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Kinetics and pathogenicity of autoantibodies induced by mercuric chloride in the brown Norway rat

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SUMMARY

Repeated low-dose injections of mercuric chloride ($HgCl_2$) in the brown Norway (BN) rat result in polyclonal activation which includes the induction of anti-glomerular basement membrane (GBM) autoantibodies. We examined the kinetics of various autoantibodies produced *in vivo*, general features of polyclonal activation such as total IgG levels and immune complex formation, and the relationship between organ specific autoimmunity and tissue injury in the kidney and thyroid. The production of immune complexes and autoantibodies to GBM and thyroglobulin was short lived, and the increase in levels of total IgG and antibodies to ssDNA and dsDNA was prolonged; the antibody response to collagen types I and II was intermediate in duration. Autoantibodies induced by $HgCl_2$ caused only mild and variable tissue injury in the kidneys and did not induce abnormalities in the thyroid. These studies demonstrate that immunostimulation by mercury may result in the formation of a range of autoantibodies, with variable kinetics and pathogenicity.

Keywords autoimmunity polyclonal activation mercuric chloride brown Norway rats

INTRODUCTION

Brown Norway (BN) rats given s.c. injections of mercuric chloride ($HgCl_2$) develop a biphasic autoimmune nephritis, characterized initially by the presence of linear deposits of IgG along the glomerular basement membrane (GBM) and subsequently by the development of granular deposits (Druet *et al.*, 1979). Circulating antibodies to GBM are present transiently in the serum of these animals (Bowman *et al.*, 1981, 1984; Bellon *et al.*, 1982) as are immune complexes (Bowman *et al.*, 1981; Bellon *et al.*, 1982). Mechanisms underlying the autoregulation of such anti-GBM antibody production are complicated, but we have found that humoral factors (putatively anti-idiotypic antibodies) (Chalopin & Lockwood, 1984) and T suppressor lymphocytes (Bowman *et al.*, 1984) are involved.

The role of $HgCl_2$ as a polyclonal activator in the BN rat has been described by Hirsch *et al.* (1982), who found that its administration *in vivo* caused enlargement of lymphoid organs (spleen and lymph nodes) and the short-lived production of antibodies to DNA, sheep red blood cells (SRBC) and trinitrophenol (TNP), in addition to the anti-GBM response. *In vitro*, $HgCl_2$ stimulated antibody responses to SRBC and TNP in normal BN spleen cell cultures (Hirsch *et al.*, 1982). Polyclonal activation, whether spontaneous or induced by parasites or chemicals, is clearly relevant in several human and animal diseases; however, the mechanisms involved are poorly understood (Fauci, Lance & Volkman, 1983).

The main aims of this study were to investigate the effects of $HgCl_2$ on the production of different autoantibodies *in vivo*; to determine whether their synthesis was self-limiting; and to study immune complex generation and overall IgG production. The relationship between organ-specific autoantibodies and development of tissue injury in the kidney and the thyroid was also examined, in order to define the pathological consequences of the generation of autoantibodies induced by non-antigenic stimuli.

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MATERIALS AND METHODS

Experimental model

BN rats were obtained from the Repgo Institute (Rijswick, The Netherlands) and a breeding colony was established. Groups of animals, aged 7–12 weeks and sex-matched in each experiment, were injected subcutaneously with 1 mg/kg $HgCl_2$ (as a 0·1% solution in distilled water) on five occasions over a 10-day period (Bowman *et al.*, 1984). Control animals were injected with equal volumes of distilled water. Serial blood samples were taken by tail artery puncture under ether anaesthesia.

