STUDIES OF SYPHILITIC ANTIBODIES*

IV. EVIDENCE OF REACTANT PARTNER COMMON FOR C-REACTIVE PROTEIN AND CERTAIN ANTI-LIPOIDAL ANTIBODIES

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Certain surface active substances produce a non-syphilitic flocculation reaction if cardiolipin is replaced by them in the VDRL test system (Tuomioja and Kajanne, 1956). The test was called the APC test because these surface active substances were products of the Atlas Powder Company (Wilmington, Delaware). The most active was Span 60, stated by the manufacturer to be mainly sorbitan monostearate. This APC test gave results parallel to the C-reactive protein (CRP) antiserum precipitation test (Tuomioja, Kajanne, Wager, and Hällström, 1956). The readings were as clearcut as those in the VDRL test for syphilis.

The exact relationship of CRP with the substances producing flocculation in the APC test remained in doubt and the present work was initiated to clarify this question. The role of CRP in the flocculation reaction was established, but it was proved that an antibody also combines with the APC antigen, and some of its properties are described.

Material and Methods

Sera Sera from patients with primary and secondary syphilis were taken from darkfield positive cases in which the date of infection was known. The majority of the late syphilitic sera were found among specimens sent to the State Serum Institute for routine treponemal tests. They all were positive in the TPI and FTA-Abs tests and had VDRL titres ranging from 2 to 128. They were supplemented by four sera with titres of 256 to 512 found among specimens sent for the standard tests for syphilis.

One serum (Case 1) with very high-titred biological false positive (BFP) sero-reactions for syphilis was obtained from Dr. J. Killander. This was the first case described with association between high Wassermann-reactivity and a monoclonal γ M component (Killander, Killander, Philipson, and Willén, 1967). A second serum (Case 2) was found in this laboratory. This had a γ M globulin content of about 1.5 g./100 ml. and an M-component, as shown by immunoelectrophoresis. The Wassermann reactivity was located entirely in the macroglobulin fraction, as evidenced by density gradient ultracentrifugation and by gel-filtration on Sephadex G-200. The other BFP sera were included in a previous study (Aho, 1968).

The sera from expectant mothers were sero-negative specimens sent for the routine syphilis tests. The CRP-containing sera were collected from among reactive routine specimens. The sera from military recruits were derived from a study dealing with rheumatoid factor and immuno-conglutinin responses following various vaccinations (Aho, Somer, and Salo, 1967).

Antisera The production of antisera against VDRL floccules has been described elsewhere (Aho, 1967; Aho, 1968). A similar immunization schedule was used to prepare antisera against APC floccules. About 40 ml. of pooled APC-positive serum with a titre of 32 was used as the starting material. The floccules were divided into two parts. Two rabbits received one half of the material after a thorough separation of the supernatant but without any washing. Two other rabbits received the remaining portion of the floccules washed four times with a large volume of buffered saline. The CRP antiserum was a commercial product of Schieffelin, New York.

Antigens Kolmer antigen and VDRL antigen were commercial products of Difco Laboratories (Detroit, Michigan) and of Burroughs Wellcome (London), respectively. Parallel testing was usually carried out with

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sitolipin antigen, in which the sitolipin is an analogue of cardiolipin prepared from wheat embryos (Uroma and Louhivuori, 1951). Lecithin for the sitolipin and APC antigens was prepared as described by Pangborn (1951).

Table I shows the composition of the antigens and gives dilutions used in the complement-fixation tests. Partial antigens (lecithin, lecithin and cholesterol, Span 60, sitolipin) were used in the concentrations in which the components were present in the complete antigens. The surveys of the occurrence of the APC antibodies and the testing of the fractions were performed with one antigen dilution. Testing of the rabbit antisera and the BFP sera was carried out with all the antigen dilutions shown in the Table. The results given refer to those obtained with the usual antigen dilution.

Serological Techniques The APC slide flocculation test was performed according to the instructions for the VDRL test in the Manual of Serologic Tests for Syphilis (USPHS, 1959). The complement-fixation tests were performed as described elsewhere (Aho, 1967). Titres are expressed as reciprocals of serum dilutions.

Fractionation Procedures Density gradient ultracentrifugations, gel filtrations on Sephadex G-200, and DEAE-cellulose column chromatography (step-wise elution system) were performed as described previously (Aho, 1967). Likewise, the measurement of human immunoglobulins in the fractions was described in that paper. In testing rabbit antisera, a rabbit anti-Salmonella DO serum was used as a marker for γ M globulin. Gel-filtration on Sephadex G-200 indicated that practically all the agglutinating antibody activity in this serum was localized in the first protein peak.

