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The Effects of a Stable Analogue of PGE_1 on the IgG Subclass Response to Particulate Bovine Tubular Basement Membrane in the Brown–Norway Rat (42567)

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Abstract. The IgG subclass and the IgM isotype response to immunization with particulate bovine tubular basement membrane (TBM) and adjuvants was studied in Brown–Norway rats receiving daily injections of a stable analogue of PGE_1 (M-PGE_1). M-PGE_1 slightly reduced the average quantity of circulating TBM antibody as well as the average quantity of eluted IgG per gram of renal tissue as compared to controls. However, M-PGE_1 did not qualitatively affect the distribution of the IgG subclass or IgM isotype response to TBM. The IgG response, which occurred predominantly in the IgG_1 and IgG_{2a} subclasses, increased from Days 8 to 14 after immunization, while the IgM response decreased over the same time period. The percentage of TBM antibody in the IgG_{2b} subclass was markedly decreased as compared to the percentage of IgG_{2b} antibody in total IgG. A substantial heterogeneity in the IgG subclass response was noted among individual rats with IgG_1 constituting from 46 to 82% of circulating TBM antibody. Although no correlation between the IgG subclass response and the severity of tubulointerstitial nephritis was noted, heterogeneity in the IgG subclass response to autoantigens may, nevertheless, theoretically play an important role in the pathogenesis of autoimmune inflammatory phenomena.

A stable analogue of PGE_1 (15S-15-methyl PGE_1; M-PGE_1) was recently demonstrated in our laboratory to abrogate or strongly inhibit the development of experimental autoimmune tubulointerstitial nephritis (TIN) in the Brown–Norway (BN) rat (13, 15) after immunization with particulate bovine tubular basement membrane (TBM) and adjuvants (9). Tubulointerstitial nephritis in the BN rat is a model of autoimmune inflammation characterized by the production of circulating anti-TBM antibody and the deposition of IgG in a linear circumferential fashion along cortical renal TBM. The deposition of IgG along renal TBM is followed by the fixation of C3 and an acute inflammatory infiltrate (9). Decomplementation strongly inhibits the development of the acute inflammatory infiltrate (14).

PGE_1 may theoretically inhibit immune-mediated inflammation by inhibiting the induction of the immune response or by inhibiting the function of inflammatory cells. In our previous experiments, TBM binding by serum from M-PGE_1-treated and control rats at 10-fold serum dilutions was equal as measured by ELISA, but TBM binding by serum from M-PGE_1-treated rats was slightly, though significantly, decreased at greater serum dilutions (15). TBM binding by pooled renal eluates from M-PGE_1-treated and control rats was approximately equal (15). The possibility that M-PGE_1 might inhibit the development of TIN by altering the IgG subclass response to TBM was not addressed in our previous study. Little information is available regarding the possible effects of prostaglandins on the IgG subclass response to immunization or, specifically, on the nature of the IgG subclass response to TBM in BN rat TIN. The purpose of the present investigation was to determine the distribution of the IgG subclass response to TBM in serum and renal eluates of M-PGE_1-treated and control BN rats immunized with TBM and adjuvants.

Materials and Methods. *Immunization and administration of prostaglandins.* BN rats (Harlan–Sprague–Dawley, Indianapolis, IN) weighing 150–200 g were immunized with particulate bovine TBM and adjuvants, as previously described (9, 15). M-PGE_1 (the generous gift of Dr. John Pike, Upjohn Co.) was administered by subcutaneous injection at a dose of 1 mg/kg/day. M-PGE_1 was stored

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in absolute ethanol (EtOH) at 10 mg/ml at −20°C and was diluted 1:5 in phosphate-buffered saline (PBS) immediately prior to injection. One group of rats received daily injections of M-PGE_1 (n = 5) or PBS (n = 5) on Days 0–14. Serum was obtained by cardiac bleeding from these rats at the time of sacrifice on Day 14. A second group of rats received daily injections of M-PGE_1 (n = 5) or 20% EtOH (n = 5) on Days 0–7 after immunization. Serum was obtained by tail bleeding on Day 8 and by cardiac bleeding at the time of sacrifice on Day 14 (15).

