0.50	2.0	0.80
Jejunum	0.03	0.15	0.04	0.10
Infarcted segment of transverse colon	0.04	0.01	0.10	0.05

* Tissues incubated in medium 199 at 7°C.

with material obtained at autopsy that the amount of C'1 produced by colon fragments was significantly greater than that produced by the ileum. In contrast, segments of an infarcted transverse colon were incapable of in vitro C'1 production. Segments of ileum but not of jejunum from this patient were capable of in vitro C'1 production (Table I). The effect of temperature on

production of C'1 by segments of sigmoid colon is shown in Fig. 2. For this experiment, portions of sigmoid colon were obtained at the time of partial colectomy and adjacent segments were incubated at 7°C and at 30°C. It can be seen that the rate of C'1 production increases with increasing temperature. Because of the rapidity of C'1 production at higher temperatures it is difficult to obtain precise measurements at closely timed intervals. For this reason, most of the studies on in vitro C'1 synthesis were performed at low temperature.

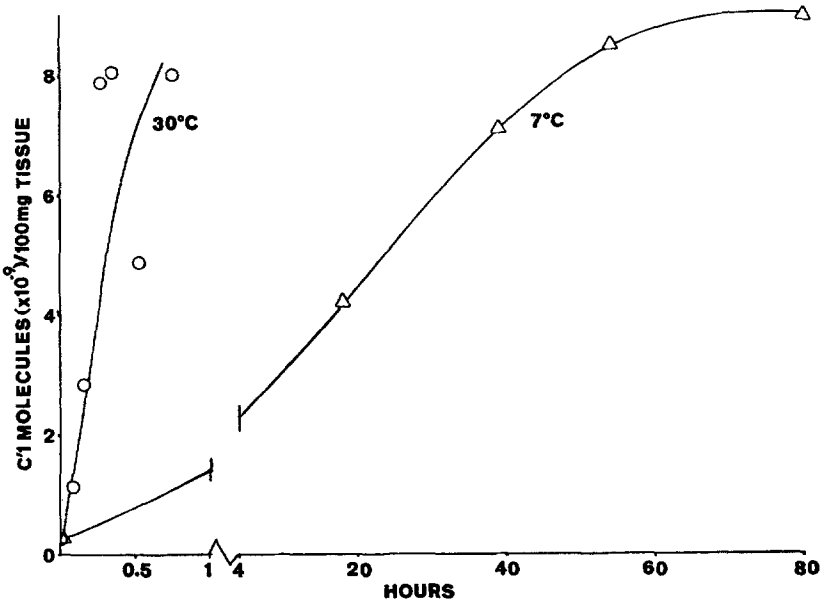

FIG. 2. Effect of temperature on the rate of in vitro production of hemolytically active human C'1 by isolated segments of sigmoid colon.

A segment of ileum obtained at surgery was capable of considerably more C'1 synthesis (Fig. 3) than ileum obtained at postmortem (Fig. 1). There was no detectable production of hemolytically active C'1 by segments of stomach, duodenum, jejunum, lung, axillary lymph nodes, liver, spleen, and kidney incubated at 30°C.

In a single experiment, fetal tissues were obtained at the time of a total hysterectomy performed at 19 wk gestation. As in the adult, segments of the fetal small intestine and colon but not of the thymus, lung, kidney, liver, spleen, or stomach were capable of in vitro production of hemolytically active C'1.

Effects of Inhibitors of Protein Synthesis.—These studies were designed to test the effects of puromycin and actinomycin D on in vitro production of hemolytically active C'1 and on the incorporation of ^{14}C-labeled amino acids into C'1-like molecules (a molecule that binds to EA but not to E and that can be removed from EA by treatment with EDTA). Adjacent segments of

