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DEPARTMENT OF THE ARMY
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IMMUNOFLUORESCENCE IN VENEREOLOGY

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There are already numerous and regularly specified applications for immunofluorescence in bacteriological and serological diagnosis.

In venereology, this technique can currently be applied for the detection of gonococcus, either on exudate smears or on cultures that are less than 24 hours old. According to Danielsson, a very sensitive and very specific diagnosis can thus be obtained which is superior to the classic methods of direct examination and culture.

We hope that the technique will also help to facilitate the diagnosis of viral urethritis.

The most important, widespread and best studied use of the technique is essentially in the serological diagnosis of treponemosis.

Our understanding of syphilis has been greatly changed in the last ten years thanks to the modern specific techniques of serological diagnosis. At the head of these is the test for treponema immobilization, both because of its priority and its incomparable specificity. Immunofluorescence has confirmed the value of this and has enabled us to acquire more precise data.

The clinical manifestations of syphilis are so varied and extend over such a protracted period interspersed with long totally quiescent phases that this disease has gradually been separated from other infectious diseases and made into a specialty, the study and knowledge of which must be restricted to specialists in the field. This attitude was reinforced by the special morphological aspect of the pathogen and the originality of the serological techniques for its detection.

However, we are gradually rediscovering what should never have been forgotten, i.e. that treponemosis is an infectious disease among the other infectious diseases and that it is probably the most complete and complex of all of them. Our modern techniques for the bacteriological and serological study of syphilis have enhanced not only our understanding of this disease but have repercussions throughout the field of immunology.

The cardiolidipic reactions of flocculation and hemolysis should be regarded solely as last resorts. They may retain some of their usefulness in current practice, but the modern specific techniques showed up too many of their deficiencies in terms of sensitivity and specificity for them to continue to serve as criteria of infection or recovery from treponemosis.

Because of its absolute specificity, the test for treponema immobilization remains the undisputed reference reaction. Its sensitivity has been greatly increased by the addition of lysozyme to the remaining milieu. However, there are some inconveniences:

The relatively delayed appearance during the primary syphilis stage of immobilizing antibodies, retardation of reacting substances and particularly the fluorescent antibodies;

The delicate technique characterized by an elevated cost price which is poorly suited for systematic detection;

The lack of standardization which necessitates comparison of the results from one technician to the other;

Finally, a manner of expressing the results, which was poorly selected initially, yields a false impression of quantitative value in the simple qualitative test that is generally performed. The level of 100% generally considered as reflecting strongly positive results, is obtained with very moderate serum antibody levels. The intensity of the positive response can be determined only by quantitative titration on progressive serum dilutions.

Technical Review of Immunofluorescence

The method was recommended by Marrack in 1934, taken up again by Coons in 1942 and applied to the diagnosis of syphilis by Dealon. It was introduced in France by Borel and Durel. This author together with Niel studied more than 18,000 serum or LCR samples comparatively in the TIT and in cardiolidipic reactions.

Let us review the principle and outlines of the reaction termed indirect. If treponemas are fixed on a slide and covered with serum containing treponemic antibodies, these will be absorbed by the corresponding antigen molecules. But these antibodies are globulins which also have the

specificity of the animal species from which they are derived. Hence, if the slide is covered again with serum from another animal species immunized against these globulins, there will be another antibody fixation. Two successive antigen-antibody complexes are thus formed. If the globulins from the second complex were previously treated with fluorescent dye, microscopic examination with an adapted light source (an ultraviolet ray in practice) will reveal fluorescent treponemas if the reaction is positive. However, there will not be any fluorescence if the serum did not contain specific antibodies when the reaction was first performed.

