Section of Clinical Immunology & Allergy

President Deborah Doniach MD

Meeting June 8 1970

Recent Advances in the Immunology of Syphilis

Dr D J M Wright[1] and
Dr D Doniach
(Department of Venereology, Guy's Hospital, London SE1 and Department of Immunology, Middlesex Hospital, London W1)

The Significance of Cardiolipin Immunofluorescence (CLF)

Syphilis is associated with a variety of auto-antibodies (Table 1). It may be seen that the auto-antigens in these systems are chiefly phospholipids (Rapport & Graf 1969), whose presence as a constituent of *Treponema pallidum* (Vaczi *et al.* 1966) provokes the cross-reacting haptenic auto-antibody (Inoue & Nojima 1967).

In particular, mitochondrial antigenicity depends upon a reaction with a diphospholipid, cardiolipin (Faure & Coulon-Morelec 1963). This is present in all tissue cells in the inner membrane of mitochondria (Fleischer *et al.* 1967). Up till now, anticardiolipins have been demonstrated by reactions in flocculation (Kahn test), complement fixation (classical Wassermann reaction (WR)) or agglutination (VDRL) test, but not by immunofluorescence. We have recently described a new anti-cardiolipin antibody which may be detected by the indirect immunofluorescent technique on tissue substrates, renal distal tubules being the most suitable. We have termed this antibody cardiolipin F (CLF) (Wright *et al.* 1970). This is quite distinct from the fluorescent treponemal antibody test (FTA) which is a specific anti-treponemal antibody.

Since completing this work we have found that Kaplan *et al.* (1961) described a diffuse myocardial staining pattern in sera with a positive WR. We confirm that this is consistent with a pattern of CLF on frozen rat and human cardiac muscle. It is not surprising that these are good tissue substrates since beef heart extract was always the source for the biological cardiolipin antigen of the older Kahn and Price tests.

A hint that the antigen for CLF lies in the mitochondrium is that the fluorescent pattern is similar to that found with sera from patients with primary biliary cirrhosis (Walker *et al.* 1965) and other auto-immune disorders (Doniach, Walker, Roitt & Berg 1970) – the so-called 'M' fluorescence.

However, M fluorescence differs from CLF in that on several tissues the pattern is more granular. The WR is negative in these sera, since the M antigen is a lipoprotein (Berg *et al.* 1969). The appearance of CLF immunofluorescence is illustrated in Figs 1 and 2. The distribution of fluorescence in different organs tends to be similar with M and CLF antibodies. However, with rat liver sections, 30 syphilitic sera which contained CLF antibodies all gave positive reactions while only one out of 20 primary biliary cirrhotic

Table 1
Auto-antibodies in syphilis

Nature of antigen	*References*
Brain tissue (probably galactolipids)	Witebsky (1929), D'Alessandro *et al.* (1950), Dupouey *et al.* (1970)
Red blood cells (glycosphingolipids):	
P group P (Tja)	Donath & Landsteiner (1904), Levine *et al.* (1965),
H group	Stäps (1965)
Mitochondrial constituents (cardiolipins):	
WR, Kahn, VDRL	Wassermann *et al.* (1906), Kahn (1928), Pangborn (1942)
Cardiolipin F	Wright *et al.* (1970)
Immune globulins:	
Rheumatoid factor	Peltier & Christian (1959), Lassus (1969)

[1]In receipt of Governors' Scholarship, Guy's Hospital

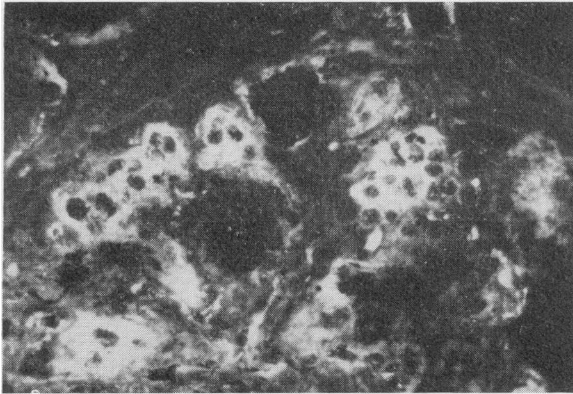

Fig 1 *Cardiolipin fluorescent antibody. Section of human thyrotoxic thyroid treated with serum from patient with secondary syphilis followed by anti-human gamma fluorescent conjugate. Normal thyroid cells are almost negative while those with eosinophilic metaplasia and hypertrophied mitochondria show a bright diffuse fluorescence.* $\times 225$

