THE EFFECT OF ANTI-MU SUPPRESSION OF γM AND γG ON THE PRODUCTION OF γE*

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The early precursors of antibody-producing cells in the mouse ("B" lymphocytes) have attached to their surface membrane immunoglobulin receptors (1, 2) specific for a very limited number of antigenic determinants (3–5). These receptors develop in fetal life before the animal is introduced to antigens and in this period are largely of the γM immunoglobulin class (6, 7). Combination of an antigen with these specific recognition units results in division and differentiation of many of these cells into mature antibody-secreting cells (plasma cells) (6, 8). In general, the class of antibody secreted by these latter cells will be the same as the class of antibody present on the surface of the B lymphocytes from which these cells were derived (9, 10). Hence, primary immune responses are largely γM in character. In humans, especially during fetal and early neonatal life, a large number of γM-bearing lymphocytes also carry γD on their surface, and it is not clear by what mechanism these cells differentiate to predominantly γM-secreting cells (11). Such a phenomenon has not been described in the mouse.

Not all γM-bearing lymphocytes stimulated by antigen become antibody-forming cells. Division of some of these cells results in an expanded pool of cells with the same recognition capacity, but with a recognition unit of the γG class of immunoglobulin (11). These cells are the precursors of antibody-producing cells secreting γG and B lymphocytes with γA class immunoglobulin receptors (11). This ontogenetic sequence can occur to some extent *in utero* where the sequential events are not driven by encounter with antigen (11, 12).

How the production of the other two major immunoglobulin classes γE and γD fit into the above scheme has not been determined. However, observation that the γM to γG to γA sequence could be blocked by anti-mu chain antisera (13, 14) prompted this study of the effect of such antisera on γE production. We have observed, as have others (15), that children with sex-linked agammaglobulinemia can have absent γM, γG, and γA production and yet have detectable levels of serum γE, γE-bearing lymphocytes, and even atopic symptoms. Such observations suggest that γE development may be independent of the ontogenetic scheme for γM, γG, and γA. In this paper we present evidence to support this concept. In the experiments described below, treatment of mice with anti-mu chain antisera failed to suppress the production of specific antibodies of the γE class.

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Materials and Methods

Animals. F_1 litter mates from C57BL/10 females mated with DBA/1 males were used as recipients of the anti-mu chain serum. As γE responsiveness in mice is variable but genetically controlled, we chose mice known to be good γE producers (16). The parent mice were obtained from the specific pathogen-free colony of the Biobehavioural Science Research Center of the University of Connecticut, Storrs, Conn. Male and female 6-wk-old Swiss mice from the Yale University Medical School colony were used to detect γG_1 antibodies using a passive cutaneous anaphylaxis (PCA)[1] technique. 200 *g* male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) were used in PCA tests to detect and titrate specific γE antibodies in mouse sera.

Antisera. Anti-mu chain sera, product B106, lot No. 40832 and product B107, lot No. 40900, were purchased from Meloy Laboratories Inc., Springfield, Va. These antisera were prepared in goats against a γM myeloma MOPC-104E. The antisera were rendered anti-mu specific by passage over immunoabsorbent columns containing MOPC-315B (γAλ). The resulting preparations, tested by diffusion in gel, were monospecific. Special attention was paid to the possible presence of antibodies to λ-light chain determinants in these antisera. No light chain activity was found in testing these preparations against AKR-J, BALB-cJ, C57BL/6J, and CBA-J mouse sera. It was necessary to remove by dialysis the sodium azide added to these antisera as a preservative before administering them to the mice.

