DIFFERENCES IN SUSCEPTIBILITY OF MATURE AND IMMATURE MOUSE B LYMPHOCYTES TO ANTI-IMMUNOGLOBULIN-INDUCED IMMUNOGLOBULIN SUPPRESSION IN VITRO

Possible Implications for B-Cell Tolerance to Self

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Antibodies to class-specific (13,17,19,22), allotypic (6,10), and idiotypic (4,9,32) determinants of immunoglobulin (Ig)[1] molecules have been widely used to inhibit B cells which carry these particular Ig determinants on their surface. These models are important not only as experimental tools and as potential therapeutic maneuvers to suppress B cells, but also in their possible relationship to B-cell immunological tolerance. In addition, suppression by endogenous anti-idiotypic antibodies may play an important homeostatic role in the normal regulation of immune responses (12,20). It is thus important to attempt to define the mechanisms involved in anti-Ig-induced suppression of B cells.

In this study we have used organ explants and dissociated cells from fetal and adult mouse lymphoid tissues to study the in vitro effects of anti-Ig antibodies on developing and mature IgM-bearing B lymphocytes. We show that anti-Ig antibodies, or their Fab fragments, completely inhibit the development of IgM-bearing B cells in explants of fetal liver, and induce the disappearance of cell surface IgM in explant or suspension cultures of late fetal or adult lymphoid tissues. Moreover, we demonstrate that while IgM-bearing B cells in fetal liver or adult bone marrow are susceptible to irreversible Ig suppression by low concentrations of anti-Ig antibody, those in adult lymph node or spleen can only be reversibly suppressed, and require much higher antibody concentrations for suppression. This suggests a fundamental difference between newly formed and more mature B cells, which may have important implications for B-cell tolerance to self-antigens.

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[1] *Abbreviations used in this paper:* anti-Ig, antibodies against Ig determinants; anti-MIg, rabbit antibodies against mouse Ig determinants; anti-μ, purified goat antibodies against mouse μ-chains; anti-MIg-Fl, purified anti-MIg conjugated with fluorescein isothiocyanate; anti-μ-Fl, purified anti-μ conjugated with fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; Fab anti-MIg, Fab fragments of rabbit anti-MIg; Fab anti-μ, Fab fragments of purified goat anti-μ; MEM, minimal essential Eagle's medium containing 15 mM HEPES.

Materials and Methods

Antibodies. Polyspecific rabbit antimouse Ig (anti-MIg) was raised against mouse IgG and purified by affinity chromatography as previously described (29,35). Purified goat antibodies to mouse μ-chains (anti-μ) were raised, adsorbed, and purified on various immunoadsorbents and tested for specificity as previously described (15). Only activity against μ-chains could be detected by Ouchterlony analysis, and after conjugation to fluorescein, by immunoflurescence staining of fixed cell preparations of various mouse myelomas. Moreover, these antibodies did not precipitate δ-like chains (1,37) from solubilized spleen cells which had been radiolabeled by lactoperoxidase-catalyzed iodination (E. Vitetta, personal communication). The methods for conjugating the antibodies with fluorescein isothiocyanate (15) and for preparing Fab monomers by papain digestion (Fab anti-MIg and Fab anti-μ) have been described (34). When tested at concentrations as high as 240 μg/ml, the Fab fragments of anti-μ and anti-MIg failed to precipitate mouse IgM (MOPC-104 E). Purified sheep antibodies to the third component of mouse complement (anti-C3), were a gift from Dr. M. B. Pepys (7).

Immunofluorescence Assays. Cell suspensions were prepared in Eagle's minimum essential medium containing 15 mM Hepes (MEM), 0.2% sodium azide and 10% fetal calf serum (FCS), and stained with fluorescein-conjugated antibodies at a concentration of 0.5 mg/ml for 30 min at 0°C. After washing, the cells were examined under cover slip for surface fluorescence using a Zeiss Ultraphot II (Carl Zeiss, Inc., New York) or a Leitz Ortholux microscope (E. Leitz Inc., Rockleigh, N. J.), both equipped with phase-contrast and incident fluorescence optics, and an Osram HBO 200 mercury arc lamp.

Organ Cultures. BALB/c mice were used in all experiments, except one involving C5-deficient AKR mice. Small fragments (1–2 mm^3) of fetal liver or adult spleen, intact fetal spleens, adult lymph node, or bone marrow plugs were cultured on top of ultrathin Millipore filters (0.4 μm pore size) floating on 1 ml of medium (RPMI 1640, or Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 50 μg streptomycin, and 50 U of penicillin) in organ culture dishes in a 5% (RPMI 1640) or 10% (DMEM) CO_2 incubator at 37°C as previously described (27).

Modulation Experiments. Approximately 2 x 10^6 nucleated cells were incubated in 50 μl of MEM (as a control), or anti-Ig diluted in MEM, for 1 h at 37°C in microprecipitation tubes, washed in MEM with 10% FCS and 0.2% sodium azide, and stained as described above.

