The University of Manitoba

ANTI-IgM SUPPRESSION IN B6AF$_1$ MICE

by

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in Partial Fulfillment of
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Degree of Master of Science.

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ABSTRACT

The objective of the present investigation was to produce a state of agammaglobulinemia in $B6AF_1$ mice with chronic administration of anti μ serum from birth in order to examine T cell mediated reactions in the absence of B cells or the immunoglobulin that they produce. The anti μ treatment resulted in a complete suppression of serum IgM and an incomplete suppression of IgG as shown by immunoelectrophoresis and immunodiffusion. Moreover, there was a complete suppression of the production of IgM antibody to SRBC detected with the direct plaque forming cell assay. An attempt was made to measure the numbers of B cells in the peripheral lymphoid organs of the suppressed mice with anti Ig (RAMIg) serum in a ^{51}Cr release cytotoxic assay. However, the anti Ig serum was unable to quantitate B cells because the serum contained antibody to mouse thymocytes and the absorbed serum did not lyse significant numbers of Ig bearing cells in the presence of complement. AKR anti C_3H θ serum (cytotoxic assay) demonstrated a statistically significant 10% increase in T cells in the lymph nodes of anti μ treated mice over their littermate controls which may indirectly reflect a corresponding B cell loss. Thus, from these results it was concluded that the anti μ suppressed $B6AF_1$ mouse may not be a good model for the study of the regulation by B cells of T cell mediated immune reactions because of the incomplete suppression of B cell activity.

ABBREVIATIONS

PFC	=	Plaque forming cell
B cell	=	Thymus independent lymphocyte
T	=	Thymus dependent lymphocyte
SRBC	=	Sheep red blood cells
PBS	=	Phosphate Buffered Saline
SAS/2	=	Saturated Ammonium Sulphate 50%
BBS	=	Borate Buffered Saline
FCA	=	Freund's Complete Adjuvant
GPC	=	Guinea Pig Complement
RAMLS	=	Rabbit Anti Mouse Lymphocyte serum
OD	=	Optical Density
$(NH_4)_2SO_4$	=	Ammonium Sulphate
ATXBM	=	Adult Thymectomized and Bone Marrow Reconstituted
poly RAMIg	=	Polyvalent Rabbit Anti Mouse Immunoglobulin
RFC	=	Rosette forming cell
ABC	=	Ag binding cells
DH	=	Delayed hypersensitivity
GVH	=	Graft versus host
MLR	=	Mixed lymphocyte reaction
FγG	=	Fowl gamma globulin
β-2-M	=	β-2-microglobulin
DMSO	=	Dimethylsulfoxide
FCS	=	Fetal calf serum
NMS	=	Normal mouse serum

ABBREVIATIONS - cont'd

NH_4Cl	=	Ammonium chloride
GPC	=	Guinea pig complement
NRS	=	Normal rabbit serum
AFC	=	Ab forming cell
H-H	=	Hanks'-Hepes
ADLC	=	Ab dependent lymphocyte mediated cytotoxicity
DLC	=	Direct lymphocyte cytotoxicity
DNP_{34} CSA	=	Dinitrophenylated chicken serum albumin

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ANTI IgM SUPPRESSION IN

$B6AF_1$ MICE

LITERATURE REVIEW

A. Introduction:

The mechanisms involved in the induction and regulation of the immune response are not yet fully understood. It is generally agreed that for the induction of the response, antigen (Ag) must meet some kind of receptor either free in the body fluids or attached to some cell surface. Studies with radioactively labelled antigens (1,2) have shown that only a small proportion of the receptor bearing lymphocytes have the ability to recognize and bind specific antigen *in vitro*.

There has been much evidence establishing the immunoglobulin (Ig) nature of these receptors on the surface of thymus independent B lymphocytes of mice (3-11). The nature of the receptor on the thymus dependent T lymphocytes is still unknown but it is likely that either these cells do not possess any surface Ig or they do so in a very low concentration. Thus, it was expected that the treatment of newborn mice with anti Ig serum would affect only the B lymphocytes. Several studies (12,14,15) have shown that *in vivo* injection of anti Ig antibodies can result in suppressed B cell Ig and Ab formation. However, this suppression is difficult to achieve in the adult animal because it's immunologically competent lymphocytes produce circulating Ig's which prevent the injected anti Ig antibodies from reaching the B lymphocyte. Thus, the injection of anti IgM antibodies into newborn mice which have immunologically incompetent lymphocyte and low serum Ig levels (placental transfer of maternal Ig) will

result in a strong immunosuppression and in some cases result in complete agammaglobulinemia (16,17,19). The objective of the present investigation was to reproduce this immunosuppression in order to examine T cell function in the absence of B cells or the Ig that they produce.

B. Anti IgM Suppression in Mice:

Many studies have been done on the suppressive effects of anti Ig serum on various immunological reactions. The most extensive studies have involved the suppression of antibody synthesis *in vivo* and *in vitro* by anti Ig serum. Anti IgM heavy chain (anti μ) serum has been the most powerful reagent suppressing all the humoral classes of Ab in the mouse strains, BALB/C (15), conventional Swiss mice (12) and nude mice (16,17). Lawton and Cooper (19) also found that in chickens, anti IgM treatment *in ovo* followed by bursectomy resulted in agammaglobulinemic chickens.

Many researchers have worked with anti IgM suppression with generally concordant results. Lawton et al. (15) subjected neonatal BALB/C mice to a series of intraperitoneal injections of purified goat anti mouse IgM serum. Specific anti IgM antibody (0.5 mg) or nonspecific globulin in 0.05 mls of PBS were given on each of the first four days after birth and 1 mg each week thereafter. Quantitation of serum Ig levels in anti u treated mice did not reveal any serum IgM although the sera were found to contain goat antibodies to mouse IgM. The suppressed mice showed significantly lowered concentrations of serum IgG ($P<0.05$), IgG_2 ($P<0.025$) and IgA ($P<0.005$) from littermate controls. Manning (14) administered a similar series of intraperitoneal injections of rabbit anti μ chain serum into newborn BALB/C mice. Injections were administered on the day of

birth and subsequent doses were given at 2-7 day intervals. Ouchterlony analysis of Ig levels in the young adult revealed a complete absence of serum IgM and a reduction of other serum immunoglobulins (IgG, IgA) to low residual levels. However, if the treatment was ceased after the first fifteen days of life and the animals were allowed to coast until day 46, serum IgM and IgG levels were seen to recover to control values.

Nude mice were found to be much more sensitive to anti μ treatment than normal littermates (17). Athymic BALB/C mice were given 0.05 mls of purified rabbit anti u globulin on day 0 and increasing doses on alternate days until a maximum of 0.20 mls of globulin was achieved on day 30. Alternate day injections of this maximum dose (0.20 mls) was continued through day 56. All of the nude mice given this suppressive treatment demonstrated an absolute loss of IgM and IgA. Serum levels of IgG_1 and IgG_2 were shown to exhibit considerable variation between litters (see Fig.I).

Manning (16) examined the suppressive effects of the dose and the injection schedule of the anti μ treatment on Ig levels in nude mice and littermate BALB/C mice (see Fig.II). The pattern of suppression induced by a slight suppressive schedule (0.47 mls in first 15 days) was similar in both groups, i.e. complete suppression of IgM and IgA and severe reduction of IgG_1 and IgG_2. However, by day 46, the littermates demonstrated considerable recovery of both IgG subclasses as well as IgM and IgA. An increased suppressive schedule (3.15 mls over 42 days) resulted in suppressed IgM and IgA levels which showed no recovery at 47 days. The suppression of the IgG classes was also maintained at 47 days.

The ability of both nude mice and their littermates to recover

Fig.I. (17) Individual Immunoglobulin and Antibody Levels in Nude Mice Injected with Anti-Ig Antisera

Mouse No. (Litter no.)	Injection	Ig or antibody levels[c] at Day 32/Day 57					
		IgM	IgG1	IgG2	IgA	Anti-rabbit	Anti-Ig
1 (1)	Anti-μ	0/0	8/16	8/8	0/0	0/0	16/16
2 (1)	Anti-μ	0/0	8/16	8/8	0/0	0/0	16/16
3 (1)	Anti-μ	0/0	8/16	8/8	0/0	0/0	16/16
4 (2)	Anti-μ	0/0	2/0	0/0	0/0	0/0	32/32
5 (2)	Anti-μ	0/0	2/8	0/0	0/0	0/0	32/32
6 (3)	Anti-μ	0/0	2/0	0/0	0/0	0/0	32/16
7 (4)	Anti-α	32/32	8/4	32/256	0/0	0/0	16/8
8 (5)	Anti-α	32/32	4/1	32/128	0/0	0/0	16/8
9 (6)	Anti-α	16/32	1/2	32/64	0/0	0/0	8/8
10 (6)	Anti-α	32/32	2/16	64/128	0/0	0/0	16/8
11 (7)	Anti-α	16/32	2/4	64/128	0/0	0/0	16/8
12 (8)	Anti-γ1γ2	32/32	0/0[a]	0/4[a]	4/4	0/0	16/4
13 (8)	Anti-γ1γ2	32/32	0/4	0/1	4/4	0/0	8/8
14 (8)	Anti-γ1γ2	32/D[b]	0/D	0/D	4/D	0/D	8/D
15 (9)	Anti-γ1γ2	16/32	0/2	0/0	4/4	0/0	8/16
16 (9)	Anti-γ1γ2	16/D	0/D	4/D	2/D	0/D	8/D

[a] All anti-γ1γ2-treated mice showed atypical, additional faint precipitin bands with both anti-γ1 and anti-γ2 at one or both assays, which differed from IgG1 and IgG2 by position in Ouchterlony gel diffusion.

[b] Died prior to second assay.

[c] Numerical averages of individual highest reciprocal serum dilutions producing distinct precipitin lines against standardized anti-Ig antisera or purified mouse Ig in Ouchterlony gel diffusion.

Fig.II. (16) Effect of Anti-μ Antiserum Dose and Injection Schedule on Immunoglobulin Levels in Mice

Group No.	Phenotype[a] (No. Mice)	Suppressive Treatment			Day of Assay		Mean Ig or Antibody Level[b] Assay 1/Assay 2				
		Serum	Days	Total ml	No. 1	No. 2	IgM	IgG1	IgG2	IgA	Anti-μ
1	BALB/c (3)	NRS	0–15	0.47	24	46	27/32	260/260	64/170	1/8	ND[c]
1a	BALB/c (3)	Anti-μ	0–15	0.47	24	46	0/27	32/150	27/64	0/8	ND
2	BALB/c (4)	NRS	0–48	1.25	42	58	32/32	1020/1540	96/160	10/10	ND
2a	BALB/c (4)	Anti-μ	0–48	1.25	42	58	0/0	96/770	9/8	2/10	ND
3	BALB/c (3)	NRS	0–42	3.15	32	47	32/32	260/1020	48/64	4/8	ND
3a	BALB/c (4)	Anti-μ	0–42	3.15	32	47	0/0	2/24	1/1	0/0	16/8
4	LM (10)	NRS	0–56	4.40	32	57	30/32	640/870	67/140	5/15	ND
4a	LM (6)	Anti-μ	0–56	4.40	32	57	0/0	64/280	6/9	0/5	27/21
5	Nu (10)	NRS	0–56	4.40	32	57	29/32	8/14	27/93	2/4	ND
5a	Nu (6)	Anti-μ	0–56	4.40	32	57	0/0	5/7	4/4	0/0	24/21

[a] LM, littermate; Nu, nude.

[b] Numerical averages of individual highest reciprocal serum dilutions producing distinct precipitin lines against standardized anti-Ig antisera or purified mouse Ig in Ouchterlony gel diffusion.

[c] Not done.

from anti μ induced suppression of Ig levels was examined on day 16 and on day 31 following the termination of treatment.(see Fig.III). After 31 days of coasting (no treatment), no recovery of IgM was seen either in nude mice or littermates despite apparent disappearance of anti μ antibodies in the sera. The IgG levels in the anti μ treated littermates of nude mice were seen to approach levels in control mice while the IgG levels in nude mice showed no recovery. Thus, it was concluded that anti μ suppression in nude mice differed from that in normal mice only quantitatively, i.e. nude mice suppressed more easily and to a greater degree with less ability to recover. The degree to which different Ig levels were suppressed by neonatally initiated anti μ treatment and the persistance of the suppression, was seen to be dependent on the total dose of antiserum used and the suppressive schedule employed.

Fig.III. (16) Recovery of Immunoglobulin Levels During Post-treatment Coasting Period in Mice Treated with Anti-μ Antiserum

Group No.	Phenotype[a] (No. Mice)	Suppressive Treatment			Day of Assay		Mean Ig or Antibody Level[b] Assay 1/Assay 2				
		Serum	Days	Total ml	No. 1	No. 2	IgM	IgG1	IgG2	IgA	Anti-μ
3	BALB/c (3)	NRS	0-42	3.15	77	107	32/32	1020/1020	130/130	8/8	ND[c]
3a	BALB/c (4)	Anti-μ	0-42	3.15	77	107	0/3	72/190	2/50	2/5	0/0
4	LM (7)	NRS	0-56	4.40	72	87	32/32	730/730	140/220	18/25	ND
4a	LM (4)	Anti-μ	0-56	4.40	72	87	0/0	340/540	7/14	7/11	12/0
5	Nu (4)	NRS	0-56	4.40	72	87	32/32	26/28	190/220	4/4	ND
5a	Nu (2)	Anti-μ	0-56	4.40	72	87	0/0	4/8	0/0	0/0	4/0

[a] LM, littermate; Nu, nude.
[b] Numerical averages of individual highest reciprocal serum dilutions producing distinct precipitin lines against standardized anti-Ig antisera or purified mouse Ig in Ouchterlony gel diffusion.
[c] Not done.

C. Anti IgG_1, IgG_2, IgA Suppression in Mice:

Sera directed to the other classes of Ig have not been as powerful in humoral Ig suppression as the anti μ serum. Treatment of nude mice with anti IgG serum produced a distinct suppression of both IgG_1 and IgG_{2a} levels (16,17). There were no detectable effects on IgM or IgA levels with anti IgG treatment (see Fig.IV). Levels of IgG_1 and

Fig.IV. (16) Immunoglobulin Levels in Mice Treated with Anti-α or Anti-γ1γ2 Antiserum During and Following Treatment.

Group No.	Phenotype[a] (No. Mice)	Suppressive Treatment			Day of Assay		Mean Ig or Antibody Level[b] Assay 1/Assay 2				
		Serum	Days	Total ml	No. 1	No. 2	IgM	IgG1	IgG2	IgA	Anti-Ig
4	LM (10)	NRS	0-56	4.40	32	57	30/32	640/870	67/140	5/15	ND[c]
4b	LM (6)	Anti-α	0-56	4.40	32	57	32/32	620/1150	240/340	0/19	8/0
4c	LM (5)	Anti-γ1γ2	0-56	4.40	32	57	26/32	230/260	82/90	4/11	0/0
4	LM (7)	NRS	0-56	4.40	72	87	32/32	730/730	140/220	18/25	ND
4b	LM (6)	Anti-α	0-56	4.40	72	87	32/32	770/850	290/290	15/21	0/0
4c	LM (5)	Anti-γ1γ2	0-56	4.40	72	87	32/32	510/610	150/260	16/22	0/0
5	Nu (10)	NRS	0-56	4.40	32	57	29/32	8/14	27/93	2/4	ND
5b	Nu (5)	Anti-α	0-56	4.40	32	57	26/32	3/5	45/140	0/0	14 8
5c	Nu (5)	Anti-γ1γ2	0-56	4.40	32	57	26/32	0/2	1/2	4/4	10 9
5	Nu (4)	NRS	0-56	4.40	72	87	32/32	26/28	190/220	4/4	ND
5b	Nu (4)	Anti-α	0-56	4.40	72	87	32/32	11/16	220/220	0/3	1 0

[a] LM, littermate; Nu, nude.
[b] Numerical averages of individual highest reciprocal serum dilutions producing distinct precipitin lines against standardized anti-Ig antisera or purified mouse Ig in Ouchterlony gel diffusion.
[c] Not done.

IgG_2 in nude mice showed little ability to recover from anti IgG suppression, whereas IgG_1 and IgG_2 levels in littermates showed substantial recovery even during treatment. Manning (16,17) also examined the ability of IgA levels in nude mice and their littermates to recover from anti IgA induced suppression (see Fig.I, IV). The anti IgA treatment did not affect IgM or IgG levels in the nude mice or their littermates. Nude mice were shown to be more sensitive to anti IgA suppression than their littermates. Levels of IgA in nude mice remained suppressed after cessation

of anti IgA treatment but IgA levels in littermates were shown to recover around 57 days after termination of the treatment.

D. Effects of Anti μ Treatment on Lymphocyte Surface Ig:

Studies with immunofluorescent antisera have further detailed the effects of anti μ on lymphoid tissue. Murgita's (13) studies on 30 day old mice given long term anti μ treatment from birth, revealed the presence of spleens completely devoid of all Ig containing cells. The spleens of 8-9 weeks old mice treated with anti μ from birth contained the same number of follicles as control animals but were completely devoid of germinal centres. When the spleens of control animals were stained with fluorescent anti Ig, they showed large clusters of brightly stained plasma cells. Spleen cells of experimental mice (anti μ treated) were more easily stained by anti IgG_1 than by anti IgM. In general there was a ten-fold reduction in the number of fluorescent stained cells in experimental versus control animals. It was a curious observation that the number of cells staining with anti IgG_2 in experimental animals was 3 times higher than the number of cells staining with anti IgG_1 despite the fact that the animals had higher levels of IgG_1 in the serum.

In the anti μ treated mice the gross appearance of the thymus showed a normal medulla and cortex. The spleen was reduced in size and the intestine appeared normal. However, further analysis of the intestine showed that there was a normal mucosa but the villi had a complete absence of plasma cells.

E. Relationship Between Surface Receptor and Secreted Ig Product:

Anti Ig reagents have been used to examine the relationship between the Ig receptor on the surface of the cell (B) membrane and the class of Ig to be secreted later. It had been formerly believed that the "receptor

equals product" (26), i.e. the class of the surface Ig will be the same class of the Ig secreted by the class. Thus, saturation of a receptor of a given class with a specific antiglobulin should suppress production of Ab of only that Ig class after antigenic stimulation. However, this prediction was found to be strictly correct only for anti IgA which suppressed only the IgA response (26). Pierce et al. (20,27) demonstrated that anti μ could reduce all classes (IgM, IgG_1, IgA) of Ab response to SRBC to less than 10% of control levels.

(a) Suppression of Direct and Indirect PFC.

Several other studies (20,21,28) have shown that in vivo anti μ treatment of newborn mice inhibits the direct and indirect PFC response of mouse spleen cells to SRBC. Treatment of newborn mice with anti IgG serum produced a partial reduction in the direct plaque response but eliminated the indirect plaque response to SRBC. In addition, either anti IgG_1 or anti IgG_2 serum could suppress the production of both classes of Ig, suggesting that the sera contained cross-reacting antibodies or the same cell had more than one type of receptor. The fact that only anti μ could suppress the direct plaque response suggests that the unprimed B cell functional receptor is almost exclusively of the μ class. Thus, virgin precursor cells could have the μ receptor and the γ receptors would appear only after differentiation events provoked by antigen.

(b) Inhibition of Rosette Forming Cells.

The effects of anti μ treatment on rosette forming cells (RFC) have also been examined. A RFC is defined as a cell that binds particulate Ag, usually heterologous red cells, or antigens coupled to red cells. Anti μ and anti γ reagents were used to examine the formation of rosettes in mouse spleen cell suspensions taken at varying intervals after SRBC

immunization (23). Rosettes examined up to 14 days after immunization are inhibited up to 65-85% in vitro with anti μ serum. The extent of the inhibition declined late in the response reaching 39% inhibition by day 28. The degree of inhibition observed by anti IgG serum rose following immunization and was maximal 28 days later at 48%. These results are further confirmed by Ada's work (29). He found that in immunized mice 70% of the antigen binding cells (ABC), failed to take up radioactive antigen after anti μ treatment.

The results of the above studies with RFC suggest that following immunization the proportion of RFC with receptors of γ specificities, increases relative to those bearing μ specificities. The sum of the inhibition achieved by antisera directed to the various classes (α,μ,γ), was found to increase to a maximum of 133% and then decline to 101% by day 14 (23). This "superaddition", suggesting that cells may bear more than one receptor, has been observed in other studies (30,31). The sum of the % inhibition of rosette formation obtained with anti IgG_1 and anti IgG_2 treatment of lymph node cells obtained from guinea pigs immunized with either SRBC or dinitrophenol bovine gamma globulin (DNP-BGG) was found to add to more than 100% in every case (30).

F. Mechanism of Anti μ Suppression:

(a) Target of Anti μ Antibodies.

The mechanism of anti μ suppression of Ig levels in serum is unknown but there is much evidence to show that the primary target of anti μ antibodies is the B lymphocyte. Examination of lymphoid tissues from anti μ treated mice showed a severe deficiency in germinal centres and primary follicles in lymph node cortex which are the major homing sites for the B lymphocytes (15). Fewer than 2% of the spleen cells from these treated

mice had membrane Ig as detected by immunofluorescence (4,32). The frequency of spleen cells from anti μ treated mice that bind ^{125}I-ferritin was less than 1 in 10^5 and was not improved following immunization (33).

(b) Lack of Cytotoxic Action.

The effects of anti μ treatment do not appear to be cytotoxic. In vitro suppressive effects of anti μ serum have repeatedly been shown to be complement independent (36,37,38). It was also shown that suppression in vitro can be achieved by non-complement fixing Fab fragment (39). Manning et al. (14) examined the response of young mice which had been lethally irradiated (900 rads) and reconstituted with various combinations of bone marrow (B) and thymus (T) cells from normal and heavily suppressed mice. All combinations of B and T cells from normal or suppressed donors made fully reconstituted responses. This implies that once cells are removed from the suppressive environment they are capable of recovering and restoring the immune response.

Pierce (20) noted that normal spleen cells which were incubated for 48 hours in a medium containing anti μ antibodies could still respond to a new Ag when separated from the medium. However, the spleen cell response was detected only when the culture period had been extended to 7 days whereas spleen cells in control cultures, (i.e. no anti μ) responded in 5 days. The addition of 1 gm myeloma protein with the new Ag improved the response, indicating that anti μ bound to the surface could be enticed from the cell by an excess of IgM in the solution. Effective suppression of PFC response requires the incubation of the spleen cells with anti μ for 24 hours or longer. This suggests that the mechanism of inhibition is more complex than just the simple saturation of Ig receptors and prevention of antigenic stimulation. Other studies (34,35) confirm this

conclusion with the observation that pronounced suppression is possible with anti μ at concentrations that are unable to effect complete antigen masking.

(c) Antigenic Modulation.

Raff (40) has shown that the resting lymphocyte has a diffuse Ig distribution on its' surface while anti Ig reagents induce a polar redistribution. Following this redistribution, the Ig molecules are pinocytosed and there are corresponding altered sensitivities at the membrane. A similar antigenic modulation was also described with thymic leukemic TL antigens (41). In this situation, when the TL+ cells are exposed to anti TL antibodies in vivo or in vitro they lose their surface TL antigens and consequently become resistant to the cytotoxic action of anti TL serum and complement.

The effects of anti Ig antibodies on human B lymphocytes seem to be just as complex. Several phenomena on B lymphocytes were observed with anti Ig antibodies, i.e. redistribution of surface complexes, pinocytosis, catabolism of complexes, shedding into extracellular environment and translational movement. All of these changes appear to result in a sterile blast cell that has lost its' differentiated surface receptors but also a cell that can recover to its' former potential, i.e. to differentiate into a plasma cell, when it is removed from the suppressive environment.

(d) Generation of Suppressor Cells.

T cells have been shown to exert a regulatory effect on Ab synthesis (43). Thus, it was thought that anti μ may generate a population of suppressor cells which could exert a negative influence on Ab synthesis. It has also been proposed that the anti μ antibodies may affect some ontogenetic regulator cell or cell product that is responsible for the further

differentiation of multipotential precursor cells to plasma cells. However, coculturing spleen cells from normal and suppressed mice did not reveal the presence of any inhibitory cells or factors (44). The observation that suppression could be achieved in athymic (nude) mice tends to invalidate theories on suppressor cell involvement.(16.17).

G. Comparison Between Allotype Suppression and Anti μ Suppression:

It was thought that the model of allotype suppression, first described by Dray (45), may be a parallel system to study the mechanism of suppression induced by anti Ig reagents. In an allotype heterozygote, chromosome (or allelic) exclusion leads to two populations of Ig producing cells, those bearing Ig with the paternal allotype and those bearing Ig with the maternal allotype. In the b^4 b^5 heterozygotes, i.e. offspring of parents homozygous at the b locus, Ig's controlled by the genes inherited from the dam and sire can be identified both on lymphocyte membranes and in the sera. If the young heterozygotes are exposed to maternal Ab directed to the paternal allotype, the appearance of circulating Ig with paternal allotype is significantly retarded and there is also a rapid and complete elimination of membrane associated Ig's of the suppressed allotype (46, 47,48).

In rabbits the suppression is severe and long term, enduring in some cases for one year (46,48,49). However, in the mouse hybrid the suppression is usually short term with a normal balance after 10-14 weeks. The SJL $\sigma^{\prime}$ x BALB/C φ mouse is an exception and has been shown to exhibit chronic suppression (20,50). It was also found that there was a compensatory increase in the expression of the non-suppressed Ig allotype on the lymphocyte membrane as well as in the secreted Ig's in the suppressed heterozygote.

The two models are similar in the absence of Ig's from lymphocyte membranes and from the sera that results from anti allotype and anti u treatment. Neither of the sera seem to involve a cytotoxic event to mediate their effects. This lack of cytotoxicity that was previously described for anti u serum, was also verified with the anti allotype suppression, i.e. neither the Fc fragment nor the bivalency of the antibody was necessary for suppression (51). The observation that the Fab fragment is efficient although less efficient than the $F(ab')_2$ fragment in allotype suppression suggests that the cross-linking of surface determinants is not essential for suppression.

The main difference between the two systems is that the allotype suppression seems to be mediated by some active regulatory process involving T cells (47,50), whereas the anti μ system does not appear to involve suppressor T cells. It has been shown that allotype suppression cannot be overcome or reversed by flooding the intact suppressed animal or the transferred suppressed animal with spleen cells or large amounts of serum Ig. This confirms the suggestion that anti allotype antibodies induce a population of suppressor cells which are capable of recognizing potential Ig producing cells and preventing the expression of the appropriate allotype.