Results

To obtain information about the interaction of CRP with the APC antigen, increasing amounts of the latter were mixed with constant volumes of pooled CRP-containing serum. Buffered saline was then added to reach the same end volume in each tube. After separation of the resulting floccules, the clear supernatants were tested against specific anti-CRP serum by double diffusion on Ouchterlony plates. No precipitin line was seen with the supernatant corresponding to the largest amount of antigen (4 parts of antigen and 1 part of serum), suggesting that there was no CRP. A line of increasing

strength was visible when using supernatants corresponding to smaller amounts of antigen.

The floccules thoroughly separated from the supernatants were washed with a small amount of pH 7.2 buffered saline, and the supernatants were tested against the anti-CRP serum as above. A precipitin line was formed suggesting that CRP is easily eluted from the floccules.

Four rabbits were then immunized with APC floccules. Two of them (Series A) received floccules thoroughly separated from the serum but without any washing, whereas the other two rabbits (Series B) received them after washing four times with a large volume of buffered saline. After absorption of the Series A antisera with a $\frac{1}{8}$ volume of normal human serum they no longer reacted with it in the double-diffusion test, but they both still gave one strong line with CRP-containing serum. This line showed a reaction of identity with the line obtained with the anti-CRP serum.

The Series B antisera contained no detectable antibodies against CRP. In immunoelectrophoresis they both reacted with immunoglobulins. In addition, there was a faint line against albumin.

Thus the immunization experiments suggested that immunoglobulins from human serum combine with the APC antigen. A search was made for the occurrence of such antibodies by the complement-fixation technique.

Table II shows that about 20 per cent. of sera from patients with early infectious syphilis (primary

TABLE II
OCCURRENCE OF COMPLEMENT-FIXING ANTIBODIES
AGAINST APC ANTIGEN

Serum Category	No. of Sera	No. Positive	Titre	
			Range	Average*
Syphilis I-II	50	11	8-128	39
Early latent syphilis	10	3	16-128	51
Late syphilis	50	1	8	8
BFP-sera	15	6	32-4,000	322
Expectant mothers	100	9	16-128	30
CRP-positive sera	50	6	16-256	51
Military recruits	100	11	8-128	36

*Calculated as logarithmic mean of titres in cases with positive reactions

TABLE I
ANTIGENS USED IN COMPLEMENT-FIXATION TESTS

Antigen	Composition (per cent.)				Dilution	
	Lecithin	Cholesterol	Cardiolipin*	Span 60	Usual	Additional
Kolmer	0.05	0.60	0.03	—	1/150	1/37.5, 1/600
VDRL	0.20	0.90	0.03	—	1/150	1/37.5, 1/600
APC	0.20	0.90	—	0.50	1/150	1/37.5, 1/600

*In sitolipin antigen, cardiolipin is replaced by 0.03 per cent. sitolipin.

or secondary) were positive. No correlation was observed between the occurrence of the APC antibodies and the VDRL titres; positive results were already recorded in sera with a negative VDRL test. Only one serum from the patients with seronegative late syphilis was weakly reactive. Forty per cent. of positive results and the highest titres were recorded in sera which had given biological false positive reactions for syphilis.

Sera of expectant mothers, CRP-positive sera, and sera from healthy military recruits were positive in about 10 per cent. Serial specimens taken from the recruits before and after the routine vaccination programme were tested; in one instance a clear titre increase was noted, while in the other cases titres remained at the same level.

Ten sera from patients with complement-fixing antibodies against the APC antigen were fractionated with sucrose gradient ultracentrifugation. Three of them were from patients with secondary syphilis and four from BFP-reactors. The remaining three sera were negative in the syphilis tests but strongly positive in the APC flocculation test.

Table III illustrates the distribution of the serological activities in one serum of the last-mentioned group. The APC antibodies were located in the 19S fraction, whereas the flocculating activity as well as the CRP detected by the antiserum precipitation test were entirely in the 7S fraction. In all the other sera the complement-fixing APC antibodies were also entirely in the 19S fraction. In one BFP serum (Case 1) the APC flocculating activity was found in the macroglobulin fraction and not associated with CRP. In the three syphilitic sera the Wassermann-antibodies were present in both the 19S and 7S fractions.