Determination of rat IgG subclass and IgM isotype in rat hybridoma supernatants. Supernatants from rat hybridomas (produced in the laboratory of G.A.G.) were screened for the presence of IgG subclasses and IgM employing an ELISA assay. Goat anti-rat Ig (American Qualex, Inc., La Mirada, CA) was incubated in 96-well microtiter plates, and in succeeding steps the following were added: rat hybridoma supernatant; rabbit anti-rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c} or IgM (American Qualex, Inc., La Mirada, CA); and peroxidase-conjugated goat anti-rabbit IgG. The chromogen *o*-phenylenediamine (0.4 mg/ml in citrate buffer) together with hydrogen peroxide was added as the final step in this and all subsequent ELISA assays. The reaction was stopped with 50 μl 1 *N* HCl, and absorbance was measured at 492 nm with a Multitek multiscanner. Hybridoma supernatants containing sufficient amounts of a single IgG subclass or IgM were chosen for use as standards.

The specificity of the rabbit antisera to the IgG subclasses and to the IgM isotype was confirmed by testing the immunoreactivity of each antiserum against hybridoma supernatants of all four IgG subclasses and IgM (Table I). The antisera were found to be highly specific for the appropriate subclasses and isotype, and little, if any, more background absorbance was noted in the assays when irrelevant hybridoma supernatants other than IgG_{2c} were employed than when PBS was employed. All antisera were found to cross-react to some degree with IgG_{2c}. Fortunately, however, this cross-reactivity did not affect the results significantly, since IgG_{2c} was found to constitute less than 1% of either TBM antibody or total eluted IgG.

Quantification of rat IgG subclass and IgM isotype standards. The concentration (μg/ml) of rat IgG subclasses and of IgM in hybridoma supernatants was determined by an ELISA inhibition assay employing a rat IgG standard. Lewis rat IgG was coated on the wells of microtiter plates and was then reacted with biotinylated monoclonal antibody OX-11 (anti-kappa specificity) that had been preincubated with either the Lewis rat IgG standard or the various rat hybridoma supernatants in twofold dilutions. Avidin–peroxidase complex was added, and the color reaction was developed as above. The data was plotted as percentage of inhibition of binding versus concentration of IgG in the standard or versus dilution of hybridoma supernatant of known isotype and subclass (Fig. 1). Percentage of inhibition of maximum binding was calculated by the following method: 1 − [Abs (experimental)/Abs (maximum)] × 100. The concentration of the standard rat IgG yielding 50% inhibition of maximum binding was multiplied by the re-

TABLE I

Subclass	Rabbit anti-IgG_1	Rabbit anti-IgG_{2a}	Rabbit anti-IgG_{2b}	Rabbit anti-IgG_{2c}	Rabbit anti-IgM
IgG_1 (187.1)	1.600	0.199	0.122	0.077	0.129
IgG_{2a} (G32/1.1)	0.252	1.345	0.102	0.060	0.142
IgG_{2b} (G26/31)	0.185	0.272	1.322	0.067	0.149
IgG_{2c} ($120a_3$)	0.622	0.458	0.581	1.447	0.492
IgM (G26/27)	0.140	0.076	0.132	0.056	1.359
PBS	0.136	0.037	0.161	0.034	0.064

Note. The specificity of the rabbit antisera to the IgG subclasses and to the IgM isotype was tested by reacting each antiserum against hybridoma supernatants of all four IgG subclasses and IgM. Wells of microtiter plates were coated with goat anti-rat Ig, and successive incubations were performed with rat IgG or IgM hybridoma supernatants, rabbit anti-isotype sera, and peroxidase-conjugated goat anti-rabbit IgG.

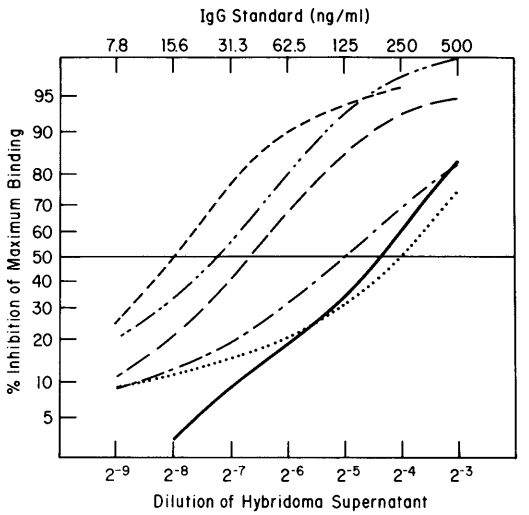

FIG. 1. The IgG subclasses and the IgM within hybridoma supernatants were quantitated by an ELISA inhibition assay employing a rat IgG standard. The microgram per milliliter of standard rat IgG yielding 50% inhibition of maximum binding was multiplied by the reciprocal of the dilution of the hybridoma supernatant required to achieve the same inhibition. Rat IgG standard —; IgG_1 (187.1) —; IgG_{2a} (G 32/1.1) ——; IgG_{2b} (G 26/31) ——; IgG_{2c} (120_{a3}) ---; (G 26/27) • • •.