It is curious that although both sera (anti allotype and anti μ) are directed to surface determinants on lymphocytes, one generates the appearance of regulatory T cells while the other does not. It was suggested (46) that allotype suppression may result from the magnification of regulation mechanisms which normally prevent the formation of self-reactive clones. In the newborn, "detector" lymphocytes would examine or monitor circulating lymphocytes and upon detection of lymphocytes that could react with self components, they would produce antibodies to these

lymphocytes. These anti Ig antibodies would result in abortive differentiation to a sterile blast cell. This surface Ag-Ab complex would initiate a second phase of suppressor T cells that would prevent the further development of these clones. However, this does not explain why anti μ antibodies which also result in a sterile blast cell do not appear to generate suppressor cells. These models have served to give some insight into the complexity of the regulatory network that results in the differentiation and clonal expansion of the lymphoid cells destined to secrete Ig.

H. Ontogenesis of the B Lymphocyte:

(a) Anti μ Panspecific Suppression.

The anti Ig reagents have been a powerful tool in defining the ontogenesis of the B lymphocyte. Most of the theories are based on the observation that anti u antibodies chronically administered to neonatal mice and chickens can affect all classes of immunoglobulins in the adult (13,14, 15,16). If anti μ is injected into 13 day old chicken embryos and the chicken is bursectomized at hatching, the bird is rendered agammaglobulinemic. The injection of anti μ Ab's at birth followed by continuous weekly injections will result in the diminished capacity of the animal to produce IgM, IgG and IgA and will result in little or no Ab response to a variety of antigens. Anti μ's panspecific effect can be best explained by the presence of a μ bearing precursor cell for all lines of Ig producing cells. This is supported by Vitetta's (5) observation that μ chains comprise some 90-95% of the heavy chains recovered from the surface of mouse spleen lymphocytes. Another study (16), showed that the number of spleen cells that could be detected with radiolabelled anti μ was as great as the number detected with labelled anti kappa, the latter representing practically the total number

of cells bearing surface Ig.

(b) Coopers Theory of B Cell Development.

Several authors (53,54,55) suggest that B cell differentiation is a discontinuous process divisible into two major stages. The first stage involves the generation of multiple clones of B cells from stem cells of haematogenous origin. In the chicken, this stage occurs in the specific microenvironment of the bursa. In mammals there is no bursa equivalent, thus the exact place of the generation of these clones is unknown. The first stage results in the production of large numbers of B cell clones, i.e. cells which express a single pair of V-region genes, which are prepared for a long journey in search of antigens with reserve energy for the synthesis and secretion of Ab. It is postulated that the diversity of Ig classes originates from an intraclonal switch of IgM to IgG and IgA which are then expressed on the cell surfaces. After a number of divisions, the switch involves repression of the gene for the C μ region and derepression of the gene for the C γ region which occurs independently of any alteration in the expression of V-region gene products.

There are several pieces of evidence that support the ontogenetic sequence of IgM→IgG→IgA. Examination of the IgM and IgG myeloma proteins produced by different cells in the same patient showed that the L chain N-terminal amino acid sequence were identical (56). Also, rabbit cells engaged in IgG synthesis were observed to have IgM on their surface(55). Moreover, as mentioned previously, treatment of mice with anti IgM antibodies from birth to maturity significantly suppressed the development of IgM, IgG and IgA producing cells.

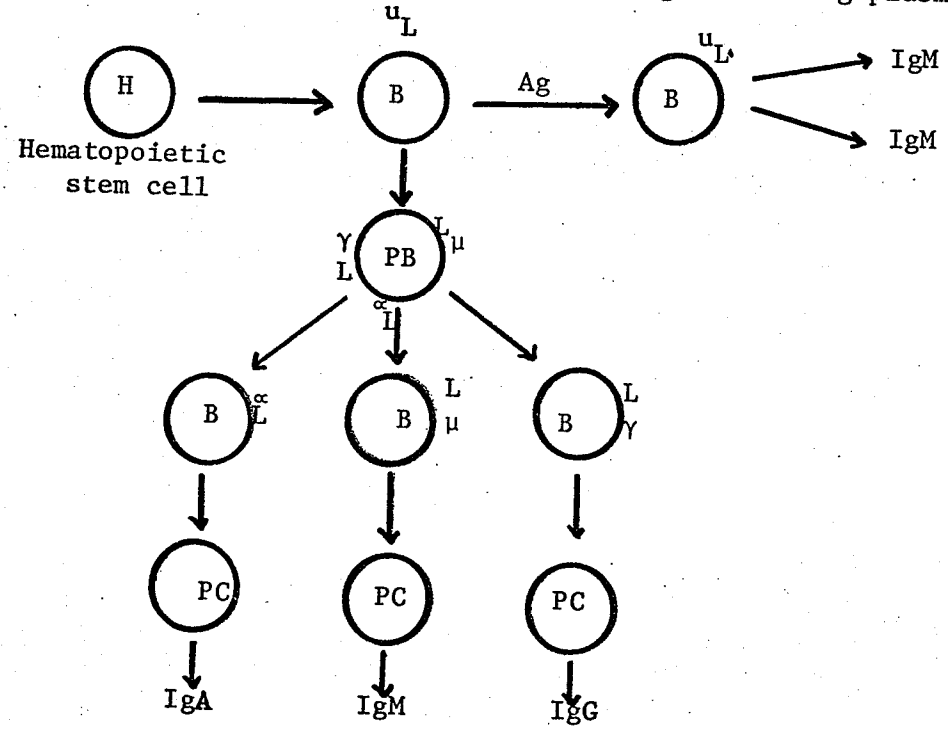
The second stage begins with the committed precursor contact with the appropriate antigen. This induces the cells to proliferate to form

germinal centres to differentiate into Ab producing plasma cells and to expand into a pool of memory cells. These steps could be further modulated by helper T cells or glass adherent cells (macrophages) and/or circulating Ab.

(c) Warner and Pierce's Theory on B cell Development.

Warner and Pierce (55,57) have proposed a model of B cell development that does not involve a sequential development of cells, i.e. IgM→IgG→IgA (see Fig.V).

Fig.V. (55) A hematopoietic stem cell (H) differentiates to a B lymphocyte having IgM receptors (light chain L and heavy chain u). Antigenic (Ag) stimulation of the B cells leads to some IgM release and to activation of other heavy chain genes (γ ∝). This pluripotential (PB) activated B cell differentiates further into either a IgM, IgA or IgG secreting plasma cell (PC).

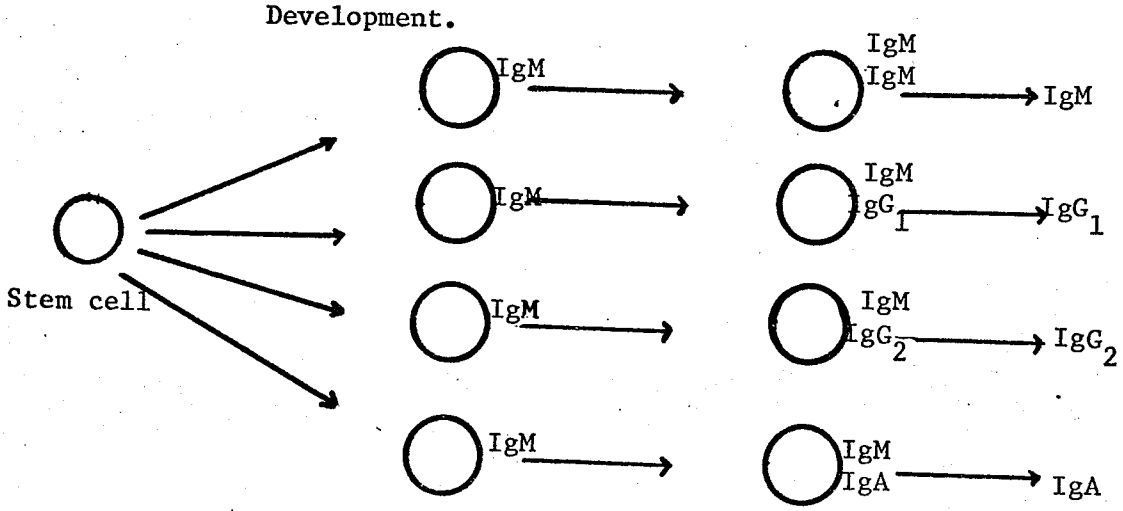


The model postulates the existence of a pluripotential B cell and several studies confirm the presence of B cells with multiple heavy chains, i.e. antiglobulin stimulation (158), inhibition of rosette forming cells (23,30) and inhibition of direct globulin staining tests (6). The final transition to plasma cell has been shown to involve the loss of the B cell surface antigenic markers (60), the loss of the cytophilic receptor for the Fc of Ig (55) and the acquisition of the Pc-1 isoantigen system (61).

(d) Manning and Jutila's Theory on B Cell Development.

Manning (14) has proposed another theory that contradicts Cooper's theory of sequential B cell development (see Fig.VI). He reasoned that if IgA forming cells are derived from IgG forming cells then injection of anti IgG into newborn mice should inhibit production of both IgG and IgA. This was not observed experimentally as anti IgG had no effect on IgA production suggesting that cells bearing IgG receptors are not the precursors of IgA producing cells. Consequently, Manning proposed the separate development of the IgM, IgA and IgG bearing cells from a common IgM bearing stem cell pool, thus accounting for the panspecific effect of anti μ and the specific suppressive effects of the other classes, i.e. anti IgA suppresses only IgA and anti IgG suppresses only IgG responses.

Fig.VI. (16) Manning and Jutila's Theory of B Cell Development.



(e) Unresolved Areas of B Cell Ontogenesis.

(i) Site of B cell development. The different models proposed to explain B cell differentiation demonstrate the deficiencies of our understanding. The site of B cell development in mammals is still controversial. Several tissues have been considered to be the central lymphoid organ analogous to the bursa of Fabricius in birds. Bone marrow, spleen, appendix and intestinal lymphoepithelial have also been examined as areas of B cell induction. However, evidence to date has been inconclusive and it is still not known if B cell development occurs in a central site or in a variety of sites.

(ii) Inducers of B cell character. The factors that induce B cell character are also unknown. Locally produced factors, hormones or accessory cells (T) may all play a role in the transformation from stem cell to Ig carrying cell. It has, however, been observed that B cells with surface IgG develop in media free of exogenous antigens as well as they do in media containing antigenic material, e.g. calf serum proteins (62). This suggests that B cell programming is able to operate without prompting from the external environment. Thus, the differentiation of stem cells into a diverse population of B cells would follow an orderly program encoded in the genes.

(iii) Relationship of receptor Ig class and secreted Ig. Only partially resolved is the relationship between the Ig class of the receptor for Ag and the secreted Ab. The clonal selection theory postulates that the receptor Ig is identical to the Ab ultimately secreted by the progeny of these precursor cells in terms of specificity, allotype and immunoglobulin class (63,64). It is difficult to reconcile this statement with observation of "superaddition" in studies on RFC inhibition (23,30) and

the ability of anti μ antibodies to suppress IgG PFC response (25,57).

(iv) Ag dependence of "receptor switch." There is much conflict on whether the receptor switch is Ag dependent or independent. Although anti μ suppressed IgM and IgG responses in vitro, IgG responses were not suppressed if anti μ was given following Ag priming in vivo (57). Another study (65) showed that 69% of ABC in unprimed mice have μ receptors while ∝ and γ receptor bearing ABC show an increase upon immunization. The most dramatic change in ABC receptors occurs with IgG, ABC receptor, i.e. from 0.2% to 9%. However, other data has shown that the proportion of μ, γ and ∝ bearing B cells is similar in germ free and conventional mice. Thus, the dependency or independency of the receptor switch on antigenic stimulation is not generally agreed upon.

(v) Role of T cells. The role of T cells in the development of B cells has not been fully clarified yet. It was suggested that T cells may function in modulating or initiating the receptor switch. However, the observation that development of B cells with each Ig class is possible in athymic mice (16) and humans (67) suggests that T cells are not a prerequisite. Further analysis of Ig levels in nude mice (53,68) suggest that IgM synthesis and secretion is thymus independent whereas IgG_1 and IgA are highly thymus dependent and IgG_2 is facilitated by the thymus but has no absolute thymus requirement.

Many antigens have been shown to activate B cells to secrete Ab only when specific T cells are assisting. In some cases where thymus deprived mice make a definite but more meager response than intact animals, the IgG response is always depressed to a greater extent than the IgM suggesting that the development of an IgG response is more dependent than the IgM response (53). This is in agreement with the

observed Ig levels in nude mice.

A study (69) on the response of C_3H.SW mice to the branched chain polymer of poly-L-(Tyr-Glu)-poly D,L-Ala-poly-D-L-Lys(TG,AL) suggests that T cells may influence the switch of IgM bearing cells to IgG. The C_3H.SW mice respond to this polymer with the production of IgM and IgG antibody while the thymus deprived C_3H.SW mice make an essentially normal IgM response, but little if any Ab of the IgG class. Thus, the IgG response to this Ag at least seems to be dependent on T cells.

Mice deficient in T cells can make a response almost equivalent to that of an intact mouse to type III pneumococcal polysaccharide (SIII) (70). However, the bulk of the response in the T deprived animal is IgM suggesting that a T cell influence is necessary for the emergence of the IgG secreting cell. The observation (71) that T deprived mice can generate a small IgG response to SIII suggests that T cells may play a role in the proliferation of the IgG bearing cells rather than initiating the switch of B cell receptors. However, several authors (194,195) have shown that SIII fails to elicit much of an IgG response even in normal animals suggesting that this polymeric nonprotein Ag is extremely poor in eliciting helper T cells even when they are available. Thus, SIII is a poor Ag to examine T dependence or independence of the IgG response.

Kishimoto's studies (72) on the response to DNP- Ascaris suggest extensive regulation of hapten specific (B) cells by carrier specific T cells. When he primed the animals with carrier (ASC) only, he found that lymph node cells formed IgG antibody whereas lymph node cells from animals primed with hapten and carrier (DNP-ASC) formed more IgM than IgG antibody. This suggests that in some way T cells have

accelerated the shift of IgM precursor to IgG producer.

Grumet (73) confirms these observations with his study on the Ab response of genetically high and low responders to synthetic peptide antigens. Both strains produced a brisk IgM response after primary immunization. However, with a secondary challenge, only high responders produced a high titer of IgG Ab. Mitchell (74) has shown that thymectomy of high responders abated the secondary IgG.

Kishimoto also showed that the optimal antigen concentration required for maximal Ab formation decreased when donors were pre-immunized with free carrier. This implies that carrier specific helper cells were involved in the emergence or selective proliferation of hapten specific memory cells (B) bearing high affinity receptors. Thus, Kishimoto concluded that the function of T cells in Ab formation is not just to increase the quantity of Ab produced by B cell progeny but also to regulate the class distribution and affinity of Ab through the exertion of some selective pressure on the generation of B memory cells.

There are Ab responses that have been shown to be completely independent of the presence of T cells, e.g. the Ab responses to Ficoll (75). That antibody responses to some Ag's are regulated by T cells and responses to other Ag's are not is a further complication in the proposed regulatory role of T cells. If T cells are not mediating regulation in some Ab responses, the question arises as to what factors are responsible for regulating the quantity of Ab produced, Ab class distribution and Ab affinities in T-independent responses. Thus, the role of T cells in the switch of Ig receptors on B cells and in the development of Ig secreting cells is complex and deserves much more study.

(f) Species Variability in B Cell Development.

A great deal of caution should be exercized in generalizing from the results on B cell development based on chicken and mouse studies because of the conflicting data found in various species. A major difference between mouse and guinea pig studies is that the major fraction of Ag binding cells in the adult unimmunized guinea pig possess surface Ig of the IgG_2 class (76), whereas the majority of ABC in the mouse possess surface Ig of the IgM class.

The IgA synthesis appears to precede the IgG synthesis in the ontogeny of B cells in the guinea pig. Thus, it is unlikely that the sequential ontogeny of B cell lines (IgM→IgG→IgA) proposed by Cooper and Kincade (54), which is based on studies in the mouse, can be generalized to all species. In addition, studies in mice, which suggest that the IgM molecule is the primordial surface receptor cannot be generalized to the human case. Pernis (77) has shown that IgD occurs first on the surface of lymphoid cells in humans and is probably the primordial receptor on human B cells. It was later shown that there is also an "IgD" like molecule on murine lymphoid cells which occurs after the presence of IgM (78). Several authors have proposed an additional receptor switch on mouse B cells from IgM to IgD (78,79).

I. Nature of the T Cell Receptor:

Anti Ig reagents have been extensively used to investigate the nature of the T cell receptor. The specificity of the cell mediated immune phenomena implies that there is recognition by T cells but the receptor for this function has not yet been established.

(a) Inhibition of T Cell Function.

(i) Delayed hypersensitivity. Delayed hypersensitivity (DH) is an immune process mediated by T cells. DH responses in bursectomized

chickens were inhibited by rabbit anti IgM and anti IgG (80). Anti L chain serum has been shown to suppress the classical tuberculin type of hypersensitivity. Greaves (81) has shown that anti L chain serum can achieve almost complete suppression of mitogenic responses of sensitive human lymphocytes to PPD. Anti L chain Ab incubated with mouse lymphocytes sensitized to $DNP_{34}CSA$; inhibited DH when transferred to irradiated syngeneic recipients (82). However, anti L chain serum treatment of mouse T cells sensitized to polymerized flagellin, was shown not to inhibit the adoptive transfer of DH (196).

(ii) Graft-versus-host disease. Graft-versus-host (GVH) is another reflection of T cell activity. Ivanyi (86) found that preincubation of lymphocytes in vitro with anti whole Ig serum did not inhibit the ability of the lymphocytes to induce either cutaneous reactions when injected into 4 day old F_1 recipients or splenomegaly when injected into 15 day old embryos. Anti L chain and anti μ antibodies were unable to inhibit the ability of lymphocytes to induce embryonic splenomegaly in chickens (133). Warner was also unable to inhibit GVH reactions in chickens (87) and in mice (82) by pretreating cells with anti L chain serum.

However, several other studies (83,84,85) have indicated that pretreatment of mouse lymphocytes with antisera to whole Ig or gamma globulin fractions did inhibit GVH splenomegaly. Mason and Warner (82,121) found that although pretreatment of mouse splenocytes with anti L chain serum resulted in reduced or abolished splenomegaly in F_1 hybrid recipients, none of the anti heavy chain sera (anti μ, anti α, anti γ_1 or anti γ_2) were able to suppress GVH. Thus, the data on the effects of anti Ig sera on GVH reactions are conflicting, but do suggest that in some cases anti L chain sera can inhibit GVH reactions.

(iii) Mixed lymphocyte reactivity. The mixed lymphocyte reactivity (MLR) is another indication of T cell reactivity. The effects of anti Ig serum on MLR are contradictory as some studies (10,89,120) have shown inhibition of MLR by pretreatment of lymphocytes with anti Ig serum and others have not (90).

(iv) Macrophage migration inhibition. Marsman (91) has shown that anti μ, anti γ or anti α antibodies did not inhibit the migration of macrophages in his test system. This suggests that this process is mediated by lymphocytes which do not seem to carry any surface heavy chain antibody (μ, γ, α) or the release of inhibitory factors from lymphocytes is not influenced by its' surface Ig.

(v) Cytotoxic T lymphocytes. The effects of anti Ig serum on the activity of "killer" cells or cells responsible for graft rejection have been examined. Manning (28) found that mice suppressed with anti μ or anti Ig did not display decreased graft rejection. Silverstein (92) demonstrated that allografted fetal sheep injected with rabbit anti sheep 7S gamma globulin and anti 19S gamma globulin, rejected grafts at a rate comparable to controls. It was also shown that chickens bursectomized and injected with anti μ were still competent to reject allografts or wattle skin (19).

Two kinds of effector cells are generated when allogeneic spleen cells are transferred into a heavily irradiated recipient - alloantibody plaque forming cell (PFC) and cytotoxic lymphocytes (CL). Anti μ was shown to inhibit the PFC response but the activity of the cytotoxic lymphocyte was independent of this and was not affected by the anti μ serum (93). The lytic activity of these CL has also been shown in other studies to be unaffected by antisera to various mouse Ig's (94,95). It was also shown that effector cells from mice presensitized in vivo against an allogeneic

tumor showed no loss of cytotoxicity for the target tumor in the presence of anti α, anti γ or anti μ Ab's (95). Polyvalent anti mouse Ig serum was also unable to impair anti tumor cytotoxic activity of activated murine T cells (96). Feldman (97) demonstrated that the ability of normal rat lymphocytes to recognize mouse fibroblasts, ultimately become sensitized to and lyze them in culture, was not impaired by the presence of rabbit anti rat L or H chain serum.

Generally there are two kinds of cell mediated cytotoxicity, (DLC) and Ab dependent lymphocyte mediated cytotoxicity (ADLC) not involving T cells (99). The effector cell in the DLC system has been shown to be a T cell (100), whereas the effector cell in the ADLC is thought to be a K cell, a nonphagocytic cell of the myeloid or lymphoid series (101, 102,103). Anti Ig serum was found to inhibit the cytotoxicity in the ADLC system but not the direct lymphocyte mediated cytotoxicity (104).

(vi) Rosette forming T cells. The ability of anti Ig serum to inhibit murine rosette forming T cells has been reported by several workers. Greaves et al. (31) using θ bearing cells enriched to 85% by filtering primed spleen cells through a cottonwool column, showed that anti u serum inhibited up to 85% of the rosettes. This suggests that immune T cells carry μ or μ-like determinants. However, other studies using congenic anti θ to identify the T cells have cast some doubt on the true T cell nature of the rosettes (105).

(vii) Helper T cells. The effects of anti μ serum on co-operating T cells have been examined in several systems. In vitro treatment of normal thymocytes with anti μ did not inhibit their ability to co-operate with splenocytes from nude mice in an in vitro response to sheep erythrocytes (106). In another study (107) educated spleen cells taken

from anti μ treated mice were able to provide helper function when reconstituted *in vitro* with normal B cells. Pierce et al. (20) also showed that the ability of lymph node T cells to co-operate in cell culture was not affected by the *in vitro* anti μ treatment that severly suppressed B cells. These studies suggest that T cells that mediate the helper activity for B cells are unaffected by anti μ treatment. However, it has been claimed that the principal target of anti L chain suppression in murine cell cultures is the helper T cell (108). Thus, although most of the studies imply that T cells do not have any surface IgM or IgG some studies affirm that T cells have surface Light chain determinants or determinants that cross-react with Light chains.

The effects of anti Ig on various functions mediated by T cells has given only indirect information on the nature of the T cell receptor. Thus, studies with anti Ig serum have not shed much light on the question of the T cell receptor except to suggest that the receptors on the population of T cells examined are not conventional immunoglobulin.

(b) Detection of T Surface Ig.

Studies on the presence or absence of surface Ig on T cells have been conflicting. Fluorescein (3,32) peroxidase (109) or ^{125}I labelled anti Ig (110) have failed to detect the presence of Ig on T cells. On the other hand, several studies (52,125) have shown that T cells bear Ig molecules in low but detectable number.

Marchalonis (114) was able to isolate Ig from murine and human thymocytes. They covalently labelled the cell surface protein with ^{125}I and then subsequently fractionated and identified the protein. They identified a protein with a similar electrophoretic mobility as μ and estimated that there was a minimum of 400 molecules per cell.

Feldman (115) also found a 7S IgM fraction that derived from activated T cells which were able to co-operate in vitro with B cells.

Bankhurst (112) examined the ability of thymocytes and thoracic duct lymphocytes (TDL) to be radioiodinated with anti L chain Ab. Ninety-five percent of the TDL were shown to have the θ marker and radioiodinated anti L chain Ab labelled 19% of the thymus cells and 50% of the TDL. This suggests that thymocytes and immunocompetent (TDL) θ bearing cells express surface L chain Ig.

Studies with indirect fluorescence (116) reveal that peripheral T cells could be labelled with polyvalent antiglobulin serum. However, the strength of the binding of the antiglobulin reagent to T cells did not exceed that of normal rabbit sera by a very large factor. However, the specificity of the reaction was confirmed by significant, though incomplete blocking of the binding through removal of antiglobulin antibodies. The observation that under the same conditions B cells bind 100-400 times more antiglobulin than T cells may decrease the significance of the binding.

Anti Ig reagents have also been shown to inhibit T cell suicide by interfering with radioactive Ag binding (29,52). Anti μ chain serum was found to be effective in blocking the binding of thymus cells to ^{125}I-labelled Ag (113). Roelants (111) was able to prevent the binding of T cells to radioactively labelled ^{125}I TG-AL by incubating them with polyvalent rabbit anti mouse Ig labelled with fluorescein isothiocyanate.

It was suggested that Ig associated with T cells may be buried in the membrane and out of the reach of antiglobulin serum. Spleen cells depleted of B cells and thymus cells were disrupted by three different methods, i.e. freeze-thaw, NP40, 9M urea-15M acetic acid and examined

for Ig content (117). However, no significant release of Ig was observed under these conditions.

(i) Presence of light chain determinant. Although studies with anti Ig question the presence of a heavy chain globulin on the surface of T cells, other studies suggest that T cells may carry a L chain determinant or a determinant that cross-reacts with a L chain (118,119). Many immune functions mediated by T cells have been reported to be inhibited by anti L chain serum (82,87,108,120).

Several authors have shown that pretreatment of mouse donor cells with rabbit anti L chain serum suppressed GVH disease as well as the adoptive transfer of DH. Anti L chain serum was shown to achieve almost complete suppression of mitogenic responses of sensitive human lymphocytes to PPD (81). However, this is not generally agreed upon as two other groups have failed to confirm this finding (86,105).

Anti L chain serum was also shown to block the ^{125}I-Ag induced suicide of T cells (52). They showed that irradiated animals receiving T cells treated with anti L chain Ab and ^{125}I-FIgG responded normally to FIgG, whereas those given T cells exposed to normal rabbit IgG and ^{125}I-FIgG failed to do so.

Leslie and Dutton (122) found that the ability of normal spleen cells to respond to sheep erythrocyte antigen (SRBC) can be inhibited by pre-treating the cells with rabbit anti L chain serum and complement. They (108) also examined the in vitro response to 2,4,6, trinitrophenyl (TNP) coupled to SRBC and found that anti L chain serum and complement prevented T cell help. It is odd that T cells can be lyzed by anti L chain serum and complement whereas the precursor to Ab forming cells were not inactivated. They did find however, that the serum prevents the

induction of an unresponsive state which occurs when B cells are incubated with polyvalent hapten or non-immunogeneic carrier (123).

