IMMUNOSUPPRESSION OF MICE INJECTED WITH HETEROLOGOUS ANTI-IMMUNOGLOBULIN HEAVY CHAIN ANTISERA*

By DEAN D. MANNING AND JOHN W. JUTILA

(From the Immunobiology Unit, Department of Botany and Microbiology, Montana State University, Bozeman, Montana 59715)

(Received for publication 22 February 1972)

There is a rapidly growing body of evidence to establish the presence of immunoglobulins on the surface of lymphoid cells in mice (1–8). The classes of these immunoglobulins and their distribution, however, are still subjects of controversy. The immunoglobulins detectable on spleen cells, for example, have been variously reported as being distributed among all of the major classes, IgM, IgG, and IgA (2, 3, 5), and as being confined primarily to IgM (4, 6, 9). Some investigators (2, 3) have detected only a single class of heavy chain on any given cell, whereas others (5, 8) have presented evidence for possible multiple heavy chain classes on each cell. It is generally agreed that many peripheral lymphoid cells, such as those in the spleen, bear surface immunoglobulins. There is conflicting evidence, however, as to whether cells of thymic origin are essentially lacking in these structures (2, 6) or bear them in low but detectable numbers (3, 7, 10).

Available evidence universally points to these surface immunoglobulins as products of the cells upon which they appear, and it is generally thought that these molecules all function somehow in antigen recognition. There is, however, some evidence that the antigen-binding or "receptor" function may actually be limited to IgM (4, 11, 12).

Several approaches for determining the biological significance of these proteins have been based upon utilization of a wide selection of antisera directed against whole immunoglobulins or their constituent chains. It has been shown, for example, that the plaque-forming response of immune cells cultured in vitro can be suppressed by treatment with antisera against whole gamma globulin, IgM, IgG, or κ–light chains (13–16). It has also been reported that pretreatment of lymphoid cells with antibodies against gamma globulin can diminish the graft-vs.-host (GVH)[1] reaction when these cells are injected into appropriate hosts (17–19). Similar pretreatment experiments using various antisera of single chain specificity have demonstrated that suppression of the GVH reaction is possible only with anti–light chain (anti-κ) antibodies (11) and that adoptive antibody production can be prevented only with anti–light chain or anti-μ antibodies (12).

---

* This research was supported in part by US Public Health Service Training Grant 5-T01-AI00131-09 and US Public Health Service Grant AI 6552-07.

[1] *Abbreviations used in this paper:* BM, bone marrow; GVH, graft-vs.-host; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; T, thymus.

There have been relatively few attempts to alter the immune responses of intact animals with anti-immunoglobulin antisera. Antibodies against human myeloma lambda chains which cross-react with mouse kappa chains have been shown to inhibit the synthesis of certain kappa determinants when injected into neonatal mice, but over-all immunoglobulin levels were not affected (20). In chickens, the administration of anti-μ antibodies during embryonation together with bursectomy and additional anti-μ at hatching has produced agammaglobulinemia (21), apparently by plasma cell line elimination (22).

Our approach has been to treat neonatal or young adult mice with heterologous antisera against specific heavy chains and then assay these animals for suppressed immune responses, both humoral and cellular. We have previously reported that such neonatal treatment of mice with anti-μ produces absolute suppression of IgM production, as reflected in serum immunoglobulin level and direct plaque formation, and severe reduction of all other serum immunoglobulins (23). This agrees with the recent observation of a lower number of antibody-forming cells in the spleen, lymph nodes, and gut of germfree mice treated neonatally with anti-μ (24). We have since reported that neither anti-μ nor anti-$\gamma 1\gamma 2$ antisera are capable of altering the course of homograft rejection in mice, even in massive doses far in excess of those required to effect profound humoral suppression (25). We report here the extension of our studies to include mice treated initially as young adults and our investigations of the mechanism and extent of this type of suppression.

Materials and Methods

Animals.—BALB/c mice were used in all of these studies; for the target cell experiments, genetically thymusless (nude) mice of the strain described by Pantelouris (26) were also used. Because these nude mice cannot suckle their young, they must be obtained by mating animals heterozygous for the nude trait. The nude and phenotypically normal littermate progeny so derived were maintained on sterilized Purina 5010C feed (Ralston Purina Co., Inc., St. Louis, Mo.) and acidified-chlorinated water. BALB/c mice were weaned on day 24 and nude mice on day 30.

Preparation of Immunoglobulins.—IgM, IgA, and IgG2 were isolated from the sera of BALB/c mice bearing, respectively, the myeloma tumors MOPC-104E ($\lambda\mu$), MOPC-406 ($\kappa\alpha$), and MOPC-173D ($\kappa\gamma 2$). IgG of both subclasses, IgG1,2 ($\kappa\lambda\gamma 1\gamma 2$), was prepared from normal BALB/c serum. Purification of IgM and IgA was achieved by successive treatments with Pevikon block electrophoresis, gel filtration on columns of Sephadex G-200 (Pharmacia Fine Chemicals Inc., Rochester, Minn.), and diethylaminoethyl (DEAE)-cellulose chromatography, using gradient elution (27). Purification of both types of IgG preparation included an initial precipitation step with ammonium sulfate (27), followed successively by treatments with DEAE-cellulose and Sephadex G-200. Purity of all preparations was checked by immunoelectrophoresis developed with high titered anti-whole mouse serum and Ouchterlony gel diffusion developed with commercial (Meloy Laboratories, Springfield, Va.) class- or subclass-specific antisera.