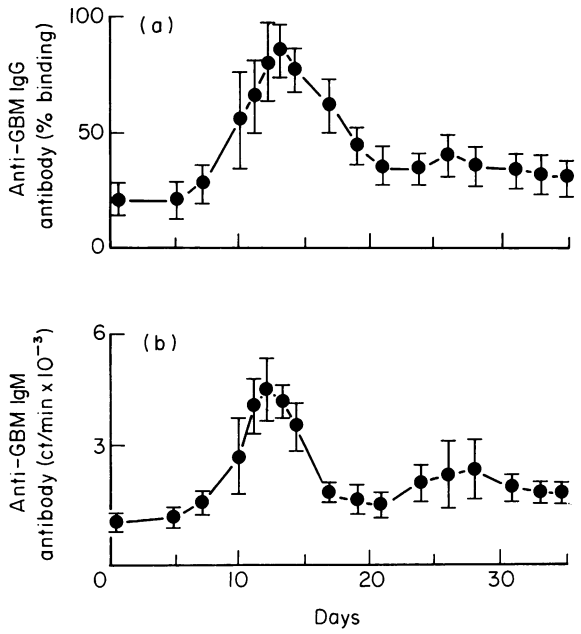

Fig. 1. Sequential levels of IgG and IgM class anti-GBM antibodies in female BN rats ($n=10$) injected with $HgCl_2$. Results show mean ± s.d. for anti-GBM antibodies of IgG class (a) and of IgM class (b).

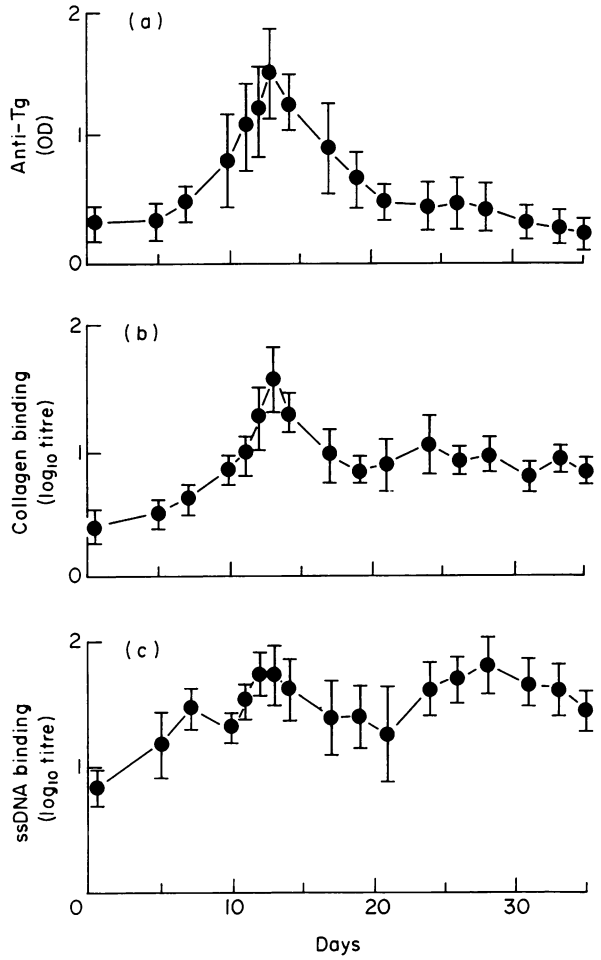

Fig. 2. Sequential levels of antibodies to thyroglobulin (Tg), type I collagen and single-stranded DNA in female BN rats ($n=5$) injected with $HgCl_2$. Results show mean ± s.d. for anti-thyroglobulin antibodies (a), collagen type I-binding (b) and ssDNA-binding (c).

