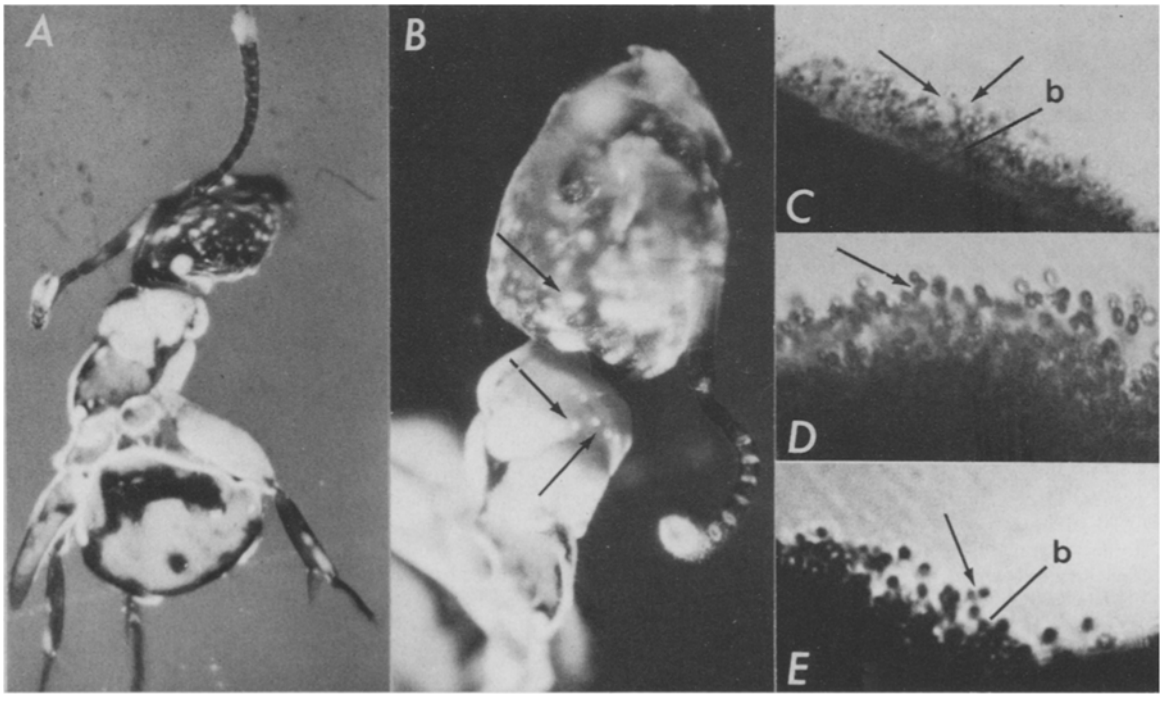

Entomogenous fungus in Dominican amber. *A* Ventral view of ant fossilized in amber, showing the white fungus covering much of the insect (× 45). *B* Dorsal view of head and thorax showing 'cottony puffs' of fungus (arrows), typical of spore bearing areas for *Beauveria* (×100). *C* Rachis with denticles (2 arrows) and globose base (b) of conidiogenous cell (× 800). *D* Geniculate rachis with spores (arrow) (×1000). *E* Spore rachis with 2 spores (arrow) and swollen base (b) of conidiogenous cell (×1000).

of the conidiogenous cells were globose (fig. E). What we interpreted as denticules appeared on the rachis (fig. C).

On the basis of its white, powdery appearance, (similar to that found in present day *Beauveria* infections; see Poinar and Thomas[1], its spore characteristics and the nature of the conidiogenous cells, we conclude that the fossil fungus belongs to the genus *Beauveria* as defined by De Hoog[2] and characterized by Samson[3]. Using De Hoog's key to the species of *Beauveria*, the fossil species is closest to *Beauveria bassiana* (Bals.) Vuill.

Present day strains of *B. bassiana* attack a wide variety of insects, including ants[4].

To our knowledge, this is the first report of a fossil entomogenous fungus belonging to the class Deuteromycetes. Previous fossil fungi associated with insects were either parasitic members of the Entomophthorales[5] or saprophytic forms[6].