Preparation of Antisera.—Antisera were prepared by subcutaneous injection of rabbits. Each animal received two weekly injections of 10–15 mg of purified immunoglobulin in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and four to six further weekly injections of 10–15 mg in incomplete Freund's adjuvant (Difco), until serum precipitin titers

ceased to rise. All antisera were routinely absorbed with BALB/c red blood cells, mouse α-globulin (fraction IV, Pentex Biochemical, Kankakee, Ill.), and mouse albumin (fraction V, Pentex), although specificities were generally lacking in the unabsorbed antisera. Light chain specificities were removed from the anti-IgM antiserum by absorption with IgA and IgG1,2. Similarly, anti-IgG1,2 antiserum was made $\gamma1\gamma2$-chain specific by absorption with IgA and IgM. A portion of the anti-$\gamma1\gamma2$ serum was further absorbed with IgG2, rendering it anti-$\gamma1$ specific. Absorptions with soluble proteins were made at 37°C for 1 hr, then overnight at 5°C, followed by centrifugation at 25,000 *g* for 30 min. Multiple absorptions were made until no further precipitate could be detected, resulting in a slight final excess of absorbing protein. The unabsorbed anti-IgG1,2, used for facilitation of indirect plaques, showed no cross-reaction with IgM or IgA in immunoelectrophoresis. Specificity of all absorbed antisera was checked by immunoelectrophoresis and Ouchterlony gel diffusion using commercial (Meloy) class-specific antiserum standards. The only antiserum not showing complete heavy chain specificity by these tests was the anti-$\gamma1$, which retained a slight trace of anti-$\gamma2$ activity. Activities of antisera used were evaluated in Ouchterlony gel diffusion plates. The antisera used for suppression were capable of producing precipitin bands at dilutions of 1:64 (anti-μ) or 1:128 (anti-$\gamma1\gamma2$ and anti-$\gamma1$) when reacted against normal or myeloma mouse sera diluted 1:4.

Suppressive Treatment.—Each litter of mice was divided between animals injected intraperitoneally with anti-immunoglobulin serum and controls similarly injected with normal rabbit serum. Neonatally treated animals were injected with 0.05 ml on the day of birth (day 0). The size of subsequent injections, given at 2- to 7-day intervals, was increased slowly until the total desired dose was achieved. At no time did a single injection exceed 0.35 ml.

Immunization.—Specific immunization was carried out by intraperitoneal injection of sheep erythrocytes which had been washed three times and resuspended in phosphate-buffered saline (PBS), pH 7.2. In the single experiment with nude mice and their littermates, immunization was accomplished by intravenous injection of 10 μg of lipopolysaccharide from *Escherichia coli*, strain 0113.

Irradiation and Reconstitution.—Young (35–38-day-old) BALB/c mice to be reconstituted with thymus and/or bone marrow cells were given 900 rads of whole body cobalt irradiation. Syngeneic donor thymuses were removed into cold PBS and minced through 80-mesh stainless steel screens. The resulting single cell suspensions were washed once and resuspended in PBS. Bone marrow from the same donors was removed from the long bones of the hind legs into cold PBS, dispersed by gentle aspiration, washed once, and resuspended in PBS. Normal donors for the reconsititution of unirradiated, antibody-suppressed recipients were all 6-wk-old females. Normal and antibody-suppressed donors for the reconstitution of irradiated recipients were 4–5 wk old and divided equally between males and females. Reconstitution was accomplished intravenously.

Immunological Assays.—Antibody-forming cells were enumerated using a slide modification of the Jerne plaque assay (28). The facilitating antiserum used for indirect plaques was unabsorbed anti-IgG1,2; in studies of $\gamma1$ suppression, plaques were also facilitated with specific anti-$\gamma1$ antiserum. The two facilitation antisera had identical precipitin activities and were used at the same predetermined optimal dilution (1:1000). The number of indirect plaques or $\gamma1$-specific plaques was calculated as the difference between numbers of facilitated and direct plaques. The response to lipopolysaccharide was measured as a plaque assay, using sheep erythrocytes conjugated with this antigen (29). A sheep erythrocyte background control was performed for each animal.

Serum immonoglobulin levels were determined semiquantitatively by the serial dilution Ouchterlony gel diffusion technique of Arnason et al. (30). These levels were reported as the reciprocal of the highest twofold serum dilution producing a distinct band against a constant dilution of commercial (Meloy) class-specific antiserum. When undiluted serum produced no reaction, a value of zero was assigned. Mean serum levels for groups were calculated as simple numerical averages of the individual values.

RESULTS

Immunosuppression of Neonatal Mice with Anti-μ Serum.—We have previously reported that mice injected for the 1st few days of life with rabbit antibodies against mouse μ-chains develop an absolute loss of ability to produce direct plaques against sheep erythrocytes, a complete loss of serum IgM, and severely depressed levels of all other serum immunoglobulins (23). Indirect plaque responses of controls to the single injection of sheep erythrocytes were not sufficient to evaluate the effect of anti-μ treatment. By administering a second erythrocyte injection, we have now been able to demonstrate that neonatal suppression with anti-μ serum also completely eliminates the indirect plaque response (litters 1–3, Table I).

Although the absolute elimination of both direct and indirect plaque responses would suggest complete suppression of both IgM and IgG antibody production, it is interesting to note that the only serum Ig which could consistently be depressed below detection limits was IgM. As shown in Table II, a short-term, low dose regimen of suppression produced complete loss of IgM but relatively little effect on IgG levels (litter 4). A prolonged, high dose regimen, on the other hand, resulted not only in the complete loss of IgM but also in the early reduction of all other serum immunoglobulins to very low residual levels. We initially believed that these early Ig residuals, at least for IgG, might represent declining levels of maternally derived antibody. A second assay on these heavily suppressed mice 3 wk later, however, revealed small but appreciable increases in serum IgG1, IgG2, and IgA. IgM remained undetectable throughout.

Because the loss of serum IgM and inability to form plaques was absolute in neonatally treated mice, we next sought to determine if the effect was reversible, that is could these mice recover immunologically upon cessation of treatment. That they do in fact recover is illustrated by litter 5 (Tables I and II). These mice, suppressed for the 1st 15 days of life, showed the usual serum pattern at day 24, including the complete absence of IgM. They were allowed to coast until day 40, at which time they were injected with 7.5×10^7 sheep erythrocytes. Assay on day 46 revealed not only a direct plaque response fully as great as that of the controls but also serum IgM and IgG1 levels recovered to near control values.

