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Hypogammaglobulinaemia in nephrotic rats is attributable to hypercatabolism of IgG

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SUMMARY

The effect of the nephrotic syndrome induced by puromycin aminonucleoside (PA) in rats on specific antibody responses to 2,4 dinitrophenyl (DNP) conjugated to either spider crab haemocyanin (MSH), a T cell-dependent antigen, or hydroxyethyl starch (HES), a T cell-independent type 2 antigen were studied. The serum IgG anti-DNP levels following immunization with both antigens were reduced in nephrotic animals compared with controls while IgM anti-DNP antibody titres were higher. The half-life of IgG anti-DNP antibodies passively transferred into non-immunized nephrotic rats was markedly reduced while the half-life of anti-DNP antibodies of the IgM class was comparable to that in controls. Low serum IgG and elevated IgM levels were seen in nephrotic animals compared to controls. Antibody-forming cells specific for DNP were demonstrated by immunohistology on rat spleens and the numbers of both IgG and IgM-producing cells were found to be significantly increased ($P < 0{\cdot}05$) in nephrotic animals in response to both DNP-HES and DNP-MSH. These data indicate that in nephrotic rats the alteration seen in the serum immunoglobulin levels is not attributable to reduced antibody production but increased catabolism of serum IgG antibodies.

Keywords hypogammaglobulinaemia nephrotic syndrome

INTRODUCTION

Although total antibody responses to pneumococcal capsular polysaccharide vaccines in children with minimal change nephrotic syndrome (MCNS) are comparable to those of control subjects (Fikrig *et al.*, 1978; Wilkes *et al.*, 1979) children with this disorder are at an increased risk of life threatening infections due to *Streptococccus pneumoniae* (Wilfert & Katz 1968). There is evidence that in mice IgG anti-pneumococcal antibody affords much greater protection against pneumococcal infections than IgM (Briles *et al.*, 1981) and therefore measurement of total specific antibodies in the nephrotic syndrome following immunization may not provide a reliable guide to the level of protective antibody.

Serum levels of IgG are depressed in patients with MCNS in relapse and the levels of IgM raised (Giangiacomo *et al.*, 1975). The glomerular leak of protein in MCNS is largely of albumin with only minor losses of IgG, making it unlikely that low serum IgG levels in this condition are attributable to urinary losses of immunoglobulin. A defect in T cell-mediated switch of IgM to IgG synthesis has been proposed as a mechanism for the immunoglobulin abnormalities seen in MCNS (Giangiacomo *et al.*, 1975) although more recent data do not support this (Yokohama *et al.*, 1985). Data on IgG synthesis by peripheral blood lymphocytes *in vitro* from patients with MCNS are contradictory (Ooi *et al.*, 1980; Heslan *et al.*, 1982; Beale *et al.*, 1983). Aro and Hardwicke (1984) suggested that in patients with MCNS, IgG was lost through the formation of immune complexes and their phagocytosis by the reticulo-endothelial system.

To identify the mechanism(s) responsible for the hypogammaglobulinaemia seen in the nephrotic syndrome we investigated the effect of the nephrotic syndrome induced in rats by the administration of puromycin aminonucleoside (PA), on their antibody class and subclass response to 2,4-dinitrophenyl hydroxyethyl starch (DNP-HES), a T cell-independent type 2 antigen and DNP-haemocyanin (DNP-MSH) a T cell-dependent antigen (Gray *et al.*, 1985).

Selective proteinuria with minor glomerular changes similar to that seen in minimal change disease are consistently produced in rats given a single parental injection of PA (Hoyer *et al.*, 1972; Lannigan *et al.*, 1969).

MATERIALS AND METHODS

Animals

Inbred male Wistar rats weighing between 250 and 300 g were

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Table 1. The development of proteinuria following the administration of puromycin aminonucleoside

Days after PA or Saline	Proteinuria mg/24 h			
	DNP-HES (group 1) Nephrotic	Controls (group 2)	DNP-MSH (group 3)	Controls (group 4)
0	30 (7·9)	32 (7·2)	34 (17·6)	39 (15·7)
7	512 (110·3)	45 (9·2)	621 (163·2)	65 (17·0)
14	723 (51·7)	35 (14·7)	861 (141·1)	48 (12·5)

Values shown are the 24 h urinary protein excretion—mean (SD). There are no significant differences for DNP-HES v DNP-MSH on days 0, 7 and 14. Significant differences exist for DNP-HES v group 2 controls ($P=0{\cdot}01$ day 7 $P=0{\cdot}01$ day 14) and DNP-MSH v group 4 controls ($P=0{\cdot}01$ day 7 $P=0{\cdot}01$ day 14).

used throughout the study and kept in metabolic cages during the 24 h urine collections. They were fed a standard laboratory pelleted diet and fluids *ad libitum*.