Recovery Experiments. Cells exposed to anti-Ig antibodies in organ culture or in suspension, were washed under sterile conditions and recultured for 24–72 h in round-bottom tubes (Falcon 2003, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), or in 16-mm Linbro plates (Linbro Chemical Co., New Haven, Conn.), in RPMI 1640, or DMEM containing 10% FCS. They were then stained for surface Ig as described.

Differential Cell Counts. Differential cell counts were made on cytocentrifuge preparations of dissociated fetal liver cells which were fixed in ethanol and stained with McNeal's tetrachrome stain.

Fluorescence Intensity Analysis Using Fluorescence Activated Cell Sorter. Anti-μ-fluorescein (Fl)-labeled cell suspensions from newborn liver and adult spleen and bone marrow were analyzed on a fluorescence activated cell sorter (FACS-1, Becton-Dickinson & Co., E. Rutherford, N. J.) (2), here used only as an analyzer.

Results

Anti-Ig Suppression of IgM-Bearing Lymphoctye Development in Explant Cultures of Fetal Liver and Spleen. The addition of 10 μg/ml of purified anti-μ or anti-MIg to 14- or 15-day fetal liver cultures completely and consistently suppressed the development of IgM-bearing cells in cultures (Table I). The suppressive effect was completely eliminated if the anti-μ or anti-MIg was first adsorbed on Sepharose-bound IgM or MIg respectively (Table I). The inhibition was not due to steric blocking of anti-μ-Fl labeling by the unfluoresceinated anti-Ig, as preincubation in up to 100 μg/ml of anti-μ or anti-MIg failed to appreciably inhibit the labeling of newborn liver B cells by anti-μ-Fl (used at the standard concentration of 500 μg/ml), even when the cells were labeled in the

TABLE I

Inhibition of the Development of IgM-Bearing Lymphocytes by Anti-Ig in Explant Cultures of Fetal Liver

No. of experiments	Age of fetus	Antibodies added (μg/ml)	Duration of culture	IgM-bearing cells*	
				Control cultures	Anti-Ig cultures
	days		*days*	*%*	*%*
3	14–15	Anti-MIg (20)‡	5–6	2.5 (0.3–3.5)	0
11	14–15	Anti-μ (10)‡	6	1.8 (0.9–2.3)	0
2	14	Fab anti-MIg (200)§	5	3.7 (1.0–4.5)	0
2	14–15	Fab anti-μ (24)‡	5	2.1 (1.8–2.4)	0
1	14	Anti-MIg (20)§	6	3.8	0
1	14	Anti-MIg adsorbed with MIg (50)§‖	5	3.8	3.7
1	15	Anti-μ adsorbed with IgM¶	6	1.9	2.0
2	14–15	Anti-C3 + anti-μ (10 + 10)**	6	2.1 (1.9–2.3)	0

* Expressed as mean (range).

‡ Immunoadsorbent purified antibodies.

§ Unpurified antibodies.

‖ Adsorption was carried out on DEAE cellulose purified MIg covalently linked to sepharose.

¶ Purified anti-μ antibodies were adsorbed with purified mouse IgM (MOPC 104E) linked to sepharose and the effluent was concentrated to a volume, that before adsorption, would have given a final concentration of 20 μg/ml when added to the cultures.

** No fetal calf serum or antibiotics were present in these cultures.

presence of the unfluoresceinated reagents. The inhibition was unaffected by the omission of fetal calf serum from the cultures, in combination with the addition of anti-C3 antibodies (Table I), and was also complete in C5-deficient AKR mice (data not shown). Thus, it is unlikely that complement-mediated lysis was involved in the suppression. Moreover, Fab fragments of anti-μ and anti-MIg completely suppressed the appearance of IgM-bearing cells (Table I), although approximately 10-fold higher concentrations were required (Fig. 1). Anti-μ inhibited the appearance of all anti-MIg-Fl-labeling cells, making it likely that all Ig-bearing cells expressed IgM on their surface at some time during the culture period. Differential counts done on cytocentrifuge preparations of dissociated 14-day fetal liver fragments, cultured for 7 days in the presence or absence of anti-μ antibodies, did not show a significant difference in the percentage of cells with lymphoid morphology (Table II).

Irreversibility of Anti-Ig-Induced Suppression of IgM-Bearing Cell Development in Fetal Liver Explant Cultures. In one experiment, 14-day fetal liver explants were cultured for 2 days in the presence of 10 μg/ml of anti-μ and then transferred to fresh filters floating on medium without antibodies. Control cultures were treated similarly except they were never exposed to anti-μ. At the end of the total culture period of 7 days, the frequency of IgM-bearing cells was similar in the cultures treated with anti-μ for the first 2 days (9/2,400) and in the untreated controls (8/2,897). Thus, exposure to anti-μ for 2 days, before the