Assay systems

Circulating anti-GBM antibodies were measured by solid-phase radioimmunoassay (RIA) as previously described (Bowman, Peters & Lockwood, 1983). Binding of IgG antibody to collagenase-solubilized rat GBM was detected by affinity-purified ^{125}I-labelled rabbit anti-rat IgG, and results expressed as percentage binding of a strongly positive reference serum. IgM autoantibodies to GBM were detected similarly, using affinity-purified rabbit anti-rat IgM (a kind gift from Dr D.W. Mason, Oxford). The anti-rat IgM was ^{125}I-labelled to a specific activity of 4 μCi/mg, and results expressed as ct/min bound. Autoantibodies to thyroglobulin were measured using a previously described ELISA, and results expressed as optical density (OD) units (Rennie *et al.*, 1983). Antibodies to collagen types I and II (Staines *et al.*, 1981), and to ssDNA and dsDNA (Morgan *et al.*, 1985), were measured by ELISA as previously described and results expressed as $\log_{10}$ of the reciprocal of the interpolated dilution giving a mid-point reading in the peroxidase titration curve (OD = 0·8). Native DNA was treated with S1 nuclease to guarantee that it was double stranded (Morgan *et al.*, 1985). To confirm the specificity of the autoantibody response, sera were absorbed with certain ligands used in the solid-phase assays and then tested for activity against other autoantigens. For anti-GBM antibodies and ssDNA-binding activity, day 14 sera were absorbed on columns of DNA (DNA-cellulose, Sigma Chemical Co., St Louis, MO) or rat GBM linked to Sepharose-4B by cyanogen bromide prior to assay in the anti-GBM RIA or ssDNA-binding ELISA.

Total IgG estimations were performed on serial serum samples using rabbit anti-rat IgG (Miles-Yeda, Rehovot, Israel) in the Mancini single radial immunodiffusion technique, and results expressed as a percentage of normal age-matched BN rat serum. Immune complexes were detected using a modification of the conglutinin binding assay (Casali *et al.*, 1977). Briefly, polyvinyl chloride microtitre plates (Dynatech, Plochingen, FRG) were coated with 100 μl conglutinin (5 μg/ml) in complement fixation diluent (CFD; Oxoid, Oxford, UK) for 18 hr at 4°C. The plates were washed three times with CFD-Tween 20 (0·05% Tween 20, Sigma) and incubated with a 1/10 dilution of test or normal sera in CFD-Tween 20, for 1 h at 37°C. After further washing, conglutinin-bound immune complexes were detected by incubation for 1 h at 37°C with 100 μl of ^{125}I-rabbit anti-rat IgG (as used in the anti-GBM assay) and results were expressed as ct/min bound. Serum samples were considered to contain elevated levels of immune complexes if levels of binding were greater than the mean (+2 s.d.) levels for day 0 samples. The 24-h urinary protein excretion was determined by trichloracetic acid precipitation followed by the biuret method.

Renal histology

This was performed on normal BN renal tissue, and on sequential wedge renal biopsies taken under ether anaesthesia on days 9, 15, 18, 20, 22, 29 and 36 following the initial $HgCl_2$ injection. Tissue for light microscopy was fixed in buffered formol saline, and processed by standard techniques. Paraffin sections were cut at 3 μm and stained using haematoxylin and eosin, periodic acid Schiff, methenamine silver, Martius scarlet blue and elastic Van Giesen methods. Tissue for immunoperoxi-

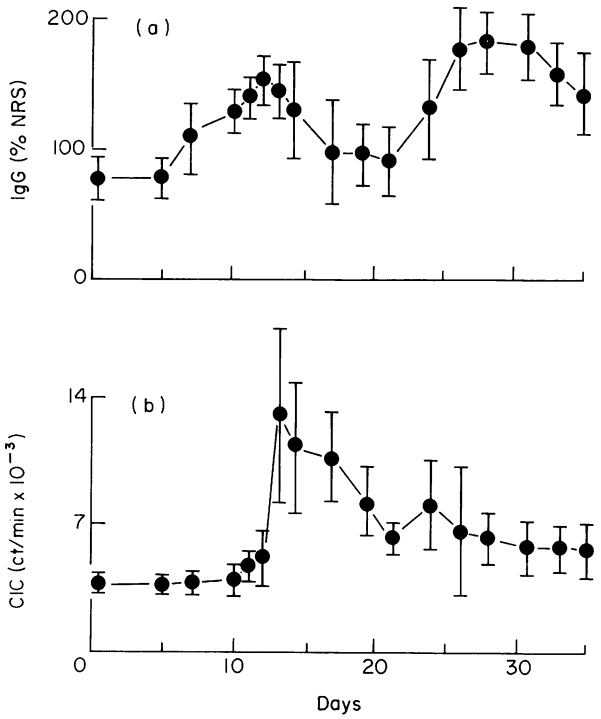

Fig. 3. Sequential levels of serum IgG and circulating immune complexes (CIC) in female BN rats ($n=5$) injected with $HgCl_2$. Results show mean ± s.d. for serum IgG (a) and CIC (b). NRS, normal rat serum.

