REVERSIBLE INHIBITION OF C1Q RELEASE FROM GUINEA PIG MACROPHAGES BY 2,2'-DIPYRIDYL

Evidence for a posttranslational hydroxylation step in the biosynthesis of C1Q, a subcomponent of the first component of complement (C1)

Wolfgang MÜLLER, Hartmut HANAUSKE-ABEL[+] and Michael LOOS
Institute for Medical Microbiology, Johannes Gutenberg-University, Obere Zahlbacher Str. 67, D-6500 Mainz, FRG

Received 11 April 1978

1. Introduction

The first component of complement C1 is a Ca^{2+}-dependent macromolecular complex consisting of three distinct proteins C1q, C1r, C1s [1]. C1 is bound to antigen–antibody complexes via the C1q subunit, which is able to interact with the Fc-region of IgM and of most IgC immunoglobulins [2,3]. As a consequence of C1q binding C1r becomes activated, which in turn converts the proenzyme C1s into its activated form, the C1-esterase [4,5]. C1q was first described as an 11 S protein [2] and has been well investigated in the last few years. It was concluded from the amino acid analysis, which revealed a high content of hydroxyproline, hydroxylysine, glycine and carbohydrates that the C1q molecule contained collagen like portions [6–9]. By reduction and alkylation 3 different peptide chains were isolated from C1q. Every chain was shown to have a collagen like region, located near the N-terminus, which was characterized by repeating X–Y–Glyc triplets often with hydroxylysine and hydroxyproline as Y and constituted about 40% of the chain [9,10]. The electron microscope pattern of the purified C1q molecule shows a unique structure in which 6 globular portions were connected by 6 strands to a fibril like central piece [11,12]. Recent studies [13,14] suggest, that the whole C1q molecule consists of 6 identical subunits, each composed of the 3 different polypeptide chains. The C-terminal regions of the 3 peptide chains constitute the globular portions, whereas the collagen like sections of the 3 chains form a triple helix and constitute the strands and the central piece. The globular portions are thought to be the binding site for antigen–antibody complexes and the central piece is believed to interact with C1r and C1s subunits [15,16]. It has been shown immunochemically that C1q is synthesized by macrophages and various other cell types [17–19]. Production of hemolytically active C1 molecules was demonstrable in the intestine, in different epithelial cells and in fibroblasts [20–23]. Recently we reported that human as well as guinea pig macrophages are able to produce C1q and C1 in a hemotlytically active form [24]. We found that C1q production exceeded C1 production by approx. 15 : 1, indicating an independent synthesis of the C1 subcomponents. Studies on collagen biosynthesis (reviewed [25]) have indicated that an intact triple helical conformation of procollagen is a prerequisite for secretion. A triple helical conformation is obtained under physiological conditions by hydroxylation of proline residues in the protocollagen molecule. When the prolyl and lysyl hydroxylases are blocked by the Fe^{2+} chelator 2,2'-dipyridyl, the unhydroxylated protocollagen peptides remain at random coil and secretion of procollagen does not occur. Because of the similarities between the collagen like

[+] Present address: St. Barbara Hospital, 4600 Dortmund 72, FRG

The nomenclature follows the recommendation of the World Health Organisation Committee on Complement Nomenclature, 1970. Immunochemistry 7: 137

portion of C1q and collagen it was of interest to study the effect of 2,2′-dipyridyl on the secretion of C1q by guinea pig macrophages.

2. Materials and methods

2.1. *Preparation of macrophage monolayers*

Starch gel-induced peritoneal exsudate cells were harvested by washing the peritoneal cavity with M199 modified (Flow Labs Ltd, Bonn) without anticoagulant. The cells were collected by centrifugation and resuspended in M199 at a concentration of 1.25×10^6/ml. Aliquots of 2 ml cell-suspension were distributed into plastic Petri dishes (35 mm diam., Greiner un Söhne, Nürtingen). After incubation at 37°C in a humidified atmosphere with 5% CO_2 for 90 min to allow attachment of adherent cells, the dishes were washed vigorously 4 times with phosphate buffered saline (PBS, pH 7.3). The macrophages were then cultured in M199 (2 ml/dish) with streptomycin and penicillin, with or without addition of 10^{-4} M 2,2′-dipyridyl (Merck, Darmstadt). Morphology of peritoneal cells was investigated after staining with May-Gruenwald and Giemsa solution, the ability to form rosettes with IgG or C3 coated SRBC and to phagocytose those cells was tested as in [26]. Viability was assessed by the trypan blue stain.

