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Mycobacteria and sarcoidosis

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Introductory article

Growth of acid fast L forms from the blood of patients with sarcoidosis

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Background. *Acid fast cell wall deficient forms (CWDF) of bacteria have been grown from blood, bronchial washings, and ocular anterior chamber fluid from patients with sarcoidosis. A monoclonal antibody raised against Mycobacterium tuberculosis whole cell antigen (H37RV) was used to characterise further CWDF grown from the blood of patients with sarcoidosis.* ***Methods.*** *Blood from 20 patients with active sarcoidosis and from 20 controls was cultured using methods favourable for the growth of CWDF. Isolates were further characterised by indirect fluorescent antibody analysis using a monoclonal antibody highly reactive with M tuberculosis.* ***Results.*** *CWDF were grown from the blood of 19 of 20 subjects with sarcoidosis. All isolates stained positively with the monoclonal antibody and with a modified Kinyoun stain. No organisms were grown from the blood of controls.* ***Conclusions.*** *These data demonstrate that CWDF can be grown from the blood of nearly all patients with active sarcoidosis. The results confirm that the organisms are mycobacterial in origin and are similar, if not identical, to M tuberculosis. Their role in the pathogenesis of sarcoidosis is unknown.* (*Thorax* 1996;**51**:530–3)

Sarcoidosis is a multisystem granulomatous disorder of unknown aetiology. As sarcoidosis commonly affects the lung and regional thoracic lymph nodes, it has been postulated that a possible aetiological agent may enter the body through the inhaled route. Since its original description,[1] many agents have been suggested as a cause for this disease but no firm consensus exists about the specific initiating antigen (box).

Possible agents in the pathogenesis of sarcoidosis

Mycobacterial infections
- *Mycobacterium tuberculosis*
- Non-tuberculous mycobacteria
- Phage transformed mycobacteria
- Cell wall deficient mycobacteria
- Viral and mycobacterial infections

Other infections
- Viruses
- Mycoplasma
- *Nocardia*
- *Corynebacterium* spp

Inorganic antigens
- Clay soil
- Talc
- Oxalosis
- Beryllium

Organic antigens
- Pine tree pollen
- Immune complexes

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These infectious agents are known to be capable of stimulating the formation of granulomas and are particulates which can be inhaled and ingested by macrophages. Some of these agents persist in tissue as a result of their resistance to intracellular bactericidal mechanisms or resistance to degradation because of their physical characteristics. Although the most extensive research has focused on an infectious aetiology and, in particular, on mycobacterial infection, other possible antigens have been investigated. It is possible that there is more than one causal agent involved and that other factors such as genetic influences may also modulate the immune response in the pathogenesis of sarcoidosis. This is supported by the high incidence of sarcoidosis in siblings when compared with the population frequency of the disease (reviewed by Brennan *et al*[2]). Studies of MHC loci have suggested genetic associations but the results have not been concordant, possibly due to ethnic differences, differences in disease type, and the technology used.[3–6]

Possible non-infective agents

Pine pollen was considered as a possible aetiological agent because of an apparent relationship between the incidence of sarcoidosis and the distribution of pine forests in south-eastern USA. Pollen grains are acid fast and can induce non-caseating granuloma in guinea pigs.[7] However, pine pollen has been discounted as further epidemiological studies did not confirm these initial observations.[8] Exposure to clay soil,[9] talc,[10] and secondary oxalosis[11] have also been postulated as possible aetiological factors. Beryllium[12] and zirconium[13] produce granulomas that are indistinguishable from sarcoid-type epithelioid granulomas in sensitised individuals but do not produce positive delayed-type hypersensitivity reactions in the skin of patients with sarcoidosis.

Evidence for an infective/transmissible agent

In support of the possibility of a transmissible agent there has been a case report of pulmonary sarcoidosis developing in the recipient of a cardiac transplant from a donor later found to have sarcoidosis at post-mortem examination.[14] There is a further report where sarcoidosis may have been transmitted through a bone marrow transplant.[15] Animal models have established that homogenates of human sarcoid tissue can induce characteristic granulomatous reactions.[16 17] These are discussed in greater detail later in this review.