Amber from the Dominican Republic (Palo Alto region) is dated around the Oligocene-Miocene boundary or at approximately 25 million years old[7].

1 Thomas, G.M., and Poinar, G.O. Jr, Hilgardia *42* (1973) 261.
2 De Hoog, G.S., Stud. Mycol., Baarn *1* (1972) 41.
3 Samson, R.A., in: Microbial Control of Pests and Plant Diseases 1970–1980, p.94. Ed. H.D. Burges. Academic Press, New York 1981.
4 Poinar, G.O. Jr, and Thomas, G.M., in: Diagnostic Manual for the identification of Insect Pathogens, p.218. Plenum Press, New York 1978.
5 Poinar, G.O. Jr, and Thomas, G.M., Mycologia *74* (1982) 332.
6 Larsson, S.G., Entomonograph *1* (1978) 1.
7 Sanderson, M.W., and Farr, T.H., Science *131* (1960) 1313.

0014-4754/84/060578-02$1.50 + 0.20/0

Pathological changes in the heterologous phase of antibasement membrane antibody mediated disease in the rat

L.C.J. Yong and J. Horn

School of Pathology, University of New South Wales, P.O. Box 1, Kensington (N.S.W. 2033, Australia), 12 July 1983

Summary. The immunological and structural changes during the heterologous phase of experimental antibasement membrane antibody mediated disease was sequentially studied in the rat following single i.v. injections of rabbit antibodies to basement membrane antigens prepared from kidney, lung and salivary gland tissues. Although each of the anti-bodies bound strongly to GBM, structural changes were initially subtle accompanied by proteinuria and hematuria. More severe structural changes related to dose and duration of the disease did not appear for several weeks.

Antibasement membrane antibody mediated disease first described by Goodpasture in 1919[1] has now become a well recognized clinical entity. It presents with pulmonary hemorrhage associated with florid proliferative glomerulonephritis. Although mediated by circulating auto anti-bodies against glomerular basement membrane (GBM) and alveolar basement membrane (ABM) the initiating factors are unknown even though an increasing number of cases associated with exposure

to toxins strengthens a suspicion of a role for environmental agents[2–4].

This paper describes an experimental time course study of the sequential immunological and structural aberrations in both kidney and lung evoked by antibody insult in the heterologous phase, as a preliminary for a comprehensive study of the possible pathogenetic role of environmental agents.

Basement membrane antigens from kidney (GBM), lung (ABM) and salivary gland (SBM) were individually prepared from 200 rats of b.wt ranging from 180 to 300 g by the method of Spiro[5]. Renal cortical tissue was sieved through graded sizes of nylon mesh to separate glomeruli from tubulo-interstitial elements. Preparations of glomeruli were concentrated by centrifugation at 1500 rpm for 2 min, then disrupted ultrasonically (M.S.E. Sonicator) for 10 min and after trypsin digestion[6] for 3 h at pH 8.2 at 37 °C was dialyzed, lyophilized and stored at −20 °C. No further attempts were made to refine the antigen. With the exception of sieving, similar methods were used to prepare basement membrane antigens from lung and salivary gland tissues. Choroid plexus was not used to prepare antigens. All tissues were obtained by clean surgical procedures using sterile instruments. Antisera were individually raised in adult New Zealand white rabbits by means of 2 or more s.c. injections of 5–mg aliquots of pooled tissue specific lyophilized antigens in Freunds complete adjuvant. Presence and specificity of antisera was screened by Ouchterlony gel diffusion as well as by methods outlined below.

In order to verify that antisera contained antibodies to basement membrane antigens, 4–μm cryostat sections of kidney, lung and salivary glands were examined by a direct immunofluorescence technique using the respective raised antisera and FITC labelled anti rabbit IgG (Dako). Results confirmed that anti-sera contained basement membrane antibodies to their respective antigens. When this procedure was repeated using antisera adsorbed with antigen or homogenates prepared from the respective organs the results were negative. Globulin fractions from good respondents were subjected to 50% saturated ammonium sulphate precipitation, dialyzed, lyophilized and stored at −20 °C.