The results of the above studies on T cell surface Ig have to be examined critically. The inhibition of T cell function by anti L chain serum is an indirect test at best and the observation that anti L chain serum binds to cells does not conclusively prove that it is binding to light chains.

The observation that there is some sequence homology between HLA antigens and IgG (124) supports this suggestion. Even if it is conclusively shown that there are light chains on the T cell surface, proving that this observed L chain is the actual T cell receptor or part of it, is another problem. The problem of examining T cell surface receptors is further complicated by the possibility of steric hinderance. The L chain may be in a position close enough to the actual receptor to prevent its' access to Ag.

Some caution should be exercized with studies that examine cell surface Ig's using Ab preparations to purified myelomas. The ascitic fluid preparations from which the various myeloma Ig's are purified, may contain additional membrane fragment contaminants. If these contaminants are not removed in the Ig purification procedure and used in the immunizing preparation, additional Ab specificities could be raised to cell surface determinants other than the desired Ig determinants. Thus, the ability of anti L chain serum to bind to T cells or prevent T cell function may be due to Ab's raised to some other cell surface determinant. Rash (197) has suggested that mouse H-2 preparations may contain a polypeptide chain homologous to the human β_2 microglobulin a low MW protein (11,600) which occurs in biological fluids (198) and is

present in high concentrations on leukocyte surfaces (199). If antibodies to a β_2-m -like protein which exist either free in the ascitic fluid or on membrane surfaces have been raised, they may seriously alter the interpretation of the previous studies. Anti β_2-m Ab's have been shown to affect human T cell function, i.e. inhibit the MLR response (200). Thus, the results of the studies with anti L chain serum must be reexamined in view of the possible interference by anti β_2-m Ab's.

(c) Association of T Cell Receptor with Histocompatibility Antigens.

In the last few years there has been an explosion of literature suggesting that the H-2 gene complex associated Ag's are involved in the effective operation of the T cell receptor for Ag. Several authors (109, 125,126,127) have been able to completely inhibit murine T-ABC by preincubating them with anti H-2 serum. However, in one of these studies (125) it was found that anti IgM and anti L chain sera partially blocked T-ABC for (TG-AL). The response of guinea pig T cells to specific Ag was also abated in vitro by sera versus histocompatibility antigens (128). Pretreatment of T cells, but not of B cells with the Fab fragment of anti H-2 Ab protected them from the suicide effect obtained with radiolabelled Ag (129).

Another approach to study the T cell receptor was to examine the T cell product. It seems unlikely that the T cell would produce a specific molecule different in terms of Ag recognition from the T cell receptor. Taussig (130) isolated a non Ig antigen specific factor from primed T cells which was capable of replacing helper T cells in vitro. He passed the media containing the factor through a Sepharose 4B column coupled to anti H-2 antibodies. After transferring the resultant material with BM and (TG-AL) into lethally irradiated recipients, he found that the factor had been

removed.

Further studies have indicated a positive association between the T cell receptor and the histocompatibility Ag's. The mixed lymphocyte response (MLR) and the PPD proliferative responses of human lymphocytes in vitro can be blocked by anti HLA serum (131,132). GVH responses were also shown to be inhibited by alloantibodies (133). The interaction of sensitized T cells with target cells occurs when at least one set of H-2 antigenic specificities is shared (134,135). Co-operation between T and B cells is generally effective only between syngeneic or semiallogeneic cells (136). There has also been a lot of work done to show that Ir genes can influence T cell response to a variety of antigens (137).

(d) Comparison of T and B Cell Receptors.

Several studies using anti histocompatibility (H-2) antisera have clearly delineated some basic differences between the T and B cell receptor. Anti H-2 serum was found to completely block the binding of TG-AL to T cells but not to B cells (125). In addition, the authors found that anti IgM could partially block the binding of naive and educated T cells to (TG-AL) whereas only anti IgG could block the B-ABC in immunized mice. Hudson (96), thus proposed that under some conditions specific B cell derived Ig or even T's natural Ab may serve to stabilize interactions between antigen and primary T cell receptor. This may explain why some authors have been successful in inhibiting T-ABC with anti Ig and others have not.

Another difference between the T and B cell receptor is reflected in the varied conditions necessary to induce T or B cell suicide and Ag binding. First T cells had to be incubated at 37°C suggesting that some metabolically active step is necessary whereas B cell suicide can be performed entirely in the cold. The necessity of metabolism is further

confirmed the ability of azide or dinitrophenol to prevent ^{125}I-(TG-AL) induced suicide with T but not B cells (126). Specific adherance of T cells to fibroblast monolayers does not occur at 4°C or in the presence of sodium azide (138). The different conditions needed to achieve B or T cell suicide may reflect some basic differences in the nature of the binding of T and B receptors to antigen.

Another study (139) has shown that the number of T-ABC and B-ABC that are detected in an assay are greatly affected by the dose of antigen presented. Antigen dilution affects the label density far more markedly on T-ABC than B-ABC suggesting that the avidity of the T cell receptor for antigen is both lower and more uniform than the B cell receptor. Whether this effect is due to an inherent property of the receptor, or to the nature of the T cell membrane cannot be concluded from this experiment. However, the experiment does show that binding curves over a wide range of antigen concentrations should be established in any of the binding studies in any valid observations are to be made.

The nature of the T cell receptor is still being hotly debated. There are several distinct possibilities open, i.e. (a) conventional serum Ig class, (b) new type of complete Ig synthesized only by T cells (IgX), (c) an incomplete molecule such as a light or heavy chain or V-region fragment of the light or heavy chain, (d) H-2 associated antigen such as Ia antigens. Thus, reviews of the work done on T cell receptors still leave open the critical question on the identity of the T cell receptor. The role of the observed Ig on the surface of T cells, in the event that they are shown not to be functional receptors is also unanswered.

J. Effect of Anti μ Treatment on Tumor Growth:

The effects of anti μ suppression in mice have been examined in

several tumor model systems with interesting results. Manning et al. (140) found that anti μ treated BALB/C mice were rendered refractory to 2.2 LD_{50} doses of Friend leukemia virus and did not develop any signs of the disease, i.e. leukocytosis, splenomegaly or spleen focus formation. Since it has been shown that the target of the anti μ treatment is the B cell (4,15,20) the above study confirms other findings that suggest that the target of the Friend leukemia virus is a relatively differentiated lymphoid cell line (141,142,143).

In another system, Manning (144) showed that BALB/C mice given a rigorous treatment of anti μ antibodies were rendered refractory to subsequent challenges with myelomas at doses that produced tumors in all mice treated with normal rabbit serum. The protection offered was found to be specific, i.e. anti μ treatment prevented the growth of IgM myeloma (MOPC-104E) and offered no protection to the growth of IgA myeloma (MOPC-406). The study served to verify that tumor cell bound proteins are serving as targets for the immune serum. However, contradictory effects were turned up in the control of serum Ig levels. Anti μ treatment produced a stable loss of serum IgM and IgA as well as offering protection against the growth of IgM myeloma. Anti IgA treatment offered protection to the growth of IgA myeloma but also allowed normal recovery of serum Ig. It is puzzling that large numbers of tumor cells deposited subcutaneously should be completely suppressed while normal IgA producing cells are not suppressed although they are all under a suppressive influence all the time. This suggests that either there are separate regulatory mechanisms or tumor cells are inherently more sensitive to anti Ig Ab than the normal cell types. Thus, further studies should be carried out to clarify the inconsistencies observed in the regulatory

mechanisms of myeloma growth.

It has been impossible to date to suppress the serum Ig levels of adult mice. This is not unexpected since these animals have a much greater serum level of Ig than newborns. This serum Ig would combine with the Ig antibodies and prevent it from reaching the immunocompetent cells. However, several studies (147,148) have shown that anti Ig treatment may have some suppressive effects in adult mice.

Reif (147) has demonstrated that the injection of rabbit antiserum directed toward mouse IgG induces a small, but statistically significant prolongation of life in mice already developing transplantable myelomas. Manning and Jutila (148) showed that young adult mice treated with anti μ showed severe reductions (26-33 times) in antibody response when they were injected with a single dose of sheep red blood cell. Thus, although the authors were unable to effect overall suppression of antibody levels, they were able to get an inhibition of clones that had not been committed (to SRBC) or possibly prevented the recruitment of new clones.

OBJECTIVE OF THE PRESENT INVESTIGATION

This study is part of an ongoing program aimed at clarifying the mechanisms which regulate T cell mediated immune responses. There has been considerable documentation on the regulatory affects of T cells on B cell differentiation (145-152) and activity (153-158) and on T cell-mediated reactions (159). However, we know very little about possible B cell regulation of T cell activity. (Dyminski (160) suggests that an accessory cell with the characteristics of a B cell is necessary for a MLR response. Several studies (145-146) suggest that B cell derived Ig may interfere with T cell recognition of tumor cells by covering up the antigenic sites on the tumor cell. Berenbaum (211) obtained a prolongation of allograft survival in H-2incompatible mice with injections of cyclophosphamide. Pretreatement of mice (212) and guinea pigs (213) with cyclophosphamide before sensitization increased the intensity and prolonged contact sensitivity reactions. Treatment with cyclophosphamide has been shown to result in a decreased humoral antibody production and a depletion of B lymphocyte areas in the lymph nodes. This suggests that this phenomenon may be another example of B cell regulation of T cell function.

The nature of the T cell receptor is still controversial but several studies show that T cells have a small amount of Ig on their surface (52, 114,115,125). This surface Ig may represent the T cell receptor or it may be that the Ig is B cell derived and functions to stabilize the interaction between Ag and the primary T cell receptor (161). However, recent studies (203,204) have shown that both T and B cells can be primed *in vivo* for an immune response by anti idiotype antibodies. Idiotypic antibodies recognize determinants on the variable region of Ig heavy chains, thus,

these studies strongly suggest that the T cell receptor is Ig. However, the possibility that the T cell Ig is derived from the B cell is not eliminated in the studies.

The chronic treatment of newborn mice with anti μ serum results in a strong suppression of the function of B cells and leaves apparently unaffected most of the T cell mediated functions (see Literature Review). Thus, the anti μ suppressed mouse offers an excellent system in which to examine T cell reactions in the absence of B cells or the antibodies that they produce. However, it should be stressed that the importance of the proposed regulatory functions of the B cells can be clarified only if it can be proven that anti μ treatment suppresses totally B cell functions and does not affect T cell function.

Although the long term objective of this study is the examination of the regulation of T cell functions by B cells or B cell products, the specific aim of the investigations reported in this study was only to establish if the claimed suppressive effects of anti μ in newborn mice could be reproduced in the $B6AF_1$ strain which is presently being used in this laboratory to study several T cell mediated immune reactions. The degree of suppression of B cell functions was studied with the measurement of circulating Ig by immunoelectophoresis and Ouchterlony gel diffusion. The ability of the B cell to make Ab upon SRBC challenge was examined by the plaque forming cell assay. Quantitation of thymus dependent (T) and thymus independent (B) cells was effected by a cytotoxic assay employing anti Θ and RAMIg sera respectively.

MATERIALS AND METHODS

Chemical and Biochemical Materials:

Radioactive chromium (^{51}Cr) was supplied by Amersham, U.K. as the saline solution of sodium chromate. Radiation was counted in a well type gamma scintillation counter (Nuclear Chicago Corp., Des Plaines, Illinois).

Agarose (Sea-Kem) was obtained from Marine Colloid, Inc., U.S.A.

Agar (Noble) was supplied by Difco Laboratories, Detroit, U.S.A.

Freund's Complete Adjuvant was also supplied by Difco Laboratories.

Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

DEAE cellulose (Cellex D) was obtained from Bio-Rad Laboratories, California, U.S.A.

Sepharose 4B was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

Cyanogen bromide was supplied by Matheson Coleman, Cincinnati, U.S.A.

Tissue Culture Plates:

Microtiter plates (V) were obtained from Cooke Laboratory Products, Virginia, U.S.A.

Plate Sealer:

Microtiter plate sealers were supplied by Cooke Laboratory Products, Virginia, U.S.A.

Petri Plates:

Sterile plastic Petri dishes (60 x 15 mm) were supplied by Falcon Plastics, Becton-Dickinson and Co., Maryland, U.S.A.

Fetal Calf Serum:

Fetal calf serum was purchased from GIBCO, Grand Island, N.Y. It was then heat inactivated at 56°C for one hour.

Tissue Culture Media:

Hanks' Balanced Salt Solution was prepared by reconstituting the dehydrated salt mixture without bicarbonate supplied by GIBCO. 0.01 M N-2-Hydroxyethylpiperazine-N-2 ethansulfonic acid (HEPES) (Calbiochem) was used as the buffering agent. The pH was adjusted to 7.1 and the solution will be referred to as Hanks'-Hepes.

RPMI-1640 with L-glutamine and without sodium bicarbonate was supplied by GIBCO. The dried powder was reconstituted with 1.0 M Hepes and the pH was adjusted to 7.2. Thus, the RPMI medium was used at a 7.2 pH and a 0.04 molality.

Myelomas:

Five BALB/C myelomas, MOPC-104E (IgM), MOPC-L4 (IgA), MOPC-21A (IgG_1), MOPC-195 (IgG_{2b}) and HOPC-1 (IgG_{2a}) were kindly suppled by Dr. B. Carter, Department of Immunology, University of Manitoba.

Immunoelectrophoretic Apparatus:

A Gelman electrophoresis chamber was used with a Gelman transistorized power supply for immunoelectrophoresis.

Columns:

Glass columns (2.4 x 35 cm) for DEAE cellulose and (2.5 x 100 cm) for Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Complement:

Guinea pig serum used as a source of complement was supplied by North American Laboratory Supplies, Gunton, Manitoba. All subsequent absorption procedures were done at 4°C to minimize complement

inactivation. The serum was diluted 1:4 with Hanks'-Hepes and mixed with agarose (Sea-Kem) at 4°C to absorb the natural anti mouse antibodies (175). The mixture was gently rotated at 4°C for 30 minutes. The agarose was removed by centrifugation at 4°C and the complement was then divided into aliquots and stored at -70°C until used in the cytotoxic assay. None of the aliquots were stored for longer than 2 months at-70°C. Lyophilized guinea pig complement was obtained from Connaught Laboratories, Toronto, Ontario.

Myeloma Protein:

Five mgs of IgM, IgG_{2a}, IgG_{2b}, IgG_1 and IgG_A were obtained as reference proteins from Litton Bionetics, Maryland, U.S.A. The purity of the proteins had been ascertained by the supplier by polyacrylamide gel electrophoresis and agar gel diffusion against class specific antisera. The commercial proteins were obtained to be used as reference proteins to confirm the identity of the myeloma immunoglobulins purified in the laboratory.

Animals:

(a) Mice. The following inbred strains(listed with their respective H-2 genotypes)were purchased from Jackson Laboratories, A($H\text{-}2^a$), C57BL/6 ($H\text{-}2^b$), BALB/C ($H\text{-}2^d$), AKR ($H\text{-}2^k$) and C3H ($H\text{-}2^k$).

(b) Rabbits. New Zealand White young adult rabbits were obtained from Canadian Breeding Laboratories, Montreal, Quebec.

Production of Myeloma Protein:

Five myeloma tumors (MOPC-104E, MOPC-L4, MOPC-21A, HOPC, MOPC-195) were obtained in the frozen form, i.e. -90°C. The vials were quickly

thawed and cell viability was examined by trypan blue. Live (9×10^6) cells of each myeloma were injected separately subcutaneously into 5 BALB/C mice. Solid tumors were removed from the mice about 1 month later. The tumors were ground up in a homogenizer with a 1 mm clearance and then passed through a stainless steel screen mesh. Cells were counted and examined for viability in trypan blue. Tumor cells (9×10^6) were injected intraperitoneally into 50 BALB/C mice. Aliquot vials (1 ml) were used to preserve the various tumor lines in liquid nitrogen, i.e. $-90^{\circ}C$ in the presence of 7.5% DMSO. When the mice had developed sufficient amounts of ascitic fluids, they were "tapped" daily for the fluid, i.e. pressure exerted on the abdomen forced the fluid out of 18 gauge needles. The ascitic fluid was then stored at $-20^{\circ}C$ until subsequent purification.

(a) Purification of Various Myeloma Classes:

The following flow diagram (Fig.VII) illustrates the purification procedure of the myeloma proteins:

IgM

SAS/2 treatment ascitic fluid
↓
dialyzed versus 0.02 M phosphate pH 8.0
↓
DEAE column
↓
dialyzed versus 0.15 M phosphate pH 8.0
↓
G-200 column
↓
dialyzed versus barbital buffer u=0.1 pH 8.6
↓
agar block electrophoresis
↓
dialyzed versus phosphate buffered saline pH 7.2
↓
purity tested by Ouchterlony and immunoelectrophoresis

IgG_1, IgG_{2a}, IgG_{2b}

SAS/2 treatment ascitic fluid
↓
dialyzed versus 0.15 M phosphate pH 8.0
↓
G-200 column
↓
dialyzed versus barbital buffer u=0.1 pH 8.6
↓
agar block electrophoresis
↓
dialyzed versus phosphate buffered saline (PBS) pH 7.2
↓
purity tested by Ouchterlony and immunoelectrophoresis

(b) Saturated Ammonium Sulfate Treatment:

Saturated ammonium sulfate was added drop by drop in the cold ($4^{o}C$) to an equal volume of ascitic fluid which was being slowly stirred. The mixture was then left slowly stirring overnight. The mixture was then poured into plastic rounded non-lipped tubes and centrifuged at 10,000 rpm for 10 minutes in a refrigerated Sorvall centrifuge using a Sorvall SS-34 rotor. The precipitate was resuspended in normal saline to the original volume and the precipitation procedure with SAS/2 was repeated three times. The sample was then dialyzed versus 0.15 M phosphate buffer pH 8.0, with 6 changes every 5 hours.

(c) DEAE (cellex D) Column Treatment:

DEAE (35 g) cellulose were activated with 1000 mls 0.5 N NaOH. The mixture was then washed with 10 liters of distilled water on a Buchner funnel under negative pressure. It was then inactivated with 1000 mls 0.5 N HCl for 15 minutes and again washed with 10 liters of distilled water on a Buchner funnel. The cellulose was again reactivated by an overnight treatment of 1000 mls of 0.5 N NaOH and again washed in 4-6 liters of distilled water on a Buchner funnel It was then washed with 0.02 M phosphate and resuspended in it. The suspension was degassed in a hot water bath and slowly poured down the side of a 2.4 x 35 cm column.

DEAE cellulose, because of its ion exchange properties can be used to separate IgM and IgG from a partially purified ascitic fluid preparation, i.e. SAS/2 treated. DEAE cellulose is negatively charged but becomes positively charged if it is prepared in a phosphate buffer pH 8.0. The IgG molecule is less negatively charged than IgM, thus it was not able to

bind to the DEAE column as strong as the IgM. Hence, at a low molality, i.e. 0.02 M phosphate, IgM will bind to the DEAE and most of the IgG will be unable to bind and will be washed through the DEAE column. The addition of a second buffer with an increased ionic strength, i.e. 0.03 M phosphate will result in the elution of IgM from the DEAE column. However, IgG_1 binds more strongly to the DEAE column than IgG_2 and was often found to contaminate the pooled fractions containing IgM.

The column was loaded with 30 mgs of SAS/2 treated ascitic fluid for every gram of DEAE cellulose in the column. The first buffer, i.e. 0.02 M phosphate, was used to elute the IgG component in the preparation. The eluate was collected in 4 ml fractions by a LKB Ultrorac Fraction Collector until the OD values of the fractions were less than 0.02. The second buffer, i.e. 0.03 M phosphate was used to elute the IgM component and 4 ml fractions were collected in the same manner as the IgG fractions. The IgM fractions were then pooled and dialyzed against 0.15 M phosphate buffer for subsequent purification on Sephadex G-200. NaCl (2 M) was then run through the column to remove any material still bound to the DEAE and the column was then washed extensively with 0.02 M phosphate.

(d) Sephadex G-200 Column:

A (2.5 x 100 cm) Pharmacia column with a bed volume of 502 cm^3 [$V = h\pi r^2 = (100)(3.14)(1.25)^2 = 502$] was used. One gram of dry Sephadex was used per 30-40 ml bed volume, thus, including a 10% excess, 23 grams of dry Sephadex were hydrated in 900 ml of 0.15 M phosphate buffer pH 8.0. The gel was shaken in the buffer and the fine particles were repeatedly decanted off, ensuring a homogenous particle size. The gel was placed in boiling water to degas it. The gel was slowly poured into a Pharmacia column to avoid trapping air bubbles in the slurry. The column

was operated at 4°C and the packing pressure was kept at a minimum by raising the exit line 10-16 cm down from the entrance. The column was washed for several days with the phosphate buffer and then 1.0 ml of 0.1% blue dextran 2000 was added to measure the void volume of the column and to examine the packing uniformity in the column.

All samples were first concentrated to 4 mls by membrane (P-30) ultrafiltration before application to the G-200 column. The eluates were collected in 4 ml fractions by a LKB Ultrorac Fraction Collector. The fractions containing IgM, i.e. the fractions excluded from the G-200 column, were pooled and dialyzed against barbital buffer pH 8.6 for further purification with agar block electrophoresis.

(e) Agar Block Electrophoresis:

An agar block (25 x 8 cm) was prepared by solidifying 300 mls of 0.85% Noble agar in barbital buffer μ=0.1 pH 8.6. Saran wrap was laid over the tray and the hot agar was poured in and allowed to solidify. Two wicks made out of gauze were embedded at both ends of the block and were held in place by the hardened gel. A transversal agar strip of 7.75 x 1 cm was cut out and the exposed bottom was covered with a small amount of gel. The small trough was then filled with 0.85% agar containing sample solution (5 ml 1.7% agar + 5 ml sample). Caution was taken to avoid introducing air bubbles which could affect the electrophoretic migration of sample components. A stable current (200 V, 100 ma) was then applied across the block for about 24 hours (36 hours for IgM separation). After the electrophoresis, the agar block was cut into transversal strips of 8 x 1 cm which were then put into numbered tubes and then subjected to freezing and thawing three times to release the contained proteins.

The protein content of the various fractions was determined by the Lowry test because the barbital buffer absorbs at 280 mμ and optical density determinations would be inaccurate. The purity of the fractions was further examined by Ouchterlony analysis.

(f) Lowry Test:

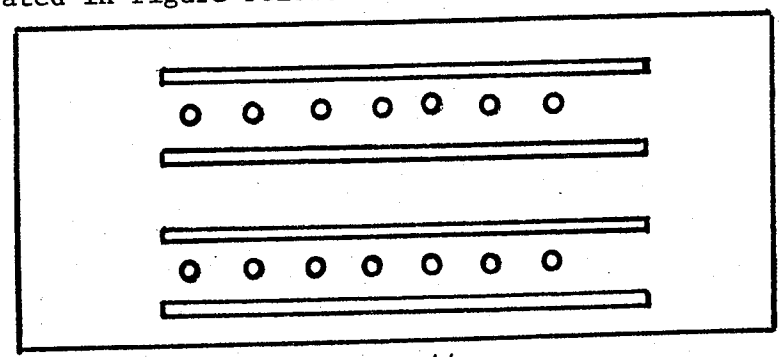
A series of tubes were set up using various amounts of bovine serum albumin (BSA) to establish a standard curve.

Tube No.	BSA ($\mu g/ml$) (Vol)	H_2O (Vol)
1	0.1	0.5
2	0.2	0.4
3	0.4	0.2
4	0.6	0.0

Three mls of Lowry reagent, obtained by mixing 100 mls of 0.1 N NaOH in 2% Na_2CO_3 with 1 ml of 1% $CuSO_4$ and 1 ml of 1% tartrate, was added to each tube and then shaken. Ten minutes later 0.4 ml of Folin reagent (IN) was added and the tubes were incubated for 30 minutes at 37°C. The optical density of the tubes was then read at 750 mμ.

Ouchterlony Analysis:

Noble agar (1%) in borate buffer 0.005 M pH 8.0 was poured on glass slides (7.5 x 5.0 cm). A series of holes (1.5 mm in diameter) were punched between two sets of parallel troughs on the same slide as demonstrated in figure below.



The eluates from each of the agar strips were placed in different holes and various antisera were placed in the troughs. The slides were then incubated in moist chamber for 24 hours and then washed 3 times in BBS. After a final wash in distilled water the slides were covered with strips of lintless paper and allowed to dry at room temperature. They were then stained for a few minutes in amido black which had been prepared with the following materials: 2.0 grams amido black + 180 ml methanol + 20 ml acetic acid. The slides were then destained in 2% acetic acid and again left to dry at room temperature and were examined for precipitin lines later.

Immunoelectrophoresis:

Noble agar (0.85%) was prepared in barbital buffer μ=0.05 pH 8.2 and poured on glass slides (2.5 x 7.5 cm) in a polystyrene frame to which silicone grease had been applied to prevent the hot agar from seeping under the slides. The frames were secured on a level table to ensure an even distribution of the agar on the slides. A Gelman punch was used to make a series of holes in the gel and the material to be electrophoresed was added to the holes with a Pasteur pipette. Paper filter wicks soaked in the barbital buffer were put at each end of the frame containing the slides. A drop of 1% thiazine red was put on each slide to act as a visual marker during the electrophoretic process. The material to be electrophoresed was then subjected to 450 V, 50 ma for 1 hour. After electrophoresis, the Gelman punch was used to cut the troughs out and the appropriate antisera were added to the troughs with a Pasteur pipette. The slides were then incubated for 18-24 hours in a moist chamber to allow the development of precipitin bands. They were then washed three times in BBS, one time in distilled water and then

covered with lintless paper strips and allowed to dry at room temperature. The slides were removed from their holders, numbered and stained in amido black for 3-5 minutes and then destained in 2% acetic acid.

Immunization of Rabbits:

Young adult New Zealand White rabbits were immunized with the myeloma proteins purified in the laboratory, i.e. IgM, IgG_{2a}, IgG_{2b} and IgG_1. The protein was emulsified in Freund's Complete Adjuvant (FCA) by forcing the mixture between two syringe barrels through an adaptor. One mg of purified immunoglobulin was injected in a one to one ratio by volume with the FCA into the four footpads and four intradermal sites on the back. The animals were boosted two weeks later with 1 mg of the same immunoglobulin preparation into four subcutaneous sites on the back. The animals were then test bled ten days later and their sera was examined electrophoretically and by Ouchterlony analysis. The animals were subsequently bled every ten days and the serum was prepared by rimming the blood, letting it stand for 1 hour at 37°C and centrifuging to collect an optically clear serum. The serum was then stored at -20°C until use.