Immunosuppression of Young Adult Mice with Anti-μ Serum. –We next attempted to determine whether the same type of severe immunosuppression produced in neonatally treated mice could be achieved by treatment of mice which had already reached the age of immunocompetence. Tables I and II present our findings for mice started on suppressive treatment at 21 days of age or older. Mice treated with a short-term, low dose schedule and immunized only once with a low dose of sheep cells (7.5×10^7) did show a very severe (26- to 33-fold) depression in direct plaque-forming cells compared to controls (litters 6 and 7). Mice given identical suppressive treatment but immunized with a

larger dose of sheep cells (15×10^7), however, showed a much less marked depression, dropping only three- to sixfold (litters 8 and 9). Increasing the sheep cell dose even more by administering a second large injection of sheep cells (15×10^7) made the relative direct plaque depression still less (twofold), even

TABLE I

Suppression of Plaque-Forming Cells (PFC) in Mice Injected with Rabbit Antiserum against Mouse μ-Chains

Litter No. (No. controls/treated)	Antiserum injected		1° SRBC given		2° SRBC given		Assayed	Mean direct PFC/spleen control/treated	Mean indirect* PFC/spleen control/treated
	day	*ml*	*day*	*(No. × 10^7)*	*day*	*(No. × 10^7)*	*day*		
1 (2/4)	0–26	(0.35)	31	(7.5)	39	(7.5)	45	3100/0	15,200/0
2 (2/2)	0–22	(0.42)	19	(7.5)	27	(7.5)	35	1200/0	2300/0
3 (3/3)	0–52	(2.40)	42	(15)	55	(15)	62	800/0	1900/0
4 (2/2)	0–9	(0.23)	18	(7.5)			24	7800/0	
5 (3/3)	0–15	(0.47)	40	(7.5)			46	17,000/25,400	
6 (3/3)	21–25	(0.40)	29	(7.5)			34	3300/100	
7 (3/3)	21–25	(0.40)	28	(7.5)			33	2600/100	
8 (3/3)	21–25	(0.40)	29	(15)			35	10,200/3100	
9 (3/3)	21–25	(0.40)	29	(15)			35	13,400/2200	
10 (3/3)	37–43	(0.70)	32	(15)	45	(15)	54	600/300	5200/600
11 (2/3)	21–34	(1.00)	28	(15)	37	(15)	44	1000/600	3600/2700

* Facilitated with anti-G1,2 antiserum and calculated as the difference between numbers of facilitated and direct plaques.

TABLE II

Suppression of Serum Immunoglobulin Levels in Mice Injected with Rabbit Antiserum Against Mouse μ-Chains

Litter No. (No. controls/treated	Antiserum Injected		Mean serum Ig level control/treated									
				IgM	IgG1	IgG2	IgA		IgM	IgG1	IgG2	IgA
	day	*ml*	*day*					*day*				
3 (3/3)	0–52	(2.40)	(41)	32/0*	850/19	32/2	4/1	(62)	32/0	1024/140	128/7	11/11
12 (2/2)	0–53	(2.40)	(42)	32/0	1024/16	128/1	12/0	(63)	32/0	1024/48	128/4	12/3
4 (2/2)	0–9	(0.23)	(24)	16/0	128/64	64/64						
5 (3/3)	0–15	(0.47)	(24)	27/0	256/32	54/27		(46)	32/27	256/150	170/64	
7 (3/3)	21–25	(0.40)	(33)	21/64	425/1365	85/85						
9 (3/3)	21–25	(0.40)	(35)	32/64	170/1365	64/64						
11 (2/3)	21–34	(1.00)	(44)	48/85	1024/2048	64/64						

* Numerical average of highest individual reciprocal serum dilutions producing precipitin bands in Ouchterlony gel diffusion.

though the suppressive dose of antiserum was about doubled and extended to include a treatment between sheep cell injections (litters 10 and 11). Caution must be exercised, however, in interpreting the direct plaques produced in response to secondary immunization as due exclusively to IgM. Indirect plaques were also depressed, but to varying degrees. Note that in litter 10, suppression

was not started until 5 days after the first sheep cell injection, allowing a full primary response before treatment with the suppressive antiserum. It was anticipated that in view of the antigen dosage effect just described, exposure to antigen before suppression should make the animals even more refractory, but such did not appear to be the case. This antigen dose dependence is in striking contrast to the independence of antigen dose reported earlier for neonatally suppressed animals, in which it was found that increasing the antigen dose from 7.5×10^7 to 3.75×10^8 could not even partially overcome the suppressive effect (23).

The serum levels of mice receiving their first anti-μ treatments as young

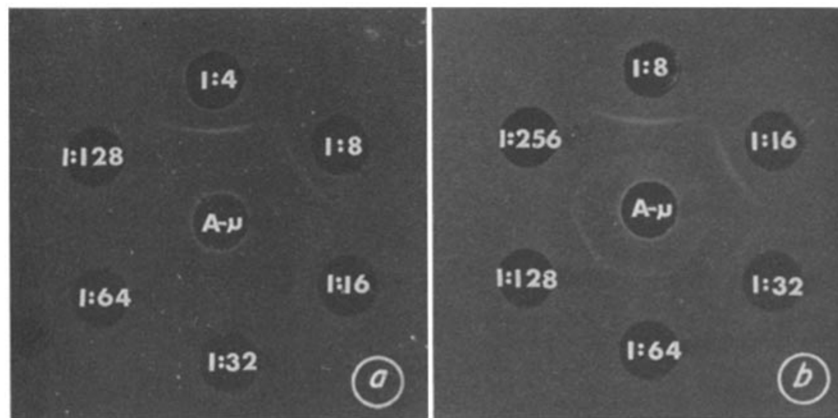

FIG. 1. Mouse serum proteins (diffusing from outer wells at dilutions shown) detectable by anti-μ antiserum (center wells). (*a*) Serum dilutions from a mouse treated as a young adult with normal rabbit serum, showing a normal IgM level of 16. (*b*). Serum dilutions from a mouse similarly treated with anti-μ antiserum, showing a normal IgM level of 32 (outer bands) and an aberrant μ-bearing protein level equal to or exceeding 256 (shown in a second plate to be 512).

adults (Table II) proved to be rather surprising. Despite generally lowered plaque-forming responses, serum levels of both IgM and IgG1 rose, sometimes strikingly, rather than falling. IgG2 levels remained constant, never falling as they did in neonatally treated animals. Even more surprising was the appearance in the serum of some substance detectable only with anti-μ antiserum of proven specificity (Meloy) but diffusing in Ouchterlony gel plates in an entirely different position from normal serum IgM. As seen in Fig. 1, this material, diffusing from the outer wells, formed a precipitin band well inside that of normal IgM, suggesting a smaller molecule, such as free μ-chains or perhaps a monomer of IgM. This substance, usually detectable in our Ouchterlony assay system at serum dilutions of 1:128 to 1:512 (serum levels of 128–512), was frequently completely absent in control animals and was never

detectable at dilutions above 1:4. The persistence of this material is illustrated by a litter of mice injected with 0.80 ml of anti-μ between days 20 and 28 and assayed periodically thereafter. At day 34 the mean serum level was 181, falling to 43 by day 47, to 24 by day 60, and to 12 by day 74. The mean littermate control level (injected with an identical regimen of normal rabbit serum) remained at three throughout the period.