Non-mycobacterial infective agents

There have been sporadic reports of viral infections in relation to sarcoidosis.[18–20] There are frequently high titres of antibodies to a variety of viruses in patients with sarcoidosis, but these findings may merely reflect B cell hyperresponsiveness with increased immunoglobulin synthesis.[21] It has also been suggested that mycobacterial and viral infections may interact and produce sarcoidosis as a result of a virally induced defective T cell response to mycobacterial infection.

Uesaka *et al*[22] described the isolation of 17 organisms from tissue samples from 36 patients with sarcoidosis and discovered that some of the organisms cultured from lymph nodes had *Nocardia*-like properties. There have been several case reports of *Nocardia asteroides* infection in cases with end stage sarcoidosis but these are likely to be an unrelated late complication.[23] Acid fast coccobacillary bacteria have been identified in tissue from cases of sarcoidosis and it has been suggested that corynebacteria may be a causal agent. Non-diphtheria corynebacteria may produce a clinical syndrome resembling sarcoidosis with lymphadenitis, pneumonitis, and skin lesions[24 25] but there have been no studies providing further evidence to implicate this organism.

Mycoplasma like organisms (MLO) are obligate intracellular cell wall deficient bacteria and are a common cause of transmissible plant diseases. Recently these organisms have been detected by electron microscopy within leucocytes in sterile aqueous and vitreous fluid from patients with uveitis secondary to sarcoidosis.[26] MLO have also been described as inducing pulmonary syndromes similar to sarcoidosis,[27] and injection of MLO from patients with uveitis into murine eyelids can produce uveitis and systemic granulomatous reactions.[28] More recently, MLO have been identified in leucocytes and endothelial cells adjacent to granulomas within transbronchial biopsy specimens from patients with sarcoidosis.[28]

Although these infective agents have been suggested as being the cause of sarcoidosis, most studies of a possible causal organism have focused on mycobacteria.

Mycobacteria

Almenoff *et al* in the introductory article[29] have recently characterised acid fast cell wall deficient forms grown in the blood of patients with active sarcoidosis and have demonstrated that these are of mycobacterial origin using an antibody raised against *Mycobacterium tuberculosis* whole cell antigen. These findings have again raised the possible link between mycobacteria and sarcoidosis. It has been postulated for some time that mycobacterial infection may play a part in the pathogenesis of sarcoidosis and these findings add to previous data implicating this. Pinner in 1938[30] proposed that sarcoidosis should, in fact, be called "non-caseating tuberculosis" because of the similarities in histological appearance.

RATIONALE FOR A MYCOBACTERIAL PATHOGENESIS

Depending on host factors, patients present clinically with a variety of responses to mycobacterial infection ranging from the pattern seen in immunocompromised subjects where there are large numbers of organisms but little evidence of an immunological response to subjects with no signs or symptoms of disease who, on the basis of their characteristic radiographs, skin reactivity and other immunological measures, have clearly been infected in the past. This group are thought to have small numbers of viable mycobacteria persisting in their tissues but these are not detectable by conventional means. It has been suggested that sarcoidosis may be a clinical manifestation of this group of patients with mycobacterial infection who have a strong granulomatous response associated with good elimination of mycobacteria.[31] As a result of their efficiency in removal of the mycobacteria, the detection of the organisms is difficult if not impossible.

Clinically, there are instances when tuberculosis is diagnosed on the basis of typical radiographic and clinical presentation without bacteriological confirmation. The diagnosis of tuberculosis may also occur when tissue reveals caseating granulomas (this having also been described in sarcoidosis), also in the absence of positive bacteriology. Typical cases of sarcoidosis may be treated for tuberculosis following the discovery of acid fast organisms that are later found to be culture negative or that are only positive in one out of numerous samples.[32 33]

There are case reports of tuberculosis preceding or following sarcoidosis and even occurring concurrently.[34–37] Although patients with sarcoidosis often have depressed type IV delayed hypersensitivity responses to recall antigens, skin testing as a discriminator for disease can be misleading as there are patients with sarcoidosis who respond to tuberculin.[34] Furthermore, there are reports of bacteriologically proven tuberculosis unresponsive to antituberculous treatment despite good in vitro sensitivities to antituberculous drugs that subsequently showed improvement following corticosteroids.[34]