TABLE III

DENSITY GRADIENT ULTRACENTRIFUGATION OF A SERUM CONTAINING CRP AND COMPLEMENT-FIXING APC ANTIBODIES

Fraction No.	γ M	γ G	CRP	APC	
				Flocc.	CF
1	<1	<1	—	<1	<2
2	1	<1	—	<1	4
3	8	1	—	<1	32
4	4	8	—	<1	16
5	1	32	+	1	2
6	<1	128	++	8	<2
7	<1	32	++	<1	<2
8	<1	<1	—	<1	<2
9	<1	<1	—	<1	<2

Fractions from two tubes were combined to obtain enough material for all tests

Table IV lists data on certain sera with BFP-reactions for syphilis. Noteworthy were two sera from patients with macroglobulinaemia in which the

pathological macroglobulin apparently was Wassermann-active. One of them was strongly positive with the APC antigen, whereas the other gave completely negative results. It is probable that the same antibodies in the former sera were responsible for the Wassermann-activity and the APC activity. The same may have been true for the antibodies in serum 3.

TABLE IV
REACTION PATTERNS OF SOME BFP SERA

Titre with Antigen		Case No.			
		1	2	3	4
Kolmer	CF	8,000	8,000	2,000	32
VDRL	F1	neg.	neg.	neg.	neg.
VDRL	CF	1,000	1,000	512	n.d.
Kahn	F1	2,000	2,000	512	4
APC	F1	1,000	neg.	8	n.d.
APC	CF	4,000	<8	1,000	256
Lecithin and cholesterol	CF	<8	<8	<8	<8
Span 60	CF	16	<8	<8	128

n.d. = not done.

All the serological activities in sera 1, 2, and 3 were in the 19S fraction. In serum 4 the Wassermann antibodies were in both the 19S and the 7S fraction and the APC antibodies in the 19S fraction only.

A prozone was frequently noted in the complement-fixation test with the APC antigen. This prozone phenomenon was observed in sera containing CRP as well as in sera that did not contain it. At least some normal human sera were found to be inhibitory in a system consisting of APC antigen and certain sera containing complement-fixing antibodies against it.

Twenty sera of various categories containing complement-fixing antibodies against the APC antigen were also tested against its components consisting of lecithin and cholesterol on the one hand and of Span 60 on the other. Negative or very weak reactions were obtained with the lecithin-cholesterol antigen, whereas some sera were definitely positive with the Span 60 antigen. Some other sera resulted in a partial inhibition of haemolysis or were completely negative.

One would expect that immunization with APC floccules would also give rise to antibody production against the APC antigen. The above four rabbit antisera were tested for such antibodies. For the sake of comparison, three antisera obtained by immunization with VDRL floccules were tested with the same systems.

As seen from Table V, each of the antisera contained complement-fixing antibodies against the APC and VDRL antigens. Titres obtained with the antigen used for immunization were somewhat higher than those obtained with the other antigen,

The antisera were not specific for cardiolipin in the VDRL antigen or Span 60 in the APC antigen, since they all fixed complement with their "control" antigen consisting of lecithin and cholesterol. The antisera against the VDRL antigen fixed complement with Span 60 alone. No reactions were noted with sitolipin alone.

TABLE V
ANTI-LIPOIDAL ANTIBODIES IN SERA OF RABBITS
IMMUNIZED WITH APC AND VDRL FLOCCULES

Serum*	Complement-Fixation Titre with Antigen				
	Kolmer	VDRL	APC	Lecithin and Cholesterol	Span 60
Series A/1 APC	128	32	512	64	128
Series A/2 APC	256	64	1,000	128	256
Series B/1 APC	8	16	128	32	64
Series B/2 APC	32	64	256	128	128
Case 5 VDRL	128	512	256	16	64
Case 6 VDRL	128	256	128	32	128
26 d VDRL	256	512	256	128	128

*Preimmunization specimens were negative

Flocculating antibodies in titres paralleling those obtained with the complement-fixation technique were detected when using the complete VDRL and APC antigens. Weak or no reactions were obtained with the "control" antigens.

All the antisera were fractionated by sucrose gradient ultracentrifugation. Antibodies were always detected in both the 19S and the 7S fraction; the former usually had somewhat higher titres. Two antisera were fractionated with the aid of DEAE-cellulose column chromatography. Antibody activity in them was present in two separate zones. The first zone contained the bulk of γ G globulin, and the antibodies in it apparently were of this type. The titres in the second zone paralleled those obtained with an indicator rabbit anti-Salmonella DO serum, and the antibodies apparently were of γ M globulin type.