Immunosuppression of Neonatal Mice with Anti-$\gamma 1$ or Anti-$\gamma 1 \gamma 2$ Serum. – The data in Tables III and IV show that suppression with antisera directed against IgG heavy chains characteristically produced less notable results than treatment with anti-μ serum. Anti-$\gamma 1 \gamma 2$ administered in a very low dose, short-term regimen produced no apparent effect on either direct or indirect plaque responses to a single injection of sheep erythrocytes (litter 13). A slight increase in the total antiserum dosage and treatment time resulted in the elimination of the indirect response but produced no effect on the direct response (litter 14). A further great increase in the suppressive dose and prolongation of treatment to cover the period needed for a secondary response to sheep cells (litters 15–17) confirmed the total elimination of the indirect plaque response. In these mice we also observed a partial suppression of the direct plaque response but never eliminated it.

Anti-$\gamma 1$ produced the same sort of complete or nearly complete suppression of the indirect plaque response as did anti-$\gamma 1 \gamma 2$ and about the same degree of inhibition of direct plaque formation (litters 18 and 19). In fact, dose considered, it seemed somewhat more effective, especially against the direct response. In control animals, facilitation with anti-$\gamma 1$ produced half to two-thirds as many developed plaques as facilitation with anti-IgG1,2 (litters 15, 18, and 19). The higher anti-IgG1,2 counts presumably represent either a difference in efficacy of the developing antisera or IgG2 plaques. Note, however, that anti-$\gamma 1$ suppression resulted in similar degrees of reduction in plaques developed with either antiserum, indicating that production of IgG2 plaque-forming cells which might occur was also suppressed by anti-$\gamma 1$ antibodies.

The effects of anti-$\gamma 1 \gamma 2$ or anti-$\gamma 1$ antisera upon serum Ig levels were less marked than those of anti-μ. IgG2 and IgA levels, for example, were rarely affected noticeably by anti-$\gamma 1 \gamma 2$, even by the largest doses and most prolonged treatments (litters 13–17, Table IV). Serum IgG1, on the other hand, frequently demonstrated a noticeable early depression (litters 13–17), although at least a partial recovery occurred even during continued suppressive treatment (litters 16 and 17). In litters 16 and 17, the two most heavily treated litters, IgM rose to levels 8- to 16-fold greater than controls. This is at variance with our preliminary observation (23) that larger doses of anti-$\gamma 1 \gamma 2$ did not seem to affect IgM levels as much as smaller doses. The most heavily treated mice maintained their elevated IgM levels but showed no evidence of any aberrant μ-containing protein such as that stimulated by anti-μ treatment of

adults. The familiar pattern of a stable IgG2 level, a slight drop in IgG1 and a slight rise in IgM was observed in animals given moderate treatment with anti-γ1.

Because of the large anti-$\gamma 1 \gamma 2$ doses required to effect neonatal suppression and because of the partial nature of the suppression of young adult mice by anti-μ, suppression of adults was not attempted with anti-$\gamma 1 \gamma 2$ or anti-γ1.

Determination of Immunosuppression Target Cells.—Antiserum against μ-chains was selected for studies of the target cells of the immunosuppressive effect reported here because among the antisera studied it consistently produced the most severe depressions with the lowest treatment doses and times. Our

TABLE III

Suppression of Plaque-Forming Cells in Mice Injected with Rabbit Antiserum against Mouse γ1 or γ1 γ2-Chains

Litter No. (No. controls/ treated)	Antiserum injected		Antibodies directed against	1° SRBC given		2° SRBC given		Assayed	Mean direct PFC/spleen control/ treated	Mean indirect* PFC/spleen control/ treated	Mean γ1‡ PFC/ spleen control/ treated
	day	*ml*		*day*	*(No. × 10^7)*	*day*	*(No. × 10^7)*	*day*			
13 (3/3)	0–12	(0.55)	$\gamma 1 \gamma 2$	16	(7.5)			24	500/600	400/400	
14 (2/2)	0–15	(0.70)	$\gamma 1 \gamma 2$	20	(7.5)			30	1900/2100	1500/0	
15 (3/3)	0–45	(1.50)	$\gamma 1 \gamma 2$	40	(7.5)	47	(7.5)	53	1200/500	1200/0	600/0
16 (3/3)	0–55	(3.00)	$\gamma 1 \gamma 2$	42	(15)	56	(15)	63	1000/300	3300/0	
17 (3/4)	0–54	(3.00)	$\gamma 1 \gamma 2$	42	(15)	56	(15)	63	2200/800	8500/0	
18 (3/4)	0–25	(0.70)	γ1	21	(7.5)	30	(7.5)	38	900/400	3600/200	2300/200
19 (3/3)	0–24	(0.55)	γ1	20	(7.5)	29	(7.5)	39	700/200	2900/0	1800/0

* Facilitated with anti-G1, 2 antiserum and calculated as the difference between numbers of facilitated and direct plaques.

‡ Facilitated with specific anti-γ1 antiserum and calculated as the difference between numbers of facilitated and direct plaques.

first effort at determining the target of suppression involved an attempt to reconstitute treated animals with bone marrow or thymus cells from normal syngeneic animals. A litter of animals was given a heavy suppressive regimen (0.65 ml) over the 1st 27 days of life and allowed to coast for 5 days, at which time they were given either 5×10^7 normal bone marrow cells or 10^8 normal thymus cells. 2 hr after intravenous administration of lymphoid cells, all animals were given 15×10^7 sheep erythrocytes intraperitoneally. 5 days later the animals were assayed. As shown in Table V, immunosuppression remained complete, with no plaque-forming cells detected and a serum pattern identical to mice receiving no lymphoid cells. The effect was most likely attributable to suppression of the donor cells by residual antiserum in circulation.