A total of 60 adult Wistar rats (male and female) weighing between 150–220 g were divided into 3 groups of 20 and each given a single dose of either 0.5 mg or 2.0 mg/kg b.wt of lyophilized anti-glomerular, anti-lung or anti-salivary gland antibodies suspended in 0.5 ml of 0.85% sterile saline. 3–4 animals from each group were sacrificed at varying intervals from 0.5 h to 10 weeks. 2 groups of control animals were prepared for comparison with the test animals. 1 group was injected with 0.5 ml of 0.85% saline whereas another group was injected with 0.2 mg/kg b.wt of lyophilized normal rabbit serum. Batches of control animals were killed at similar time intervals to test animals.

Cryostat sections of kidney, lung, salivary gland and choroid plexus were examined for antibody binding by direct immunofluorescence using FITC labelled anti rabbit IgG (Dako) and by an immunoperoxidase technique on paraffin sections. As the results will to some extent depend on the staining intensity of products of peroxidase, the staining procedure had to be carefully standardized. In brief, the modified method[7] included the following essential steps: Deparaffinized sections were treated with 0.1% trypsin and 0.1% sodium fluoride in Tris buffered saline (pH 7.6) for 1 min. After rinsing in buffer normal swine serum was applied for 30 min followed by swine anti-rabbit immunoglobulin (1:20) for 30 min at 20 °C. Slides were rinsed in buffer and PAP applied for 30 min. After rinsing, sections were emersed in a freshly prepared solution of diamino-benzidine-4-hydrochloic acid (5 mg/10 ml) with 0.01% H_2O_2 in 0.05 M Tris buffer (pH 7.6). Maximal staining time was 8 min. Sections were washed, counterstained with hematoxylin and mounted for examination. For ultrastructural studies, small pieces of tissue fixed in 2.5% freshly prepared glutaraldehyde in cacodylate buffer were routinely processed, sectioned and examined by a Philips 300 transmission electron microscope. Light microscopic and electron microscopic aberrations of target organs were sequentially correlated.

Rats injected with heterologous antibodies to GBM exhibited early and strong antibody binding to GBM (fig. 1) but weak and transient binding to ABM and no binding to either salivary gland or choroid plexus membranes. On the other hand anti-lung antibodies bound strongly to GBM, weakly and transiently to ABM and again no binding to either salivary or choroid plexus membranes. Anti-salivary basement membrane antibodies bound only moderately to GBM (fig. 2) but not to

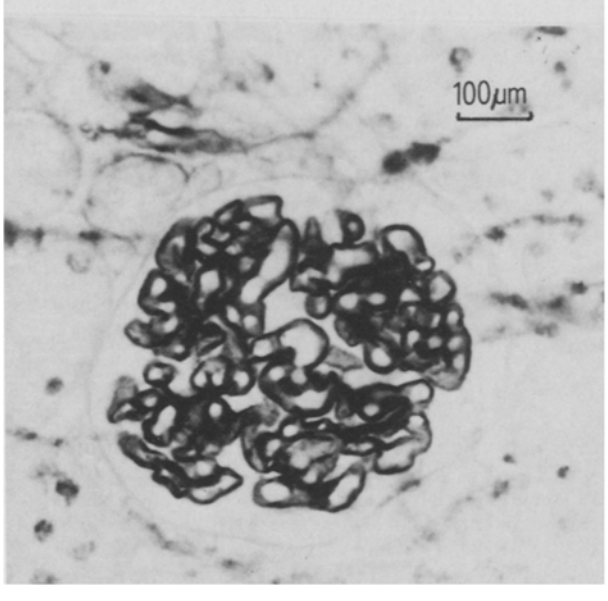

Figure 1. Glomerulus from rat given single dose of 0.2 mg/kg b.wt of lyophilized anti-lung antibodies 14 days previously. Note the localization of IgG in linear continuous fashion along GBM and not in tubular or Bowman's capsular basement membrane.

