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CORRESPONDENCE

This department is for the publication of informal communications that are of interest because they are informative and stimulating, and for the discussion of controversial matters. The mandate of this JOURNAL is to disseminate information relating to leprosy in particular and also other mycobacterial diseases. Dissident comment or interpretation on published research is of course valid, but personality attacks on individuals would seem unnecessary. Political comments, valid or not, also are unwelcome. They might result in interference with the distribution of the JOURNAL and thus interfere with its prime purpose.

A Claim for a *Mycobacterium leprae* Specific Antigen

TO THE EDITOR:

The availability of armadillo-grown *Mycobacterium leprae* has made it possible to study its antigenic structure in greater detail than before, and several investigators are currently attempting to develop new diagnostic reagents and techniques based on the use of *M. leprae* isolated from armadillo tissues. Two recent papers in the INTERNATIONAL JOURNAL OF LEPROSY by Caldwell, *et al.* report on such studies.

In the first paper ([1]) Caldwell and Buchanan studied surface proteins of *M. smegmatis* iodinated by the lactoperoxidase method. When surface labeled *M. smegmatis* was mixed with armadillo liver tissue and separated from tissue using a method formerly employed by the WHO Immunology of Leprosy (IMMLEP) Programme for purification of *M. leprae* ([4]), "as much as 50% of the surface proteins of *M. smegmatis* was either released or destroyed. In addition, another twenty distinct proteins were released from *M. smegmatis* after treatment with Triton X-100." They pointed out that "similar losses of proteins from *M. leprae* may also occur using this procedure for *M. leprae* purification. Separation techniques employing surfactants and enzymatic treatment should be carefully evaluated since proteins lost during these procedures may prove relevant to human immune responses to *M. leprae*."

In the second paper ([2]) Caldwell, *et al.* studied acetone-killed *M. leprae* separated from infected armadillo liver tissue without the use of proteases ([11]) and extracted these bacilli with 0.2 M lithium acetate, 20 mM EDTA at pH 8.8, in a procedure considered to be particularly suitable for extraction of surface components of bacteria. The concentrated antigen extract was analyzed by double diffusion in gel, and the authors concluded: "In this study we report confirmation of a protein antigen(s) specific for *M. leprae* that was solubilized from organisms that were separated from armadillo liver tissue without employing any proteases. The antigen is strongly precipitated by treated lepromatous leprosy (LL) patients' sera and also recognized by serum from patients with tuberculoid leprosy. This indicates its possible importance for the serodiagnosis of leprosy."

We have attempted to reproduce the findings reported by Caldwell, *et al.* This work was greatly facilitated by a kind gift of a small amount of the antigen purified by Caldwell, *et al.* and a sample of their absorbed lepromatous leprosy serum. Thus we could directly compare the results obtained on our own isolated antigen preparations with those obtained on the antigen preparation made by Caldwell, *et al.* Due to the great importance of the matter, we think that the largely negative results of our experiments should be made known.

We extracted purified *M. leprae* obtained as freeze-dried cobalt irradiated bacilli from

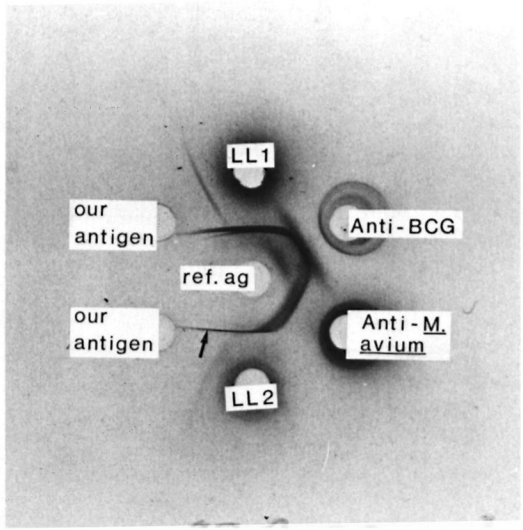
P. Draper through the IMMLEP Programme with lithium acetate. Three different batches were extracted separately, closely following the procedures described by Caldwell, *et al. M. leprae* antigens were demonstrated in the extracts, antigens no. 4, 5, and 7 ([6]) being positively identified. Lepromatous sera precipitated with these cross-reacting antigens in crossed immunoelectrophoresis (CIE), but no precipitate line corresponding to the antigen of Caldwell, *et al.* could be demonstrated in CIE or double diffusion tests in gel.