Table 1. Proteinuria and anti-GBM antibodies in $HgCl_2$-treated BN rats

Rat no.	Peak proteinuria (mg/24 h)	Time (day)	Peak anti-GBM antibody (% positive binding)
1	143	15	73
2	61	17	55
3	44	12	93
4	28	13	64
5	8	—	64
6	8	—	54
7	9	—	42

dase studies was snap frozen in OCT compound (Tissue-Tek II, Raymond Lamb) using liquid nitrogen. Sections were cut at 4 μm, dried in air, fixed in acetone at 4°C and then incubated with biotinylated rabbit anti-rat IgG for 30 min. After washing in three changes of PBS at 4°C, the sections were blotted dry and incubated with horseradish peroxidase conjugated avidin (Biotin/avidin system, Vector Laboratories, Burlingame, CA). IgG was revealed after further washing in PBS, by standard techniques using diamino-benzidine and H_2O_2. The sections were counter-stained with haemotoxylin, dehydrated and mounted in DPX (BDH Chemicals, Poole, UK).

Thyroid histology

This was performed on thyroid from normal BN rats, and from rats killed by cervical dislocation on days 14 and 21 after $HgCl_2$. Tissue for light microscopy was fixed in 10% phosphate-buffered formalin and immobilized in paraffin. Sections were cut at 5 μm and stained with haematoxylin and eosin. Tissue for immunofluorescence studies was snap-frozen in OCT compound using liquid nitrogen, and processed as described previously (Noble *et al.*, 1976).

RESULTS

Circulating autoantibodies, IgG levels and immune complexes

The kinetics of anti-GBM antibody synthesis are shown in Fig. 1. This represents pooled data from two similar experimental groups; one group ($n=5$) was used for studies of other autoantibodies, and the other ($n=5$) was used for studies of IgG and immune complex levels. Anti-GBM antibodies of both G and M class were first detectable in the circulation after day 7. Levels were maximal between days 12 and 14 and thereafter fell. A subsequent smaller rise occurred between days 26 and 28, and by day 36 anti-GBM antibodies of IgG class were undetectable; those of IgM class were present only at low levels. Control animals did not develop detectable anti-GBM antibodies during the course of the experiment.

Antibodies to thyroglobulin were first detectable after day 7, peaked at day 13, and fell to become undetectable by day 31 (Fig. 2a). Antibodies to collagen type I could be detected by day 7, reached maximal levels at day 13 and then fell, although remaining elevated at lower levels until the end of the experiment at day 36 (Fig. 2b). Antibodies to collagen type II showed similar kinetics and levels. Antibodies to ssDNA showed an initial peak at day 13 and then fell slightly, before rising again by day 26 and remaining high at the end of the experiment (Fig. 2c). Antibodies to dsDNA showed similar kinetics and levels. Control animals did not develop autoantibodies detectable in these assays. In specificity studies, absorption of day 14 sera with ssDNA removed antibody activity against ssDNA (76%), but not against GBM. Similarly, absorption of day 14 sera with GBM removed antibody activity against GBM (55%), but not against ssDNA.

Total IgG levels rose in response to $HgCl_2$, showing two major peaks at days 12–13 and 26–31. Levels remained elevated at day 36 (Fig. 3a). Control animals showed no significant increase in IgG levels during the experiment, although the normal concentration of IgG rises slowly with age in BN rats (unpublished observations). Immune complexes were first detectable in the circulation by day 12, at which time levels peaked and thereafter gradually fell (Fig. 3b). Subsequently, in individual animals, elevated levels of circulating complexes were detected sporadically.

Proteinuria

Proteinuria (> 10 mg protein excreted/24 h) occurred in four out of seven female rats examined, with peak levels ranging from 28 to 143 mg. Maximum urinary protection excretion occurred at the same time as, or shortly following the peak of anti-GBM antibody (Table 1). However, levels of urinary protein excretion did not correlate with levels of anti-GBM antibody or immune complexes (see Fig. 4). Control animals did not develop proteinuria.

