Experimental Autologous Immune Deposit Nephritis in Rats Associated with Mercuric Chloride Administration*

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Summary. Serial administration of mercuric chloride to rats was followed by development of antibodies to tubular basement membrane and renal tubular epithelial antigen (RTE) and glomerulonephritis characterized by granular deposits of host IgG, C_3 and RTE along the glomerular capillary walls. The glomerular fixed antibody was directed against RTE. These studies suggest that tubular injury by mercury may lead to release of RTE and autosensitization and subsequent antibody production to this antigen result in formation of and glomerular deposition of circulating immunopathogenic complexes (RTE-anti-RTE) and glomerular morphologic alterations.

Exposure to heavy metals is known to be associated with renal damage in both man and experimental animals. Many of these agents cause tubular injury, however, glomerular alterations have also been observed with heavy metal exposure. Recently an immune deposit nephritis has been described in rats following intramuscular administration of mercury salts[4]. Antigenic components present in renal tubules have been implicated in immune complex glomerulonephritis and tubulointerstitial nephritis in man and experimental animals[5–21]. Since mercury is a tubular toxin it is possible that one or several of these antigens may be of immunopathogenic significance in the immune mediated glomerular disease associated with this compound. The present study was designed to elucidate immunopathogenic mechanisms in mercurial nephritis in rats.

Materials and methods. 35 male Lewis rats of approximately 375 g body weight were used in these studies. 20 animals (Group I) received s.c. injections of mercuric chloride ($HgCl_2$) 0.15 to 0.3 mg per 100 g of body weight 3 times weekly for approximately 7 weeks. The other 15 animals were used as controls (Group II) and received i.m. injections of saline in a volume equal to that of the mercury treated group. All animals were placed in metabolic cages, and fed on Purina rat chow and water ad libitum.

Prior to the onset of the study, and weekly thereafter, 24 h urine collections were obtained on all animals. Urine volume was recorded and urinary excretion of protein, sodium, potassium, chloride, calcium and phosphorus quantitated. Urine osmolality was measured and qualitative blood, proteine and glucose determined on random urine samples using Hemacombistix (Ames Labs.). The dosage of mercuric chloride was varied in relationship to the severity of the tubular disease as evidenced by glycosuria, phosphaturia, polyuria and urinary excretion of sodium, potassium and chloride. In animals showing severe tubular damage, the lower dosage was used, whereas those showing mild or no tubular functional impairment, received the higher dosage. Thus, each injection varied in relationship to the laboratory parameters. Serum was obtained prior to beginning the study and at the time of sacrifice. Sodium, potassium, chloride, calcium and phosphorus were measured. Serum levels of IgG were quantitated by radial immunodiffusion. Serum total hemolytic complement was determined by hemolytic assay and C_3 was quantitated by radial immunodiffusion.

Globulins isolated from the serum of each animal were conjugated with Fluorescein isothiocyanate (FITC). FITC conjugated globulins were used to stain 4 μm sections of normal human, Brown Norway (BN), and Lewis rat kidneys. Antibody to renal proximal tubular epithelial antigen (RTE) and renal tubular basement membrane (TBM) was measured in the serum by ouchterlony immunodiffusion in agar gel complement fixation and agglutination[20,22–25]. RTE 5 was prepared by the method of GLASSOCK and EDGINGTON[6,7]. TBM was prepared according to the method of MAHIEU[26]. These procedures have been described by us in earlier communications[20,22–25].

At the time of sacrifice, kidneys were removed and a portion was placed in isopentane and snap frozen in liquid nitrogen for immunohistologic studies. Another part of the kidney was placed in Bouin's solution and 3 μm parrafin sections stained with hematoxylin and eosin (H and E) and periodic acid Schiff (PAS) and examined by light microscopy. Immunohistologic studies were performed on 4 μm cryostat frozen sections of renal tissue utilizing monospecific FITC conjugated rabbit antisera to rat IgG, C_3, fibrinogen and albumin. The preparation of antisera and immunohistologic methods have been detailed previously[25]. In addition, immunohistologic studies were

