

STUDIES OF SYPHILITIC ANTIBODIES*†

I. ANTI-LIPOIDAL ANTIBODIES IN VARIOUS STAGES OF SYPHILIS

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There is good evidence to indicate that the syphilitic anti-lipoidal "reagins" are true antibodies. They appear in demonstrable amounts in the serum 4 to 6 weeks from the date of infection and at about the same time as the most sensitive treponemal test, the FTA-ABS test, becomes positive (Deacon, Lucas, and Price, 1966; Lassus, Mustakallio, Aho, and Putkonen, 1967). Uniformly high titres are recorded in patients with secondary syphilis, whereafter they usually start to decline. Antibodies with the serological properties of syphilitic anti-lipoidal antibodies have been induced in rabbits by immunization with alcoholic heart extracts (Sachs, Klopstock, and Weil, 1925) or with killed *Treponema pallidum* (Eagle and Fleischman, 1948). In fractionation experiments the anti-lipoidal antibodies have proved to be heterogeneous. They have been recovered in both 7S and 19S fractions obtained by ultracentrifugation (Davis, Moore, Kabat, and Harris, 1945) and in both γ G globulin- and γ M globulin-containing fractions obtained with the aid of column chromatography on DEAE-cellulose (Laurell and Malmqvist, 1961).

There is divergence in opinion whether these antibodies result from immunization against treponemal lipids or whether the stimulus is by autologous lipid constituents liberated from organs affected by the syphilitic infection. Immunization experiments with killed *T. pallidum* favour the first hypothesis. However, one wonders how to explain in these terms the occurrence of positive reactions in several other infectious diseases and in diseases of unknown aetiology such as systemic lupus erythematosus. It is also somewhat difficult to explain the reasons for the failure to induce anti-lipoidal antibodies by immunization with cultivable treponemes (Eagle and Fleischman, 1948) although these do contain the

lipid antigen (D'Alessandro and Dardanoni, 1953).

A series of experiments was designed to characterize the immunochemical and serological properties of various anti-lipoidal substances. The present paper deals with anti-lipoidal antibodies in different stages of syphilis. Evidence is sought for possible differences in the distribution between the early and late antibodies revealed by fractionation procedures and for differences in the serological properties of the antibodies in the fractions.

Material and Methods

Syphilitic Sera Three types of syphilitic sera were used:

(a) Sixteen from patients with primary and ten from patients with secondary syphilis.

(b) Sixteen from patients with latent or late symptomatic or congenital syphilis which were positive in the TPI and FTA-ABS test and had been selected to have VDRL titres corresponding to those seen in primary syphilis.

(c) Eight sera with very high titres from patients with late syphilis which were found within a 3-year period among samples sent for the standard serological tests for syphilis.

The VDRL titres ranged from 4 to 16 in the sera from patients with primary syphilis, from 32 to 128 in those from patients with secondary syphilis, and from 256 to 1024 in the high-titred sera from patients with late syphilis.

Antisera The production of specific anti- γ G and anti- γ M globulin sera has been described elsewhere (Aho, 1967). Samples of anti- γ A globulin sera were obtained from Prof. O. Mäkelä and Dr O. Wager. The anti-whole human serum was a commercial product from the Pasteur Institute, Paris.

Fractionation Procedures Density gradient ultracentrifugations were performed in a 10–40 per cent. sucrose gradient. The SW 50 L rotor and a Spinco Model L 50 ultracentrifuge were employed in a 16-hr run at 40,000 rpm. Nine successive 0.5 ml. fractions were collected by puncturing the tubes through the

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bottom in a fractionating apparatus (Beckman Instruments, Palo Alto, California). Gel filtrations were performed on 100×1.5 cm. column of Sephadex G-200 (Pharmacia AB, Uppsala, Sweden). The flow rate was about 1 ml./hr. Column effluents were collected usually in 1 ml. fractions and the protein concentration was registered at $254 \text{ m}\mu$ in a Uvicord absorptiometer (LKB-produkter AB, Stockholm, Sweden). DEAE-cellulose chromatography was carried out on columns of 30×1.5 cm. An initial buffer of 0.02 M phosphate, pH 7.5, was used to equilibrate column and sample. Absorbed proteins were removed either by gradient elution by increasing the phosphate molarity while maintaining the pH at 7.5, or by stepwise elution using increasing concentrations of sodium chloride in 0.02 M phosphate buffer, pH 7.2.