ciprocal of the dilution of the hybridoma supernatant required to achieve the same inhibition to determine the concentration of IgG or IgM in each hybridoma supernatant. Once the concentrations of IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c} and IgM were known, these hybridoma supernatants were employed as standards in the antigen-specific IgG subclass and IgM binding assays to be described below.

Quantitation of serum anti-TBM antibody of IgG subclass and IgM isotype. Anti-TBM antibody of specific IgG subclass and of IgM isotype was quantitated by an antigen-specific serum binding assay compared to a standard curve generated with hybridoma supernatants in a similar binding assay. The antigen-specific serum binding assay was performed by coating the wells of microtiter plates with particulate TBM followed by incubation with BN rat anti-TBM serum; rabbit anti-rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, or IgM; and, finally, peroxidase-conjugated goat anti-rabbit IgG. Background binding was determined by substituting PBS in place of BN rat anti-TBM serum. Standard curves were performed by coating the wells of microtiter plates with goat anti-rat Ig followed by incubation with known quantities of hybridoma supernatants of known subclass and, finally, by incubation with rabbit anti-rat isotype sera and peroxidase-conjugated goat anti-rabbit IgG in the same concentrations as used for the TBM binding assays. Background binding was determined by substituting PBS in place of the hybridoma supernatant. A representative TBM binding assay with standard curve is plotted in Fig. 2 with percentage of binding calculated by the following method: [Abs (experimental) − Abs (background)/Abs (maximum of standard) − Abs (background)] × 100.

Quantitation of renal eluate antibody of IgG subclass and IgM isotype. Renal eluates were obtained with citrate buffer, 0.02 *M*, pH 3.2, from renal homogenates washed in PBS (18). The IgG subclasses and IgM antibody from renal eluates were quantitated in parallel binding assays with hybridoma standards. Wells were coated with goat anti-rat Ig, incubated with dilutions of renal eluate or hybridoma standards, incubated with rabbit anti-

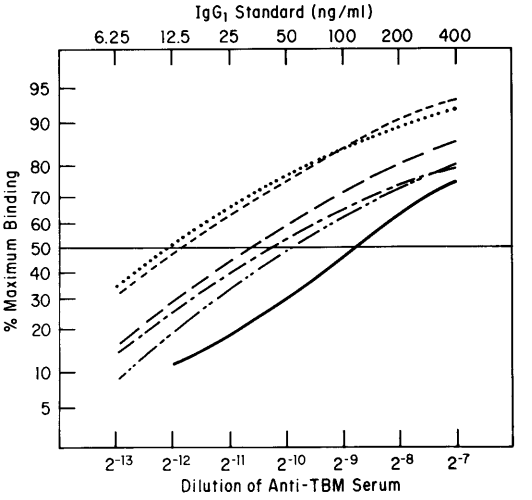

FIG. 2. The concentration of serum anti-TBM antibody of the IgG_1 subclass was quantitated by an ELISA binding assay employing an IgG_1 hybridoma supernatant standard. The microgram per milliliter of the standard IgG_1 yielding 50% of maximum binding was multiplied by the reciprocal of the dilution of the anti-TBM serum required to achieve the same binding. IgG_1 standard (187.1) —; Control anti-TBM serum No. 1 ---; No. 2 ——; No. 3 • • •; No.4 ---; No. 5 ——.

rat isotype sera, and, finally, incubated with peroxidase-conjugated goat anti-rabbit IgG.

Quantitation of total antibody of IgG subclass and IgM isotype in immune serum. The IgG subclasses and IgM antibody from pooled control immune serum were quantitated in parallel binding assays with hybridoma standards. Wells were coated with goat anti-rat Ig, incubated with dilutions of immune serum or hybridoma standards, incubated with rabbit anti-rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, or IgM, and, finally, incubated with goat peroxidase-conjugated anti-rabbit IgG.