Immunoadsorbent Column Preparation:

Both direct (IgG_{2a}, IgG_1) and indirect (anti IgG_{2a}) immunoadsorbent columns were prepared. Cyanogen bromide (1 gram) was slowly added to 30 mls distilled water in the fume hood. Thirty mls of packed Sepharose 4B were poured into the above solution and the pH was constantly adjusted to 11 by 2 N NaOH. The suspension was then washed with 500 mls of PBS in a Buchner funnel under negative pressure in the fume hood. The purified protein (90 mg) to be coupled to the Sepharose, was added to the mixture and

stirred slowly at 4°C for 24 hours. The Sepharose mixture was then poured through a filter and the optical density of the filtrate was examined to determine the amount of protein that did not couple to the Sepharose. The Sepharose was then washed with 1.5 liters of PBS on a Buchner funnel and then put in a beaker with 140 mls of 0.1 M ethanolamine for 30 minutes in the cold (4°C) to inactivate any free sites on the gel. The mixture was then washed with 1 liter of PBS on a Buchner funnel until the optical density of the eluate was less than 0.01. The solution was then washed with 400 mls 0.2 M glycine-HCl to remove the loosely bound protein. The Sepharose was again washed with PBS until the pH of the eluate was 7.2 and then poured into glass columns.

Direct Plaque Forming Cell Assay:

The ability of anti μ treated $B6AF_1$ mice to form an immune response to red sheep blood cells (SRBC) was examined by Jerne's direct plaque forming cell assay. The animals were given an intraperitoneal injection of 5×10^8 SRBC on day 0. Petri plates (60 x 15 mm) were prepared with 2.5 mls of 0.8% Sea Kem agarose in phosphate buffered saline pH 7.2 solution and then stored at 4°C. Three days later the mice were killed by cervical dislocation and their spleens were removed and suspended in ice-cold Hanks' Hepes supplemented with 10% fetal calf serum.

The spleens were homogenized with a homogenizer with a 1 mm clearance and the crude suspension was filtered through a stainless steel mesh screen. The suspension was allowed to stand in ice for about 5 minutes to let the clumps settle. The clump-free suspension was then washed once in ice-cold Hanks'-Hepes (without FCS) and adjusted to the desired concentrations. Three separate dilutions were made up for each spleen cell suspension. Pyrex glass culture tubes (13 x 100 mm) were placed in

a rack in a water bath at 46°C with 0.4 mls of 2X Hanks'-Hepes and 0.4 mls of 0.8% agarose made in distilled water. Then 0.1 ml of a 25% solution of SRBC was added to groups of three tubes at a time. Then 0.1 ml of a single dilution of one of the spleen cell suspensions was added to each of the three tubes. The tube was rapidly shaken and the resulting mixture was poured on the previously prepared Petri plates which contained 2.5 mls of 0.8% agarose in PBS. The plates were then incubated for 60 minutes at 36°C. Then 2 ml of a 1:10 dilution of guinea pig complement were added to each plate. The plates were agains incubated at 37°C for 30 minutes. The guinea pig complement was then poured off and the resultant plaques on the plates were either counted immediately or the plates were stored at 4°C to be counted the next day. The total cell number of the spleen was determined with a haemocytometer. Thus, the number of plaques produced by a known spleen cell concentration was extrapolated to calculate the number of plaques that the total spleen cell population would produce, i.e. PFC/spleen.

Breeding Program:

Young A males and C57BL/6 females were obtained from Jackson Laboratories. Twenty-five plastic breeding cages each including one A male mated with two C57BL/6 females were set up. The mice were examined periodically and when the females showed obvious signs of pregnancy the cages were examined daily to ensure the offspring were injected on the day of their birth.

Production of T Deprived Mouse, i.e. ATXBM Mouse:

Ten A/J mice were thymectomized by Tony Wong, Lab. Technician, Department of Immunology and when recovery from the operation was complete, i.e. 3-4 weeks later, the animals were lethally irradiated with 900 rads. The following day the mice were reconstituted (iv) with 1×10^6 syngeneic BM cells. Some of the animals from this group were sacrificed and a histological

examination using serial sections of the mediastinum of the animal was performed by the Histology Department. Dr. Sabbadini examined the slides and confirmed the completeness of the thymectomy. Approximately one month from the day of the bone marrow reconstitution, the animals were sacrificed and the numbers of T and B cells in their spleen and lymph nodes were measured in the cytotoxicity assay with anti θ and RAMIg serum respectively.

Suppressive Treatment:

Littermates were designated as control animals which received normal rabbit serum, or experimental animals which received rabbit anti u serum by toe markings. The control animals had the second toe on a front leg cut off and the experimental animals had the second toe on a back leg cut off. A wide range of suppressive regimes which altered the dose of anti u and the schedule of injection were examined. Anti μ serum that produced precipitin bands with 30 μg/ml of purified IgM and nms diluted 1:32 in Ouchterlony diffusion analysis, was diluted with PBS, i.e. 1:2, 1:4 or 1:8. These various dilutions were administered either on alternate days or every 5th day from the day of birth. The following sequence of intraperitoneal injections employing a 1:2 PBS dilution of anti μ represents the most successful schedule for the suppression of serum Ig levels as determined by immunoelectrophoresis of the adult sera: day 0 - 0.05 mls, day 2 - 0.06 mls, day 4 - 0.08 mls, day 6 - 0.10 mls, day 8 - 0.15 mls, day 10 - 0.20 mls and then 0.20 mls every second day until day 40.

Antibody Mediated Cytotoxicity Test:

The number of T and B cells in mice that had received the anti μ suppressive treatment were studied with the cytotoxic assay using chromium 51 (^{51}Cr) labelled cells. The mice were killed by cervical dislocation and their spleen and lymph nodes were removed. Spleen cell

suspensions were freed of red blood cells with tris buffered NH_4Cl (201) and washed twice in Hanks' Hepes.

(a) Labelling of Target Cells:

Radioactive chromium was added to washed lymph node and spleen suspensions. Chromium was added in the ratio of 200 uci per 1×10^7 spleen cells and 250 μCi per 1×10^7 lymph node cells. The mixture (1 ml) in Falcon round bottom platic tubes (17 x 100 mm), was incubated at 37^oC for 45 minutes with occasional agitation. The cells were then washed five times with the medium (Hanks'-Hepes) and the concentration was adjusted to 1×10^6/ml for use in microtiter V-shaped plates.

(b) Production of RAMIg Serum:

Rabbit anti mouse IgG_1, IgG_{2a}, IgG_{2b}, IgA and IgM which had been heat inactivated at 56^oC for 30 minutes were mixed in equal proportions to make a polyvalent rabbit anti mouse Ig serum.

(c) AKR Anti C_3H Theta Serum:

Thymuses from twenty young C_3H mice were collected. These were then injected intraperitoneally into sixty AKR mice, about 3×10^7 cells per mouse. After a period of 4 weeks the mice were injected once a week for five weeks in the same manner as the first injection. One week after the final injection the mice were bled and the sera were pooled and heat inactivated at 56^oC for 30 minutes. The serum was then stored at -70^oC until use. A high titered anti θ serum, which was necessary for the lysis of peripheral T lymphocytes in the cytotoxic assay, was obtained from this extended immunization schedule.

(d) Rabbit Anti Mouse Lymphocyte Sera (RAMLS):

On day 0, 2.5×10^7 mouse spleen and lymph node cells were injected in a 1:1 volume ratio with Freund's Complete Adjuvant into the four foot-

pads of a New Zealand White rabbit. On day 28, 29, and 30 2.5 x 10^7 lymphocytes were administered by an intravenous injection. After a period of 7 days the animal was bled and the sera was stored at -70°C until used. The serum was not absorbed , thus, it was not specific for lymphocytes.

(e) Titration of Xenoantisera (RAMIg) and Alloantisera (anti θ) with the Two-step Assay:

Micro-tissue culture plates with V-shaped wells and holding a maximum of 0.3 ml were used. Duplicates of xenoantisera and alloantisera were serially diluted in two-fold dilutions in 0.1 ml of Hanks'-Hepes. To each well, 0.1 ml of 1 x 10^5 ^{51}Cr-labelled cells was added using an Oxford sampler. The plates were then covered with a microtiter plate sealer and gently agitated to ensure adequate mixing of sera and cells. The plates were incubated at either 37°C for 30 minutes in some experiments or at 4°C for 60 minutes in other experiments. They were then spun at 250 g for 10 minutes. A portion (0.15 ml) of the supernatant was removed with a Pipetteman variable volume sampler and 0.15 ml of guinea pig complement (GPC), at an appropriately tested concentration, was added to the wells. The control wells had cells and GPC alone, cells and antisera alone and cells and medium alone. The plates were then again agitated to disperse the pellet and incubated at 37°C for 30 minutes. The plates were then spun at 250 g for 10 minutes and 0.1 ml of the supernatant was removed from each well with an Oxford sampler and put into (13 x 100 mm) Falcon glass culture counting tubes. The radioactivity was then counted for 1 minute with a well-type gamma scintillation counter.

(f) Titration of Xenoantisera (RAMIg) and Alloantisera (anti θ) with the One-step Assay:

V-shaped micro-tissue culture plates holding a maximum of 0.3 ml were used. Xenoantisera and alloantisera were two-fold serially diluted in 0.1 ml volumes with Hanks'-Hepes. ^{51}Cr labelled cells (0.05 mls with 1×10^6 cells) were added to each well with an Oxford sampler. A previously determined, maximal concentration of agarose absorbed GPC (0.05 mls) was added to the wells after letting the plate stand for 15 minutes at room temperature. The plates were sealed with microtiter plate sealers, gently agitated to ensure maximal mixing and then incubated at 37°C for 60 minutes. They were then spun at 250 g for 10 minutes and 0.1 mls of the supernatant was removed with an Oxford sampler and put in culture tubes to be counted. The released radioactivity was counted for 1 minute per tube with a well-type gamma scintillation counter.

(g) Calculation of Percent Lysis:

Cytotoxicity was calculated according to the formula:

$$L = \frac{E-C}{R_{100} - C}, \text{ where}$$

L = corrected percent lysis.

E = counts in the supernatant of experimental wells.

C = counts in the supernatant of the control wells. The control values, derived from the incubation of the target cells and guinea pig complement, were recorded as the mean value of cpm triplicates.

R_{100} = 100% releasable radioactivity, i.e. counts in the supernatant in the presence of ALS and complement. The ALS was used to determine the R_{100} value because it effects chromium release

from the target cells in a similar manner as the anti θ and RAMIg serum, i.e. complement attachment to Ag-Ab complexes on the cell surface with the subsequent lysis. The use of physical agents, e.g. freeze-thaw, ultrasound which disrupt cells more extensively could result in an additional chromium release. This would inflate the R_{100} value and decrease the % lysis values determined for anti θ and anti RAMIg serum. The ALS serum had been previously shown to induce 100% lysis of the target cells by the trypan blue exclusion cytotoxic test (202). The dilution of ALS serum (1:10, 1:100, or 1:1000) that yielded the maximum release of radioactivity, varied from experiment to experiment. Thus, all three dilutions were included in each cytotoxic assay.

Absorption of Polyvalent RAMIg Serum:

The RAMIg serum was found to have a strong cytotoxicity activity for thymocytes (see Fig.9). Thus, a series of absorptions were carried out in an attempt to reduce this toxicity.

(a) Mouse Liver Extract Absorption:

Whole livers from A mice were ground up with a homogenizer of a 1 mm clearance. They were then mixed with 5-6 times their volume with absolute acetone. This was repeated 4-5 times and the residue was then left for 4-6 hours at $4^{o}C$ to settle out. The residue was then put into equal volumes of acetone and ether to remove the lipid content. This was repeated twice for about 5-6 hours. The residue was then put on filter paper and left at $37^{o}C$ to dry out. The dried residue was ground up with a mortar and pestle into a very fine powder. The powder was reconstituted with physiological saline pH 7.2 , and repeatedly washed until the

supernatant no longer showed the presence of protein, i.e. OD<0.04. The fixed liver powder was then mixed with the serum and slowly rotated for 1 hour at 4°C. The serum was then spun at 1000 g for 10 minutes to clear the serum.

(b) Glutaraldehyde Fixed and Live Thymocytes Absorption:

Thymuses were removed from young $B6AF_1$ mice taking care to avoid contamination with blood. The fixative effects of various glutaraldehyde concentrations (1%, 2%, 3%) on thymocytes were examined and 1% glutaraldehyde was found to produce the best results. All three concentrations fixed the thymocytes, as was evidenced by a brownish colour change, but the 1% concentration gave the least amount of cell clumping. Fifty mls of 1% glutaraldehyde were added to 2 mls of packed thymocytes (from 50 yound $B6AF_1$ mice) and then gently rotated at 4°C for 2 hours. The thymocytes were then spun down at 1000 g for 10 minutes and extensively washed with PBS until the supernatant was clear of protein, i.e. OD <0.04. For the absorption of the RAMIg serum the fixed thymocytes were mixed with polyvalent RAMIg serum in a 1:1 ratio by volume and left slowly rotating for 3 hours at 4°C. The thymocytes were spun out at 2000 rpm for 10 minutes and the absorbed RAMIg serum was stored at 4°C until use. The adsorbed antibodies were removed from the fixed thymocytes by washing with glycine-HCl pH 2.8. The thymocytes were then washed several times in PBS to remove the glycine-HCl and were stored at 4°C for further use.

RESULTS

This section is divided into five main parts: (A) the purification of myeloma proteins and the production of monospecific rabbit anti mouse IgM serum, (B) immunoelectrophoretic analysis of circulating Ig in $B6AF_1$ mice treated with anti μ serum, (C) analysis of the degree of immunosuppression in anti μ treated $B6AF_1$ mice determined by the direct plaque cell assay, (D) examination by the antibody dependent cytotoxicity assay of the effects of anti μ suppression on the level of immunoglobulin (Ig) bearing cells and (E) theta (θ) bearing cells in $B6AF_1$ spleen and lymph nodes.

(A). Production and Specificity of Rabbit Anti-Mouse μ Chain Serum:

(a) Preparation of Purified Mouse IgM:

Ascitic fluid with high concentrations of IgM globulin was obtained by injecting the MOPC-104E plasma myeloma tumor into BALB/C mice. The resultant ascitic fluid was collected and subjected to 50% saturated $(NH4)_2$ SO4 treatment (see METHODS) to remove albumins and haemoglobin. However, ascitic fluid preparation was further contaminated by the animals normal serum immunoglobulin which were not removed by SAS/2 treatment. Thus, the ascitic fluid preparation was run through an anionic cellulose exchange column, i.e. DEAE. The column was equilibrated with 0.02 M tris phosphate buffer pH 8.0 in the cold ($4^{o}C$). The $(NH4)_2SO4$ treated ascitic fluid was loaded on the column and washed with several column volumes of buffer to remove the unabsorbed protein. The material bound to the column (IgM) was eluted with a 0.03 M phosphate buffer. However, not only IgM bound to the DEAE column at high ionic strength as was evidenced by a severe skewing in the elution pattern. Other serum proteins,

i.e. IgG_1, transferrin, which have a similar electrophoretic charge as IgM were likely contaminants.

The IgM containing fractions eluted from the DEAE column were then concentrated using a PM-30 ultrafiltration membrane which excludes materials with a MW under 30,000 and applied to the G-200 column. The G-200 column provided an excellent method to separate out the 7S contaminant fractions because the 7S IgG fraction has a MW 160,000 and is not excluded in the void volume while the 19S IgM fraction has a MW 900,000 and is excluded in the void volume. Unfortunately, other large MW compounds are also excluded in the void volume. The first fractions eluted from the G-200 column were pooled (F_{27}-F_{47}) and in Fig.1 were referred to as the F_1 fraction. An examination of this pooled F_1 fraction by immunoelectrophoresis revealed the presence of more than one component. Rabbit anti mouse gamma globulin reveals the presence of another class of gamma globulin and the rabbit anti MWS serum reveals the presence of some other non gamma globulin serum protein (see Fig.2). Thus, the pooled concentrated F_1 fraction from the G-200 column was then applied to an agar block electrophoresis to further purify the IgM globulin. The agar block was run for 48 hours to effect maximal separation of the IgM from its contaminants. Mouse IgM protein has a very low electrophoretic mobility, thus the eluates from the origin and slightly to the left i.e. cathode side of the origin, were examined by immuno-electrophoresis for purity (see Fig.3). The eluates from all three strips (0,-1,-2) contained only one serum protein, as determined by anti MWS and that protein was IgM immunoglobulin, as determined by anti IgM. The eluates from all three strips were then pooled, concentrated and dialyzed versus PBS to remove the barbital buffer.

Fig.1. Separation of BALB/C MOPC-104E ascitic fluid from DEAE on Sephadex G-200. Four mls of 19 mg/ml of protein was loaded on the column and 4 ml fractions were collected. Fraction 1 (F_1) was pooled and concentrated by diaflo ultrafiltration.

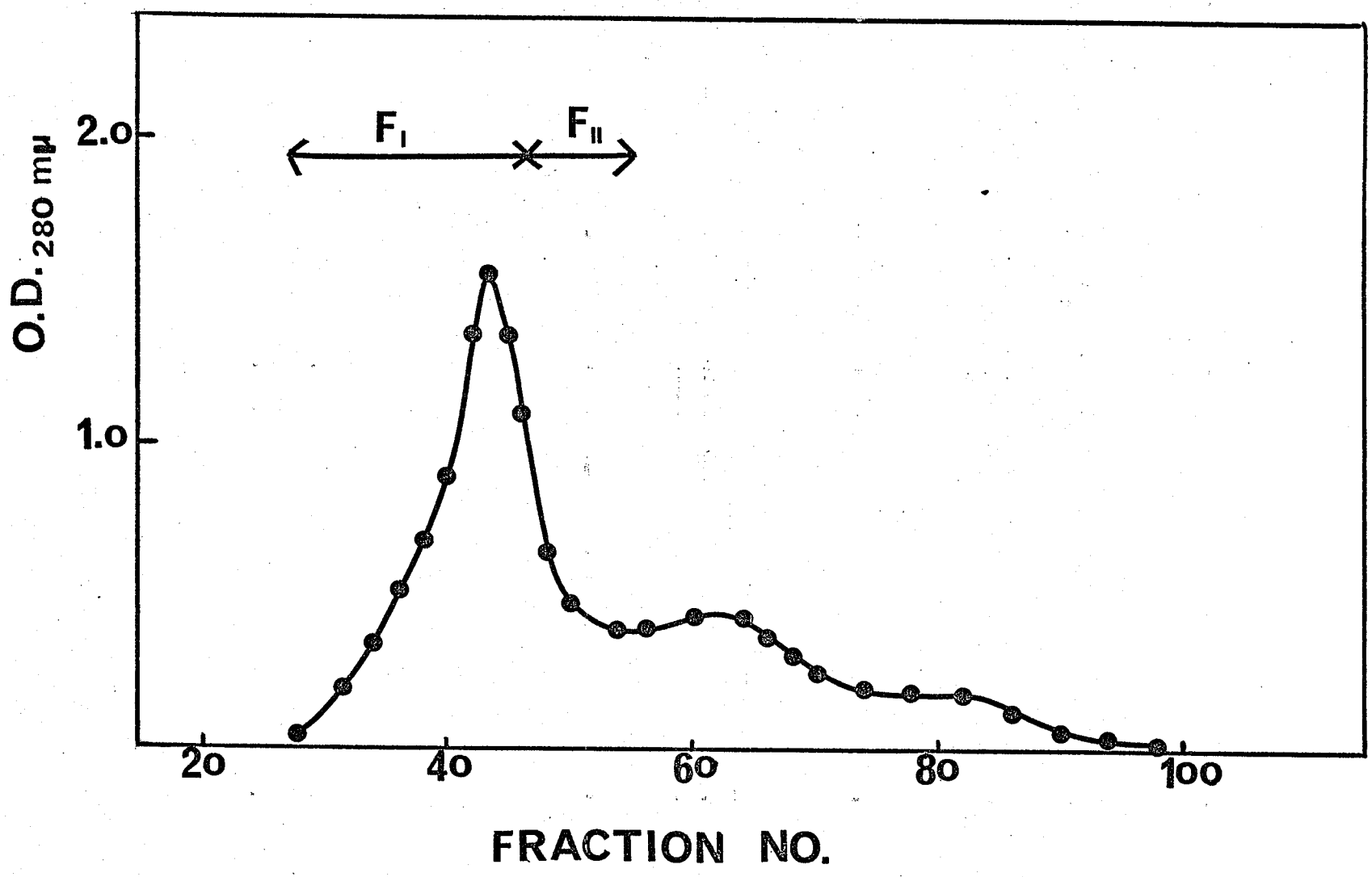
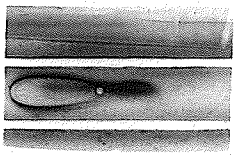

F_I
F_II
O.D. 280 mμ
2.0
1.0
20
40
60
80
100
FRACTION NO.

Fig.2. Immunoelectrophoresis of the pooled concentrated F_1 fraction of IgM, i.e. in the origin, from the Sephadex G-200 column.

NOTE: The terminal portions of this and all subsequent electrophoresis slides have been blanked out for photographic purposes and contained no additional precipitin lines.

Fig.2



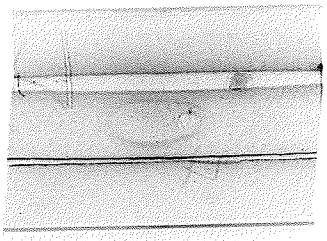
anti mouse whole sera

anti gamma globulin

Fig.3. Immunoelectrophoretic analysis of mouse IgM myeloma protein after agar block electrophoresis. The central well of A has eluate from the origin strip, B has eluate from the first strip toward the cathode, C has eluate from the second strip toward the cathode.

NOTE: Rabbit anti mouse IgM was kindly supplied by Dr. S. Fujimoto.

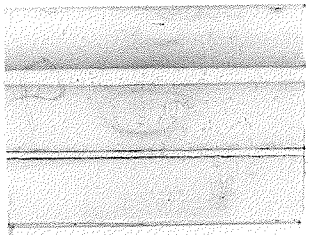
Fig.3A



← Anti-MWS

← AIgM

Fig.3B



← Anti-MWS

← Anti-IgM

Fig.3C

← Anti-MWS

← Anti-IgM

(b) Production of Rabbit Anti IgM serum:

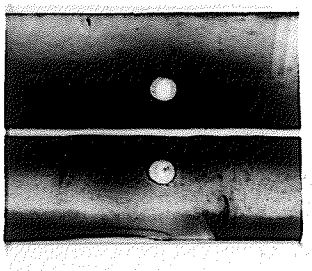
Three New Zealand White rabbits were then immunized with the purified IgM preparations (see METHODS). The rabbits were bled at two weeks and the sera was stored at -70°C. The rabbits responded with high titered sera to the injected material as demonstrated by the strong bands the sera produced with purified IgM immunoglobulin and normal sera in immunoelectrophoresis (see Fig.4). The presence of a shorter band with the purified IgM myeloma may be due to their monoclonal nature, i.e. move to a more restricted area. Ab's to other serum components in the anti IgM serum were demonstrated by the additional precipitin lines against nms.

(c) Production of Rabbit Anti μ Serum:

To produce anti μ serum the cross-reacting anti light chain activity was absorbed out by immunoadsorbent columns, i.e. IgG_{2a} and IgG_1 immunoglobulins coupled to Sepharose 4B. The IgG_{2a} and IgG_1 preparations had been purified using a similar protocol to the IgM purification except that the DEAE column was eliminated as IgG subclasses cannot be separated on DEAE columns. The purity of both these classes was ascertained by immunoelectrophoresis (see Figs. 5,6). The anti IgM serum was extensively absorbed on the IgG_1 and IgG_{2a} Sepharose 4B column after absorption. The anti IgM serum produced no bands against other Ig classes (IgG_1, IgG_{2a}, IgG_{2b}) when tested immunoelectrophoretically or by double diffusion analysis. The anti μ serum was then titrated against normal mouse serum and purified IgM to assess the level of anti μ activity. The anti μ serum produced precipitin bands with normal mouse serum diluted to 1:16 and with purified IgM containing 0.031 mg/ml diluted to 1:32. The anti μ serum was then diluted in an equal volume with PBS and administered to newborn $B6AF_1$ mice (see METHODS).

Fig.4. Immunoelectrophoretic analysis of rabbit anti mouse IgM. Purified IgM supplied from Litton was used as the reference protein

Fig.4



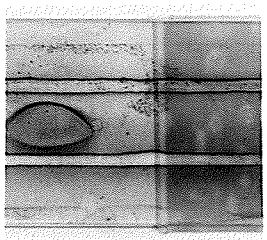
nms

MIgM

Fig.5. Immunoelectrophoretic analysis of mouse IgG_1, i.e. origin contains mouse IgG_1

Fig.6. Immunoelectrophoretic analysis of mouse IgG_{2a}, i.e. origin contains mouse IgG_{2a}

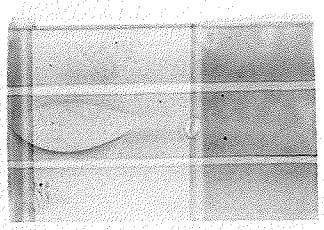
Fig.5



← Anti-IgG_1

← Anti-MWS

Fig.6



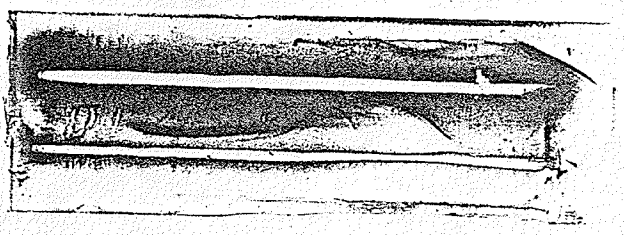
← Anti-MWS

← Anti-IgG_{2a}

(B) Immunoelectrophoretic Analysis of Immunoglobulins in the Serum of Anti μ Treated Mice:

Several test animals (12) given the anti μ treatment, were weaned on day 20 and on day 40, their sera was examined by immunoelectrophoresis for circulating Ig. The sera from the anti μ treated mice produced no precipitin bands with anti μ when tested either by immunoelectrophoresis or by Ouchterlony diffusion tests, although the anti μ serum was able to detect IgM in untreated mice (see Fig.4). The sera were then examined for the presence of other Ig's and Fig.25, Fig.26 represent typical serum electrophoretic profiles observed in the mice. Serum from a control mouse (NRS treated) developed several precipitin lines of gamma globulin nature as defined by the anti Ig serum (Fig.26), whereas the serum from the anti μ treated mice produced only one strong precipitin line of gamma globulin nature (Fig.25). The precipitin lines from the serum of anti μ treated mice developed against anti IgG_1 (Fig.27) were similar to the precipitin lines against anti IgG_1 from the serum of control mice (see Fig.28). However, the precipitin lines from the serum of anti μ treated mice developed against anti IgG_{2a} (Fig.27) were barely visible, while strong precipitin bands were produced with a normal serum and against purified IgG_{2a} (see Fig.28). Thus, it was concluded that the anti μ treatment had effected a complete suppression of serum IgM and a partial suppression of serum IgG subclasses, i.e. IgG_2 was strongly reduced and IgG_1 had recovered. The observation that IgG_2 was the least able to recover is supported by other workers (15,17). Lawton (15) has proposed that the difference observed in the concentrations of IgG_1 and IgG_2 may actually reflect differences in rates of synthesis because of the relatively long half-life of IgG immunoglobulins.