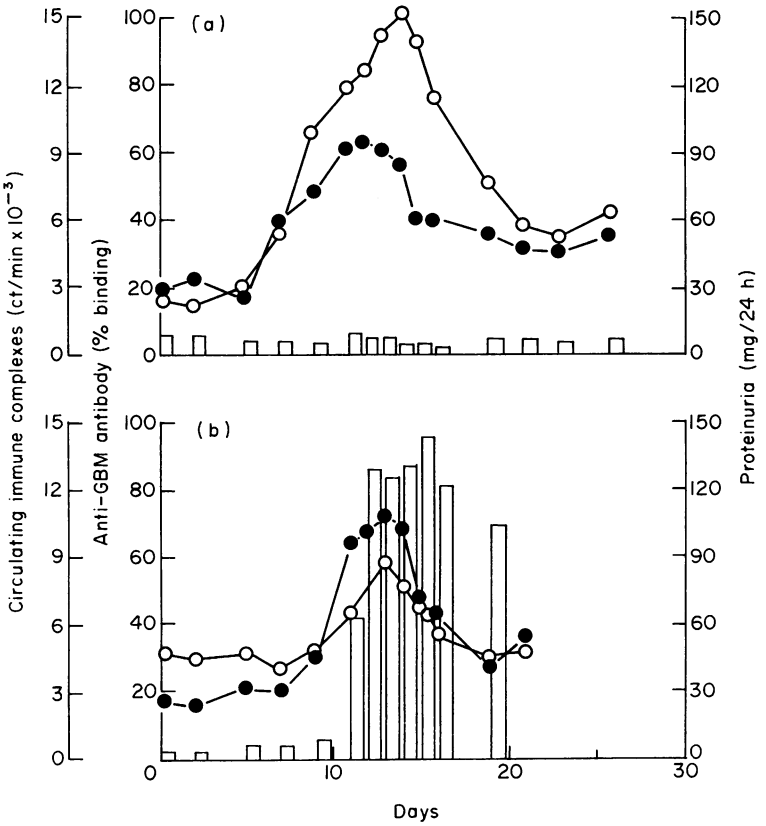

Fig. 4. Relationship between anti-GBM antibodies, immune complexes and proteinuria in individual rats injected with $HgCl_2$. Data shown are for two animals (a, b), with sequential measurements of anti-GBM antibody (●), immune complexes (○) and proteinuria (open bars). Only animal (b) developed proteinuria, despite similar levels of anti-GBM antibody and immune complexes in animal (a).

Renal histology

Light microscopy. No significant changes were noted in the tubules or interstitium of animals injected with $HgCl_2$ ($n=3$). Most glomeruli also showed no changes during the course of the experiment; however, rare focal abnormalities were observed. These were first seen in the day 15 biopsy and comprised global capillary dilatation and segmental double contouring of capillary walls as seen in silver preparations (Fig. 5a). Further fresh lesions were seen in later biopsies, for example global tuft thrombosis with segmental double contouring. By day 36 residual wrinkling and collapse and mesangial proliferation were all that remained. Vascular changes characterised by endothelial cell swelling were noted in arteries and arterioles from day 18.

Immunoperoxidase studies. (i) GBM: moderate, diffuse, global, linear staining for IgG was present at day 9, and had increased in intensity by day 15. A small amount of segmental, granular staining was present at day 9 and had increased by day 15. By day 18 the granular deposits were global and so intense that they merged with the linear staining, reaching a peak between days 20 to 22 (Fig. 5b). By day 36 linear and granular deposits were markedly decreased in intensity and segmentally distributed.

(ii) Tubular basement membrane: at day 9 segmental linear staining was present. By day 15 granular staining was seen in peritubular capillaries, and this was maintained through to day 36. The granular peritubular capillary staining made linear tubular basement membrane staining difficult to assess at this stage.