2.2. *Hemolytic assays of complement components*

Hemolytic activity of C1, C4, C2 was measured as in [27]. The methods for preparation of EA, EAC4, EAC14 cell intermediates and appropriate buffers were given in [27]. Partially purified guinea pig C1 was obtained by zonal ultracentrifugation according to [28]. C2 component was purchased from Cordis (Cordis Corp., Miami, FL). Guinea pig serum was used as source for C-EDTA. Supernatants from monolayers were centrifuged to remove any cell fragment and diluted in veronal buffered saline sucrose (VBS-S, $\mu = 0.065$, pH 7.35). The hemolytic assay for C1q based on the ability of C1q to reconstitute the C1 molecule with C1r and C1s. As source for C1r and C1s a C1q depleted human serum (RC1q) was used, kindly provided by Dr H. Isliker (Lausanne). The method for RC1q preparation has been described [29]. Supernatant dilutions, 0.1 ml, were incubated with 0.1 ml diluted and recalcified RC1q and 0.1 ml EAC4 (1.3×10^8 cells/ml) for 10 min at 30°C. The cells were washed twice and resuspended in 0.2 ml VBS-S. The following steps were performed as described for C1 assay. It was confirmed that 2,2′-dipyridyl up to 10^{-3} M did not affect the hemolytic assay of the complement components.

3. Results

3.1. *Morphology*

At zero time of culture monolayers consisted of about 90–96% macrophages. After 72 h incubation about 50–60% were still adherent and viable. In monolayers cultivated in the presence of 10^{-4} M 2,2′-dipyridyl the number of adherent viable cells was as high as in controls and the spreading of the macrophages was even enhanced. The number of hemolytically active complement molecules found at different times in the supernatant was always related to the number of viable cells/dish counted at zero time of culture (on an average $1.25–1.4 \times 10^6$ cells).

3.2. *Effect of 2,2′-dipyridyl on the release of hemolytically active complement components*

Guinea pig macrophage monolayers were incubated in M199 or in M199 with addition of 10^{-4} M 2,2′-dipyridyl for 72 h. At zero time and different intervals thereafter samples were removed and investigated for hemolytically active C1q and C1 molecules. As a control, to rule out inhibition of protein synthesis or nonspecific blocking of secretion by 2,2′-dipyridyl, the number of C4 and C2 molecules in the supernatant were also determined hemolytically. Both components have been described to be synthesized by macrophages [30]. The results are shown in table 1. In the supernatants from monolayers cultivated with 2,2′-dipyridyl strikingly lower amounts of hemolytically active C1q and also C1 molecules were detected, whereas the number of C4 and C2 molecules did not essentially differ from control values. In subsequent experiments cultures were incubated with 10^{-4} M 2,2′-dipyridyl for 24 h, washed 3-times in PBS and then to half the cultures M199 containing 2,2′-dipyridyl was again added and to the other half M199 alone. Figure 1 shows the time course of C1q release into the supernatant. In cultures with 10^{-4} M 2,2′-dipyridyl, after an initial increase during the first 6 h, the concentra-

Table 1
Effect of 2,2'-dipyridyl on the release of complement components from guinea pig macrophages[a]

		Incubation time (h)			
		0	24	48	72
C1q eff. mol ($\times 10^{-2}$) per Mϕ[b]	Control	0.13 ± 0.16	64.5 ± 5.8	179.1 ± 28.5	238.0 ± 30.5
	+ 10^{-4} M 2,2'-dipyridyl	0.11 ± 0.10	9.9 ± 3.8	19.6 ± 8.3	27.8 ± 4.9
C1 eff. mol per Mϕ[b]	Control	0.19 ± 0.18	42.5 ± 28.3	420.9 ± 123.5	801.3 ± 112.8
	+ 10^{-4} M 2,2'-dipyridyl	0.43 ± 0.15	10.6 ± 5.6	54.2 ± 22.9	55.0 ± 30.3
C4 eff. mol ($\times 10^{-2}$) per Mϕ[c]	Control	0.16	15.38	46.06	57.2
	+ 10^{-4} M 2,2'-dipyridyl	0.12	11.8	31.20	47.9
C2 eff. mol ($\times 10^{-2}$) per Mϕ[c]	Control	0.12	49.6	69.3	75.7
	+ 10^{-4} M 2,2'-dipyridyl	0.08	58.6	65.5	78.4