Although this reaction is very attractive in terms of its principle and technical execution, it, like the TIT, is also characterized by drawbacks. It requires reagents of a very high quality. The treponema suspension of the Nichols strain, which is the same as that used in the immobilization test, must be concentrated, pure and fresh. The human anti-globulin immune rabbit serum must have a very high titer. The globulins must be purified and conjugated with fluorescein isothiocyanate according to a very precise procedure. If the conditions are not fulfilled and observed, the essential aspects of the reaction are lost. Unfortunately, these conditions are not always fulfilled and many of the studies published on this topic are inadequate and run the risk of jeopardizing the reputation and future of the reaction. Nevertheless, these technical requirements concern only the reagent manufacturers. It is likely that there will be some agreement on these requirements and that the WHO will take an active interest in the matter.

Nonetheless, by comparison with the TIT, immunofluorescence presents one shortcoming. It does not have the absolute specificity. The antigenic structures of the microbes and treponemas are so complex, and the reaction is so sensitive that if the test sera or the fluorescent immunosera are not adequately diluted, unspecific treponema antigen-antibody complexes are demonstrated, which are probably common to large groups of microbes. Hence, only a very limited area of nonspecificity is acceptable from the start.

In practice, this area of unreliability is of negligible importance because the specific reactional zone is much greater. Still this is a theoretic deficiency which is bothersome and provides grist for the opposing factions.

The technique has remarkable advantages for the practicing biologist who has or will soon have flawless reagents at his disposal.

The method is extremely simple and rapid and is suitable for systematic detection in current practice. It lends itself very well to quantitative titration and the reproducibility is entirely satisfactory. The results should be expressed only by quantitative values which represent the extreme dilution of the test serum that still yields a positive reaction. Cross notations indicating the intensity of fluorescence of the

treponemas placed in contact with a single dilution of the serum are as inadequate as they are deceptive. Since we know that the nonspecificity area extends from the pure serum to a dilution of 1/150, we must also realize that the area of positivity extends from 1/150 to 1/100,000.

With regard to both immunofluorescence and the TIT, we must say that the biologist and clinical practitioner who use these reactions only as qualitative techniques for purposes other than detection are robbing them of their essential utility.

The extremely broad scale of titers shows that immunofluorescence is very sensitive and that it indicates the intensity of treponemic multiplication, i.e. the degree of evolution of the infectious disease. However, it has an inherent and extremely valuable feature which is the early stage at which it yields a positive reaction. It reveals the first perceptible humoral modification during the development of syphilis and this makes it an indispensable diagnostic aid. We shall take it up again.

Before examining the results obtained in the various stages of the disease, we should like to state that, despite their very different technical execution, these two reactions, TIT and immunofluorescence, have two essential characteristics in common:

Both use the specific antigen of syphilis, the pathogenic *Treponema pallidum*.

Both are microscopic reactions, i.e. they detect the formation of the antigen-antibody complex directly at its source. Let us bear in mind that the antigen-antibody complex is a molecular phenomenon and that there is a greater chance for its sensitive and specific observation if it is seen closer to this stage. Macroscopic visualization of the reactions of course necessitates the phenomenon to reach a more elevated sensitivity. Moreover, the reactions may be sensitized by artifices which impair their specificity.

Development of Antibodies in the Course of an Untreated *Treponema* Infection

We can now coherently follow the organic modifications brought about by treponemic infection. During the incubation period, i.e. the interval when the pathogen adapts to the host, there is already microbial multiplication and the start of extension. As a matter of fact, when the primary cutaneous lesion appears, the treponemas are already locally widespread and virulent. The visible ulceration following microbial development reflects a conflict the exact nature of which we do not know. The treponemas destroyed by the organism liberate their antigenic molecules which precipitate the formation of corresponding specific antibodies at the level of the "immunologically competent" lymphoid cells. It is apparently the antigen corresponding to the fluorescent antibody which is most reactive in this stage. We have to date observed more than one hundred untreated chancres. The fluorescent antibodies generally appear as of the second

or third day, which clearly shows that the immunological process has started before the appearance of the clinical lesion. On the eighth day, the titers are already 900 or above, while the cardiolidipidic reactions are still negative and doubtful and the TIT with lysozyme just begins to show positive traces. We recently had occasion to follow the development of the disease very closely in a subject who was suspected to have been contaminated on a certain date. While the patient was totally negative on the ninth day after contamination, immunofluorescence was at 100 the third day and at 300 on the sixth without any clinical lesion. On the 20th day, when the immunofluorescence titer was 450, there appeared a small ulceration in which microscopic examination demonstrated the presence of treponemas.