Injection of Anti-Mu Chain Antisera and Immunization. Mice were given their first injection of anti-mu chain sera or bovine gamma globulin (BGG) (control animals) within 24 h of birth. 0.05 cm^3 of either agent was injected intraperitoneally (i.p.) daily for the first 4 days of life, while 0.1 cm^3 was injected 9, 16, 23, and 29 days after birth. 21 days after birth both experimental and control animals were given an i.p. injection of 0.1 μg of twice-recrystallized ovalbumin (OA) (Worthington Biochemical Corp., Freehold, N. J.) plus 1.12 mg of alum ($Al(OH)_3$ gel) in a total volume of 0.1 cm^3. Alum was prepared as previously described (17) and had a dry weight of 28 mg/ml. 34 days after birth, all animals were lightly anesthetized and bled. Approximately 0.2 cm^3 of serum was obtained per animal.

Antibody Measurements.

PCA REACTIONS. Mouse sera contains two immunoglobulin classes, γG_1 and γE, that can bind to the surface of mast cells (18). Antigen binding to these surface immunoglobulins releases histamine and other vasoactive substances from these cells. To measure anti-OA antibody of the γG_1 class, sera from control and anti-mu-suppressed mice were diluted (commencing with a 1:5 dilution) and tested as follows. 0.03 cm^3 of diluted sera was injected intradermally into the shaved skin on the back of Swiss mice. Four injections of variously diluted antisera were given per back. All dilutions were tested simultaneously in three mice. 2 h later under ether anesthesia 0.15 cm^3 of saline with 0.7% OA and 0.4% Evans blue (Matheson, Coleman & Bell, Rutherford, N. J.) was injected intravenously via the retro-orbital plexus. After 30 min the animals were sacrificed, the skin removed from the back, and the diameter of blueing at the injection sites measured. The final titer of antibody activity was the highest dilution of serum causing blueing of 5 mm or more in diameter in at least two of three mice.

Quantitation of anti-OA antibodies of the γE class were measured by a PCA technique in rats. Mouse γE, but not γG, will bind rapidly to rat mast cells (19). Male Sprague-Dawley rats were anesthetized with an i.p. injection of 0.8 cm^3/kg of sodium pentabarbital (Diabutal, Diamond Labs, Des Moines, Iowa). The backs of the rats were shaved, and 24 injections of 0.05 cm^3 of variously diluted sera were injected intradermally. 2 h later the rats were reanesthetized, and 1 cm^3 of saline containing 0.4% Evans blue and 0.7% OA was injected through the dorsal vein of the penis. After 30 min, the animals were sacrificed and the skin of the back removed and read as described above for γG_1 measurements.

QUANTITATION OF γM AND γG ANTI-OA ANTIBODIES. A passive red cell hemagglutination technique was used. Washed sheep red blood cells (SRBC) were washed and mixed for 4 min at room temperature with 3 mg of 0.1% $CrCl_3 \cdot 6H_2O$ (Mallinckrodt Inc., St. Louis, Mo.) and 3 ml of OA at 10 mg/ml. The treated cells were washed five times and resuspended in normal saline at a

[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; OA, ovalbumin; PCA, passive cutaneous anaphylaxis.

concentration of 5% and then diluted 1:5 in phosphate-buffered saline. The ability of serial two-fold dilutions of test sera (commencing at a dilution of 1:10) to agglutinate these treated SRBC was used to quantitate total antibody levels against OA. A hyperimmune rabbit antisera to OA, supplied by Dr. P. Askenase, was used as a control. To measure γG antibody to OA, test sera were reacted with mercaptoethanol (Pierce Chemical Co., Rockford, Ill.). To 0.1 ml of sera to be tested, 0.3 ml of 0.133 M mercaptoethanol was added and allowed to react for 2 h at room temperature before the sera were tested. Several dilutions of sera were added to autotiter plates containing the treated SRBC and antibody titers measured by detecting the dilution at which agglutination ceased. 15 dilutions were tested for each sample.

DETECTION OF γG_1, γG_2, AND γA. The presence of γG_1, γG_2, and γA was sought in sera by a gel diffusion technique using class-specific antibodies. Precipitation lines were sought with sera diluted 1:5 in saline.