PA induced nephritis

Animals were anaesthetized with diethyl ether. The nephrotic syndrome was induced by a single intraperitoneal injection of PA (Sigma Chemicals Co., UK) dissolved in 0·9% saline at a dose of 150 mg/kg. Control animals received an equal volume of 0·9% saline.

Antisera

Rabbit antibodies respectively specific for rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, IgA and IgM were prepared and their specificity tested as described by Bazin *et al.* (1978). These antibodies were a kind gift from Professor H. Bazin, Experimental Immunology Unit, University of Louvain, Belgium. Sheep antibody to total rat immunoglobulin (specific to both heavy and light chains) was prepared in the Immunodiagnostic Research Larboratory, Department of Immunology, University of Birmingham (IDRL). Sheep anti-rabbit and swine anti-sheep immunoglobulin antisera were also obtained from IDRL and labelled with ^{125}I using the chloramine-T method (Hunter & Greenwood, 1962). Swine immunoglobulins to rabbit immunoglobulins, horseradish peroxidase and rabbit anti-horseradish peroxidase (Rabbit PAP) and normal swine serum were obtained from Dakopatts, Denmark.

Substrates of Immunohistology

Levamisole, naphthol As-Mx phosphate, fast blue BB salt and 3-amino-9-ethylcarbazole were all obtained from Sigma Chemical Company, UK.

Antigens

Dinitrophenylated hydroxyethyl starch (DNP-HES) was a gift from the late Professor J. H. Humphrey, Royal Postgraduate Medical School, London and was prepared as described previously (Humphrey, 1981). Spider crab (*Maia squinada*) haemocyanin (MSH) was also supplied by Professor J. H. Humphrey and was dinitrophenylated to produce a substitution ratio of 150 DNP-MSH as described by Gray *et al.* (1985).

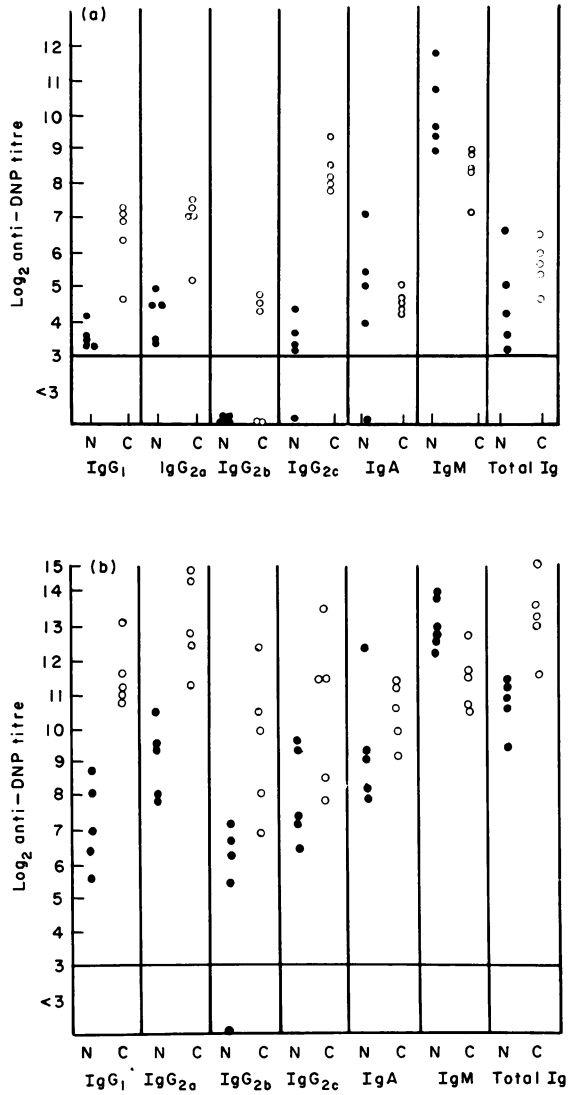

Fig. 1. Serum anti-DNP antibody titres in nephrotic and control animals of groups 1, 2, 3 and 4 following immunization with (a) DNP-HES or (b) DNP-MSH. Values are assessed 7 days after immunization. Nephrotic rats had received PA 7 days before immunization. C and ○ = control, N and ● = nephrotic rats.