Calculations based upon the figures provided by Caldwell, *et al.* ([2]) concerning the number of *M. leprae* in the armadillo tissue used for extraction and the amount of protein in their extracts indicated to us that a major part of the extracted protein might be of non-mycobacterial origin. Therefore, experiments were made in parallel, starting with the same amount of armadillo liver, one containing *M. leprae* and the other not. Our yield of *M. leprae* from the first liver tissue was very similar to the yield of Caldwell, *et al.* The two preparations obtained looked virtually identical and were then extracted exactly following the procedure described. The two extracts contained virtually the same amount of protein, as determined by the Folin assay. Since many armadillo serum proteins and liver antigens cross-react with the corresponding human antigens ([10]), the preparations were examined by CIE using rabbit antisera against human serum proteins and armadillo liver homogenate. Both extracts were shown to contain armadillo liver components and armadillo plasma protein antigens giving similar patterns in these tests. The extract from the *M. leprae*-containing preparation again contained cross-reacting antigens, *M. leprae* antigens no. 2, 4, 5, and 7 being positively identified. The other extract was completely negative in parallel tests with various anti-*M. leprae* reagents. With the *M. leprae* antigen-containing extract we did not get any precipitate line in double diffusion tests similar to that reported by Caldwell, *et al.* It was concluded from these experiments that a major part of the protein in the lithium extract prepared by us was of armadillo origin.

Additional gel diffusion experiments using an array of anti-immunoglobulin reagents proved that the *M. leprae* antigen-containing extracts also contained armadillo immunoglobulins. Anti-*M. leprae* antibodies have been demonstrated in sera of *M. leprae* inoculated armadillos ([8]). The presence of immune complexes in the antigen extracts is then expected. They may cause aberrant reactions in gel diffusion tests where the antigen concentration employed is as high as in the experiments described by Caldwell, *et al.* This matter has not been further pursued.

The armadillo liver tissue provided for our experiments by Dr. R. J. W. Rees and the tissues used by Caldwell, *et al.* might conceivably be different after the procedures used for killing of *M. leprae,* and this might have led to destruction of "the Caldwell/Buchanan antigen" in the livers used by us before purification of bacilli and extraction. To try to exclude this possibility, we prepared *M. leprae* from non-cobalt irradiated armadillo liver under strict precautions. The number of bacilli obtained and extracted was larger than in our previous experiments and larger than the amount used by Caldwell, *et al.* Again, the extract obtained contained easily identifiable cross-reacting *M. leprae* antigens, but we were still unable to get a precipitate line in double diffusion tests with the absorbed lepromatous serum.

Finally, the double diffusion experiment shown in the Figure was made. The center well contained the reference antigen kindly provided by Caldwell and Buchanan. Strong precipitate lines were obtained against lepromatous sera (LL1 and LL2) in the top and the bottom well. One of these lines (indicated by an arrow) was very sharp and straight with the typical appearance of the precipitate line demonstrated by Dr. Buchanan at an IMMLEP meeting in Geneva, February 1979. The precipitate line of Caldwell and Buchanan was thus definitely confirmed in our laboratory. A strong anti-*M. avium* antibody ([6]) and anti-BCG ([3]) in neighboring wells interfered with and bent *all* the precipitate lines between the lepromatous sera and the reference antigen preparation. To us, this proves that there is a cross-reaction between the claimed *M. leprae* specific antigen and *M. avium* and BCG. The antigen thus contains cross-reactive determinants, but the exper-



THE FIGURE. Double diffusion test in gel. The center well contained the reference antigen isolated and provided by Caldwell and Buchanan. Lepromatous leprosy sera LL1 and LL2 gave strong precipitate lines with the reference antigen. The arrow points to the sharp precipitate line with the distinctive appearance previously demonstrated by Dr. T. Buchanan. Bending of this line by anti-*M. avium* and anti-BCG proves cross-reactivity. "Our antigen" means antigen extracted by us from *M. leprae* as described by Caldwell, *et al.*

iment does not indicate or exclude that the antigen contains additional *M. leprae* specific determinants.