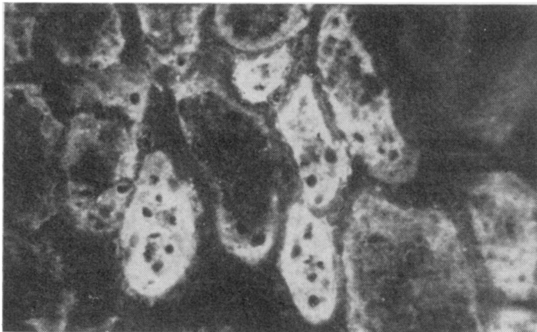

Fig 2 *Cardiolipin fluorescent antibody. Human kidney treated as in Fig 1. Distal tubules are brightly stained while proximal tubules are almost negative.* $\times 225$

sera produced a convincing degree of fluorescence on this organ.

Recently, mitochondrial fluorescent staining of low titre was found in some patients with chronic BFP reactions (Doniach, Delhanty, Linqvist & Catterall 1970). By definition, they are associated with a positive WR. Similarly, syphilitic fluorescence occurs with positive cardiolipin serology. However, these two fluorescence mitochondrial antibodies behave quite differently, in that cardiolipin F can be abolished by absorbing the serum with pure VDRL antigen, while the tissue fluorescence in both BFP reactors and in primary biliary cirrhosis remains unaffected. The other constituents of the VDRL, namely cholesterol and lecithin, do not affect either the titre or the brightness of the syphilitic tissue fluorescence.

When sub-cellular fractions from rat liver mitochondria were examined as a substrate using the indirect immunofluorescent technique, succinic dehydrogenase being the enzyme marker, we found that the organic solvents, ethanol, methanol, acetone and ether, did not affect the antigenicity of the mitochondria for CLF, whereas the use of lipase and in particular phospholipases destroyed the antigenic sites. This suggests that a diphospholipid of the cardiolipin type is the antigen (De Haas *et al.* 1966). β-glucuronidase had no effect on the fluorescent reaction, implying that mucopolysaccharides present in mitochondria play no part in the reaction.

Although it seems possible that the cardiolipin fluorescent antibody is provoked in infections by treponemes containing the diphospholipid antigen, specific absorption with *T. pallidum* could not be satisfactorily demonstrated as these organisms are contaminated with testicular tissue containing mitochondria. Therefore, controls using uninfected material likewise abolished the fluorescence.

We also tried unsuccessfully to absorb out the CLF with Reiter treponemal antigens and sorbent. Our failure is not surprising since it is doubtful if these treponemes provoke true WR antibodies (but see Tringali *et al.* 1968). This is presumably due to these treponemes not containing the specific cardiolipin.

Clinical Associations

The two types of cardiolipin antibodies found in syphilis behave differently in that the classical WR and VDRL reagins tend to persist into the chronic stages of the disease, while the CLF antibody is present mainly in the acute infectious phase. The maximal incidence and the highest titres of CLF were found in early active syphilis, especially in the secondary stage of the disease (Fig 3). The early latent cases gave similar titres, since many of the patients had hidden chancres.

Fig 3 *Distribution of cardiolipin fluorescence (CLF) antibody in untreated infectious syphilis*

After penicillin injections, the cardiolipin fluorescent reaction disappeared rapidly, usually within 3 months. The rate of disappearance of tissue fluorescence was related to the initial titre and became negative in all cases within a maximum of 14 weeks.

We also examined sera from a 6-month-old congenital syphilitic with hydrocephalus, hepatosplenomegaly, skin rash, osteitis and syphilitic nephritis. The cardiolipin fluorescence in this case was present in a titre of 1:10 a month after the commencement of treatment.

In general, there was a lack of correlation between the CLF antibody and VDRL. This was even more striking when sera from late symptomless syphilitics were examined. Only 8% had slight cardiolipin fluorescence whereas over 80% gave a positive WR and some had a VDRL titre up to 1:128 (Figs 4 & 5).