B-Lymphocyte Quantitation. At the time the anti-mu or BGG-treated mice were sacrificed, some spleens were removed for determination of their B-cell content. Spleens were gently teased between two glass slides into a Petri dish containing modified Eagle's medium and 10% fetal calf serum. The cells so obtained were layered onto a Ficoll-Hypaque gradient and centrifuged for 45 min at 4°C. The single cell suspension removed from the Ficoll-Hypaque interface was washed and reacted with a polyvalent mouse anti-immunoglobulin serum raised in rabbits (Meloy Laboratories Inc.). After washing, these cells were reacted for 30 min at room temperature with a fluoresceinated antirabbit immunoglobulin raised in goats (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). After washing, these cells were examined under ultraviolet light, and the percentage of fluorescent lymphocytes ("B" cells) was quantitated after a differential leukocyte count of the preparation was performed.

Statistical Analysis. Comparison of the weights of the mice in the control and anti-mu sera-treated groups, as well as the number of B lymphocytes in the spleens of mice from each group, were made by Student's t test for unpaired data. For the antibody studies, the reciprocal of the highest titers measured were converted to the Log_2, and from these values the mean Log_2 values were obtained and compared by Student's t test for unpaired data. All calculations were performed on a Monroe statistical calculator, Model 1930.

Results

Tables I and II contain all the data obtained from control and anti-mu-treated mice; Table III contains statistical comparison of the data obtained from both groups. The protocol used did not cause significant runting in anti-mu-treated mice. Two mice, 3A and 4D (Table II), were clearly not immunosuppressed, and the results in these mice were excluded from the statistical analysis. The administration of anti-mu chain sera, but not BGG, was generally immunosuppressive causing a significant reduction in B lymphocytes in the spleen and anti-OA antibodies of the γM, γG, and γG_1 class. However, there was no significant reduction in anti-OA antibodies of the γE class. Nonspecific γG_1 and γG_2 antibodies were frequently detected in both groups by a gel diffusion technique and probably represent maternally transmitted γG. γA was not detected in the anti-mu-treated litter in which it was sought.

Discussion

The suppression of γM and γG production by anti-mu chain sera observed in the present studies is similar to that reported in other in vivo studies (13, 14), some of which have used germ-free animals (11). Suppression has also been observed in in vitro studies (20, 21). Such data strongly supports the concept that the virgin precursor "B" lymphocytes from which the humoral immune system develops express a surface receptor of γM character. When germ-free mice were treated with anti-γ chain sera they were unable to develop a γG or γA response

TABLE I
Antibody Production in Control Mice

Litter	Mouse*	Weight	Antibodies to OA				Serum Immunoglobulins‖			Splenic "B" lymphocytes
			γE‡	γG_1‡	γM + γG	γG§	γG_1	γG_2		
		g								%
1	C	18.2	320	5	1,280	80	+	+		NT¶
	D	16.9	160	0	640	40	+	+		NT
2	B	13.6	640	20	120	1,280	+	+		NT
	E	15.7	80	40	10,240	1,280	+	−		NT
	F	14.9	80	40	5,120	320	+	−		NT
3	B	15.8	1,280	160	5,120	160	+	+		NT
4	A	13.0	1,280	10	2,560	1,280	NT	NT		NT
	B	15.0	640	40	5,120	5,120	NT	NT		NT
5	A	16.0	640	10**	2,560	1,280	+	+		NT
	B	15.7	80	10**	640	160	+	+		NT
	D	15.0	640	10**	1,280	320	+	+		NT
6	B	9.3	320	40	640	160	+	+		57
	C	11.1	160	10	1,280	640	+	+		63
	D	12.8	160	40	640	160	+	+		49
	E	11.1	80	80	2,560	1,280	+	−		NT
7	A	16.0	640	80	5,120	320	−	−		43
	B	16.0	0	0	0	0	+	−		29
	C	16.8	640	80	5,120	320	+	+		62
8	A	16.5	80	10	160	0	−	−		47
	B	15.7	640	160	10,240	160	+	+		55
	C	15.8	640	160	5,120	320	+	+		38
	D	16.9	640	80	2,560	320	+	+		47
	E	16.0	320	160	1,280	160	+	+		39
9	A	17.4	640	40	10,240	5,120	+	+		NT
	B	18.6	320	40	5,120	640	+	+		NT
	C	16.7	1,280	40	2,560	640	+	+		NT
	D	16.9	320	40	2,560	640	+	+		NT
									γA‡‡	
10	A	15.5	320	80	5,120	640	+	+	+	58
	D	14.9	1.280	160	5,120	320	+	+	+	42
	E	14.5	1,280	40	640	160	+	+	+	59

