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C-reactive protein and apoB containing lipoproteins are associated with *Mycobacterium leprae* in lesions of human leprosy

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SUMMARY

Skin biopsies from patients with leprosy across the spectrum from tuberculoid (TT) to lepromatous (LL), including histoid lepromas and erythema nodosum leprosum (ENL) reactions, were stained immunohistochemically for the presence of C-reactive protein (CRP) and the apolipoprotein, apoB. *Mycobacterium leprae* bacillary material comprising cell walls, cytoplasmic and soluble components was present with increasing abundance towards the lepromatous end of the spectrum and always stained positively with anti-CRP. *M. leprae* from armadillos did not stain with anti-human CRP indicating that the staining of *M. leprae* in human tissues was not due to a cross-reaction between anti-CRP and the organism itself. When CRP was present in large amounts apoB was also demonstrated in the same distribution. CRP was detected on bacilli and their degradation products within the cytoplasm of macrophages even in the absence of a raised serum CRP level in some ENL patients and also in two cases of advanced resolving lepromas. These findings demonstrate remarkable persistence of CRP in association with *M. leprae in vivo*, and raise intriguing questions about the possible role of CRP in relation to the handling of leprosy bacilli.

Keywords leprosy C-reactive protein apoB lipoprotein

INTRODUCTION

Leprosy is a chronic bacterial infection in which the low cytotoxicity of *Mycobacterium leprae* may lead to a clinicopathological spectrum of disease that is largely a reflection of complex host responses to this organism (Ridley, 1974). The nature of the immunological defect, whether primary due to T cell deficiency or secondary due to prolonged persistence in the tissues of *M. leprae*, remains unresolved (Godal, 1978). Recently we showed that there was a common defect in borderline and lepromatous leprosy (LL) patients which was not present in tuberculoid leprosy (TT): thus in the spectrum from borderline tuberculoid to lepromatous leprosy, antibody, complement and other mediators of inflammation were detected within the lesions only in amounts which paralleled the antigenic load, whilst in TT, where the antigen was scarcely demonstrable, these mediators were present in abundance (Ridley, Russell & Ridley, 1982; Ridley & Ridley, 1983a). The only protein present at the lepromatous but not the tuberculoid end of the spectrum was C-reactive protein (CRP).

Human CRP is the classical acute phase protein, the circulating concentration of which

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increases rapidly and extensively in response to most forms of tissue injury, infection and inflammation (Pepys, 1981; Kushner, Volanakis & Gewurz, 1982). CRP is a member of a unique plasma protein family, the pentaxins, the structure and calcium-dependent ligand binding properties of which have been stably conserved throughout vertebrate evolution (Baltz *et al.*, 1982). A homologous protein has even been demonstrated in the invertebrate, *Limulus polyphemus*, the horseshoe crab (Liu, Robey & Wang, 1982). No individual lacking CRP has yet been described and these features, together with the remarkable adaptive regulation of gene expression which underlies the acute phase response to tissue injury, suggest that CRP has an important function *in vivo* which contributes to survival. Despite much recent progress in understanding of the structure of CRP, identification of its ligand binding specificities and description of various secondary effects of its ligand binding, the precise biological role of CRP remains unknown (Kushner *et al.*, 1982; Pepys & Baltz, 1983).

In experimental model systems CRP binds to some micro-organisms, particularly certain pneumococci, modulates their clearance *in vivo* and can confer effective protection against otherwise lethal pneumococcal infection (Edwards *et al.*, 1982; Mold *et al.*, 1981, 1982; Yother, Volanakis & Briles, 1982). On the other hand tissue injury or inflammation in the absence of microbial infection are also potent stimuli to CRP production and CRP binds to the membranes and other constituents of damaged but not healthy autologous cells (Narkates & Volanakis, 1982). CRP may thus participate in the handling of abnormal materials both of extrinsic and autologous origin. The capacity of complexed or aggregated human CRP to efficiently activate the classical complement pathway suggests that the complement system may be involved in such an *in vivo* role of CRP (Volanakis, 1982). However, CRP of other species tested so far, including rat (de Beer *et al.*, 1982a), rabbit (Pepys & Baltz, 1983) and dog, does not activate its own autologous complement, indicating that CRP may be able to function without triggering the complement system. A more general property of CRP's in different species seems to be their capacity to interact with plasma low density and very low density lipoproteins, but this has hitherto only been demonstrated *in vitro* (Pontet *et al.*, 1979; de Beer *et al.*, 1982b; Nagpurkar & Mookerjea, 1981; Cabana, Gewurz & Siegel, 1982; Rowe *et al.*, 1984). It is therefore of interest to determine whether these lipoproteins, which share apoB as their major apoprotein, may be associated with CRP *in vivo*.