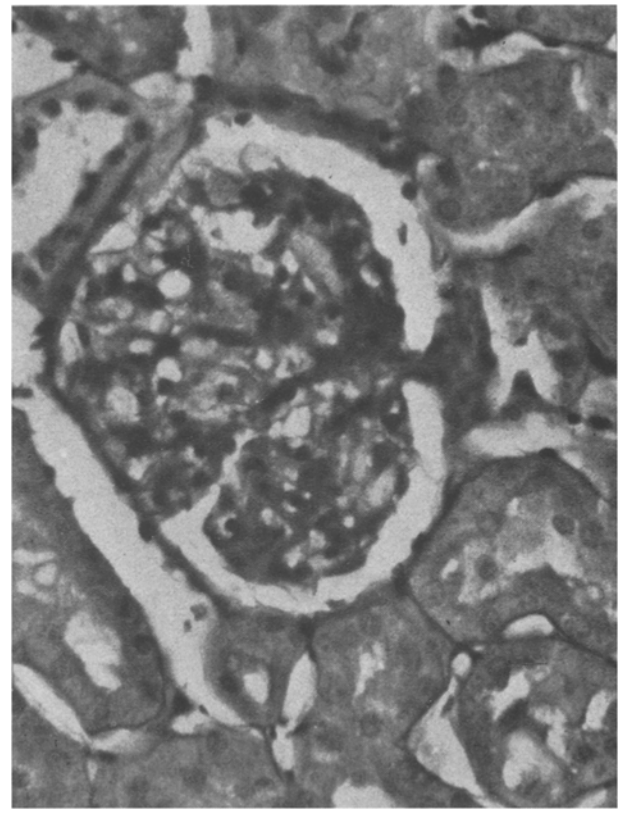

Fig. 1. Representative glomerulus from mercury treated animal No. 345 showing increase in mesangial matrix mild hypercellularity and basement membrane thickening (PAS).

done using FITC conjugated rabbit antisera to rat renal tubular epithelial antigen before and after pretreatment of the slides with 0.01*M* citrate buffer pH 3.2 and before and after absorbtion with RTE and TBM. Antisera to RTE was prepared by serial immunizations of rabbits with this antigen. These methods have been detailed previously[24, 25, 27].

The remainder of the kidneys were frozen in saline at −70 °C for elution studies. Kidneys from animals showing immune renal deposits were pooled. Glomeruli were isolated and glomerular fixed antibody eluted as described previously[5, 24, 25, 27]. The eluate was tested for the presence of rat serum proteins by immunodiffusions and immunoelectrophoresis. The eluate was also tested for anti-TBM and anti-RTE antibody activity by double layer immunofluorescence on normal BN and Lewis rat and normal human kidneys and jejunal mucosa, using FITC rabbit anti-rat immunoglobulins as the second layer[24]. Control slides were stained with normal rabbit serum and the labeled antisera without eluate. In addition, eluates were tested for antibody activity to these antigens by immunodiffusion complement fixation and agglutination.

Results. Animals in Group I received a total of 15 to 18 mg mercuric chloride (Mean = 16.8 mg). All animals in this group developed varying degrees of glycosuria and phosphaturia during the course of the experiment. The saline treated animals, Group II, showed no urinary abnormalities. Hematuria and mild proteinuria were detected in 25% of the animals in Group I. Hematuria was not found in any animals in Group II. Urine protein excretion, while not markedly elevated in the experimental animals, was significantly higher than in controls. No differences were observed in serum IgG, C_3 or total hemolytic complement levels between the mercury and saline treated rats. Similarly no significant differences in serum electrolytes were observed between the two groups.

FITC conjugated serum globulins obtained from all the mercury treated animals at sacrifice, stained the tubules but not the glomeruli of normal Lewis, BN and human kidney. No glomerular staining was observed. The immunofluorescent staining was not greatly diminished by absorption with either RTE or TBM, but was no longer observed by absorption with a combination of both of these antigens. No staining was detected using FITC labelled globulins from control rats. Antibodies to both RTE and TBM were demonstrated in the sera of the animals in Group I while antibodies to these antigens were not detected in sera of control animals.