Tests for Anti-lipoidal Antibodies The VDRL slide flocculation test and the Kahn standard test were performed according to the "Manual of Serologic Tests for Syphilis" (1959). The complement-fixation tests were performed with one volume of a 2-fold dilution of the fraction under test and one volume of each of antigen, complement (2 full units), haemolysin, and 2 per cent. sheep red cells in a total volume of 0.5 ml. When possible, dilutions were made in bulk and 0.1 ml. volumes were distributed into the appropriate rows of tubes. Bacto Kolmer antigen (Difco Laboratories, Detroit, Michigan) or cholesterol-sensitized alcoholic heart extracts were used as antigens. A 1-hr fixation at 37°C . was used before addition of the sensitized cells. The titres are expressed as reciprocals of serum (or fraction) dilutions.

Tests for Treponemal Antibodies The TPI and FTA-ABS tests were performed according to standard techniques as described elsewhere (Salo, Valtonen, Jokipii, and Aho, 1967).

Measurement of Immunoglobulins The γG globulin was determined by measuring the inhibition of the Coombs reaction. The immunoglobulins γM and γA were determined by making 2-fold dilution series of the fractions and by testing the dilutions against specific anti- γM and anti- γA globulin sera on Ouchterlony plates.

Results

Ultracentrifugation Experiments

All the fifty syphilitic sera included in the study were fractionated by density gradient ultracentrifugation. The fractions were titrated by the Kolmer complement-fixation test. When the titres in the sera were low, the titrations were started from undiluted fractions. When the titres were higher, the titrations were started from a dilution 1:2, and the fractions were frequently tested with some other system as well. In no instances were the fractions anticomplementary and therefore unsuitable for complement-fixation tests. To obtain an estimate of the amounts of immunoglobulins in the fractions, they were always titrated for γG globulin and were usually tested also for γM globulin.

Three sera from patients with primary syphilis and one serum from a patient with late syphilis yielded only weakly active fractions; the strongest fraction had two reactive tubes (a titre of 2). This was considered insufficient for a picture of the distribution of the antibody activity in the various fractions, and these sera were excluded from the series. In all other sera there was at least one fraction with three reactive tubes (a titre of 4).

Fig. 1 illustrates different types of elution diagrams seen in the study. In Fig. 1a the antibodies were located solely in the 19S fraction (primary syphilis) and in Fig. 1b typically in both the 19S and the 7S fractions (early latent syphilis).

The distribution of the antibody activity in the whole series by the stage of syphilis is shown in Table I. There were three sera from patients with primary syphilis with detectable antibodies only in the 19S fraction, and in ten out of the thirteen sera of this group there were higher titres in the 19S fraction than in the 7S fraction. In patients with secondary syphilis the reverse was true: six of the

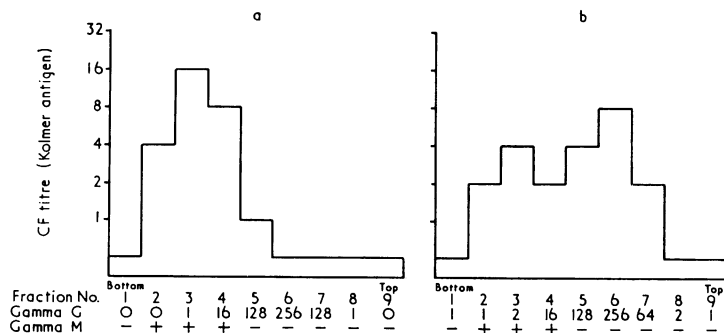


FIG. 1(a).—Ultracentrifugation of a serum from a patient with primary syphilis. Antibodies are present only in the 19S fraction.