Thyroid histology

Light microscopy. Thyroids from normal control and $HgCl_2$-treated animals were comparable by light microscopy, with no cellular infiltrates at days 14 and 21 ($n=3$).

Immunofluorescence studies. Thyroids from normal and $HgCl_2$-treated animals showed no abnormalities on immunofluorescence.

DISCUSSION

Our findings confirm the action of $HgCl_2$ as a polyclonal activator *in vivo* in the BN rat (Hirsch *et al.*, 1982). Autoantibodies to GBM, thyroglobulin, collagen types I and II, and ssDNA and dsDNA were generated, and there was a rise in total IgG levels. However, of additional interest were differences in the regulation of synthesis of the individual autoantibodies produced. Whereas the antibody response to GBM and thyroglobulin was self-limiting, that to DNA (both ssDNA and dsDNA) was persistent and cyclical. Whether this reflects the differing involvement of T cell regulatory mechanisms remains unknown. It has been reported that polyclonal responses to $HgCl_2$ *in vitro* and *in vivo* are T dependent (Hirsch *et al.*, 1982), but potent stimulation of B cells may circumvent the requirement for T cells in the response to some normally T dependent antigens (Chiller & Weigle, 1973). The overall polyclonal response to $HgCl_2$ was clearly persistent, as shown by measurement of total IgG concentrations, which remained markedly elevated at a time when organ-specific autoantibody responses had fallen towards