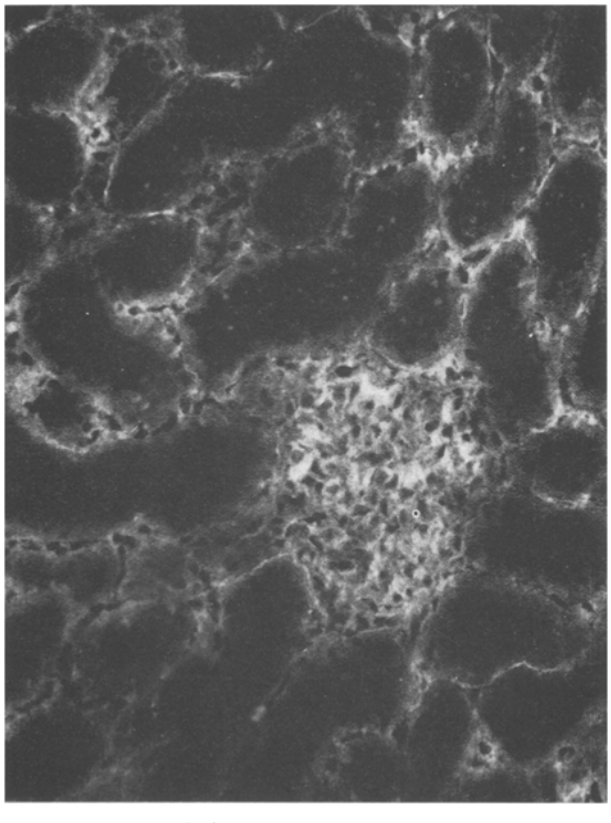

Fig. 2. Immunohistologic staining of renal tissue from the same animal with FITC rabbit anti-rat IgG. Glomerular basement membrane and mesangial as well as proximal tubular staining.

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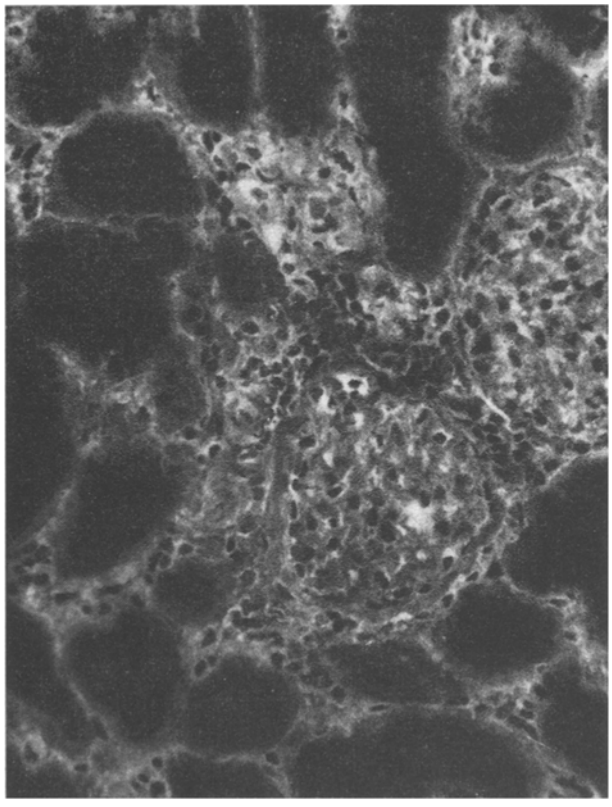

Fig. 3. Staining of renal tissue with FITC rabbit antisera to RTE. The immunofluorescent pattern in the glomeruli and tubules is similar to that observed with antisera to IgG.

The results of light microscopic studies on mercury treated animals are summarized in Table I. Hypercellularity and increase in mesangial matrix were the most prominent histologic alterations (Figure 1). Glomerular basement membrane thickening was observed in only a few animals and tubular alterations were not prominent. No morphologic abnormalities were seen in the saline treated group.