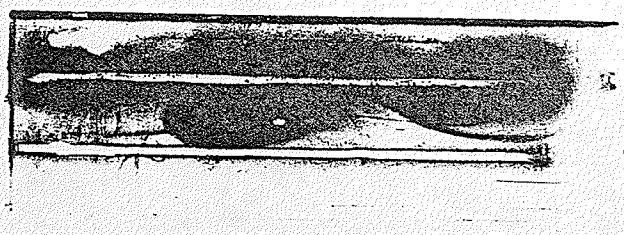
Fig. 25



(-) (+)

Immunoelectrophoretic analysis of the serum of an anti μ treated mouse, i.e. origin contains whole serum

Fig. 26



(-) (+)

Immunoelectrophoretic analysis of serum from a control mouse (nrs treated), i.e. origin contains whole serum.

Fig.27. Immunoelectrophoretic analysis of the serum of an anti u treated mouse, i.e. origin contains whole serum.

Fig.28. Immunoelectrophoretic analysis of anti IgG_{2a} serum, i.e. trough contains anti IgG_{2a}.

Fig. 27

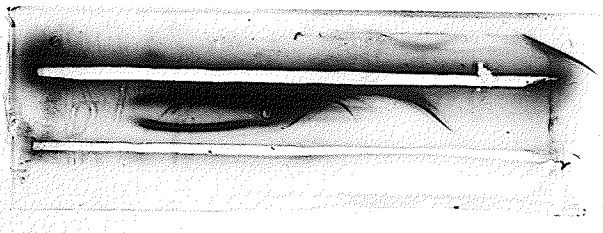


Fig. 28

(C). Analysis of Anti μ Treated $B6AF_1$ with the Direct Plaque Forming Cell Assay:

The anti μ treatment was shown to effect a complete suppression of serum IgM levels. Thus, the ability of anti μ treatment to suppress the animals IgM response upon antigen stimulation (SRBC) was examined using Jerne's (167) antibody plaque assay in agarose. The indirect or IgG response to antigenic stimulation was not examined because the serum IgG levels demonstrate the ability of the B cell to produce IgG immunoglobulin. Thus, the ability of anti μ treated mice to produce IgM Ab after an injection of 5×10^8 SRBC cells was compared with their littermate controls, i.e. normal rabbit serum treated. (see Table I). Group 2, 3 and 4 demonstrate a significant inhibition, $P < 0.005$, $P < 0.005$, $P < 0,005$ respectively, of the PFC response of anti μ treated mice when compared with their littermates. Background plaque values contributed by spleen AFC, i.e. Ab response due not to the test Ag priming, but due to a previous exposure of the Ag or a cross-reacting Ag, were not subtracted from the recorded PFC responses of the control or experimental animals. Thus, the low PFC values, largely background, in these groups demonstrate that the anti μ treated mice are suppressed in their ability to form an IgM response to SRBC. In group 1, one of the control mice failed to respond to the SRBC Ag and its' PFC value accounted for the high S.E. This was thought to represent an error either in the immunization procedure or in the assay conditions as these results are not repeated with any other group. One of the experimental mice responded with an unusually high PFC value, reflecting a partial suppression of the anti μ treatment. However, the PFC values from this group are questionable in view of the poor response from control mice. Thus, based on the results from group 2,3 and 4 it was

Table I. Direct plaque formation (IgM) response to SRBC by experimental (anti-μ treated) and littermate controls, (normal rabbit serum treated) $B6AF_1$ mice.

Group 1 (39 days)	No. of Animals	[a]mean PFC/spleen	SE	[b]P
Control	2	2117	1711	---
Experimental	2	477	314	.25 < p < .20
Group 2 (38 days)	**No. of Animals**	**mean PFC/spleen**	**SE**	**P**
Control	3	1,460	532	---
Experimental	4	48	14	p < 0.0005
Group 3 (44 days)	**No. of Animals**	**mean PFC/spleen**	**SE**	**P**
Control	3	2,707	211	---
Experimental	4	43	11	p < 0.0005
Group 4 (43 days)	**No. of Animals**	**mean PFC/spleen**	**SE**	**P**
Control	2	1,147	125	
Experimental	2	37	68	p < 0.005

[a] Mean PFC/spleen values were derived from the mean PFC/spleen from three spleen dilutions each performed in triplicate.

[b] P values determined by Student t Test.

concluded that the B cell in the anti μ treated mouse was suppressed in its ability to form Ab and the antibody mediated cytotoxicity test was employed to determine if this functional loss of B cell activity could be correlated with a corresponding loss of B cells. T cell help is necessary to form an Ab response to SRBC thus, T cells were also examined to see if there were any changes in their numbers, i.e. increased or decreased T cell population.

(D) Antibody Mediated Cytotoxicity Assay for the Quantitation of Ig Bearing Cells:

The original cytotoxic test as devised by Gorer and O'Gorman (168) was performed with the use of a dye to act as an indicator of cell death. The use of ^{51}Cr-Na chromate as an indication of cell death in the cytotoxic test was first introduced by Sanderson (162) and later modified by Brunner (163) and Wigzell (164). The first cytotoxicity tests using ^{51}Cr labelled cells have been used in the mouse H-2 alloantisera titrations (170,171). Although measuring isotope release is a simple method it enables the use of higher numbers of cells per plate, thus, increasing the accuracy of the technique over the former conventional assays using supravital dyes. The labelling has been shown not to increase the fragility of the cells and once ^{51}Cr is bound intracellularly to a cell it does not interchange with other cells (169). The amount of label released into the supernatant is proportional to the amount of cytolysis and is an objective and reproducible representation of cell death (170). Thus, it was decided to use the cytotoxic ^{51}Cr release assay to quantitate the numbers of Ig bearing and θ bearing cells with RAMIg and anti θ sera respectively.

(a) Choice of Guinea Pig Complement:

The cytotoxic assay was used to examine the levels of theta (θ) bearing cells (T) and immunoglobulin (Ig) bearing cells (B) in the lymph

nodes and spleens of $B6AF_1$ anti μ treated mice. Use of the cytotoxic assay requires the establishment of several parameters, i.e. concentration of GPC, concentration of RAMLS and specificity of test sera (RAMIg, AKR anti C_3H θ) Obviously the GPC should be used at a concentration that will lyze all target cells that have attached the appropriate antibodies.

However, the GP serum contained some natural antibodies to mouse cell surface protein and an attempt was made to dilute out the natural antibodies to mouse protein in the serum while still employing a dilution that is able to lyze all the appropriate lymphocytes. Since the natural antibodies in the guinea pig serum could not be diluted out without reducing the strength of the complement below an effective concentration, the GP serum was absorbed with agarose before use. Agarose is believed to have a determinant which cross-reacts with a mouse component, thus, could act to absorb out natural anti mouse antibodies (172). Thus, the GPC absorbed with agarose was titrated with various concentrations of rabbit anti mouse lymphocyte serum (RAMLS) on young $B6AF_1$ lymph node and spleen cells. The cytotoxic assay was initially a two-step assay with a 30 minute first incubation at $37^{o}C$ and a 30 minute second incubation at $37^{o}C$ (see Figs.7,8).

The ^{51}Cr release detected with the incubation of spleen (170 cpm) and lymph node (65 cpm) cells with Hanks'-Hepes medium only was similar to the release detected when the cells (133 cpm and 78 cpm respectively) were incubated with the agarose absorbed GPC (1:10), whereas high chromium release (483 cpm and 357 cpm respectively) had been obtained in their incubation with unabsorbed GPC (:10). Thus, the natural antibodies appear to have been absorbed out with the agarose. All four GPC dilutions (1/10, 1/20, 1/40, 1/80) produced maximal lysis for both spleen and lymph node cells thus

Fig.7. Cytotoxic test for the titration of Rabbit anti mouse lymphocyte serum (RAMLS) using ^{51}Cr labelled $B6AF_1$ lymph node cells. The effect of four guinea pig complement (agarose absorbed) concentrations (1:10, 1:20, 1:40, 1:80) on RAMLS lysis was examined. The determination of the % lysis was based on the following values: lymph node + medium = 65 cpm, lymph node + GPC = 78 cpm, lymph node + $ALS_{R_{100}}$ = 566 cpm. For the calculation of the % lysis refer to METHODS.

◆ = GPC 1:10

▲ = GPC 1:20

◇ = GPC 1:40

○ = GPC 1:80

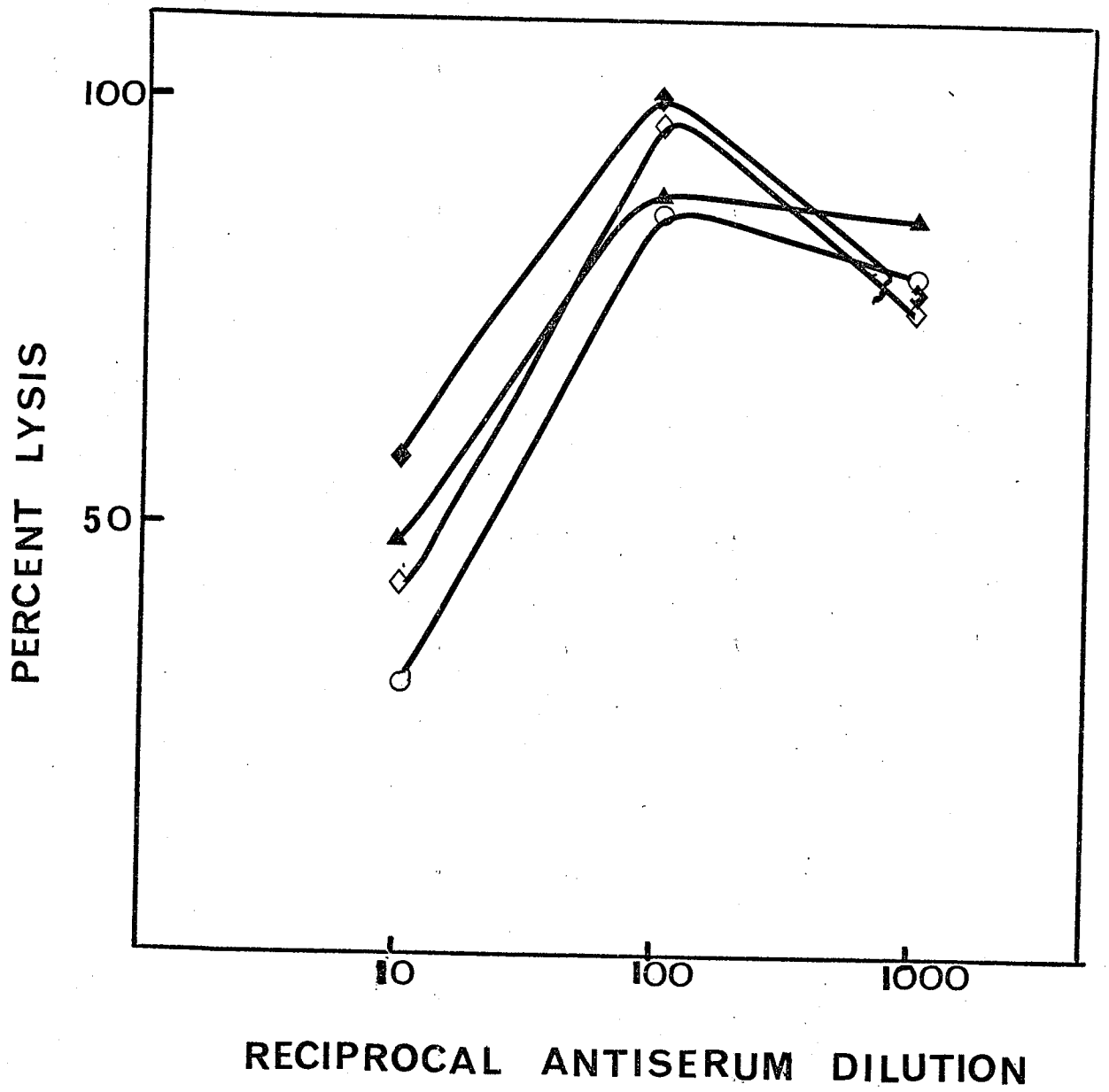

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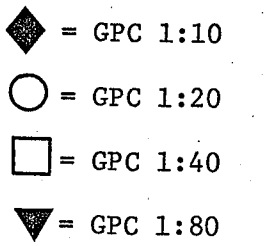
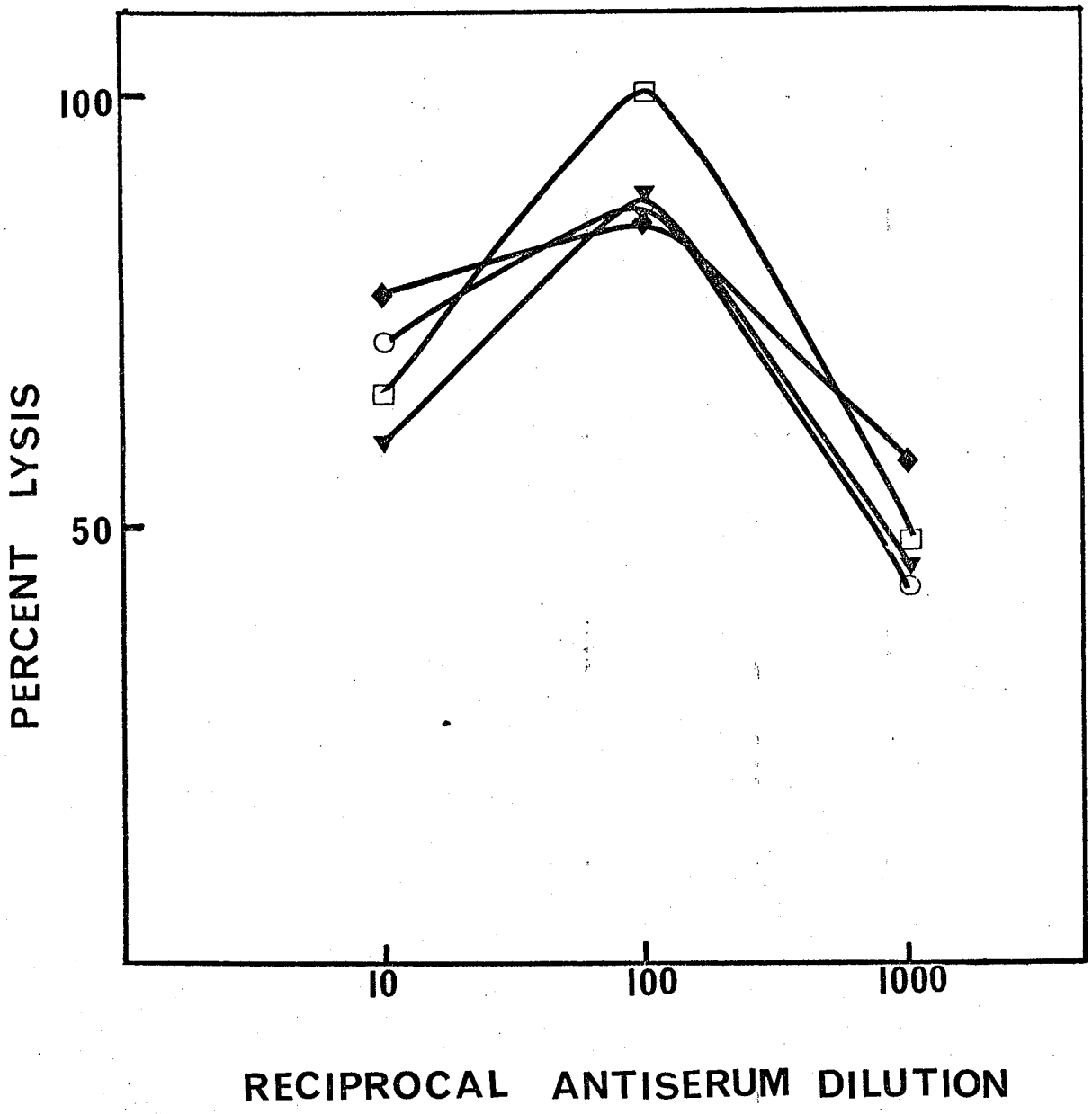
Fig.8. Cytotoxic test for the titration of rabbit anti mouse lymphocyte serum (RAMLS) using ^{51}Cr labelled $B6AF_1$ spleen cells. Effect of four guinea pig complement (agarose absorbed) concentrations (1:10, 1:20, 1:40, 1:80) on RAMLS lysis was examined. The determination of the % lysis was based on the following control values: spleen + medium = 17 cpm, spleen + GPC = 133 cpm, spleen + $ALS_{R_{100}}$ = 604 cpm.

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the 1:10 dilution which represents an excess of complement was chosen for use in the subsequent assays.

(b) Activity of Polyvalent RAMIg Serum:

The polyvalent rabbit anti mouse Ig serum (see METHODS) used to determine the number of Ig bearing cells produced strong precipitin bands against all purified classes (IgG_1, IgG_{2a}, IgG_{2b}, IgM) of immunoglobulins tested by immunoelectrophoresis analysis (see Figs. 12,13,14). However, the RAMIg serum produced no precipitin lines when tested against IgA. Then the polyvalent rabbit serum was also examined to determine if the serum contained any natural antibodies to mouse protein that would yield false results in the cytotoxic assay. The serum was first tested for its cytotoxicity on thymocytes (see Fig.9). The test revealed the marked cytotoxicity of the RAMIg serum for thymocytes which may have been due to either the presence of Ig on the T cell surface or it may have been due to the presence of antibody to other cell surface structures. The second possibility is more likely since numerous studies have failed to detect any T cell surface Ig (3,32,110) and others have found only low levels of surface Ig (52,114,125). Further tests were done with thymocytes to see if the antibodies were present in other rabbit anti mouse Ig preparations, i.e. anti IgG_1, anti IgG_{2a}, anti IgM, anti IgG_{2b}, anti IgA and anti $F(ab')_2$ sera (see Figs.10,11). Anti $F(ab')_2$, anti IgG_{2b} and anti IgG_{2a} were found to have a much higher toxicity for thymocytes than the anti IgM, anti IgG_1 and anti IgA sera. The results clearly demonstrated a strong thymocyte toxicity in anti Ig sera taken from at least six New Zealand White rabbits.

(c) Attempt to Absorb out Thymocyte Toxicity of RAMIg Serum:

(i) Agarose and liver extract adsorption

The RAMIg serum was first absorbed with agarose in an attempt to remove

Fig.9. Cytotoxic test for the titration of poly rabbit anti mouse Ig serum using ^{51}Cr labelled $B6AF_1$ thymocytes. The determination of the % lysis was based on the following control values: thymus + medium = 206 cpm, thymus + GPC = 276 cpm, thymus + $ALS_{R_{100}}$ = 746 cpm.

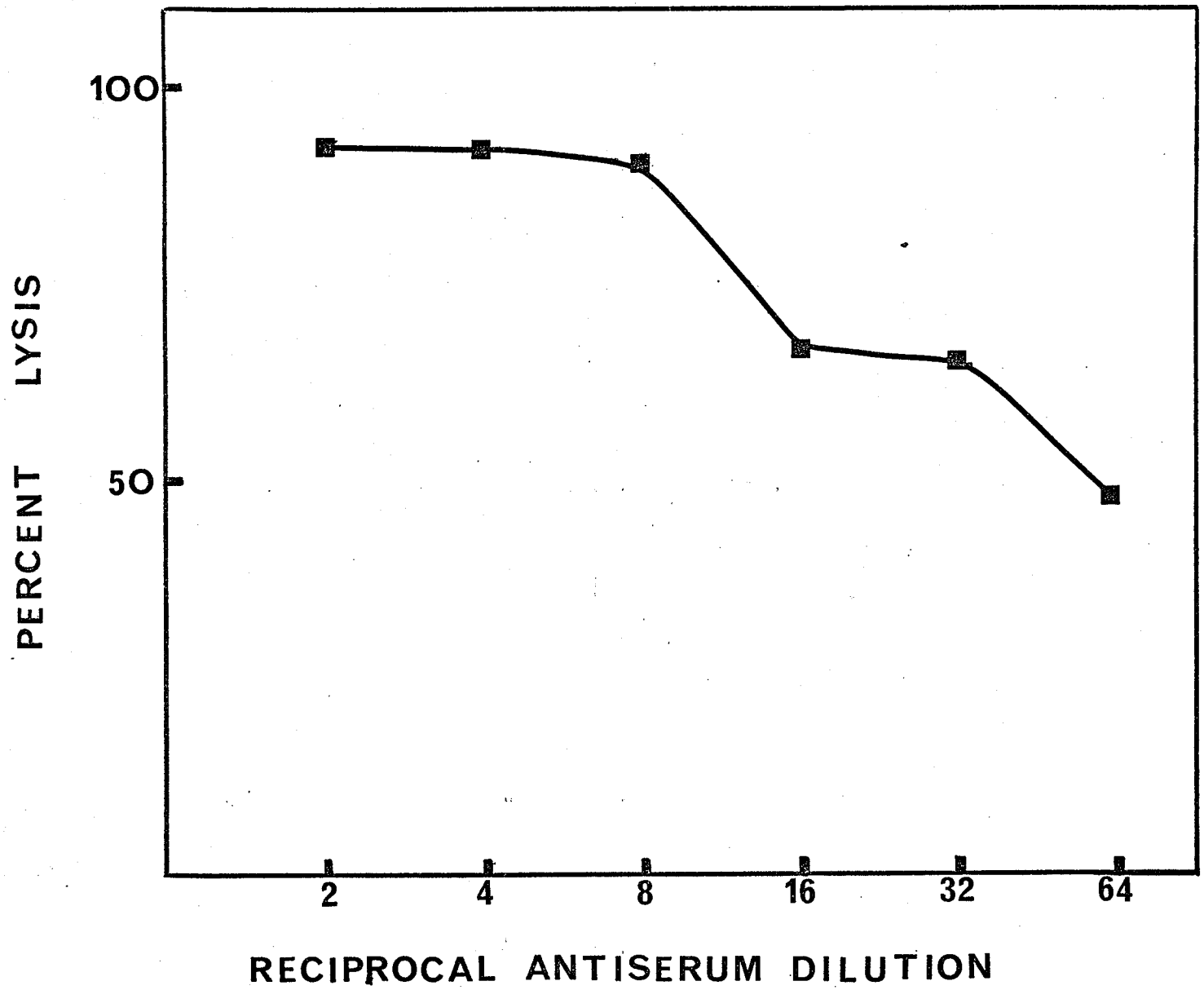
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Fig.10. Cytotoxic test for the titration of various rabbit anti mouse Ig classes using ^{51}Cr labelled $B6AF_1$ thymocytes. The determination of the % lysis was based on the following control values: thymus + GPC = 120 cpm, thymus + heated GPC = 114 cpm, thymus + medium = 114cpm thymus + $ALS_{R_{100}}$ = 429 cpm.

▲ = anti $F(ab')_2$

▽ = anti IgA

◆ = anti IgM

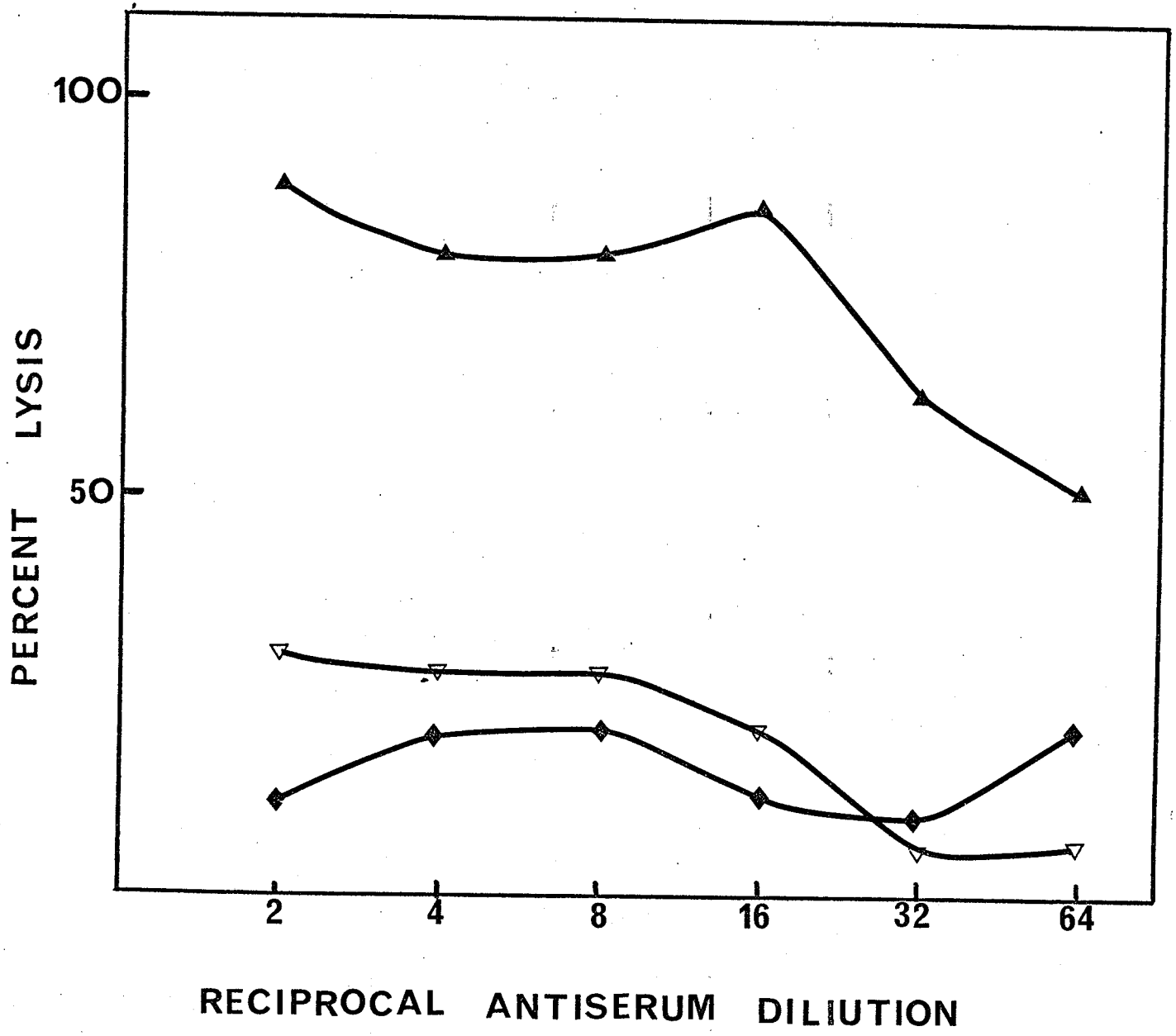
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Fig.11. Cytotoxic test for the titration of various rabbit anti mouse Ig classes using ^{51}Cr labelled $B6AF_1$ thymocytes. The determination of the % lysis was based on the following control values: thymus + heated GPC = 114 cpm, thymus + GPC = 120 cpm, thymus + medium = 114 cpm, thymus + $ALS_{R_{100}}$ = 429 cpm.