Our earlier immunoperoxidase study of the leprosy spectrum (Ridley *et al.*, 1982) had suggested that CRP may play a role in leprosy which has yet to be elucidated. We report here further studies which confirm that CRP becomes associated with leprosy bacilli and their degradation products *in vivo*. Furthermore we show for the first time that apoB, the major apolipoprotein of low density and very low density lipoproteins, is also present together with the CRP.

MATERIALS AND METHODS

Tissue biopsies. Skin biopsies from 87 patients were studied, including eight active lepromatous leprosy (LLA), five with histoid lepromas, 10 with lepromatous leprosy treated over 5 years and in remission (LLR), 20 with borderline lepromatous leprosy (15 untreated, five treated for up to 2 years), eight with untreated mid-borderline (BB), eight with untreated borderline tuberculoid leprosy (BT), eight with untreated tuberculoid leprosy (TT) and 20 with erythema nodosum leprosum (ENL) reactions. Patients were classified according to the criteria of Ridley (1974). Biopsies from positive Mitsuda skin tests in four patients with BT leprosy were taken 30 days after intradermal injection of 0·1 ml heat killed *M. leprae* suspended in saline. Sixteen slit skin smears were taken from patients with treated lepromatous leprosy and five smears prepared from armadillos infected with *M. leprae* were also studied. The biopsies were obtained from the Medical Research Council Units at Sungei Buloh, Malaysia and Addis Ababa, Ethiopia; from Papua, New Guinea and the Hospital for Tropical Diseases, London. Tissues were fixed in a formalin–mercuric chloride–acetic acid (FMA) mixture (10 ml formalin, 2 g mercuric chloride, 3 ml acetic acid in 100 ml distilled water) for 2 h and transferred to 70% alcohol before processing. This fixative has been used routinely for leprosy for many years. It meets fully the requirements for the preservation of cytoplasmic detail and of antigenicity (Curran & Gregory, 1980; Ridley & Ridley, 1983b).

Antisera. Rabbit anti-human CRP serum was raised by repeated immunization with isolated pure human CRP (de Beer & Pepys, 1982). It was monospecific for CRP by immunoprecipitation testing and all positive staining of biopsies was completely abolished by prior absorption of the serum with isolated pure CRP. Rabbit anti-human low density lipoprotein, which was a monospecific anti-apoB serum, was purchased from Miles-Yeda Ltd., Rehovoth, Israel. Rabbit anti-BCG serum was obtained without Freund's complete adjuvant (DAKO-Mercia Brocades, Copenhagen, Denmark). Anti-BCG is known to cross-react with *M. leprae* and positive staining can be abolished by absorption with BCG. Other reagents were from DAKO whilst diamino benzidine hydrochloride (DAB) was purchased from Sigma Chemical Co Ltd, Poole, Dorset, UK.

Staining procedures and enumeration of bacilli. Apart from the specific immunoperoxidase staining all biopsies were also examined after standard haematoxylin & eosin staining, PAS, a modified Fite–Faraco acid fast stain and silver methenamine impregnation (Ridley, 1983). The bacilli were estimated on a logarithmic scale from 0 to 6+ (1,000+ bacilli per 1/12 oil immersion field).

Analysis of results. A semi-quantitative method of scoring positively stained cells and exudate was used. Positive cells were expressed as a percentage of the total macrophage population in the granuloma. In the case of ENL, polymorphs and macrophages in the reaction area only were counted. Extracellular CRP and apoB were assessed as a percentage area of the granuloma. Three assessors scored the results the means of which are recorded in the figures.