Several mechanisms by which undetected mycobacteria may produce the clinical and histological changes of sarcoidosis have been put forward. Following the discovery that a large number of patients with either tuberculosis or sarcoidosis had mycobacteriophage infection, Mankiewicz[38] suggested that tuberculosis infected patients normally produce mycobacteriophage neutralising antibodies and that patients incapable of producing these antibodies – that is, patients with sarcoidosis – may develop non-caseating granulomas. Although there were some further experimental data from the same group in support of this hypothesis, other investigators were not able to demonstrate an identifiable deficiency in neutralising antibody production to mycobacteriophages. Burnet[39] suggested that mycobacteria may be present in a protoplast or cell wall deficient L form persisting as an intracellular organism in mesenchymal cells which may be present in the epithelioid cells of non-caseating granulomas. Hanngren reiterated the concept of interaction of virus and mycobacteria by suggesting that concurrent viral infection depressed T cell function and therefore altered the immune response to mycobacteria.[40]

The T lymphocyte may play a central role in the initial immune response to a presumptive antigenic stimulus in sarcoidosis. T lymphocytes are capable of producing the characteristic granulomatous changes seen in sarcoidosis by elaboration of various immune mediators.[41–43] T lymphocytes bearing $\gamma\delta$ receptors have also been found to be increased in the peripheral blood of patients with sarcoidosis.[44] These T lymphocytes appear to proliferate in the presence of mycobacterial antigen and, in particular, show an expansion of the $V\delta2+$ subsets[45] in both patients with tuberculosis and sarcoidosis and may therefore reflect undetected infection in sarcoidosis.

EXPERIMENTAL ANIMAL MODELS

An infective or transmissible cause for sarcoidosis has been supported by animal experiments where injection of sarcoid lymph node material into footpads of mice resulted in formation of granulomas when biopsy samples were taken 6–8 months later. Notably, acid fast organisms were also found in some of these tissues.[17] The same response and detection of acid fast bacilli could also be produced by injecting pooled homogenates or supernatants of mouse granulomatous tissue into further mice. These preparations had been filtered through a 0.2 µm filter therefore implying that the "transmissible" agent had to be the size of a virus or protoplast and notably could be inactivated by irradiation or autoclaving. Mycobacteria having the characteristics of *Mycobacterium tuberculosis* were also grown in Löwenstein-Jensen medium from some of these homogenates. Other animal studies have confirmed the production of slow developing granulomas after the injection of sarcoid homogenates.[46] However, there still remains some uncertainty about these experiments implicating mycobacterial infection as other investigators have not been able to reproduce them.[16]

ANTIMYCOBACTERIAL ANTIBODY STUDIES

A number of investigators have compared the level of antimycobacterial antibodies in patients with sarcoidosis, mycobacterial infection, and control subjects[47 48] but these investigations have led to differing results.[49 50]

MOLECULAR STUDIES

Several studies investigating the presence of mycobacterial DNA employing molecular biological techniques have been carried out in sarcoidosis. A review of the techniques and various molecular studies have been comprehensively described.[31]

In a study using liquid in situ hybridisation Mitchell *et al*[51] extracted RNA from five normal subjects and five patients with sarcoidosis. Using a DNA probe specific for ribosomal RNA of *Mycobacterium tuberculosis* they demonstrated a significantly higher expression of mycobacterial nucleic acid in splenic tissue of subjects with sarcoidosis than normal controls. Bocart *et al*[52] used the polymerase chain reaction (PCR) to detect DNA encoding for the 65 kD mycobacterial antigen and detected this in three out of 14 biopsy samples. Popper *et al*[53] also found similar results in a group of sarcoidosis patients with two out of 15 cases demonstrating positive signals (compared with all 24 tuberculosis subjects). Thakker *et al*[54] reported a single positive result from lymph node biopsy specimens but the authors suspected that this was due to contamination.