[a] Macrophages were cultured in M199 with or without addition of 10^{-4} M 2,2'-dipyridyl for 72 h. The complement components were measured in the supernatant by the hemolytic assay.
[b] Values represent means from 3 expt ± SD
[c] Values represent means from 2 expt

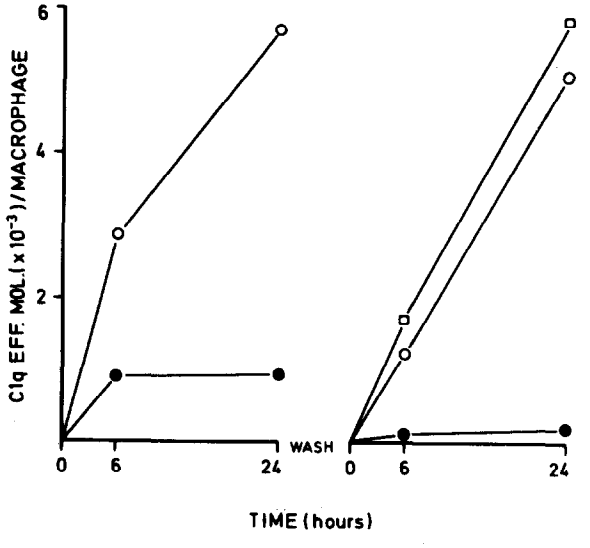

Fig.1. Reversible effect of 2,2'-dipyridyl on the release of hemolytically active C1q molecules. Macrophage monolayers were incubated in M199 with 10^{-4} M 2,2'-dipyridyl for 24 h (●——●) and after washing incubated again in M199 with (●——●) or without (□——□) 2,2'-dipyridyl. (○——○) controls.

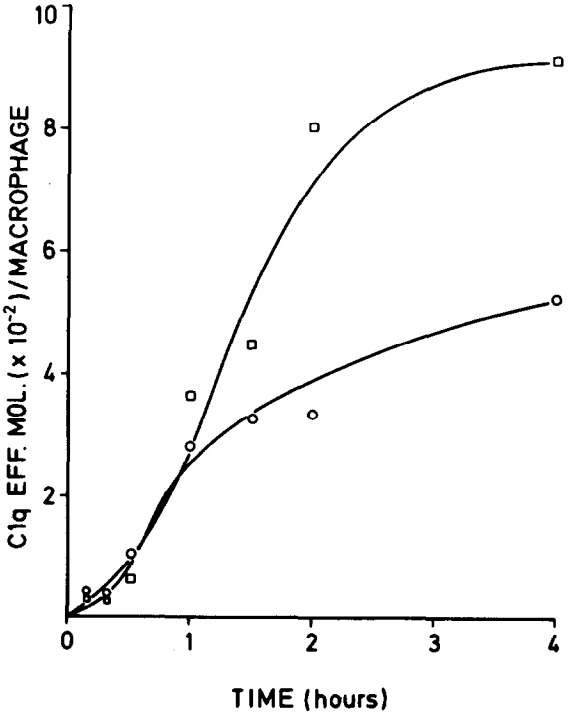

Fig.2. Release of hemolytically active C1q molecules after preincubation of macrophage monolayers in M199 with (□——□) or without (○——○) 10^{-4} M 2,2'-dipyridyl for 24 h.

tion of C1q remains almost constant. After washing and repeated incubation in 2,2′-dipyridyl only residual amounts of C1q molecules were detectable. When 2,2′-dipyridyl was removed its inhibitory effect on C1q secretion was fully reversible. During the first 6 h after removal of 2,2′-dipyridyl the increase in C1q concentration even exceeded the control values. To investigate the latter finding further monolayers were preincubated with 2,2′-dipyridyl for 24 h in the same way as outlined above. After washing and adding fresh M199 without inhibitor, samples were removed at short time intervals. Figure 2 demonstrates that after a lag of 30 min a higher rate of C1q secretion occurs from cells which had been preincubated with 2,2′-dipyridyl. After an incubation period of 2 h the rate of C1q secretion returned to that of the control value.