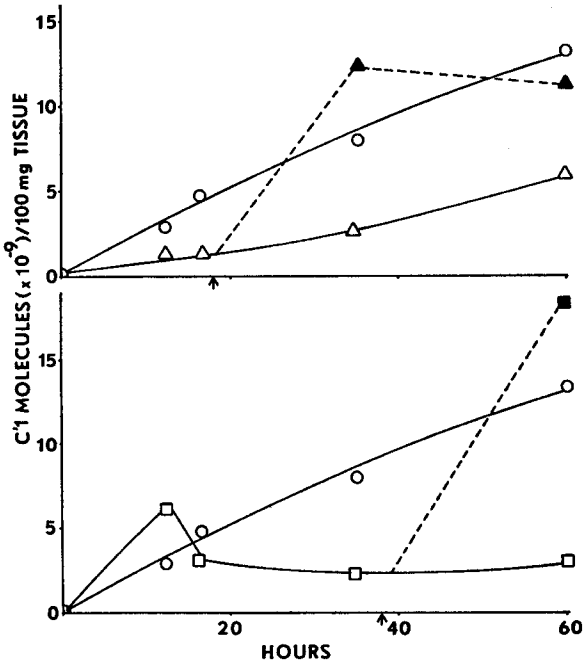

FIG. 3. Effect of inhibitors of protein synthesis on the production of hemolytically active human C'1 by segments of ileum at 7°C in medium 199. Upper figure: ○, no inhibitor; △, puromycin 10 μg/ml; ▲, tissue removed at ↑ and reincubated in medium 199 lacking puromycin. Lower figure: ○, no inhibitor; □, actinomycin D 10 μg/ml; ■, tissue removed at ↑ and reincubated in medium 199 lacking actinomycin D.

transverse colon were incubated at 10°C in medium 199 alone, in medium 199 containing 15 μg/ml of puromycin, in medium 199 containing 15 μg/ml of actinomycin D, and in medium 199 containing 10 μg/ml of puromycin plus 10 μg/ml of actinomycin D. At 23 hr of incubation, portions from each of the tissues incubated in the presence of inhibitors were removed and incubated in medium 199 lacking inhibitors. In addition at that time, portions of tissues incubated in the presence of both inhibitors were transferred to medium 199 containing either 10 μg/ml of puromycin or 10 μg/ml of actinomycin D. Table

II summarizes the results of these experiments. The in vitro production of C'1 was reduced by about 90% in the presence of actinomycin D or in the presence of both actinomycin D and puromycin. Puromycin alone reduced C'1 production by about 60%. Portions of tissues incubated in the presence of both inhibitors and then further incubated in puromycin alone resumed C'1 production; the amount of C'1 produced was about 30% of that produced by tissues incubated in medium 199 alone. Segments of an ileum were incubated at 7°C in medium 199, medium 199 containing 10 μg/ml actinomycin D, and

TABLE II

Effect of Puromycin and Actinomycin D on the Production of Hemolytically Active C'1 by Segments of Human Transverse Colon

	C'1 molecules ($\times 10^{-6}$)/100 mg of tissue			
	Time of incubation, *hr*			
	0	20	42	65
Colon*	0.052	46.7	42.2	24.5
Colon with puromycin (15 μg/ml)		18.7	6.1	10.0
Puromycin removed at 23 hr			15.5	25.0
Colon with actinomycin D (15 μg/ml)	0.052	6.2	2.8	5.2
Actinomycin D removed at 23 hr			14.0	9.4
Colon with puromycin (10 μg/ml) and actinomycin D (10 μg/ml)	0.052	2.4	1.6	3.3
Puromycin removed at 23 hr			2.5	4.5
Actinomycin D removed at 23 hr			9.9	11.5
Puromycin and actinomycin D removed at 23 hr			12.6	22.3

* Tissues incubated in medium 199 at 10°C.

medium 199 containing 10 μg/ml puromycin. As with the colon, C'1 production by the ileum was reversibly inhibited by puromycin and actinomycin D (Fig. 3).

The data presented in Table III show that puromycin and actinomycin D are effective in blocking incorporation of ^{14}C-labeled amino acids into C'1-like molecules. Furthermore, it can be seen that tissues from ascending, transverse, and descending portions of the colon and ileum are capable of incorporating ^{14}C-labeled amino acids into C'1-like molecules. As we have shown, these tissues were also capable of producing hemolytically active C'1. Un-

sensitized erythrocytes (E), mixed with fragments of descending or transverse colon, bound more than twice as much radioactivity as fragments of ascending colon. A portion of the counts taken up by the unsensitized erythrocytes could be removed by EDTA and therefore may be radiolabeled C'1.