Immunization

DNP-HES was injected i.v. in aqueous solution. Preliminary experiments confirmed the optimum dose to be 5 μg per rat with a peak response at 7 days (Gray, 1983). This dose and the 7 day response was used throughout. Boosting with this antigen does not produce a hightened secondary response or shift in isotype profile (Gray, 1983).

The primary injection of alum-precipitated DNP-MSH (50 μg) was given by intraperitoneal injection with 5×10^9 chemically killed *Bordatella pertussis* as adjuvant (Pertussis Vaccine, Wellcome, UK). Secondary immunization with 50 μg of DNP-MSH in an aqueous solution was given by intravenous injection 3 weeks after priming. Dose-response and time course experiments revealed the above dose to be optimal, the response reaching plateau levels by 7 days after secondary immunization (Gray *et al.*, 1983).

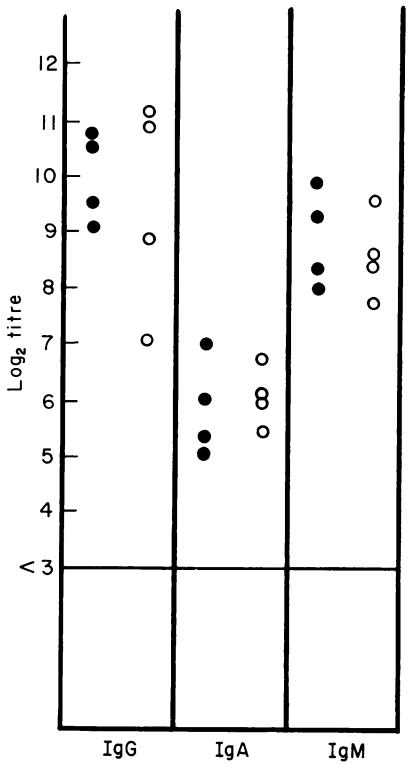

Fig. 2. The effect of puromycin aminonucleoside (PA) without the nephrotic state on specific antibody response. Post immunization titres 5 days after (PA)+DNP-HES. (○=Control animals and ●=PAN treated).

Radioimmunoassay for anti-DNP antibodies
This was performed as described previously (Gray *et al.*, 1985). Briefly, polystyrene multiwell plates (Linbrow, Flow Laboratories) were coated overnight at 4°C with 50 μg/ml DNP-ovalbumin (substitution ratio of 9:1 which was used consistently throughout this study) in 0·1 M carbonate buffer pH 9·5. Sequential incubations were performed for 1 h at room temperature with doubling dilutions of test sera, then rabbit anti-rat antibody or sheep anti-rat antibody and finally ^{125}I-sheep anti-rabbit IgG or ^{125}I-swine anti-sheep IgG. Between incubations the plates were washed five times with PBS supplemented with 0·2% bovine serum albumin. Titres were calculated by taking the dilution of serum giving 40% of maximum binding obtained with a positive control serum used consistently throughout the study. Pre-immunization titres were also measured and their arithmetic titre was subtracted from the arithmetic post-immunization titre if the increase in titre was less than four-fold.

Measurement of anti-DNP antibody half-life ($T_{\frac{1}{2}}$)
High titres of anti-DNP antibody were raised by repeated immunizations with DNP-MSH (50 μg) in healthy rats. One millilitre of the high-titre pooled sera was injected into four control and four nephrotic animals (7 days post PA injection). Daily venous sampling allowed the clearance of IgG and IgM anti-DNP antibodies to be determined using the radioimmunoassay described. Urine samples were examined for specific IgG anti-DNP using the radioimmunoassay described.

Proteinuria
Twenty-four-hour urine collections were obtained while the rats were housed in metabolic cages. Protein was measured on Days 1, 7 and 14 by the Biuret method (Wolfson *et al.*, 1948).

Serum Immunoglobulins
Serum levels of rat IgG, IgA and IgM were measured by single radial immunodiffusion using plates supplied by BDS Biologicals, Birmingham, UK.