FIG. 1(b).—Ultracentrifugation of a serum from a patient with early latent syphilis. Antibodies are present in both the 19S and 7S fractions.

ten sera had higher titres in the 7S fraction. A similar type of antibody distribution was seen in patients with early latent syphilis.

TABLE I
DISTRIBUTION OF ANTIBODY ACTIVITY INTO THE 19S AND 7S FRACTIONS

Syphilitic Serum	No. of Sera with Titres			Average Titre Difference 19S/7S (log ₂)
	19S > 7S	19S = 7S	19S < 7S	
Primary	10	0	3	+1.4
Secondary	2	2	6	-0.6
Early latent	0	1	5	-1.2
Late low-titred	5	1	3	+0.7
Late high-titred	8	0	0	+3.3

All the patients with late syphilis who were selected because of their high VDRL titres had more antibody activity in the 19S than in the 7S fraction. Marked variations were noted in the patients with late syphilis and with lower titres. In one patient (with congenital syphilis) antibodies were detected only in the 19S fraction. In five sera higher titres were seen in the 19S fraction, whereas in three the titres were higher in the 7S fraction.

The ultracentrifugal fractions of six sera from patients with secondary syphilis and four high-titred sera from patients with late syphilis were tested with the VDRL test. The results were compared with those obtained in the Kolmer complement-fixation test performed on the same fractions. The average titre values in the 19S and 7S fractions obtained with the two test systems are shown in Table II.

TABLE II
COMPARISON OF THE KOLMER AND VDRL TITRES IN THE 19S AND 7S FRACTIONS IN THE SERA OF PATIENTS WITH SECONDARY AND LATE SYPHILIS

Syphilitic Serum	Test System	Average log ₂ Titre		Titre Difference
		19S	7S	
Secondary	Kolmer	5.5	6.4	-0.9
	VDRL	3.0	2.7	+0.3
Late high-titred	Kolmer	9.0	5.5	+3.5
	VDRL	5.5	4.0	+1.5

It appears that in the high-titred sera the titre difference between the 19S and the 7S fractions was greater in the complement-fixation tests than in the flocculation tests. Thus the late 7S antibodies may be relatively poor complement-fixing antibodies (or relatively good flocculating antibodies) as compared with the 7S antibodies in secondary syphilis.

Other Fractionations

Five high-titred sera from patients with late syphilis and one from a patient with secondary syphilis were fractionated by gel filtration on Sephadex G-200. Fig. 2 (overleaf) shows two elution diagrams.

Fig. 2a (secondary syphilis) shows a clear-cut separation of the antibodies into two fractions. Five of the six sera showed this pattern. Fig. 2b (cardiovascular syphilis) shows a considerable amount of complement-fixing antibody activity in the fractions preceding the bulk of γ G globulin. These fractions did not contain measurable amounts of γ M globulin, but they did contain considerable amounts of γ A globulin.

DEAE-cellulose column chromatography was employed to complement the fractionation procedures based on differences in molecular weights. Four high-titred sera were fractionated by using a continuous gradient. Some anti-lipoidal antibody activity was always recovered in the first protein peak. The concentrated fractions corresponding to this peak were tested against whole human serum on Ouchterlony plates. Only one line was seen which gave a reaction of identity with the specific anti- γ G globulin serum. The antibodies were eluted in two separate zones. The fractions in the first antibody-containing zone always contained some γ G globulin and in the second zone there was γ M globulin. Attention was focused on the first zone. Three sera yielded only one distinct antibody peak in this region, and the titres in the fractions paralleled their γ G globulin concentration. In the fourth serum (Fig. 2b) there was a second peak of antibody activity in the fractions containing the bulk of γ A globulin. However, these also contained a considerable amount of γ G globulin, so that it was not possible to decide whether the antibody activity was related to γ A or γ G globulin.