Higher detection rates have been found in two other studies. Saboor *et al*,[55] in a study of 22 subjects with sarcoidosis, using PCR, found expression of IS6110 (an insertion element specific for *M tuberculosis*) in bronchoalveolar lavage fluid from half of their subjects but also demonstrated a high false positive rate in their normal control subjects and therefore raised the possibility that some of the positive results may be due to contamination. Using a different primer they also showed evidence for the presence of non-tuberculous mycobacteria in 20% of these samples. Fidler *et al*,[56] this time examining tissue from 16 subjects with sarcoidosis, 16 control subjects (squamous cell carcinoma, Hodgkin's disease), and four lung samples from tuberculosis subjects, were able to demonstrate *M tuberculosis* DNA in seven of the 16 sarcoidosis samples but also in one of the 16 matched control group. Notably, only two of the four positive control samples were positive. They based a positive result on the demonstration of amplification of both IS6110 and the 65 kD gene material. Detection of the 65 kD antigen gene is less sensitive than that of IS6110 and hence this study may potentially be underestimating the presence of *M tuberculosis* material.[31]

Although in general these studies using sensitive molecular tools have shown some evidence for the presence of mycobacteria in sarcoidosis, two studies have not demonstrated any mycobacterial nucleic acid in sarcoid tissue. Ghossein *et al*,[57] using a target sequence for the 65 kD antigen, did not detect any positive signal in fixed tissues from 10 patients with sarcoidosis and 10 control subjects although they were able to demonstrate positive signals from six patients with tuberculosis. Similarly, Gerdes *et al*[58] found no evidence for a target sequence not specific for *M tuberculosis* (16S rRNA) in 14 samples although all of their control group of six tuberculosis subjects were positive.

Specific studies have also attempted to address directly the issue of whether these mycobacteria are atypical[59 60] but these have not been positive. It is notable, however, that the study by Saboor *et al*[55] detected that 20% of their sample may have represented atypical mycobacteria by using a separate non-specific primer.

Introductory article

In the introductory article Almenoff *et al*[29] have cultured acid fast cell wall deficient forms (CWDF) or "L forms" from blood of patients with active sarcoidosis and have characterised these as being likely to be *Mycobacterium tuberculosis* in origin by using a monoclonal antibody against whole cell antigen ($H_{37}RV$).

"L form" is a term used to describe spheroblasts (atypical morphological forms derived from classical bacteria that have lost their rigidity due to cell wall deficiency) that are capable of giving rise to typical "L colonies" with a "fried egg" appearance on agar.[61] These CWDF may have the capacity to persist for longer as a possible consequence of loss of membrane receptors for phagocytosis. There is no definite evidence that these L forms are pathogenic but they may revert back to the parent form at a slow rate and therefore maintain disease at a subclinical level.[62] An additional factor favouring the role of CWDF in sarcoidosis is that they are harder to detect by conventional culture and are also capable of producing filterable sized organisms. CWDF[63] have been described in tissue from subjects with sarcoidosis for some time[64–66] and have been thought most likely to be mycobacterial in nature. In addition, these CWDF have been cultured from blood,[67] skin,[60] bronchial washings, and ocular anterior chamber fluid from patients with sarcoidosis.[68] Previous experimental studies of

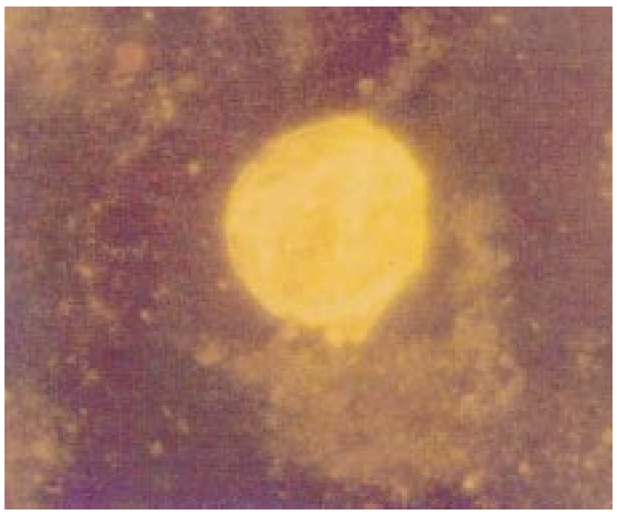

Figure 1 Photomicrograph showing cell wall deficient forms (CDWF) grown from the blood of a patient with active sarcoidosis using the Mycobacterium tuberculosis $H_{37}RV$ monoclonal antibody. A large yellow fluorescing L body is seen in the centre, and small yellow fluorescing microcolonies (×400) are seen elsewhere.[29]

intraperitoneal injections of CWDF, grown from blood of patients with sarcoidosis, demonstrated production of granulomatous lesions in lung, liver, kidney, spleen, and eyes in mice, gerbils, and guinea pigs.[69] More recently a study by Alavi *et al*[70] has characterised these CWDF in sarcoidosis tissues as being mainly *M tuberculosis* in origin by the use of specific antibodies.