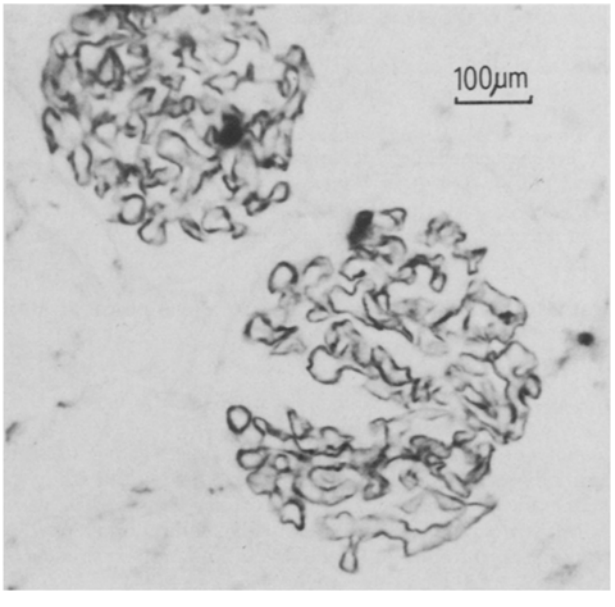

Figure 2. Glomeruli from rat following administration of single dose of 0.2 mg/kg of b.wt of lyophilized anti-salivary gland antibodies. Note the localization of antibodies in GBM of reduced intensity compared to figure 1.

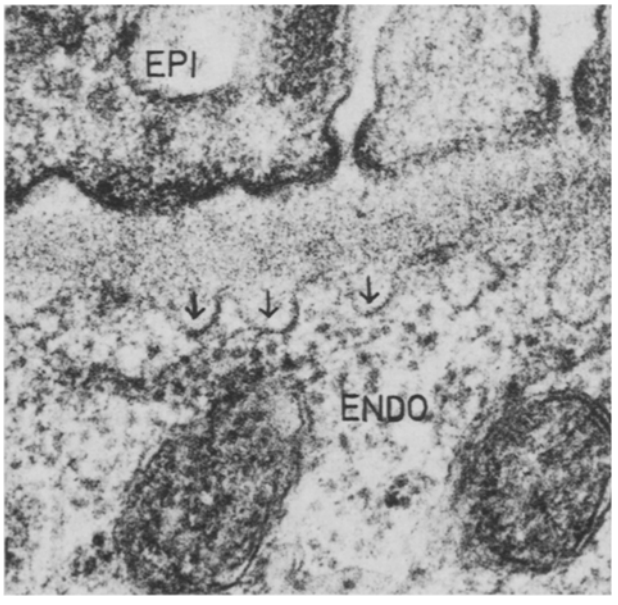

Figure 3. Electronphotomicrograph of part of a glomerulus from rat 10 weeks after injection of anti GBM antibody. Note thickening of GBM with 'bulbous' projections (arrows) into the endothelium (ENDO). The epithelial cell (EPI) shows foot process swelling.

any other tissue membranes. Antibody binding to target organs persisted for up to 10 weeks. In those experiments in which GBM labelling occurred, no binding was concurrently detected in either Bowman's capsule or tubular basement membranes. No antibody localization was found in basement membranes of all target organs of control animals injected with normal saline or normal rabbit serum. Only those animals which exhibited proteinuria and hematuria were subsequently found to show antibody binding to GBM. It is important to emphasize that the binding to antibodies to basement membranes of target organs was linear and continuous. However in order to exclude the possibility that the observed positive staining was due to aggregated immunoglobulin or immune complexes, tissues were found to be negative when tested for the binding of C_3 by direct immunofluorescence or immuno peroxidase technique. Results of injections of single dose of 0.2 mg/kg b.wt are summarized in the table.

In the first 30 days, ultrastructural changes in the glomerulus subjected to anti GBM antibody insult were unimpressive but the changes became more marked with time especially in rats receiving larger doses of antibody. By the end of the first week glomeruli were congested and swollen and in the period 4–10 weeks exhibited mild focal mesangial hypercellularity with ultrastructural evidence of patchy foot process fusion but without evidence of endothelial detachment previously reported[8,9]. By 10 weeks hypertrophy of mesangial and endothelial cells were overt, the GBM was clearly thickened and exhibiting protrusions[10] into the endothelial fenestrae (fig. 3).