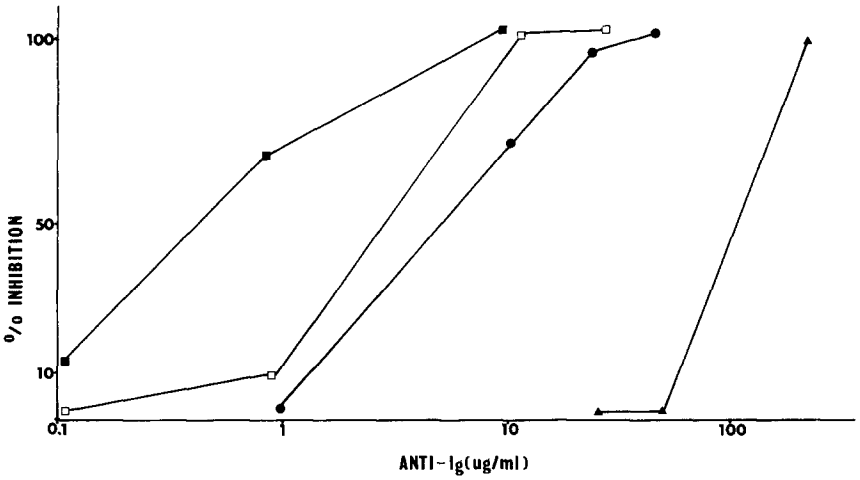

FIG. 1. Dose-response curves for the inhibition of the development of IgM-bearing cells in explant cultures of fetal liver by purified anti-μ (■—■) and Fab anti-μ (●—●), and unpurified anti-MIg (□—□) and Fab anti-MIg (▲—▲) antibodies. 14-day fetal liver explants were cultured for 6 days in the presence or absence of the appropriate concentrations of antibodies.

TABLE II

Differential Cell Counts on Control and Anti-μ Inhibited Explant Cultures of 14-day fetal Liver

Cell type	Control cultures	Anti-μ inhibited cultures
	%	%
IgM-bearing cells	2.4	0
Small lymphocytes	2.1	1.6
Granulocytes	73	74
Monocytes/macrophages	12.6	10
Erythroid series	8.5	9
Blast cells	3.8	5.4

* Explants of 14-day fetal liver were cultured for 7 days with or without anti-μ (10 μg/ml) antibodies, after which explants were dissociated and, either labeled with anti-μ fluorescein, or centrifuged onto a slide, fixed in ethanol, and stained with McNeal's tetrachrome stain.

detectable expression of surface IgM, did not irreversibly inhibit the ability of fetal liver explants to generate IgM-bearing B lymphocytes in vitro.

On the other hand, when 14- to 17-day fetal liver explants were treated with anti-μ, anti-MIg, or their Fab fragments for 5–7 days and then dissociated into cell suspensions (to prevent or slow down further differentiation of Ig^- precursors to Ig^+ B cells), washed and recultured for 24–72 h, usually no recovery of IgM-bearing cells was seen (Table III). In 4/21 experiments a small number of IgM-bearing cells appeared during the period of reculture (Table III), which could have been B cells that had recovered after the inhibition, or that had

TABLE III

Inhibition and Recovery of IgM-Bearing Cells in Anti-Ig-Treated Explant Cultures of Fetal Liver

No. of Experiments	Age of fetus	Antibodies added* (μg/ml)	Duration of culture	IgM-bearing cells‡		IgM-bearing cells after 24 h recovery‡§	
				Control cultures	Anti-Ig cultures	Control cultures	Anti-Ig cultures
	days		*days*	%	%	%	%
5	14–15	Anti-MIg (20)	5–7	2.4 (0.8–6.5)	0	2.0 (0.7–4.4)	0
1	17	Anti-MIg (20)	7	4.4	0	6	0
1	20	Anti-MIg (20)	5	3.0	0	3	0
4	14	Anti-μ (10)	5–7	0.9 (0.3–2.5)	0	0.8 (0.3–2.4)	0
2	17–18	Anti-μ (10)	5–7	3.0 (2–4)	0	4.0 (2–6)	0
2	14–15	Fab anti-μ (24)	6	4.5 (3–6)	0	4.0 (2.5–5.5)	0
2	18	Fab anti-μ (24)	5	2.5 (2–3)	0	3.0 (2–4)	0
1	14	Anti-μ (10)	7	4.5	0	4.4	0.4
2	16	Anti-μ (10)	5	5.5 (3–8)	0	5.0 (3–7)	1 (0.5–1.5)
1	19	Anti-MIg (20)	1	3.0	0	2.8	2.5
1	19	Anti-MIg (20)	2	2.8	0	2.7	0
1	19	Anti-MIg (20)	3	3.0	0	3.0	0

* Immunoadsorbent purified antibodies.
‡ Expressed as mean (range).
§ Cells in explants were dissociated and recultured for a further 24 hours and then stained for surface IgM.

differentiated from Ig^- precursors during reculture.