The following conclusions have been made.

1. We have not been able to reproduce the lithium acetate extraction from *M. leprae* of an antigen with similar properties to the antigen extracted by Caldwell and Buchanan. We do not know the reason for this failure.

2. Based on experiments on antigenic material isolated and provided by Caldwell and Buchanan we have found that their antigen cross-reacts with *M. avium* and BCG.

Cross-reacting antigenic components of *M. leprae* may be very useful for antibody assays in leprosy ([9, 12]), and further studies are needed to establish the value of Caldwell and Buchanan's antigen in this regard. However, strict criteria should be used to accept a component of *M. leprae* as "*M. leprae* specific" ([5, 7]). The antigen reported by Caldwell and Buchanan is precipitated by anti-*M. avium* and by anti-BCG and should therefore not be termed *M. leprae* specific.

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Reply to Dr. Harboe's and Dr. Closs' Letter to the Editor

TO THE EDITOR:

We have no explanation why Harboe and Closs were unable to reproduce our lithium acetate extraction procedure to obtain an extract containing antigens with reactivities that we described as *M. leprae* "specific" in 1979 ([2]). We have reproduced this extraction procedure with comparable results more than 15 times and would welcome Harboe or Closs trying the same procedure in our laboratory. Perhaps small but relatively important differences in technique would prove responsible for their inability to detect the antigen(s) that we described.

Harboe and Closs state that our "antigen" cross-reacts with *M. avium* and BCG. These authors have demonstrated that our antigenic extract reacts with antiserum to *M. avium* and BCG without demonstrating that the antigen(s) with *M. leprae* "specific" activity react with the BCG and *M. avium* antiserum. We have never claimed antigenic purity of the lithium acetate extract, and an SDS polyacrylamide gel of this extract reveals that more than ten separate proteins as well as carbohydrate and glycolipid molecules are present. One would estimate therefore that there may be 10 to 20 separate antigens in this extract, considerably more than just the antigens 4, 5, and 7 recognized with CIE by Harboe and Closs. The basis for the claim of "specificity" of protein antigenic determinants for *M. leprae* was the use of a pool of sera from LL patients adsorbed by Abe, *et al.* ([1]) with BCG, *M. vaccae*, cardiolipin, and lecithin. This adsorption made the serum pool "specific" for *M. leprae* in an IFA test and specific for *M. leprae* as compared to four other mycobacterial species using double diffusion in gel. The proof that the *M. avium* or BCG antisera recognized the same *M. leprae* antigen(s) in our extract as that recognized by Abe's adsorbed serum pool would require using Abe's adsorbed serum pool in the same double diffusion in gel experiment. This critical experiment was not performed by Harboe and Closs. Therefore, their claim of cross-reactivity between the antigen(s) that we described and antigens of *M. avium* and BCG, while potentially correct, is not proven.

We have extended our studies by reacting Abe's adsorbed serum with lithium acetate extracts from 21 species of mycobacteria in double diffusion in gels. In September 1979 we reported that Abe's adsorbed serum recognized a shared antigenic determinant between *M. leprae* and *M. lepraemurium* ([3]). Subsequent studies with Abe's adsorbed serum also showed shared reactivity between *M. leprae* and *M. bovis* (BCG), *M. gordonae*, *M. nonchromogenicum*, *M. flavescens*, and *M. gastri* and no shared reactivity with 15 other mycobacterial species including significant human pathogens such as *M. tuberculosis*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, and *M. marinum*. These results are summarized in an abstract ([4]) which has appeared since the letter of Harboe and Closs was written.

It is our opinion that definite proof of whether or not *M. leprae* contains unique antigenic determinants will require monoclonal antibodies or extensive antigen purification. It cannot be answered by double diffusion in gel or CIE experiments.

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