Four sera from patients with lower antibody titres were fractionated with a step-wise elution system. Two were from early latent syphilis and two from late syphilis. In each instance, some antibody activity was found in the fall-through fraction consisting of γ G globulin.

Immunization Experiments

A rabbit was immunized with VDRL floccules prepared from the serum of a patient with secondary syphilis (12 intravenous injections over a 6 weeks' period). Immunoelectrophoresis carried out against an infectious mononucleosis serum revealed antibodies only against the immunoglobulins, but not against any other serum protein constituents.

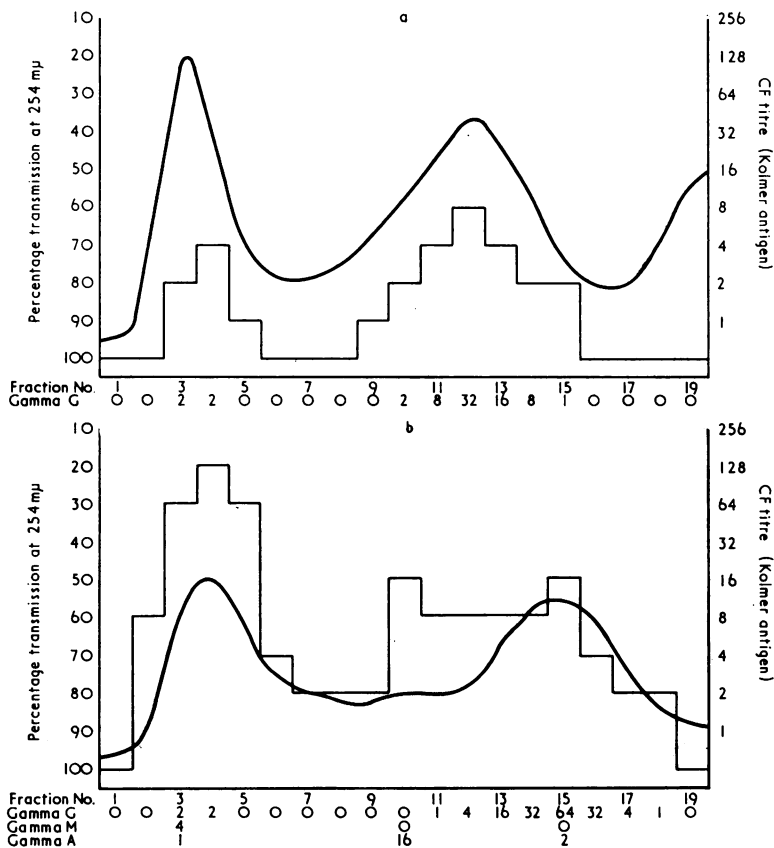


FIG. 2(a).—Gel filtration (Sephadex G-200) of a serum from a patient with secondary syphilis. Antibodies are present in the γ M globulin- and γ G globulin-containing fractions. Curve: Protein concentration in effluents.

FIG. 2(b).—Gel filtration (Sephadex G-200) of a high-titred serum from a patient with late syphilis. Antibodies are present also in the intermediate fractions between the peaks of γ M and γ G globulin. Curve: Protein concentration in effluents.

Discussion

The present experiments confirm the earlier ultracentrifugal findings of Davis and others (1945) that syphilitic anti-lipoidal antibodies can usually be recovered in both 7S and 19S fractions. However, in some sera from patients with primary syphilis, antibodies were detected in the 19S fraction only and the majority of these had higher antibody titres in the 19S than in the 7S fraction. In secondary syphilis the reverse seemed to be true, since the sera frequently contained more antibodies in the 7S fraction. Thus it is probable that the first antibodies produced during syphilitic infection belong to the 19S fraction, and that thereafter a gradual shift takes place to the 7S antibodies. However, the shift was never found to be complete. The patients with early latent syphilis had the same type of slight 7S antibody predominance as those with secondary syphilis. Sera from patients with late syphilis

frequently had more antibodies in the 19S fraction, and this was the situation in all the sera which were selected for the study because of their high titres.