Anti-Ig-Induced Ig Suppression of IgM-Bearing Cells in Explants of Liver, Bone Marrow, Spleen, and Lymph Node. In BALB/c mice, IgM^+ cells first appear in the liver and spleen at 17 days of gestation (27). When liver fragments from 18–20 day fetuses were cultured in the presence of 10 to 20 μg/ml of anti-MIg or anti-μ, or their Fab fragments, no IgM^+ cells were detected after 1–7 days of culture (Table III). If such liver fragments were dissociated after 24 h exposure to anti-MIg, washed, and recultured for a further 24 h in the absence of antibody, the same number of IgM^+ cells were found as in control fragments never exposed to anti-IG, indicating complete recovery had occurred (Table III). However, exposure of the fragments for $\geq$48 h to anti-MIg almost always led to irreversible suppression (Table III). Most B cells in cultured plugs of young adult bone marrow behaved similarly to those in fetal liver, in that no, or few, IgM^+ cells were found after 7 days incubation with anti-MIg or anti-μ antibodies, and little or no recovery was seen after the plugs were dissociated and recultured for a further 24 h in the absence of antibody (Table IV). On the other hand, cultured fragments of adult spleen or lymph nodes behaved very differently to liver or bone marrow when exposed to anti-μ or anti-MIg antibodies. In these tissues, even 10 times higher concentrations of anti-Ig failed to completely suppress IgM-bearing cells (Table IV), and complete recovery always occurred after dissociation and reculture without antibody (data not shown).

Anti-μ-Induced Modulation of B-Cell IgM in Dissociated Cell Suspensions of Newborn Liver, Adult Bone Marrow, and Spleen. Incubation of Ig^+ B cells with anti-Ig antibodies at 37°C has been shown to redistribute the membrane Ig molecules into patches and caps and to induce vigorous pinocytosis of the Ig-anti-Ig complexes (5,18,34–36). If sufficient antibody is present and adequate time is allowed, most of the Ig can be specifically removed from the cell surface

TABLE IV

Inhibition and Recovery of IgM-Bearing Cells in Anti-Ig Treated Explant Cultures of Spleen, Bone Marrow and Lymph Node

No. of experiments	Tissue	Age of mouse	Antibodies added* (μg/ml)	Duration of culture	IgM-bearing cells‡		IgM-bearing cells after 24 h recovery‡§	
					Control cultures	Anti-Ig cultures	Control cultures	Anti-Ig cultures
		days		*days*	%	%	%	%
1	Spleen	120	Anti-MIg (200)	6	40	30	—	—
1	Spleen	120	Anti-MIg (100)	6	40	38	—	—
3	Lymph node	11	Anti-μ (20)	7	8.3 (5–10)	4.3 (3.2–5.2)	—	—
3	Lymph node	11	Anti-MIg (20)	7	8.3 (5–10)	3.7 (1.9–5.8)	—	—
2	Lymph node	60	Anti-MIg (200)	6	32 (24–40)	25 (20–30)	—	—
2	Lymph node	60	Anti-MIg (100)	6	32 (24–40)	30 (22–38)	—	—
2	Bone marrow	11	Anti-μ (20)	7	3.7 (3.5–3.8)	0	7.5 (4–11)	0
2	Bone marrow	11	Anti-MIg (20)	7	3.7 (3.5–3.8)	0	7.5 (4–11)	0
1	Bone marrow	60	Anti-μ (20)	7	25	0.3	15	0.4
2	Bone marrow	60	Anti-MIg (20)	7	18.5 (12–25)	0	11 (7–15)	0.25 (0–0.5)

* Immunoadsorbent purified.
‡ Expressed as mean (range).
§ Recovered as described in Table III.

(18,34,36). This antibody-induced disappearance of a cell surface antigen is referred to as antigenic modulation (25).

The striking differences in the susceptibility to anti-μ-induced modulation of B-cell IgM of cells in the newborn liver and adult bone marrow on the one hand, and adult spleen on the other, are shown in Figs. 2 and 3. While the former modulate rapidly and at a low concentrations of anti-μ, the latter require approximately 10 times as much antibody and a longer period of time for comparable modulation to occur. IgM-bearing B cells in adult lymph nodes behaved similarly to spleen B cells (data not shown). In all these cell types, complete recovery was seen, if cells, treated with anti-μ for up to 24 h, were washed and cultured for a further 24 h without antibody. Since treating cells with up to 100 μg/ml of anti-μ for 60 min at 0°C did not cause any reduction in the percentage of cells labeled with anti-μ-Fl (used at 500 μg/ml), it is unlikely that the suppression seen at 37°C was related to steric blocking. When newborn fetal liver cells were incubated with anti-μ-Fl (in the absence of sodium azide) at 37°C, rapid capping and massive pinocytosis was readily seen within 2 min.

To exclude the possibility that the differences in susceptibility to anti-μ-induced IgM modulation reflected differences in the amount of IgM on the surfaces of these different B cells, adult bone marrow and spleen and newborn liver cells were labeled with anti-μ-Fl in azide at 0°C and analyzed for intensity of surface fluorescence on the fluorescence activated cell sorter (2). As can be seen in Fig. 4, a continuous spectrum of intensities was seen in cells from all three tissues, with no significant difference between newborn liver and adult spleen cells. More heavily labeled cells were present in the bone marrow than in liver or spleen. Thus, differences in the amount of membrane IgM cannot explain the differences in susceptibility to IgM modulation.