△ = anti IgG_{2b}

▲ = anti IgG_{2a}

□ = anti IgG_1

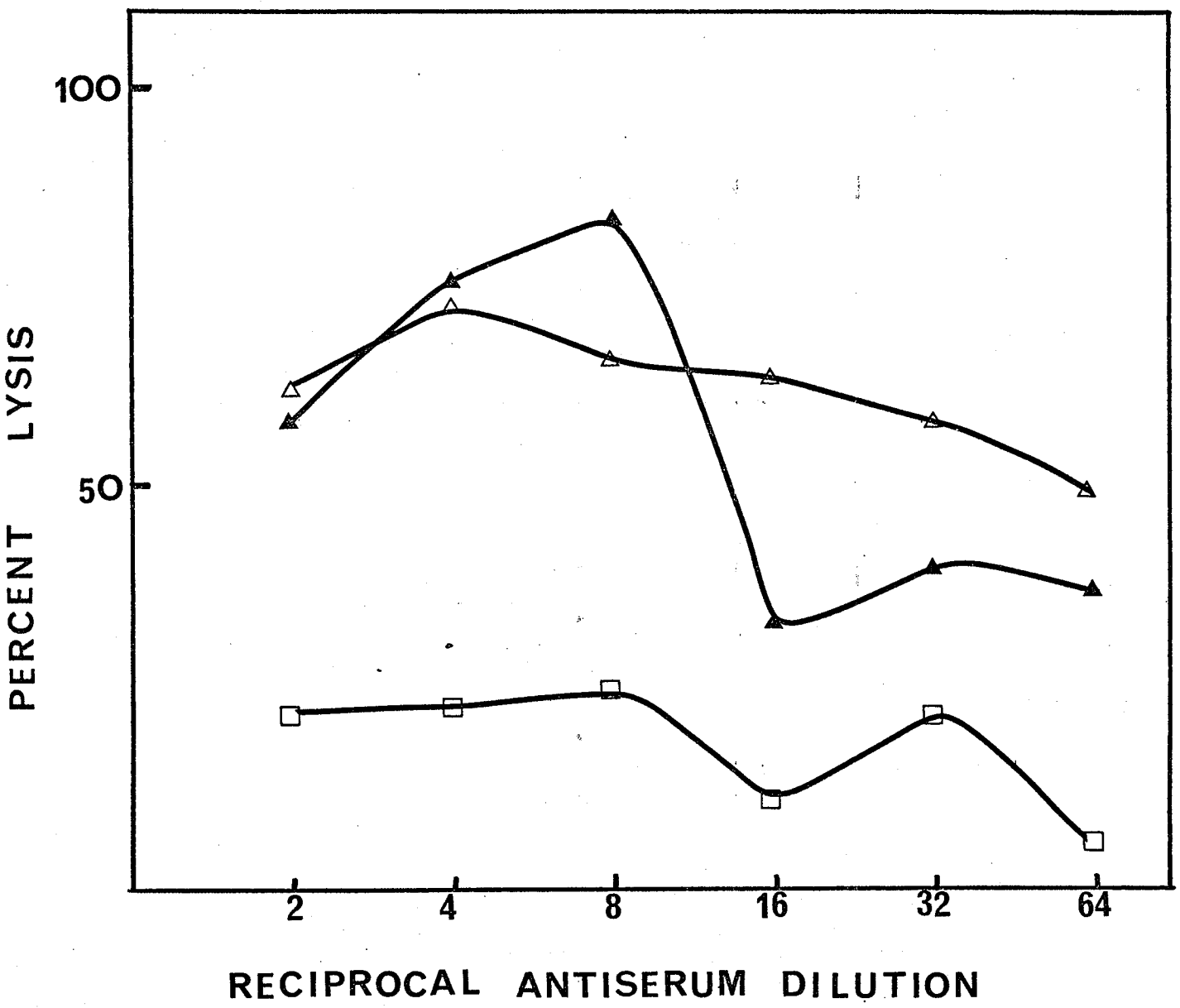
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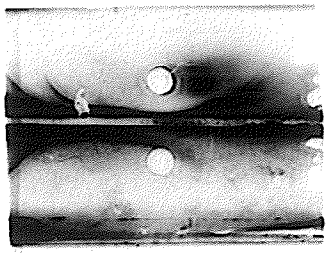
Fig.12,13,14 Immunoelectrophoretic analysis of the poly rabbit anti mouse immunoglobulin serum (poly RAMIg) used in the cytotoxicity assay. Each trough of Fig. 12,13, 14 contains the RAMIg serum.

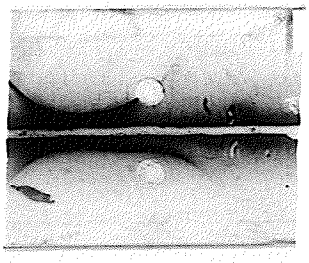
Fig.12



nms

IgG_{2b}

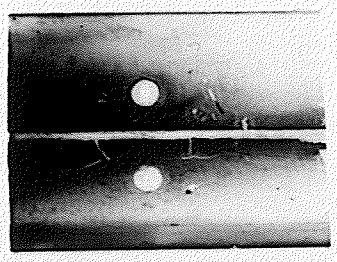
Fig.13



IgG_1

IgG_{2a}

Fig.14



IgM

IgA

the toxicity for mouse thymocytes although agarose was originally used to absorb out thymocyte activity only in guinea pig serum (172). The titration of agarose absorbed poly anti Ig with thymocytes revealed that the thymocyte toxicity had not been eliminated for there was still extensive lysis of thymocytes with the anti Ig sera (see Fig.15). Although increasing the time of the agarose absorption decreased the toxicity for thymocytes there was still a high degree of toxicity observed in the serum. The RAMIg serum could not be absorbed with larger amounts of agarose because the agarose took up the serum and swelled to such an extent that the serum could not be removed by centrifugation. The poly anti Ig serum was absorbed with various dilutions of liver extract (see METHODS) in an attempt to absorb out the antibody (see Fig.16). The liver absorption procedure was also tried with highly specific rabbit anti mouse $F(ab')_2$ serum (see Fig.17). Again the titration of both the absorbed RAMIg serum and the anti $F(ab')_2$ serum against thymocytes demonstrated the persistence of a marked toxicity for thymocytes.

(ii) Live and glutaraldehyde-fixed thymocyte absorption. Absorption with thymocytes was carried out in a further attempt to reduce the thymocyte toxicity in the RAMIg serum. The absorption reduced the toxicity of the serum for thymocytes but unfortunately, its activity for lymph node and spleen cells was also significantly reduced (see Table II). However, the presence of anti Ig activity in the serum after the absorption precedure was confirmed by Ouchterlony analysis, i.e. showed definite precipitin bands with purified Ig (see Fig.18). The possibility was considered that thymocytes which are known to secrete a vast number of substances (177), may have released a factor that interfered with the ability of either the anti Ig to attach to the target cell surface or with the subsequent activity of complement. This possibility was supported by the observation

Fig.15. Cytotoxicity test for the titration of agarose absorbed poly rabbit anti mouse Ig using ^{51}Cr labelled $B6AF_1$ thymocytes. The determination of the % lysis was based on the following control values: thymus + medium = 206 cpm, thymus + GPC = 276 cpm, thymus + $ALS_{R_{100}}$ = 746 cpm.

◆ = unabsorbed RAMIg

■ = agarose absorbed (1 hr) RAMIg

▼ = agarose absorbed (3 hr) RAMIg

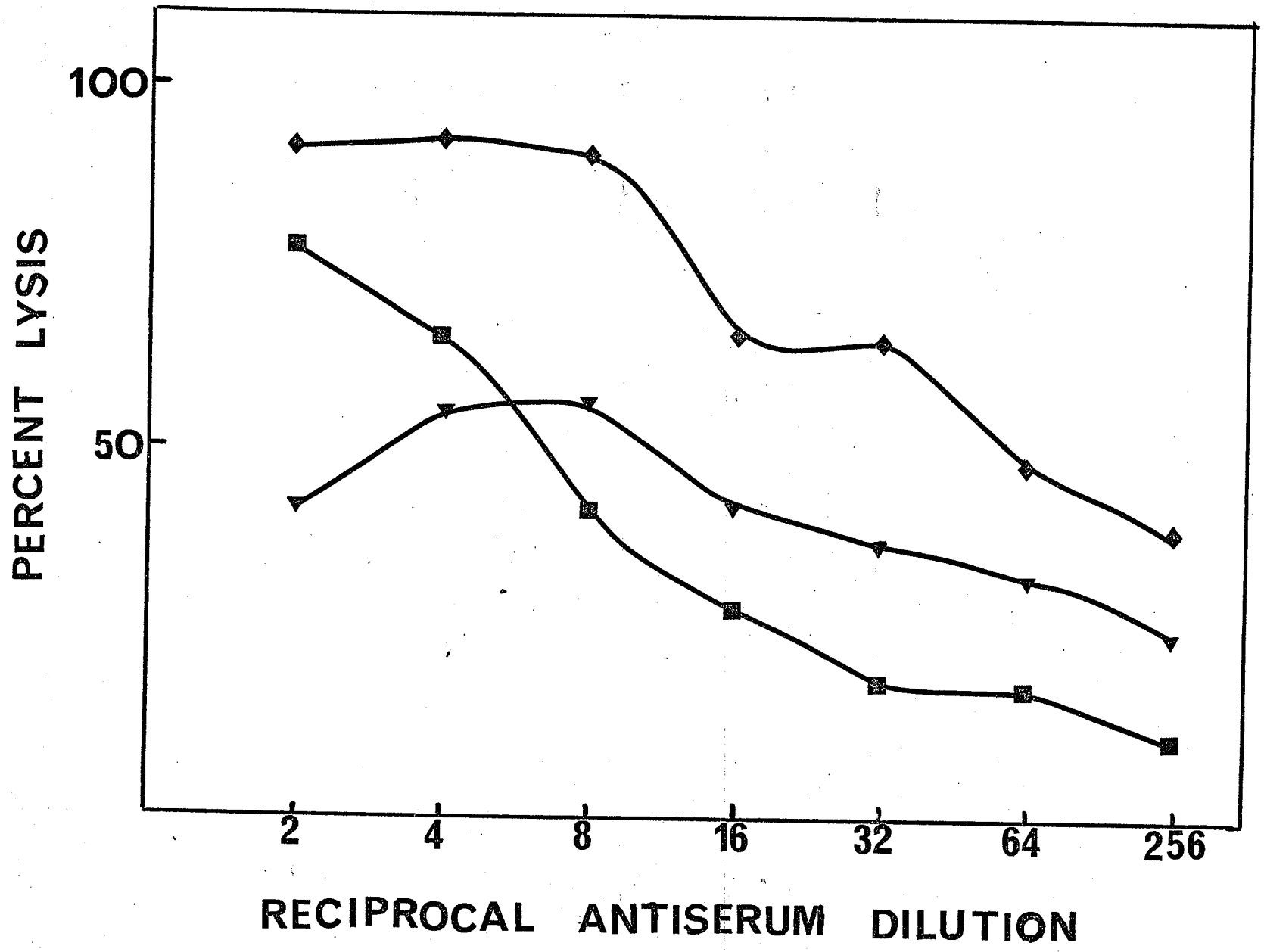

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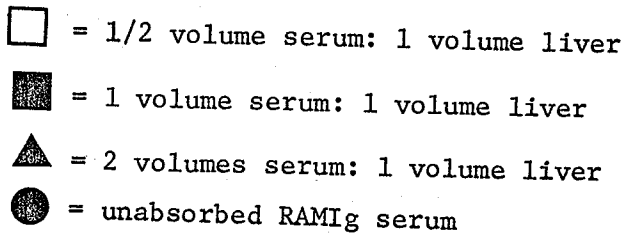
Fig.16. Cytotoxicity test for the titration of liver extract absorbed poly rabbit anti mouse Ig using ^{51}Cr labelled $B6AF_1$ thymocytes. The determination of the % lysis was based on the following control values: thymus + medium = 206 cpm, thymus + GPC = 276 cpm, thymus + $ALS_{R_{100}}$ = 746 cpm.



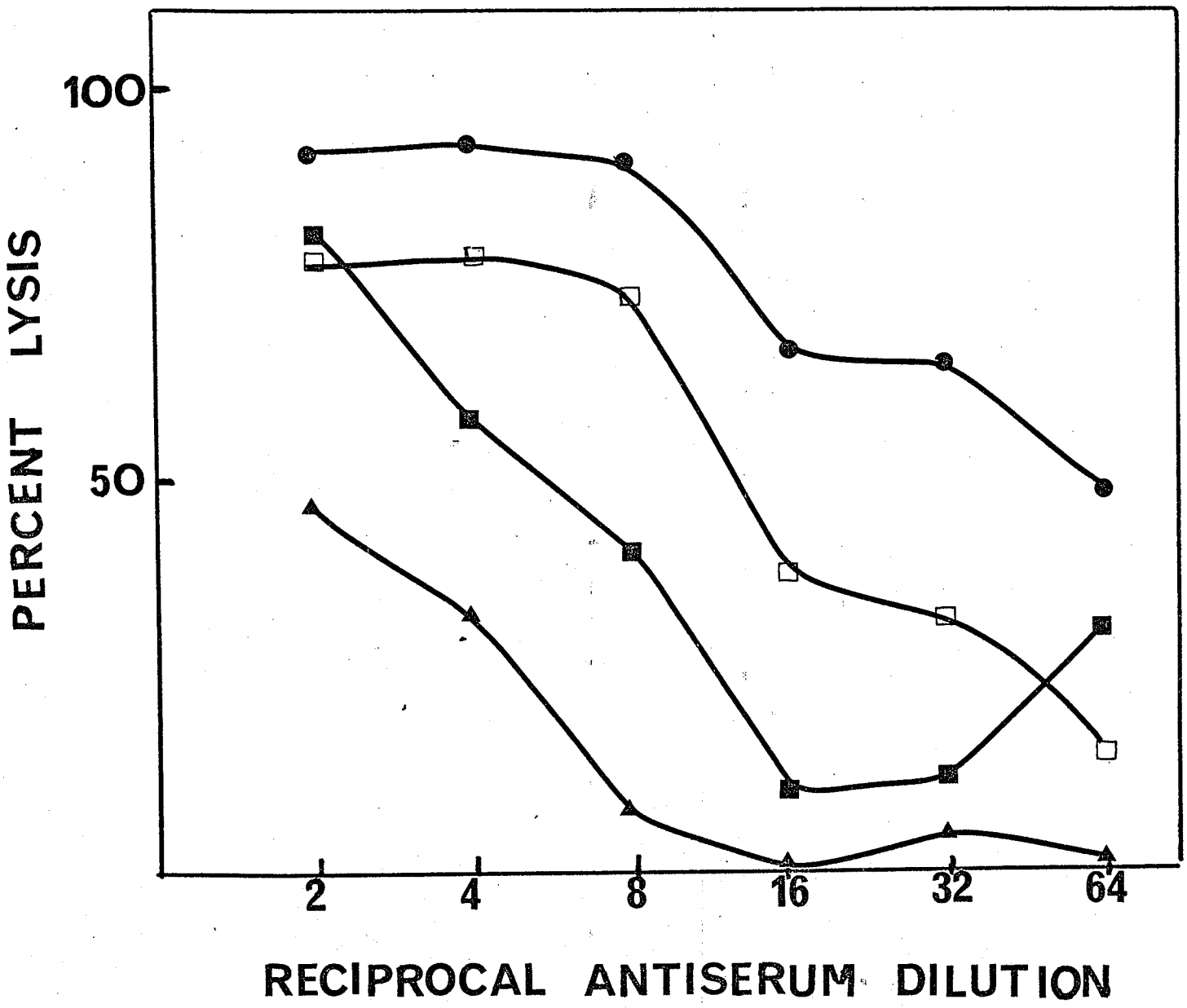

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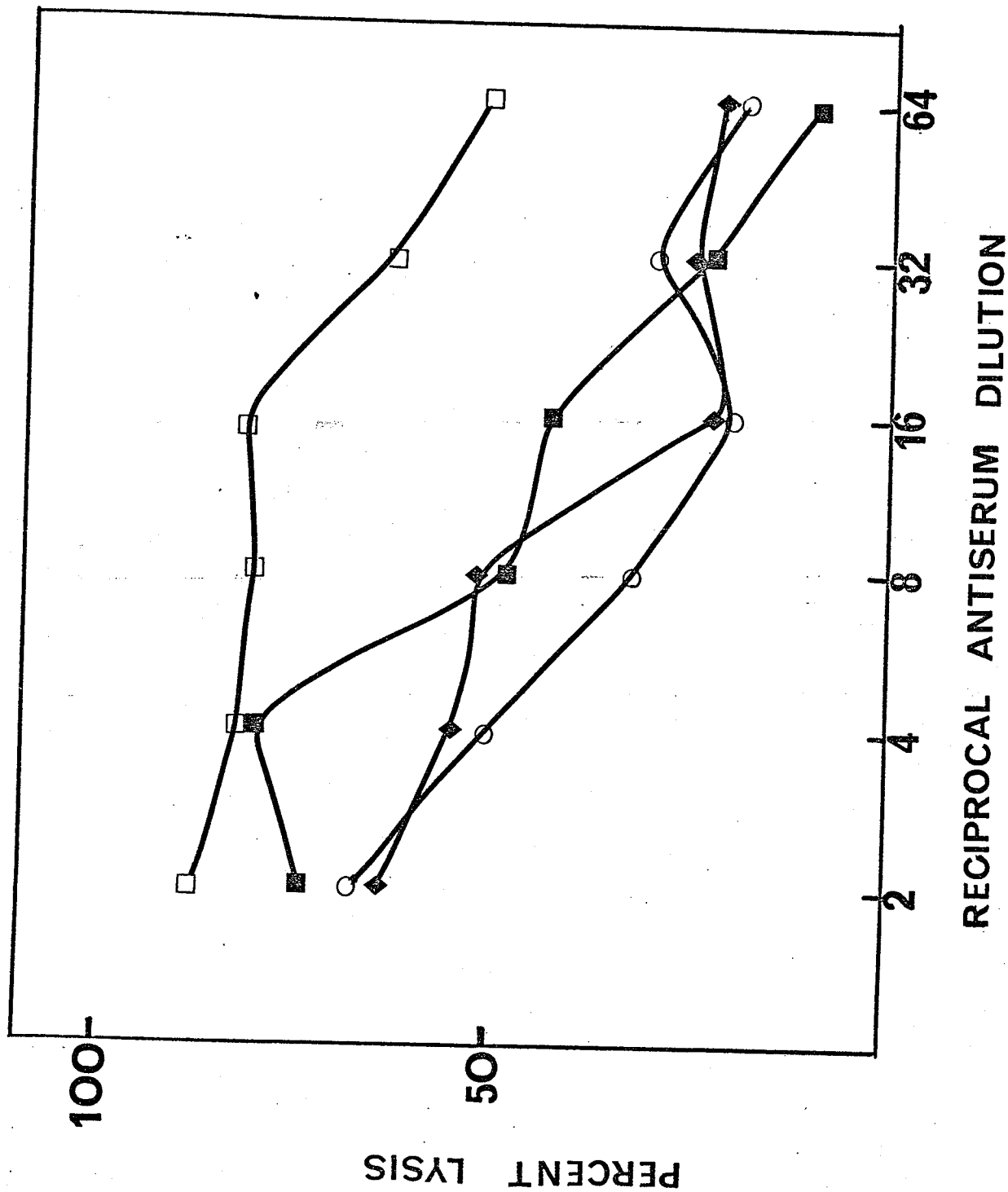
Fig.17. Cytotoxicity test for the titration of liver extract absorbed specific rabbit anti mouse $F(ab')_2$ using ^{51}Cr labelled $B6AF_1$ thymocytes. The determination of the % lysis was based on the following control values: thymus + medium = 206 cpm, thymus + GPC = 276 cpm, thymus + $ALS_{R_{100}}$ = 746 cpm

■ = 1/2 volume serum: 1 volume liver

○ = 1 volume serum: 1 volume liver

◆ = 2 volumes serum: 1 volume liver

□ = unabsorbed $F(ab')_2$ serum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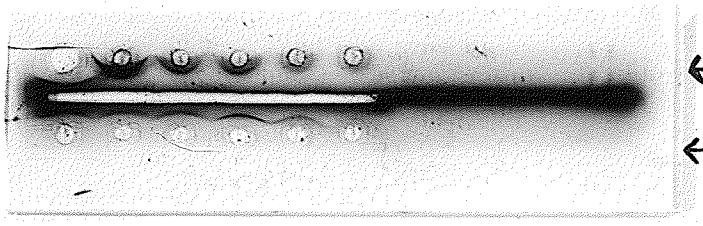
Tabel II. Cytotoxicity test for the titration of live thymocyte absorbed RAMIg using ^{51}Cr labelled $B6AF_1$ thymocytes, lymph node and spleen cells.

Thymus		Lymph Node		Spleen	
Dilution of RAMIg	Percent Lysis	Dilution of RAMIg	Percent Lysis	Dilution of RAMIg	Percent Lysis
1/2	2	1/2	1	1/2	11
1/4	0	1/4	-1	1/4	12
1/8	5	1/8	-2	1/8	11
1/16	4	1/16	-1	1/16	11
1/32	-4	1/32	6	1/32	6
1/64	-3	1/64	-	1/64	-
Thymus + medium = 315 cpm		Lymph Node + medium = 220 cpm		Spleen + medium = 300 cpm	
Thymus + GPC = 400 cpm		Lymph Node + GPC = 308 cpm		Spleen + GPC = 252 cpm	
Thymus + ALS_{R100} = 1168 cpm		Lymph Node + ALS_{R100} = 852 cpm		Spleen + ALS_{R100} = 974 cpm	

Fig.18. Analytical (double) diffusion analysis of poly RAMIg after absorption with live thymocytes. The top series of wells contains purified IgM and the bottom series of wells contains purified IgG_{2a} in halving dilutions from an original concentration of 1 mg/ml.

Fig.18



that several cytotoxic assays using RAMIg serum absorbed with live thymocytes released less chromium from spleen and lymph nodes that was released when the target cells were incubated only with medium or GPC. Thus, to eliminate the possible complications that may have been introduced with live thymocytes, glutaraldehyde fixed thymocytes were then used in the absorption (see METHODS) of the RAMIg serum (see Table III). Although the RAMIg serum is absorbed with glutaraldehyde fixed thymocytes, it is titrated against live thymocytes and the possibility that they interfere with either GPC activity or AIg attachment is again supported by the observed percent lysis values which are suppressed below zero percent. Thus, although the RAMIg serum detected some Ig bearing cells in the LN (13%) and spleen (19%), the values were too low to represent actual numbers of Ig bearing cells.

(d) Attempt to Increase the Sensitivity of the Polyvalent RAMIg Serum:

(i) Two-step antibody assay with a $4^{o}C$ first incubation. The assays presented above have all employed a two-step procedure with a $37^{o}C$ first incubation. It was thought that capping of Ig may have occurred during this first incubation. The capping or internalization of Ag-Ab complexes would have caused decreased lysis as the complement would have less of a chance to attach and lyze target cells. Thus, for this reason the cytotoxic assay was repeated with the RAMIg serum absorbed with fixed thymocytes using a $4^{o}C$ incubation in the first step. The change of temperature for the first incubation did not seem to affect the degree of cytotoxicity (see Fig.19) of the RAMIg serum for lymph node or spleen target cells. However, the $4^{o}C$ temperature was adopted in the first incubation for the subsequent assays described in this section since it would minimize any potential capping or shedding of Ag-Ab complexes from the cell surface.

Table III. Cytotoxic test for the titration of glutaraldehyde fixed thymocyte absorbed RAMIg serum using ^{51}Cr labelled thymus, lymph node, and spleen cells.

Thymus		Lymph Node		Spleen	
Dilution of RAMIg	Percent Lysis	Diltuion of RAMIg	Percent Lysis	Diltuion of RAMIg	Percent Lysis
1/2	-23	1/2	13	1/2	13
1/4	- 7	1/4	10	1/4	19
1/8	- 6	1/8	6	1/8	18
1/16	-27	1/16	1	1/16	19
1/32	-18	1/32	6	1/32	14
1/64	-27	1/64	13	1/64	11
Thymus + medium = 145 cpm Thymus + GPC = 111 cpm Thymus + ALS_{R100} = 360 cpm Thymus + RAMIg (1/2) = 51 cpm		Lymph Node + medium = 162 cpm Lymph Node + GPC = 123 cpm Lymph Node + ALS_{R100} = 480 cpm		Spleen + medium = 230 cpm Spleen + GPC = 202 cpm Spleen + ALS_{R100} = 891 cpm	

An ATXBM mouse was used in order to assess the ability of the RAMIg serum absorbed with fixed thymocytes, to detect a change in the percentage of B cells in a lymphoid organ. A higher percentage of B cells is expected in the lymph nodes and spleens of these animals because of the absence of T cells. However, although the RAMIg serum was able to detect an increase of B cells in the lymph nodes of the ATXBM mouse, it was not able to detect significant increase of B cells in the spleen cell population of the ATXBM mouse over the spleen cell population of an untreated mouse (see Fig.19). The results indicate that the lysis with the RAMIg serum in the cytotoxic test is affected by variations in lymphoid B cell populations. However, the assay does not provide a reliable percentage of B cells as the B cell levels determined in the spleen and lymph node are below levels cited by others (178,179).