Immunohistology
Class and subclass specific DNP-binding plasma cells were identified using techniques based upon those described by Claassen, Kors & van Rooijen (1986). Briefly, spleens were snap-frozen in liquid nitrogen and 5 μm sections prepared. Following fixation in acetone, DNP binding cells were detected using a DNP-alkaline phosphatase conjugate which was visualized using naphthol-AS-MX-phosphate, levamisole and fast blue BB salt. The isotype under investigation was detected using rabbit anti-rat antibody followed by a swine anti-rabbit immunoglobulin and a rabbit peroxidase anti-peroxidase complex. The peroxidase activity was visualized using 3-amino-9-ethylcarbazole. While single stained cells were either blue or red, double stained cells had a purple colour. The number of stained cells in the red pulp of spleens was determined using the point counting technique of Weible (Weible, 1963).

Experimental design
Pre-immunization samples were obtained from all animals by tail vein sampling. Groups of matched rats were treated as follows:

Group 1 ($n=5$) received PA on day 0 and DNP-HES on day 7 when preliminary experiments had shown that proteinuria was significantly increased and the animals nephrotic.
Group 2 ($n=5$) received 0·9% saline on day 0 and DNP-HES on Day 7.
Group 3 ($n=5$) received PA on day 0 and DNP-MSH on Day 7.
Group 4 ($n=5$) received 0·9% saline on Day 0 and DNP-MSH on Day 7. Groups 3 and 4 were primed with DNP-MSH 3 weeks before giving PA or saline.
Group 5 ($n=4$) received PA on day 0 and DNP-HES 12 h later.

Groups 1–4 were bled 7 days post-immunization to determine the immunoglobulin levels and both the class and subclass specific anti-DNP titres.

Statistical analysis
The Wilcoxon sum of ranks test was used as a statistical measure of the significance of differences between groups in this study. A two-tailed P value $>0·05$ was taken to be non-significant.

RESULTS

Proteinuria induced by PA
The mean (±1 s.d.) 24 h proteinuria for control and nephrotic animals is shown (Table 1).

Table 2. The number of antigen-specific plasma cells in the spleens of nephrotic and control rats

	DNP-HES-immunized rats		DNP-MSH-immunized rats	
	DNP binding cells/ 1·5 mm² of red pulp	Weight of spleen (mg)	DNP binding cells/ 1·5 mm² of red pulp	Weight of spleen (mg)
Nephrotic	547	780	768	810
groups 1 and 3	409	810	718	737
	495	765	802	888
	512	800	780	903
	532	770	820	760
Median	512†	780*	780†	810*
Control	394	770	296	789
groups 2 and 4	403	720	471	873
	290	800	466	777
	291	690	608	850
	349	760	310	817
Median	349†	760*	466†	817*

* No significant difference in spleen weight between nephrotic and control group.
† Nephrotic spleens contain significantly larger numbers of DNP binding cells than controls ($P=0·01$).

Table 3. The numbers of antigen-specific plasma cells of the different class and subclass in nephrotic and control rats

	Percentage of DNP-binding plasma cells in the spleen						
	DNP-HES-immunized rats					DNP-MSH-immunized rats	
Animal	IgM	IgG_1	IgG_{2a}	IgG_{2b}	IgG_{2c}	IgM	$IgG_1+IgG_{2a}+IgG_{2b}+IgG_{2c}$
Nephrotic	49	4	4	1	36	94	5
groups 1 and 3	68	3	2	0	30	90	5
	47	2	5	2	28	96	3
	43	4	2	0	41	91	4
	53	2	1	1	36	93	2
Median	49	3	2	1	36	93	4
Control	50	3	2	0	24	93	2
groups 2 and 4	48	2	3	0	40	95	3
	55	4	2	2	30	91	2
	52	4	0	3	32	94	4
	48	1	4	1	37	94	5
Median	50	3	2	1	32	94	3

Observations were made 5 days after immunization. The results are the number of class or subclass-specific hapten-binding plasma cells. There are no significant differences between the nephrotic and control groups.

The effect of PA on pre-immunization serum immunoglobulin levels

At the time of maximal proteinuria (day 14) the mean (±s.d.) serum IgG levels were depressed in nephrotic animals 0·48±(0·134) g/l compared to controls 1·84±(0·439) g/l ($P=0·01$). The IgA levels showed no significant difference between the groups with nephrotic levels of 0·14±(0·038) g/l and controls 0·14±(0·014) g/l. IgM levels were significantly elevated in nephrotic 1·63±(0·196) g/l compared to control animals 1·0±(0·149) g/l ($P=0·01$). No significant difference was seen in the serum IgG, IgA and IgM levels between the two groups of animals prior to the induction of the nephrotic syndrome.