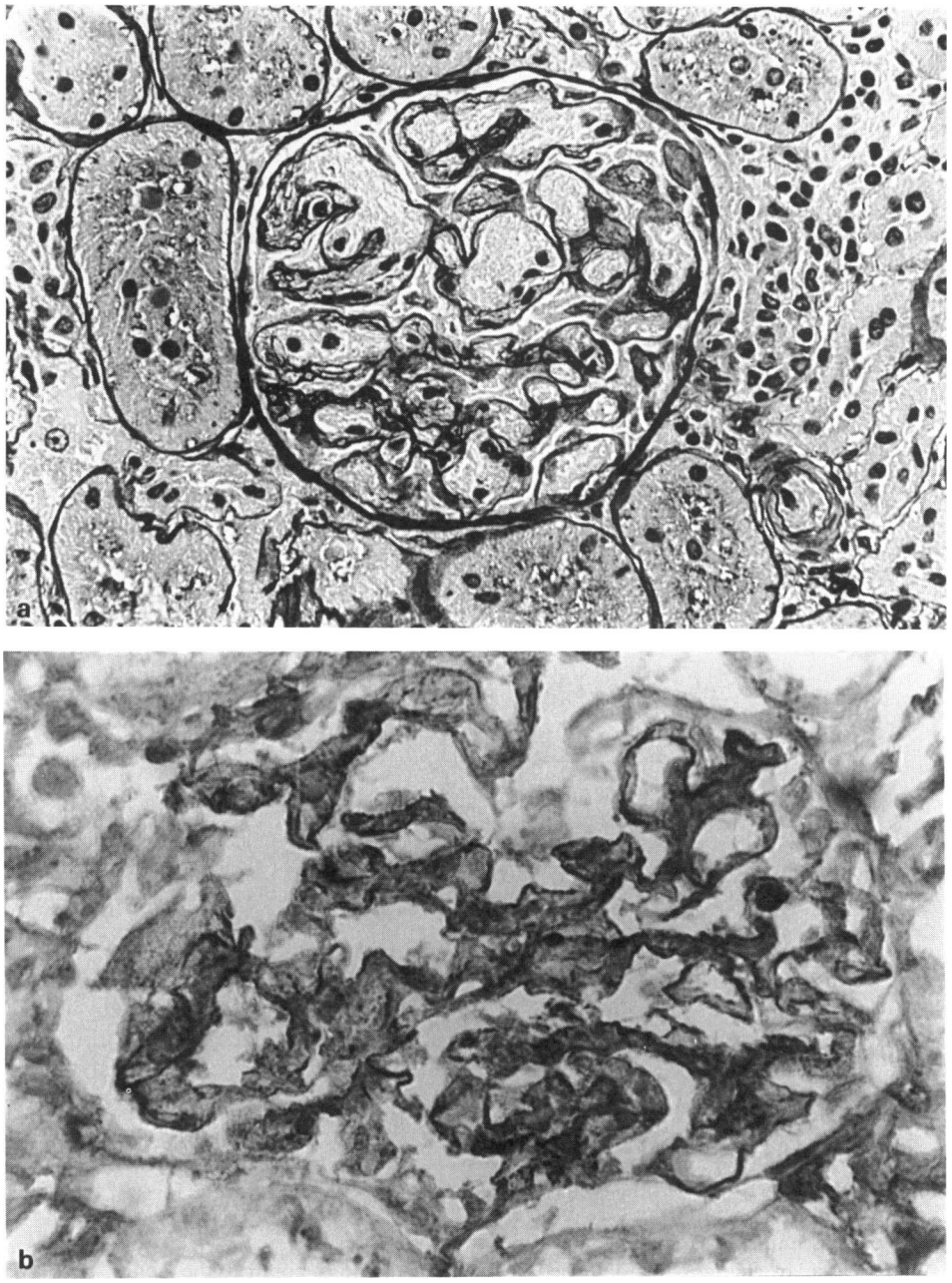

Fig. 5. (a) Glomerulus from biopsy performed at day 15 showing capillary lumens dilated by granular material, and frequent double contours of capillary walls (silver methenamine × 325). (b) Heavy granular localisation of IgG in capillary walls at day 22; it is still possible to distinguish some linear staining (peroxidase anti-rat IgG × 325).

background. Both transient and prolonged effects of drugs which induce autoantibodies in humans have been reported; for example, procainamide-induced cold-reactive lymphocyte antibodies decline quickly after stopping therapy (Bluestein *et al.*, 1979), but methyldopa-induced antibodies to red cells may persist for months after discontinuation of the drug (Kirtland, Mohler & Horwitz, 1980).

The mechanism by which $HgCl_2$ initiates polyclonal activation remains unclear; a possible explanation is inhibition of T suppression and/or induction of T help. Polyclonal immunoglobulin production has been shown to follow inhibition of T suppressor cell activity by agents such as methyldopa (Kirtland *et al.*, 1980), procainamide (Ochi *et al.*, 1983) and prostaglandin E (Ceuppens & Goodwin, 1982). Penicillamine is another polyclonal activator which induces autoimmune syndromes in both humans (Jaffe, 1981) and BN rats (Donker *et al.*, 1984), and which may act by direct B cell stimulation and/or augmentation of T help; recent work suggests selectivity for certain autoantigens in such responses (Rubin *et al.*, 1986). $HgCl_2$ has been shown to impair T suppressor cell function in the PVG rat (Weening *et al.*, 1981), and although polyclonal activation has not been demonstrated, the resulting membranous glomerulonephritis involves immune complexes composed in part of antinuclear autoantibodies. In the BN rat, $HgCl_2$ administration results in transient decreases in T suppressor cells in peripheral blood (Bowman *et al.*, 1987). However, direct stimulation of T

help is suggested by Pelletier *et al.* (1985) who demonstrated that T cells from $HgCl_2$ injected BN rats may induce proliferation of normal B and T cells *in vivo*. They also reported that $HgCl_2$ may induce autoreactive anti-Ia helper T cells (Pelletier *et al.*, 1986) which could lead to polyclonal autoantibody production and autoimmune disease. Furthermore, they have been able to demonstrate transfer of autoimmunity to normal BN rats using T cells from $HgCl_2$-injected animals (Pelletier *et al.*, 1988).