Additional experiments were then performed to test whether inactive C1q molecules were secreted when the monolayers were cultivated in the presence of 10^{-4} M 2,2′-dipyridyl for 24 h, 48 h and 72 h. Culture supernatants from monolayers were incubated with EA or EAC4 for 20 min at 30°C and after washing the erythrocytes they were reincubated with purified hemolytically active C1 or C1q molecules. It was found by the hemolytical assay that the binding of purified C1 or C1q molecules was uneffected by prior incubation of the EA or EAC4 with the culture supernatant.

4. Discussion

In the present study we investigated whether the inhibitory effect of 2,2′-dipyridyl on the posttranslational hydroxylation in collagen biosynthesis is also true for a similar step in the biosynthesis of the collagen like triple helical portions of C1q, a subunit of the first component of complement. The data presented here support our assumption. We found that 2,2′-dipyridyl at a concentration of 10^{-4} M drastically reduced secretion of hemolytically active C1q molecules into the supernatant. It has been demonstrated that 2,2′-dipyridyl up to 3×10^{-4} M does not inhibit protein synthesis in short term experiments [31]. In our experiments it affected neither cell viability nor production and secretion of C4 and C2 molecules. Therefore, we conclude that the effect of 2,2′-dipyridyl is restricted to the inhibition of hydroxylation of the proline and lysine residues in the collagen like region of the C1q molecule. In collagen biosynthesis it has been shown that hydroxyproline is crucial for triple helix conformation under physiological conditions and that the unhydroxylated procollagen molecules, which contain polypeptides with a random coil formation, are not secreted [32–35]. Thus, a 'pro-C1q molecule' containing only peptides with a random coil formation might not be secreted and would perhaps be accumulated intracellularly. This possibility seems to be supported by the finding that macrophages after preincubation with 2,2′-dipyridyl for 24 h followed by washing and cultivation in fresh M199, released more C1q molecules during the first hours than controls. This effect might be due to rapid hydroxylation and secretion of accumulated 'pro-C1q' molecules, provided that translational protein synthesis is the rate limiting step in the biosynthesis of C1q under our experimental conditions. However, considering that 2,2′-dipyridyl does not effect protein synthesis the number of C1q molecules which are released after removal of 2,2′-dipyridyl are relatively small. This suggests that other mechanisms are probably involved in the further metabolism of the unhydroxylated C1q molecule. Procollagen peptides of random coil conformation are readily digested by proteolytic enzymes and thus rapid degradation of unhydroxylated molecules probably might occur, both intra- and extracellularly, as suggested by studies of [25,34]. The fate of unhydroxylated C1q molecules might be a similar one. Other data indicate that when hydroxylation of protocollagen is blocked by 2,2′-dipyridyl in fibroblast cultures underhydroxylated procollagen is secreted at a low rate after an incubation period of 2 h [36]. In our experiments there were no hemolytically inactive C1q molecules present which via their intact globular portions could possibly have bound to EA or EAC4 and blocked the subsequent binding of hemolytically active C1 or C1q molecules. Two explanations may be considered for this finding, either unhydroxylated C1q molecules are not present in the supernatants in substantial amounts or binding of the globular portions is influenced by the triple helical structure of the collagenous regions of the molecule. Attempts to clarify these questions are in progress. The effect of 2,2′-dipyridyl on C1q production may provide the opportunity to obtain

more information on the different steps of C1q biosynthesis and perhaps on the mode of interaction of C1q with immunaggregates and the C1r and C1s subunit.

Acknowledgements

The authors wish to thank Professor Dr H. Isliker, Lausanne, for the gift of the RC1q-reagent. The expert technical assistence of Mrs D. Bauer, is gratefully acknowledged. M.L. was supported by grant of the Deutsche Forschungsgemeinschaft (SFB 107, A2 Mainz).