Our next experiment designed to determine the target of suppression employed genetically thymusless mice. These mice have been shown to possess

TABLE IV

Suppression of Serum Immunoglobulin Levels in Mice Injected with Rabbit Antiserum against Mouse $\gamma 1$ or $\gamma 1\gamma 2$-Chains

Litter No. (No. controls/treated)	Antiserum injected	Antibodies directed against	Mean serum Ig level control/treated									
				IgM	IgG1	IgG2	IgA		IgM	IgG1	IgG2	IgA
	day *ml*		*day*					*day*				
13 (3/3)	0–12 (0.55)	$\gamma 1\gamma 2$	(24)	21/32*	256/256	85/64						
14 (2/2)	0–15 (0.70)	$\gamma 1\gamma 2$	(30)	16/16	256/32	16/8						
15 (3/3)	0–45 (1.50)	$\gamma 1\gamma 2$	(53)	32/128	1365/1024	85/85						
16 (3/3)	0–55 (3.00)	$\gamma 1\gamma 2$	(42)	8, 128	2048/512	64/64	8/8	(63)	32/128	2048/1024	64/64	16/16
17 (3/4)	0–54 (3.00)	$\gamma 1\gamma 2$	(42)	16/128	2048/512	64/64	10/10	(63)	32/128	2048/1024	64/64	16/16
18 (3/4)	0–25 (0.70)	$\gamma 1$	(38)	32/56	512/384	107/112						
19 (3/3)	0–24 (0.55)	$\gamma 1$	(39)	32/64	850/425	128/128						

* Numerical average of highest individual reciprocal serum dilutions producing precipitin bands in Ouchterlony gel diffusion.

an embryonic abnormal thymic rudiment which never becomes populated with lymphoid cells (31). Although unable to respond normally to thymus-dependent antigens such as sheep erythrocytes (32, 33), these mice have been shown by our colleagues, Manning et al. (34), to respond well to the thymus independent lipopolysaccharide (LPS) antigen of *E. coli*.

Using this assay system, we attempted anti-μ suppression of thymusless mice. This experiment, presented in Table VI, provides definitive evidence that at least part of the target cells of anti-μ suppression are of bone marrow origin. Thymusless control animals, treated only with normal rabbit serum, responded to LPS about as well as their phenotypically normal littermates and showed

TABLE V

Attempted Reconstitution of Anti-μ Suppressed Mice with Cells from Normal Bone Marrow or Thymus

Litter No. (No. animals)	Antiserum injected		SRBC given		Assayed	Reconstitution No. and type of cells (day 32)	Direct PFC/spleen	Indirect PFC/spleen	Mean serum Ig level		
									IgM	IgG1	IgG2
	day	*ml*	*day*	*(No. × 10⁷)*	*day*						
20 (2)	0–48	(1.25)	54	(15)	59	None	0	0	0	128	8
21 (2)	0–27	(0.65)	32	(15)	37	5×10^7 BM*	0	0	0	64	8
21 (2)	0–27	(0.65)	32	(15)	37	10^8 T‡	0	0	0	48	6

* Bone marrow cells.
‡ Thymus cells.

serum Ig levels comparable in all respects to normal animals except for a lower IgG1 value. Thymusless mice receiving anti-μ antiserum, however, showed the complete elimination of plaque response typical of anti-μ suppressed animals and exhibited an even greater degree of serum Ig suppression than their phenotypically normal littermates. Although these data clearly indicate suppression of bone marrow–derived cells, they tell nothing about the possibility that thymus-derived cells might also be affected.

To try to answer the question of suppression of thymus-derived cells, a group of young adult mice was lethally irradiated (900 rads) and reconstituted with various combinations of bone marrow (BM) and thymus (T) cells from normal and heavily anti-μ suppressed (0.70 ml, 28 days) mice. These results are presented in Table VII. As expected, mice receiving antigen only or antigen plus a single cell type, either BM or T, from normal mice made virtually no response. All combinations of both BM and T cells from either normal or suppressed donors, however, made similar, fully reconstituted responses. Whatever cell types may be affected in the intact suppressed animal, it is clear that both BM and T cells, once removed from the suppressed animals and placed into an

TABLE VI

Suppression of Genetically Thymusless (Nude) Mice with Rabbit Antiserum against Mouse μ-Chains

Type of mice (No.)*	Type of serum injected		10 μg LPS‡ given	Assayed	Mean plaque‡ response	Mean Serum Ig level			
						IgM	IgG1	IgG2	IgA
		ml	*day*	*day*					
Littermate normal (3)	Normal rabbit	(0.90)	31	35	4400	64	850	43	3
Littermate normal (2)	Anti-μ	(0.90)	31	35	0	0	320	16	0
Thymusless (4)	Normal rabbit	(0.90)	31	35	5700	64	40	32	2
Thymusless (3)	Anti-μ	(0.90)	31	35	0	0	5	1	0

* Pooled from two litters.

‡ Response to LPS determined by plaque assay using sheep cells conjugated with LPS.

TABLE VII

Reconstitution of Lethally (900 rads) Irradiated Mice with Bone Marrow and Thymus Cells from Normal and Anti-μ Suppressed Donors

No.* of animals	No. of cells used in reconstitution				Mean direct plaques/spleen§
	Normal bone marrow	Normal thymus	Anti-μ suppressed‡ bone marrow	Anti-μ suppressed‡ thymus	
2	—	—	—	—	0
2	—	2×10^8	—	—	0
2	2×10^7	—	—	—	100
3	2×10^7	2×10^8	—	—	2900
3	2×10^7	—	—	2×10^8	1400
2	—	2×10^8	2×10^7	—	3100
2	—	—	2×10^7	2×10^8	2000

* Pooled from four litters, each litter being distributed equally among test groups.

‡ Anti-μ suppressed donors were treated from day 0–28 with a total of 0.70 ml; the animals were sacrificed on day 29.

§ All animals were given 15×10^7 sheep erythrocytes intraperitoneally on the day of reconstitution and assayed 9 days later.

environment free of anti-μ antibodies, are fully capable of restoring immune competence.