The results of immunohistologic studies on the mercury treated animals are summarized in Table II. IgG and C_3 were observed in a granular pattern along the glomerular basement membranes and in the mesangium (Figure 2). Tubular staining, when present, was seen only in proximal tubules. RTE was detected in the glomeruli in the same pattern as IgG and C_3. In addition, RTE was present on all proximal tubules (Figure 3). Glomerular staining with antisera to RTE was more prominent after pretreatment of the slides with citrate buffer. Immunofluorescence was no longer observed after absorption with RTE, but was not affected by absorption with TBM. Albumin and fibrinogen were not localized in the kidney of any animals. No immunofluorescent deposits of IgG or C_3 were found in any of the control animals. However rabbit antisera to RTE stained proximal tubules but not glomeruli of control animals. Glomerular eluates contained rat IgG. No other serum proteins were detected in the eluates by the methods used. Eluates stained proximal tubular brush border but not glomeruli of normal human, Brown Norway and Lewis rat kidney (Figure 4). Antibody activity, in addition, eluates fixed to normal rat and human jejunal mucosa. Antibody activity to RTE was present in the glomerular eluate. However, no antibody to TBM was detected by the methods employed in these studies.

Discussion. The effects of mercury on the kidney are well recognized[28–34]. Renal lesions include proximal tubular and Fanconi syndrome due to mercurial diuretics,

Table I. Morphologic studies on kidneys of mercury treated animals

No.	Glomeruli				Interstitium			Vessels	Tubules
	Cell↑	Mesang matrix↑	GBM↑	Exudate	Sclerosis	Edema inflam.	Fibrosis	Vascular Injury	
284	2 +	1 +	0	0	0	0	0	0	1 + tubular atrophy tubular casts
310	1 +	1 +	0	0	0	0	0	0	0
311	1 +	1 +	0	0	0	0	0	0	0
323	0	0	0	0	0	0	0	0	0
324	0	0	0	0	0	0	0	0	0
325	2 +	1 +	0	1 +	0	0	0	0	0
327	0	0	0	0	0	0	0	0	0
328	0	0	0	0	0	0	0	0	0
339	2 +	4 +	2 +	0	1 +	0	0	0	0
340	2 +	3 +	1 +	0	0	0	0	0	0
341	1 +	1 +	0	0	0	0	0	0	0
342	1 +	1 +	0	0	0	0	0	0	0
344	2 +	2 +	1 +	0	0	0	0	0	0
345	2 +	2 +	1 +	0	0	0	0	0	0
346	2 +	3 +	0	1	0	0	0	0	0
347	2 +	1 +	1 +	0	0	0	0	0	focal tubular necrosis
348	1 +	1 +	0	0	0	0	0	0	focal tubular necrosis
349	1 +	1 +	0	0	1 +	1 +	0	0	0
350	1 +	2 +	0	0	0	0	0	0	severe tubular necrosis
353	3 +	2 +	1 +	+	0	0	0	0	tubular casts and atrophy

Graded by intensity 0–4 +.

calomel and ammoniated mercury ointments; proliferative glomerulonephritis due to mercurial teething powders, ammoniated mercury and mercury paint additives; albuminuria and basement membrane thickening due to inorganic or phenyl mercuries; and the nephrotic syndrome from seed disinfectant. The administration of mercuric chloride to rats and guinea-pigs has usually been associated with tubular lesions[28–30]. Recently immune deposit diffuse membranous glomerulonephritis and segmental and focal membranous glomerulonephritis has been observed in rats receiving mercuric chloride[4]. However, the immunopathogenic events and the nature of the antigen was not defined.

The demonstration of autologous immune complex membranous nephropathy in rats with renal tubular epithelial antigen[6,7] and the demonstration of the immunopathogenic significance of this antigen in membranous nephropathy[19] has stimulated investigations as to the role of this antigen in human renal disease. We have demonstrated an immunopathogenic role for this antigen in several histologic forms of glomerulonephritis associated with diseases which affect renal tubules[5,22,24,25]. Several recent publications have implicated tubular basement membrane antigen in tubulointerstitial nephritis in experimental animals as well as in man[8–18]. Several of the reports in man have been associated with drugs[9,15]. TBM antigen appears to be related to species as well as strain[16,17]. The demonstration that RTE cross reacts in man and rats and the finding of immunoreactive RTE in only one other anatomic site, the small bowel mucosa[6,7] in addition to the observation in human studies of the presence of immunocrossreactive TBM in Brown Norway but not in Lewis rats[17], has aided differentation between these two tubular antigens.