Immunoperoxidase technique. The peroxidase–anti-peroxidase (PAP) technique used was carried out according to established procedures (Ridley, 1983; Ridley *et al.*, 1982; Ridley & Ridley, 1983a, 1983b; Ridley & Russell, 1982) as follows. Endogenous peroxidase was blocked by 1% hydrogen peroxide in methanol for 30 min at room temperature. Non-specific binding sites were blocked for 45 min by normal swine serum diluted 1/5 and, without washing, the optimally diluted antiserum was then applied to the section which was incubated in a moist chamber at room temperature for 30 min. After washing in Tris-buffered saline the sections were treated with swine anti-rabbit Ig diluted 1/20. Then they were washed and treated with PAP at 1/50 for 30 min. The final product was obtained by exposure to DAB in hydrogen peroxide for 5 min. The sections were counterstained in Mayer's haemalum. Optimal dilutions of antisera and reagents used were obtained by reference to control sections of two active lepromas.

Control of specificity. Many haem containing proteins react with hydrogen peroxide and DAB so that strict control of the technique is necessary. Controls in this study included one section for endogenous peroxidase, one section treated for endogenous peroxidase after blocking for this enzyme and one section treated with normal rabbit serum in place of the antiserum of the test. Anti-CRP absorbed with isolated pure human CRP was also tested. Finally it was felt that since the study was being carried out on a spectrum of disease manifestations, this in itself, provided a control for the technique.

RESULTS

Number and distribution of M. leprae

M. leprae organisms were only rarely detected in the lesions of tuberculoid leprosy. In all cases across the spectrum from BT to BL, organisms were present and in each biopsy the same objects were detected by silver staining of cell walls, by acid fast staining and by staining with anti-BCG. Only a granular staining of solid acid fast bacilli was observed with anti-BCG. In BL cases a high proportion of macrophages also stained diffusely with anti-BCG. The diffuse staining corresponded to fragmented or granular acid fast organisms and was especially pronounced after chemotherapy. In active lepromatous lesions a large number of intact bacillary cell walls were seen by silver impregnation and a smaller proportion of solid or fragmented acid fast bacilli which also stained with anti-BCG were present. The number of acid fast bacilli in the lesions studied is given in Fig. 1.

Immunohistochemical staining with anti-CRP antibodies

There was little or no positive staining with anti-CRP antibodies in biopsies from lesions of patients with TT or BT leprosy. However, in tissue from patients with BB, BL and LL leprosy, anti-CRP

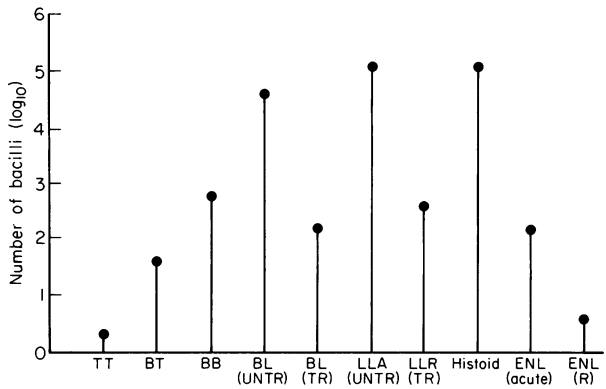


Fig. 1. Mean number of acid fast bacilli in lesions of leprosy across the spectrum. ENL included 15 acute and five resolving cases.

antibodies stained bacilli within macrophages (Figs 2–4). The staining pattern was finely granular without any diffuse staining. The amount and intensity of staining increased across the spectrum from BB to the active LL group, coinciding with breakdown of bacilli as revealed by fragmented or granular acid fast staining. Bacilli within macrophages situated in nerves or blood vessels stained with anti-CRP just as did those within macrophages in granulomata. After chemotherapy, staining with anti-CRP was still demonstrable in all BL cases but after prolonged chemotherapy it was variable, being present in six out of 10 cases of regressing lepromatous leprosy. In biopsies from two cases of advanced resolution, residual foam cells contained aggregated material which stained diffusely with anti-CRP. This material could be seen by silver impregnation but was neither acid fast nor positive with anti-BCG. Extracellular CRP was detected in blood vessels, in the subcutis and basal lamina of the BL and active LL lesions.