* Litter mates are compared in Table II.
‡ As measured by PCA reactions.
§ As measured by passive hemagglutination after mercaptoethanol treatment of serum.
‖ As measured by gel diffusion.
¶ Not tested.
** Levels may have been higher than 10; insufficient serum available to test further.
‡‡ γA present in serum measured by gel diffusion in litter 10.

TABLE II
Antibody Production in Mice Treated with Anti-mu Serum

Litter	Mouse*	Weight	Antibodies to OA				Serum immunoglobulins‖			Splenic "B" lymphocytes
			γE‡	γG_1‡	γM + γG	γG§	γG_1	γG_2		
		g								%
1	B	9.3	40	0	0	0	+	−		NT¶
2	A	15.0	160	0	80	0	+	+		NT
	C	19.0	40	0	0	0	+	+		NT
3	A**	15.0	320	0	320	80	+	+		NT
	C	14.2	1,280	10	160	0	−	−		NT
	D	16.3	320	10	0	0	−	−		NT
4	C	17.0	640	0	0	0	+	−		NT
	D**	15.9	640	40	2,560	160	+	−		NT
5	D	9.2	160	0	0	0	−	−		NT
6	A	11.6	0	0	0	0	+	+		7
	F	10.9	160	0	0	0	+	−		5
7	D	14.5	80	0	0	0	−	+		27
	E	13.5	160	5	10	0	+	−		17
	F	16.0	160	0	0	0	+	+		8
	G	15.7	160	5	0	0	+	+		4
8	F	13.3	320	5	40	10	+	+		6
9	E	16.0	0	10	0	0	−	−		NT
	F	15.5	160	0	0	0	+	+		NT
	G	16.2	80	0	0	0	+	−		NT
	H	15.2	640	40	0	0	+	+		NT
									γA‡‡	
10	B	14.8	160	0	0	0	+	+	−	4
	C	15.2	1,280	0	20	0	+	−	−	15
	F	11.8	320	10	40	0	+	+	−	3
	G	10.7	320	0	0	0	+	−	−	7
						32				

* Litter mates are compared in Table I.
‡ As measured by PCA reactions.
§ As measured by passive hemagglutination after mercaptoethanol treatment of serum.
‖ As measured by gel diffusion.
¶ Not tested.
** Mice not considered to be immunosuppressed and not included in statistical comparison of controls detailed in Table III.
‡‡ γA absent from sera of litter 10 as measured by gel diffusion.

to antigen, but could produce γM (22). Such experiments, coupled with the observation that the order of appearance of the major immunoglobulin classes in both ontogeny and phylogeny follows the sequence γM, γG, γA, led to the hypothesis that γG-bearing cells, the precursors of γG-producing plasma cells,

TABLE III

Comparison of the Antibody Response to OA of Control Mice and Mice Treated with Anti-Mu Chain Sera