Pulmonary morphological aberrations were subtle. Numerous small scattered hemorrhages were noted soon after antibody injection. Significant numbers of eosinophils and monocytes appeared in the peribronchial and perivascular connective tissues by 5 days. This subsided within the following 7–10 days. Apart from mild oedema of the ABM no other significant ultrastructural changes were noted.

It is commonly accepted that in Goodpasture's syndrome injury to either kidney or lung basement membranes triggers production of antibodies to either or both localized membranes, the antibodies then react or cross react with such membranes to evoke structural changes. The present results are in accord with this general postulate but on the evidence of the effect of anti-salivary basement membrane antibody on GBM, injury to any basement membrane of non vascular origin may act as the origin of the triggering altered immunizing antigen. Whereas McIntosh[11–13] described antibody localization to choroid plexus basement membrane in human and experimental antibasement membrane disease, the present results failed to demonstrate any localization in the choroid plexus despite the fact that all 3 antisera reacted strongly with GBM. Although this difference may result from differing experimental details, difference in cross reactivity of the heterologous antibodies and animal susceptibility, it is reasonable to postulate that the difference between glomerular and aveolar basement compared with choroid plexus and salivary gland relate to a morphological difference between the endothelial and epithelial relationships between glomerulus and aveolus on the one hand, the salivary gland on the other. The absence of membrane covering the fenestrae of the glomerulus together with the larger fenestrae compared to sparcely occurring fenestrae in the lung and small membrane covered fenestrae in the choroid plexus may well explain the greater susceptibility of the glomerulus by allowing relatively easier antibody contact with the GBM. However if this be the case it is surprising that in vitro studies with isolated glomeruli and choroid plexus incubated with anti GBM antibody have demonstrated persistently more avid binding to GBM (unpublished data).

It is concluded from these studies that although heterologous GBM antibody binding to GBM is immediate accompanied by proteinuria and hematuria significant renal ultrastructural changes took several weeks to evolve i.e. immunological insult proceeds morphological aberrations. Furthermore whereas heterologous antibody provoked a strong glomerular response, lung lesions are only slight and transient. The latter has yet to be explained on a reasonable pathological basis[14–15].

1 Goodpasture, E.W., Am. J. med. Sci. *158* (1919) 863.
2 Klavis, G., and Drommer, W., Arch. Tox. *26* (1970) 40.
3 Kleinknecht, D., Morel-Maroger, L., Callard, P., Adhémar, J., and Mahieu, P., Archs intern. Med. *140* (1980) 240.
4 Beirne, G.J., and Brennan, J.T., Archs envir. Hlth *25* (1972) 365.
5 Spiro, R.G., J. biol. Chem. *242* (1967) 1915.
6 Shibata, S., Miyahawa, Y., Naruse, T., Nagasawa, T., and Takuma, T., J. Immun. *102* (1969) 593.
7 Sternberger, A.L., Immunocytochemistry, 2nd edn. John Wiley, New York 1979.
8 Churg, J., Grishman, E., and Mantner, W., Am. J. Path. *37* (1960) 727.
9 Shibata, S., Sakaguchi, H., and Nagasawa, T., Lab. Invest. *38* (1978) 201.
10 Kühn, K., Ryan, G.B., Hein, S.J., Salaske, R.G., and Karnovsky, M.J., Lab. Invest. *36* (1977) 375.
11 McIntosh, R.M., and Griswold, W.R., Archs Path. *92* (1971) 329.
12 McIntosh, R.M., Koss, M.N., Chernack, W.B., Griswold, W.R., Copack, P.B., and Weil, R., Proc. Soc. exp. Biol. Med. *247* (1974) 216.
13 McIntosh, R.M., Copack, P., Chernack, W.B., Griswold, W.R., Weil, R., and Koss, M.N., Archs Path. *99* (1975) 48.
14 Shigematsu H., and Kobayashi, Y., Virchows Arch. Abt. B. Zellpath. *11* (1972) 111.
15 Hagadorn, J.E., Vasquez, J.J., and Kinnery T.R., Am. J. Path. *57* (1969) 17.

0014-4754/84/060579-03$1.50 + 0.20/0