DISCUSSION

Our data indicate that the injection of neonatal mice with rabbit antiserum directed against mouse μ-chains completely prevents these animals from developing direct or indirect plaque-forming cells to sheep erythrocytes. This inability to form plaques is accompanied by the total loss of detectable serum IgM and, if the treatment is continued for several weeks, by severe decreases

in serum levels of IgG1, IgG2, and IgA. The depression in serum IgG1 and IgG2 does not become fully apparent until sometime shortly after weaning, presumably because of the presence of maternal antibody Ig obtained by nursing. Despite the loss of ability to respond to at least certain specific immunogens and nearly complete loss of serum immunoglobulins, there was no indication whatsoever at any time of wasting or loss of vigor among treated animals.

The suppression of more than one Ig class in mice treated with anti-μ serum, as observed by us and by Lawton et al. (24), is in accord with the dual IgM and IgG agammaglobulinemia achieved by Kincade et al. (21) with anti-μ treatment of chickens. This progressive suppression of all classes of serum Ig in mice by anti-μ serum suggests that the production of all of these proteins depends upon cells bearing μ-chains on their surfaces. There are several plausible variations of this interpretation. First, some nonproducing cell involved in the immune response, such as the macrophage or thymocyte, might be μ-bearing. Alternatively, all mature lymphoid cells which actually produce antibody could possess surface μ-chains. This would agree with the observation of Vitetta et al. (4) that μ-chains compose some 90–95% of the total heavy chains recoverable from the surface of mouse splenic lymphocytes. Such an interpretation would also be consistent with the finding by Bankhurst and Warner (5) that the number of mouse spleen cells detectable with radiolabeled anti-μ antibodies is as great as the number detectable with labeled anti-κ, the latter representing the total number of cells bearing any surface Ig.

Another alternative is the occurrence of μ-bearing precursors for all antibody-forming cells. This interpretation implies that all immunoglobulin-forming cell lines possess μ-containing receptors in their early stages of development, making them susceptible to suppression, but during subsequent maturation may lose all or part of these receptors. They could, during maturation, retain or even add surface immunoglobulins of other classes. Such a universal role for a μ-containing receptor is supported by the work of Warner et al. (12), who found that among a battery of antisera directed against heavy and light chains, only anti-κ and anti-μ could prevent antigen binding by splenocytes or suppress antibody production by treated cells when adoptively transferred. Similarly, Greaves (35) has established that in the early stages of an immune response to sheep erythrocytes many antigen-binding cells bear multiple heavy chains but later usually become heavy chain class "restricted." Significantly, he found that in the early stages rosette formation by almost all cells can be blocked by anti-IgM but later many can be blocked only by anti-IgG sera. The related concept of an actual switch from IgM production to IgG production, as suggested by the presence of double producers, has been discussed at some length by Nossal et al. (36).

Our data most nearly support this latter concept of early possession of μ-containing receptors and subsequent partial or complete loss or replacement during maturation. This support is based partly upon the dramatic suppressive

effect of anti-μ on all serum immunoglobulin levels in neonatally treated animals as contrasted to the relatively minor influence on animals first treated as adults. The failure of anti-μ treatment to ever completely suppress IgG1 or IgG2 may be due to early (prenatal) maturation of a few cells in these lines with the consequent loss of μ-bearing receptors. The partial recovery of serum IgG1 and IgG2 levels which we observed even among animals given continuous, massive anti-μ treatment well into adulthood could then be due to the further development of these early maturing cells.

That such differentiation can in some cases occur even during continued suppression is best illustrated by the changes in IgA serum levels which we observed. Neonatal treatment with anti-μ serum consistently depressed serum IgA far below normal, frequently to undetectable levels. Even during continuous treatment, however, IgA levels invariably recovered, often to normal values. If, as Walters and Wigzell (37) have suggested, surface immunoglobulins of lymphocytes involved in humoral antibody production represent the productive potential of the cell, then the IgM line might be expected to bear no surface immunoglobulins other than those containing μ. This would account for the absolute suppression achieved in neonates and failure of IgM levels to recover during treatment.

The difficulty of suppressing young adult animals with anti-μ may in part be explained by their larger size and the presence of greater amounts of serum IgM. Both would make it difficult to achieve, with the small doses used, an adequate circulating concentration of unreacted anti-μ antibodies. Perhaps even more importantly, maturation of cell lines other than IgM could, in the view of our working hypothesis, lead to the loss of surface IgM receptors and thus to loss of susceptibility to anti-μ suppression.

There may be some cells of the IgM line which instead of being inactivated by anti-μ are triggered to production of some sort of nonantibody, unassembled IgM product. This would account for the soaring levels of aberrant μ-containing protein observed in animals first treated as adults. The decline in indirect plaques but rising levels of IgG1 suggests a similar, but lesser, stimulatory activity of anti-μ serum on the IgG1 line.

Some support for the concept of changing surface immunoglobulins during cell line maturation can be gained from observations of the suppressive effects of anti-$\gamma 1 \gamma 2$ and anti-$\gamma 1$. A low dose, early treatment was seen to produce little or no effect on plaque responses, whereas higher doses and prolonged treatment seemed to result in elimination of the indirect response and diminished serum IgG1. This is consistent with the idea that surface $\gamma 1$-bearing structures become more abundant during maturation. The continued absence of an indirect plaque response accompanying the partial recovery of serum IgG1 levels during prolonged anti-$\gamma 1 \gamma 2$ treatment suggests the possibility that the protein produced during "recovery" is a nonantibody Ig such as the aberrant μ-protein stimulated by anti-μ treatment of young adults. The significance of the ele-

vated IgM levels in animals neonatally treated with anti-$\gamma 1 \gamma 2$ is not clear. It does not seem to be entirely analogous to the stimulatory effect of anti-μ because the aberrant μ-protein was never detected. How much of the IgG1 serum fluctuation is attributable to simple antigen-antibody complexing and clearance is not certain.

The apparent suppression of IgG2 plaque-forming cells by anti-$\gamma 1$ might be explained by the known residual of anti-$\gamma 2$ activity in this antiserum. It could also be explained by the report of Lee et al. (8) that among all mouse spleen cells showing any IgG determinants, nearly all have $\gamma 1$ specificity, about two-thirds also have $\gamma 2$ specificity, and only a very few have $\gamma 2$ specificity only.