Treatment	Weight*	"B" cells‡	Geometric mean titer: $\log_2$ ± SD ± SEM			
			γG + γM	γG	γG_1	γE
	g					
BGG	15.27 ± 0.38	49.14 ± 10.22	10.86 ± 2.51 ± 0.44	8.12 ± 2.71 ± 0.49	4.9 ± 1.94 ± 0.34	7.96 ± 3.05 ± 0.55
Anti-mu sera	14.24 ± 0.5	9.36 ± 2.16	1.83 ± 2.61 ± 0.54	0.14 ± 0.70 ± 0.14	0.61 ± 1.19 ± 0.24	7.01 ± 2.62 ± 0.54
BGG/Anti-mu						
ti§	1.65	10.9	12.55	13.41	9.16	1.16
P	NS	<0.0001	<0.0001	<0.0001	<0.0001	NS

§ ti, *t* value for unpaired data.
* Mean ± SEM.
‡ Mean ± SD.

may arise from γM-bearing cells (12). In a further sequential ontogenetic step, γA-bearing cells would arise from precursor cells bearing γG receptors (12). While all studies agree on the potent immunosuppressive ability of anti-mu chain sera, experiments using anti-γ chain sera in nongerm-free states have not demonstrated an equally potent suppressor effect (20, 21, 23). The reason for this is not certain, but at least in vivo is probably related to the large amounts of maternal γG present in the circulation of newborn mice which may result in rapid removal of administered anti-γG before it can act at a cellular level (24). It is this maternally derived γG that explains the inability of anti-γM to eliminate serum γG_1 and γG_2. In the present study the profound effect of anti-mu sera on the endogenous production of γG_1 to OA in the presence of detectable γG_1 in serum supports this idea. In vitro studies with anti-γ_1 or anti-γ_2 sera have resulted in suppression of both γ_1 and γ_2, but not γM or γA plaque-forming cell responses (20). In such studies the anti-immunoglobulin sera must simultaneously compete with antigen stimulation, and this may reduce the effectiveness of the anti-γ sera. The fact that anti-mu chain sera is completely suppressive in these experiments while anti-γ chain sera is less potent could be explained if the affinity of γG receptors for antigen is greater than that of γM receptors. It is well established that treatment with anti-γA affects only γA production (22, 24). Thus, for the three major immunoglobulin classes, a γM to γG to γA sequence or a selective γM to γG to γM development remain viable alternatives. The latter alternative seems likely to be true at least for the gastro-intestinal tract where evidence for γM precursors differentiating directly into γA cells has been reported (22).

The placement of γE and γD within an ontogenetic scheme for the development of humoral immunity has not been previously attempted. It is certain, at least for man, that a γM to γG to γA to γE sequence is not likely, as many patients with absent γA production have normal or even high levels of γE (25).

As we have observed patients without detectable γA-bearing lymphocytes who have normal levels of γE, it is unlikely that the former observation is explained by a failure of γA-bearing cells to become γA-secreting plasma cells while still being able to differentiate to γE-bearing precursor cells. There is considerable clinical evidence to suggest that γE production and development may be independent of factors controlling the other major immunoglobulin classes. The presence of detectable quantities of γE and even atopy has been observed by us and others (15) in children with an otherwise marked agammaglobulinemia.

The production γE in vitro by primed lymphocytes exposed to anti-mu chain sera has been studied. Such lymphocytes were able to produce γE despite the presence of anti-mu chain sera (26). However, as this study utilized lymphocytes that had been exposed to antigen before antisera treatment, it indicates only that cells producing γE are unaffected by anti-mu chain sera and does not answer questions about possible ontogenetic precursors of γE-bearing cells. The data presented here strongly suggests that profound suppression of γM and γG production by anti-mu chain sera does not significantly reduce γE production. At least three explanations for this observation can be forwarded. Firstly, stem cell precursors of B lymphocytes thought to at least partially differentiate in the yolk sac and/or fetal liver (27) may develop either γE- or γA-bearing cells. Thus, anti-mu suppression would leave γE development intact. Secondly, γE-bearing cells may develop early in fetal life from γM-bearing cells by a genetically controlled differentiation pathway which is independent of antigen (12) and so escape the effects of anti-mu sera given at birth. Thirdly, γE-bearing cells may develop from γM-bearing cells unable to differentiate along the γG to γA pathway, but perhaps with T-cell help able to differentiate directly to γE-bearing cells.