(ii) One-step antibody cytotoxicity assay: Other authors (173,174) have shown that incubation of anti L chain serum with lymph node or myeloma target cells and the later addition of complement resulted in decreased or abolished sensitivity to anti L chain serum, i.e. no lysis. However, they did detect lysis when all the components, i.e target cells, anti L chain serum and complement, were incubated together. Thus, it was decided to use the one-step cytotoxicity assay in an attempt to increase the numbers of B cells detected by the RAMIg serum absorbed with fixed thymocytes. However, the one-step assay did not result in improved toxicity of the RAMIg serum for either lymph node or spleen B cell populations. The chromium release from target cells incubated with various dilutions of the RAMIg serum and GPC was often comparable with the chromium release of target cells incubated with GPC alone. Thus, all attempts to raise the toxicity of the RAMIg serum were unsuccessful.

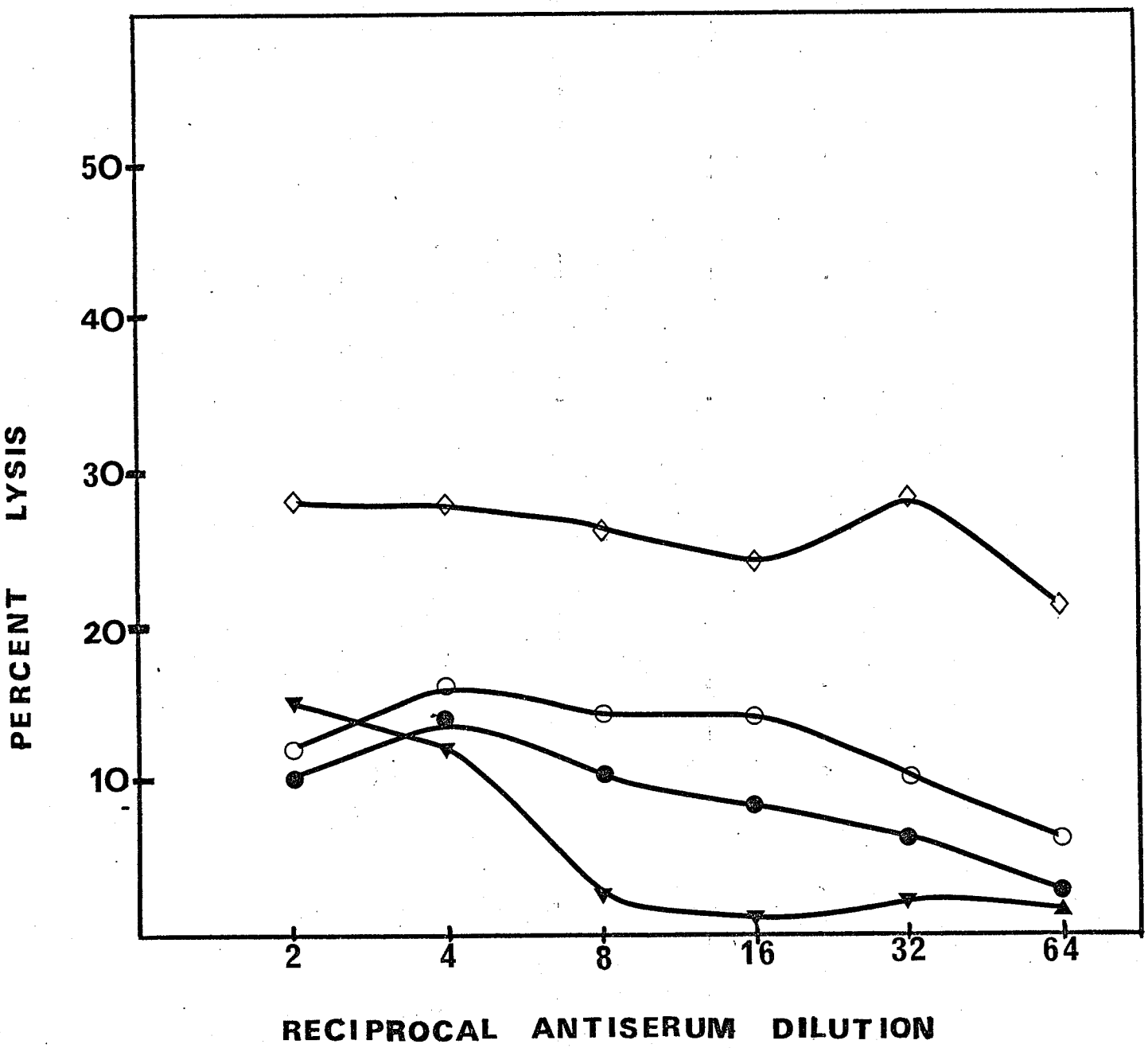
Fig.19. Cytotoxic test for the titration of glutaraldehyde fixed thymocyte absorbed poly RAMIg serum using ATXBM A and $B6AF_1$ ^{51}Cr labelled spleen and lymph node cells. A 60 minutes $4^{o}C$ first incubation was employed. The determination of the % lysis was based on the following control values: ATXBM spleen + medium = 330 cpm, spleen + GPC = 379 cpm, spleen + $ALS_{R_{100}}$ = 1111 cpm, lymph node + medium = 309 cpm, lymph node + GPC = 340 cpm, lymph node + $ALS_{R_{100}}$ = 1035 cpm, $B6AF_1$ lymph node + medium = 168 cpm, lymph node + GPC = 205 cpm, lymph node + $ALS_{R_{100}}$ = 941 cpm, spleen + medium = 308 cpm, spleen + GPC = 391 cpm, spleen + $ALS_{R_{100}}$ = 1698 cpm.

● = ATXBM spleen

◇ = ATXBM lymph node

▼ = $B6AF_1$ lymph node

○ = $B6AF_1$ spleen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(E). Determination of Levels of θ Bearing Cells with the Cytotoxic Assay:

The cytotoxic assay using AKR anti C_3H θ serum was employed to determine the number of θ bearing (T) cells in the spleen and lymph node of the anti μ treated mice. A percentage T cell increase was expected if the anti μ treatment had decreased or eliminated B cells in the lymph node and spleen.

(a) Titration of GPC with AKR Anti C_3H θ Serum:

The anti θ serum was titered against lymph node and spleen cells with various concentrations (1:10, 1:20, 1:40, 1:80) of GPC absorbed with agarose to determine the most effective concentrations of GPC to use (see Figs. 20,21). Since all GPC concentrations gave similar levels of cytotoxicity a 1:10 GPC dilution was adopted for all subsequent assays to ensure that the complement activity is in excess of the amount required for maximal lysis.

(b) Two-step Anti θ Cytotoxicity Assay:

Cytotoxic tests with anti θ serum were then carried out using a two-step procedure comparing a first incubation of $4^{o}C$ with $37^{o}C$ (see Fig.22). The use of the $4^{o}C$ temperature in the first incubation resulted in decreased percent lysis for corresponding antisera dilutions with a $37^{o}C$ first incubation. Thus, it was concluded that $37^{o}C$ is the optimal temperature for the first incubation.

(c) One-step Anti θ Cytotoxic Assay:

The one-step assay was then performed titrating the anti θ serum with thymocytes and splenocytes. The percent lysis from the one-step method was considerably less than the values derived from the two-step assay for both thymocytes and splenocytes (see Fig.23). These results suggest that removal of anti-complementary factors from the serum by the two-step method is more important than any antigenic surface modulation that may be occurring. This

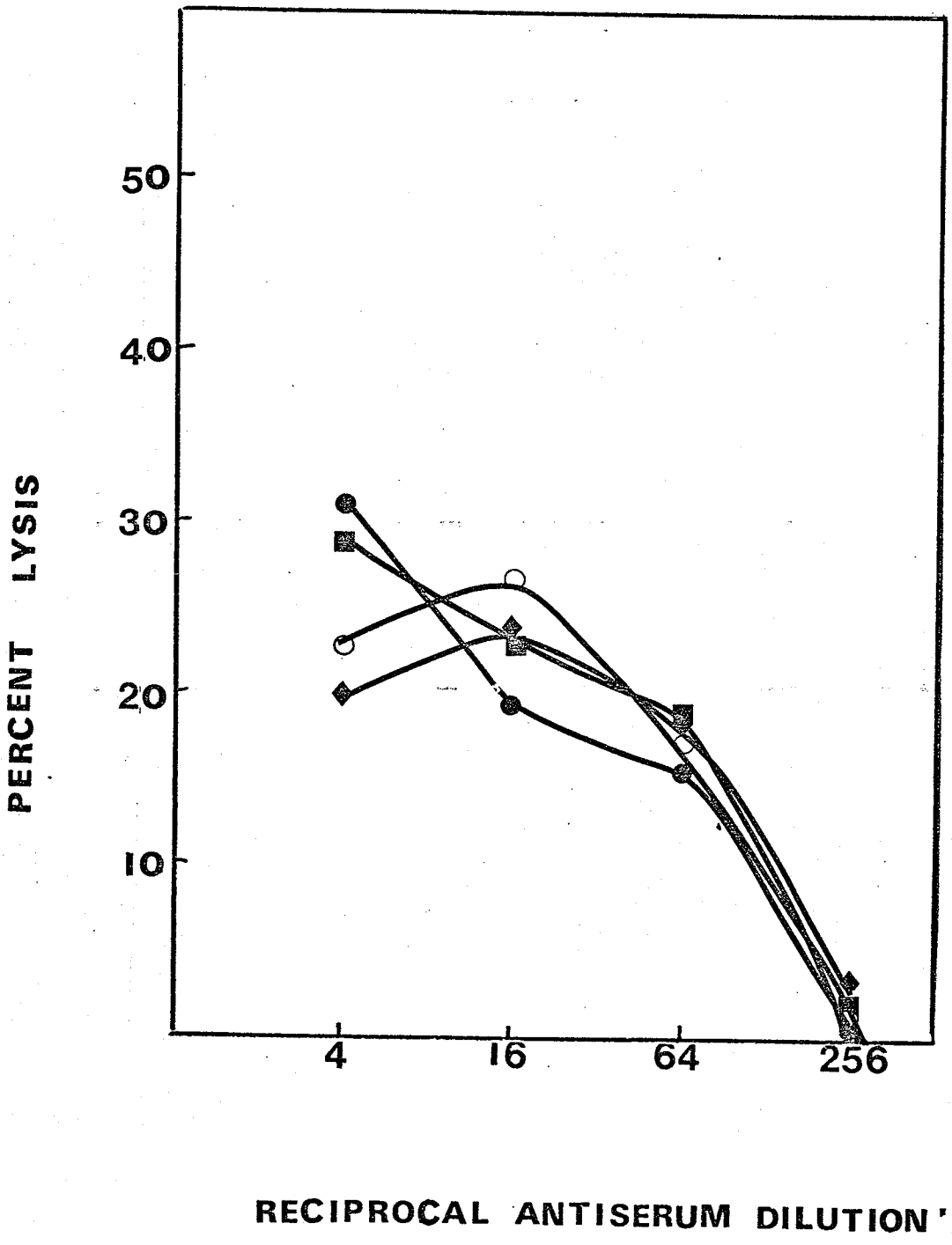
Fig.20. Cytotoxic test for the titration of AKR anti C_3H Θ serum on $B6AF_1$ ^{51}Cr labelled spleen cells using various concentration of agarose absorbed guinea pig complement. The determination of the % lysis was based on the following control values: spleen + medium = 43 cpm, spleen + GPC = 54 cpm, spleen + $ALS_{R_{100}}$ = 383 cpm.

■ = GPC 1:10

● = GPC 1:20

○ = GPC 1:40

◆ = GPC 1:80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RECIPROCAL ANTISERUM DILUTION

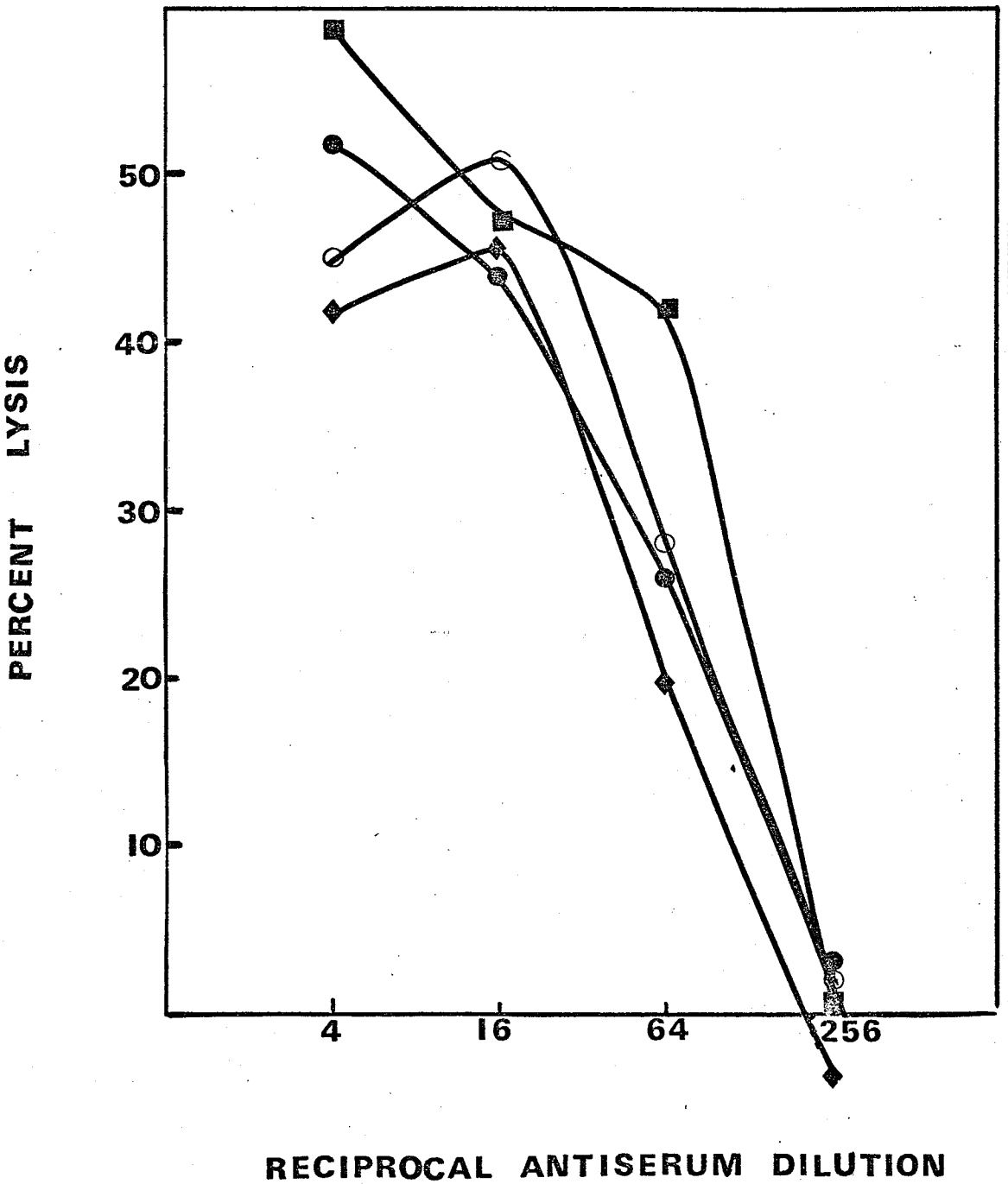
Fig.21. Cytotoxic test for the titration of AKR anti C_3H θ serum on $B6AF_1$ ^{51}Cr labelled lymph node cells using various concentrations of agarose absorbed guinea pig complement. The determination of the % lysis was based on the following control values: lymph node + medium = 42 cpm, lymph node + GPC = 45 cpm, lymph node + $ALS_{R_{100}}$ = 345 cpm.

■ = GPC 1:10

● = GPC 1:20

○ = GPC 1:40

◆ = GPC 1:80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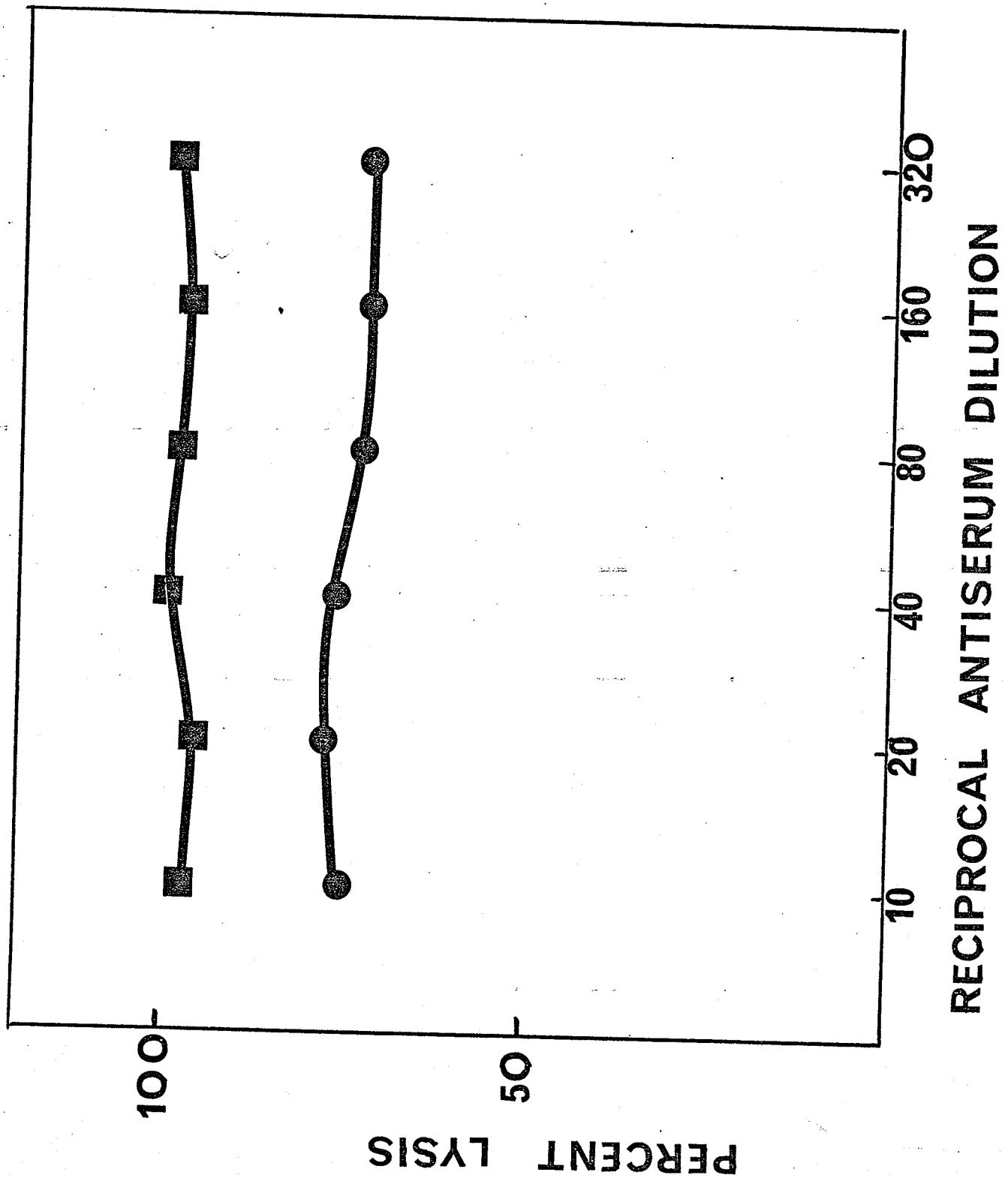
RECIPROCAL ANTISERUM DILUTION

Fig.22. Cytotoxic test for the titration of AKR anti C_3H θ serum on ^{51}Cr labelled thymocytes using a 60 minutes $4^{\circ}C$ first incubation period and a 30 minutes $37^{\circ}C$ first incubation period. The determination of the % lysis was based on the following control values: thymus + medium = 111 cpm ($4^{\circ}C$), 125 cpm ($37^{\circ}C$), thymus + GPC = 155 cpm ($4^{\circ}C$), 180 cpm ($37^{\circ}C$), thymus + $ALS_{R_{100}}$ = 622 cpm ($4^{\circ}C$), 700 cpm ($37^{\circ}C$).

■ = 30 minutes $37^{\circ}C$ first incubation period

● = 60 minutes $4^{\circ}C$ first incubation period


PERCENT LYSIS
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320
RECIPROCAL ANTISERUM DILUTION

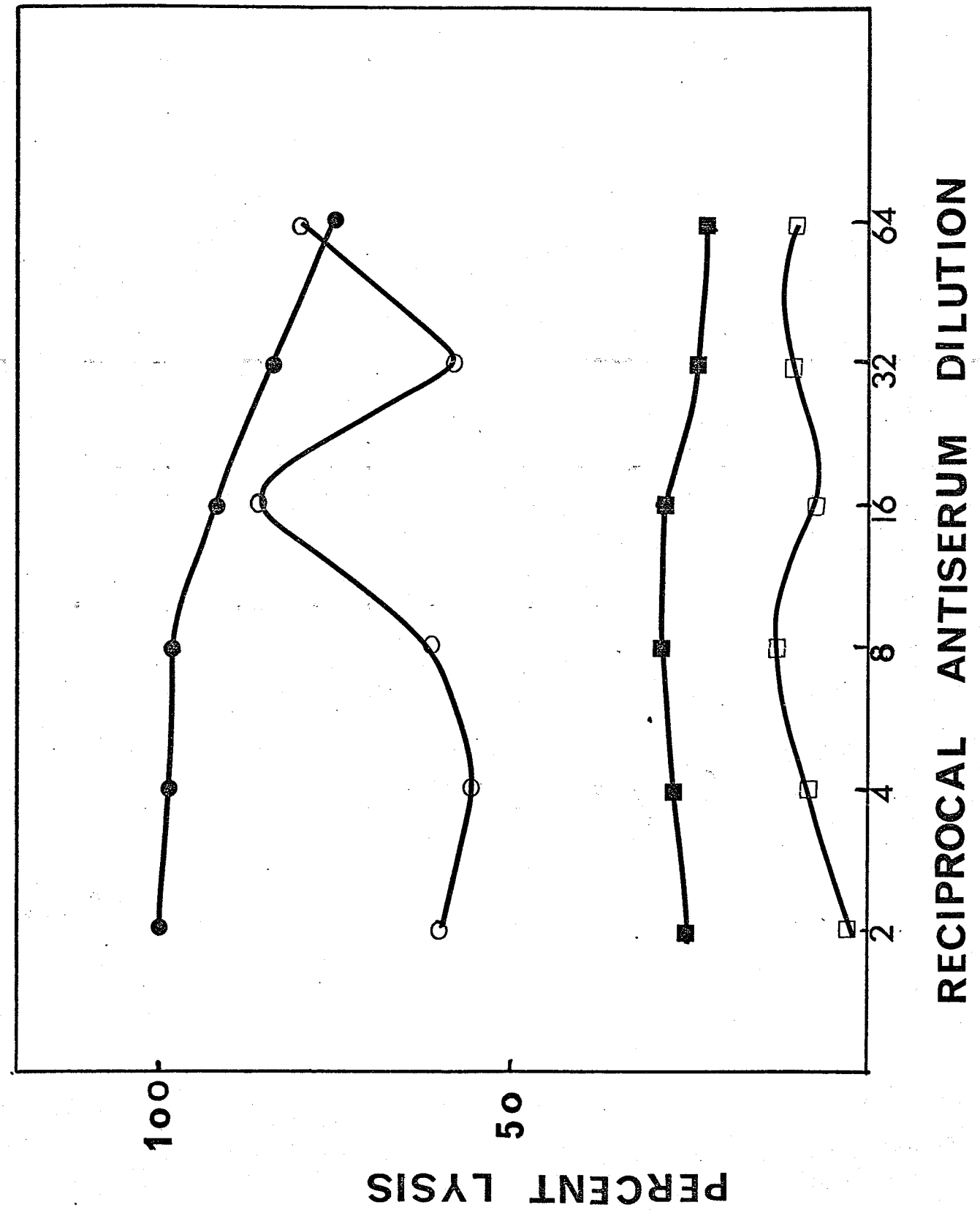
Fig.23. Cytotoxic test for the titration of AKR anti C_3H θ serum on ^{51}Cr labelled thymus and spleen cells using the one-step and the two-step methods (see METHODS). The determination of the % lysis was based on the following control values: thymus + medium = 203 cpm, thymus + GPC = 301 cpm, thymus + $ALS_{R_{100}}$ = 785 cpm, spleen + medium = 264 cpm. spleen + GPC = 251 cpm, spleen + $ALS_{R_{100}}$ = 887 cpm.