Statistics. The microgram IgG per milliliter and microgram IgM per milliliter within control rats and the pooled serum of Day 0–14 M-PGE_1-treated rats were compared using the unpaired *t* test for a difference between two independent means with the assumption that the relative standard deviation is identical in both groups.

Results. *Quantitation of anti-TBM IgG subclasses and IgM isotype in serum.* The serum of TBM-immunized rats who received M-PGE_1 on Days 0–14 had a lower total anti-TBM IgG content (186 μl/ml) than the serum of control rats (428 ± 239 μg/ml) ($P < 0.25$). The decrease in the absolute quantity of circulating anti-TBM antibody in M-PGE_1-treated rats is consistent with our previous observation that the TBM-binding of sera from these rats at increasing serum dilutions drops off more rapidly than the TBM-binding of the control group (15). However, the IgG subclass distribution in M-PGE_1-treated and control groups was the same. The majority of the TBM antibody was in the IgG_1 subclass (61% in controls vs 64% in the M-PGE_1 group), a lesser amount in the IgG_{2a} subclass (36% in controls vs 34% in the M-PGE_1 group), and very small amounts in the IgG_{2b} (approximately 2%) and IgG_{2c} (<1%) subclasses. Thus, M-PGE_1 does not affect the distribution of the IgG subclass response to immunization, at least in the present system of the BN rat immunized with TBM (Table II).

The anti-TBM IgG subclasses in the sera of control rats were individually quantitated to discern any possible heterogeneity in the subclass response to immunization among a group of inbred rats. The range of IgG_1 anti-TBM produced was from 46 to 82% of the total circulating IgG anti-TBM. The percentages of the total IgG anti-TBM in the remaining IgG subclasses ranged from 18 to 46% for IgG_{2a}, <1 to 7% for IgG_{2b}, and <1% for IgG_{2c}. Thus, a substantial heterogeneity existed within the percentage of total IgG found within each subclass. The subclass heterogeneity was not merely a reflection of the overall magnitude of the immune response, as seen, for example, by the fact that rat No. 3 produced four times as much IgG_1 as rat No. 5 (481 μg/ml vs 104 μg/ml), but exactly the same amount of IgG_{2a} (103 μg/ml vs 103 μg/ml) (Table II).

The total circulating IgG anti-TBM ranged from 224 to 707 μg/ml, but these differences may have been due to unrecognized differences in immunization. Comparison of the histologic severity of nephritis in individual rats with the quantity of circulating anti-TBM IgG or IgG subclasses could not reveal any correlation since all rats had severe interstitial nephritis involving the entire circumference

TABLE II. IgG SUBCLASS (μg IgG/ml AND % OF TOTAL IgG) AND IgM ISOTYPE (μg IgM/ml) COMPOSITION OF TBM ANTIBODY IN SERUM

Experimental group:	IgG_1 (%)	IgG_{2a} (%)	IgG_{2b} (%)	IgG_{2c} (%)	Total IgG	IgM
Controls (days 0–14)						
1	411 (54)	338 (44)	16 (2)	<1 (<1)	767	35
2	173 (53)	141 (43)	13 (4)	2 (<1)	329	14
3	481 (82)	103 (18)	1 (<1)	<1 (<1)	586	18
4	134 (58)	93 (40)	4 (2)	<1 (<1)	231	17
5	104 (46)	103 (46)	16 (7)	<1 (1)	224	38
Average ±1 STD	261 ± 173 (61)	156 ± 104 (36)	10 ± 7 (2)	<1 (<1)	428 ± 239	24 ± 11
M-PGE_1 (days 0–14)						
Pooled serum	120 (64)	62 (34)	3 (2)	<1 (<1)	186	38
P value Control vs M-PGE_1-treated	<0.25	<0.25	<0.25	N.S.	<0.25	<0.25

TABLE III. IgG SUBCLASS (mg IgG/ml AND % OF TOTAL IgG) AND IgM ISOTYPE (mg IgM/ml) COMPOSITION OF IMMUNE BN RAT SERUM POOLED CONTROL (DAYS 0–14)

Experimental group:	IgG_1 (%)	IgG_{2a} (%)	IgG_{2b} (%)	IgG_{2c} (%)	Total IgG	IgM
Pooled immune serum	3.82 (29)	3.66 (28)	5.39 (41)	0.38 (3)	13.25	0.29

of the renal cortex. The IgM isotype response to TBM in M-PGE_1-treated and control sera was similar (24 ± 11 μg/ml in controls vs 38 μg/ml in M-PGE_1-treated group) (Table II).