To exclude the possibility that the high susceptibility of liver IgM-bearing cells to modulation by anti-μ antibodies was due to a secreted product of liver

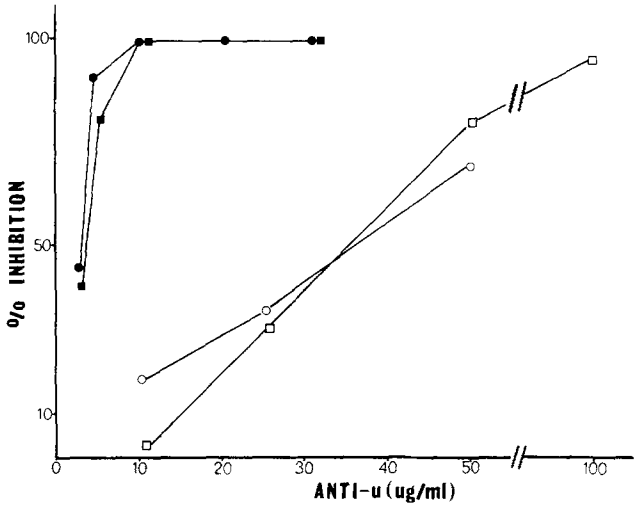

FIG. 2. Dose-response curves for the modulation of IgM-bearing cells in fetal liver (●—●), young adult spleen (□—□) and bone marrow (■—■) with anti-μ antibodies, and in fetal liver with Fab fragments of anti-μ antibodies (○—○). Cells were treated for 60 min at 37°C with anti-μ, or with medium, washed and stained with anti-μ-fluorescein at 4°C in the presence of sodium azide.

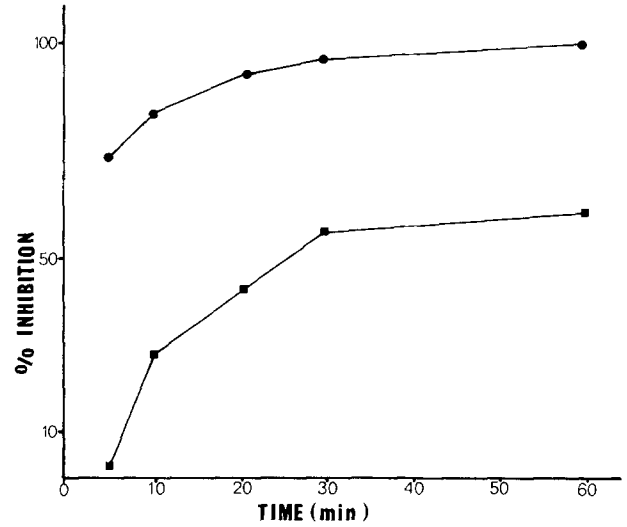

FIG. 3. Time-course of modulation of IgM-bearing cells in fetal liver (●—●) and adult spleen (■—■) by anti-μ antibodies. Cells were incubated with anti-μ (50 μg/ml) at 37°C, and at the indicated time, cold medium containing 0.2% sodium azide was added to stop the modulation.

cells, such as a proteolytic enzyme, 10^6 spleen cells (40% IgM^+ cells) were mixed with 3 x 10^6 18-day fetal liver cells (0.1% IgM^+ cells) and modulated with anti-μ. No difference in the dose-response curve of spleen cell modulation was seen compared to when 10^6 spleen cells alone were modulated. As can be seen in Fig. 2, Fab fragments of anti-μ were able to modulate IgM on newborn liver cells but

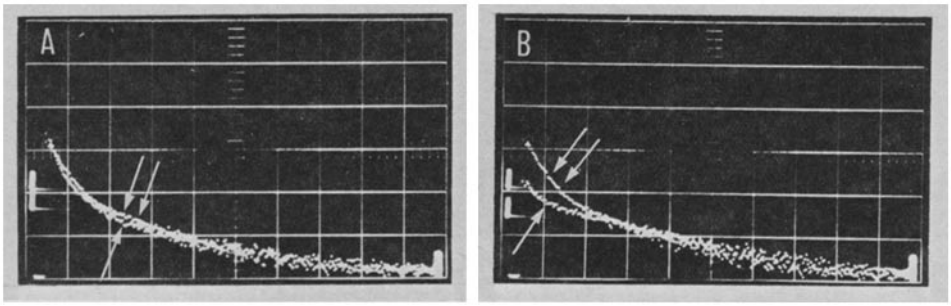

FIG. 4. Intensity analysis of immunofluorescence labeling of IgM-bearing cells in (A) newborn liver (single arrow) and adult spleen (double arrow) and in (B) adult bone marrow (single arrow) and adult spleen (double arrow). Cells were labeled with anti-μ-Fl and analyzed in the fluorescence activated cell sorter. The relative intensity of fluoresence (abscissa) is plotted against the relative number of cells (ordinate). In each sample, 10,000 cells were counted.

more than 10 times the concentration was required to induce the same degree of modulation as intact anti-μ.