TABLE III

Incorporation of ^{14}C-Amino Acids into C'1-Like Molecules: Detection by Uptake on Sensitized Sheep Erythrocytes

	CPM on cells washed in VBS-sucrose buffer, μ = 0.065			CPM on cells washed in 0.01 M EDTA buffer		
	EA	E	EA-E	EA	E	EA-E
Ascending colon	116	30	86	53	35	18
Ascending colon + actinomycin D (15 μg/ml)	22	23	0	36	18	18
Descending colon	131	79	52	43	44	0
Descending colon + puromycin (15 μg/ml)	86	95	0	32	33	0
Transverse colon	130	82	48	58	47	11
Transverse colon + puromycin (15 μg/ml)	94	81	13	43	31	12
Ileum	40	21	19	22	11	11
Kidney	2	4	0	0	4	0
Liver	11	6	5	2	0	2

E, sheep erythrocytes; EA, sheep erythrocytes sensitized with rabbit anti-Forssman antibody. CPM, counts per min.

All tissues were assayed for C'1-like molecules at 40 hr except the ileum which was assayed after 60 hr incubation.

A control for nonspecific uptake of ^{14}C-amino acids consisted of colon that contained preformed C'1 incubated for less than 5 min at 0°C with the ^{14}C-amino acids. No significant radiolabel was transferred to EA from tissue treated in this manner.

DISCUSSION

This is the first report of the in vitro synthesis of a hemolytically active complement component in human tissue. We have found that, as in the guinea pig, hemolytically active C'1 is produced in the tissues of the intestinal tract. In guinea pigs, the small intestine was the only tissue capable of in vitro synthesis of C'1. In contrast, the human C'1 is synthesized in both the large intestine and the ileum. We were unable to demonstrate synthesis of significant amounts of human C'1 in the jejunum, stomach, liver, kidney, lung, spleen, lymph node, and thymus.

Several lines of evidence indicate that in these experiments we measured synthesis and not release of preformed C'1. After elimination of C'1 from tissues by treatment with EDTA, we found between a 50–1000-fold increase in C'1 activity. The production of C'1 is highly temperature dependent and reversibly inhibited by puromycin and actinomycin D. C'1 production was not observed in infarcted colon or in tissues obtained more than 4–5 hr after death although these tissues contained preformed C'1. Furthermore, incorporation of ^{14}C amino acids into C'1-like molecules accompanied the production of hemolytically active C'1. Stecher, Morse, and Thorbecke (10) reported that peritoneal macrophages and thoracic duct lymphocytes of humans incorporated ^{14}C-labeled amino acids into C'1q (11s fragment of C'1); no attempts were made to demonstrate biological activity. Inhibitors of protein synthesis were not used in their studies and they pointed out that "the possibility of artifacts created by the complexing of serum proteins with other labeled tissue products cannot be ruled out." Complexing may occur between gamma globulin and the 11s molecule (11). Thus, the results of our study and those of Stecher, Morse, and Thorbecke are not comparable.

Some patients with congenital agammaglobulinemia have reduced serum C'1 (12, 13) and C'1q levels (11–15); it is not known, however, whether the low C'1 levels are the result of a defect in synthesis or an increased catabolic rate. Our studies demonstrate the feasibility of a direct study of synthesis of C'1 in such patients. These studies would be of interest since associated intestinal pathology has been found in some patients with agammaglobulinemia (16).

We have not yet identified the cell in the human intestine synthesizing C'1. It has been suggested that different parts of the C'1 molecule may be synthesized in different cell types (13). At present, however, there is no direct evidence to support this hypothesis. Our data clearly show that portions of the intestine are capable of synthesizing and assembling the C'1 molecule. In the guinea pig, isolated epithelial cells of the small intestine were capable of producing hemolytically active C'1. Work is in progress to identify the cell or cell types producing human C'1.

SUMMARY

Isolated segments of human colon and to a lesser extent ileum were capable of synthesizing hemolytically active C'1. This conclusion was based on the following evidence:

After elimination of C'1 from tissue with EDTA, we found that segments of the intestinal tract in short-term organ culture showed a 50–1000-fold increase in C'1 activity. The rate of production of C'1 in human intestine was highly temperature dependent; C'1 production was reversibly inhibited by puromycin and actinomycin D. Furthermore, ^{14}C-labeled amino acids were incorporated

into molecules which behaved like C'1. No significant $C'1^{hu}$ synthesis was observed in isolated segments of jejunum, stomach, liver, kidney, lung, spleen, lymph node, and thymus.

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