It is difficult to explain this 19S antibody predominance in late syphilitic sera. It may be related to some difference in the immunogenic stimulus. In patients with primary and secondary syphilis, the number of treponemes is great, whereas in those with late syphilis they are few. It is possible that living treponemes or some treponemal material is necessary for continuing antibody production and that a small amount of immunogenic material favours the production of 19S antibodies. A second possibility is that the antibodies in patients with infectious syphilis are directed mainly against treponemal lipids, whereas in late syphilitic sera they may be directed mainly against altered tissue constituents, or it may be a question of altered (allergic) response.

It is probable that the syphilitic anti-lipoidal antibodies found in the 19S fraction are of γ M globulin variety. It is also probable that the antibodies found in the 7S fraction were mainly of γ G globulin variety. To confirm the presence of γ G globulin antibodies, fractionation experiments were conducted with the aid of DEAE-cellulose column chromatography. In each of the syphilitic sera selected for this purpose some antibodies were found in the first protein peak consisting of γ G globulin. The presence of syphilitic anti-lipoidal antibodies was also looked for in the γ A globulin fraction. However, it is not easy to detect small amounts of γ A globulin antibodies if the sera simultaneously contain both γ M and γ G globulin antibodies. Furthermore, the testing of the fractions was usually limited to complement-fixation because of its greater sensitivity as compared with flocculation. In one serum fractionated by gel filtration on Sephadex G-200, there was a considerable amount of antibody activity in the intermediate fractions between the peaks of γ M and γ G globulin. This antibody may have been of γ A globulin variety.

Summary

Fifty syphilitic sera were fractionated by ultracentrifugation. Some sera from patients with primary syphilis contained detectable antibodies in the 19S fraction only and the majority of them had higher titres in the 19S than in the 7S fraction. In contrast, sera from patients with secondary and early latent syphilis usually had slightly higher titres in the 7S fraction. Sera from patients with late syphilis frequently had again a predominance of 19S antibodies. The 7S antibodies in late syphilitic sera were relatively poor complement-fixing antibodies. Fractionation experiments with the aid of DEAE-cellulose chromatography indicated that the 7S antibodies were at least mainly of γ G globulin variety. Gel filtration on Sephadex G-200 revealed in one serum the presence of antibody activity in the fractions between the peaks of γ M and γ G globulin. Immunization of a rabbit with VDRL floccules gave rise to antibody production against immunoglobulins but not against other serum proteins.

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Les études des anticorps syphilitiques I. Les anticorps anti-lipides dans les différents stages de la syphilis

RÉSUMÉ

Cinquante sérums syphilitiques ont été fractionnés par l'ultra-centrifugation. Certains sérums des malades atteints de syphilis primaire contenaient des anticorps perceptibles dans la fraction 19S seulement et la majorité d'entre eux avait des titres plus élevés dans la fraction 19S que dans la fraction 7S. Par contraste le sérum des malades atteints de syphilis secondaire et de syphilis précoce latente avait généralement des titres légèrement plus élevés dans la fraction 7S. Le sérum des malades atteints de syphilis tertiaire avait aussi montré assez souvent une prédominance des anticorps 19S. Les anticorps 7S du sérum de la syphilis tertiaire étaient des anticorps relativement médiocres pour fixer le complément. Les expériences de fractionnement avec l'aide de la chromatographie (DEAE-cellulose) ont indiqué que les anticorps 7S étaient principalement de la variété de la globuline γ G. Un des sérums a révélé la présence de l'activité des anticorps dans les fractions entre les sommets de la globuline γ M et de la globuline γ G après filtration colloïdale sur Sephadex G-200. L'immunisation d'un lapin par des floccules VDRL avait donné lieu à la production d'anticorps contre les immunoglobulines mais non contre les autres protéines du sérum.