Twenty-four patients with tertiary syphilis have been tested and significant CLF titres were found in only 2 cases of gumma. Both were female, one having had her gumma treated 20 years previously, while the other maintained the same fluorescent titre for one year following treatment.

In view of the predominance of CLF antibodies in infectious syphilis, we thought that the antibody might represent 'syphilitic activity'. This impression was heightened by the results from testing syphilitic sera from 104 infected asymptomatic mice. It was found that the CLF was only weakly positive at a titre of 1:5 in 3 animals, despite the fact that they all had significantly positive FTA (absorbed) tests. It is of interest that the classical reagin tests are also rarely positive in mice infected with syphilis (Ovcinnikov 1955). However, when we followed up patients undergoing treatment with the ESR (Westergren) and fluorescence tests, in early syphilis both returned to normal, whereas in late syphilis both the VDRL and the tissue fluorescence tests remained static, while the ESR fell.

When the class of immunoglobulin in CLF was examined it was found that, in primary syphilis, IgM antibodies were detected more often than IgG. This was most evident in a group of 8 cases diagnosed by direct isolation of treponemes before the appearance of positive serological tests. Weakly positive tissue fluorescence could be detected with anti-IgM in 6 instances, while none reacted with anti-IgG or polyvalent anti-Ig. Of 5 primary syphilitics with positive serological tests, 4 reacted with anti-IgG in titres of 1:1 to 1:5 while all 5 gave titres of 1:5 to 1:20 with anti-IgM. In secondary and early latent cases, cardiolipin F titres were roughly equal with both conjugates. It is also of interest that several cases of infectious relapse were first detected by using the CLF with anti-IgM conjugate.

CLF Fluorescence in Other Diseases

Cardiolipin fluorescence is not absolutely confined to syphilis but may occur in low titres, up to 1:40, in other treponemal diseases. For instance, in experimental infections in rabbits and monkeys, significant CLF antibodies were found with *T. cuniculi*, *T. pertenue* and *T. Fribourg-Blanc*, as well as *T. pallidum*. This type of fluorescence has also recently been found in high titres (1:300) in two rare cases, one with malignant reticulosis, the other with an obscure clotting diathesis; in

Fig 4 *Distribution of cardiolipin fluorescence (CLF) and VDRL titres in patients with untreated syphilis*

Fig 5 *Distribution of cardiolipin fluorescence (CLF) and VDRL titres in patients with treated syphilis*

both patients the VDRL titre was of the order of 1:4,000. These cases with exceptionally high VDRL agglutination represent a rare form of BFP reaction and it appears that the CLF antibodies are not always present. We had an opportunity of retesting a case reported by Wilkinson (1954). While a high VDRL titre had persisted for 18 years, we were unable to demonstrate any cardiolipin fluorescence, but there were antinuclear antibodies to a titre of 1:2,000. The more usual patients with chronic BFP reactions suffer from collagen disorders and tend to have rather low VDRL results. In the cases in which mitochondrial fluorescence was present, it could not be absorbed out with cardiolipin.

The cardiolipin fluorescence test may be clinically useful in deciding if a latent syphilitic should be regarded as having early (infectious) or late latent (non-infectious) syphilis. This may be important in tracing sexual contacts. The test can also be helpful in the diagnosis of early seronegative primary syphilis and likewise infectious relapsing cases. The rapidity of fall of the CLF titre may give an indication of the efficacy of treatment.