The constituents of the circulating immune complexes generated by $HgCl_2$ are unknown. However, the close proximity of their appearance to the fall in anti-GBM antibody levels suggests that they might be implicated in regulation of the autoantibody response, perhaps via the idiotypic network (Chalopin & Lockwood, 1984). If this is the case, then several other antigen/antibody systems could be involved, since our data show that $HgCl_2$ induces autoantibodies to thyroglobulin and collagen with similar kinetics. The relationship between circulating and deposited immune complexes in this model is not clear, and an alternative explanation for the granular deposits seen in the second phase of the disease is that they represent the generation of autoantibodies to discrete glomerular antigens, analogous to the antibodies to the epithelial cell glycoprotein antigen gp330 in Heymann's nephritis (Camussi *et al.*, 1985). Some support for this hypothesis comes from our unpublished observation that certain monoclonal antibodies derived from the spleen cells of $HgCl_2$-injected BN rats react in a granular pattern along the GBM (S.J. Cashman, personal communication).

Since polyclonal activation has been implicated in the pathogenesis of disease via autoantibodies (Fauci *et al.*, 1983), it is important to know whether these antibodies necessarily cause tissue damage. In $HgCl_2$-induced autoimmunity, we have confirmed the transient presence of both circulating and tissue-fixed anti-GBM antibodies and immune complexes at a time closely associated with the presence of proteinuria (Druet *et al.*, 1979; Bellon *et al.*, 1982). However, proteinuria occurred only in a proportion of rats and was not as severe as that reported by Druet *et al.* (1979). Histological changes in sequential renal biopsies, which have not been studied previously, were minor. In addition, there was no consistent relationship between levels of circulating anti-GBM antibodies or immune complexes and the degree of tissue injury as reflected by proteinuria. These findings could be explained by differences in the characteristics of the antibodies induced by $HgCl_2$, or by the composition of the complexes generated and it is of interest that no proteinuria was observed as a result of anti-GBM antibodies induced by penicillamine in the BN rat (Donker *et al.*, 1984). Our recent experiments, using BN rats from different sources, have shown more consistent proteinuria following $HgCl_2$ (unpublished observations), raising the possibility that genetic differences even within an inbred strain are involved. An alternative explanation is that environmental factors such as infection may increase the tissue injury provoked by fixation of a certain amount of antibody. This possibility was suggested by clinical observations in anti-GBM disease (Rees, Lockwood & Peters, 1977), and has subsequently been demonstrated in animal models of nephrotoxic nephritis (Tomosugi *et al.*, 1989). Although circulating anti-thyroglobulin antibodies were also readily detectable, light microscopy showed no injury in the thyroid gland. Failure to detect immunoglobulin deposition in thyroid could be related to poor accessibility of the antigen concerned. A similar lack of correlation between the autoantibody response and thyroiditis in various rat strains has been found by other investigators (Lillehoj & Rose, 1982).

As well as humoral components of the autoimmune response, cellular mechanisms may be important in mediation of tissue injury in glomerulonephritis, as shown in rat models of the autologous phase of nephrotoxic nephritis (Tipping, Neale & Holdsworth, 1985); Hinglais *et al.* (1979) noted an influx of monocytes in the glomeruli in $HgCl_2$ nephritis, which slightly preceded the onset of proteinuria. In our study, scant evidence of mononuclear cell infiltrates was seen in the kidney (although immunohistochemistry using antibodies specific for different leucocyte populations was not performed), and this could explain the lower incidence and severity of proteinuria. The minor vascular changes observed in our animals could be related to the development of disseminated intravascular coagulation, as previously demonstrated in BN rats treated with $HgCl_2$ (Michaud *et al.*, 1983) and penicillamine (Donker *et al.*, 1984); the cause of this remains unknown.

We have shown here that polyclonal activation and a range of autoantibodies are induced *in vivo* by the administration of $HgCl_2$ to BN rats. Autoantibody responses continue for a variable length of time, some undergoing rapid autoregulation and others persisting long-term; whether this reflects differences in autoregulatory mechanisms is not yet known. We failed, however, to show a consistent correlation between levels of organ-specific autoantibodies or immune complexes and tissue injury, and this indicates the need for further studies to define the relationship between the induction of autoimmunity and the production of target organ damage. Mercury is well recognized as a cause of glomerulonephritis in humans (Wedeen, 1984), although the mechanisms involved remain obscure, and our studies should contribute to the understanding of environmentally induced autoimmune disease.

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