The first explanation is the simplest, is consistent with the observation of γE production in congenital sex-linked agammaglobulinemia, and is strongly suggested, but not established, by the present data, since we were unable to eliminate all B lymphocytes from the spleen of anti-mu-treated mice. Increasing either the dose or frequency of injections caused severe runting and such poor survival as to be impractical. As both problems were encountered in the control mice as well, this may well be a function of too severe or frequent manipulations of such young mice. It is not currently possible to determine directly the number of residual B lymphocytes that have surface γE, but it seems unlikely that our mean residual 9% splenic B cells would all have γE recognition units.

Anti-mu chain sera administered at birth could not affect γE differentiation occurring earlier in fetal life. The human fetus can synthesize γM as early as 10.5 wk of gestation and γG by 12 wk of gestation, while γA synthesis has not been demonstrated in the human conceptus. Recently, γE synthesis in the human fetus was noted as early as 11 wk (28) in fetal lung and liver. While such studies have not been reported in the mouse, it is at least possible that very early development of γE-producing precursor cells may allow the escape from anti-mu suppression reported here.

Although the surface of the B lymphocyte is the obvious site of action for anti-mu chain sera, its mode of action is uncertain. There is a natural recovery of immune competence, occurring in one study between 9 and 31 days after

cessation of suppressive treatment (22). It is possible that anti-mu-affected cells are alive but unable to differentiate along their γG to γA pathway. However, they may be capable of differentiation along pathways less dependent on the integrity of the γM receptor. So strong is the evidence for a T-cell role in γE production and regulation that it must at least be considered that T cells are helping cells with totally or partially blocked mu receptors to differentiate along a normal pathway to γE production. It has recently been shown that a solubilized fraction of T cells can serve a helper function in γE production, and that this helper activity can be removed by absorption of the solubilized material with anti-mu chain sera (29). Our in vivo observations indicate that an anti-mu susceptible helper function is not essential for normal γE production. Conversely, many observations report high γE levels in those situations where at least some T-cell functions are reduced. In diseases such as Hodgkin's disease (30), Wiskott-Aldrich syndrome (31), and infectious mononucleosis (32) where T-cell function is suppressed and after T-suppressive manipulations such as thymectomy (33), irradiation (34), and treatment with antilymphocyte serum (35), γE production is enhanced. Indeed, a soluble factor apparently obtained from thymocytes has been isolated that can suppress γE responses (36). Thus, although the data at this stage is confusing, there can be little doubt that T cells are involved in the ontogeny of γE.

The observation that T cells can both enhance and suppress γE production can only be explained by T-cell subpopulations with different functions, and it remains reasonable that one such subpopulation could be involved in the ontogeny of γE-bearing cells. γE antibody function is able to bridge the gap between pharmacological and immunological mechanisms and must certainly play a constructive, if elusive, biological role. Better understanding of the relationship of γE to the rest of the immune system will improve our understanding of this role.

Summary

Newborn mice were treated from the day of birth with either bovine gamma globulin or anti-mu chain sera. The latter was administered using a protocol known to produce suppression of γM, γG, and γA production. Subsequent immunization with ovalbumin (OA) in alum was attempted to see if suppression of γM, γG, and γA classes of antibody would also be accompanied by suppression of γE-producing capacity. γG and γM antibody to OA and mercaptoethanol-resistant (γG) antibody to OA were measured by passive hemagglutination; γG and γE anti-OA antibodies were measured by passive cutaneous anaphylaxis. Anti-mu suppression was achieved with significant reduction in γM and γG antibodies. γE antibodies were not affected, suggesting an ontogenetic development for γE-bearing lymphocytes independent of the previously described γM to γG to γA ontogenetic sequence.

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