Quantitation of the total IgG subclass and IgM isotype antibodies in pooled control immune serum demonstrated that the immune response to TBM appeared to be proportionately increased in the IgG_1 and IgG_{2a} subclasses and proportionately decreased in the IgG_{2b} subclass. Thus, IgG_1 and IgG_{2a} anti-TBM antibodies comprised 61 and 36% of total anti-TBM IgG, whereas the IgG_1 and IgG_{2a} subclasses comprised only 29 and 28% of total IgG (Table III). Conversely, IgG_{2b} anti-TBM antibody comprised only 2% of total anti-TBM IgG, whereas total IgG_{2b} comprised 41% of total IgG. The total IgG content of the pooled immune serum was 13.25 mg/ml and the average serum IgG anti-TBM content was 428 μg/ml. Circulating TBM antibody can thus be calculated to have comprised approximately 3% of total circulating IgG antibody 14 days after immunization.

In the second group of experiments, the IgG subclass and IgM isotype response to TBM was measured on Days 8 and 14 after immunization in rats who received M-PGE_1 on Days 0–7 after immunization (Table IV). The IgG anti-TBM response again occurred predominantly in the IgG_1 and IgG_{2a} subclasses and increased from Day 8 to Day 14. The amount of IgG_{2b} and IgG_{2c} anti-TBM antibody was less than 3 μg/ml in all groups. The average total concentration of anti-TBM IgG in M-PGE_1-treated rats was somewhat lower than controls on Day 8 (50 μg/ml in M-PGE_1 group vs 86 μg/ml in controls) but somewhat higher than controls on Day 14 (232 μg/ml in M-PGE_1 group vs 164 μg/ml in control group). The IgM anti-TBM response was similar in both M-PGE_1 and control groups and decreased from Day 8 (32 μg/ml in M-PGE_1 group vs 43 μg/ml in control group) to Day 14 (13 μg/ml in M-PGE_1 group vs 9 μg/ml in control group).

Quantitation of anti-TBM IgG subclasses and IgM isotype in renal eluates. Quantitation of the IgG subclasses in renal eluates demonstrated a subclass distribution generally similar to that of serum anti-TBM antibody (Table V). The percentage of IgG_{2b} in the renal eluates, however, was slightly higher than expected by comparison with serum. IgG_{2b} constituted as much as 21% of total eluted IgG in one group and ranged around 10% of eluted IgG in several other groups.

IgM, which on Day 14 constituted from 5 to 17% of total circulating IgG plus IgM in terms of micrograms per milliliter, contributed to only approximately 1% of total eluted renal IgG plus IgM on the same day. Thus, the IgM isotype does not appear to bind significantly to TBM *in vivo*, as is consistent with its role as a predominantly intravascular immunoglobulin.

TABLE IV. IgG SUBCLASS AND IgM ISOTYPE COMPOSITION (μg/ml) OF TBM ANTIBODY ON DAYS 8 AND 14 IN SERUM OF RATS RECEIVING M-PGE_1 FOR 7 DAYS AFTER IMMUNIZATION

Experimental group:	Day 8						Day 14					
	IgG_1	IgG_{2a}	IgG_{2b}	IgG_{2c}	Total IgG	IgM	IgG_1	IgG_{2a}	IgG_{2b}	IgG_{2c}	Total IgG	IgM
Control (days 0–7)	44	39	3	<1	86	43	120	42	<1	<1	164	9
M-PGE_1 (days 0–7)	38	11	1	<1	50	32	215	16	<1	<1	232	13

TABLE V. IgG SUBCLASS (μg Ig/g KIDNEY AND % OF TOTAL ELUTED IgG) AND IgM (μg IgM/g) ISOTYPE COMPOSITION OF DAY 14 RENAL ELUATES

Experimental group	IgG_1 (%)	IgG_{2a} (%)	IgG_{2b} (%)	IgG_{2c} (%)	Total IgG	IgM
M-PGE_1 (days 0–14)	25 (50)	14 (29)	11 (21)	<1 (<1)	50	<1
Controls (days 0–14)	72 (72)	18 (18)	10 (10)	<1 (<1)	100	1
M-PGE_1 (days 0–7)	9 (59)	5 (35)	1 (6)	<1 (1)	15	<1
Controls (days 0–7)	20 (75)	6 (22)	1 (3)	<1 (<1)	27	<1
Negative control eluate	<1	<1	<1	<1	<2	<1

Discussion. The stable PGE analogue M-PGE_1, injected daily at a dose of 1 mg/kg, reduced the average quantity of circulating antigen-specific antibody 2 weeks after immunization but did not qualitatively alter the nature of the IgG subclass response. M-PGE_1, administered for only the first week after immunization, resulted in a decreased total antigen-specific IgG response on Day 8 but not at 2 weeks.