REFERENCES
Berg P A, Doniach D & Roitt I M
(1969) *Klin. Wschr.* 47, 1297
D'Alessandro G, Oddo F G & Dardanoni L
(1950) *J. vener. Dis. Inform.* 31, 314
De Haas G H, Bonsen P P M & Van Deenen L L M
(1966) *Biochim. biophys. Acta (Amst.)* 116, 114
Donath J & Landsteiner K
(1904) *Munch. med. Wschr.* 51, 1590
Doniach D, Delhanty J, Lindqvist H J & Catterall R D
(1970) *Clin. exp. Immunol.* 6, 871
Doniach D, Walker J G, Roitt I M & Berg P A
(1970) *New Engl. J. Med.* 282, 86
Dupouey P, Coulon-Morelec M J & Marechal J
(1970) *C. R. Acad. Sci. (Paris)* 270, 1541
Faure M & Coulon-Morelec M J
(1963) *Ann. Inst. Pasteur* 104, 246
Fleischer S, Rouser G, Fleischer B, Casu A & Kritchevsky G
(1967) *J. Lipid Res.* 8, 170
Inoue K & Nojima S
(1967) *Biochim. biophys. Acta (Amst.)* 144, 409
Kahn R L
(1928) The Kahn Test: A Practical Guide. Baltimore
(1950) Serology with Lipid Antigen. London
Kaplan M H, Meyeserian M & Kushner I
(1961) *J. exp. Med.* 113, 17
Lassus A (1969) *Int. Arch. Allergy* 36, 515
Levine P, Celano M F & Falkowski F
(1965) *Ann. N. Y. Acad. Sci.* 124, 456
Ovcinnikov N M (1955) Experimental Syphilis. Moscow
Pangborn M C (1942) *J. biol. Chem.* 143, 247
Peltier A & Christian C L (1959) *Arthr. and Rheum.* 2, 1
Rapport M M & Graf L (1969) *Progr. Allergy* 13, 274
Stäps R (1965) *Derm. Wschr.* 151, 52
Tringali C, Zaffiro P & Buttitta C
(1968) *WHO Document WHO/VDT/RES* 68, 151
Vaczi L, Kirley K & Rethy A
(1966) *WHO Document WHO/VDT/RES* 66, 93
Walker J G, Doniach D, Roitt I M & Sherlock S
(1965) *Lancet* i, 827
Wassermann A, Neisser A & Bruck C
(1906) *Dtsch. med. Wschr.* 32, 745
Wilkinson A E (1954) *Brit. J. vener. Dis.* 30, 38
Witebsky E Z (1929) *Immun. Forsch. exp. Ther.* 62, 35
Wright D J M, Doniach D, Lessof M H, Grimble A S,
Turk J L & Catterall R D (1970) *Lancet* i, 740

Dr A E Wilkinson and Miss C C Wiseman
(Venereal Diseases Reference Laboratory (PHLS), The London Hospital, Ashfield Street, London E1)

The Role of Sorbent in the Absorbed Fluorescent Treponemal Antibody (FTA-ABS) Test

During infection with syphilis, two types of antibody detectable by indirect immunofluorescence tests with treponemes are produced. One is specific for *Treponema pallidum* and the other pathogenic treponemes; the other is a group-reactive antibody which reacts with *T. pallidum* and with a wide variety of cultivable and commensal treponemes because of shared antigens. The group-reactive antibody is also found in most normal, non-syphilitic sera at a low titre, probably being produced in response to the normal flora of commensal treponemes. In the fluorescent treponemal antibody (FTA-200) test described by Deacon *et al.* (1960), sera were tested at a dilution of 1:200 to get above the normal threshold of group antibody. This test had a good specificity but was relatively insensitive and has been largely replaced by the absorbed fluorescent treponemal antibody (FTA-ABS) test described by Hunter *et al.* (1964). In this, sera are tested at a dilution of 1:5 after absorption of group antibody. Reports on the performance and scope of the test have been reviewed by Hunter *et al.* (1968). Deacon *et al.* (1966), evaluating the test on 2,252 defined sera in parallel with the treponemal immobilization (TPI) test, considered it to be more sensitive than and as specific as the TPI test.

Originally, ultrasonically disintegrated Reiter treponemes were used to remove group antibody. These were later replaced by a heated and concentrated culture filtrate of Reiter treponemes, and this reagent (sorbent) is now generally used. It was thought to owe its activity to the presence of antigenic material set free from the treponemes, but Cannefax *et al.* (1968) reported that the uninoculated medium in which the treponemes were grown would also block the union of group antibody in normal serum with *T. pallidum*. This was confirmed by Wilkinson & Ferguson (1968) and by Rathlev (1968) and led to a re-examination of the means by which sorbent exerts its effect.

Methods

The technique of the FTA-ABS test and of the standardization of reagents for use in it were those described in the Manual of Tests for Syphilis (US Dept of Health, Education and Welfare 1969). All reagents were prepared in the laboratory. Reiter treponemes for preparation or sorbent and ultrasonicate were grown in Rajkovic's (1966) medium. Sonicates were standardized on the lines laid down for sorbent.