Discussion

We have previously reported that Ig-bearing B lymphocytes develop in vitro in explants of 14- or 15-day mouse fetal liver (27), spleen (28) and bone marrow,[2] providing direct evidence for multifocal development of B cells in the hemopoietic tissues of mice. In this study, we have shown that the presence of purified anti-μ or anti-MIg antibodies in the culture medium completely inhibits the appearance of IgM-bearing cells in 14-day fetal liver cultures and causes the disappearance of cell surface IgM in cultures of late fetal or newborn liver. Since complete inhibition also occurred in culture medium containing anti-C3 antibodies, in the absence of fetal calf serum, and with Fab fragments of anti-μ and anti-MIg, it is unlikely that the suppression involves opsinization, complement-mediated phagocytosis or lysis, or K-cell-mediated killing of IgM-bearing cells. Our previous demonstration that θ-bearing lymphocytes do not develop in these cultures (27), also makes T-cell suppression an unlikely mechanism. By exclusion, "antigenic modulation," the specific and metabolically dependent disappearance of a cell surface antigen induced by the binding of antibody (25), would seem to be the most likely explanation. Our finding that anti-μ-Fl is rapidly pinocytosed following binding to IgM-bearing cells in newborn liver suggests that modulation is due largely to anti-Ig-induced pinocytosis, as has been previously shown for adult spleen B cells (34,36).

By definition, antigenic modulation is reversible (25), the antigen reappearing on the surface within 4–12 h when the cells are washed free of antibody and cultured at 37°C (18,25). The reappearance of antigen has been shown to require protein synthesis (18). While IgM-bearing cells in 19-day fetal liver fragments, which had been suppressed by the presence of anti-Ig for 24 h in the culture

[2] Owen, J. J. T., M. C. Raff, and M. D. Cooper. 1975. Generation of B cells in fetal bone marrow. Manuscript submitted for publication.

medium, completely recovered when they were washed, dissociated and recultured for an additional 24 h, IgM-bearing cells in fragments treated for ≥48 h rarely showed any recovery. Thus, while the suppression probably begins as repeated cycles of Ig modulation and resynthesis, another mechanism (such as the shut-off of Ig synthesis) must take over after 24 h, to make the process irreversible. A hint that Ig-suppressed B cells may survive, at least for a number of days, after irreversible Ig suppression, is provided by the finding that approximately the same number of cells with lymphoid morphology were found in 14-day fetal liver explants cultured for 7 days in the presence or absence of anti-μ antibodies; however, the use of an independent B-cell marker will be required to establish this point.

Our finding that Fab fragments of anti-μ and anti-MIg antibodies were able to modulate IgM from the surface of B cells (and irreversibly suppress their development) is of interest but not without precedent. Fab fragments of alloantibodies directed against the thymus-leukemia (TL) alloantigens have been reported to modulate TL from the surface of TL^+ leukemia cells (14), and Fab fragments of anti-Ig antibodies have been shown to induce pinocytosis of Ig in B lymphocytes (5). In view of the tendency for Fab fragments to aggregate, one must be cautious before concluding that monovalent interactions of antibody with membrane antigens can induce these reactions. The greater difference in the dose-response curves for B-cell inhibition between Fab fragments and their divalent parent antibodies in the case of unpurified anti-MIg compared to immunoadsorbent-purified anti-μ (Fig. 1) is compatible with the notion that aggregates are involved in the inhibitory activity of the Fab fragments: in the case of the purified antibodies, all aggregates can bind multivalently to Ig, while only those aggregates containing two or more IgG molecules with anti-Ig specificity can do so in unpurified antibody preparations.

The most important observations of this study are those concerning the differences between IgM-bearing cells in fetal and newborn liver and adult bone marrow on one hand, and those in adult lymph node and spleen on the other. While low concentrations of anti-Ig antibody or their Fab fragments, induced Ig modulation and irreversible Ig inhibition in the former cells, suppression of the latter required much higher (~10 times) concentrations of these antibodies, was incomplete, and was always reversible. In addition, modulation occurred much more rapidly in the former cells. These differences in susceptibility to Ig modulation and irreversible Ig suppression by anti-Ig antibodies cannot be explained by differences in the amount of IgM on the surface of these cells, as analysis of the intensity of fluorescence-labeling with anti-μ-fluorescein failed to find such quantitative differences.

The simplest interpretation of these findings is that the differences in susceptibility to anti-Ig-induced Ig modulation and irreversible suppression reflect differences in the maturation of the IgM-bearing B cells. It is clear that all of the B cells in mouse fetal and newborn liver are newly developed and there is increasing evidence that the same is true for the majority of Ig-bearing cells in adult bone marrow (26,31). Whether this apparent maturation from "high Ig suppressibility" to "low suppressibility" is antigen driven, and whether it requires T cells and/or migration from the sites of production to the microenvironment of

the peripheral lymphoid tissues, remains to be determined. It is also unclear how it relates to other parameters of B-cell maturation that have been described, such as changes in generation time (33), cell density and size (21,33), Ig receptor turnover (21), response to B-cell mitogens (21), or the acquisition of surface IgD (1,37) or complement receptors (8).