● = thymus (2 step)

○ = thymus (1 step)

■ = spleen (2 step)

□ = spleen (1 step)

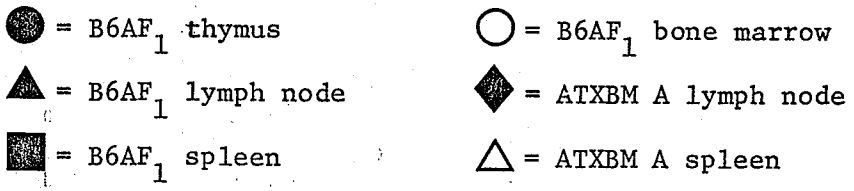

PERCENT LYSIS
100
50
RECIPROCAL ANTISERUM DILUTION
2
4
8
16
32
64

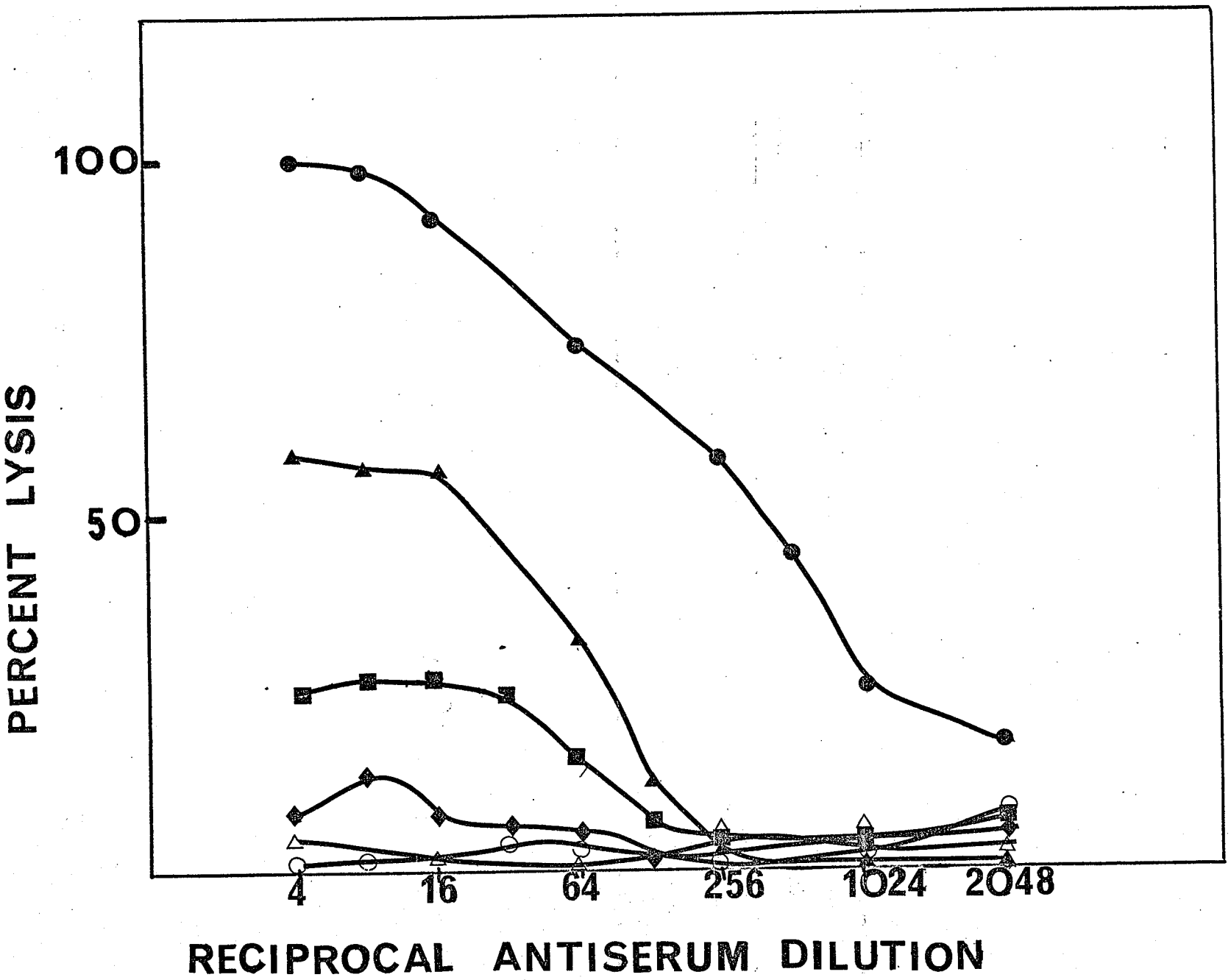
agrees with Schlesinger's (175) work who found mouse serum highly anti-complementary. Reif and Allen (176) were unable to lyze θ bearing cells in peripheral lymphoid tissue when they did not wash away the antisera before adding complement. Thus, the two-step methods using a 37°C first incubation temperature was employed for all subsequent anti θ cytotoxic assays.

(d) Reliability of AKR Anti C_3H θ Serum:

The basic two-step method employing a 37°C first incubation, was used to detect the toxicity of the anti θ serum for bone marrow cells. The level of T cells detected in the spleen and lymph node of ATXBM mice was compared with the T cell levels in the untreated $B6AF_1$ mice (see Fig.24). Negligible levels of toxicity were observed at all dilutions of the anti θ serum with bone marrow target cells. The anti θ serum was seen to have a high toxicity for thymocytes and was capable of producing a 50% lysis at a 1:640 dilution. The ability of the anti θ serum to detect a change, i.e. decrease in T cell levels was confirmed using an adult thymectomized, irradiated and bone marrow reconstituted (ATXBM) mouse. The anti θ serum demonstrated low levels of T cells both in the spleen and lymph node of the ATXBM mouse. However, the anti θ serum also detected low levels of T cells (20%) in the spleen of untreated mice suggesting that either, splenic T cells are inherently more resistant to lysis with anti θ serum and complement or they are a more heterogeneous population with respect to the density of θ surface Ag than lymph node or thymocyte T cell populations. Thus, these results demonstrated that the anti θ serum used in these studies was specific for T cells and also demonstrated that the ATXBM mice had been properly thymectomized.

Fig.24. Cytotoxic test for the titration of AKR anti C_3H θ serum on $B6AF_1$ and ATXBM A ^{51}Cr labelled spleen, lymph node, bone marrow and thymus cells. The determination of the % lysis was based on the following control values: $B6AF_1$ thymus + medium = 242 cpm, $B6AF_1$ thymus + GPC = 200 cpm, $B6AF_1$ thymus + $ALS_{R_{100}}$ = 1202 cpm, $B6AF_1$ lymph node + medium = 168 cpm, $B6AF_1$ lymph node + GPC = 205 cpm, $B6AF_1$ lymph node + $ALS_{R_{100}}$ = 941 cpm, $B6AF_1$ spleen + medium = 308 cpm, $B6AF_1$ spleen + GPC = 391 cpm, $B6AF_1$ spleen + $ALS_{R_{100}}$ = 1698 cpm, $B6AF_1$ bone marrow + medium = 120 cpm, $B6AF_1$ bone marrow + GPC = 135 cpm, $B6AF_1$ bone marrow + $ALS_{R_{100}}$ = 815 cpm, ATXBM A lymph node + medium = 309 cpm, ATXBM A lymph node + GPC = 340 cpm, ATXBM A lymph node + $ALS_{R_{100}}$ = 1035 cpm, ATXBM A spleen + medium = 330 cpm, ATXBM A spleen + GPC = 379 cpm, ATXBM A spleen + $ALS_{R_{100}}$ = 1576 cpm.




100
50
PERCENT LYSIS
4
16
64
256
1024
2048
RECIPROCAL ANTISERUM DILUTION

(e) Comparison of θ Bearing Cells between Normal Control and Experimental $B6AF_1$ Mice:

T cells have been shown to contain some surface Ig (29, 52,111,125), thus it was thought that anti u treatment may affect the number of T cells. The theta (θ) antigen determined by a single locus with two alleles has been shown to be a marker for all thymus dependent lymphocytes (189,190, 191). The AKR θ is found in AKR and RF strains of mice whereas the C_3H θ is found in most other inbred strains of mice. Thus, the AKR anti $C_3H\theta$ serum was used to quantitate θ bearing cells in the peripheral lymphoid organs of normal (untreated) control (nrs treated) and experimental (anti μ treated) $B6AF_1$ mice.

Six groups of animals ranging from 39-98 days old were examined for levels of θ bearing cells in their spleens and lymph nodes (see Table IV). No significant differences were found in the percentage of θ bearing cells between the normal or the control groups ($0.20 < p < 0.15$, $0.35 < p < 0.40$ respectively) in either the spleen or the lymph node. No significant difference of T cell numbers were detected in the spleen between control and experimental animals ($0.25 < p < 0.20$) but a 10% statistically significant ($.0025 < p < .0005$) increase in T cell number was observed in the lymph nodes of the experimental animals from the control animals. The number of θ bearing cells detected in the cytotoxic assay (see Table IV) correlates with values reported by other authors, i.e. 60%-85% θ positive lymph node cells and 25%-45% θ positive spleen cells (178,192,193).

Table IV. Precent lysis values from cytotoxic titration of AKR anti-C_3H θ serum on ^{51}Cr labelled spleen and lymph node cells of $B6AF_1$ anti-μ treated (experimental) and normal rabbit serum treated (control) and untreated (normal) mice.

Lymph Node	No. of animals	mean % lysis[a]	SD	SE	p[b]
Normal (untreated)	4	50	12	4	—
Control (NRS treated)	7	48	13	3	$0.35 < p^c < 0.40$
Experimental (anti-μ treated)	10	58	10	2	$.0025 < p^d < .0005$
Spleen					
Normal (untreated)	4	13	9	2	—
Control (NRS treated)	7	17	10	2	$0.20 < p < 0.15$
Experimental (anti-μ treated)	10	15	6	2	$0.25 < p < 0.20$

[a] The mean precent lysis was derived as the mean of percent lysis from several dilutions (1:2 - 1:32) that defined the upper plateau of the cytotoxicity curve.

[b] P values determined by Student t Test.

[c] Control P values determined by a comparison with the normal cell populations.

[d] Experimental P values determined by a comparison with the control cell populations.

DISCUSSION

The objective of this thesis was to reproduce with the $B6AF_1$ strain, the anti μ mediated suppression of serum Ig previously shown with the BALB/C strain of mice (14,15,17). Chronic anti μ treatment of newborn mice was shown to result in some cases in complete agammaglobulinemia. Thus, the anti μ treated mice could be used to examine T cell mediated phenomena in the absence of B cells or the immunoglobulin they produce.

A. Anti μ Suppression of Serum Ig:

The long term anti μ treatment of newborn $B6AF_1$ mice was seen to result in a complete suppression of serum IgM and an incomplete suppression of IgG. Several suppressive schedules, involving variations in dose and administration schedule (see METHODS), had been tried and it was thought unlikely that the incomplete suppression was due to an inadequate schedule. The rabbit anti IgM serum had been extensively absorbed on purified IgG_{2a} and IgG_1 adsorbent columns and was specific for IgM as demonstrated by the inability of the serum to form precipitin lines against other Ig classes in electrophoretic or Ouchterlony studies. Thus, circulating maternally derived IgG in the newborn would not have reacted with the anti μ antibodies and prevent them from reaching the immunocompetent cells. Basten (52) has shown that bone marrow derived cells are susceptible to radioactively labelled Ag only when they have been peripheralized to the spleen suggesting that natural recovery may occur by this pool of inaccessible stem cells. A pool of BM cells that escape anti μ would produce Ab's to the rabbit anti μ Ab's and the removal of the latter would ensure recovery of Ig levels.

Several other studies have suggested that antibodies of different classes ma exert different and opposite effects on immune responses. The suppression

or activation of the production of mouse Ab bearing a particular idiotype by guinea pig anti idiotype Ab has been shown to be depressed on the class of Ab used, i.e. IgG_1 class enhances the production of the Ab with the idiotype and IgG_2 class suppresses the production of the Ab with the idiotype (206,207). Jansen (208) showed that the different classes of Ig within the mouse itself exhibited opposite biological activties. The 7S IgG_1 ($B6AF_1$ anti B10.D2) Ab showed a clearcut acceleration of B10.D2 skin graft destruction whereas the 7S IgG_2 Ab induced both enhancement and hyperacute destruction of grafts. Thus, these studies suggest that the use of antibody fractions of different classes, i.e. IgG_1 class or IgG_2 class of rabbit anti μ serum because of their opposing regulatory activities, may have resulted in the incomplete suppression.

A panspecific suppression of Ig classes has only been shown with the BALB/C mice (14,15,17). The dose of anti μ and the injection schedule employed in the present investigations using the $B6AF_1$ mouse were similar to those used in these previous studies so it was concluded that serum IgG levels of the $B6AF_1$ mouse are not as easily suppressed as the BALB/C IgG levels. A strain specific restricted phenomenon has also been shown in allotype suppression, i.e. chronic allotype suppression occurred only in the SJL x BALB/C mouse (20,50).

The present investigations showed that serum IgM levels were suppressed and serum Ig G levels although reduced were present. This tends to refute theories (53,54,55) involving sequential development of B cells and support the theories (14,55,57) involving a separate line of development for each Ig class. The mechanism whereby anti μ antibodies mediate a suppression of IgM bearing cells is still unknown although several studies (see Literature Review) suggest that the effects of anti μ are not cytotoxic. It has also

been shown that simple prevention of antigenic stimulation (20) and simple saturation of Ig receptors (34,35) is inadequate for anti μ suppression of PFC response. The possibility that Ag receptors are lost by capping is unlikely in view of the ability of monovalent Ab's which do not induce capping, to induce heavy chain isotype suppression (39). The formation of Ag-Ab complexes on the surface of immature lymphocytes may have resulted in opsonization. This has been shown to occur in mice (52) and this removal of lymphocytes would explain the suppressive effects of anti μ *in vivo* but not *in vitro*. Thus, the anti Ig antibodies are thought to interact directly with the cell surface Ig of immature lymphocytes and either transmit a signal or effect membrane changes that result in the inability of the lymphocyte to produce Ab.

B. Inhibition of Direct Plaque Formation:

A previous study (209) has shown that a few B cell clones stimulated by Ag will differentiate and their subsequent loss of cell surface μ receptor enables them to escape from anti μ mediated suppression. Thus, it was shown that serum Ig levels are a relatively insensitive index of suppression as a few B cells may produce many plasma cells which may result in normal serum Ig levels. The effects of anti μ treatment on B cells were examined more directly with the plaque forming cell assay which measures the ability of the B cell to mount an adaptive immune response. Anti μ treated animals when compared with their littermate controls were suppressed in their ability to form IgM antibody to the SRBC antigens. This unresponsiveness has been shown not to occur at the T cell level as T cells from anti μ treated mice could still provide helper function (20,106,107). Studies employing fluorescent labelled antibodies (13,15) showed that splenic B cells from anti μ treated mice had little or no surface Ig. This would

suggest that the B cell is unable to respond to antigen because it lacks adequate surface receptors. It is unlikely that the injected anti μ Ab's are interfering in the assay (complexing with IgM and preventing plaque formation) because all injections were ceased 10 days prior to the assay and the presence of residue anti μ Ab's would not be expected to completely suppress IgM plaque formation.

C. Quantitation of Ig Bearing Cells with the Cytotoxic Assay:

Polyvalent RAMIg serum was used to quantitate Ig bearing cells in peripheral lymphoid organs using the antibody mediated cytotoxicity assay. Unfortunately, the RAMIg serum demonstrated a strong toxicity for ^{51}Cr labelled thymocyte target cells. It was thought that the RAMIg toxicity may be due either to (a) the presence of Ig on the T cell surface or (b) the presence of other induced Ab's in the RAMIg serum, (c) the presence of natural antibody. Different sera demonstrated varied degrees of toxicity for thymocytes, i.e. anti IgG_{2b} and anti IgG_{2a} had a greater toxicity than anti IgM, anti IgG and anti IgA suggesting that IgG_{2b} and IgG_{2a} may occur on the T cell surface in higher concentration than IgM, IgG_1 and IgA. Since numerous studies have failed to detect any T cell surface Ig (3,22,110) and other only low levels of surface Ig (54,114,125),it was unlikely that the RAMIg toxicity for thymocytes was due to the presence of cell surface Ig. The RAMIg serum has been raised by the immunization of rabbits with purified myeloma proteins and antibodies raised to contaminant membrane fragments or β -2 microglobulin-like antigens, may be responsible for the toxicity of the RAMIg serum for thymocytes. However, this is also unlikely as $F(ab')_2$ serum, produced in rabbits immunized with normal serum immunoglobulin, was also toxic for mouse thymocytes. In addition, serum obtained from unimmunized rabbits often demonstrated considerable toxicity when

incubated with thymocyte target cells in the presence of complement suggesting that natural antibody in rabbit serum is responsible for the toxicity. Thus, the New Zealand White rabbit should not be used to raise Ab's to study mouse T cell surface receptors because of the interference from its strong natural antibody to mouse thymocytes.

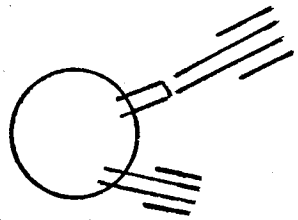
The RAMIg serum was subjected to a series of absorption in an attempt to remove the natural antibody. Absorption with glutaraldehyde fixed thymocytes removed the RAMIg toxicity for thymocytes but, it was also insufficiently toxic for lymph node and spleen cells, i.e. 13% of lymph node cells and 19% of spleen cells. All attempts to increase the toxicity of the RAMIg serum were unsuccessful. The inability to detect significant levels of toxicity of the RAMIg serum for normal B cell population (spleen and lymph node) can be explained in several ways.

Whole immunoglobulin was used as the immunizing antigen for production of RAMIg thus a greater spectrum of Ab specificities would have been raised. If the Ab specificity was directed mainly or partly to the Fc fragment, the RAMIg antibodies may not have been able to attach to the cell surface Ig receptors as the surface Ig Fc piece has been suggested to be largely buried in the cell membrane (108). The class of antibody raised in the RAMIg serum is important as not all rabbit Ig classes can fix complement (210). Thus, if the main class of antibodies raised in the rabbit is not a complement fixing class, then although the RAMIg antibodies will be able to attach to Ig bearing target cells they will be unable to lyse them in the presence of complement. Although gel diffusion tests indicated that the absorbed RAMIg serum combined with purified serum immunoglobulin, i.e. produced precipitin bands, it does not give any information on the ability of the sera to bind surface membrane immunoglobulins. The affinity of the RAMIg antibodies may also be

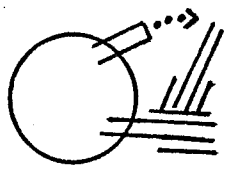
critical as it has been shown that high affinity antibodies are better able to induce conformational changes that trigger complement activity than low affinity antibodies. Thus the serum may contain low affinity antibodies which are either unable to attach to surface Ig or unable to activate complement efficiently.

It was also thought that the cell surface Fc receptors may directly interfere with complement lysis (see diagram below). Thus, this surface complex, i.e. antibody attached to cell surface by its Fc fragment, may not effect the conformational changes necessary to activate complement. The cell Fc receptor may also interfere in an indirect fashion by attaching to the Fc fragment of RAMIg antibodies that have attached to cell surface Ig with their $F(ab')_2$ fragment (see diagram below). This may constrain additional conformational changes that inhibit complement activation or attachment. However, both mechanisms necessitate that the Fc receptor and the Ig receptor are in close proximity to each other. This is supported by Lawson's (181) capping studies that show that both Ig and Fc tend to aggregate over the same pole suggesting at least that they are in the same area on the cell surface. Thus, the Fc receptor interference may be a significant factor for the observed weak cytotoxicity of the RAMIg serum. However, this interference would be difficult to avoid because the Fc fragment of the antibody is necessary for complement attachment.

Fc Receptor Direct Interference



Fc Receptor Indirect Interference



= Fc receptor

= RAMIg, surface Ig

It is known that lymphocytes will resist immune cytolysis unless there are minimum surface receptor sites that can bind the relevant antibodies. If optimal conditions for complement activation are met, immune cytolysis will provide quantitative evidence of the immune reaction. Thus, the density and distribution of Ig receptors on the B cell surface are critical. However, B lymphocytes have been shown to have $10^4 - 10^5$ Ig molecules on their surface (3,32,182). Unanue (182) using a freeze-etching technique showed that surface Ig molecules occur as a continuous network of molecules close to each other forming a lacy pattern. This pattern was also confirmed in a study by Karnovsky's (183) in which appropriately labelled antibody was seen as a fairly continuous layer all around the cell. Thus, the density and distribution of Ig molecules on the B lymphocyte appear to create optimal conditions for immune lysis.

Lymphocyte surface Ig is not rigidly attached to the plasma membrane and anti Ig antibodies result in the formation of Ig anti Ig complexes that have been shown to follow four basic pathways subsequent to or during redistribution: (a) dissociation, (b) persistance for finite period on the cell membrane, (c) shedding off into the extracellular milieu or (d) interiorization by the cell, i.e. endocytosis (184,185,186). Three of the above pathways would act to reduce complement activity in the cytotoxic assay. The modulating effects of the anti Ig serum that results in an apparent phenotypic loss of surface immunoglobulin has been observed in other systems. Modulation described for the thymus leukemia (TL) antigens by the anti TL antibodies (187) and for myeloma and lymph node Kappa determinants produced by anti Kappa antibodies (173) are similar to the anti Ig induced modulation in that the treated cells are rendered insensitive to complement mediated lysis. The capping phenomena may be resposible for the decreased toxicity of the RAMIg

serum in the 45 minute 37°C first incubation assay as Raff (40) has shown that capping can occur very rapidly at 37°C, i.e. 50% of the cells will be capped in 1-5 minutes. Endocytosis of Ig-anti Ig complexes which can occur without capping (188) can also occur very rapidly. Thus, both of these processes (capping and endocytosis) which result in altered or decreased Ig-anti Ig complexes on the cell surface, could account for the decrease toxicity of the RAMIg serum by preventing complement activity.

Inhibitors of glycolysis or oxidative phosphorylation (Na azide or iodoacetamide) have been shown to inhibit anti Ig induced capping (182). However, the use of these inhibitors in the cytotoxic assay is limited because although they allow the formation of surface Ig-anti Ig complexes (182) and prevent their subsequent capping, they would also inhibit complement activity. Both capping and endocytosis have been shown to be dependent on the degree of cross-linking of surface determinants which requires a bivalent ligand (40,184). The surface Ig-anti Ig complexes formed by a monovalent whole antibody would not effect cross linking of surface molecules and although complexes formed by monovalent ligands can be interiorized they are done so at a much slower rate and to a lesser degree (182). However, it is not known if the monovalent antibodies would be as efficient as the bivalent antibodies in inducing the conformational changes necessary to active complement or if it would offer an adequate site for complement attachment.

Thus, the cytotoxic assay using rabbit RAMIg serum was unsuccessful in quantitating Ig bearing cells. However, the assay did detect the strong toxicity of the RAMIg serum for mouse thymocytes probably due to natural antibody. The RAMIg serum would only have indicated if the anti μ antibodies had effected an elimination or alteration of surface immunoglobulin. The

use of the mouse specific B lymphocyte antigen (MBLA) defined by heterologous antibodies raised in rabbits against mouse B cells may further detail the effects of anti μ treatment on B cells. Raff (178) has shown that all B cells including Ab forming precursors and Ab- forming cells, all carry the MBLA marker. Thus, as anti μ antibodies would not be expected to alter the surface MBLA marker, a decreased percentage of MBLA bearing cells would in indicate an actual elimination of B cells.

D. Quantitation of θ Bearing Cells with the Cytotoxic Assay:

The cytotoxicity assay was used to examine the effects of anti μ treatment on the number of T cells in the peripheral lymphoid organs (spleen and lymph node). The comparison of the number of T cells in control and normal groups of animals suggest that lymph nodes have more T cells than spleens. The lower levels of θ bearing cells in the spleen were thought to be due to a greater difficulty in lyzing splenic T cells with anti θ and complement because of a low level of θ Ag on their surfaces. However, it has been shown (205) that the subpopulations of T cells (T_1) that preferentially homes to the spleen have a higher θ Ag concentration on the cell surface and are more sensitive to anti θ and complement in vitro than the T cell subpopulation (T_2) that preferentially homes to the lymph node. It was thought that the lower levels of T cells in the spleen likely reflects a percentage difference, i.e. the spleen has more cells than the lymph node, thus, it may actually have a higher number of T cells than the lymph node although it registers a lower percentage of T cells. Thus, on a percentage basis, the lymph node is a richer source of T cells than the spleen.

The anti μ treatment resulted in a 10% increase in T cell number in the lymph nodes of experimental animals (58%) when compared with control animals (48%). However no significant differences ($0.25 < p < 0.20$) were detected in

the T cell numbers of their spleens. The % of θ bearing cells detected in controls (17%) or normal (13%) spleens appeared to be depressed when compared to values (25%-45%) cited in other studies (179,192,193). Thus, the anti θ serum may not be able to discriminate between T cell variances, i.e. increase of decrease in the spleen because of the relatively small percentage of T cells that exist or can be detected because of the heterogenous cell population existing in the spleen cell population. The observation that anti μ treatment effects a 10% increase in T cell numbers in the lymph node may be interpreted several ways. The increase in the percentage of T cells may represent an absolute increase in T cell number or it may represent a relative increase of T cells due to a B cell loss.

E. General Discussion:

The results of the present investigation with $B6AF_1$ mouse confirm the results obtained with anti μ chronically treated BALB/C mice (15,16,17). Although serum IgM levels were completely suppressed in the $B6AF_1$ mouse, the suppression of the $B6AF_1$ serum IgG levels differed qualitatively from that obtained in the BALB/C mouse. Immunoelectrophoretic studies indicated that $B6AF_1$ serum IgG_1 levels were unaffected and serum IgG_{2a} levels were partially suppressed by anti μ treatment whereas previous authors (15,16) demonstrated a severe (residual level) suppression of both IgG_1 and IgG_2 in the BALB/C mouse. The direct plaque cell assay demonstrated that the anti μ treated $B6AF_1$ mouse was completely suppressed in its ability to produce IgM Ab to SRBC. However, the study of B cell regulation of T cell mediated reactions required the suppression of both IgG and IgM. The suppression of the IgG class is particularly important for studies on tumor grafts because of the protective or enhancing activity of the IgG class (214). Thus, it was concluded that because of the incomplete suppression of serum Ig levels, the

anti μ treated $B6AF_1$ mouse may not be a good model to study the regulation by B cells of T cell functions.

The present investigations employing the cytotoxic assay demonstrated a 10% increase in T cell numbers in the lymph nodes of anti μ treated mice when compared with their littermate controls. Unfortunately, the RAMIg serum used in the cytotoxic assay was unable to quantitate B cells to directly confirm a B cell loss because the serum was strongly toxic formouse thymocytes and the absorbed serum did not lyse significant numbers of B cells. The thymocyte absorbed RAMIg serum demonstrated anti Ig activity when tested by Ouchterlony indicating that the antibody activity removed by absorption was not anti Ig. Sera from immunized NZW rabbits was strongly toxic for mouse thymocytes indicative of natural antibody to mouse thymocytes. Thus, rabbits should not be used as a serum source to study T cell receptors or T cell functions because of the interference from this natural antibody.

CONCLUSIONS

From the observations presented in this thesis, the following conclusions can be made:

(1) Chronic anti μ treatment of $B6AF_1$ mice from birth resulted in a complete suppression of serum IgM and an incomplete suppression of IgG.

(2) Anti μ treatment effectively suppressed the ability of young $B6AF_1$ mice to mount an IgM antibody response to SRBC.

(3) The anti μ suppressed $B6AF_1$ mouse is not a good model for the study of B cell regulation of T cell mediated immune reactions because the suppression of serum Ig levels is incomplete.

(4) RAMIg sera contains antibodies to a mouse cell surface protein, particularly thymocytes, and may not be suitable for the study of the T cell receptor.

(5) The antibody mediated cytotoxicity assay is not suitable for the quantitation of Ig bearing cells.

(6) No gross differences of theta bearing cells were detected in the spleens of control (NRS treated) and experimental (anti μ treated) mice. However, a 10% increase of theta bearing cells was detected in the lymph nodes of experimental mice over control mice.

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