Almenoff *et al*[29] studied 20 subjects with clinical evidence of sarcoidosis whose sputum was negative for acid fast bacilli on smear and culture. All these subjects had pulmonary disease but three also had skin disease, one joint disease, and one had neurological involvement. Seven were receiving systemic corticosteroids. Twenty healthy control subjects were also studied and none had a history of either HIV or tuberculosis.

Cultures of peripheral blood from all subjects were examined by microscopy using a modified Kinyoun stain by blinded investigators. Cell wall deficient bacteria were identified in 19 out of the 20 sarcoidosis subjects whereas none were identified in the control subjects. The authors then went on to characterise these CWDF by the use of a mouse monoclonal antibody developed against *M tuberculosis* $H_{37}RV$ whole cell antigen (fig 1). Previous experiments had shown that this antibody was relatively specific for $H_{37}RV$, BCG, and $H_{37}RA$ antigens with little binding to atypical mycobacteria and no binding to other Gram positive or negative bacteria. By the use of a second fluorescein isothiocyanate-conjugated goat anti-mouse antibody they identified positive binding of $H_{37}RV$ by fluorescence microscopy. All the identified CWDF appeared to stain positively with the antibody and were therefore felt to be mycobacterial in origin and most likely to be *M tuberculosis*. The authors point out that further DNA analysis of these L forms may allow further characterisation and differentiation of *M tuberculosis* and other atypical mycobacteria.

FACTORS AGAINST A MYCOBACTERIAL AETIOLOGY

Although there appears to be some evidence for the presence of mycobacteria in patients with sarcoidosis, this does not necessarily imply a causal pathogenetic relationship and therefore these findings should still be interpreted with caution pending further confirmatory studies. Currently the most positive molecular studies have only detected mycobacterial DNA in 50% of cases, raising the possibility of some other uncharacterised agent being involved. However, it is notable that in the introductory article Almenoff *et al*[29] were able to culture mycobacterial L forms from 19 of their study population of 20. A possible confounding factor in these studies is the lack of control subjects for the effect of corticosteroids. It may be argued that corticosteroids are merely allowing increased proliferation of mycobacteria in these subjects and that the increased detection of mycobacteria or L forms is a "bystander" effect.

The treatment of sarcoidosis with corticosteroids has not resulted in an increase in mycobacterial infection. Furthermore, previous studies examining the effect of antituberculous therapy in sarcoidosis have not shown any efficacy, although the role of mycobacterial infection may be through an initiation of an "overexuberant" immune process and may not be required to continue this process. There has not been any consistent epidemiological evidence for an increase in sarcoidosis in areas with an increased prevalence of tuberculosis.[34 71] Additionally, BCG vaccination has not been shown to reduce the incidence of sarcoidosis.[72]

Conclusion

The aetiology of sarcoidosis still remains undetermined. However, given the recent molecular studies and the findings of the introductory article, the evidence for a possible role of mycobacteria in the pathogenesis of this disease is still emerging and warrants further evaluation. In the introductory article Almenoff *et al* add support for the role of mycobacteria in the pathogenesis of sarcoidosis. The studies to date have been variable in their findings and more extensive controlled studies are required with standardisation of the molecular tech-

LEARNING POINTS

- **The aetiology of sarcoidosis is still uncertain.**
- **There is accumulating evidence for the role of mycobacteria in patients with sarcoidosis.**
- **The finding of cell wall deficient forms goes some way to explaining previous conflicting data on the role of mycobacteria in sarcoidosis.**
- **Use of molecular biological techniques may be helpful in further characterising whether these organisms are atypical or tuberculous mycobacteria.**
- **An appreciation of the epidemiological and genetic basis of the susceptibility to cell wall deficient forms (CWDF) would support their role in the aetiology of sarcoidosis.**

niques employed. The precise mechanism by which cell wall deficient mycobacterial forms initiate a granulomatous response in susceptible individuals also still awaits clarification. This hypothesis would be advanced by evaluations of genetic susceptibility, an understanding of how CWDF initiate the T cell mediated immune response (believed to be the cornerstone of sarcoidosis), and DNA analysis of isolated CWDF.

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