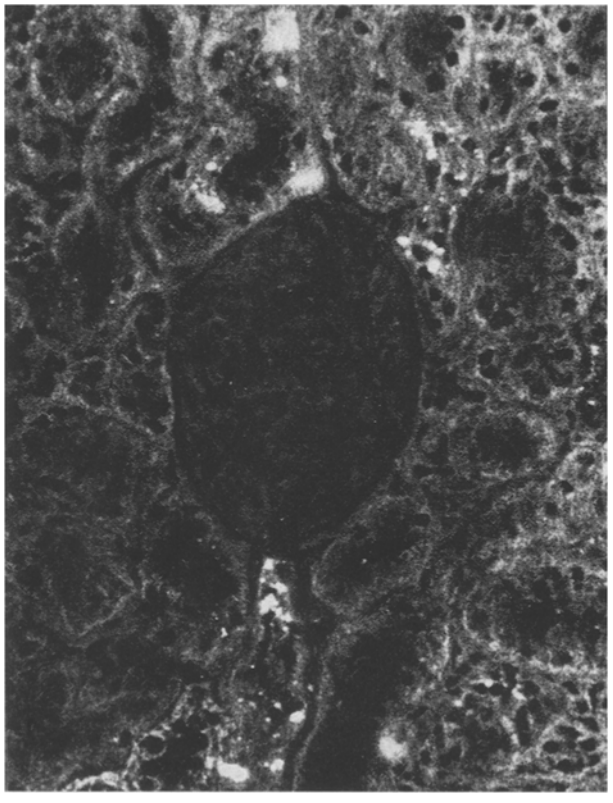

Fig. 4. Staining of normal human kidney with IgG eluted from diseased glomeruli from diseased mercury treated rats. Proximal tubular staining is prominent. No staining is observed in glomeruli.

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Table II. Immunohistologic studies on kidneys of mercury treated rats

No.	Antisera to rat									
	IgG		C_3		RTE		Fibrinogen		Albumin	
	Glom.	Tub.	Glom.	Tub.	Glom.	Tub.	Glom.	Tub.	Glom.	Tub.
284	+	+ +	+	+ +	+	+ +	0	0	0	0
310	+	+ +	+	+ +	+	+ +	0	0	0	0
311	+	+ +	+	+ +	+	+ +	0	0	0	0
323	0	0	0	0	+	+ +	0	0	0	0
324	0	0	0	0	0	+ +	0	0	0	0
325	+	+ +	+	+ +	0	+ +	0	0	0	0
327	0	0	0	0	0	+ +	0	0	0	0
328	0	0	0	0	0	+ +	0	0	0	0
339	+	+ +	+	+ +	+	+ +	0	0	0	0
340	+	+ +	+	+ +	+	+ +	0	0	0	0
341	+	+ +	+	+ +	+	+ +	0	0	0	0
342	+	+ +	+	+ +	+	+ +	0	0	0	0
344	+	+ +	+	+ +	+	+ +	0	0	0	0
345	+	+ +	+	+ +	+	+ +	0	0	0	0
346	+	+ +	+	+ +	+	+ +	0	0	0	0
347	+	+ + +	+	+ + +	+	+ +	0	0	0	0
348	+	+ + +	+	+ + +	+	+ +	0	0	0	0
349	+	+ +	+	+ +	+	+ +	0	0	0	0
350	+	+ + +	+	+ + +	+	+ +	0	0	0	0
353	+	+ + +	+	+ + +	+	+ +	0	0	0	0

+, positive; + +, proximal tubular staining; + + +, diffuse staining of all tubules.

Since several heavy metals, drugs and toxins, as well as other factors, such as hemodynamic alterations, damage the renal tubules it is attractive to speculate that such injury may lead to release of one or more tubular antigens resulting in antibody formation. Antibody may induce tubular damage resulting in tubulointerstitial disease or form immune complexes with antigen in the circulation. Deposition of antigen-antibody complexes may result in an immune complex glomerulonephritis. The morphologic and immunohistologic alterations apparently are related to the nature of the tubular antigen or antigens involved.

In these studies, mercuric chloride administration was associated with an immune complex nephritis apparently secondary to deposition of RTE-anti-RTE immune complexes. Although antibodies to TBM were detected in the serum of these animals, tubulointerstitial disease usually associated with this antigen was not prominent and TBM could not be implicated in the pathogenesis of the glomerular lesions.