After the completion of the primary period and during the florid secondary period, all the reactions are obviously very positive, showing high titers which increase constantly. At this stage, the specific tests are not of any great diagnostic importance. Nevertheless, it is necessary to know the exact titers before or at the start of therapy because they constitute a standard of reference for the effectiveness of the treatment and the evolution of the treponemosis.

Hence, it is a strict rule for the clinical practitioner that even if the clinical or bacteriological diagnosis is a formality and there is every reason to suppose that the reactions will be negative, they are indispensable because they serve as a prognostic element and therapeutic guide.

The fluorescent antibodies precede the immobilizing ones during the entire primary and secondary period. The gap between the titers of the two reactions is an excellent indication of the proximity of contamination. Titers are between 12,000 and 36,000 for IF at the end of the eruptive period, and between 500 and 1000 for TIT.

Latent syphilis. We then see the spontaneous disappearance of the secondary lesions, in the same way that the chancre subsided alone and the treponemosis enters its phase of clinical latency. The immunofluorescence tests stop rising and slowly drop, while the immobilizing antibodies continue to increase for some time before they, too, reach a ceiling and slowly decrease.

This latent period is amazingly quiet and stable in the vast majority of the cases, and the subject may live and die without anyone suspecting that he has the microbial infection.

Let us consider the biological mechanism of this equilibrium. We have observed that biological defenses appear from the onset of treponemic multiplication. It is very likely that the tissue defenses which we cannot detect are also organized and are partially responsible for the spontaneous subsidence of the chancre.

The antibodies revealed by immunofluorescence are not treponemicidal in vitro, nor do they appear to be in vivo, and the real humoral defense is organized solely with the immobilizing antibodies. Prior to their appearance, the treponemas find an excellent culture medium in the lymph and extracellular fluid and develop almost freely. They diffuse throughout the organism after crossing the ganglionic barrier. We must be aware of this general dissemination period. It is not only the mucosa and skin which are affected. Despite the moderate nature of the clinical manifestations, careful examination sometimes reveals subicterus, moderate albuminuria, a meningeal reaction and arthralgia, which reflect this diffusion.

Latent Treponemic Forms

At this stage, the immobilizing antibodies increase and their action stops treponemic multiplication. We now see a very remarkable metabolic alteration of the treponemas. While the majority is killed and eliminated, a certain number of pathogens adapts and resists. These are the latent treponemic forms with retarded processes. They lose most of their pathogenic and extending power, as though in a state of "hibernation", and along with this lose their sensitivity to antibodies and therapeutic agents.

We can assume the existence of these latent forms on the basis of clinical determination of delayed treponemic flare-ups and the persistence of elevated specific antibody titers in treated or untreated subjects who were contaminated a long time ago.

We obtained a preliminary in vitro test of this by demonstrating that, if the survival conditions of the treponemas are modified by an inadequate medium, traces of oxygen or an unfavorable temperature, the surviving treponemas will show remarkable resistance to doses of antibodies or penicillin which are normally treponemicidal.

Bacteriological proof was obtained by Collart et al. These investigators found morphologically indisputable treponemas in the ganglia of humans or animals with syphilis that had been treated for years with large doses of bismuth or penicillin.

We must therefore be aware of the imperative necessity for rapid action when the first therapy is administered. Syphilis should be considered among the medical emergencies. If therapy is started before the appearance of the resistant treponemic forms, clinical and serological recovery is assured. If this is not the case, the inflammatory lesions will be healed but the latent infection will persist and the serological future of the subject will be characterized by a perpetually positive reaction.