Immunoglobulin class and subclass anti-DNP antibody titres following immunization with DNP-HES and DNP-MSH

In normal animals both antigens DNP-HES and DNP-MSH induced an antibody response of IgM, IgA and IgG isotypes. The IgG subclass response, however, was different for the two antigens (Fig. 1). The subclass titres are not absolute values.

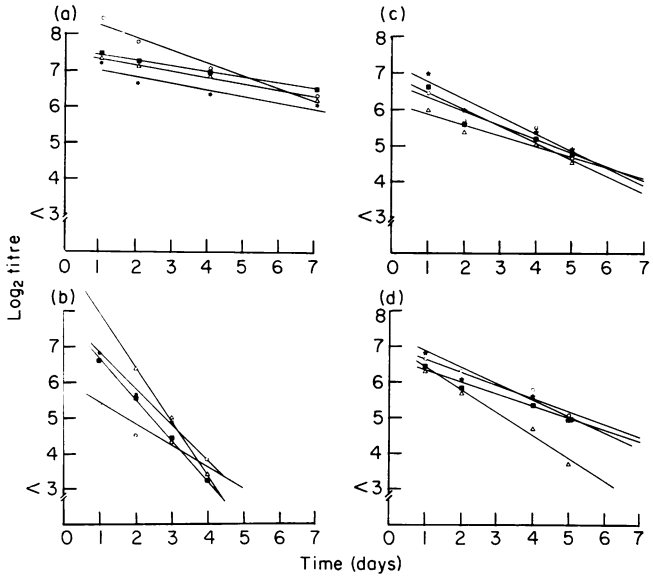

Fig 3. The clearance kinetics of IgG anti-DNP antibodies in control animals (a), IgG anti-DNP antibodies in nephrotic animals (b), IgM anti-DNP antibodies in control animals (c) and, IgM anti-DNP antibodies in nephrotic animals (d).

However, it is valid to compare the titres of each subclass in the different experimental groups. In nephrotic animals immunized with DNP-HES the titre of IgG_1, IgG_{2a} and IgG_{2c} anti-DNP antibodies were significantly lower than the titre of controls ($P=0{\cdot}01$). The IgG_{2b} anti-DNP titre was not significantly reduced, IgA titres of the two goups were similar and IgM titres in nephrotic animals significantly higher ($0{\cdot}01 < P < 0{\cdot}05$).

Following immunization with DNP-MSH, significantly lower anti-DNP titres were achieved in nephrotic as compared to control animals for IgG_1, IgG_{2a} ($P=0{\cdot}01$) and IgG_{2b} ($0{\cdot}01 < P < 0{\cdot}05$). Although IgG_{2c} titres were also lower in nephrotic rats this difference did not reach significant levels in this experiment. Again IgA antibody titres were similar in nephrotic animals whilst IgM antibody titres were higher ($P=0{\cdot}05$) as compared with the control group of animals.

Effect of puromycin on specific IgG anti-DNP levels prior to the onset of the nephrotic syndrome

In order to exclude a direct immunosuppressive effect of PA upon the serum antibody response, four animals were injected with PA followed by DNP-HES 12 h later. Five days after immunization and before the animals had developed a nephrotic syndrome, the specific antibody titres of the IgG, A and M class were not significantly different from those of controls (Fig. 2). Further evidence indicating that immunosuppression induced by PA was not responsible for the reduced IgG titres is provided by the assessment of plasma cell numbers in the spleens of these rats.

The numbers of antibody forming cells in the spleen of nephrotic and control rats following immunization with DNP-MSH and DNP-HES

Preliminary experiments showed that the time of the peak splenic plasma cell response following primary immunization with DNP-HES and secondary imunization with DNP-MSH was 5 days after antigenic challenge. This time, therefore, was used to see if the number of plasma cells induced in nephrotic rats was different from controls. The total number of cells binding DNP-alkaline phosphatase to their cytoplasm was elevated in nephrotic rats compared with controls (Table 2). Simultaneous analysis of anti-DNP-containing cells by the class and subclass of antibody produced using immunoperoxidase showed no significant alteration in the proportion of antigen binding plasma cells expressing any of the heavy chain isotypes in nephrotic animals (Table 3).