Discussion

The experiments carried out in this work showed that CRP binds to APC floccules and that immunization of rabbits with such floccules provides an easy means to prepare antisera against CRP. However, it proved also that a human antibody combines with the APC antigen. No correlation was found between the occurrence of this APC antibody and the APC-flocculating substances. Antibodies were detected in about the same frequency in CRP-positive (and APC-flocculating) sera and in sera negative for CRP. The antibody

was found to be located in the 19S fraction, whereas the flocculating substances were usually in the 7S fraction, paralleling the occurrence of CRP as detected by the antiserum precipitation test.

The above observations suggest that the antibody only occasionally brings about flocculation. Since the absorbed antisera against APC floccules contained detectable antibodies only against CRP, it apparently is the only immunogenic component in CRP-containing sera participating in the flocculation reaction. This can be taken to mean that the APC flocculation test is a measure of CRP and can be used with certain precautions as a substitute for the antiserum precipitation test.

The human antibodies combining and fixing complement with the APC antigen presented certain puzzling features. Very high titres were observed in some high-titred BFP sera. The probable reason for this was that in these sera the same antibodies were responsible for the Wassermann activity and the APC complement-fixing as well as for the flocculating activity. With the exception of these few sera no correlation was observed between the occurrence of APC antibodies and Wassermann antibodies. This strongly suggests that the APC antibodies and the syphilitic antilipoidal antibodies were separate from each other.

The absence of complement-fixing APC antibody activity in the 7S fraction does not necessarily imply the absence in that fraction of antibodies with the property to combine with the antigen. The serological reactivity may be related to some surface property of the APC antigen. If the density of the antigenic determinants is small, it may not allow the attachment of two γ G globulin molecules close enough for the complement fixation to occur, whereas a single molecule of γ M globulin antibody is sufficient to bind complement (Borsos and Rapp, 1965).

The present experiments do not justify any far-reaching conclusions concerning the specificity of the APC antibodies. Some of the sera were observed to fix complement with Span 60 alone. According to the manufacturer's statement, Span 60 is a complicated mixture of fatty acid esters of sorbitol anhydrides, chiefly sorbitan monostearate. Sorbitol itself is a hexahydric alcohol with formula $C_6H_8(OH)_6$. It is produced from natural sugars by a hydrogenation process. Experiments carried out to determine the components responsible for the APC flocculation reaction (Tuomioja, Kajanne, and Junnila, 1961) suggested that the reactivity was not a property of a single chemical substance. Rather it seemed that compounds of different fatty acids and sorbitan were active provided that certain

structural conditions were fulfilled. No corresponding experiments were conducted to delineate the specificity of the APC antibodies.

In no instances were the human sera observed to give clear reactions with the lecithin-cholesterol part of the antigen or with lecithin alone. Among others, this was the situation with the macroglobulinaemia serum 1. However, in another laboratory this serum was found to fix complement with lecithin alone (Killander and others, 1967). This suggests that subtle physicochemical differences in lecithin might be responsible for the reactivity. The observation that another macroglobulinaemia serum 2 with very high BFP titres was negative in the APC complement-fixation test probably reflects differences in the serological specificity of Wassermann-active pathological macroglobulins.

The APC complement-fixation system with human sera was somewhat difficult to work with. In some instances a prozone was observed suggesting the presence of an inhibitor which was then diluted out in higher serum dilutions. Some non-reactive sera were inhibitory for the system. These observations bear a certain resemblance to the behaviour of rheumatoid factors with human γ -globulin (Grubb and Laurell, 1956; Fudenberg, 1967), where the prozone or even a complete inhibition is due to the competition by γ -globulin of an appropriate genetic type.

Antibodies with many of the properties of syphilitic anti-lipoidal antibodies can be induced in rabbits by immunization with alcoholic heart extracts (Sachs, Klopstock, and Weil, 1925) or with purified cardiolipin antigens (Fowler and Allen, 1962). The fractionation experiments carried out in the present work showed that antibody activity in such immune sera was present in both the 19S (γ M) and the 7S (γ G) fraction. Thus the sera resembled in this respect syphilitic sera (Aho, 1967) and differed from BFP sera, in which the serological activity is only occasionally found in the γ G globulin fraction (Aho, 1968). However, the antisera induced in rabbits were not specific for cardiolipin, since they reacted also with the "control" antigen consisting of lecithin and cholesterol alone. On the other hand, the absence of reactivity with cardiolipin alone does not imply that antibodies were not produced against it, since the syphilitic sera fail to react with it if lecithin and cholesterol are not present. It has already been pointed out by Sachs and others (1925) that rabbit immune sera differ from syphilitic sera in that they are specific for lecithin, although no further data were given by them.