We consider the most likely explanation of the partial suppressive effect of anti-$\gamma 1 \gamma 2$ and anti-$\gamma 1$ on the direct plaque response as being due to trace contamination with anti-μ. Although not detected by immunoelectrophoresis or Ouchterlony gel diffusion, such a contaminant might be detectable by the more sensitive radioimmunoprecipitation test recommended by Nossal et al. (36). In view of the massive doses of anti-$\gamma 1 \gamma 2$ and anti-$\gamma 1$ used and the extreme suppressive efficiency of anti-μ in neonatal animals, even an undetectable trace contamination of anti-μ could account for this effect.

The steady serum IgG2 levels observed constitute the most difficult aspect of the study to explain. Only massive neonatal treatment with anti-μ appreciably affected IgG2 levels. Neither adult treatment with anti-μ nor neonatal treatment with either anti-$\gamma 1$ or anti-$\gamma 1 \gamma 2$ moved these levels at all, although the latter antiserum had an extremely high precipitin activity against $\gamma 2$. The complete elimination of indirect plaque formation by neonatal treatment with either anti-μ or anti-$\gamma 1 \gamma 2$ establishes that either IgG2 plaques are not formed in response to sheep erythrocytes or else the IgG2 line is susceptible to suppression. It is not immediately apparent why serum IgG2 levels should remain so constant.

Our success in suppressing genetically thymusless mice with anti-μ serum clearly indicates that at least some of the target cells in the type of suppression reported here are bone marrow derived. We have no information bearing on the question of suppression of thymus-derived cells involved in humoral antibody responses other than the indirect evidence afforded by the homograft studies reported previously (25). In that case we found absolutely no evidence of prolongation of survival of homografted skin resulting from the use of either anti-μ or anti-$\gamma 1 \gamma 2$, even when the grafted animals were massively treated from the day of birth on through the entire rejection period. It is apparent, therefore, that the population of thymus cells responsible for cellular immune responses (38–40) is not noticeably affected by these antisera.

Our evidence indicating that the target cells of anti-μ antibodies in vivo are bone marrow derived agrees with the conclusion of Doria et al. (41) that the cells inhibited by exposure of mouse splenocyte cultures to anti-whole mouse serum were of nonthymic origin. It is an interesting contrast, however, to the

finding by Lesley et al. (42) that the primary target of anti–light chain antibodies (anti-κ) in vitro is the thymus-derived cell.

There is nothing in our study upon which a mechanism can be based to explain the actual suppressive effect of antisera on immune cells. We have no basis for deciding whether cells contacting antibody are reversibly inhibited, irreversibly inhibited, or killed outright. Both the natural recovery of immune competence, occurring between 9 and 31 days after cessation of suppressive treatment, and the reconstitution of irradiated animals with thymus and bone marrow cells from anti-μ suppressed mice suggest that either suppression is reversible or the cells within the bone marrow and/or thymus are not affected by antibody. Basten et al. (10) have demonstrated that bone marrow–derived cells are susceptible to lethally radioactive antigen only when peripheralized to the spleen, indicating that while still in the bone marrow the cells are either inaccessible to the antigen or lack receptors for it. We believe that this sort of cellular inaccessibility and/or lack of differentiated receptors offer plausible support for the idea that cells within the marrow remain unaffected by anti-μ, thus explaining both natural recovery and the ability of these cells to effect reconstitution of irradiated animals.

SUMMARY

Neonatal injection of mice with rabbit anti-μ antiserum has been shown to produce complete loss of direct and indirect plaque-forming responses to sheep erythrocytes as well as loss of serum IgM and severe depressions of all other serum immunoglobulins. Similar injection of anti-$\gamma 1\gamma 2$ or anti-$\gamma 1$ antibodies effects a loss of the indirect response but induces relatively minor alterations in serum Ig levels. Delaying initiation of anti-μ treatment until young adulthood results in a somewhat diminished effect on plaque-forming responses and serum Ig levels but triggers the release of high serum levels of an aberrant μ-bearing protein.

Anti-μ suppression of genetically thymusless mice indicates that at least part of the target cells for suppression are bone marrow derived. A working hypothesis for the maturation of humoral antibody-producing cell lines as it relates to these data is discussed.

We wish to thank Dr. J. A. Rudbach for supplying the lipopolysaccharide antigen. We also thank Ms. J. K. Manning for assistance in the assay of thymusless mice, and Dr. N. D. Reed for consultation and review of the manuscript.

REFERENCES

1. Greaves, M. F., and N. M. Hogg. 1971. Immunoglobulin determinants on the surface of antigen binding T- and B-Lymphocytes in mice. *In* Progress in Immunology. B. Amos, editor, Academic Press, New York. 111.
2. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1971. Immunoglobulins

on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* **133:**156.