Histoid leproma lesions were comparable to highly active lepromas with large numbers of solid acid fast bacilli. Most macrophages contained bacilli which stained strongly with anti-CRP and in addition there was marked diffuse staining in the subepidermal zone and around most of the smaller blood vessels and in intercellular spaces of the granuloma.

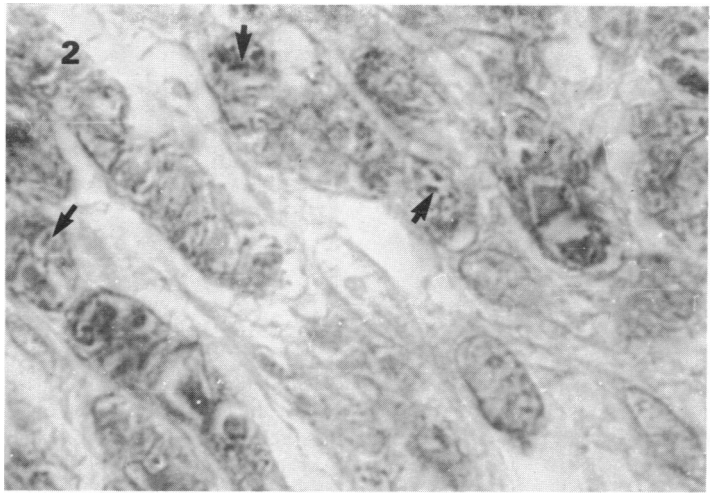
In contrast to histoid lesions, those of ENL involved the break up of small foci of effete foam cells with release of degenerating bacterial products. In new lesions, at the time of the earliest polymorph infiltration, there was usually little or no staining with anti-CRP at the reaction site, but in the acute phase of the reaction there was strong staining of coarse aggregates located both intracellularly within macrophages and extracellularly within the reactive area. Bacteria in macrophages in non-reacting peripheral granulomata also stained with anti-CRP. In addition the connective tissue of the lower dermis, intercollagenous septa of subcutaneous fat and the subepidermal zone also stained positively, as did the endothelium and adventitia of blood vessels. When the reaction had subsided there was no longer any positive staining with anti-CRP at the site although some residual foam cells with acid fast debris remained.

Biopsies of Mitsuda skin test reaction sites did not stain with anti-CRP, nor did smears containing leprosy bacilli taken from the armadillo. In contrast the bacilli in all the slit skin smears obtained from patients with lepromatous leprosy stained weakly positive with anti-CRP.

Fig. 2. Immunoperoxidase staining with anti-CRP of BL leprosy lesion. Macrophages are filled with solid or fragmented *M. leprae* organisms (arrowed) which are CRP positive (magnification × 750).

Fig. 3. Immunoperoxidase staining with anti-CRP of LLA leprosy lesion. Macrophages and a giant cell contain *M. leprae* organisms (arrowed) which are CRP positive (magnification × 750).