Delayed Clinical Manifestations

The specific serological techniques have made it possible to dis-

tinguish between two fundamentally different types of delayed clinical manifestations; the treponemic recurrences and the cicatricial or tissue sensitization factors. This distinction is essential because it serves as a guide for therapeutic management.

Local Treponemic Recurrences

These are actually very rare and have become almost exceptions in our country. They are localized almost exclusively in the neuraxis. They are reflected biologically by extremely high antibody titers. These are the highest ever encountered, reaching 5 to 20,000 in the TIT and 30 to 100,000 in the IF. We shall take up subsequently the value of examining the cerebrospinal fluid in these cases. These cases are easily explained biologically. The treponemic antibodies require the action of complement, which is normally present in very small amounts in human serum. It may be entirely absent in the localized foci where the virulent treponemas may multiply, but from which they cannot diffuse freely. The antigenic attraction is therefore considerable and the secreting cells of the antibodies, which have been sensitized for years, react violently, which is the reason for the extremely high titers of specific antibodies. It is an irrefutable law for us that there can be no treponemic recurrence with low antibody titers.

Delayed Cicatricial Manifestations

This topic represents one of the strongest disagreements between the clinical practitioner and the biologist. Patients contaminated a long time ago often exhibit minor visceral disorders termed fixed tabes, aortitis, keratitis or arreflexia, etc., and we are asked for confirmation of treponemic development. However, the antibody titers seen in these patients are generally moderate and do not differ from those seen in the course of latent treponemosis. Or they may even be very low, i.e. they fluctuate between 10 and 500 for the TIT and between 450 and 4000 for immunofluorescence.

In our opinion, these delayed manifestations are not due to treponemic development but to cicatricial processes in certain visceral foci, which have been organized during the primary-secondary florid period. We are beginning to recognize secondary mechanisms which appear in the wake of various acute diseases, and which are characterized by the formation of auto-antigen-auto-antibody complexes. In the cicatricial foci, the neoformation proteins act like foreign proteins and, if they are mobilized, precipitate the secretion of antibodies which will create a pathologic conflict. We must bear in mind that the action of an antibody is not necessarily beneficial. In all the infectious diseases, we still do not know what elements are due to direct microbial aggression and what elements are the result of secondary reactions caused by cellular wastes produced by the pathogen or inflammatory reaction. The future will

clarify this difficult problem. However, the current investigation methods for these complexes are too rudimentary.

It is useless to mention the inadequacy of the cardiolipidic reactions in the stages of serologically asymptomatic syphilis and in the delayed manifestations. The reactions are generally either negative or dissociated or fluctuating and yield very low titers. However, they may at times also be excessively positive and associated with very moderate specific antibody titers. In this case, they probably reflect undetectable tissue sensitization. Despite efforts to discover common antigenic structures between the treponema and cardiolipine, we must admit that it is much more reasonable to consider extract of beef heart as an antigen that is closer to tissue structures than to the microbial ones.

Development of Antibodies in the Course of Treated Syphilis

While the effect of treatment, which is now reduced to penicillin with fewer contraindications, is always spectacular on the clinical lesions, this is not the case in terms of the evolution of antibodies according to the date of onset.

So long as the latent forms have not developed, complete serological recovery may be expected. But if these forms have become established in their ganglionic or visceral locations, negativization can no longer be expected. The increase of the titers is always checked and this aspect of "photography" of the initial titers which is found almost systematically unchanged for years and years is very striking. We have used the TIT for eight years and immunofluorescence for three in following several hundred patients with latent treponemosis, where the titers are amazingly stable despite the most intensive therapy.

Nevertheless, during latent recurrences and cases of latent treponemosis with high titers, we can and must expect to obtain at least a slight drop in titers, making them recede to the levels that are normal for fixed treponemosis, i. e. below [numbers illegible] for the TIT and 8000 for the IF.