Over 90% of the cells in the red pulp of rats 5 days after boosting with DNP-MSH contained IgM in both nephrotic and control animals. Following immunization with DNP-HES a different isotype profile was found (Table 3). Here 30–40% of the hapten-binding plasma cells were of the IgG_{2c} subclass in both the nephrotic and control groups.

Half-life ($T_{\frac{1}{2}}$) of anti-DNP antibodies in nephrotic compared with control rats

The mean $T_{\frac{1}{2}}$ of IgG anti-DNP antibodies in normal animals was 5·3 days $\pm$ 1·5 days which was significantly reduced ($P<0.05$) in the nephrotic animals to 1·1 $\pm$ 0·4 days (Fig. 3a, b). The $T_{\frac{1}{2}}$ of IgM anti-DNP antibodies was not significantly different with a mean of 2·5 $\pm$ 0·5 for the controls and 2·4 $\pm$ 0·6 for the nephrotic animals (Fig. 3c, d). We were unable to raise sufficiently high titres of IgA anti-DNP antibodies to measure the $T_{\frac{1}{2}}$ of this isotype. We failed to detect urinary IgG anti-DNP antibodies using the radioimmunoassay described.

DISCUSSION

This study demonstrates that the experimental nephrotic syndrome causes both a selective depression of total IgG immunoglobulin levels and a reduction of the levels of specific IgG serum antibodies achieved following imunization with DNP-HES and DNP-MSH. As IgM levels are elevated and there is no effect on IgA levels, measurement of total specific antibody titres will not necessarily identify the selective hypogammaglobulinaemia associated with the nephrotic syndrome. This may explain why studies in humans looking at total titres of antibodies against pneumococcal capsular polysaccharides (Fikrig *et al.*, 1978; Wilkes *et al.*, 1979) have failed to identify a reduction in these antibody titres in patients with minimal change disease. IgG_{2c} is the major IgG subclass produced in response to DNP-HES in rats (Gray *et al.*, 1985). This was more markedly depressed in nephrotic animals compared with controls than any of the other subclasses. Evidence exists for structural and functional homology between rat IgG_{2c} and mouse IgG_3, based on serological cross-reactivity, and their dominant position in responses to polysaccharide antigens in both species (Der Balian *et al.*, 1980). Briles *et al.* (1981) demonstrated a different degree of protection against pneumococci afforded by anti-pneumococcal polysaccharide antibodies of different heavy chain classes. They found that mouse IgG_3 anti-pneumococcal polysaccharide antibodies were 40 times more protective than IgM antibodies on a weight for weight basis. If the response in nephrotic patients is comparable then the alteration of IgG and IgM isotypes seen in the nephrotic rats to these antigens may explain the susceptibility of nephrotic individuals to pneumococcal infection.

Puromycin does not itself directly depress the humoral antibody response to sheep red blood cells as measured by haemagglutinin IgM titres (Garin, Sausville & Richard, 1983)

until the nephrotic state has developed. We have confirmed this by demonstrating that following puromycin administration but prior to the development of the nephrotic state normal IgG, IgA and IgM anti-DNP antibody titres were achieved. Also, our analysis of specific antibody producing cell numbers in nephrotic and control rats indicate that antibody responses were not impaired in rats with a puromycin-induced nephrotic syndrome.

The low serum levels of IgG and elevated levels of IgM in rats with PA-induced nephrotic syndrome in this study are identical to those seen in patients with MCNS (Giancomo *et al.*, 1975). In this model of the nephrotic syndrome the immunoglobulin class and IgG subclass response to a T cell-independent and T cell-dependent antigen were broadly comparable and consisted of a low IgG subclass response, a normal IgA and a raised IgM response as compared with control animals. There is no reason, therefore, to postulate that the serum imunoglobulin abnormalities in puromycin nephropathy or the isotype pattern of antibody response are mediated by a defect in T cell help as has been suggested for MCNS (Giangiacomo *et al.*, 1975).

In animals with puromycin-induced nephrotic syndrome there was an increased loss of IgG anti-DNP antibodies presumably due to increased catabolism of IgG, as we were unable to detect specific IgG anti-DNP antibodies in the urine, a feature reflecting the selective nature of the proteinuria.

In conclusion the present study suggests that the low serum IgG found in the nephrotic syndrome is due to increased catabolism. We found no evidence of impaired antibody responses in these nephrotic rats. The global finding of reduced IgG antibody levels in all types of nephrotic syndrome in man as well as in experimental nephrotic syndrome in rats makes it tempting to postulate that a common mechanism is responsible for this phenomenon.

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