Fig. 4. Immunoperoxidase staining with anti-CRP of LLR leprosy lesion. Foamy macrophages contain residual aggregated material from *M. leprae* organisms (arrowed) which is CRP positive (magnification × 500).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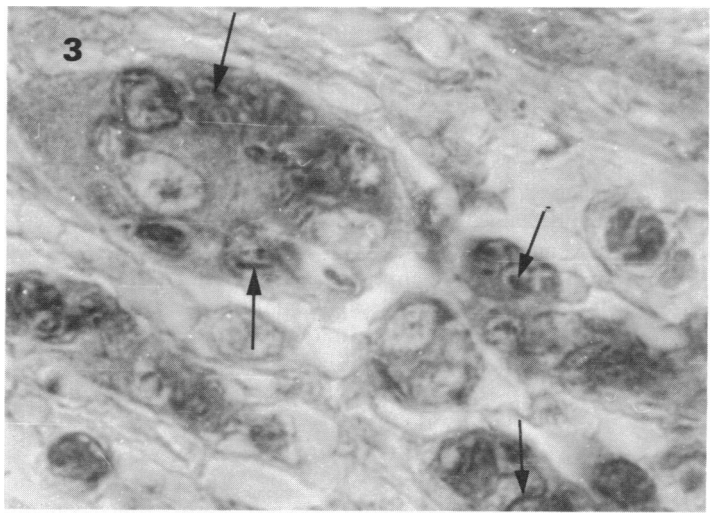
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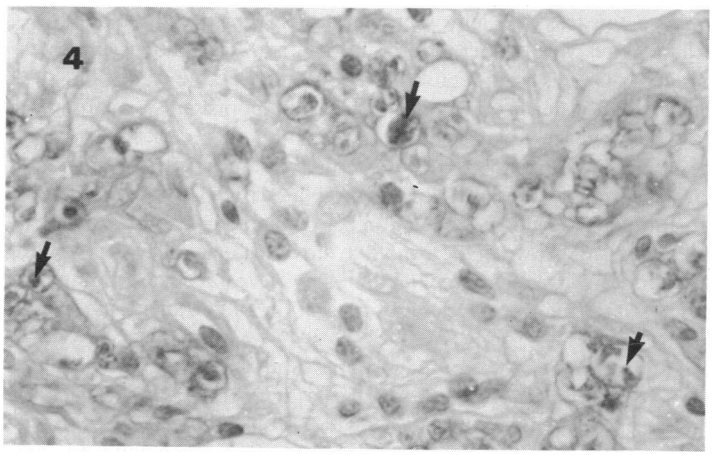
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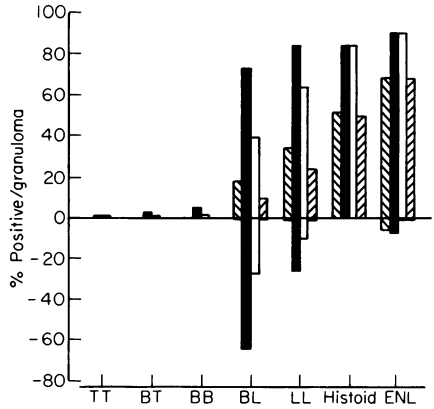

Fig. 5. Presence and quantity of immunoperoxidase staining with anti-CRP and anti-apoB in leprosy lesions and in ENL. Untreated cases across the spectrum are shown above the line and treated cases below. Staining of intracellular material (■ = CRP; □ = apoB) is expressed as percentage of macrophages which were positive; extracellular staining (▧ = CRP, ▨ = apoB) is expressed as a percentage of the area of the granulomas which is positive.

When normal rabbit serum was substituted for rabbit anti-CRP serum no staining at all was observed and absorption of the anti-CRP with isolated pure CRP completely abolished all positive staining.

Immunohistochemical staining with anti-apoB antibody

Anti-apoB antibody generally stained bacilli within the various lesions in the same pattern and distribution as the anti-CRP antibody although it was at a lower intensity. Thus bacilli and their remnants were stained most intensely in active lepromatous lesions and to a lesser extent in BL lesions and in regressing lepromas (Fig. 5). BB lesions and the two cases of advanced resolution did not stain at all with anti-apoB despite the presence of CRP. Lesions of ENL stained with anti-apoB in exactly the same pattern as with anti-CRP.

DISCUSSION

We show here for the first time that the cutaneous lesions of leprosy which contain demonstrable bacilli, that is those towards the lepromatous end of the spectrum, contain CRP which is predominantly associated with intact organisms or their breakdown products. Furthermore wherever the amount of CRP is considerable apoB is also detectable at the same sites. Since apoB is the major apoprotein of low density and very low density lipoproteins this observation suggests an *in vivo* association between these classes of lipoprotein and CRP.