The observation in the present work that anti-

bodies induced in rabbits by immunization with VDRL floccules reacted with Span 60 adds a further dimension in the complex interplay between Wassermann antibodies, APC antibodies and CRP.

Summary

C-reactive protein produces a flocculation reaction if cardiolipin is replaced by Span 60 in the VDRL test system. Immunization with such APC floccules provides an easy means of preparing antisera against CRP.

A human antibody also combined and fixed complement with the APC antigen. The antibody was detected in about the same frequency in sera from patients with syphilis, in CRP-containing sera, and in sera from healthy persons. It was found to be located in the γ M globulin fraction. The antibody-active fractions were usually negative in the flocculation system, whereas the flocculating activity paralleled the occurrence of CRP as detected by the antiserum test.

Immunization of rabbits with APC and VDRL floccules gave rise to antisera reactive in the usual syphilis tests. Antibody activity was present in both the γ M and γ G globulin fraction. The antisera differed from human syphilitic sera in that they showed reactivity with the lecithin-cholesterol part of the antigen. Antisera raised against VDRL floccules gave cross-reactions with Span 60 alone.

REFERENCES

- AHO, K. (1967). *Brit. J. vener. Dis.*, **43**, 259.
- (1968). *Ibid.*, **44**, 49.
- , SOMER, T., and SALO, O. P. (1967). *Proc. Soc. exp. Biol. (N.Y.)*, **124**, 229.
- BORSOS, T., and RAPP, H. J. (1965). *Science*, **150**, 505.
- FOWLER, E., and ALLEN, R. H. (1962). *J. Immunol.*, **88**, 591.
- FUDENBERG, H. H. (1967). *Clin. exp. Immunol.*, **2**, 1.
- GRUBB, R., and LAURELL, A.-B. (1956). *Acta path. microbiol. scand.*, **39**, 390.
- KILLANDER, A., KILLANDER, J., PHILIPSON, L., and WILLÉN, R. (1967). In "Gamma Globulins, Nobel Symposium 3", ed. J. Killander, p. 359. Almqvist and Wiksell, Stockholm.
- PANGBORN, M. C. (1951). *J. biol. Chem.*, **188**, 471.
- SACHS, H., KLOPSTOCK, A., and WEIL, A. J. (1925). *Dtsch. med. Wschr.*, **51**, 589.
- TUOMIOJA, M., and KAJANNE, P. (1956). *Ann. med. exp. Fenn.*, **34**, 209.
- , —, and JUNNILA, R. (1961). *Ibid.*, **39**, 29.
- , —, WAGER, O., and HÄLLSTRÖM, K. (1956). *Ibid.*, **34**, 211.
- UROMA, E., and LOUHIVUORI, A. (1951). *Ibid.*, **29**, 227.
- USPHS (1959). "Serologic Tests for Syphilis", US Communicable Disease Center, US Department of Health, Education, and Welfare. Public Health Service Publ. 411, Washington, D.C.

L'étude des anticorps syphilitiques**IV. La démonstration du partenaire réactif commun de la protéine C-réactive et de certains anticorps lipoides****RÉSUMÉ**

La protéine C-reactive produit une réaction de floculation si la cardiolipine est remplacée par le Span 60 dans le test VDRL. L'immunisation avec tels flocons APC procure un moyen facile de préparer l'antisérum contre le CRP.

Un anticorps humain a aussi combiné et fixé le complément avec l'antigène APC. L'anticorps a été discerné avec presque la même fréquence dans le sérum des patients atteints de syphilis, dans le sérum con-

tenant le CRP, et dans le sérum des personnes saines. On l'avait trouvé localisé dans la fraction de la globuline γ M. Les fractions d'anticorps actifs étaient généralement négatives dans le système de floculation, tandis que l'activité de floculation suit la présence du CRP comme il est indiqué par le test d'antisérum.

L'immunisation des lapins avec les flocons APC et VDRL avait donné lieu à des antisérums réactifs dans les tests usuels de la syphilis. L'activité des anticorps était présente dans les deux fractions des globulines γ M et γ G. Les antisérums différaient du sérum syphilitique humain en ce qu'il montraient une réactivité avec la partie lécithine-cholestérol de l'antigène. Les antisérums en contact avec les flocons VDRL donnaient des réactions croisées seulement avec le Span 60.