3. Jones, G., G. Torrigiani, and I. M. Roitt. 1971. Immunoglobulin determinants on mouse lymphocytes. *J. Immunol.* **106:**1425.
4. Vitetta, E. S., S. Baur, and J. W. Uhr. 1971. Cell surface immunoglobulin. II. Isolation and characterization of immunoglobulin from mouse splenic lymphocytes. *J. Exp. Med.* **134:**242.
5. Bankhurst, A. D., and N. L. Warner. 1971. Surface immunoglobulins on mouse lymphoid cells. *J. Immunol.* **107:**368.
6. Takahashi, T., L. J. Old, K. R. McIntire, and E. A. Boyse. 1971. Immunoglobulin and other surface antigens of cells of the immune system. *J. Exp. Med.* **134:**815.
7. Bankhurst, A. D., N. L. Warner, and J. Sprent. 1971. Surface immunoglobulins on thymus and thymus-derived lymphoid cells. *J. Exp. Med.* **134:**1005.
8. Lee, S. -T., F. Paraskevas, and L. G. Israels. 1971. Cell surface associated gamma globulins in lymphocytes. II. The pluripotentiality of mouse spleen lymphocytes. *J. Immunol.* **107:**1583.
9. Baur, S., E. S. Vitetta, C. J. Sherr, I. Schenkein, and J. W. Uhr. 1971. Isolation of heavy and light chains of immunoglobulin from the surfaces of lymphoid cells. *J. Immunol.* **106:1133.**
10. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labeled antigen. *Nature* (*London*). **231:**104.
11. Mason, S., and N. L. Warner. 1970. The immunoglobulin nature of the antigen recognition site on cells mediating transplantation immunity and delayed hypersensitivity. *J. Immunol.* **104:**762.
12. Warner, N. L., P. Byrt, and G. L. Ada. 1970. Blocking of the lymphocyte antigen receptor site with anti-immunoglobulin sera in vitro. *Nature* (*London*). **226:**942.
13. Fuji, H., and N. K. Jerne. 1969. Primary immune response in vitro: reversible suppression by anti-globulin antibodies. *Ann. Inst. Pasteur* (*Paris*). **117:**801.
14. Hartmann, K. -U., S. Reeg, and C. Mehner. 1971. The induction of haemolysis producing cells in vitro: inhibition by antiglobulin antisera. *Immunology.* **20:**29.
15. Lesley, J., and R. W. Dutton. 1970. Antigen receptor molecules: inhibition by antiserum against kappa light chains. *Science* (*Washington*). **169:**487.
16. Sjöberg, O., and M. Greaves. 1971. Inhibition of the immune response of mouse spleen cells to sheep erythrocytes *in vitro* by anti-immunoglobulin sera. *Eur. J. Immunol.* **1:**157.
17. Tyan, M. L. 1971. Modification of bone marrow induced GVH disease with heterologous antisera to γ globulin or whole serum. *J. Immunol.* **106:**586.
18. Cole, L. J., and S. E. Maki. 1971. Differential inactivation of lymphocytes and bone marrow stem cells by heterologous anti-mouse γ-globulin serum. *Nature* (*London*). **230:**244.
19. Riethmüller, G., E. -P. Rieber, and I. Seeger. 1971. Suppression of graft versus host reaction by univalent anti-immunoglobulin antibody. *Nature* (*London*). **230:**248.
20. Ruffilli, A., A. Compere, and C. Baglioni. 1970. Repression of the synthesis of immunoglobulins in new born mice by antibodies directed against the variable region of light chains. *J. Immunol.* **104:**1511.

21. Kincade, P. W., A. R. Lawton, D. E. Bockman, and M. D. Cooper. 1970. Suppression of immunoglobulin G synthesis as a result of antibody-mediated suppression of immunoglobulin M synthesis in chickens. *Proc. Nat. Acad. Sci. U.S.A.* **67**:1918.
22. Kincade, P. W., A. R. Lawton, and M. D. Cooper. 1971. Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line. *J. Immunol.* **106**:1421.
23. Manning, D. D., and J. W. Jutila. 1972. Immunosuppression in mice injected with heterologous anti-immunoglobulin antisera. *J. Immunol.* **108**:282.
24. Lawton, A. R., R. Asofsky, M. B. Hylton, and M. D. Cooper. 1972. Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to μ-chain. *J. Exp. Med.* **135**:277.
25. Manning, D. D., and J. W. Jutila. 1972. Effect of anti-immunoglobulin antisera on homograft rejection in mice. *Nature (London).* In press.
26. Pantelouris, E. M. 1968. Absence of thymus in a mouse mutant. *Nature (London).* **217**:370.
27. Potter, M. 1967. The plasma cell tumors and myeloma proteins of mice. *In* Methods in Cancer Research. H. Busch, editor. Academic Press, New York. **2**:105.
28. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
29. Neter, E., O. Westphal, O. Lüderitz, E. A. Gorzynski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte-modifying, antigenic toxic and pyrogenic properties. *J. Immunol.* **76**:377.
30. Arnason, B. G., C. DeVaux St-Cyr, and E. H. Relyveld. 1964. Role of the thymus in immune reactions in rats. IV. Immunoglobulins and antibody formation. *Int. Arch. Allergy Appl. Immunol.* **25**:206.
31. Pantelouris, E. M., and J. Hair. 1970. Thymus dysgenesis in nude (nu nu) mice. *J. Embryol. Exp. Morphol.* **24**:615.
32. Wortis, H. H. 1971. Immunological responses of "nude" mice. *Clin. Exp. Immunol.* **8**:305.
33. Pantelouris, E. M. 1971. Observations on the immunobiology of "nude" mice. *Immunology.* **20**:247.
34. Manning, J. K., N. D. Reed, and J. W. Jutila. 1972. Antibody response to *Escherichia coli* lipopolysaccharide and type III pneumococcal polysaccharide by congentially thymusless (nude) mice. *J. Immunol.* In press.
35. Greaves, M. F. 1971. The expression of immunoglobulin determinants on the surface of antigen-binding lymphoid cells in mice. I. Analysis of light and heavy chain restrictions on individual cells. *Eur. J. Immunol.* **1**:186.
36. Nossal, G. J. V., N. L. Warner, and H. Lewis. 1971. Incidence of cells simultaneously secreting IgM and IgG antibody to sheep erythrocytes. *Cell. Immunol.* **2**:41.
37. Walters, C. S., and H. Wigzell. 1970. Demonstration of heavy and light chain antigenic determinants on the cell-bound receptor for antigen. Similarities between membrane-attached and humoral antibodies produced by the same cell. *J. Exp. Med.* **132**:1233.

38. Sloboda, A. E., and J. Landes. 1970. The comparative immunosuppressive effects of heterologous antisera to various C_3H mouse tissues. *J. Immunol.* **104:**185.
39. Blomgren, H., M. Takasugi, and S. Friberg, Jr. 1970. Specific cytotoxicity by sensitized mouse thymus cells on tissue culture target cells. *Cell. Immunol.* **1:**619.
40. Cerottini, J. C., A. A. Nordin, and K. T. Brunner. 1970. Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. *Nature (London).* **228:**1308.
41. Doria, G., G. Agarossi, and S. Di Pietro. 1971. Effect of blocking cell receptors on an immune response resulting from in vitro cooperation between thymocytes and thymus-independent cells. *J. Immunol.* **107:**1314.
42. Lesley, J. F., J. R. Kettman, and R. W. Dutton. 1971. Immunoglobulins on the surface of thymus-derived cells engaged in the initiation of a humoral immune response. *J. Exp. Med.* **134:**618.