The ability of Fab fragments of anti-Ig antibodies to induce Ig modulation and irreversible Ig suppression in immature Ig-bearing B cells indicates that the Fc part of the anti-Ig antibody is not required for the suppression and makes it likely that antigen binding to immature B-cell Ig receptors would be similarly suppressive. Nossal and Pike have recently reported that IgM antihapten-forming precursor B-cells in adult mouse bone marrow are much more sensitive to hapten-specific tolerance induced in vitro by deaggregated dinitrophenyl-human gammaglobulin than are similar cells in adult spleen (24). Their observations, together with our own, lend support to the old, but presently unfashionable hypothesis, that tolerance to self, at least in B cells, may be related to B cells passing through a stage in their normal differentiation where they are highly susceptible to tolerance induction (21,23).

Recent studies in adult animals, indicating that B cells require much higher concentrations of antigen for tolerance induction than do T cells (3,11), have led to the currently more popular view that tolerance to self-antigens, present in relatively low concentrations, exclusively involves T cells (38). However, such experiments in adults may be misleading in terms of their implications for B-cell self-tolerance, if immature and mature B cells behave differently when ligands bind to their surface Ig receptors, as suggested by our findings. An interesting example of probable B-cell tolerance to a self antigen (hemoglobin) which would not be expected to be present in sufficiently high concentration to tolerize mature B cells is that recently described by Reichlin (30). He found that rabbits immunized with the human α-chain of hemoglobin were unable to make antibodies against those determinants shared by their own α-chains; since they could make antibodies against other α-chain determinants, their failure to do so against self-determinants must reflect B-cell tolerance (11). A clear, and easily tested prediction that follows from all of these observations is that while low zone tolerance induced in adult animals appears to involve only T cells, B cells as well as T cells should be tolerant when low zone tolerance is induced in neonates.

The high susceptibility to Ig suppression of immature B cells when ligands bind to their membrane Ig receptors, may also explain the recent observations of Strayer and his colleagues (32), who have shown that anti-idiotype antibody induces prolonged clonal deletion when injected into neonatal mice, but when injected into adults, reversible receptor blockade is produced. While these authors postulate that these differences may relect different rates of depleting antibody-coated cells by antibody-dependent cell-mediated cytotoxicity in neonatal and adult animals, our observations suggest the possibility that intrinsic differences between immature and mature B cells may be at least partly responsible. The in vitro models described in this report provide unusually accessible systems for studying the molecular mechanisms involved in the process of Ig suppression.

Summary

Purified goat antibodies against mouse μ-chains and rabbit antibodies against mouse Ig determinants, and their Fab fragments, inhibited the development of IgM-bearing B cells in explant cultures of 14-day mouse fetal liver, and caused the disappearance of cell surface IgM in explant and dissociated cell cultures of more developed lymphoid tissues. While treatment of cultures of fetal or newborn liver, or adult bone marrow, with low concentrations (<10 μg/ml) of anti-Ig for ≤ 24 h caused the complete, but reversible, disappearance (modulation) of cell surface IgM, treatment for ≥ 48 h produced irreversible IgM suppression. In contrast, anti-Ig-induced suppression of cell surface IgM in cultures of adult spleen or lymph nodes required much higher concentrations of antibody (≥ 100 μg/ml) and was always reversible. These differences between immature and mature IgM-bearing cells could not be related to differences in the amount of surface IgM on the cells. The remarkable sensitivity of newly formed B cells to IgM modulation and irreversible IgM suppression when ligands bind to their Ig receptors, may have important implications for B-cell tolerance to self antigens.

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Note Added in Proof. Similar observations have recently been made by C. L. Sidman and E. R. Unanue [Receptor-mediated inactivation of early B lymphocytes. *Nature* (*Lond.*). In press.].

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References

1. Abney, E. R., and R. M. E. Parkhouse. 1974. Candidate for immunoglobulin D present on murine B lymphocytes. *Nature* (*Lond.*). **252**:600.
2. Bonner, W. A., H. R. Hulett, R. G. Sweet, and L. A. Herzenberg. 1972. Fluorescence activated cell sorting. *Rev. Sci. Instrum.* **43**:404.
3. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science* (*Wash. D. C.*). **171**:813.
4. Cozenza, H., and H. Kohler. 1972. Suppression of the antibody response by antibodies in receptors. *Proc. Natl. Acad. Sci. U.S.A.* **69**:2701.
5. de Petris, S., and M. C. Raff. 1973. Normal distribution, patching and capping of lymphocyte surface immunoglobulin studied by electron microscopy. *Nat. New Biol.* **241**:257.
6. Dray, S. 1962. Effect of maternal isoantibodies on the quantitative expression of two allelic genes controlling the γ-globulin allotypic specificities. *Nature* (*Lond.*). **195**:677.
7. Feldmann, M., and M. B. Pepys. 1974. Role of C3 in *in vitro* lymphocyte cooperation. *Nature* (*Lond.*). **249**:159.
8. Gelfand, M. C., G. J. Elfenbem, M. M. Frank, and W. E. Paul. 1974. Ontogeny of B lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. *J. Exp. Med.* **139**:1128.
9. Hart, D. A., A. L. Wang, and A. Nisonoff. 1972. Suppression of idiotypic specificities in adult mice by administration of anti-idiotypic antibody. *J. Exp. Med.* **135**:1292.
10. Herzenberg, L. A., R. C. Goodlin, and E. C. Rivera. 1967. Immunoglobulin synthesis in mice. Supression by antiallotype antibody. *J. Exp. Med.* **126**:701.