References

[1] Lepow, J. H., Naff, G. B., Todd, E., Pensky, J. and Hinz, C. F. (1963) J. Exp. Med. 117, 983–1008.
[2] Müller-Eberhard, H. J. and Kunkel, H. G. (1961) Proc. Soc. Exp. Biol. NY 106, 291–295.
[3] Augener, W., Grey, H. M., Cooper, N. R. and Müller-Eberhard, H. J. (1971) Immunochemistry 8, 1011–1020.
[4] Borsos, T., Rapp, H. J. and Walz, U. L. (1964) J. Immunol. 92, 108–112.
[5] Loos, M., Borsos, T. and Rapp, H. J. (1972) J. Immunol. 108, 683–688.
[6] Müller-Eberhard, H. J. (1968) Adv. Immunol. 8, 1–80.
[7] Calcott, M. A. and Müller-Eberhard, H. J. (1972) Biochemistry 11, 3443–3450.
[8] Yonemasu, K., Stroud, R. M., Niedermeier, W. and Butler, W. T. (1971) Biochem. Biophys. Res. Commun. 43, 1388–1394.
[9] Reid, K. B. M., Lowe, D. M. and Porter, R. R. (1972) Biochem. J. 130, 749–763.
[10] Reid, K. B. M. (1976) Biochem. J. 155, 5–17.
[11] Shelton, E., Yonemasu, K. and Stroud, R. M. (1972) Proc. Natl. Acad. Sci. USA 69, 65–68.
[12] Knobel, H. R., Villiger, W. and Isliker, H. (1975) Eur. J. Immunol. 5, 78–82.
[13] Reid, K. B. M. and Porter, R. R. (1976) Biochem. J. 155, 19–23.
[14] Brodsky-Doyle, B., Leonard, K. R. and Reid, K. B. (1976) Biochem. J. 159, 279–286.
[15] Knobel, H. R., Heusser, C., Rodrick, M. L. and Isliker, H. (1974) J. Immunol. 112, 2094–2101.
[16] Reid, K. B. R., Sim, R. B. and Faiers, A. P. (1977) Biochem. J. 161, 239–245.
[17] Stecher, V. J., Morse, J. H. and Thorbecke, G. J. (1967) Proc. Soc. Exp. Med. 124, 433–438.
[18] Lai A Fat, R. F. M. and Van Furth, R. (1975) Immunology 28, 359–368.
[19] Al-Adnani, M. S. and O'D Mc Gee, J. (1976) Nature 263, 145–146.
[20] Colten, H. R., Borsos, T. and Rapp, H. J. (1966) Proc. Natl. Acad. Sci. USA 56, 1158–1163.
[21] Colten, H. R., Gordon, J. M., Borsos, T. and Rapp, H. J. (1968) J. Exp. Med. 128, 595–604.
[22] Bing, D. H., Spurlock, S. E. and Bern, M. M. (1975) Clin. Immunol. Immunopathol. 4, 341–351.
[23] Reid, K. B. M. and Solomon, E. (1977) Biochem. J. 167, 647–660.
[24] Loos, M., Müller, W. and Storz, R. (1978) J. Immunol., abstr. in press.
[25] Prockop, D. J., Berg, R. A., Kivirikko, K. I. and Uitto, J. (1976) in: Biochemistry of collagen (Ramachandran, G. N. and Reddi, A. H. eds) pp. 163–273, Plenum Press, NY.
[26] Wellek, B., Hahn, H. and Opferkuch, W. (1975) Eur. J. Immunol. 5, 378–382.
[27] Rapp. H. J. and Borsos, T. (1970) Molecular Basis of Complement Action, Appleton-Century-Crofts, NY.
[28] Colten, H. R., Borsos, T. and Rapp, H. J. (1969) J. Immunol. 102, 1336.
[29] Heusser, Ch., Boesmann, M., Nordin, J. H. and Isliker, H. (1973) J. Immunol. 110, 820–828.
[30] Wyatt, H. V., Colten, H. R. and Borsos, T. (1972) J. Immunol. 108, 1609–1614.
[31] Uitto, J. and Prockop, D. J. (1974) Eur. J. Biochem. 43, 221–230.
[32] Juva, K., Prockop, D. J., Cooper, G. W. and Lash, J. (1966) Science 152, 92–94.
[33] Prockop, D. J. (1971) Fed. Proc. Fed. Am. Soc. Exp. Biol. 30, 984–990.
[34] Jimenez, S. A., Dehm, P., Olsen, B. R. and Prockop, D. J. (1973) J. Biol. Chem. 248, 720–729.
[35] Fessler, L. I. and Fessler, J. H. (1974) J. Biol. Chem. 249, 7637–7646.
[36] Ramaley, P. B., Jimenez, S. A. and Rosenbloom, J. (1973) FEBS Lett. 33, 187–191.