Just as it is a matter of great delicacy to establish a precise margin between the titers for development and complete latency, it is equally difficult to fix, on the other pole, the borderline with the residual antibodies deriving solely from the humoral balance.

The problem is not of great practical importance. The clinical practitioner must know that there is little chance for achieving negative findings in these subjects, but he must also know that treponemic flares-ups do not occur in patients who are supervised and who receive brief cycles of penicillin at long intervals.

Congenital Syphilis

Our knowledge of congenital syphilis has benefitted remarkably from modern specific techniques. The condition breaks down into two fundamentally different factors.

The fetus is either not contaminated in utero by the maternal treponemas. In this case, at birth his serum will contain the same antibody levels, transmitted passively, as the maternal serum. They will disappear entirely within three to six months without any treatment.

Or the fetus is contaminated in utero or during the delivery by the maternal treponemas. The infant's treponemosis will then take on very different elements which are governed by the virulence of the maternal treponemas.

Immune systems do not appear in the infant before the sixth month. It is therefore understandable that, since he has no defense against the treponemic infection, the evolution of the condition depends on the state of virulence or latency of the treponemas, i.e. on the phase of the maternal syphilis. If the mother was contaminated shortly prior to the birth without yet having developed antibodies, the treponemosis in the infant will be severe and fatal if the diagnosis is not established rapidly. On the other hand, if the mother was contaminated at the start of her pregnancy, the infant's treponemas will already be in a latent nonvirulent form and the maternal antibodies, which are transmitted passively, will assure an interval of protection until the child develops its own protective antibodies.

There are of course many intermediate states between these two extreme forms. The study of hereditary syphilis confirms very well the existence of these two treponemic forms, the virulent and the latent.

Reinfections

Modern techniques also permit us to acquire more specific knowledge in the area of reinfection. Antibodies classically assure protection against recontamination. Nevertheless, we observed three indisputable cases of reinfection in subjects who still showed positive results in the TIT and IF. Two of these had rather elevated readings at 300 and 1350. It should be noted that in these three cases the new chancre did not exhibit any special characteristics.

Even if the subject was previously entirely negative, the re-elevation of specific antibodies is extremely rapid and intense in all cases of recontamination. This is the classic fluid response.

Specific Antibodies in the Cerebrospinal Fluid

Detection yields very useful information. It is generally considered that the antibodies contained in the cerebrospinal fluid come from the serum by way of a break in the meningeal barrier and merely reflect the alteration of this membrane. We think this explanation is wrong for two reasons:

Antibodies are gamma globulins. They are therefore the most voluminous of all the serum protein molecules. Since there can be no question of elective permeability of the meninges, antibody levels in the CSF would e.g. reach ten times those in the serum and would have to be accompanied by albuminorachia close to 10 g/l. This is obviously absurd. On the other hand, in certain very rare but indisputable cases of neuraxial treponemosis, the levels of immobilizing or fluorescent antibodies in the cerebrospinal fluid are extremely high, close to those in the serum. In one case, we even found them to be higher. We see this as proof of the presence of cells which secrete antibodies in the interior of the neuraxis and we believe that the confirmation of specific antibodies in the cerebrospinal fluid indicates the presence of local foci of virulent or latent treponemosis.

Let us say further that it is impossible to see positive reactions in the cerebrospinal fluid and negative ones in the serum and that the detection of local antibodies should be limited to the search for cerebral complications from treponemosis that has been diagnosed.

Conclusions

Thanks to the introduction of modern specific techniques, our knowledge of syphilis has made great progress during the last ten years.

Progress in the bacteriological and immunological understanding of the development of treponemosis has enabled us to make it the pilot microbial infection in the study of all infectious processes.

Progress in the discrimination of clinical manifestations has made it possible to differentiate those which are directly due to treponemosis and those caused by secondary tissue processes where the treponema is merely an irritative factor.

Progress in therapy has made it possible to act more rapidly and effectively, and on the other hand, to limit treatment that is often useless during the latent phases of the disease.