11. Howard, J. G., and N. A. Mitchison. 1975. Immunological tolerance. *Prog. Allergy*. **18**:43.
12. Jerne, N. K. 1975. The immune system: a web of V-domains. *Harvey Lect*. In press.
13. Kincade, P. W., A. R. Lawton, D. E. Bockman, and M. D. Cooper. 1970. Suppression of immunoglobulin M synthesis in chickens. *Proc. Natl. Acad. Sci. U.S.A.* **67**:1918.
14. Lamm, M. E., E. A. Boyse, L. J. Old, B. Lisouska-Bernstein, and E. Stockert. 1968. Modulation of TL (thymus-leukemia) antigens by Fab-fragments of the antibody. *J. Immunol.* **101**:99.
15. 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.* **15**:277.
16. Lederberg, J. 1959. Genes and antibodies: (do antigens bear instructions for antibody specificity or do they select cell lines that arise by mutation?) *Science (Wash. D.C.)*. **129**:1649.
17. Leslie, G. A., and L. N. Martin. 1973. Modulation of immunoglobulin ontogeny in the chicken: effect of purified antibody specific for μ-chain on IgM, IgG, and IgA production. *J. Immunol.* **110**:959.
18. Loor, F., L. Forni, and B. Pernis. 1972. The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *Eur. J. Immunol.* **2**:203.
19. Manning, D. D., and J. W. Jutila. 1972. Immunosuppression of mice injected with heterologous anti-immunoglobulin heavy chain antisera. *J. Exp. Med.* **135**:1316.
20. McKearn, T. J., F. P. Stuart, and F. W. Fitch. 1974. Anti-idiotypic antibody in rat transplantation immunity. I. Production of anti-idiotypic antibody in animals repeatedly immunised with alloantigens. *J. Immunol.* **113**:1876.
21. Melchers, F., H. Von Boehmer, and R. A. Phillips. 1975. B lymphocyte subpopulations in the mouse: organ distribution and ontogeny of immunoglobulin-synthesizing and of mitogen-sensitive cells. *Transplant. Rev.* In press.
22. Murgita, R., C. Mattioli, and T. B. Tomasi, Jr. 1973. Production of a runting syndrome and selective IgA deficiency in mice by the administration of anti-heavy chain antisera. *J. Exp. Med.* **138**:209.
23. Nossal, G. J. V. 1958. The induction of immunological tolerance in rats to foreign erythrocytes. *Aust. J Exp. Biol. Med. Sci.* **36**:235.
24. Nossal, G. J. V., and B. L. Pike. 1975. Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J. Exp. Med.* **141**:904.
25. Old, L. J., E. Stockert, E. A. Boyse, and J. H. Kim. 1968. Loss of TL antigens from cells exposed to TL antibody. Study of the phenomenon in vitro. *J. Exp. Med.* **127**:523.
26. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. *Cell. Immunol.* **13**:132.
27. Owen, J. J. T., M. D. Cooper, and M. C. Raff. 1974. *In vitro* generation of B lymphocytes in mouse fetal liver, a mammalian bursa equivalent. *Nature (Lond.)*. **249**:361.
28. Owen, J. J. T., M. C. Raff, and M. D. Cooper. 1975. Studies on the generation of B lymphocytes in the mouse embryo. *Eur. J. Immunol.* In press.
29. Raff, M. C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology*. **19**:637.
30. Reichlin, M. 1972. Localizing antigenic determinants in human haemoglobin with mutants. Molecular correlations of immunological tolerance. *J. Mol. Biol.* **64**:485.
31. Ryser, J.-E., and P. Vassalli. 1975. Mouse bone marrow lymphocytes and their differentiation. *Eur. J. Immunol.* In press.

32. Strayer, D. S., W. M. F. Lee, D. A. Rowley, and H. Kohler. 1975. Anti-receptor antibody. II. Induction of long-term unresponsiveness in neo-natal mice. *J. Immunol.* **114**:728.
33. Strober, S. 1975. Maturation of B lymphocytes in the rat. II. Subpopulations of virgin B lymphocytes in the spleen and thoracic duct lymph. *J. Immunol.* **114**:877.
34. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. de Petris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by antiimmunoglobulin antibody. *Nat. New. Biol.* **233**:225.
35. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Ligand-induced movement of lymphoctye membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* **136**:885.
36. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Endocytosis by lymphocytes of complexes of anti-Ig with membrane bound Ig. *J. Immunol.* **108**:569.
37. Vitetta, E. S., U. Melcher, M. McWilliams, M. Lamm, J. M. Phillips-Quagliata, and J. W. Uhr. 1975. Cell surface immunoglobulin. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. *J. Exp. Med.* **141**:206.
38. Weigle, W. O. 1971. Recent observations and concepts in immunological unresponsiveness and autoimmunity. *Clin. Exp. Immunol.* **9**:437.