These studies support the hypothesis that tubular injury induced by mercuric chloride may be followed by release of renal proximal tubular antigen, subsequent formation of antibody to this antigen, followed by formation and glomerular deposition of immune complexes of RTE-anti-RTE resulting in an autologous immune complex glomerulonephritis. This etiopathogenic mechanism may occur in man and may also be a factor in the development of several cases of nephritis associated with agents which produce tubular alterations. The significance of TBM in this study is unclear.

Ultrastructural Identification of Human Tonsil T-Lymphocytes by Peroxidase-Conjugated Anti-HTLA Serum[1]

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Summary. A horse anti-serum rendered specific for human T-lymphocytes was conjugated with peroxidase and used for ultrastructural identification of human tonsil T-lymhocytes. With T- and B-enriched suspensions, virtually all T-lymphocytes were labelled with Po-anti-HTLA, whereas no B-cells were stained with this conjugate. The labelling was found to be irregularly distributed on the plasma membrane of T-cells. Direct identification with specific Po-anti-HTLA conjugate confirm the ultrastructural characteristics of thymus-dependant cells.

Human T-lymphocytes are often identified by the formation of rosettes with sheep erythrocytes[5] (E), although the mechanisms and specificity of the binding are still unclear. Demonstration of an antigen specific for the T-cell lineage by using specific antisera[6] provides a new approach for enumeration and characterization of T-lymphocytes in cell suspensions of various origins. We have prepared a horse antiserum specific for human T-lymphocyte antigen (HTLA). Criteria of specificity of this serum are described in another publication[7]. We report here its use for ultrastructural studies of T-lymphocytes after conjugation with peroxidase.

Material and Methods. Lymphocyte suspensions: tonsils freshly obtained from operation were teased with forceps into Hanks, washed twice, centrifuged (20 min, 400 *g*, 4 °C) on Ficoll-Isopaque (6.3%–9.9% final concentration; density 1.080) and washed 3 times. T- and B-enriched suspensions were prepared by centrifugation on Ficoll-Isopaque after T-cells had been allowed to form E rosettes: T-enriched cells were recovered at the bottom and used after E lysis by NH_4Cl (0.87%, 10 min) and B-enriched cells at the interface.

Anti-HTLA serum: anti-HTLA serum was prepared from anti-human thymocyte globulins (Institut Mérieux, Lyon) sequentially absorbed on AB red cells, polymerized Ig, placental tissue and lymphoblastoid cell lines[7]. Cytotoxicity tests were performed as previously described[8].

Peroxidase-labelling of anti-HTLA Ig: peroxidase-conjugated anti-HTLA (Po-anti-HTLA) Ig were prepared according to AVRAMEAS' technique[9]. Lymphocytes were fixed in 2% paraformaldehyde (20 min, 4 °C), washed with PBS and incubated with Po-anti-HTLA Ig for 3 h at 37 °C. After washing with PBS, cells were fixed again with 2% glutaraldehyde, washed with PBS and incubated with Graham-Karnowsky medium for 30 min at 37 °C. Cells were washed with *tris*-HCl buffer (0.2 *M*, pH 7.6), post-fixed with OSO_4 1% in cacodylate buffer, and included in epoxy medium. Sections were observed unstained under the electron microscope (Philips EM 300).

Other techniques for T- and B-cell identification: T-cells were also characterized by the E rosette test[10] and B-lymphocytes by the detection of membrane Ig (S Ig) with a fluoresceinated anti-human Ig (Behring). In addition a B-lymphocyte and monocyte common antigen (HBLMCA) was identified by mean of a rabbit antiserum as previously described[7]. In electron microscopy studies, B-cells were identified by reaction with peroxidase-conjugated Fab anti-human Ig (Institut Pasteur, Paris) according to the technique described above.

Table I. Percent of T- and B-cells in tonsillar lymphocytes

	Anti-HTLA	E rosettes	Anti-HBLMCA	S Ig
No. of donors	47	14	40	7
Cells ± SE (%)	47.1 ± 1.7	42.8 ± 4.0	47.4 ± 1.8	40.7 ± 5.4

Table II. Percent of T- and B-lymphocytes in purified suspensions

	Anti-HTLA	Po-anti-HTLA	Anti-HBLMCA	Po-Fab anti-Ig
T-enriched (%)	91	96	4	5
B-enriched (%)	5	3	93	90