Endogenous prostaglandin has also been postulated to decrease antigen-specific antibody production *in vivo* (2). In support of the negative feedback role of prostaglandins in the regulation of antigen-specific antibody production are the findings of Webb and Osheroff that injection of mice with sheep red blood cells leads to a dramatic increase in splenic $PGF_{2\alpha}$ and that prior administration of a prostaglandin synthesis inhibitor results in an increased number of splenic plaque-forming cells (17). Similarly, the administration of prostaglandin synthesis inhibitors in humans and rats has been reported to augment the humoral immune response in some circumstances (5, 6). M-PGE_1, at the dose of 1 mg/kg also used in the present study, has been reported to cause a dramatic decrease in splenic B-lymphocytes (3). In the same study, somewhat paradoxically, M-PGE_1 did not appear to affect serum hemagglutination titers to soluble schistosomal egg antigens after embolization of schistosomal eggs, but the need to study the effect of prostaglandins on immunoglobulin isotype was discussed.

The effect of exogenous prostaglandins on the antigen-specific IgG subclass response has not, to our knowledge, been previously reported. The observation that M-PGE_1 does not qualitatively affect the antigen-specific IgG subclass response to TBM is of interest, firstly, to preclude the theoretical possibility that changes in the IgG subclass distribution (e.g., from complement- to noncomplement-fixing IgG subclasses) could contribute to the anti-inflammatory effects of M-PGE_1 in actively induced models of humoral autoimmune inflammation and, secondly, as evidence against the possibility that PGE is involved in the endogenous mechanisms regulating the nature of the IgG subclass response to immunization.

Three percent of the total circulating IgG in TBM-immunized control rats was estimated to represent TBM-specific antibody, a result that is concordant with the findings of Zanetti, *et al.* (19) who showed that 4.8% of splenic lymphocytes in TBM-immunized BN rats express TBM-specific immunoglobulin as probed with anti-idiotypic antiserum by the immunofluorescent technique. The seemingly disproportionate representation of anti-TBM antibody in the IgG_1 and IgG_{2a} subclasses compared to the IgG_{2b} subclass is of interest in regard to other reports of IgG subclass restrictions. Susceptibility to type II collagen arthritis in rats, for example, may be related to the preferential production of IgG_{2b} antibody (12). The human IgG_3 subclass restriction in the response to the P blood group system may be of immunopathogenetic significance to the development of hemolytic disease of the newborn (11). The nature of the IgG subclass response has also been reported to be dependent on the adjuvants employed for immunization (8). The disproportionate representation of anti-TBM antibody in the IgG_1 and IgG_{2a} subclasses is consistent with the recent observation of Clayman *et al.* (4) that all 22 monoclonal anti-TBM antibodies derived in their laboratory from five BN rats were of the IgG_1 or IgG_{2a} isotype. The humoral immune response to autoantigens does not always dem-

onstrate evidence of subclass restriction. The antigen binding capacity for thyroglobulin in the IgG subclasses of serum from patients with Hashimoto's disease, for example, closely reflects the IgG subclass composition of normal serum (7).

The heterogeneity in the IgG subclass response to immunization is also of note, since heterogeneity in the IgG subclass response to exogenous or autoantigens may play a role in the pathogenesis of autoimmune inflammatory phenomena. E. R. Arquilla and his colleagues, for example, recently reported heterogeneity in the IgG subclass response to insulin as well as significant differences in the *in vivo* localization and catabolism of subclass specific insulin immune complexes (1, 16). Similarly, the IgG subclass composition of human antinuclear antibodies has been studied, and a correlation between complement-fixing antibody and lupus nephritis has been reported (10).

In summary, M-PGE_1 administered daily at pharmacologic doses was found to quantitatively diminish but not qualitatively alter the humoral immune response. Quantitation of the IgG subclass response revealed evidence of subclass selectivity as well as heterogeneity in the humoral response to tubular basement membrane. Selectivity and heterogeneity in the IgG subclass response to antigens may be of immunopathogenic significance.

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