The staining of bacilli with anti-CRP was definitely immunospecific since it was completely abolished by prior absorption of the anti-CRP with isolated pure CRP. Furthermore it could not be attributed to a fortuitous antigenic cross-reaction between human CRP and an intrinsic constituent of *M. leprae* since organisms grown in the armadillo did not stain at all with anti-CRP. We conclude that, following its growth *in vivo* in man, CRP binds to *M. leprae*. The binding occurs early in the disease and may persist till the last stages of disintegration and absorption of residual cell walls in lepromatous disease.

Serum CRP levels are known to be raised in leprosy, possibly to a greater extent in lepromatous than in the tuberculoid form (Rabson, 1955; Srivasta *et al.*, 1975; Chakrabarty *et al.*, 1983) and especially in reactive episodes (Languilloon, Ndiage & Roux, 1981; Malaviya *et al.*, 1972). Serum CRP values were available in 12 of the cases studied here, all of which had ENL, and were raised in nine (median 45 mg/l, range 6–83 mg/l). It was notable that whilst extracellular material stained

more strongly in the cases with high serum CRP concentrations, there was still positive intracellular material in those with normal levels of CRP.

We could find no previous work on apoB containing lipoproteins in leprosy but *in vitro* work in man and experimental animals has recently documented selective binding of these lipoproteins by CRP (de Beer *et al.*, 1982a; Cabana *et al.*, 1982; Rowe *et al.*, 1984). It was therefore of interest to investigate a possible *in vivo* interaction in the context of *M. leprae* infection and the positive results reported here were obtained. Although plasma lipoproteins could conceivably bind to *M. leprae* on their own account our study did not show any evidence of this, suggesting that the presence of apoB was secondary to the uptake of CRP.

Since CRP is synthesized only by the liver and apoB is synthesized by the liver and intestine and neither is known to be sythesized by mononuclear phagocytic cells, the source of these proteins detected on the intracellular bacilli must be the plasma. It is not clear how the CRP and apoB containing lipoproteins come into contact with the bacilli, whether it is by pinocytosis into the phagolysosome within which the organisms are either growing or degenerating, or whether these proteins bind to the organisms in the extracellular environment and then persist in antigenically recognisable form after phagocytosis. Whichever mechanism is involved there is clear evidence of persistence of CRP and apoB antigens inside macrophages so that lack of correlation with the serum CRP level, which can alter very rapidly, is not unexpected.

Although much remains to be learned of the mechanisms involved, the present findings suggest firstly, that *M. leprae* contains ligand(s) for CRP and, secondly, that CRP binding and the apparently secondary interaction with apoB containing lipoproteins may participate in some way in the *in vivo* handling of *M. leprae*. Activation of complement by the bound CRP seems less likely to be involved since staining of the leprosy biopsies with antisera to C1q, C3 and C3d did not identify an association of these proteins with bacillary material (Ridley & Ridley, 1983b; Ridley *et al.*, 1982); nor is antibody bound to intracellular *M. leprae* except after secondary phagocytosis by freshly recruited macrophages. On the other hand, CRP is known to bind to certain polysaccharides (Pepys & Baltz, 1983), and the presence of these substances in *M. leprae* cell wall components is indicated by positive reactions with PAS and methenamine silver. Moreover it is of interest that it is the polysaccharide fraction that is shown to be antigen specific (Harboe *et al.*, 1977), and antibodies to polysaccharide that demonstrate variations in individual specificity (Harboe *et al.*, 1981). It is also the cell walls which accumulate in the lepromatous patient and are most resistant to clearance.

These points may be important as it seems highly likely that it is the nature of the cell wall that is responsible for the low immunogenicity of *M. leprae*. An intriguing possibility is that the coating of *M. leprae* and its breakdown products with CRP and lipoproteins, that is the host's own plasma proteins, may mask the microbial antigenic and/or toxic constituents thereby preventing recognition and interfering with clearance. This could contribute to the apparently low immunogenicity of *M. leprae* in susceptible humans, and the known difficulty of its detection in nerves by immunological means (Barnetson *et al.*, 1975).

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